Edited by Uwe T. Bornscheuer
in collaboration with the German Society
for Fat Science (DGF)

Enzymes in Lipid Modification

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Prof. Dr. Uwe T. Bornscheuer
Institute for Chemistry and Biochemistry
Dept. of Technical Chemistry and Biotechnology
University of Greifswald
Soldmannstraße 16
D-17487 Greifswald
Germany

German Society for Fat Research (DGF)
Varrentrapstraße 40–42
D-60486 Frankfurt/Main
Germany

Cover illustration: Three-dimensional structure of the *Rhizopus delemar* lipase, determined by X-ray crystallography. The graphic of an oil drop was kindly provided by Peter Laurat, tangram documents, Bentwisch, Germany.

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Foreword

Industrial-age society has been – or at least is being – transformed into an information-age society. The plethora of information now available down to molecular "genomic" levels still does continues to grow. With justification those working in such areas feel part of a biosociety; and professional organizations have responded accordingly. For instance, in the 1970s our own *Deutsche Gesellschaft für Fettwissenschaft* (DGF) created a *Division of Biochemistry and Biotechnology* which for some time now has been co-chaired by Uwe Bornscheuer.

Uwe Bornscheuer is an authoritative colleague in the field of enzyme technology. Together with Romas Kaslauskas he wrote a highly acclaimed book on *Hydrolases in Organic Synthesis* published by WILEY-VCH. The present book *Enzymes in Lipid Modification* is also a publication of WILEY-VCH, and I am pleased that this renowned publishing house is becoming increasingly engaged in lipids, fats, and oils. By launching the new monthly *European Journal of Lipid Science and Technology* in January 2000, indeed, DGF and WILEY-VCH are already a successful collaboration.

Scientific interest in enzymes acting on lipids has a long history. The first focus was on lipases. Then phospholipases came to the fore, and lately lipoxygenases and monooxygenases have garnered attention. I wish to thank *Uwe Bornscheuer* for gathering acknowledged experts to write succinct chapters pertaining to this groups of enzymes. Their informative and critical reviews discuss how to use and improve properties of proteins such as reversibility, chirality, and stability in the synthesis of products. The aim and scope of the book is thus truly biotechnological.

DGF happily cooperates in this endeavor!

Münster, April 2000

Friedrich Spener
President of DGF

Preface

In the last two decades, our understanding of biocatalysts has increased considerably, as have the number of applications of biocatalysts for synthesis. The success of biocatalysts stems from better availability of enzymes – mainly due to the vast progress in genetic engineering –, advances in bioreaction engineering, increasing demands for environmentally-friendly processes, which makes a biocatalytic route more attractive and often more cost-effective than a chemical one.

Most of the approximately 90 million metric tonnes of fats and oils produced worldwide are used in human nutrition. However, not all fats and oils obtained from animals or plants are necessarily ideal for the human diet, e.g., high contents of saturated fatty acids can cause cardiovascular diseases. Beside physico-chemical modifications and the introduction of genetically engineered plants producing "designer-oils", biocatalysts offer an alternative way to concert lipids into suitable edible products as well as their conversion into "basic-chemicals" useful for, e.g., synthesis of detergents or emulsifiers.

From all enzymes available in nature, hydrolases are probably most easy-to-use, because they do not require cofactors and are usually rather stable under process conditions. This holds especially true for lipases and phospholipases. A large number of lipases are commercially available, and several industrial processes use lipases. As lipids are the natural substrates of lipases it is not surprising that most chapters in this book review their application in lipid modification, such as hydrolysis to produce free fatty acids, synthesis of partial glycerides, enrichment/isolation of polyunsaturated fatty acids which are important for the human diet. Also covered are their cloning, expression and mutagenesis as well as attempts to understand the molecular basis for their specificity and stereoselectivity. In addition, engineering aspects and the choice of suitable solvent systems are addressed.

Availability and applications of phospholipases are still less developed compared to lipases. The two chapters in this book allow the conclusion, that in the near future drawbacks such as stability under process conditions and difficult expression in suitable hosts will be overcome.

Other enzymes frequently studied in lipid modification are lipoxygenases and P450-monooxygenases, which are reviewed in the remaining chapters. They are very attractive for organic synthesis, because they allow functionalization of fatty acids in order to generate, e.g., flavors and emulsifiers such as sophorose lipids.

I am convinced that this book – reflecting the state-of-the-art of enzymatic lipid modification written by leading experts in their field – will provide the reader with guidelines how to select suitable enzymes and how to apply them efficiently.

Comments and suggestions are welcome and will be posted together with updates on a website at: *http://www.chemie.uni-greifswald.de/~biotech/lipid-book.html*.

Finally, I would like to express my thanks to all contributors to this book. The untiring support and patience of Ms. Karin Dembowsky and her staff at Wiley-VCH is gratefully acknowledged.

Greifswald, April 2000 Uwe Bornscheuer

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About the Editors

Uwe Bornscheuer (born 1964) studied Chemistry at the University of Hannover, Germany, where he graduated with a Diploma in 1990. After receiving a Ph.D. in Chemistry at the Institute of Technical Chemistry at the same University in 1993, he spent a postdoctoral year at the University of Nagoya, Japan, with Professor Tsuneo Yamane. He then joined Professor Rolf Schmid at the University of Stuttgart, Germany, where he finished his Habilitation in Technical Biochemistry in 1998. In 1999, he became Professor at the Department of Technical Chemistry and Biotechnology at the University of Greifswald, Germany. He is married and has one daughter.

The German Society for Fat Science (DGF, Deutsche Gesellschaft für Fettwissenschaft) was founded in 1936 by the late Professor Hans Paul Kaufmann. DGF is a non-profit organization dedicated to the promotion of fat science and technology. Consisting of several divisions and working groups, DGF ensures an exchange of state-of-the-art knowledge in the area of lipids, fats and oils. DGF dissiminates expert knowledge, organizes international and national conferences, training courses, and ring tests. The Society's former journal *Fett/Lipid* has now been transformed to the new *European Journal of Lipid Science and Technology.* DGF also publishes the *German Standard Methods for the Analysis of Fats and Other Lipids* (DGF Standard Methods).

Professor Friedrich Spener, University of Münster, Germany, is the current president of DGF.

Further information can be found at *http://www.gdch.de/dgf/*.

List of Editors and Corresponding Authors

Editors:

Prof. Dr. Uwe T. Bornscheuer
Institute for Chemistry and
Biochemistry, Dept. of Technical
Chemistry and Biotechnology
University of Greifswald
Soldmannstraße 16
D-17487 Greifswald
Germany
Tel.: (0 38 34) 86 43 67
Fax: (0 38 34) 86 43 73
bornsche@mail.uni-greifswald.de

Deutsche Gesellschaft
für Fettwissenschaft
(German Society for Fat Research)
Varrentrappstraße 40–42
D-60486 Frankfurt/Main
Germany
Tel.: (0 69) 79 17 5 29, -5 33
Fax: (0 69) 79 17 5 64
F.Amoneit@gdch.de

Authors:

Dr. Patrick Adlercreutz
Dept. of Biotechnology
Center for Chemistry and Chemical
Engineering
Lund University
P.O. Box 124
S-22100 Lund
Sweden
Patrick.Adlercreutz@biotek.lu.se

Dr. John Bosley
Unilever Research
Colworth House, Sharnbrook
Bedford, MK44 1LQ
United Kingdom
John.Bosley@unilever.com

Dr. Rob M. M. Diks
Unilever Research Laboratorium
Vlaardingen
Olivier van Noortlaan 120
NL-3133 AT Vlaardingen
The Netherlands
rob.diks@unilever.com

Dr. Ivo Feussner
Institute for Plant Biochemistry
Weinberg 3
D-06120 Halle/Saale
Germany
ifeussne@ipb.uni-halle.de

Dr. Peter Grunwald
Institute for Physical Chemistry
University of Hamburg
Bundesstraße 45
D-20146 Hamburg
Germany
grunwalp@cip.chemie.uni-hamburg.de

Dr. Michael J. Haas
USDA, ARC, ERRC
600 East Mermaid Lane
Wyndmoor, PA 19038
USA
mhaas@ARSERRC.Gov

Prof. Dr. Gudmundur G. Haraldsson
Science Institute
University of Iceland
Dunhaga 3
107 Reykjavik
Iceland
gghar@raunvis.hi.is

Prof. Douglas G. Hayes
The University of Alabama
in Huntsville
Dept. of Chemical Engineering
MSB, Room 307
Huntsville, AL 35899
USA
dhayes@ebs330.eb.uah.edu

Prof. Dr. Gilles Iacazio
Laboratoire de Microbiologie,
UDESAM
Faculté des Sciences de Saint Jérôme
Avenue Escadrille Normandie Niemen
F-13397 Marseille Cedex 13
France
Gilles.IACAZIO@VMESA12.U-3
MRS.FR

Dr. Kumar D. Mukherjee
Institute for Biochemistry and
Technology of Lipids
H. P. Kaufmann Institute
Piusallee 68
D-48147 Münster
Germany
ibtfett@uni-muenster.de

Dr. Jürgen Pleiss
Institute for Technical Biochemistry
University of Stuttgart
Allmandring 31
D-70569 Stuttgart
Germany
jpleiss@tebio1.biologie.uni-stuttgart.de

Prof. Dr. Mark Rüsch gen. Klaas
Neubrandenburg University of Applied
Sciences
Department of Technology
Brodaer Straße 2
D-17033 Neubrandenburg
Germany
ruesch.gen.klaas@fh-nb.de

Prof. Dr. Manfred Schneider
Bergische Universität
Gesamthochschule Wuppertal
FB Chemie
Gaußstraße 20
D-42097 Wuppertal
Germany
schneid@uni-wuppertal.de

Prof. Dr. Yuji Shimada
Osaka Municipal Technical Research
Institute
1-6-50 Morinomiya, Joto-ku
Osaka 536-8553
Japan
shimaday@omtri.city.osaka.jp

Prof. Dr. Friedrich Spener
(President of DFG)
Institute for Biochemistry
University of Münster
Wilhelm-Klemm-Straße 2
D-48149 Münster
Germany
spener@uni-muenster.de

Prof. Dr. Christoph Syldatk
Institute for Bioprocess Engineering
University of Stuttgart
Allmandring 31
D-70569 Stuttgart
Germany
syldatk@ibvt.uni-stuttgart.de

Dr. Ulrich Schwaneberg
California Institute of Technology
Division of Chemistry and Chemical Engineering 210-41
Pasadena, 91125, CA
USA

Prof. Dr. Renate Ulbrich-Hofmann
Institute for Biotechnology
University of Halle-Wittenberg
Kurt-Mothes-Straße 3
D-06120 Halle (Saale)
Germany
ulbrich-hofmann@biochem-tech.uni-halle.de

Prof. Dr. Siegfried Warwel
Institute for Biochemistry and Technology of Lipids
H. P. Kaufmann Institute
Piusallee 68
D-48147 Münster
Germany
ibtfett@uni-muenster.de

Dr. Xuebing Xu
Dept. of Biotechnology
Technical University of Denmark
Building 221
DK-2800 Lyngby
Denmark
xx@IBT.DTU.DK

Prof. Dr. Tsuneo Yamane
Laboratory of Molecular Biotechnology
Graduate School of Bio- and Agrosciences
Nagoya University
Chikusa-ku, Nagoya 464-8601
Japan
yamanetu@agr.nagoya.u.ac.jp

Color Plates

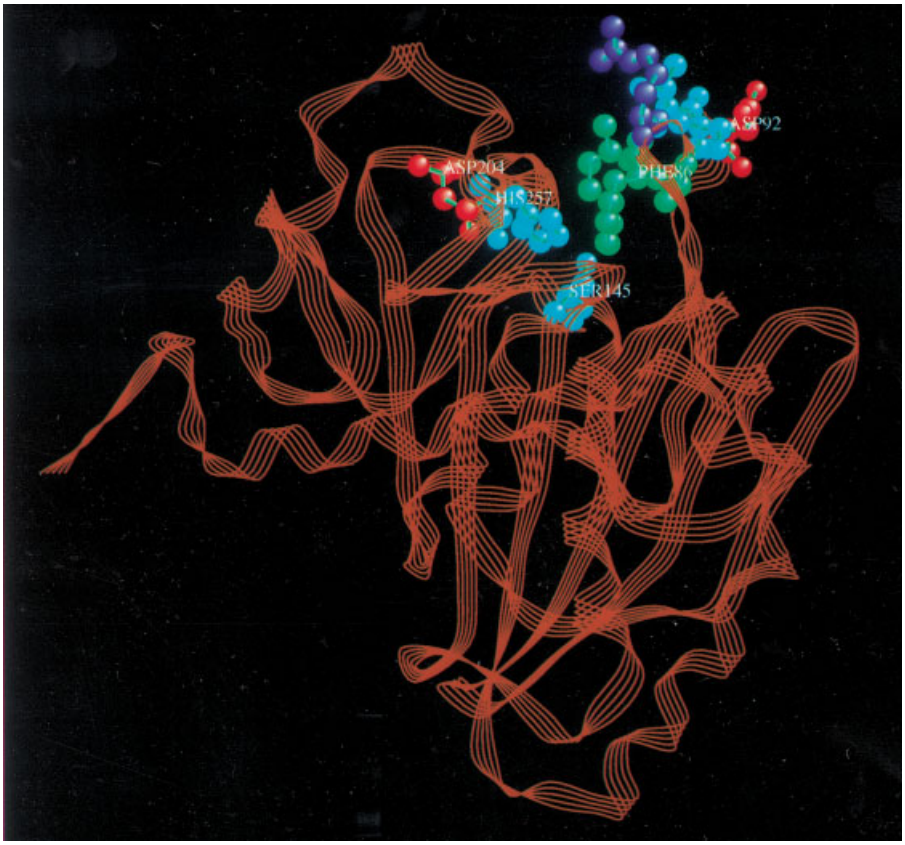

Figure 3 (Chapter 4). Three-dimensional structure of the *Rhizopus delemar* lipase, determined by X-ray crystallography (Derewenda et al., 1994a,b,c; Swenson et al., 1994). (A) The 'lid', residues 86–92, is in the closed conformation and occludes the active site. (B) Lid is partially opened, opening the active site for access by substrates to the catalytic triad residues: Ser145, Asp204, and His257.

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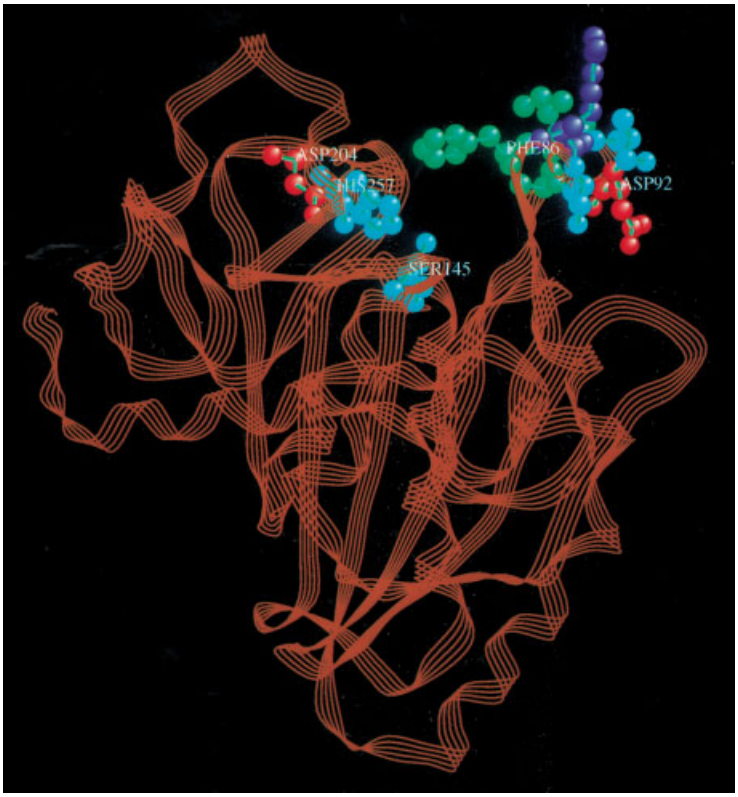

Figure 3B (Chapter 4)

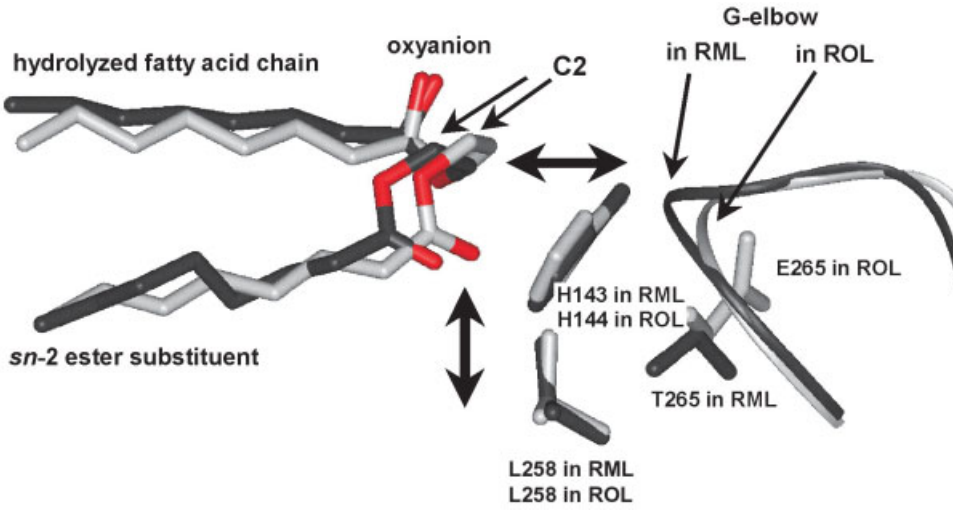

Figure 6 (Chapter 5). Ester substrate in *sn*-1 orientation in *Rhizopus oryzae* lipase (ROL, light gray) and *Rhizomucor miehei* lipase (RML, dark gray) (Scheib et al., 1999). The position of the G266 varies in both lipases ($C_\alpha - C_\alpha$ distance = 1.3 Å) due to the different position of E265 in ROL and T265 in RML, respectively (horizontal arrow). Therefore, in ROL, the substrate binds deeper into the binding site than in RML ($C2_{RML} - C2_{ROL} = 0.9$ Å). Hence, steric interactions between the *sn*-2 substituent and L258 are more pronounced in ROL rather than RML (vertical arrow).

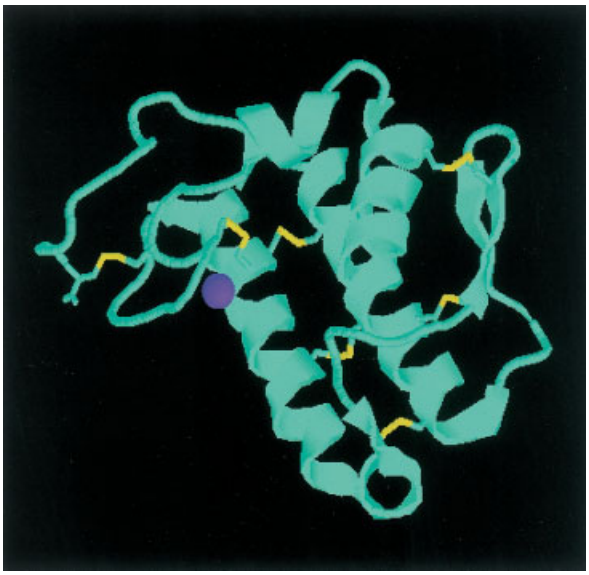

Figure 3 (Chapter 12). Tertiary structure of PLA_2 from bovine pancreas with seven disulfide bridges and one Ca^{2+} ion. The structure was taken from the Brookhaven Protein Data Bank, file 1BP2, and drawn by RasMol.

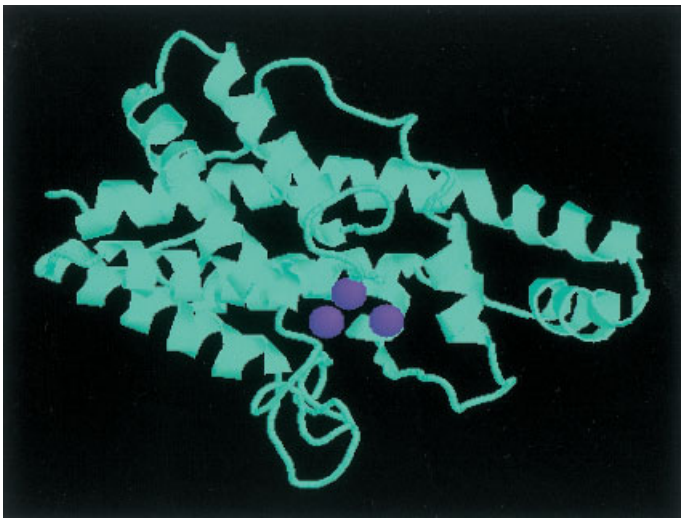

Figure 5 (Chapter 12). Tertiary structure of PLC from *Bacillus cereus* with three Zn^{2+} ions. The structure was taken from the Brookhaven Protein Data Bank, file 1AH7, and drawn by RasMol.

Bovine PKC	GLSDPYVKLKL	(19)	NPRWDESF		
Arabidopsis PLDα	GETRLYATIDL	(15)	NPKWYESF	HQKIVVVDSEMPSRGGSEMRRI	HTKMMIVDDEYIIIGS
Arabidopsis PLDβ	ITSDPYVSVSV	(16)	NPVWMQHF	HQKNVIVDADAGGNRRKI	HSKGMVVDDEYVVIGS
Arabidopsis PLDγ	ITSDPYVTVSI	(15)	NPVWIEHF	HEKTVIVDSEAAQNRRKI	HSKGMVVDDEFVLIGS
Arabidopsis PLDγ2	FTSDPYVTVSI	(16)	ILCGMPAF	HQKTMIVDAEAAQNRRKI	HSKGMVVDDEFVLIGS
Cabbage PLD1	GETQLYATIDL	(16)	NPKWYESF	HQKIVVVDSEMPSRGGSQMRRI	HTKMMIVDDEYIIIGS
Cabbage PLD2	GETQLYATIDL	(16)	NPKWYESF	HQKIVVVDSEVPSQGGGSEMRRI	HSKMMIVDDEYIIVGS
Castor bean PLD	GVSKLYATIDL	(16)	NPRWYESF	HQKIVVVDSAMPNGDSQRRRI	HTKMMIVDDEYIIIGS
Cowpea PLD	GVTKLYATIDL	(16)	NPKWNESF	HQKIVVVDSALPGGGGSDKRRI	HTKMMIVDDEYIIIGS
Craterostigma PLD1	GTPKIYASIDL	(16)	NPRWYESF	HQKIIVVDSDLPSGGSDKRRI	HAKMMIVDDEYIIIGS
Craterostigma PLD2	GTPKIYASIDL	(16)	NPRWYESF	HQKIIVVDSDLPSGGSDKRRI	HAKLMIVDDEYIIIGS
Maize PLD	GATKIYATVDL	(16)	NPRWYESF	HQKIVVVDHEMPNQGSQQRRI	HTKMMIVDDEYIIIGS
Pimpinella PLD	GTPKIYASIDL	(16)	NPKWNESF	HQKIVVVDSEMPTSGSENRRV	HAKMMIVDDEYIIIGS
Rice PLD1	GATKVYSTIDL	(16)	NPRWYESF	HQKIVVVDHELPNQGSQQRRI	HTKMMIVDDEYIIIGS
Rice PLD2	GATRLYATIDL	(16)	NPRWYEVF	HQKTVIVDHDMPVPRGGGSRRI	HSKMMIVDDEYIIVGS
Tobacco PLD	GTPAIYATVDL	(16)	NPRWYESF	HQKIVVVDSELPSGESEKRRI	HSKMMIVDDEYIIVGS
Upland cotton PLD	ITSDPYVTIAV	(15)	NPVWMQHF	HQKTVIVDADAGNNHRKI	HSKGMIVDDEFIIVGS
Candida albicans PLD				HEKLCIIDHTYAFLGGIDL	HAKTMIVDDRSVIIGS
Saccharomyces cerevisiae PLD				HEKFVVIDETFAFIGGTDL	HAKILIADDRRCIIGS
Streptomyces antibioticus PLD	ADGKPYALHHK	(16)	YPAWLQDF	HSKLLVVDGKTAITGGING	HHKLVSVDDSAFYIGS
Streptoverticillium cinnamoneum PLD	ADGHPYALHHK	(16)	YPSWLQDF	HSKLLVVDGESAVTGGINS	HHKLVAVDSSAFNIGS

Figure 6 (Chapter 12). HKD motifs and sections of the C2 domain in PLDs from plants and microorganisms. Amino acid sequences are taken from the NCBI Genbank. From the C2 domain the sequences of the β3 and β5 sheets are shown and compared with the well-known sequence of the C2 domain in protein kinase C (PKC). The numbers in parentheses indicate the number of amino acids between β3 and β5.

Lipases

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1 The Exploitation of Lipase Selectivities for the Production of Acylglycerols

R.M.M. Diks and J.A. Bosley

1.1 Introduction

Triglyceride modification using lipases nowadays is a well-known principle. During the past 20 years, numerous papers have been published describing the production of mono-, di- and triglycerides, as well as other lipid derivatives, mostly fatty acid esters. Throughout the literature, many different types of lipases have been described for enzymatic modification. Based on their specificity or selectivity these lipases can be divided into three classes (Table 1):

1. Regio- or positional specific;
2. Fatty acid type specific;
3. Specific for a certain class of acylglycerols, i.e. mono-, di- or triglycerides.

The reactions catalyzed by these lipases include hydrolysis, glycerolysis, esterification, acidolysis and interesterification. Using any of these reactions the aim is to produce specific end products at highest yield and purity. Obviously careful selection of the process conditions, such as water activity, temperature or substrate composition is required. However, selection of the correct lipase with the most appropriate selectivity can also be of the highest importance in achieving commercially interesting products. The following overview aims to highlight the exploitation of the specificity of these lipases in the production of various acylglycerols.

It is important to realize that most process descriptions given below are generally based on laboratory or bench-scale studies. Very few are run as full-scale commercial processes, especially in relation to foods. This is mainly due to the high cost and relatively low operational productivity of most of the lipases applied. The growing ability to exploit the high selectivity of enzymes, yet retain mild processing conditions means that interest in biocatalysis is expected to grow during the coming years. Moreover, as a consequence of modern biotechnology, the cost of enzymes is likely to fall, thus permitting wider economic application.

1.2 Free fatty acids

1.2.1 Nonselective production of free fatty acids

The simplest process involving lipases is hydrolysis of oils and fats. This reaction actually reflects the natural function of lipases. However, only few lipases – all from *Candida rugosa* – are actually able to completely hydrolyze triglycerides into gly-

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Table 1. Major specificities of lipases and their applications.

Specificity	Lipases	Production of	References
Regio specificity			
1,3-Regio specific	*Rhizomucor miehei* *Rhizopus oryzae*	triglyceride synthesis	Section 1.2.2
	Rhizopus arrhizus	1,2(2,3)-diglycerides by triglyceride hydrolysis	Section 1.3.2
	Rhizopus delemar	1,3-diglyceride by fatty acid (directed) esterification	Section 1.3.3
	Rhizopus niveus		
	Porcine pancreatic lipase	2-monoglycerides by triglyceride hydrolysis	Section 1.4.2
		1(3)-monoglycerides by fatty acid esterification	Section 1.4.3
Non-specific	*Candida rugosa* *Chromobacterium viscosum*	fatty acid production by hydrolysis	Section 1.2.1
	Pseudomonas fluorescens	mono- and diglycerides by directed glycerolysis	Section 1.3.3
	Pseudomonas cepacia		Section 1.4.3
Fatty acid specific			
Long chain poly-unsaturated acids	*Geotrichum candidum* *Candida rugosa*	Selective hydrolysis	Section 1.2.2
Saturated acids	*Fusarium oxysporum*	Selective hydrolysis	Section 1.2.2
cis-Δ9 unsaturated acids	*Geotrichum candidum B*	Selective hydrolysis	Section 1.2.2
Short acids	*Cuphea* sp.	Selective hydrolysis	Section 1.2.2
Acylglycerol specific			
Monoacylglycerols	Potato acylhydrolase (patatin)	Monoglycerides by fatty acid esterification	Section 1.4.3
Mono- and diacylglycerols	*Penicillium camembertii*	Mono- and diglycerides by fatty acid esterification	Section 1.3.3
	Penicillium cyclopium M1 *Fusarium* sp.		Section 1.4.3
Triacylglycerols	*Penicillium roquefortii*	1,2-Diglycerides by triglyceride hydrolysis or alcoholysis	Section 1.3.2
	Penicillium cyclopium M1 *Penicillium expansum*		

cerol and free fatty acids (Bühler and Wandrey, 1987; Macrae and Staines, 1994; Plou et al. 1996). These lipases are all non-specific and allow for hydrolysis of the fatty acids on all positions on the triglycerides. Moreover, as opposed to other lipases, they are not inhibited by high fatty acids concentrations, allowing for near-complete hydrolysis (Macrae and Staines, 1994). Continuous lipase-based fat-splitting processes have been designed, in which the lipase was recovered by either in-line centrifugation (Bühler and Wandrey, 1987) or membranes (Hoq et al., 1985). Despite the cost reduction thus achieved, enzymatic hydrolysis is not applied in practice to a significant extent.

1.2.2 Hydrolysis using fatty acid-selective lipases

Reduction of the saturated fatty acid content in oil

Another application of a specific lipase in triglyceride hydrolysis is the production of a low-SAFA oil. For many decades, the lowering of blood cholesterol by changing dietary fat consumption has been a hot issue. Nowadays, it is recommended that the intake of total fat is limited, and especially that from saturated fats (American Dietetic Association, 1998); hence, there is considerable interest in low saturated fat products.

Based on the typical selectivity of lipases from *Fusarium oxysporum*, it should be possible to lower the level of saturated fatty acids in oil via selective hydrolysis. Both intracellular and extracellular lipases from this microorganism have been described which are reported to have a high affinity towards saturated fatty acids.

The extracellular lipase was shown to hydrolyze tristearin at a higher rate as compared to triolein, both as single substrates. However, when mixed triglycerides were tested, no significant selectivity for the saturated fatty acids was actually observed (Hoshino et al., 1992).

Using an intracellular lipase from *Fusarium oxysporum*, hydrolysis of cotton seed oil or groundnut oil yielded high levels of saturated fatty acids in the free fatty acid fraction (Joshi and Dhar, 1987). Recalculation of the experimental data suggests that this lipase even has an overall specificity factor in the range of 40[1].

Unfortunately, no more data are available on any further attempts to exploit this rare fatty acid selectivity.

Another option for producing a low-SAFA oil is a two-step process based on the high selectivity of Lipase B (Charton and Macrae, 1992) or Lipase I (Holmqvist et al., 1997) from *Geotrichum candidum*. This B-lipase has a very distinct preference for handling unsaturated free fatty acids with a *cis*-Δ-9 double bond, e.g., oleic or linoleic acid.

For example, taking sunflower oil [$\pm$ 88 % (w/w) unsaturated fatty acids] as substrate, $>$ 99 % unsaturated fatty acids were obtained upon hydrolysis in a 1 : 1 oil/water emulsion (Diks and Lee, 1999). From the experimental data it was derived that the so-called 'specificity factor' was approximately 30. It was shown that the lipase maintained its high selectivity up to 70 % degree of hydrolysis.

Of practical importance is the rather low stability of the lipase observed during hydrolysis in emulsion. In solution, the lipase appeared quite stable, even at 40 °C. Once added to the oil/water emulsion, the lipase lost most of its activity within hours at only 30 °C. This discrepancy was attributed to a reduced stability of the lipase at the oil/water interface (Diks and Lee, 1999).

Immobilization was shown not to effect the selectivity of the *Geotrichum* B-lipase (Charton and Macrae, 1993). Moreover, the catalyst was active in organic solvents.

[1] Specificity factor defined as $S = (S/U)_{FFA}/(S/U)$ (Diks and Lee, 1999), in which S is the fraction of saturated fatty acids and U the fraction of unsaturated fatty acids. The specificity factor reflects the relative net rate of hydrolysis of the saturated over the unsaturated fatty acid, corrected for their concentration difference in the starting oil.

As observed for other lipases (Balcao et al., 1996) its stability may have increased upon immobilization, though no data are available for this.

It should be noted that all wild-type *Geotrichum candidum* strains are known to produce at least two different lipases (isozymes) of which only the B-lipase is highly specific. The other lipase, generally referred to as Lipase-A (Charton and Macrae, 1992) or Lipase-II (Holmqvist et al.,1997) is totally nonspecific. Purification and isolation of the wild-type B-lipase therefore requires laborious and multi-step separations (Sidebottom et al., 1991; Diks and Lee, 1999). Alternatively, the Lipase-B can also be produced in *Pichia pastoris* (Catoni et al., 1997) by applying genetic modification techniques.

Separating the free fatty acids by distillation, they were re-esterified with glycerol (molar ratio 3 : 1). This reaction was catalyzed by immobilized *Rhizomucor miehei* lipase (Lipozyme) at 60 °C under continuous removal of water. Under these conditions the product consists of > 95 % (w/w) triglycerides (Ergan et al., 1990; McNeill et al., 1996; Diks and Lee, 1999). Although the *Rhizomucor* lipase is 1,3-regiospecific, the high reaction temperature promotes acyl migration of partial glycerides (Lortie et al., 1993; Dudal and Lortie, 1995). As a result 1(3)-monoglycerides and 1,3-diglycerides will be converted in their 2-position isomers, resulting in subsequent conversion into triglycerides. Moreover, acyl migration is promoted by the ionic carrier material (Duolite) applied (Millqvist Fureby et al., 1996).

Enrichment of DHA and EPA from fish oil

It is now recognized that polyunsaturated fatty acids (PUFA) play an essential role in human nutrition, as well as having important biomedical properties. Both the ω-3 and ω-6 families play important roles in human metabolism, but they cannot be interconverted in the body. Despite the fact that there is continuing debate among nutritionists on the ideal dietary ratio of ω-6 to ω-3 acids, it is generally accepted that the intake of ω-6 acids is adequate in developed countries. Consequently, nutritionists now recommend that the intake of ω-3 acids, particularly the long-chain PUFA (LCPUFA) docosahexaenoic acid (DHA, $C_{22:6}$) and eicosapentaenoic acid (EPA, $C_{20:5}$), is increased (Ashwell, 1992).

There are a number of sources of these acids, e.g., fish oils, marine algae and some micro-organisms. Of these, fish oils are the most commercially significant although the levels are usually only low to moderate. Oils derived from microbial fermentation can contain high levels but only at a relatively high cost. This has led to a great deal of interest in processes for the enrichment of DHA and EPA from commodity fish oils. Many physical methods have been developed for extracting and enriching these fatty acids, including crystallization, distillation, and the use of supercritical carbon dioxide.

The use of lipase biotechnology now offers an alternative method which has the advantage of mild processing conditions that minimize the degradation/oxidation of these acids (see also Chapters 8-10). The enrichment processes are based on the principle that, in general, lipases show low reactivity towards LCPUFA compared to more common fatty acids ($< C_{20}$). This can be exploited to enrich LCPUFA from commodity fish oils. Many lipases also show low reactivity towards γ-linolenic acid

(GLA) and this technology has been used to enrich this biologically important fatty acid from evening primrose or borage oil (Rahmatullah et al., 1994; Huang et al., 1997).

Two approaches have been widely studied; hydrolysis of fish oil triglycerides (or simple esters), and (trans)esterification of fish oil fatty acids. Hydrolysis of fish oil triglycerides leads to partial glycerides enriched in EPA and DHA. The lipase from *Candida rugosa* (formerly classified as *C. cylindracea*) has been shown to enrich EPA and DHA from sardine oil, cod liver oil (Hoshino et al., 1990) and tuna oil (Tanaka et al., 1992). Lipase from *Geotrichum candidum* has also been used (Shimada et al., 1994), although the overall yields were lower unless a second step involving removal of free fatty acids and a second hydrolysis was employed (see also Chapter 8). As a result of their inability to hydrolyze fatty acids present at the *sn*-2 position of a triglyceride, 1,3-regioselective lipases are less widely used, although *Rhizomucor miehei* lipase has been reported to enrich LCPUFA from anchovy oil (Uston et al., 1997). The overall concentration of LCPUFA in the glyceride fraction was raised from 27 % to 40 %. Selected data for the enrichment of LCPUFA are shown in Table 2.

Although lipases from a number of sources have been shown to be effective in these enrichment processes, the lipase from *Candida rugosa* is particularly interesting as it has been shown to have relatively lower activity against DHA as compared to EPA (Tanaka et al., 1992; McNeill et al., 1996). This observation has been exploited to generate LCPUFA fractions enriched in these fatty acids. Using Chilean fish oil the DHA:EPA ratio was raised to approximately 5 : 1 from 1 : 1 in the starting oil (McNeill et al., 1996). If required the products of the enrichment process can be re-esterified using immobilized *Rhizomucor miehei* lipase to generate triacylglycerols enriched in these fatty acids (Moore and McNeill, 1996). The reaction scheme is outlined in Figure 1.

Typically, a solution of *C. rugosa* lipase was added to fish oil at a ratio between 0.5 : 1 and 1 : 1. The enzymatic hydrolysis was allowed to proceed at 25 °C under a nitrogen blanket until the required level of free fatty acids was obtained. The reaction was stopped by heating the mixture to 90 °C. The aqueous phase was then allowed to separate and the oil phase removed and dried under vacuum. The free fatty acids were then separated from the glycerides by distillation.

Table 2. Enrichment of EPA and DHA from fish oils by lipase-catalyzed hydrolysis.

Oil	Lipase	Start, wt%		Enriched, wt%		Reference
		EPA	DHA	EPA	DHA	
Chilean fish	*C. rugosa*	16	12	10	36	McNeill et al., 1996
Tuna	*C. rugosa* (two-step)	8	30	7	58	Shimada et al., 1994
Tuna	*G. candidum* (two-step)	8	30	11	47	Shimada et al., 1994
Tuna	*C. rugosa*	6	25	4	53	Tanaka et al., 1992
Cod liver	*A. niger*	9	20	12	38	Hoshino et al., 1990
Refined sardine	*C. rugosa*	15	10	10	37	Hoshino et al., 1990

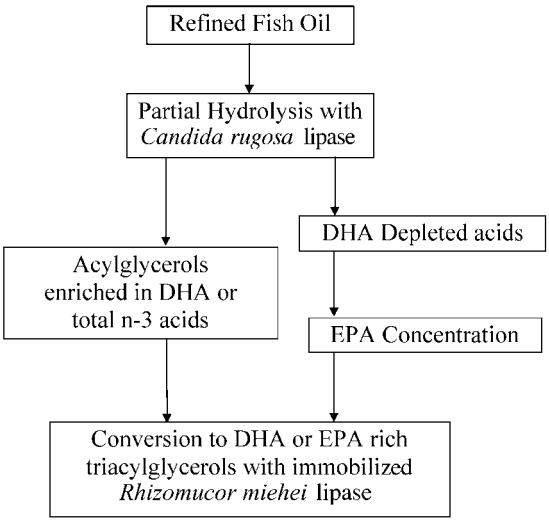

Figure 1. Surface active compounds from plant materials.

It is interesting to note that selective hydrolysis of fish oil triglycerides leads to unexpectedly high residual levels of triglyceride, even at degrees of conversion above 70 % (Tanaka et al., 1992; Moore and McNeill, 1996). It is believed that *Candida rugosa* lipase recognizes the whole triglyceride structure and discriminates more strongly against triglycerides containing increasing numbers of LCPUFA. This 'compositional specificity' has been studied using chemically synthesized triglycerides comprising only of oleic acid or DHA (Tanaka et al., 1993). The selectivity was less in the case of diglycerides, and monoglycerides were hydrolyzed relatively easily. This results in the triglyceride becoming the dominant species.

A wider range of lipases has been used for selective (trans)esterification using fish oil fatty acids as starting material. This overcomes the problems associated with 1,3-regioselective lipases (see above) as simple aliphatic alcohols can be used (e.g. methanol, ethanol, *n*-butanol). The desired LCPUFA is concentrated in the fatty acid fraction and the esters of other fatty acids are removed by distillation. Examples of lipases used include: *Rhizopus arrhizus*, *Rhizomucor miehei* (Mukherjee et al., 1993), *Rhizopus delemar* (Shimada et al., 1997), and *Pseudomonas* sp. (Breivik et al., 1997; Haraldsson et al., 1997).

Separation of CLA isomers

Conjugated linoleic acid (CLA) is used as a general term to describe a family of compounds derived from linoleic acid ($C_{18:2}$, n-6) containing conjugated double bonds. These double bonds can occur in either the *cis* or *trans* configuration. CLA occurs naturally in meat and dairy products obtained from ruminant animals where the predominant isomer (> 75 %) is *c*-9, *t*-11 CLA (Chin et al., 1992; Lin et al., 1995). Anti-cancer activity associated with beef has been attributed to the pre-

sence of this isomer (Pariza and Hargraves, 1985) and, subsequently, additional biological activities have been identified. These include prevention of body fat deposition during growth of some animal species, decreasing atherosclerosis and boosting immune function (Nicolosi et al., 1997; Park et al., 1997; Belury, 1995; Miller et al., 1994).

CLA in ruminant animals is believed to be generated by rumen bacteria during the digestive process followed by incorporation into milk and body fat. However, it can also be directly produced from linoleic acid or high-linoleate triglycerides such as safflower oil. Under carefully controlled alkaline conditions the product comprises of two main isomers; *c*-9, *t*-11 CLA and *t*-10, *c*-12 CLA, in approximately equal amounts (Ip et al., 1991). It is believed that not all CLA isomers have equivalent biological effects, and consequently there is now a great deal of interest in evaluating the individual isomers. As the properties of these isomers are similar, they are difficult to separate by traditional physical or chemical means. However, the use of lipases has proved effective in separating CLA isomers. Lipases obtained from *Geotrichum candidum* have been shown to be particularly useful (McNeill et al., 1999; Haas et al., 1999; Chen and Sih, 1998), especially the B isoform (Haas et al., 1999), although the use of lipase from *Aspergillus niger* has also been reported (Chen and Sih, 1998). *G. candidum* B lipase has a very unusual selectivity as it shows an extremely high preference for fatty acids containing a *cis*-Δ9 bond (see above). This enzyme has been used in both hydrolysis and esterification reactions to generate products containing > 90 % of the *c*-9, *t*-11 isomer (based on total CLA content) (see Table 3). The hydrolysis reactions have typically used CLA methyl esters as substrate, whereas the esterification reactions are based on the use of primary aliphatic alcohols (e.g., methanol, ethanol, octanol, dodecanol). *Geotrichum candidum* lipase has been used for the selective esterification of CLA with dodecanol on the multi-kilo pilot plant scale (McNeill et al., 1999).

The enrichment process is shown schematically in Figure 2, and yielded CLA fractions containing 92 % *c*-9, *t*-11 isomer and 81 % *t*-10, *c*-12 isomer based on total CLA content. If required the CLA fractions can be incorporated into triglycerides by interesterification using a non-selective lipase such as that obtained from *Rhizomucor miehei*.

Plant lipases with unusual selectivities

Compared with microbial or mammalian lipases, only limited information has been published on the fatty acid selectivity of plant lipases (Mukherjee and Hills, 1994). However, the data that are available suggest that some lipases derived from germi-

Table 3. Enrichment of *c*-9, *t*-11 CLA isomer by hydrolysis of mixed CLA methyl esters (adapted from Haas et al., 1999).

Hydrolysis time (h)	CLA 9,11 in ester fraction (%)		CLA 9,11 in free fatty acid fraction (%)	
	G. candidum (GC-4)	*G. candidum* B	*G. candidum* (GC-4)	*G. candidum* B
0	34	34	0	0
4	17	8	77	94

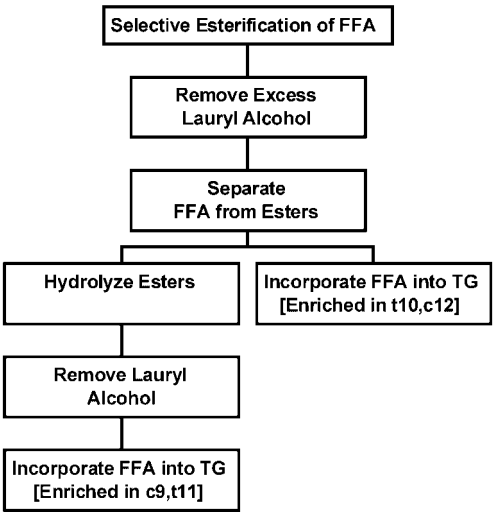

Figure 2. Surface active compounds *via* enzymatic acyl transfer.

nating seeds have unusual fatty acid selectivities with strong preference for the dominant fatty acid(s) present in the seed. Examples include; castor bean lipase with a preference for ricinoleic acid (Lin et al., 1986); oil palm lipase (Lin et al., 1986), elm lipase (Lin et al., 1986) and *Cuphea* lipases (Hellyer et al., 1999) for capric acid, and *Vernonia* lipase for vernolic acid (Ncube et al., 1995). The majority of other seed lipases studied are non-selective, i.e. they are able to act on a wide range of common fatty acids, even if the fatty acid is not normally present in the seed. Although the lipase obtained from oilseed rape (*Brassica napus*) is non-selective towards a wide range of common fatty acids it has been shown to discriminate against *cis*-4 and *cis*-6 unsaturated fatty acids (Hills et al., 1990a). Some of the selectivities reported are also dependent on the variety used; this is illustrated by *Cuphea* lipases. The lipase obtained from *Cuphea racemosa* was found to be non-selective, whereas those obtained from *Cuphea procumbens* and *Cuphea llavea* both showed approximately 20-fold preference for capric acid over other fatty acids (Hellyer et al., 1999).

One drawback to the application of plant lipases is that they are usually only present at very low levels in germinating seeds; castor bean lipase is one of the few present in dormant seed. Consequently there are few examples of their use as biocatalysts. However, de-fatted oat caryopses have been used for the hydrolysis of edible oils (Piazza, 1991) and oilseed rape lipase has been used for the enrichment of GLA from evening primrose oil (EPO) (Hills et al., 1990b). The esterification of EPO fatty acids with *n*-butanol was conducted at 30 °C with immobilized oilseed rape lipase and resulted in an approximately 7-fold enrichment of GLA in the residual fatty acid fraction (65 % from 10 % in starting oil).

1.3 Diglycerides

1.3.1 Introduction

Diglycerides are known for their retarding effect on fat crystallization, e.g., as anti-blooming agent in cocoa butter (Okiy, 1978; Wähnelt et al., 1991), and have large potential as building block for the chemical synthesis of phospholipids, glycolipids and drugs (El Kihel et al., 1996). However, their actual application is rather limited due to the lack of a selective low-cost production process. Conventional chemical production as described above lacks any positional selectivity; however, their use as pro-drug building block requires the diglycerides to be available as pure 1,2(2,3)- or 1,3-isomers.

Many processes for the preparation of concentrated partial glycerides have been developed in recent years. Using various different lipases they are all based on the three principal reactions catalyzed by lipases, i.e., hydrolysis, esterification or transesterification. Various researchers have been investigating partial glyceride production, focusing on product yield and purity enhancement. Mostly the reaction conditions such as temperature, solvents system, molar ratio of substrates have been varied. However, also lipase selectivity's have been exploited to optimize target product formation (Table 4).

1.3.2 1,2-Diglycerides

The most straightforward route for the production of 1,2(2,3)-diglycerides is the hydrolysis of triglycerides using a 1,3-regioselective lipase, such as porcine pancreatic lipase. Using this lipase in free powdered form, $>$ 90 % diglycerides (fraction of the total of partial glycerides) were produced at an overall conversion of 50 % (Plou et al., 1996). However, this process is based on kinetic resolution and hence the degree of conversion and incubation time are important parameters determining product composition and purity. At low degree of conversion, high levels of triglycerides still remain, whereas at high conversions significant amounts of monoglycerides will form.

The latter can be avoided using a specific lipase from *Penicillium* sp. strains. These fungi are known to produce several lipases, one of which displays a reduced hydrolytic activity on partial glycerides in comparison with triglycerides (*Penicil-* sp. UZLM-4 (Gulomova et al., 1996), *P. cyclopium* (Ibrik et al., 1998), *P. expansum* (Stöcklein et al., 1993) and *P. roquefortii* (Millqvist Fureby et al.,1997). Thus, in the hydrolysis of triolein by *Penicillium* UZLM-A lipase a 1 : 1 w/w mixture of mono- and diglycerides was obtained with 64 % degree of conversion (Gulomova et al., 1996). It was shown that the specific hydrolytic activity of the lipase on diolein and mono-olein was about a factor of 5 to 10 respectively lower as compared to its activity on triolein.

Further optimization of the reaction may perhaps enhance the selectivity of the lipase to discriminate between diglycerides and monoglycerides. Optimization of the water activity during glyceride synthesis was shown the enhance the selectivity of a

Table 4. Typical data for diglyceride production by lipase-catalyzed processes.

Process	Lipase	T (°C)	Yield[1] (%)	Major isomer	Product composition[2] (% w/w)				Reference
					FFA	MG	DG	TG	
Substrate: triglycerides									
Hydrolysis	Porcine pancreatic lipase	40	67	1,2(2,3)	18	3	30	49	Plou et al., 1996
	Penicillium sp.	–	42	–	5	31	28	36	Gulomova et al., 1996
	P. roquefortii (solvent)	25	75	1,2(2,3)					Millqvist Fureby et al., 1997
Ethanolysis	*P. roquefortii* (solvent)	25	75	1,2(2,3)					Millqvist Fureby et al., 1997
Directed glycerolysis	*Pseudomonas* sp.	60–48	87	1,3	1	3	87	9	Yamane et al., 1994
	Pseudomonas fluorescens	60–40	75–92	1,3	1	22	75	2	McNeill and Berger, 1993
Substrate: free fatty acids/ glycerol									
1,3-Selective esterification	*Rhizomucor miehei*	40	70–85	1,3	2–21	6–16	61–85	1–8	Rosu et al., 1999
	Rhizopus arrhizus	40	70		n.a.	21	79	< 1	Millqvist Fureby et al., 1996
	R. arrhizus (directed)	40	82		18	5	77	0	Weiss, 1990
	P. camembertii (directed)[3]	40	> 98		< 1	1	> 98	0	Weiss, 1990
Acylglycerol specific esterification	*Penicillium camembertii*[4]	40	60	n.d.	40	25	35	–	Yamaguchi and Mase, 1991

[1] Maximum yield on molar basis.
[2] Product composition on weight basis, recalculated from experimental data reported in literature.
[3] Molar ratio glycerol/fatty acid 3:1 to 4:1.
[4] 20% (w/w) water in glycerol.
n.a., not available; n.d., not detected.

partial-glyceride selective lipase from *Penicillium camembertii* (Yamaguchi and Mase, 1991) (see Section 1.4.3).

Though selective enzymes were used in the processes described above, product yields were rather low. Much better results were obtained using *P. roquefortii* lipase (Lipase R) for hydrolysis in an organic solvent. This lipase is 1,3-regiospecific and also displays a low activity towards diglycerides. Hydrolysis of trilaurin using this lipase was shown to yield nearly pure 1,2-diglycerides (Millqvist Fureby et al., 1997). Similar results were also obtained by alcoholysis instead of hydrolysis. Using ethanol at 25 °C, approximately 80 % diglycerides were obtained, 95 % of which were the 1,2(2,3)-isomer (Millqvist Fureby et al., 1997).

It should be noted that the relatively low reaction temperature applied above is essential with respect to the suppression of acyl migration. At temperatures > 40 °C, acyl migration of fatty acids in diglycerides (Kodali et al., 1990; Millqvist Fureby et al., 1996; Xu et al., 1998) as well as monoglycerides (Boswinkel et al., 1996) will result in significant amounts of the 1,3-isomers. Moreover, at thermodynamic equilibrium the 1,3-isomer will be the dominant diglyceride at a ratio of 1.3 : 1 to 2 : 1 over the 2-position isomer (Kodali et al., 1990; Millqvist Fureby et al., 1996).

Solvent interactions also appear to effect the rate of acyl migration. The lower the polarity, the more important acyl migration (Sjurnes and Anthonsen, 1994). Alcoholysis in ethers (diisopropylether, methyl-*tert*-butylether) thus appeared to give the highest product yield, whereas in alkanes (hexane, isooctane) low yields were obtained due to acyl migration and subsequent hydrolysis of the 1,3-position isomers (Millqvist Fureby et al., 1997).

Apart from the typical selectivities of the *P. roquefortii* lipase described above, this lipase also appears to discriminate between the two diglyceride isomers themselves. During ethanolysis a very low activity was found on the 1,2(2,3)-isomer as compared to the 1,3-diglyceride. Surprisingly this difference was lost during hydrolysis. This phenomenon was therefore attributed to a specific interaction of the ethanol with the active site of the lipase, inhibiting the binding of the 1,2(2,3) diglyceride (Millqvist Fureby et al., 1997).

1.3.3 1,3-Diglycerides

Several routes have been described for the production of 1,3-diglycerides. Starting from triglycerides as the substrate, high-yield production was reported by directed glycerolysis (Yamane et al., 1994). Starting with a molar ratio glycerol/hydrogenated beef tallow of 1 : 2, approximately 90 % diglycerides were obtained, 95 % being the 1,3-isomer. During this process temperature programming was applied. Thus, starting at 62 °C the temperature was stepwise decreased to reach an end temperature of 48 °C, thereby promoting preferential crystallization of the diglycerides (Yamane et al., 1994). Comparable results were obtained for the production of dipalmitin at 40 °C, starting with palm stearin at 60 °C (McNeill and Berger, 1993).

Although very successful, this principle can be applied only for diglycerides with high melting point, i.e., those consisting of long saturated fatty acids (Rosu et al.,1999). Using oils of lower melting point and hence higher levels of unsatura-

tion, the glycerolysis reaction continues to form predominantly monoglycerides (McNeill and Berger, 1993; McNeill et al., 1991).

With regard to the biocatalysts applied for directed glycerolysis, the highest yields were obtained using non-specific lipases from *Pseudomonas* sp. or *Chromobacterium viscosum* (Rosu et al., 1997, Bornscheuer et al., 1994; McNeill et al., 1991).

An alternative approach for 1,3-diglyceride synthesis is the esterification of free fatty acids and glycerol. Here the use of a 1,3-specific lipase, e.g., from *Rhizomucor miehei* (Rosu et al., 1999; Kim and Rhee, 1991) or *Rhizopus arrhizus* (Millqvist Fureby et al.,1996; Weiss, 1990) can effectively be exploited.

Experimental data show that diglyceride yields between 60 % and 85 % have been obtained using both fatty acids and ethyl esters as acyl donor. Higher yields (> 90 %) were even obtained applying continuous removal of water, e.g., under vacuum (Rosu et al., 1999), or again temperature programming (Weiss, 1990; Millqvist Fureby et al.,1996).

Similar to the production of 1,2-diglycerides described above, the low reaction temperature (15–40 °C) applied in these processes is important to control acyl migration. At higher temperatures, racemization of mono- and diglycerides will lead to acylation of the 2-position and subsequent production of triglycerides by esterification, especially when applying vacuum (Ergan et al., 1990; McNeill et al., 1996; Diks and Lee, 1999).

It should be noted that directed glycerolysis as well as directed esterification are preferably carried out using free lipases. Due to solidification of the reaction mixture the rate of mass transfer is severely limited, and depending on the actual enzyme input applied, this may result in reaction times of several days. Moreover, because of process economics recovery of the free enzyme in an active form is often required. This is generally rather difficult, but immobilization of the enzyme on a finely dispersed carrier can solve this issue. Good recovery of *Pseudomonas* sp. or *Chromobacterium viscosum* lipase on $CaCO_3$ was obtained by filtration of the biocatalyst, having dissolved the entire reaction mixture in acetone (Rosu et al., 1997).

The esterification of diglycerides to triglycerides, as mentioned above, can also be avoided by using a mono- and diacylglycerol specific lipase, e.g., from *Penicillium camembertii* (Yamaguchi and Mase, 1991), *Penicillium cyclopium* M1 (Okumura et al., 1980) or *Fusarium* sp. (Mase et al., 1995). These lipases strongly discriminate against triglycerides, and hence partial glycerides will accumulate during synthesis from fatty acids and glycerol.

Starting with a glycerol/oleic acid weight ratio of 4 : 1, approximately 60 % conversion was thus obtained after 5 days of incubation, yielding 60 % diglycerides and 40 % monoglycerides (Yamaguchi and Mase, 1991). This work also showed that selectivity of the lipase for either monoglyceride or diglyceride synthesis could be controlled by the water activity applied during synthesis. The higher the a_w, the higher the rate of diglyceride production, especially for a water activity > 0.05.

1.4 Monoglycerides

1.4.1 Introduction

Monoglycerides are widely applied as emulsifiers or surfactants in the food, cosmetics and pharmaceutical industries (Boyle, 1997). They have excellent emulsifying properties, have low odor and taste, are biodegradable, and are generally recognized as safe (GRAS).

Conventional production involves high-temperature glycerolysis (180–220 °C) using an inorganic catalyst. The result is a crude mixture of mono- and diglycerides (roughly equal amounts) and some unreacted triglycerides (overall conversion ± 90 %). Further separation is generally carried out by molecular distillation yielding high-purity monoglycerides (> 90 %).

Due to the high temperature applied during glycerolysis, decomposition and oxidation reactions take place which often result in a dark-colored, burnt-flavor product. Obviously this requires extensive purification during further downstream processing. Because of the ambient reaction temperature applied, biocatalysis can potentially yield a higher quality product with lower energy consumption in a more 'natural' type process. Various lipase-catalyzed routes are discussed below, and major data from these processes have been summarized in Table 5.

1.4.2 2-Monoglycerides

From the point of view of yield, synthesis of monoglycerides from glycerol and free fatty acids would clearly be the most attractive route. However, lipases with a high selectivity for the 2-position on the glycerol backbone in esterification do not exist, and hence triglycerides are the necessary starting material to produce 2-monoglycerides by lipase-catalyzed synthesis.

Direct hydrolysis using the 1,3-regiospecific porcine pancreatic lipase has been reported as a means to produce 2-monoglycerides (Plou et al., 1996) in a solvent-free system. It was shown that hydrolysis of triolein at 40 °C, using Celite-immobilized lipase, resulted in a product mixture with a maximum monoglyceride content of 68 % (w/w), predominantly being the 2-position isomer. It should be noted that the product composition is controlled by kinetic resolution, and hence enzyme input and incubation time are rather important.

It was shown that immobilization of the lipase significantly changed its selectivity towards monoglycerides. As discussed above, use of the free lipase predominantly gave diglycerides during the early stages of the reaction, whereas monoglycerides were the main product when using the immobilized enzyme.

A considerable monoglycerides yield was also reported for hydrolysis of palm oil in an AOT/isooctane microemulsion system (Holmberg and Osterberg, 1988). Catalyzed by *Rhizopus delemar* lipase at 35 °C, an 80 % degree of conversion was obtained (on a molar basis) towards monoglycerides. Due to the low reaction temperature and high water activity in the apolar solvent, acyl migration takes place only slowly; thus mainly the 2-position isomer will be obtained. Similar results were

Table 5. Typical data for lipase-catalyzed monoglyceride synthesis.

Process	Lipase	T (°C)	Yield[1] (%)	Major isomer	Product composition[2] (% w/w)				Reference
					FFA	MG	DG	TG	
Substrate: triglycerides									
Hydrolysis	Porcine pancreatic lipase[3]	40	67	2	60	26	4	8	Plou et al., 1996
	R. delemar (microemulsion)	35	80	2	55	31	13[4]	–	Holmberg and Osterberg, 1996
	R. arrhizus (solvent)	35	95	2					Millqvist et al., 1994
Alcoholysis	*R. arrhizus* (solvent)	35	60–81	2	70[5]	25	5	0	Millqvist Fureby et al., 1997 Soumanou et al., 1998
Directed glycerolysis	*Pseudomonas fluorescens*	60–40	75–92	1(3)	1	22	75	2	McNeill and Berger, 1993
	Pseudomonas cepacia	55–25	96	1(3)	n.a.	96	2	2	Bornscheuer et al., 1994
	Chromobacterium viscosum	25–10	90						Rosu et al., 1997
Substrate: fatty acids/glycerol									
Esterification	Patatin	50	86	n.d.	6	89	5	0	Macrae et al., 1998
	Penicillium camembertii	40	76	1(3)	20	72	8	0	Yamaguchi and Mase, 1991
	P. cyclopium (directed)[6]	40	90	1(3)	10	90	0	0	Weiss, 1990
	Rhizopus arrhizus (directed)	20	80	1(3)	8	80	12	0	Weiss, 1990

[1] Maximum yield on molar basis.
[2] Product composition on weight basis in the lipid fraction, as recalculated from experimental data reported in literature.
[3] Lipase immobilised on Celite.
[4] Sum of diglycerides and triglycerides.
[5] Sum of free fatty acids and ethylesters.
[6] 2–5% (w/w) water in glycerol.

reported for tripalmitin hydrolysis in ethers using *Rhizopus arrhizus* lipase at 35 °C, with over 95 % molar yield of 2-monopalmitin being obtained at a water activity > 0.5 (Millqvist et al., 1994).

Alcoholysis of triglycerides in polar solvents has been described as an alternative means to hydrolysis for the production of 2-monoglycerides. Better control over acyl migration is claimed for alcoholysis compared to hydrolysis.

Using ethanol either in pure form (Millqvist Fureby et al., 1997) or dissolved in ethers (Millqvist et al., 1994; Soumanou et al., 1998), high-purity 2-monoglycerides (97–99 %) were thus obtained. After purification by crystallization, the molar yield was between 60 % and 81 %. A high water activity during alcoholysis ($a_w > 0.75$) was found to be essential to suppress acyl migration and to maximize 2-monoglyceride production.

1.4.3 1(3)-Monoglycerides

Synthesis of 1(3)-monoglycerides by esterification of fatty acids with glycerol is rather simple using one of the many 1,3-regiospecific lipases. However, high-purity monoglyceride can be produced only when minimizing the subsequent esterification reaction towards diglycerides.

This may easily be achieved by using patatin, an acylhydrolase from potato tubers, which has an almost absolute specificity towards hydrolysis of monoglycerides (Macrae et al., 1998; Huang, 1987; Racusen, 1984).

Using the enzyme from the Santé variety of potato, it was shown that patatin displays the same specificity during synthesis (Macrae et al., 1998). In the presence of a small molar excess of glycerol, > 95 % (w/w) pure monoglycerides were produced, even using a crude protein extract. Although the free enzyme has an optimum temperature of approximately 50 °C, at higher temperatures the lipase deactivated rather quickly, making it difficult to esterify saturated fatty acids longer than myristic acid, which are solid at 50 °C.

Less selective for monoglycerides, but at least strongly discriminating against triglyceride formation are the lipases from several strains of *Penicillium*.

In particular, the lipase from *Penicillium camembertii* (Lipase G) gave appreciable results in fatty acid esterification. By carefully controlling water activity and substrate ratio, almost 90 % pure monoglycerides were obtained at 76 % degree of conversion (molar basis) (Yamaguchi and Mase, 1991). The conversion was even increased to 97 % (w/w) by removal of water via molecular sieves. However, under the conditions applied, a somewhat reduced monoglyceride purity (74 %) was obtained.

Control of the water activity appeared of major importance, as it determined the maximum conversion as well as the selectivity of the *P. camembertii* lipase. The higher the water activity, the lower the selectivity for monoglyceride formation and hence the higher the diglyceride content in the final product mixture. Despite the fact that the rate of reaction was significantly reduced below 5 % water in the glycerol, the highest conversion towards monoglycerides was obtained at a water content around 2 % (w/w) ($a_w \sim 0.1$).

It should be noted here that the *Penicillium* sp. lipase referred to above, is a different lipase from the 'triglyceride-specific' lipase described above for 1,2-diglyceride production. Several strains of *Penicillium* spp. are known to produce both types of lipases, i.e., one selective for, and the other selective against triglyceride hydrolysis and synthesis (Gulomova et al., 1996).

By combining the selectivity of the biocatalyst with adjustment of the reaction conditions, and especially the reaction temperature, much better monoglyceride yields were obtained. Using the partial glyceride-specific lipase from *Penicillium cyclopium* at 40 °C, up to 90 % (w/w) degree of conversion towards monoglycerides was achieved, even when using palmitic acid (Weiss, 1990). This result is quite remarkable, considering the fact that both the fatty acid substrate and the monoglyceride product are essentially solid at reaction temperature. Under these conditions, dissolution of the fatty acid becomes the rate-limiting step, and hence the process takes 10 – 15 days to complete. However, crystallization of the monoglycerides is promoted, resulting in very high yield of near-pure product. Clearly, the particle size and initial mixing are very important in starting the reaction and obtaining a high yield (Weiss, 1991). Patatin could presumably be used in a similar process.

The selectivity of the *P. cyclopium* lipase for mono- or diglyceride formation during directed esterification could be changed in a similar way as described earlier for the *P. camembertii* lipase. Increasing the water content in the glycerol from 5 % (w/w) up to 20 % (w/w), the product composition changed from monoglycerides to near-pure diglycerides (Weiss, 1990).

Temperature control, as applied above, also appeared crucial during the directed glycerolysis of triglycerides. Using the nonspecific lipases from *Pseudomonas fluorescens* sp. or *Chromobacterium viscosum* (Rosu et al., 1999; McNeill and Berger, 1993; McNeill et al., 1991), high-yield monoglyceride synthesis was obtained, simply by controlling the reaction temperature below a certain upper limit – referred to as the 'critical temperature' (McNeill et al., 1991). It was shown that this 'critical temperature' was dependent on the saturation level and the type of triglyceride substrate involved. Monoglyceride yields between 70 % (w/w) and 90 % (w/w) were reported, predominantly consisting of the 1-isomer (Bornscheuer et al., 1994; Rosu et al., 1997).

As discussed earlier, recovery of the lipase during directed glycerolysis is rather difficult. Applying solvent-based systems, this problem can easily be solved using other means of directing synthesis towards monoglyceride production. In these cases the reaction mixture is circulated over an enzyme reactor and a separator vessel, removing the monoglycerides from the product mixture by selective adsorption (Padt et al., 1990), crystallization (Berger and Schneider, 1993) or extraction (Stevenson et al., 1992). As the removal system is monoglyceride selective a conventional non-selective lipase can be applied.

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2 Fractionation of Fatty Acids and Other Lipids Using Lipases

Kumar D. Mukherjee

2.1 Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) from various organisms have been used successfully as biocatalysts in bioorganic synthesis (Chen and Sih, 1989; Klibanov 1990; Haraldsson, 1992; Kötting and Eibl, 1994; Jaeger and Reetz, 1998; Kazlauskas and Bornscheuer, 1998) and modification of fats and other lipids via hydrolysis, esterification and interesterification (Macrae, 1983; Mukherjee, 1990; Gandhi, 1997). Since most of the commercially available lipase preparations are rather expensive, lipase-catalyzed reactions are industrially attractive mainly for the manufacture of products of good commercial value (Vulfson, 1994). Processes for the preparation of purified fatty acids and lipids from inexpensive starting materials using lipase-catalyzed reactions should be commercially attractive.

This chapter covers the specificity of lipases in view of their application in the fractionation of fatty acids and their derivatives via kinetic resolution.

2.2 Fractionation of fatty acids and other lipids by lipase-catalyzed reactions

The ability of certain lipases to discriminate against or prefer particular fatty acids or acyl moieties of lipids has been utilized for the enrichment of such fatty acids or their esters from natural fats and oils via selective hydrolysis, esterification and interesterification, i.e. via kinetic resolution (Figure 1) (Mukherjee, 1995; 1996). Moreover, the regioselectivity of certain triacylglycerol lipases for the cleavage of fatty acids esterified at, for example *sn*-1,3-positions of triacylglycerols, has been utilized to enrich those fatty acids via kinetic resolution through regioselective hydrolysis, esterification or transesterification.

2.2.1 Lipase specificities

Fatty acid selectivity and positional specificity of triacylglycerol lipases are well documented in the literature (Mukherjee, 1990; 1998; Kazlauskas and Bornscheuer, 1998). Fatty acid selectivity and activity of several commercially important lipase preparations have been evaluated (Rangheard et al., 1989; Mukherjee

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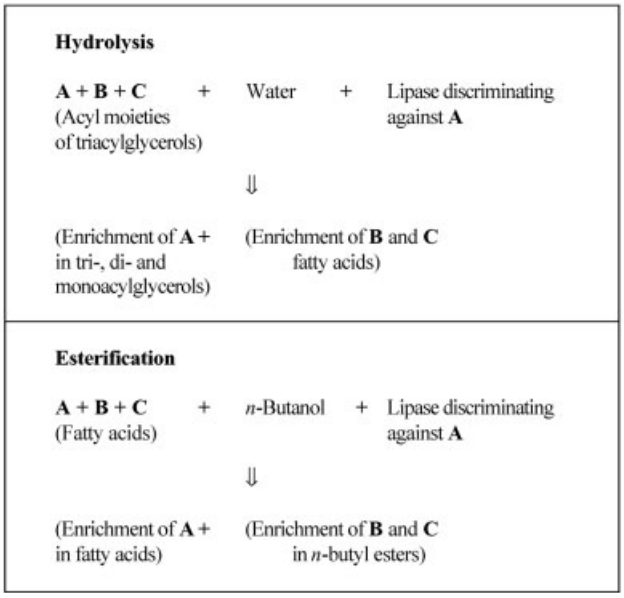

Figure 1. Scheme of kinetic resolution for the enrichment of fatty acids/acyl moieties via lipase-catalyzed hydrolysis of triacylglycerols or esterification of fatty acids with *n*-butanol.

et al., 1993) with a view to assess their potential as biocatalyst for the enrichment of definite fatty acids that are of considerable interest due to their biomedical (Innis, 1991) and technical properties (Vulfson, 1994; Gandhi, 1997).

Specificity of lipases towards individual fatty acids can be determined from the overall reaction rates in hydrolysis, esterification and interesterification. Using mixtures of triacylglycerols or fatty acids as substrates, preference for or discrimination against definite acyl moieties/fatty acids can be determined by comparing their relative rates of conversion.

Kinetic analysis under competitive conditions can be carried out according to Rangheard et al. (1989). For example, kinetic analysis of lipase-catalyzed esterification of fatty acids with *n*-butanol is carried out by reacting 250 mM of each of the fatty acids, individually, together with 250 mM of myristic or oleic acid, the reference standard, and 500 mM *n*-butanol in 1 mL hexane in the presence of 5 – 10 % (w/w) of substrate of the lipase preparation (Mukherjee et al., 1993). The reaction products consisting of butyl esters and unesterified fatty acids, are treated with diazomethane to convert the unreacted fatty acids to their methyl esters. Subsequently, the mixtures of butyl esters and methyl esters are analyzed by gas chromatography. Overall reaction rates are calculated from the composition of the reaction products as µmol butyl esters formed per g lipase per min.

Specificity constants are calculated according to Rangheard et al. (1989) as follows. For two substrates competing for the lipase, the ratio of the reaction rates of each substrate ($v1$ and $v2$) is derived from

$$v1/v2 = \alpha \cdot (\mathrm{Ac1X})/(\mathrm{Ac2X})$$

where (Ac1X) and (Ac2X) are concentrations of the two substrates at time X and α is the competitive factor which is defined by the following equation:

$$\alpha = (V\text{Ac1X}/K\text{Ac1X})/(V\text{Ac2X}/K\text{Ac2X})$$

where V is the maximal velocity and K is the Michaelis constant. The competitive factor is calculated from the substrate concentrations Ac1X0 and Ac2X0 at time zero as follows:

$$\alpha = \text{Log}[\text{Ac1X0}/\text{Ac1X}]/\text{Log}[\text{Ac2X0}/\text{Ac2X}].$$

Specificity constant is calculated from the competitive factor as 1/α with reference to the specificity constant of the reference standard, e.g., myristic or oleic acid, taken as 1.00. The higher the specificity constant for a particular fatty acid, the higher is the preference of the lipase for that particular fatty acid as substrate. Figure 2 shows the specificity constants for common and unusual fatty acids in the esterification with *n*-butanol, catalyzed by lipases from a mammalian tissue (porcine pancreas), plant (rapeseed, *Brassica napus*) and microorganisms (*Rhizomucor miehei*, *Rhizopus arrhizus* and *Candida cylindracea* = *C. rugosa*). Despite being of different origin, some common features of substrate specificities of these lipases are evident from Figure 2.

Table 1 summarizes the fatty acid selectivity of triacylglycerol lipases from various sources. Lipases from *C. rugosa, R. miehei, R. arrhizus* (Mukherjee et al., 1993; Jachmanián et al., 1996; Figure 2), *Penicillium cyclopium* and *Penicillium* sp. (Lipase G) (Mukherjee et al., 1993) and those from germinating rapeseed (Hills et al., 1990a; Jachmanián et al., 1996; Figure 2), papaya (*Carica papaya*) latex (Mukherjee and Kiewitt, 1996b) and bromelain as well as *Rhizopus* sp. (Mukherjee and Kiewitt, 1998) have closely resembling substrate specificities in esterification with *n*-butanol. All these lipases strongly discriminate against unsaturated fatty acids having the first double bond from the carboxyl end at an even number carbon, i.e. *cis*-4, e.g. all-*cis*-4,7,10,13,16,19-docosahexaenoic (n-3 22 : 6), *cis*-6, e.g. γ-linolenic (all-*cis*-6,9,12-octadecatrienoic, n-6 18 : 3), petroselinic (*cis*-6-octadecenoic, *cis*-6-18 : 1) and stearidonic (all-*cis*-6,9,12,15-octadecatetraenoic, n-3 18 : 4) or *cis*-8, e.g. dihomo-γ-linolenic (all-*cis*-8,11,14-eicosatrienoic, n-6 20 : 3) acid.

The selectivity of the lipase from porcine pancreas towards fatty acids having a *cis*-4, *cis*-6 and *cis*-8 double bond is, however, not as pronounced as the corresponding selectivities of the above lipases (Mukherjee et al., 1993; Jachmanián et al., 1996; Figure 2).

The lipase from *Chromobacterium viscosum* exhibits different substrate specificity compared to the above lipase preparations. This lipase utilizes fatty acids with a *cis*-6 double bond, e.g., *cis*-6-18 : 1, n-6 18 : 3 and n-3 18 : 4, as substrates equally well or even better than myristic acid or α-linolenic acid (n-3 18 : 3) (Mukherjee et al., 1993).

In interesterification reactions, lipase from *R. miehei* discriminates against *cis*-6–18 : 1 (Osterberg et al., 1989), n-6 18 : 3 (Rangheard et al., 1989; Osterberg et al., 1989) and n-3 22 : 6 (Langholz et al., 1989). Lipases from *C. rugosa*, porcine pancreas and *Geotrichum candidum* also discriminate against n-6 18 : 3 in interesteri-

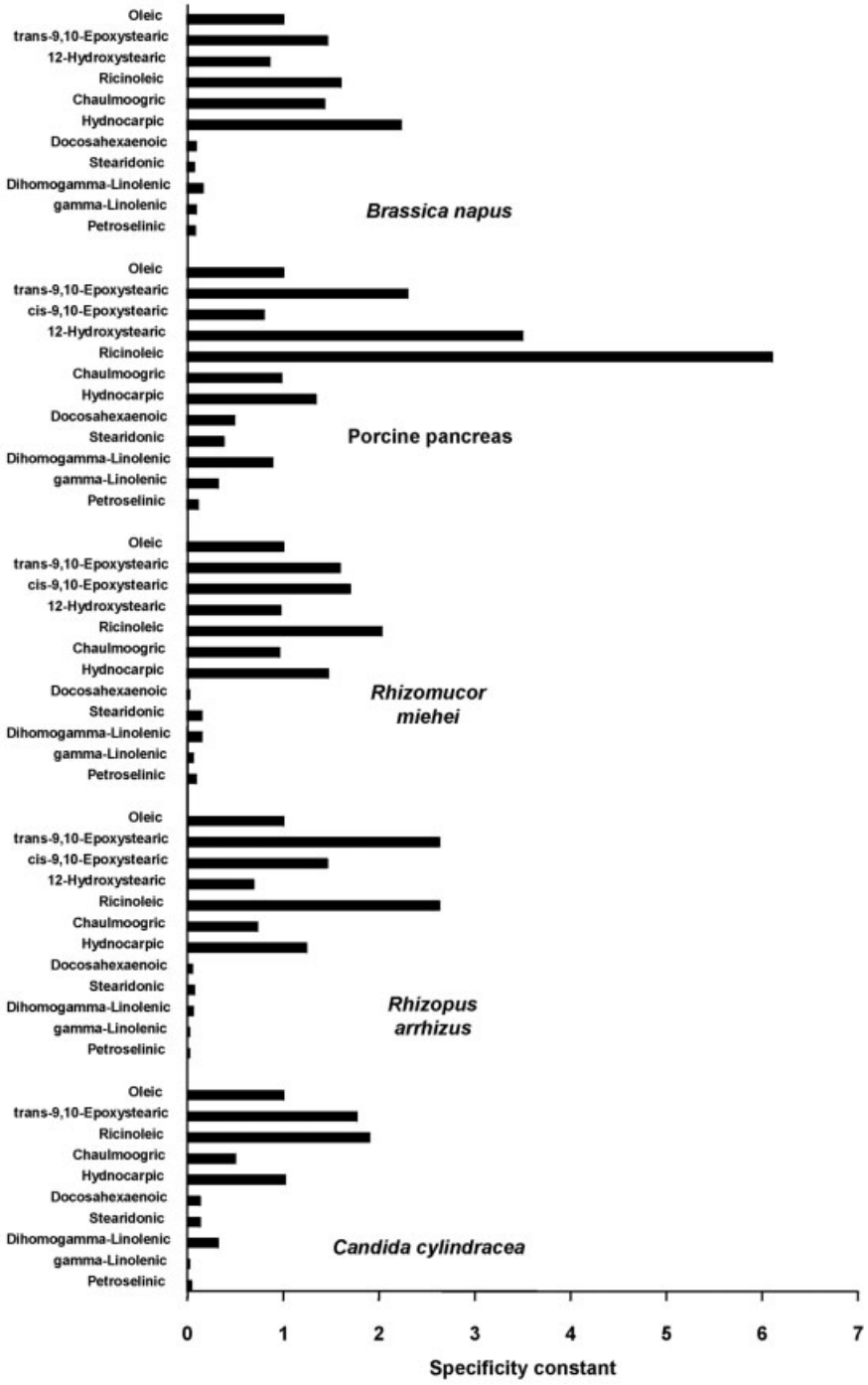

Figure 2. Specificity constants of lipases from various sources in the esterification of fatty acids with *n*-butanol under competitive conditions. (From Jachmanián et al., 1996).

Table 1. Specificity of lipases in view of kinetic resolution of fatty acids and other lipids.

Lipase from	Reaction	Specificity	Reference
Porcine pancreas	Hydrolysis of whale oil triacylglycerols	Discrimination against n-3 22 : 6 as compared to C_{16} and C_{18} acyl moieties	Bottino et al., 1967
Porcine pancreas	Hydrolysis of seed oil triacylglycerols	Discrimination against acyl moieties having a *trans*-3 olefinic bond	Kleiman et al., 1970
G. candidum	Hydrolysis of triacylglycerols containing isomeric *cis*-octadecenoyl moieties	Strong preference for *cis*-9 – 18 : 1	Jensen et al., 1972
Porcine pancreas	Hydrolysis of triacylglycerols containing isomeric *cis*-octadecenoyl moieties	Discrimination against *cis*-2-18 : 1 to *cis*-7-18 : 1	Heimermann et al., 1973
G. candidum	Hydrolysis of triacylglycerols	Distinct preference for C_{18} acyl moieties having *cis*-9- or *cis*-9, *cis*-12-bonds	Jensen, 1974
G. candidum	Hydrolysis of triacylglycerols	Distinct preference for C_{18} acyl moieties having *cis*-9- or *cis*-9, *cis*-12-bonds and discrimination against *cis*-13-22 : 1	Baillargeon and Sonnet, 1991
G. candidum (lipase B)	Hydrolysis of fatty acid methyl esters	Highly selective for *cis*-9 – 18 : 1 as compared to *cis*-11-18 : 1 or *trans*-9-18 : 1	Sidebottom et al., 1991
Oat (*Avena sativa*) seed lipase	Hydrolysis of triacylglycerols	Discrimination against tripetroselinin as compared to triolein, trilinolein and tri-α-linolenin	Piazza et al., 1992
G. candidum	Esterification of fatty acids with *n*-butanol	Distinct preference for C_{18} acyl moieties having *cis*-9- or *cis*-9, *cis*-12-bonds and discrimination against *cis*-13-22 : 1, *trans*-9-18 : 1, n-6 18 : 3, ricinoleic and 9-docosynoic acids	Sonnet et al., 1993b
G. candidum (lipase B)	Hydrolysis of triacylglycerols and esterification of fatty acids with *n*-octanol	Highly selective for triolein and for *cis*-9-18 : 1 as compared to *cis*-6-18 : 1, *cis*-11 – 18 : 1 or *trans*-9-18 : 1	Charton and Macrae, 1993
Pythium ultimum	Hydrolysis of triacylglycerols	Preference for tri-α-linolenin > trilinolein > triolein	Mozaffar and Weete, 1993
Oat caryopses	Hydrolysis of milk fat triacylglycerols	Preferential hydrolysis of C_6-C_{10} acids	Parmar and Hammond, 1994

Table 1. continued

Lipase from	Reaction	Specificity	Reference
R. miehei	Alcoholysis of fatty acid ethyl esters with *n*-propanol	Preference for *cis*-11-20 : 1 and *cis*-13-22 : 1, strong discrimination against n-3 22 : 6, and moderate discrimination against n-3 20 : 5	Bech Pedersen and Holmer, 1995
Candida parapsilosis	Hydrolysis of fatty acid methyl esters	Substrate preference: *cis*-9–16 : 1 ≈ *cis*-9-18 : 1 >; n-6 18 : 3 > *cis*-11-18 : 1 > *cis*-6-18 : 1	Briand et al., 1995
C. rugosa	Hydrolysis of borage oil	Discrimination against n-6 18 : 3	Ergan, 1996
R. miehei	Hydrolysis of fatty acid ethyl esters	Discrimination against n-3 22 : 6 as compared to n-6 18 : 2 and n-3 18 : 3	Kosugi et al., 1997
Flavobacterium odoratum	Hydrolysis of *p*-nitrophenyl esters of fatty acids	Substrate preference: n-6 18 : 2 > *cis*-9-18 : 1 ≈ n-6 18 : 3 > *cis*-6-18 : 1 > *trans*-9-18 : 1	Labuschagne et al., 1997
R. delemar	Interesterification of randomized triacylglycerols with caprylic acid	Strong discrimination against n-6 18 : 3 and n-3 22 : 6; moderate discrimination against n-6 20 : 4 and n-3 20 : 5	Shimada et al., 1997b
C. rugosa, *Pseudomonas cepacia*, porcine pancreas	Esterification of acetylenic fatty acids with *n*-butanol	Preference for 10-undecynoic acid and strong discrimination against 6-octadecynoic acid	Lie Ken Jie and Xun, 1998a
C. rugosa, *R. miehei*, porcine pancreas	Esterification of medium- and long-chain olefinic and acetylenic alcohols with pentanoic acid and stearic acid	Discrimination against medium- and long-chain olefinic alcohols in the esterification with pentanoic acid; olefinic and acetylenic alcohols well accepted in the esterification with stearic acid catalyzed by lipase from porcine pancreas	Lie Ken Jie and Xun, 1998b
Burkholderia sp.	Hydrolysis of *t*-butyl esters	Strong preference for *t*-butyl octanoate as compared to *t*-butyl palmitate and stearate	Yeo et al., 1998
C. rugosa, *R. miehei*, *C. antarctica* lipase A	Esterification of *cis*- and *trans*-octadecenoic acids with *n*-butanol	Preference for oleic acid over elaidic acid by *C. cylindracea* and *R. miehei*, whereas strong preference for elaidic acid by *C. antarctica* lipase A	Borgdorf and Warwel, 1999

fication reactions (Rangheard et al., 1989). In hydrolytic reactions catalyzed by lipases from *R. arrhizus* and porcine pancreas discrimination against *cis*-6-18 : 1 (Mbayhoudel and Comeau, 1989) and n-3 22 : 6 (Yang et al., 1990) moieties, respectively, has been observed. Similarly, lipase from rape hydrolyzes tripetroselinin and tri-γ-linolenin at much lower rate than triolein (Hills et al., 1990a).

It appears from the above findings that discrimination against fatty acids having the first double bond from the carboxyl end as a *cis*-4, *cis*-6 or a *cis*-8 is a common feature of many lipases. It has been suggested that the lipase from rape discriminates against *anti*-oriented *cis*-4 and *cis*-6 unsaturated fatty acids due to the direction of twist of the carbon chain after the first double bond which might hinder binding of the reactive group to the lipase (Hills et al., 1990a). It is conceivable that the same argument is valid for the selectivity of many lipases against fatty acids having a *cis*-4, *cis*-6 or a *cis*-8 double bond as the first olefinic bond at the carboxyl end of the fatty acid.

In the esterification of common and unusual fatty acids with *n*-butanol, lipases from rape, porcine pancreas, *C. rugosa, R. miehei* and *R. arrhizus* (Jachmanián et al., 1996), papaya (Mukherjee and Kiewitt, 1996b) and pineapple as well as *Rhizopus* sp. (Mukherjee and Kiewitt, 1998) have been shown to have strong preference for fatty acids having hydroxy groups, e.g., ricinoleic (12-hydroxy-*cis*-9-octadecenoic) and 12-hydroxystearic acid, epoxy groups, e.g. *trans*-9,10-epoxystearic acid, and cyclopentenyl fatty acids having saturated alkyl chains, e.g. hydnocarpic [11-(cyclopent-2-en-1-yl)undecanoic] and chaulmoogric [13-(cyclopent-2-en-1-yl)tridecanoic] acid, whereas a cyclopentenyl fatty acid having a *cis*-6 olefinic bond, i.e., gorlic [13-(cyclopent-2-en-1-yl)tridec-6-enoic] acid is strongly discriminated against by several lipases (Jachmanián et al., 1996; Jachmanián and Mukherjee, 1996).

A partially purified lipase from *Vernonia galamensis* seeds has been shown to catalyze the hydrolysis of trivernolin (tri-*cis*-12,13-epoxy-*cis*-9-octadecenoin), the predominant constituent of the seed oil of *V. galamensis*, much faster than triolein or other triacylglycerols (Ncube et al., 1995). Similarly, in the transesterification of tricaprylin with fatty acids, catalyzed by purified *V. galamensis* lipase, a strong preference for vernolic (*cis*-12,13-epoxy-*cis*-9-octadecenoic) acid has been observed (Ncube et al., 1995).

The above substrate specificities of various lipases have been utilized for the enrichment of specific unsaturated fatty acids or derivatives via kinetic resolution from their mixtures, obtained from naturally occurring fats and other lipids.

2.2.2 Enrichment of n-6 polyunsaturated fatty acids

The applications of lipase-catalyzed kinetic resolution for the enrichment of n-6 polyunsaturated fatty acids are summarized in Table 2.

γ-Linolenic acid is of considerable commercial interest due to its beneficial biomedical properties (Horrobin, 1992). Seed oils of evening primrose, *Oenothera biennis* (Hudson, 1984; Mukherjee and Kiewitt, 1987), borage, *Borago officinalis* (Whipkey et al., 1988) and *Ribes* spp. (Traitler et al., 1984) are some common

Table 2. Enrichment of n-6 unsaturated fatty acids and their derivatives via kinetic resolution catalyzed by lipases.

Lipase from	Reaction	Fatty acid enriched	Reference
Rapeseed (*Brassica napus*), *R. miehei*, *G. candidum*, *C. rugosa*	Selective esterification of fatty acids of evening primrose oil or borage oil with *n*-butanol	γ-Linolenic acid in unesterified fatty acids	Hills et al., 1989; 1990b; Syed Rahmatullah et al., 1994a; Foglia and Sonnet, 1995; Jachmanián and Mukherjee, 1996; Huang et al., 1997b; Schmitt-Rozieres et al., 1999; Van Heerden and Litthauer, 1999
Rapeseed, *R. miehei*, *C. rugosa*	Selective hydrolysis of evening primrose oil or borage oil	γ-Linolenoyl moieties in tri-, di- and monoacylglycerols	Hills et al., 1989; Ergan et al., 1992; Syed Rahmatullah et al., 1994b; Jachmanián and Mukherjee, 1995; Huang et al., 1997a; 1999; Shimada et al., 1998d
R. miehei	Selective esterification of fatty acids of fungal oil from *Mortierella* sp. with *n*-butanol	γ-Linolenic acid in unesterified fatty acids	Mukherjee and Kiewitt, 1991; Carvalho and Pastore, 1998
C. rugosa	Selective hydrolysis of triacylglycerols of fungal oil from *Mortierella* sp.	Arachidonoyl moieties in tri-, di- and monoacylglycerols	Shimada et al., 1995a
R. delemar	Selective esterification of fatty acids of borage oil with lauryl alcohol	γ-Linolenic acid in unesterified fatty acids	Shimada et al., 1997c; 1998a
C. rugosa	Selective esterification of fatty acids of fungal oil from *Mortierella* sp. with lauryl alcohol	Arachidonic acid in unesterified fatty acids	Shimada et al., 1998b

sources of γ-linolenic acid. Fungal oils containing γ-linolenic acid, such as those from *Mortierella* spp. (Hansson and Dostálek, 1988) and *Mucor ambiguus* (Fukuda and Morikawa, 1987) are now commercially available. γ-Linolenic acid has been enriched from natural resources via urea adduct formation (Traitler et al., 1988), separation on Y-Zeolite (Arai et al., 1987), and solvent winterization (Yokochi et al., 1990).

The ability of the lipases from rapeseed, *R. miehei, C. rugosa*, and *G. candidum* to discriminate against γ-linolenic acid has been utilized for the enrichment of this acid from the mixture of fatty acids derived from evening primrose seed oil or borage seed oil via lipase-catalyzed selective esterification with *n*-butanol (Figure 3; Table 2). Most of the fatty acids from evening primrose oil or borage oil are converted to butyl esters, with the exception of γ-linolenic acid, which is thus obtained as a concentrate in the unesterified fatty acid fraction (Hills et al., 1989; 1990a; Syed Rahmatullah et al., 1994a). Selective hydrolysis of the triacylglycerols of evening primrose oil or borage oil, catalyzed by lipases from rapeseed (Hills et al., 1989), *R. miehei* and *C. rugosa* (Syed Rahmatullah et al., 1994b) also leads to enrichment

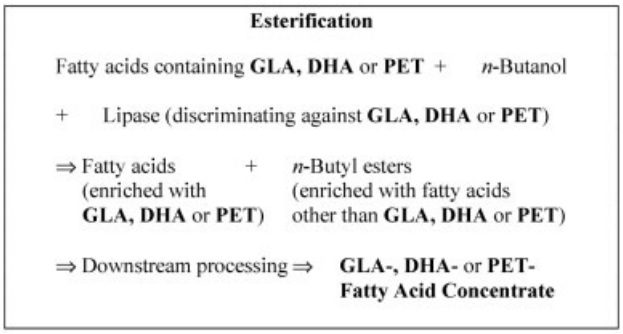

Figure 3. Scheme of kinetic resolution for the enrichment of γ-linolenic (GLA), n-3 docosahexaenoic (DHA) or petroselinic (PET) acid via lipase-catalyzed selective esterification of fatty acid mixtures with *n*-butanol.

of γ-linolenoyl moieties in the unhydrolyzed acylglycerols, i.e., tri-, di- and monoacylglycerols, whereas the other acyl moieties are cleaved to fatty acids (Figure 4; Table 2).

Fatty acid concentrates containing about 75 % γ-linolenic acid have been prepared via selective esterification of fatty acids of evening primrose oil and borage oil with *n*-butanol, catalyzed by lipase from *R. miehei* (Syed Rahmatullah et al., 1994a).

Partial hydrolysis of evening primrose oil using lipase from *C. rugosa* leads to enrichment of γ-linolenic acid from about 10 % in the starting material to about 47 % in the unhydrolyzed acylglycerols (Syed Rahmatullah et al., 1994b). Similarly, hydrolysis of borage oil using lipase from *C. cylindracea* results in enrichment of γ-linolenic acid from about 20 % in the starting material to about 48 % in the unhydrolyzed acylglycerols (Syed Rahmatullah et al., 1994b).

The selectivity of the lipases from *R. miehei* and *C. rugosa* towards γ-linolenic acid has been utilized to concentrate this fatty acid from fatty acids of fungal oil via

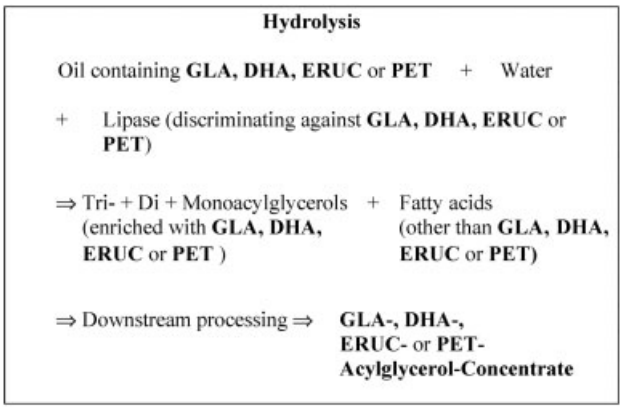

Figure 4. Scheme of kinetic resolution for the enrichment of γ-linolenic (GLA), n-3 docosahexaenoic (DHA), erucic (ERUC) or petroselinic (PET) acid via lipase-catalyzed selective hydrolysis of triacylglycerols.

selective esterification with *n*-butanol (Mukherjee and Kiewitt, 1991). Using the lipase from *C. rugosa*, after a 1–h reaction period as much as 92 % of the fungal oil fatty acids are esterified, and concomitantly the level of γ-linolenic acid in the unesterified fatty acids is raised from 10 % to about 47 %, whereas virtually none of the γ-linolenic acid is converted to butyl esters (Mukherjee and Kiewitt, 1991). With the lipase from *R. miehei*, 91 % esterification of the fungal oil fatty acids occurs after 4 h of reaction; this results in an increase in the level of γ-linolenic acid in the unesterified fatty acids to 69 % (yield of γ-linolenic acid 59 %) (Mukherjee and Kiewitt, 1991). With both lipases the enrichment of γ-linolenic acid is paralleled by a decrease in the levels of palmitic, oleic and linoleic acid in the fatty acid fraction, and some increase in the proportion of these acids in the butyl esters.

It has also been shown using several lipases that selective esterification of fungal oil fatty acids with *n*-butanol, rather than selective hydrolysis of fungal oil triacylglycerols, should be the method of choice for enrichment of γ-linolenic acid (Mukherjee and Kiewitt, 1991).

2.2.3 Enrichment of n-3 polyunsaturated fatty acids

n-3 Polyunsaturated fatty acids are extensively used in nutraceutical preparations due to their interesting biomedical properties (Innis, 1991).

The ability of the lipase from rape to discriminate against n-3 docosahexaenoic acid has been utilized for the enrichment of this acid from a mixture of fatty acids derived from cod liver oil via lipase-catalyzed selective esterification with *n*-butanol (see Figure 3) (Hills et al., 1990b). Moreover, the ability of the lipase from *R. miehei* to discriminate against n-3 22 : 6 has been utilized for the enrichment of this fatty acid from fish oil fatty acids via selective esterification with methanol (Langholz et al., 1989). For example, esterification of fish oil fatty acids with methanol, catalyzed by *R. miehei* lipase, raises the level of n-3 22 : 6 from about 8 % in the initial fatty acid mixture to about 48 % in the unesterified fatty acids (Langholz et al., 1989). Shortly thereafter, selective hydrolysis of fish oil triacylglycerols, catalyzed by lipases from *Aspergillus niger* and *C. rugosa*, has been employed to concentrate n-3 polyunsaturated fatty acids, i.e. n-3 20 : 5 and n-3 22 : 6, that are enriched in the tri- + di- + monoacylglycerol fraction (see Figure 4) (Hoshino et al., 1990) (see also chapters 8-10).

Numerous publications have appeared since the above studies were published (Table 3) on the selective hydrolysis of marine oil triacylglycerols for the enrichment of n-3 polyunsaturated fatty acids in the unhydrolyzed acylglycerols (see Figure 4) and selective esterification of marine oil fatty acids for the enrichment of the n-3 fatty acids in the unesterified fatty acid fraction (see Figure 3).

Thus, two successive hydrolyses of tuna oil triacylglycerols, catalyzed by lipase from *G. candidum*, result in enrichment of n-3 fatty acids, i.e., n-3 20 : 5 and n-3 22 : 6, from about 32 % in the untreated oil to about 49 % in the fraction consisting of tri-, di- and monoacylglycerols (Shimada et al., 1994). Similarly, in a commercial process, fish oil is partially hydrolyzed by *C. rugosa* lipase to yield an acylglycerol fraction enriched in n-3 20 : 5, and especially in n-3 22 : 6; the acylglycerol fraction is subsequently

Table 3. Enrichment of n-3 unsaturated fatty acids and their derivatives via kinetic resolution catalyzed by lipases.

Lipase from	Reaction	Fatty acid enriched	Reference
R. miehei	Selective esterification of fatty acids of marine oil with methanol	n-3 Docosahexaenoic acid in unesterified fatty acids	Langholz et al., 1989
Rapeseed, *R. miehei*	Selective esterification of fatty acids of fish oil with *n*-butanol	n-3 Docosahexaenoic acid in unesterified fatty acids	Hills et al., 1990b; Jachmanián and Mukherjee, 1996
C. rugosa	Selective hydrolysis of fish oil triacylglycerols	n-3 Docosahexaenoyl moieties in tri-, di- and monoacylglycerols	Hoshino et al., 1990; Tanaka et al., 1992; 1993; Moore and McNeill, 1996; McNeill et al., 1996; Wanasundara and Shahidi, 1998; Rice et al., 1999
Rhizopus niveus	Selective hydrolysis of fish oil triacylglycerols	n-3 Docosahexaenoyl moieties in monoacylglycerols	Yadwad et al., 1991
R. miehei	Interesterification of cod liver oil with a fatty acid mixture or its ethyl esters enriched in n-3 20 : 5 and n-3 22 : 6	n-3 Eicosapentaenoyl and n-3 docosahexaenoyl moieties in triacylglycerols	Haraldsson and Almarsson, 1991
R. miehei	Interesterification of cod liver oil with a fatty acid mixture enriched in n-3 20 : 5 and n-3 22 : 6	n-3 Docosahexaenoyl moieties in acylglycerols	Yamane et al., 1992; 1993
R. miehei	Interesterification of vegetable oils with n-3 20 : 5 and n-3 22 : 6 fatty acids or their methyl or ethyl esters	n-3 Eicosapentaenoyl and n-3 docosahexaenoyl moieties in triacylglycerols	Sridhar and Lakshminarayana, 1992; Zu-yi and Ward, 1993; Huang and Akoh, 1994; Huang et al., 1994
Pseudomonas sp.	Interesterification of sardine oil with a fatty acid mixture enriched in n-3 20 : 5 and n-3 22 : 6	n-3 Eicosapentaenoyl and n-3 docosahexaenoyl moieties in acylglycerols	Adachi et al., 1993
Pseudomonas sp.	Interesterification of fish oil with alcohols	n-3 Eicosapentaenoyl and n-3 docosahexaenoyl moieties in monoacylglycerols	Zu-yi and Ward, 1993a; 1993b
C. antarctica	Esterification of glycerol or alkylglycerols with n-3 20 : 5 and n-3 22 : 6 fatty acids	n-3 Eicosapentaenoyl and n-3 docosahexaenoyl moieties in triacylglycerols and alkylacylglycerols, respectively	Haraldsson et al., 1993; Haraldsson and Thorarensen, 1994

Table 3. continued

Lipase from	Reaction	Fatty acid enriched	Reference
C. viscosum	Esterification of acylglycerols, enriched in n-3 22 : 6, with a fatty acid fraction enriched in n-3 22 : 6	n-3 Docosahexaenoyl moieties in triacyl-glycerols	Tanaka et al., 1994
Pseudomonas sp.	Selective hydrolysis of fish oil triacylglycerols	n-3 Docosahexaenoyl moieties in tri-, di- and monoacylglycerols	Maehr et al., 1994
G. candidum	Selective hydrolysis of fish oil triacylglycerols	n-3 Docosahexaenoyl moieties in tri-, di- and monoacylglycerols	Shimada et al., 1994; 1995b
C. antarctica	Selective interesterifica-tion of cod liver oil with ethanol in supercritical carbon dioxide	n-3 Docosahexaenoyl moieties in monoacyl-glycerols	Gunnlaugsdottir and Sivik, 1995
Rapeseed	Selective hydrolysis of fish oil triacylglycerols	n-3 Docosahexaenoyl moieties in tri-, di- and monoacylglycerols	Jachmanián and Mukherjee, 1995
R. delemar	Selective esterification of fatty acids of fish oil with lauryl alcohol	n-3 Docosahexaenoic acid in unesterified fatty acids	Shimada et al., 1997e
R. delemar	Selective interesterifi-cation of fatty acid ethyl esters of fish oil with lauryl alcohol	n-3 Docosahexaenoyl moieties in fatty acid ethyl esters	Shimada et al., 1997d
Pseudomonas sp.	Interesterification of fish oil triacylglycerols with ethanol	n-3 Eicosapentaenoyl and n-3 docosahexaenoyl moieties in acylglycerols	Breivik et al., 1997; Haraldsson et al., 1997
R. miehei	Selective hydrolysis of phospholipids	n-3 Docosahexaenoyl moieties in unhydrolyzed phospholipids	Ono et al., 1997
R. miehei	Interesterification of ethyl esters of tuna oil fatty acids with lauryl alcohol	n-3 Docosahexaenoyl moieties in fatty acid ethyl esters	Shimada et al., 1998c
R. miehei	Interesterification of fish oil triacylglycerols with ethanol	n-3 Docosahexaenoyl moieties in acylglycerols	Haraldsson and Kristinsson, 1998

isolated by evaporation and converted to triacylglycerols via hydrolysis and reesterification, both catalyzed by *R. miehei* lipase (Moore and McNeill, 1996).

Using *R. delemar* lipase, selective esterification with lauryl alcohol of fatty acids from tuna oil has yielded an unesterified fatty acid fraction containing 73 % n-3 22 : 6 as compared to 23 % in the starting material (Shimada et al., 1997e). Using the same lipase, selective esterification of tuna oil fatty acids with lauryl alcohol, extraction of the unreacted fatty acids and their repeated esterification with lauryl alcohol has resulted in an unesterified fatty acid fraction containing as much as 91 % n-3 22 : 6 (Shimada et al., 1997a).

Interesterification (acidolysis) of marine oil triacylglycerols with marine oil fatty acid fractions, that have been enriched with n-3 polyunsaturated fatty acids, has been carried out using lipase from *Pseudomonas* sp. to raise the level of total n-3 polyunsaturated fatty acids in the triacylglycerols to 65 % (Adachi et al., 1993).

Moreover, lipase-catalyzed selective alcoholysis of marine oil triacylglycerols or alkyl esters of marine oil fatty acids with short- or long-chain alcohols has resulted in the enrichment of n-3 polyunsaturated fatty acids in the acylglycerol fraction or alkyl ester fraction, respectively. Thus, selective alcoholysis of ethyl esters of tuna oil fatty acids with ethanol using *R. delemar* lipase and *R. miehei* lipase as biocatalyst leads to enrichment of n-3 22 : 6 in the ethyl ester fraction from 23 mol% to about 50 mol% (Shimada et al., 1997d) and from 60 mol% to 93 mol% (Shimada et al., 1998c). However, with *R. miehei* lipase as biocatalyst selective interesterification of tuna oil triacylglycerols with ethanol yields an acylglycerol fraction containing 49 % n-3 22 : 6, whereas selective esterification of tuna oil fatty acids with ethanol yields an unesterified fatty acid fraction containing 74 % n-3 22 : 6 (Haraldsson and Kristinsson, 1998).

2.2.4 Enrichment of very long-chain monounsaturated fatty acids (VLCMFA)

Very long-chain monounsaturated fatty acids (VLCMFA), such as gondoic (*cis*-11-eicosenoic, 20 : 1) acid, erucic (*cis*-13-docosenoic, 22 : 1) acid (Mikolajczak et al., 1961; Knowles et al., 1981), nervonic (*cis*-15-tetracosenoic, 24 : 1) acid (Mukherjee and Kiewitt, 1986) and *cis*-5-eicosenoic acid (Joliff, 1981) are abundant constituents of seed oils, especially from Cruciferae. Erucic acid and its derivatives are extensively used for the manufacture of a wide variety of oleochemical and technical products (Sonntag, 1991), and other VLCMFA might also find novel applications in hitherto less explored areas.

Erucic acid is currently isolated from high-erucic oils by fat splitting followed by fractional distillation, both of which are rather energy-consuming processes. Lipase-catalyzed selective hydrolysis of high-erucic triacylglycerols has been proposed as alternative process for the isolation of erucic acid (Baillargeon and Sonnet, 1991; Ergan et al., 1992; Kaimal et al., 1993). Hydrolysis of high-erucic oils, catalyzed by lipases from *G. candidum* (Baillargeon and Sonnet, 1991) and *C. rugosa* (Ergan et al., 1992; Kaimal et al., 1993) leads to enrichment of erucic acid in the tri-, di- and monoacylglycerols (see Figure 4). Thus, the level of erucic acid is raised from 43 %

in the high-erucic rapeseed oil to 66 % in the tri-, di- and monoacylglycerols (Baillargeon and Sonnet, 1991). In the esterification of fatty acids of high-erucic rapeseed oil with *n*-butanol, catalyzed by lipase from *G. candidum*, strong discrimination against erucic acid has been reported, which leads to enrichment of erucic acid from 47 % in the fatty acid mixture to 83 % in the unesterified fatty acids (Sonnet et al., 1993a). Partial hydrolysis of high-erucic rapeseed oil, catalyzed by *C. rugosa* lipase yields dierucin in 73 % purity which has been used for lipase-catalyzed synthesis of trierucin (Trani et al., 1993).

Selective hydrolysis of triacylglycerols of meadowfoam (*Limnanthes alba*) oil containing over 60 % *cis*-5-eicosenoic acid (*cis*-5-20 : 1) results in enrichment of *cis*-5-20 : 1 in tri- + di- + monoacylglycerols when lipase from *Chromobacterium viscosum* is used as biocatalyst (Hayes and Kleiman, 1993). Selective esterification of meadowfoam oil fatty acids with *n*-butanol, catalyzed by *C. viscosum* lipase, leads to enrichment of *cis*-5-20 : 1 in the unesterified fatty acids in excellent yield (> 95 %) and purity (> 99 %) (Hayes and Kleiman, 1993).

Partial hydrolysis and transesterification of high-erucic oils catalyzed by several lipases have been studied with the objective to develop strategies for the enrichment of VLCMFA utilizing the selectivity of these enzymes (Mukherjee and Kiewitt, 1996a). Lipase-catalyzed selective hydrolysis of high-erucic oils from white mustard (*Sinapis alba*) and oriental mustard (*Brassica juncea*), has revealed that the lipases studied can be broadly classified into three groups according to their substrate selectivity.

The first group includes lipases from *C. cylindracea* and *G. candidum* that selectively cleave the C_{18} acyl moieties from the triacylglycerols; this results in enrichment of these fatty acids in the fatty acid fraction. Concomitantly, the level of erucic acid and the other VLCMFA is raised in the mono- + di- + triacylglycerol fraction from 51 % in the starting oil to about 80 % and 72 %, respectively, when lipases from *C. rugosa* and *G. candidum* are used as biocatalyst (Mukherjee and Kiewitt, 1996a).

The second group includes lipases from porcine pancreas, *C. viscosum*, *R. arrhizus* and *R. miehei*, with their known regioselectivity towards acyl moieties at the *sn*-1,3 positions of triacylglycerols (Mukherjee, 1990). With these lipases the VLCMFA, esterified almost exclusively at the *sn*-1,3 positions of the triacylglycerols of the high-erucic oils, are selectively cleaved and the resulting VLCMFA enriched in the fatty acid fraction to levels as high as 65–75 % (Figure 5); simultaneously the level of the C18 fatty acids is increased in the acylglycerol fraction (Mukherjee and Kiewitt, 1996a).

The third group includes lipases from *Penicillium* sp. (Lipase G) and *Candida antarctica* (Lipase B), which do not seem to exhibit any pronounced specificity towards either C_{18} fatty acids or VLCMFA (Mukherjee and Kiewitt, 1996a).

The lipases with strong fatty acid selectivity or regioselectivity have been tested for the selective hydrolysis of the triacylglycerols of honesty (*Lunaria annua*) seed oil which is rich in VLCMFA, including nervonic acid. The *C. rugosa* lipase cleaves preferentially the C_{18} fatty acids, which are enriched from 36 % in the starting oil to 79 % in the fatty acids, while the VLCMFA are enriched in the di- and triacylglycerols. In particular, the diacylglycerols were almost exclusively (> 99 %) composed of VLCMFA, whereas barely traces of monoacylglycerols were formed (Mukherjee and Kiewitt, 1996a).

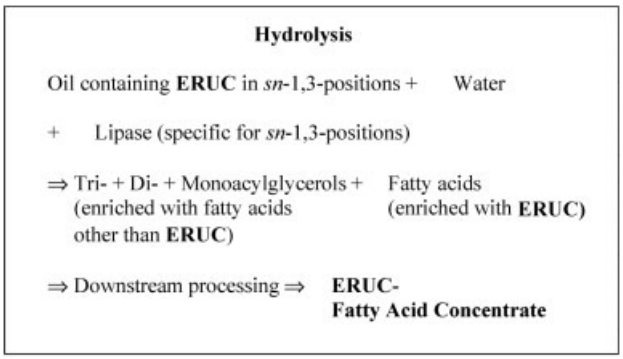

Figure 5. Scheme of kinetic resolution for the enrichment of erucic (ERUC) acid via lipase-catalyzed regioselective hydrolysis of triacylglycerols.

The lipases from porcine pancreas and *R. miehei* selectively cleave the VLCMFA, esterified almost exclusively at the *sn*-1,3-positions of the triacylglycerols (Mukherjee and Kiewitt, 1986), and consequently, the VLCMFA are extensively enriched in the fatty acid fraction, whereas the C_{18}-fatty acids, esterified predominantly at the *sn*-2 position, are enriched in the mono- and diacylglycerols (Figure 5) (Mukherjee and Kiewitt, 1996a).

The *sn*-1,3-regioselectivity of the *R. miehei* lipase has also been utilized for the enrichment of VLCMFA from high-erucic oils via selective transesterification of the triacylglycerols with alkyl acetates or selective alcoholysis of the triacylglycerols with *n*-butanol.

Transesterification of triacylglycerols of high-erucic mustard oil with ethyl, propyl and butyl acetate, catalyzed by the lipase from *R. miehei*, yields alkyl esters (ethyl, propyl and butyl, respectively) of fatty acids and a mixture of acetylacylglycerols (e.g., monoacetyldiacyl- + diacetylmonoacylglycerols) as well as acylglycerols (tri- + di + monoacylglycerols) (Mukherjee and Kiewitt, 1996a). Regioselective transesterification of the triacylglycerols at the *sn*-1,3 positions leads to enrichment of the VLCMFA in the alkyl ester fractions and decrease in the level of these fatty acids in acetylacylglycerols and acylglycerols; concomitantly, the level of the C18 fatty acids is increased in the acetylacylglycerols and acylglycerols, and decreased in the alkyl ester fractions (Mukherjee and Kiewitt, 1996a).

Similarly, alcoholysis of high-erucic mustard oil with *n*-butanol, catalyzed by *R. miehei* lipase, yields butyl esters and mixtures of acylglycerols (mono- + di- + triacylglycerols) and minor proportions of fatty acids (Mukherjee and Kiewitt, 1996a). The VLCMFA, esterified at the *sn*-1,3 positions of the triacylglycerols, are selectively transesterified, resulting in enrichment of these fatty acids in the butyl ester fraction and decrease of their level in the acylglycerols (Figure 6). Simultaneously, the C_{18} fatty acids are enriched in the acylglycerols.

Transesterification of triacylglycerols with alkyl acetates or *n*-butanol is nearly as effective for the enrichment of VLCMFA in the alkyl ester fraction as selective hydrolysis of triacylglycerols for the enrichment of VLCMFA in the fatty acids or acylglycerols (Mukherjee and Kiewitt, 1996a). The ultimate choice of a process for the enrichment of VLCMFA depends on selectivity, cost and reusability of the

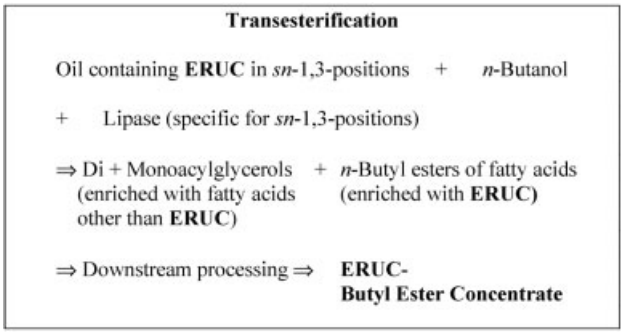

Figure 6. Scheme of kinetic resolution for the enrichment of erucic (ERUC) acid via lipase-catalyzed regioselective transesterification (alcoholysis) of triacylglycerols with *n*-butanol.

lipase preparation, as well as efficiency of downstream processing for the separation of the desired products from the reaction mixture, i.e., fatty acids from acylglycerols in the case of hydrolysis and alkyl (ethyl, propyl or butyl) esters from acylglycerols in the case of transesterification with alkyl esters or *n*-butanol. Acylglycerols and acetylacylglycerols could be useful byproducts of such processes.

Selective hydrolysis of high-erucic rapeseed oil, catalyzed by lipases from *G. candidum* results in the enrichment of erucic acid in diacylglycerols to an extent of 85 % (McNeill and Sonnet, 1995). When hydrolysis, catalyzed by *C. rugosa* lipase, is carried out below 20 °C, the reaction mixture solidifies and the diacylglycerols formed contain as much as 95 % erucic acid (McNeill and Sonnet, 1995).

2.2.5 Enrichment of other fatty acids

Lipase-catalyzed reactions have been employed for the enrichment of several common and unusual fatty acids via kinetic resolution. For example, oleic acid has been incorporated into a few selected plant oils by interesterification of the triacylglycerols with methyl oleate, catalyzed by *R. miehei* lipase (Sridhar et al., 1991). By this process oleoyl moieties replace the saturated acyl moieties and linoleoyl moieties of the triacylglycerols, yielding an oil with improved stability and nutritional properties.

Interesterification (glycerolysis) of animal fats such as beef tallow and lard with glycerol, catalyzed by *Pseudomonas fluorescens* lipase, results in monoacylglycerols enriched with saturated acyl moieties in high (45–70 %) yields, when the reaction is carried out at or below a 'critical' temperature of 40 °C (McNeill et al., 1992). Similarly, lipase-catalyzed glycerolysis of low-erucic rapeseed oil or soybean oil at 5 °C, results in monoacylglycerols enriched with palmitoyl and stearoyl moieties (McNeill et al., 1992). Short-chain fatty acids with desirable flavor have been produced by selective hydrolysis of butter fat fraction, catalyzed by *Penicillium roqueforti* lipase (Lencki et al., 1998).

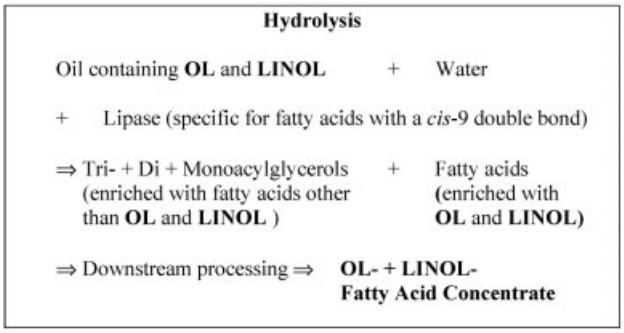

Figure 7. Scheme of kinetic resolution for the enrichment of oleic (OL) and linoleic (LINOL) acids via selective hydrolysis of triacylglycerols, catalyzed by a lipase specific for fatty acids/acyl moieties having a *cis*-9 olefinic bond.

A process aimed at the production of very low saturate oil is based on selective hydrolysis, catalyzed by *G. candidum* lipase B, of sunflower oil, containing 12 % saturated fatty acids, to yield a fatty acid fraction containing > 99 % unsaturated fatty acids (Figure 7) (Diks and Lee, 1999). The unsaturated fatty acids are recovered by evaporation and esterified to glycerol using *R. miehei* lipase with constant removal of the water formed by sparging with nitrogen under vacuum to produce unsaturated triacylglycerols containing < 1 % saturated fatty acids in a yield of > 95 %.

Selective hydrolysis of fennel (*Foeniculum vulgare*) oil using a lipase from *R. arrhizus* leads to enrichment of petroselinic acid in the acylglycerols (Mbayhoudel and Comeau, 1989). Separation of the fatty acids using an ion-exchange resin followed by hydrolysis of the resulting acylglycerols by the lipase from *C. cylindracea* yields a fatty acid concentrate containing 96 % petroselinic acid.

Selective esterification of fatty acids of coriander (*Coriandrum sativum*) oil with *n*-butanol, catalyzed by rape lipase leads to enrichment of petroselinic acid from about 80 % in the starting material to > 95 % in unesterified fatty acids (Jachmanián and Mukherjee, 1996). Using the same biocatalyst, selective esterification with *n*-butanol of fatty acids of *Hydocarpus wightiana* oil containing about 10 % gorlic acid yields an unesterified fatty acid fraction containing almost 50 % gorlic acid (Jachmanián and Mukherjee, 1996).

Hydroxy acids, e.g., lesquerolic (14-hydroxy-*cis*-11-eicosenoic) and auricolic (14-hydroxy-*cis*-11-*cis*-17-eicosadienoic) acid, have been selectively cleaved from lesquerella oil triacylglycerols by hydrolysis catalyzed by lipases from *R. arrhizus* (Hayes and Kleiman, 1992a,b) or *R. miehei* (Hayes and Kleiman, 1992b) yielding fatty acid concentrates containing 85 % hydroxy acids.

Two polyunsaturated fatty acids with unusual structure, having a *cis*-5-olefinic bond, e.g., all-*cis*-5,11,14-octadecatrienoic and all-*cis*-5,11,14,17-octadecatetraenoic acid, have been enriched from fatty acids of *Biota orientalis* seed oil via selective esterification with *n*-butanol, catalyzed by lipase from *C. rugosa*; thereby the content of total *cis*-5-polyunsaturated fatty acids is raised from 15 % in the starting material to 73 % in the unesterified fatty acids (Lie Ken Jie and Syed Rahmatullah, 1995). Similarly, selective hydrolysis of the *Biota orientalis* seed oil, cata-

lyzed by *C. rugosa* lipase, leads to enrichment of total *cis*-5-polyunsaturated fatty acids in the acylglycerols to 41 % (Lie Ken Jie and Syed Rahmatullah, 1995). It appears from these results and those reported by Hayes and Kleiman (1992a,b) that fatty acids or acyl moieties having a *cis*-5 double bond are also discriminated against by some lipases.

Butter oil has been enriched with conjugated linoleic acids to an extent of 15 % by interesterification of the triacylglycerols with conjugated linoleic acids, catalyzed by lipase from *C. antarctica* (Garcia et al., 1998).

2.3 Perspectives

A wide variety of applications of lipase-catalyzed reactions for the enrichment of particular fatty acids or lipids via kinetic resolution utilizing the fatty acid specificity and regioselectivity of lipases has become known in recent years. Fatty acid concentrates containing well over 70 % to 80 % of one particular fatty acid can be easily prepared in the laboratory and pilot plant scale by low energy-consuming processes. Further enrichment of such fatty acid concentrates can be carried out by repeated kinetic resolutions. Several commercial applications of such processes, e.g., production of n-3 fatty acid concentrates from marine oils, have become known. Further commercial applications will probably be governed by the cost and reusability of lipases, economy of downstream processing, and the market value of the product. One potential market for fatty acid concentrates, prepared enzymatically, is the area of nutraceuticals and cosmetics. In particular, the fatty acid concentrates can be used for the preparation of structured lipids using lipase-catalyzed esterification and interesterification.

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3 Lipid Modification in Water-in-Oil Microemulsions

Douglas G. Hayes

3.1 Introduction

Water-in-oil microemulsions (w/o-MEs), also known as reverse micelles, provide what appears to be a very unique and well-suited reaction medium for hosting lipid modification reactions involving lipases and other enzymes. The medium consists of small aqueous/polar nanodroplets dispersed in an apolar bulk phase by surfactants (Figure 1). Moreover, the droplets are on the same order of magnitude as the encapsulated enzyme molecules. Typically, the medium is quite dynamic, with droplets spontaneously coalescing, exchanging materials, and reforming on the order of microseconds. Such small droplets yield a large amount of interfacial area. Several reviews have been written which provide more detailed discussion of the physical properties of microemulsions (Eicke, 1987; Luise et al., 1988; Sjöblom et al., 1996).

Microemulsions have been employed in a variety of applications, such as tertiary oil recovery, hosting inorganic and organic syntheses, including the degradation of nerve gas, and the formation of ultra-fine metallic and ceramic superconductive materials (Holmberg, 1994; Pillai et al., 1995; Sjöblom et al., 1966). In addition, a large body of literature exists on microemulsion-based enzymatic reactions, and the use of the medium to purify biomolecules, refold denatured proteins, and serve as drug delivery vehicles (Bru et al., 1995; Luisi et al., 1988; Oldfield, 1994; Nicot and Waks, 1995; Sjöblom et al., 1996).

Microemulsions are well-suited for hosting lipase-catalyzed reactions, especially those involving ester synthesis or transesterification. For example, w/o-MEs solubilize lipase, water, and both polar and nonpolar substrates, yielding an optically transparent medium. In addition, its low water content promotes ester synthesis. Furthermore, its large amount of interfacial area is advantageous since many lipases are activated upon adsorption at an interface. The adsorption promotes the opening of an α-helix lid that covers the enzymatic active site (Lawson et al., 1994). In addition, the difference in interfacial partitioning of substrates and products with the microemulsion interface can be used to drive a reaction in the forward direction, and to control the product distribution.

These inherent advantages has motivated several groups to investigate w/o-MEs as host for lipases, yielding a large body of literature. In general, research in this field has not resulted in the degree of success that was anticipated. There are many disadvantages associated with this medium, including the difficulty in recovering products and enzyme from reactions in batch mode (due to the presence of surfactant) and its adaptation into continuous reactor schemes. However, important knowledge has been gained from this research, relating to nonaqueous biocatalysis and the importance of the interaction of substrates, product, and enzyme with the host system.

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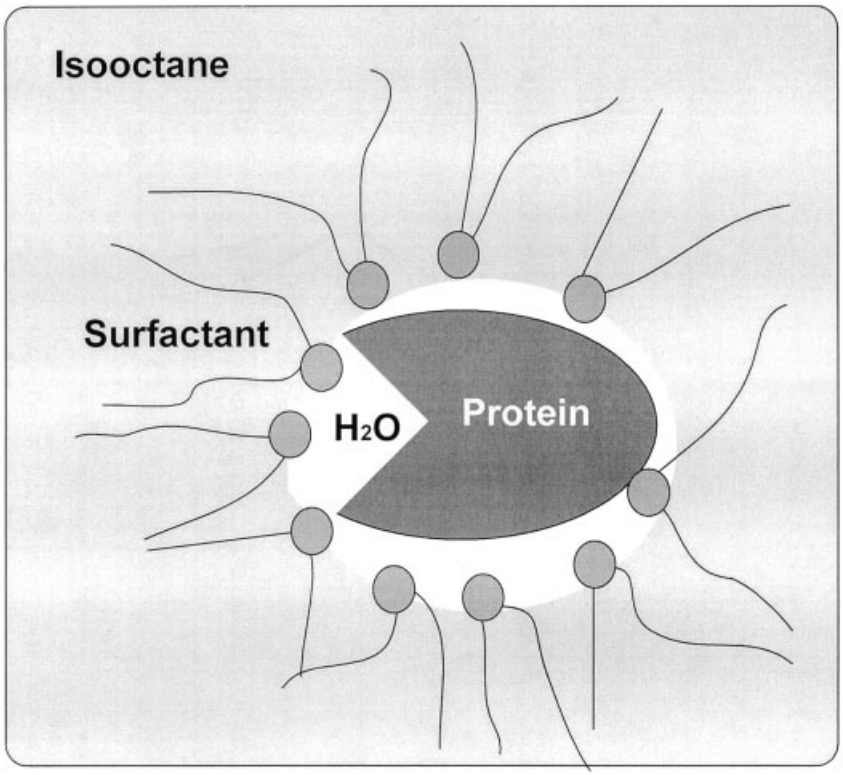

Figure 1. Illustration of an enzyme-containing water-in-oil microemulsion.

This will be the main focus of the chapter. In addition, recent efforts on the development of continuous bioreactors for microemulsion-encapsulated lipases, including the use of ultrafiltration membranes and the formation of microemulsion gels, will be reviewed here. Furthermore, a short review of lipid modification by other microemulsion-encapsulated enzymes will be provided.

3.2 Lipases encapsulated in water-in-oil microemulsions

Three early reviews have been written which describe lipases in microemulsions (Walde, 1989; Ballesteros et al., 1995; Sjöblom et al., 1996). Here, we will expand and update these efforts.

3.2.1 Applications

A wide variety of reactions have been investigated in w/o-MEs (Table 1). Microemulsions have hosted both hydrolysis and esterification reactions, demonstrating their versatility. Many of the applications focus on the ability of w/o-MEs to cosolubilize polyhydric alcohols (e.g., glucosides, glycerol, cholesterol, and glycols) fatty acyl substrates, lipase, and water in an optically transparent medium, with sufficient interfacial area between nanophases. Most alternate reaction systems for polyol ester syntheses involve biphasic/heterogeneous media. With care, lipases can exhibit quite high turnover rates and remain reasonably stable, as will be discussed in Sections 3.2.3 and 3.2.5. In addition, the enantioselectivity reported for w/o-ME-encapsulated lipases is quite high, and can surpass that occurring in alternate nonaqueous lipase media (Hedström et al., 1993). A unique application of w/o-MEs is for hosting the formation of lactones from long-chain ω-hydroxy fatty acids (Rees et al., 1995a). Chemical means to form lactones from these molecules is quite slow. The w/o-ME interface attracts both the carbonyl and hydroxyl termini of the substrate, bringing both in close contact to the active site of lipase. Although w/o-ME-encapsulated lipases have formed structured triglycerides via acidolysis or esterification, yields have been less than desired due to a significant extent of hydrolysis, which also occurred (Holmberg and Österberg, 1987; Hayes and Gulari, 1992). Structured lipids consist of triglycerides containing a long-chain acyl group in the 2-glycerol position and medium chain groups in the outer positions. Applications include infant formulae and nutritional agents (Soumanou et al., 1998).

The optical clarity and high reaction rates make w/o-MEs well-suited for assaying lipase activity in situ. For instance, *in situ* lipase assays based on FTIR (Walde and

Table 1. Examples of lipase-catalyzed reactions performed in microemulsions

Reaction type	Reference
Hydrolysis of triglycerides	Han and Rhee, 1985b
Esterification of glycerol	Fletcher et al., 1998 Hayes and Gulari, 1991
Esterification of ethylene glycol	Hayes and Gulari, 1992
Esterification of diols	Yang and Gulari, 1994 Stamatis et al., 1996
Esterification of glucoside	Skagerlind et al., 1997
Structured triglyceride synthesis	Hayes and Gulari, 1992
Lactonization of ω-hydroxy-hexadecanoic acid	Rees et al., 1995a
Glycerolysis of triglycerides	Chang et al., 1991
Acidolysis and Triglycerides	Holmberg and Österberg, 1987
Interesterification between triolein and tributyrin	Bello et al., 1987
Cholesterol oleate ester synthesis and hydrolysis	Hedström et al., 1992
Acidolysis of phosphatidylcholine	Holmberg and Eriksson, 1992
Enantioselective esterification of 2-octanol	Rees and Robinson, 1995
Enantioselective esterification of ibuprofen	Hedström et al., 1993
Enantiomeric esterification of menthol	Stamatis et al., 1993b
Transesterification of butter fat	Kermasha et al., 1995
Lipase hydrolysis coupled with other enzymatic reactions	Hochkoeppler and Palmieri, 1990 Yang and Russell, 1994

Luisi, 1989; 1990; O'Connor and Cleverly, 1994), NIR (Hanna et al., 1996), and FT-^{1}H-NMR (Rees and Robinson, 1995) have been developed. Furthermore, Walde has developed an assay to measure the formation of free fatty acids (FFA) during lipolysis by encapsulating the fluorescent dye phenol red in the w/o-MEs (Walde, 1990). The dye's absorbance spectrum undergoes a 100 nm blue shift upon binding with FFA. Alternatively, the release of FFA was determined *in situ* through a lipase-lipoxygenase coupled reaction scheme, yielding hydroperoxides, which were detected spectrophotometrically (250 nm) (Hochkoeppler and Palmieri, 1990). Microemulsion-encapsulated lipases have been used to screen for lipase type in their activity toward unusual free fatty acids (Österberg et al., 1989; Derksen and Cuperus, 1992; Hayes and Kleiman, 1993a; 1993b).

3.2.2 Substrate partitioning controls the kinetics

There is sufficient circumstantial evidence that indicates that lipases catalyze reactions at the interface. In other words, the partitioning of substrates and products between the interface, the bulk solvent, and the dispersed aqueous phase plays a strong role in controlling the observed kinetics. For instance, w/o-ME-encapsulated lipase hydrolyzed the wax ester 1-butyl laurate to a small extent, but esterified 1-butanol and lauric acid strongly in the forward direction (Figure 2) (Hayes, 1991). These results reflect the greater degree of interfacial acticity of lauric acid and 1-butanol relative to butyl laurate, demonstrated by dynamic light scattering data results (w/o-ME size and attractive interaction parameters) (Hayes, 1991). Note the agreement of equilibrium concentrations from hydrolysis and esterification reac-

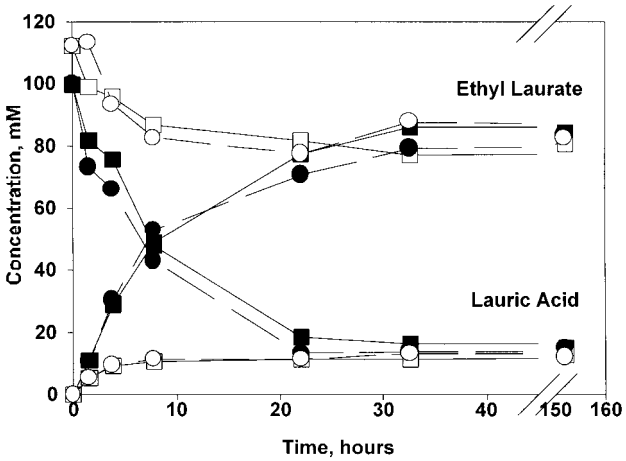

Figure 2. Synthesis (smid symbols) and hydrolysis (open symbols) of 1-butyl laurate yield the same equilibrium position, independent of lipase type. 100 mM AOT and 970 mM water in isooctane (i.e., w_0 = 9.7), 23 °C; all substrates present at 100 mM. *Candida rugosa* (●, ○) and *Rhizopus delemar* (■, □) lipase at micro-aqueous concentration of 2.0 and 1.0 mg mL, respectively. One lipase Unit is defined as the amount to produce 1 mM of ester per hour at w_0 = 8.0. (Reproduced with permission from Hayes, 1991).

tions performed by two different lipases in Figure 2. The fact that water is present at a 10-fold concentration higher than any substrate, but does not promote hydrolysis to a large extent, suggests that lipase may be performing biocatalysis at the w/o-ME interface rather than in the interior of the nanodroplets.

A second example of the influence of substrate/product partitioning is glycerol-fatty acid esterification. Most reports reflect a product distribution consisting of monoglyceride and diglyceride, but not triglyceride (Hayes and Gulari, 1991; 1992). The hypothesis is that diglyceride, being less interfacially active than monoglyceride and free fatty acid (Hayes, 1991), preferentially solubilizes in the bulk solvent rather than the interfacial region, hence reducing their availability as substrate. A unique solution was provided by Holmberg and co-workers to improve triglyceride yield (Oh et al., 1996). It is well known that hydrocarbon solvent molecules of long chain length cannot penetrate into the surfactant tail layer. By using such a hydrocarbon rather than isooctane, the partitioning of diglyceride to the interface increased (due to the poor solubility of diglyceride in the bulk solvent and the improved opportunity for diglyceride molecules to penetrate the tail region), hence leading to an increased production of triglyceride (Oh et al., 1996).

An alternative hypothesis is that substrates which are not strongly adsorbed by the surfactant layer are more readily available, and hence effective, substrates (Hossain et al., 1996; Yang and Gulari, 1994). Hayashi et al. (1996) employed this hypothesis in a kinetic model, which describes the experimental data well.

3.2.3 Kinetics

In general, given that the lipase has been encapsulated in an active conformation (discussed in Section 3.2.5), its inherent activity (e.g., pH and fatty acyl substrate specificity) is quite similar to that encountered in more traditional aqueous or oil-in-water (o/w) emulsion media. However, short-chain acyl groups generally are poor substrates, presumably because they partition weakly to the interface. One must be careful when comparing fatty acyl (and fatty alcohol) selectivity between systems, since such differences may be due to the systems' interfacial properties or to inherent differences in biocatalytic behavior. Regarding the effect of pH, a decrease at high pH was reported for encapsulated *Rhizomucor miehei* and *Humicola lanuginosa* lipase due to the ionization of FFA, in contrast to what occurs in aqueous media (Crooks et al., 1995a).

Temperature affects the activity of w/o-ME-encapsulated lipase differently than encountered in aqueous or heterogeneous nonaqueous systems. Generally, in the latter, an Arrhenius model describes the temperature–activity relationship within a lipase's thermostable region. However, for w/o-MEs, the indirect effect of temperature by its control of w/o-ME behavior is often more significant. Generally, between the upper and lower temperature boundaries of the one-phase microemulsion region, the temperature has very little effect on rate, with activation energies being much smaller than aqueous and heterogeneous nonaqueous systems (Oliveira and Cabral, 1993; Crooks et al., 1996b). For instance, w/o-ME-encapsulated lipases are quite active, even at 273 K or below (Ayyagari and John, 1995; Crooks et al., 1995b).

There are two reported cases where an upper temperature limit for activity is reached within the one-phase region and below the thermostable limit of the lipase. The first is for glycerol-laurate esterification catalyzed by *R. delemar* lipase (Hayes and Gulari, 1991). The second is for *C. rugosa* catalyzed esterification of oleic acid and octanol (Ayyagari and John, 1995). In addition, enhanced thermostability has been reported for lipases encapsulated in lecithin w/o-MEs (Chen and Chang, 1993).

Enzyme kinetics for esterification and hydrolysis reactions employing several surfactant systems have been described by the Ping Pong Bi-Bi mechanism (Prazeres et al., 1993b; Stamatis et al., 1993c; 1995; Yang and Gulari, 1994; Nagayama et al., 1996; Jenta et al., 1997b), which is applicable for lipase reactions in several media types (Malcata et al., 1992). This mechanism features sequential steps: the formation of an acyl-enzyme intermediate, followed by the reversible attack by various nucleophiles (e.g., alcohols, polyol monoesters, water). The model, when applied to w/o-ME systems, frequently requires incorporation of competitive inhibition term for FFA (Tsai and Chiang, 1991; Prazeres et al., 1993b; Yang and Gulari, 1994; Stamatis et al., 1995; Jenta et al., 1997b) and alcohol (Stamatis et al., 1995), and a noncompetitive inhibition term for surfactant (Marangoni et al., 1993). For most cases, in agreement with kinetic models, the reaction rate is proportional to the overall enzyme concentration (Han et al., 1987a; Hayes and Gulari, 1990; Miyake et al., 1993a; Crooks et al., 1995a; Rees and Robinson, 1995).

The optimal alkanol chain length for fatty acid-n-alkanol esterification for reactions catalyzed in w/o-MEs can be quite different from that encountered in alternate reaction media such as o/w emulsions (Hayes and Gulari, 1990). The differences reflect the partitioning of the alkanol molecules in the respective reaction media. Of great interest, the optimal chain length varies greatly with lipase type in a given w/o-ME system. For instance, lipases from the *Rhizopus* and *Pseudomonas* families prefer 1-propanol (Hayes and Gulari, 1990; Stamatis et al., 1993a; 1993d; 1995), *C. rugosa* prefers 1-butanol or 1-hexanol, depending on surfactant system (Morita et al., 1994; Hayes and Gulari, 1990), and *Penicillium simplicissimum* prefers 1-nonane (Stamatis et al., 1993d). It has been suggested that this difference may reflect the position of the enzyme in the w/o-ME droplet. For instance, from light scattering experiments (Hayes and Gulari, 1990; Hayes, 1991), it is speculated that 1-propanol solubilizes in all three nanodomains (aqueous core, interfacial region, and bulk solvent), 1-butanol solubilizes strongly in the interfacial region, and that the alkanols partition more strongly to the bulk solvent and penetrate the surfactant layer less strongly as the chain length increases further. According to this hypothesis, *Rhizopus* and *Pseudomonas* lipases solubilize more strongly in the interior water pools and *P. simplicissimum* solubilizes to a greater extent in the surfactant tail region. The alkanol chain length-activity profile is reported to change with w_o (Schlatmann et al., 1991), surfactant type (Stamatis et al., 1995; Avramiotis et al., 1996) and substrate type (Hayes and Gulari, 1990).

The composition of the w/o-ME system greatly impacts the enzyme kinetics. For instance, a bell-shaped relationship between the parameter w_o, the water-surfactant molar ratio, and the observed enzymatic activity, commonly occurs for several encapsulated enzymes, including lipases. Numerous examples are provided in the technical literature. An example is given in Figure 3 for *C. rugosa* lipase. The parameter w_o for many surfactant systems is proportional to the size of the w/o-MEs. The op-

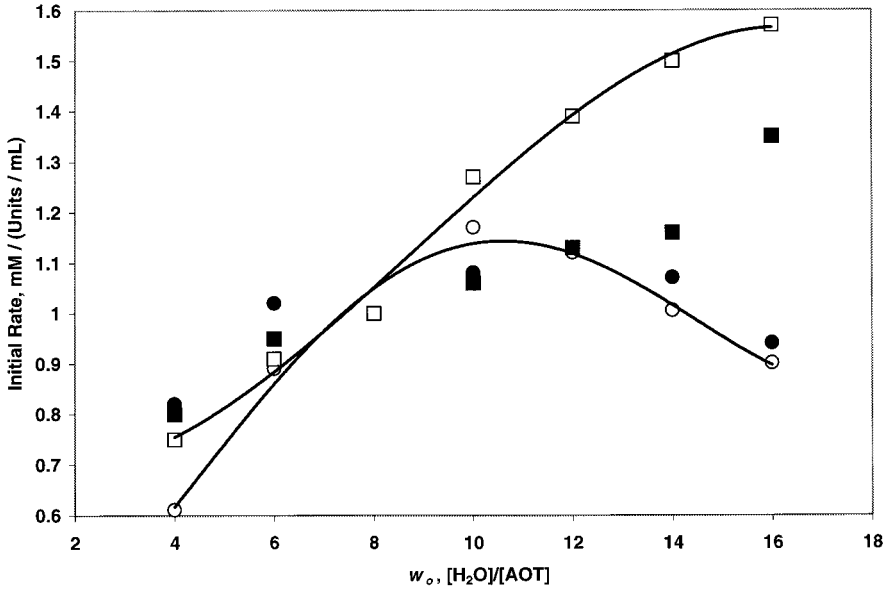

Figure 3. Effect of w_0 on the activity of *C. rugosa* (●, ○) and *R. delemar* (■, □) lipase. Solid symbols: [AOT] held constant at 100 mM (in isooctane); open symbols: water concentration held constant at 800 mM. Reaction: lauric acid (100 mM) + 1-butanol (250 mM). (Reproduced with permission from Hayes, 1991).

timal w_o value for *C. rugosa* lipase occurs at a w_o value of about 9, which corresponds to a w/o-ME water pool diameter of about 6 nm (Zulauf and Eicke, 1979). A similar w_o value has been obtained for this enzyme undergoing alternate reactions (Han and Rhee, 1985b; Chen and Chang, 1993; Hedström et al., 1993; Yang and Russell, 1995) and for other lipases (Prazeres et al., 1992; Stamatis et al., 1993b; 1995; Crooks et al., 1995a) and surfactant systems (Valis et al., 1992). Close agreement between the optimal water pool diameter and the size of the encapsulated protein has been noted for several different biomolecules (Oldfield, 1994). However, despite several different theoretical approaches, the underlying reason for the bellshaped curve and a means for predicting the optimal w_o value are still not well understood. The reader must bear in mind that a difference in size between protein-containing, or filled, w/o-MEs and empty w/o-MEs may exist. In a typical w/o-ME system, the population of empty w/o-MEs is 100–1000 times larger than the filled w/o-ME population, making discernment of the properties of filled micelles by conventional techniques (e.g., quasi-elastic light scattering) challenging. Possible reasons for low activity at low w_o include an insufficient supply of water molecules for hydrating both surfactant and enzyme, and the presence of strong surfactant–protein interactions. At large w_o, activity may be lower due to unfavorable partitioning of substrate and/or enzyme away from the interface. Exceptions to the bell-shaped relationship exist, such as *R. delemar* lipase in the Aerosol-OT (AOT) surfactant system (Figure 3) and *H. lanuginosa* lipase encapsulated in lecithin w/o-MEs (Svensson et al., 1996). Also, the position of the w_o-activity profile can be shifted by pH, substrate and re-

action type, enzyme concentration (Han et al., 1990; Shiomori et al., 1996), and whether the surfactant or water concentration is held constant for a series of reactions (e.g., Figure 3) (Hayes, 1991; Shiomori et al., 1996). Also, theoretically, it is not clear whether the overall enzyme concentration, or the enzyme concentration in the dispersed aqueous phase should be held constant (Han et al., 1990). Of interest, when water was added to a lipase-encapsulated w/o-ME solution at a w_o value that is below optimal, the anticipated increase in activity did not occur (Han et al., 1990). This may reflect the role of interfacial curvature on the activity retention achieved during encapsulation, as will be discussed in Section 3.2.5.

For w/o-ME employing AOT, the most commonly employed surfactant, the specific activity often decreases with AOT concentration (at a constant w_o) (Prazeres et al., 1992; Marangoni, 1993; Miyake et al., 1993a; 1993b; Patel et al., 1996). Since an increase in AOT (and water, at constant w_o) should just increase the concentration of w/o-MEs without any other change in medium properties, the decrease in activity reflects that AOT must inactivate lipase (Section 3.2.5). [An exception to this rule is for hydrolysis under conditions where the additional water substrate is required for the reaction to proceed (Han et al., 1987b).]

Reactions catalyzed by w/o-ME-encapsulated lipases are quite sensitive to substrate (and product) concentrations due to their roles as inhibitors (discussed above) and their effect on the properties of the interface and the bulk solvent. For instance, the degree of hydrolysis decreases with triglyceride concentration for the AOT surfactant system due to the presence of increasing levels of product (FFA), acting as inhibitor (Han and Rhee, 1985a; Chang et al., 1991; Tsai and Chiang, 1991; Hayes and Kleiman, 1993a; Prazeres et al., 1994; Patel et al., 1995). This makes w/o-MEs impractical for hosting lipolysis. Regarding fatty acid–fatty alcohol esterification, an optimal concentration of each substrate exists; and, the optimal levels vary with enzyme type (Hayes and Gulari, 1990; Rao et al., 1991; Oliveira and Cabral, 1993; Stamatis et al., 1993d). For polyol-fatty acid ester synthesis, the concentrations of both substrates control the success of the reaction by their strong influence on the phase behavior of the w/o-ME system (Hayes and Gulari, 1991; 1992; 1995). To form fatty acid ethyl esters, it is best to deliver the substrate ethanol slowly during the course of reaction, as ethanol/water mixtures are known to inactivate lipases (Rao et al., 1991).

Several comparisons have been made between w/o-MEs and alternate reaction systems for hosting lipase-catalyzed reactions. Russell and co-workers screened several nonaqueous enzymology systems for lipase-catalyzed hydrolysis (Yang and Russell, 1995). They report that w/o-ME systems yielded the highest specific lipase activity, but also the lowest rate of product formation and product concentration, due in part to the low enzyme and substrate loadings achievable in w/o-ME systems. A similar finding was also determined when screening reaction systems for lipase-catalyzed esterification (Borzeix et al., 1992; Bornscheuer et al., 1994; Ayyagari and John, 1995). The most discouraging attributes of w/o-ME reaction systems are the difficulty in product and enzyme recovery, due to the presence of surfactant, and the poor suitability for continuous reactor schemes. Approaches to address these flaws are given in Section 3.4

3.2.4 Effect of surfactant and solvent type

Most of the early studies of w/o-ME-encapsulated lipases employed AOT [sodium bis(2-ethylhexyl sulfosuccinate], a two-tailed anionic surfactant, because of its demonstrated ability to readily form monodisperse w/o-MEs in a one-phase medium for a large variety of components without the requirement of a co-surfactant. Moreover, a great body of literature exists on the physico-chemical properties of AOT w/o-ME systems (Eicke, 1987). More recent efforts have sought substitutes for AOT due to the frequent lipase activity loss occurring with AOT, the surfactant's non-biocompatibility, and complications it induces for downstream product recovery. Examples of alternate surfactant systems are given in Table 2. Many of the alternative systems consist of AOT mixed with less-harsh, more biocompatible surfactants such as Tweens or Spans (polyoxyethylene sorbitan fatty alcohol esters) (Yamada et al., 1993; 1994; Hossain et al., 1999), lecithin (Nagayama et al., 1998), taurocholate and bile salts (Kuboi et al., 1992), or polar cosurfactants (Hayes and Gulari, 1994). It is believed that the presence of the second surfactant or co-surfactant reduces the strong interactions between AOT and proteins (Schomaecker et al., 1988) which probably reduce activity and stability upon encapsulation.

Regarding cationic surfactants, the single-tail amphiphile CTAB (cetyl trimethylammonium bromide) yields much less complication for downstream separations and improved lipase activity retention; however, it promotes a much slower specific catalytic rate (Rees and Robinson, 1995). [Double-tail cationic surfactants are reported to form non-specific aggregates with lipase (Skagerlind and Holmberg, 1994).]

Several groups have employed lecithin, which contains mostly phosphatidylcholine, as a natural biocompatible zwitterionic surfactant (Hochkoeppler and Palmieri, 1990; Chen and Chang, 1993; Marangoni et al., 1993; Oliveira and Cabral, 1993; Kermasha et al., 1995; Avramoiotis et al., 1996; Svensson et al., 1996; Nagayama et al., 1998). Lipase reactions have been reported to yield biocatalytic rates superior to AOT with good activity retention (Oliveira and Cabral, 1993; Svensson et al., 1996). The performance of the lecithin is sensitive to its head group and fatty acyl composition, which can vary significantly between commercial sources (Marangoni et al., 1993). The structure of lecithin w/o-MEs consists of a series of interconnected tubes or rods rather than typical discrete, spherically shaped w/o-MEs (Walde et al., 1990). Lecithin w/o-MEs can also form gels, as will discussed in Section 3.5. A minor problem with lecithin is that lipases are reported to hydrolyze it to a small extent (Morita et al., 1984). A second natural surfactant is the use of fatty acid soaps (see Table 2).

Nonionic surfactants, particularly those based on commercially available polyethylene glycol (PEG) fatty alcohol ethers (e.g., Brij) or PEG sorbitan fatty acid esters, are attractive biocompatible surffactants. They can readily form w/o-MEs in the absence or presence of co-surfactants given that the surfactant system's hydrophilic–lipophilic balance, or HLB value, is between 8 and 15. Early work by Holmberg's group and others demonstrated PEG ether & w/o-ME systems were effective in hosting lipase-catalyzed acidolysis of triglycerides (Holmberg and Österberg, 1987) and esterification of glycerol and fatty alcohols (Stamatis et al., 1995), but less successful for hydrolysis (Holmberg and Österberg, 1988; Stark

Table 2. Microemulsion systems employed for hosting lipase-catalyzed reactions

Microemulsion system	Reference
Anionic surfactants	
Aerosol-OT (sodium bis[2-ethylhexyl] sulfosuccinate)	Hayes and Gulari, 1990
Cationic surfactants	
Cetyl trimethylammonium bromide (CTAB)/1-Pentanol	Valis et al., 1992
Didodecyldimethylammonium bromide	Skagerlind and Holmberg, 1994
Nonionic surfactants	
Poly(ethylene glycol) fatty alcohol ethers)	Bello et al., 1987 Holmberg and Österberg, 1987
Tween 80 (polyoxyethylene sorbitan fatty acid esters)	Österberg et al., 1985 Kermasha et al., 1993
Octa(ethylene glycol) mono(2-butyloctyl) ether	Skagerlind and Holmberg, 1994
Nonidet P-40 (octylphenoxy polyethoxyethanol)	Yang and Russell, 1994
surfactants derived from natural sources	
Phosphatidylcholine (lecithin)/Alkanol	Morita et al., 1984 Schmidli and Luisi, 1990
C_{10}-C_{12} acid/C_{10}-C_{12} FA soap	Oh et al., 1996 Skagerlind et al., 1997
Mixed surfactant systems	
AOT/Polyoxyethylene sorbitan fatty acid ester (Tween 85)	Yamada et al., 1993 Hossain et al., 1999
AOT/Taurodeoxycholate	Kuboi et al., 1992
AOT/Lecithin	Nagayama et al., 1998
Winsor III microemulsion systems	
Branched-tail oxyethylene phosphonate surfactant	Sonesson and Holmberg, 1991

et al., 1990). The inability to hydrolyze triglycerides was attributed to the presence of the PEG palisade layer at the interface, which is known to repel proteins (Holmberg and Österberg, 1990; Stark et al., 1990). In addition, a side reaction occurred: the free hydroxyl on the free end of PEG acted as an acyl acceptor for free fatty acid (Holmberg and Österberg, 1987; Stark et al., 1990). Likewise, when PEG-sorbitan fatty acid esters were employed as surfactant to host lipolysis, they were more readily hydrolyzed than the triglyceride substrate (Österberg et al., 1985; Yamada et al., 1993). In subsequent work, Holmberg and co-workers screened several surfactants for their ability to hydrolyze palm oil (Skagerlind and Holmberg, 1994). Their work demonstrated that the two-tailed, or branched, PEG-fatty acid ether, octa(ethylene glycol) mono(2-butyloctyl)ether, was better suited for encapsulating lipase than single-tail nonionic surfactants (Skagerlind and Holmberg, 1994). In agreement, Wiencek and co-workers have demonstrated that the best surfactant structure for w/o-ME formation consists of two tail groups of nonequal length, with an HLB value of about 10 (Vasudevan et al., 1995).

An additional novel approach was to employ Winsor III microemulsion systems, which consist of a middle bicontinuous microemulsion phase that contains the surfactant and enzyme, and excess aqueous and apolar (isooctane) phases. Holmberg and co-workers developed an oxyethylene ether phosphonate surfactant that readily formed Winsor III systems over a 30 °C temperature range (Sonesson and Holmberg,

1991). The organic and aqueous excess phases contained only the hydrolysis reaction products, monoglyceride and fatty acid soaps, respectively (Sonesson and Holmberg, 1991). The rate of reaction was slightly slower than hydrolysis in the AOT system (Sonesson and Holmberg, 1991). The drawbacks of this approach were the high ionic strength of the aqueous phases (1.5 M) and the low substrate loading (Sonesson and Holmberg, 1991). When the substrate (trimyristin) concentration in isooctane was greater than 0.07 (w/w), the microemulsion system transformed into a Winsor II system, i.e., consisting of a w/o-ME phase in equilibrium with an excess aqueous phase (Sonesson and Holmberg, 1991).

With regard to solvent types, hydrocarbon solvents that can penetrate the surfactant layer have been demonstrated to yield the highest rate of enzymatic reaction. Typically, isooctane, and C_6–C_8 alkanes perform optimally (Han and Rhee, 1985b; Hayes and Gulari, 1990). Esterification has also been catalyzed by lipase encapsulated in AOT/near-critical liquid propane w/o-MEs (Murakata et al., 1996).

3.2.5 Stability and conformation of encapsulated lipases

As mentioned above, lipases encapsulated in AOT w/o-MEs can rapidly inactivate, with strong activity loss occurring almost immediately (Han and Rhee, 1985b; Hayes and Gulari). However, the activity loss is greatly reduced when w_o is small (Fletcher and Robinson, 1985; Han and Rhee, 1985b; Hayes and Gulari, 1990; Shiomori et al., 1996) and when fatty acyl substrate is present (Hayes and Gulari, 1990; Rao et al., 1991; Patel et al., 1995). The improvement of stability with low w_o also holds true for CTAB (Valis et al., 1992). Perhaps the lower interfacial curvature at small w_o reduces enzyme–surfactant interaction. It is believed that fatty acyl groups lessen inactivation due to the conformational changes in lipase upon formation of an acyl–enzyme intermediate. In addition, it has been demonstrated that short-chain alcohols (1-butanol, glycerol) improve the activity and stability of encapsulated lipases (Hayes and Gulari, 1990; 1994). Hayes and Gulari speculate that the improvement occurs because of the reduction of interfacial tension and disruption of surfactant packing. Both events would reduce enzyme adsorption at the interface (Hayes and Gulari, 1994). In agreement, the presence of long-chain co-surfactant, known to promote ordered surfactant packing, reduced enzyme stability (Hayes and Gulari, 1994). Hayes and Gulari designed a series of experiments that allowed determination of lipase stability in the presence of both fatty acid and glycerol. Their results demonstsrated that the encapsulated lipase possessed remarkable stability (Figure 4). When tetradecane, which promotes similar structural changes as 1-butanol, was employed as bulk solvent, lipase possessed a half-life of ca. 3 weeks (Figure 4). [In agreement, Freedman and co-workers also reported enhanced stability for w/o-ME-encapsulated enzymes in long-chain oils (Skrika-Alexopoulos et al., 1987).] Furthermore, it appears that solubilization of lipase by a liquid–liquid extraction process (Section 3.3) yields a more stable lipase than that encapsulated by the injection of aqueous lipase buffer in organic media (Prazeres et al., 1992).

The loss of activity retention in AOT systems coincides with changes in the secondary structure of encapsulated lipases, as observed using circular dichroism and

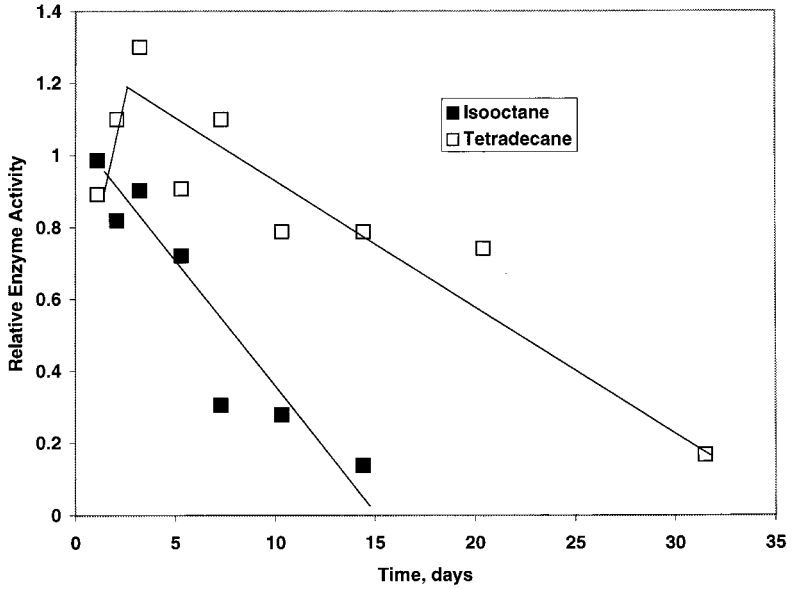

Figure 4. Effect of solvent on *R. delemar* stability in glycerol and fatty acid-containing microemulsions of the AOT system. (Reproduced with permission from Hayes, 1991).

fluorescence spectroscopy (Brown et al., 1993; Marangoni, 1993; Miyake et al., 1993a; Walde et al., 1993; Otero et al., 1995). Furthermore, the presence of FFA was reported to reduce the degree of structural change (Walde et al., 1993). The same study demonstrated the need of the protein co-lipase to yield an active w/o-ME-encapsulated porcine pancreatic lipase (Walde et al., 1993).

In contrast to AOT, enzyme stability in CTAB and lecithin w/o-ME systems is significantly improved compared to AOT (Rees and Robinson, 1995; Avramiotis et al., 1996).

3.2.6 Influence of lipase on microemulsion structural properties

It would be expected that the presence of lipase, a molecule with geometric dimensions on the same order of magnitude as the diameter of the w/o-MEs, would perturb the structure and behavior of w/o-MEs. In addition, the occurrence of lipases near interfaces *in vivo* would support this hypothesis. In agreement, small angle X-ray scattering results demonstrate that the lipases reduce w/o-ME size, presumably by acting as a surface-active agent (Papadimitriou et al., 1995). Further evidence of w/o-ME structural changes were detected using time-resolved fluorescence energy transfer experiments (Avramiotis et al., 1996).

3.3 Recovery of lipases by microemulsion-based extraction

Water-in-oil microemulsion solutions have been employed successfully to purify and recover proteins from complex aqueous solutions through specific (electrostatic or bioaffinity) or nonspecific (hydrophobic) interactions in a process known as microemulsion-based liquid–liquid extraction. This body of research has been recently reviewed (Pires et al., 1996). Cabral and co-workers have employed this method to separate two different lipases from a crude protein mixture derived from *Chromobacterium viscosum* (Camarinha Vicente et al., 1990; Aires-Barros and Cabral, 1991). Lipase A and B differed in their molecular weight (120 kDa and 30 kDa, respectively) and their pI values (3.7 and 7.3, respectively). These workers employed AOT/isooctane solution as extractant, indicating an electrostatic driving force. The protein mixture was solubilized in a pH 6 buffer in order for lipase B to have a net positive charge to promote interaction with the anionic sulfonate groups of AOT. Upon contact with the w/o-ME solution (forward extraction), most of the Lipase B molecules were extracted away. However, all of the Lipase A remained in the aqueous phase, presumably because the AOT w/o-MEs were too small to host the large proteins. Hence, the degree of separation was quite good.

However, recovery of the w/o-ME-encapsulated lipase B was difficult. Cabral and co-workers adopted a typical back-extraction procedure, consisting of contact with an aqueous stripping solution at a pH that encourages electrostatic repulsion between the protein's charged surface groups and the surfactant head group, and high ionic strength to induce Debye shielding of the surfactant head groups. This approach was not successful, probably because hydrophobic interactions between lipase and AOT were not interrupted. Hence, they included ca. 5 % polar alcohol (e.g., ethanol) to disrupt the interfacial packing of the w/o-ME interface. This led to the release of encapsulated lipase. However, the presence of polar alcohol in aqueous medium is known to promote inactivation. Two approaches have been applied to improve the recovery of lipases from w/o-ME solution: (i) the employment of a mixed AOT/polyoxyethylene fatty acid ester (Tween 85) system (Yamada et al., 1994); and (ii) the inclusion of guanidine hydrochloride or high ionic strength in the dispersed aqueous phase (Nagayama et al., 1999). With regard to the first approach, the authors hypothesize that Tween improves back-extraction by reducing the surface charge density of the interfacial sulfonate surfactant head groups (Yamada et al., 1994). Regarding the second approach, although guanidine is a well-known denaturant, its presence at low-to-moderate amounts in the w/o-ME system did not reduce the activity of encapsulated or recovered lipases (Nagayama et al., 1999).

3.4 Membrane bioreactors and continuous processes

Several research groups have assembled membrane bioreactor systems for continuous steady-state lipolysis. For a typical process (depicted in Figure 5), the effluent from a tank reactor is fed to an ultrafiltration membrane module. Molecules of product (FFA), mono-, di-, tri-glycerides, and solvent are sufficiently small to permeate the membrane; however, w/o-MEs in theory are too large to permeate. The flow of nonpermeable materials, or retentate, is recycled directly back into the reactor. The permeate can be collected as product and/or recycled back to the reactor. In addition to continuous operation, a second advantage of this system is the *in situ* removal of FFA, a competitive inhibitor, suggesting an increase in the rate and extent of reaction (Chiang and Tsai, 1992b). Such an increase of rate for bioreactors has been reported (Prazeres et al., 1993c). However, in general, bioreactor performance did not meet expectations, a major problem of all bioreactor systems being the significant permeation of w/o-MEs (the surfactant AOT and water) (Chiang and Tsai, 1992a; Prazeres et al., 1993a; 1994). This occurrence leads to complications in downstream product recovery and the need to replenish the w/o-ME supply in the reactor to maintain steady state. In addition, a significant amount of the lipase and surfactant adsorbed to the membrane surface, leading to a decreased permeation flux (Prazeres et al., 1993a; Nakamura and Hakoda, 1995). Furthermore, a trade-off existed between degree of conversion and productivity (moles of FFA generated per time per mass of enzyme) (Prazeres et al., 1994). A second trade-off existed for w/o. A larger w/o-ME size generally decreased the permeability of w/o-MEs (Nakamura and Hakoda, 1995), though not always (Prazeres et al., 1993a), but led to an increase in the rate of inactivation, in agreement with the material presented in Section 3.2.5 (Nakamura and Hakoda, 1995; Hakoda et al., 1996a). Furthermore, although FFA permeated through the membrane, its concentration in the retentate was greater than or equal to its permeate concentration, indicating a strong degree of association of FFA molecules with the w/o-ME interface, as discussed in Section 3.2.2, hence leading to product inhibition (Prazeres et al., 1993c).

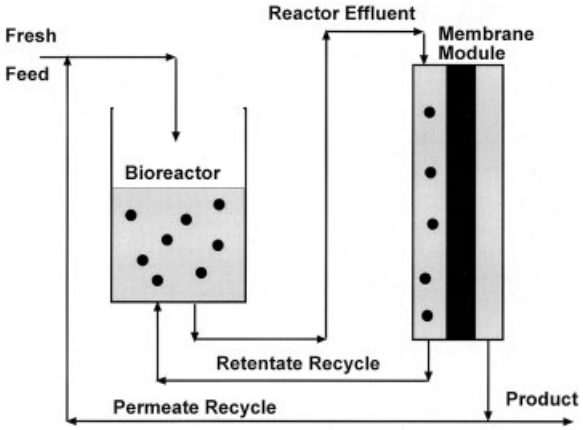

Figure 5. Membrane reactor scheme for w/o-ME systems.

To improve the performance of the continuous membrane bioreactors, two approaches were adopted. The first was to introduce an electric field across the membrane surface. This induced electrophoretic and electroosmotic motion of the w/o-MEs away from the membrane surface. Such treatment improved the permeation flux rate, leading to a slight improvement in reactor performance (Hakoda et al., 1996a). However, the presence of a voltage drop led to further lipase inactivation, (Hakoda et al., 1996a, b). The second approach was to immobilize the lipase in liposomes, then dissolve the complexes in w/o-ME media. Immobilization reduced the permeability and adsorption of lipases on the membrane surface (Chang et al., 1991). This approach led to a similar rate of glycerolysis as achieved in a batch system, indicating that immobilization did not promote mass transfer limitations (Chang et al., 1991). Importantly, the immobilized lipases were quite stable, with a half-life of 45 days reported (Chang et al., 1991). Similarly, Cabral and co-workers reported the enhancement of bioreactor operation by the addition of lecithin (Prazeres et al., 1993c). However, the reactor productivity was plagued with the same problem that occurs in batch systems, namely, decreased yields at higher substrate concentrations or molar flow rates (Chang et al., 1991).

3.5 Microemulsion gels

It was discovered during the late 1980s that the solid-phase microemulsion gels can be formed by adding the protein gelatin to AOT-based w/o-ME solutions, adding water at certain levels to lecithin w/o-ME systems, or adding phenolic compounds to AOT (Luisi et al., 1990; Yu et al., 1993). Microemulsion gels have since received much attention, particularly as transdermal drug delivery agents (Dreher et al., 1997). Generally, the gels are believed to contain a series of networked aqueous rods stabilized by a surfactant layer, and perhaps co-existing with normal w/o-MEs.

3.5.1 AOT/gelatin gels

Robinson and co-workers demonstrated that AOT-gelatin gels were a particularly useful form of immobilized lipase (de Jesus et al., 1995; Nascimento et al., 1992; Rees et al., 1991; 1993). The gels are prepared by combining a w/o-ME solution containing lipase with an aqueous gelatin solution (or equivalently, mixing AOT/solvent with aqueous lipase and gelatin solutions) at 55 °C, and then allowing the mixture to slowly cool below the gelation temperature (30–35 °C) (Rees et al., 1991). The gel can then be carved into small particulates using a scalpel, or mechanically ground using a mortar and pestle in liquid nitrogen, the later yielding a more reactive, less diffusion-limited product (Jenta et al., 1997a). The gel particulates can then be suspended in stirred tank reactors, or employed in a packed bed. A typical gel composition would consist of 24 % water, 9 % AOT, and 14 % gelatin (Rees et al., 1993). ‘Soft’, optically-transparent gels, containing a minimal amount of gelatin (5–8 %), exhibit the highest rate of biocatalysis, but are not stable and are susceptible to

dissolution in the bulk solvent (Jenta et al., 1997a; Karlsson et al., 1998). 'Hard', opaque gels (8–14 % gelatin) are less active, presumably due to diffusional limitations caused by the additional gelatin, but are much more stable than soft gels (Jenta et al., 1997a). As the w_o value of the gel increased from 40 to 200, the gel underwent a soft-to-hard transition. The gels must be stored in closed containers to reduce the evaporation of solvent and water, which renders the gels ineffective (Uemasu and Hinze, 1994).

The gels were successfully applied to catalyze a variety of reactions, particularly enantioselective esterification of racemic alcohols and acids, even on a preparative scale and at $-20\,^{\circ}C$ (Rees et al., 1991; 1993; 1995b; Uemasu and Hinze 1994). Surprisingly, even polyols were successful substrates. The polyols were adsorbed by the gels, rendering them as swollen; but, as esterification proceeded, the swelling decreased due to the consumption of polyol (Rees et al., 1993). The only substrates not tolerated were short-chain (C_1–C_3) and branched fatty acyl substrates (Rees et al., 1991), which induced a phase transition of the gel to a liquid phase (Rees et al., 1993). In addition, the presence of substrates (fatty acids and alcohols) during gel preparation prevented gel formation (Jenta et al., 1997b). Substrates that accumulated in the gel phase were readily extracted away by solvent (Rees et al., 1991). An attempt to employ the gels to catalyze acidolysis (racemic 2-octanol + vinyl acetate) yielded the hydrolysis side reaction, producing acetic acid to a significant extent (Karlsson et al., 1998). Several different lipases were active in the gel-phase, with the exception of *C. rugosa* lipase (Nascimento et al., 1992; Rees et al.; 1995b; Karlsson et al., 1998). [However, one group reported the successful resolution of polyphenolics by *C. rugosa* lipase encapsulated in AOT–gelatin gel (Parmar et al., 1996).]

Importantly, the leakage of AOT, water, and gelatin from the gels is minimal, simplifying downstream separations (Rees et al., 1993). The gels demonstrated excellent stability, allowing them to be reused over a period of months (Rees et al., 1991; Nascimento et al., 1992; Backlund et al., 1996). The majority of the activity loss that occurred was due to the accumulation of water, an ester synthesis product, in the gels (Jenta et al., 1997a; Rees and Robinson, 1995). Moreover, the gel activity decreased with w_o because of the soft-to-hard gel transition (Jenta et al., 1997a). However, the activity loss was reversed when water was extracted from the gels by low-w_o microemulsion solution (Jenta et al., 1997a).

Surprisingly, the gels exhibited minimal diffusional limitations. Moreover, the turnover number and activation energy for esterification, determined by applying a Ping Pong Bi-Bi model, was very similar to the value achieved in a w/o-ME solution (Jenta et al., 1997b). However, many of the transformations took several days to complete, due to a limit in lipase loading. Moreover, a concentration of lipase over 200 μg/liquified the gel (Jenta et al., 1997a).

The solvent type employed in the gel formation and in the reaction medium only slightly affected the performance on the gels (Nascimento et al., 1992; Jenta et al., 1997a). [The solvent type contained in the gel did not necessarily have to be the same as that in the bulk reaction medium. Of interest, solvent molecules transported between the solid and bulk liquid phases (Karlsson et al., 1998).] In addition, the gels successfully esterified a liquid mixture of substrates in the absence of solvent.

3.5.2 Lecithin gels

Luisi and co-workers combined lipase, triglyceride substrate, cyclooctane, and water at determined proportions to produce a gel (Scartazzini and Luisi, 1990). Hydrolysis occurred in the solid phase until a critical concentration of fatty acid was reached, which led to a solid-to-liquid phase transition.

The research group from Turku, Finland, formed lecithin–gelatin gels using a similar approach to that developed for the AOT–gelatin gels. They combined a bicontinuous lecithin/ethanol/water/1-hexadecane system with aqueous gelatin solution to form a stable gel. The gel esterified 2-octanol with good enantioselectivity; however, the performance of this gel was inferior to that of AOT–gelatin gel (Backlund et al., 1995). In addition, the ethanol existing in the gel phase served as an acyl donor, producing fatty acid ethyl ester byproduct (Hedström et al., 1997). Xenakis and co-workers employed a similar aproach; except that several gelation reagents were screened (Stamatis and Xenakis, 1999). Indeed, it was found that agar outperformed gelatin (Stamatis and Xenakis, 1999). Their gels exhibited excellent activity retention, but as the concentration of lipase in the gel was increased, the observed reacstion rate did not increase proportionally (Stamatis and Xenakis, 1999).

3.6 Lipid modification catalyzed by other microemulsion-encapsulated enzymes

A brief survey of other enzyme-catalyzed lipid transformations in w/o-MEs will now be given.

3.6.1 Phospholipases

Phospholipase A_2 has been employed to hydrolyze phosphatidylcholine (yielding lyso-phosphatidylcholine and fatty acid as product) and to perform the reverse reaction, esterification, in mixed AOT–lecithin w/o-MEs (Na et al., 1990; Holmberg and Eriksson; Morgado et al., 1995). The important parameters in the operation of these reactions include the presence of calcium, a necessary co-factor, and the procedure employed to form w/o-ME solution (Na et al., 1990; Holmberg and Eriksson, 1992; Morgado et al., 1995). Cabral and co-workers conducted the hydrolytic reaction in a ultrafiltration membrane bioreactor (Morgado et al., 1996). Phospholipase A_2 was unable to catalyze acidolysis of phosphatidylcholine (Holmberg and Eriksson, 1992). In addition to phospholipase A_2, the hydrolysis of phosphatidylcholine was conducted using Phospholipase D in Triton X-100/phosphatidylcholine w/o-MEs in reasonably good yield (Subramani et al., 1996). The products from this reaction are choline and phosphatidic acid.

3.6.2 Cutinase

Cabral and co-workers have investigated the use of a recombinant cutinase from *Fusarium solani* to hydrolyze triglycerides and to form esters in w/o-MEs. Their work has demonstrated that AOT systems, although successful in hosting reactions, rapidly inactivated cutinase (Sebastiao et al., 1993; Melo et al., 1998). Improved stability (although not activity) have been observed for CTAB and lecithin surfactant systems (Pinto-Sousa et al., 1996; Melo et al., 1998).

3.6.3 Lipoxygenases

The formation of hydroperoxides from unsaturated fatty acids (e.g., linoleic acid, $18{:}2^{9cis,12cis}$) and dissolved oxygen catalyzed by soybean lipoxygenase has been conducted in AOT w/o-MEs by several groups (Luisi et al., 1984; Kurganov et al., 1989; Hochkoeppler and Palmieri, 1990; Shkarina et al., 1992; Rodakiewicz-Nowak et al., 1996). On the positive side, the reaction occured readily in this system, providing an optically clear medium well-suited for *in situ* monitoring of the reaction. However, the reaction rates observed were quite slow, presumably due to unfavorable partitioning of substrate and irreversible inactivation of the enzyme (Rodakiewicz-Nowak et al., 1996). A nonionic surfactant system did not fare any better (Piazza, 1992).

3.7 Conclusions

Water-in-oil microemulsions provide a simple reaction system to employ for evaluating the modification of lipids via enzymology in screening experiments. A major advantage of w/o-MEs solutions is the ability to analyze reactions in situ due to their optical transparency. Although currently not employed on a large scale, the development of microemulsion gels appears to be an important process-scale application resulting from w/o-ME/enzyme technology.

3.8 References

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4 Cloning, Mutagenesis, and Biochemical Properties of a Lipase from the Fungus *Rhizopus delemar*

Michael J. Haas, David G. Bailey, Wilber Baker, Thomas R. Berka, David J. Cichowicz, Zygmunt S. Derewenda, Robert R. Genuario, Rolf D. Joerger, Robert R. Klein, Karen Scott and Deborah J. Woolf

4.1 Introduction

Enzymes are attractive catalysts for the conduct of chemical reactions. Compared to nonbiochemical catalysts, they can offer advantages of improved or unprecedented substrate and product specificity, activity under gentle reaction conditions, easy product clean-up, reduced use of toxic chemicals, and ready production of the catalyst from renewable resources. However, the development of enzymes for biotechnological catalysis has been hindered by such disadvantages as scarce supply (which translates to high cost), instability, relatively low reaction rates, and large gaps in the areas of basic and applied knowledge of the best ways in which they may be used.

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are enzymes that, in aqueous systems, hydrolyze the ester bonds of water-insoluble substances. As such, they offer an alternative to high-pressure/high-temperature methods for lipid hydrolysis. The fact that in systems with low water activity they can also synthesize ester and related bonds has made them attractive catalysts in other applications. Furthermore, the discovery that they remain catalytically active in organic solvents, in which the high solubility of hydrophobic substrates and the low solubility of water foster ester synthesis and interchange, has greatly expanded the range of applications of these now popular catalysts (Rubin and Dennis, 1997a,b; Kazlauskas and Bornscheuer, 1998).

We have conducted a program to characterize and modify an extracellular lipase produced by the mycelial fungus *Rhizopus delemar.* [Now more correctly known as *Rhizopus oryzae*, as many individually named isolates in this genus are now recognized to be the same organism (Schipper, 1984)]. *Rhizopus* lipases are attractive catalysts for lipid modification because they are members of a group of lipases that are active only against esters of primary alcohols. Thus, in the hydrolysis and synthesis of glycerides, enzymes in this group are positionally selective, acting only at the 1- and 3- locations. Such a specificity is useful because alteration of the fatty acid occupancy at these sites can change the physical properties of a lipid quite markedly. For example, enzyme-catalyzed, positionally specific, interesterification allows the synthesis of high-value cocoa butter analogs from low-value tallow (Macrae, 1983), of novel glycerides possessing readily digested medium-chain fatty acids at their termini (Huang and Akoh, 1996; Soumanou et al., 1998), and of triglycerides that are optimally suited for infant nutrition because their fatty acid com-

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position and arrangement mimic those of human milk fat lipids (Quinlan and Moore, 1993). However, at the time that the research described here was initiated, the *R. delemar* (Rd) lipase and other 1,3- specific enzymes were not generally available commercially, and very little was known concerning the basic biochemical features or capabilities of lipases in general. The research program described here was undertaken to fill these gaps. It was one of the first of what have now become several thorough studies of lipolytic enzymes, and illustrates the strengths of combining biochemical and molecular biological approaches to achieve advances in basic and applied enzymology.

4.2 Simplification of lipase production

Lipases are enzymes of secondary metabolism, designed by nature to initiate the metabolism of lipids when they become available in the environment. Therefore, lipase production is often regulated in response to the presence of lipids in the growth medium. In the production of microbial lipases it is standard practice to grow the microorganism in liquid medium containing fats or oils to induce lipase production. This approach has disadvantages, among them the limitation of nutrient and energy availability due to the low solubility of the carbon source, difficulties in maintaining the lipid in an emulsified state in the culture, and the fact that the lipids make the growth medium turbid, which hinders optical or spectral assessment of the growth of the organism. In addition, residual glycerides and free fatty acids from the medium contaminate the lipase preparation and can complicate characterization or purification. We postulated that metabolites of glycerides might serve as inducers of lipase production, and explored the ability of glycerol, a fully water-soluble ultimate product of glyceride hydrolysis, to foster lipase production. Using *R. delemar* ATCC 34612 (American Type Culture Collection, Rockville, MD, USA), it was found that the use of glycerol as a carbon source not only supported lipase production, but stimulated it by as much as three-fold relative to the use of glucose or triglyceride (Haas and Bailey, 1993). This provided a simple method for the ready production of crude lipase preparations that were uncontaminated by lipid and thus optimally suited for subsequent biochemical characterization of the lipase.

4.3 Purification and characterization of the Rd lipase

Complete purification of an enzyme may not be necessary for its use as an applied catalyst. However, purification and characterization can identify reaction conditions that give optimal activity. Such information facilitates efficient biotechnological application of the enzyme. Thus, the development and implementation of a purification scheme for the Rd lipase was undertaken (Haas et al., 1992).

Affinity chromatography played a key role in purification of the enzyme. An affinity resin was produced by coupling oleic acid molecules through their carboxylic acid groups to free amino groups of a polyacrylamide gel resin. The result-

ing resin had a high affinity and capacity for lipase. A small column (20 mL bed volume) quantitatively bound lipase from 20–30 L of culture supernatant. Lipolytic activity was released from this column as a single peak by elution with a gradient of Triton X-100 from 0 to 0.5 % (v/v), resulting in a seven-fold increase in specific activity. Denaturing gel electrophoresis followed by silver staining indicated that this step reduced the number of peptides in the sample from about 20, in the crude enzyme preparation, to four (Figure 1).

Ion-exchange chromatography of the affinity-purified lipase on carboxymethyl-Sephadex, with elution by a gradient of sodium chloride from 0 to 0.25 M, produced a lipolytically active sample containing a single polypeptide band of molecular mass 30.3 kDa (Figure 1). Nondenaturing isoelectric focusing, followed by activity staining, verified that this polypeptide was lipolytically active, and had an isoelectric point of 8.6 (Figure 2).

The pure lipase displayed maximum hydrolytic activity between pH 7.5 and 8.5. It was most active between 25 and 35 °C, and was rapidly inactivated above these temperatures. The purified enzyme contained less than one saccharide molecule per molecule of enzyme. This is unusual, in that extracellular fungal proteins often contain significant degrees of glycosylation. However, it is possible that endogenous hydrolases are present in the crude enzyme preparation, and that these deglycosylated the lipase during the affinity chromatography step, which was conducted at room temperature and took several days. The enzyme was not inhibited by disulfide reducing agents. Cations such as calcium, barium or manganese were required for full activity, which was manifested above a cation concentration of 10 mM. This cation requirement is a general feature of lipases, and is often attributed to a masking by the ion of electrostatic repulsions between the enzyme and either its emulsified substrate or product fatty acids.

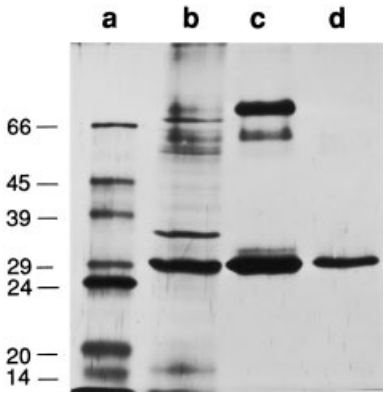

Figure 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the extracellular Rd lipase at various stages of purification (Haas et al., 1992). Proteins were detected by silver staining. Lane a, 2.25 µg of protein molecular mass marker preparation; lane b, 37.5 µg of culture filtrate; lane c, 3 µg of pooled lipase-positive fractions eluted from oleic acid affinity chromatography column; lane d, 1 µg of pooled lipolytic material obtained by carboxymethyl–Sephadex column chromatography. The masses of the molecular weight marker proteins (kDa) are indicated in the left margin.

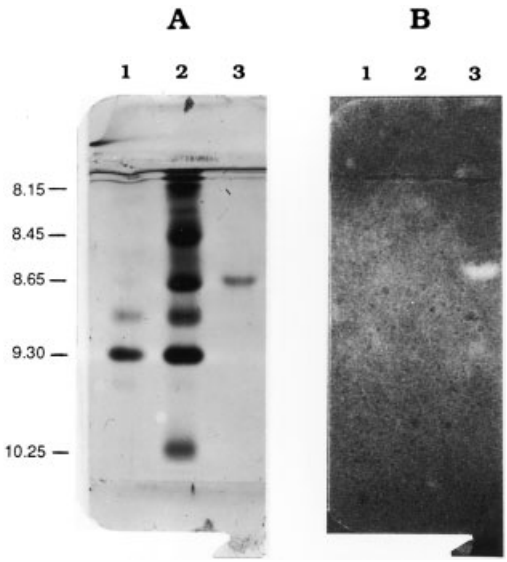

Figure 2. Isoelectric focusing (IEF) of the purified lipase (Haas et al., 1992). (A) Silver-stained IEF gel. Lane 1, 1.0 µg of trypsinogen standard; lane 2, 0.433 µg of IEF standard protein preparation; lane 3, 98 ng of pure lipase. The isoelectric points of the standard proteins are indicated in the left margin. (B) Detection and localization of lipolytic activity in the purified lipase preparation by activity stain. An IEF gel identical to that in Panel A was inverted onto lipase indicator media containing olive oil and rhodamine B, incubated, and photographed under ultraviolet light. The bright band indicates the site of lipase activity.

Among the most useful information obtained from the purified lipase was amino acid sequence data. The sample was sufficiently pure that unambiguous identification of the first 28 amino-terminal residues was possible, with a 'best guess' possible for the amino acid sequence of an additional 12 positions. This information was vital to subsequent characterization of the cloned lipase gene (see Section 4.5).

4.4 Crystallization and determination of three-dimensional structure

Just as the development of the purification scheme was being completed, the first high-resolution three-dimensional structures of lipases appeared (Brady et al., 1990; Winkler et al., 1990). The availability of pure enzyme fostered a similar attempt to determine the structure of the Rd lipase. Several large lipase crystals were gratifyingly obtained within months. However, none of these proved suitable for the collection of diffraction data. Continued efforts over the course of more than two years were required to identify crystallization conditions that produced diffracting crystals (Swenson et al., 1994). Subsequently the structure of the lipase was determined to a resolution of 2.6 Å, the fourth fungal lipase structure to be published (Derewenda et al., 1994a,b,c).

The resulting three-dimensional model of the lipase (Figure 3) showed an extremely high degree of homology with the similarly 1,3-specific lipase from the related organism *Rhizomucor miehei*. Both enzymes are monomeric proteins, globular in shape, and possess three disulfide bonds. Examination of the *Rhizomucor miehei* (Rm) lipase model, and determination of its structure following the binding of an active site-directed inhibitor, identified a serine (Ser)-histidine (His)-aspartic acid (Asp) triad that served as the catalytic center, an oxyanion hole appropriately positioned to stabilize tetrahedral intermediates formed during catalysis, and a hydrophobic channel postulated to be the substrate binding region (Brady et al., 1990; Brzozowski et al., 1991). Analogous structures were identified in the Rd lipase model (Derewenda et al., 1994a,b). Studies of the Rm lipase model had also identified a surface peptide loop that covered the active site and appeared to prevent access of substrate (Brady et al., 1990). Inhibitor studies indicated that, in the presence of substrate, a physical movement of this 'lid' occurred, exposing the active site to

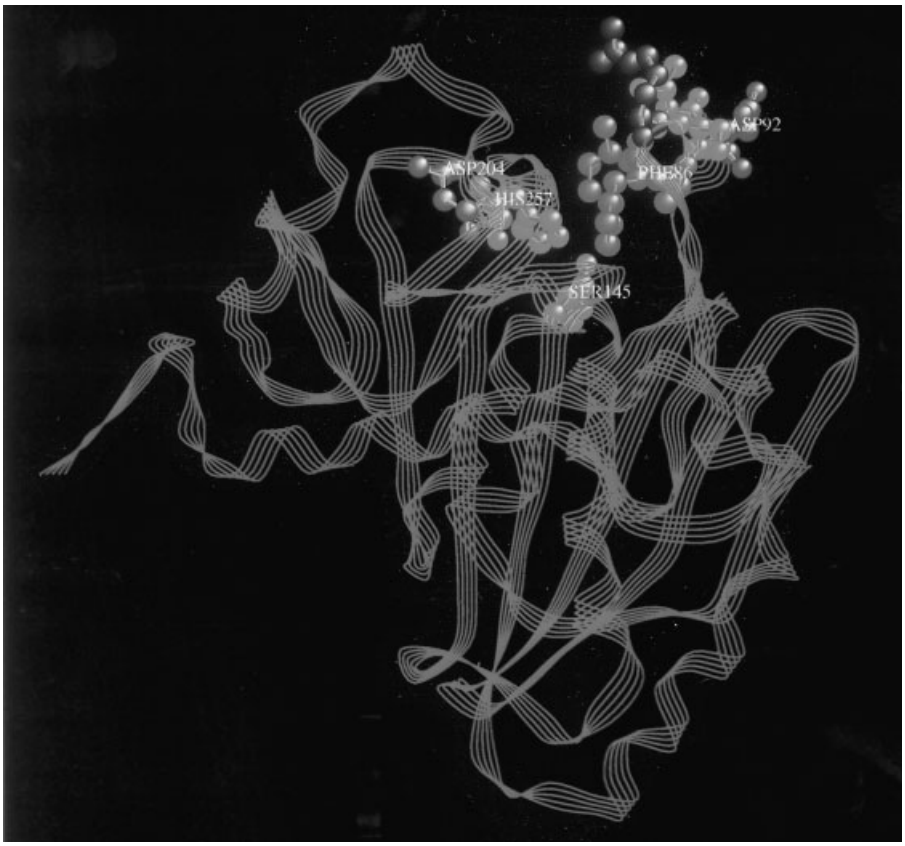

Figure 3. Three-dimensional structure of the *Rhizopus delemar* lipase, determined by X-ray crystallography (Derewenda et al., 1994a,b,c; Swenson et al., 1994). (A) The 'lid', residues 86–92, is in the closed conformation and occludes the active site. (B) Lid is partially opened, opening the active site for access by substrates to the catalytic triad residues: Ser145, Asp204, and His257. (See color plate, page XVII).

substrate (Brzozowski et al., 1991; Derewenda et al., 1992). This was proposed to be the functional basis of 'interfacial activation', the phenomenon whereby lipases are inactive in the absence of lipid-water interfaces, and become active in their presence (Brzozowski et al., 1991; Derewenda et al., 1992). This postulate led to the notion that there were two basic states for the lid, namely 'open' and 'closed'. The Rd lipase model largely agreed with these interpretations of lipase structure and function. However, it expanded appreciation of the nature of interfacial inactivation. As crystallized, the Rd lipase asymmetric unit contained two lipase molecules. In one of these the lid was down, atop the active site (Figure 3A). In the other molecule, the lid was in a conformation intermediate between closed and open (Figure 3B), and appeared to be stabilized there by detergent molecules added during crystallization. This implies that there is much more conformational fluidity to the lid structure than a simple case of 'open in lipid, closed in water'. Rather, it appears that the nature of the substrate, and the presence and chemical features of other molecules in the

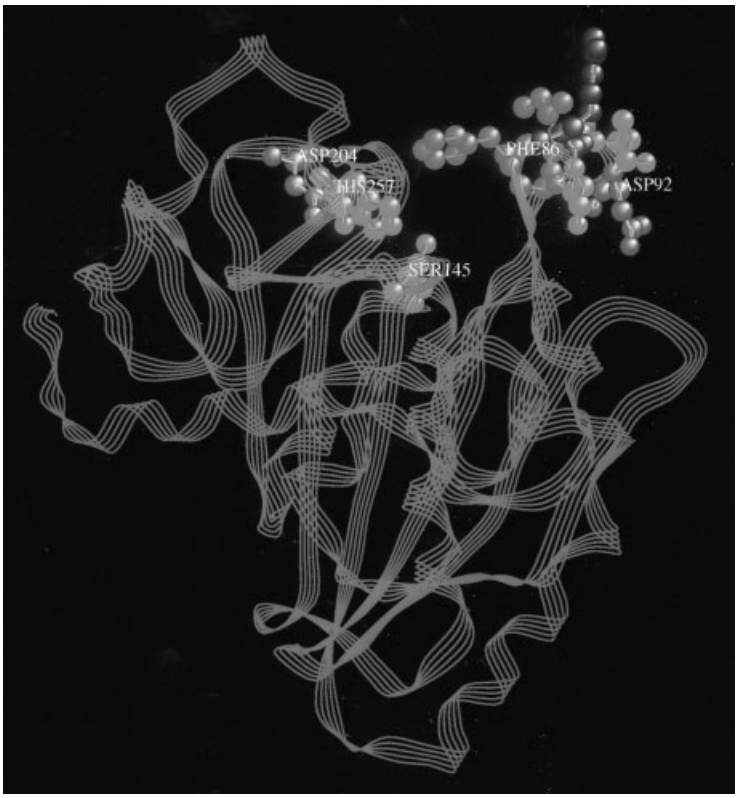

Figure 3B. (See color plate, page XVIII).

solution, can affect the lid conformation. The lid appears to be in dynamic motion, exposing the active site even in the absence of an oil-water interface. Stabilization of the open configuration by such an interface, or by similar chemical structures, results in the appearance of appreciable lipolytic activity. Thus, these studies of the Rd lipase structure refined and expanded notions of the dynamics of lid conformation in the lipases, and helped to unify previously disparate theories regarding the roles and importance of enzyme structure and substrate structure in regulating lipase activity.

4.5 Cloning and characterization of an expressed *R. delemar* lipase gene

Perhaps the most effective method of increasing the availability of an enzyme is through the cloning and expression of its corresponding gene. Cloned genes are also the basis of enzyme modification via directed mutagenesis. For these reasons, the cloning of the Rd lipase gene was undertaken. From the outset, the goal was not only to isolate the gene but also to achieve its expression, and in this the project differed from other lipase cloning projects, which achieved cloning but lacked expression.

The initial approach involved construction, in *E. coli*, of a library of total *R. delemar* DNA and screening for direct expression of the lipase gene. However, direct expression was not detected (Haas et al., 1990). This most likely was due to an inability of *E. coli* to read the transcriptional or translational control signals of the fungus, since subsequent work (W. Friesen, and W. Baker, unpublished results) demonstrated the absence of intervening sequences in the lipase gene.

Subsequently, a complementary DNA (cDNA) cloning strategy was undertaken (Haas et al., 1991). Bulk RNA was prepared from an *R. delemar* culture growing on glycerol and producing lipase. The messenger fraction isolated from this RNA by affinity chromatography served as the template for synthesis of double-stranded cDNA. DNA linkers containing recognition sites for the restriction enzyme *Eco*RI were ligated onto the ends of these cDNA fragments, which were then joined by ligation to bacteriophage λgt11 arms. *E. coli* Y1090r$^-$ was infected with intact bacteriophage produced by *in vitro* packaging of these DNA molecules, and plated as a lawn on solid growth media containing olive oil and rhodamine B. [Rhodamine B is a pink fluorescent dye. In the presence of free fatty acids its fluorescence wavelength is shifted and the fluorescence intensity increases, resulting in a strikingly 'hot pink' signal that is an unmistakable indication of the presence of lipolytic activity (Kouker and Jaeger, 1987)]. Since the color intensity increases with continued enzyme activity, it is useful for the detection of very low levels of lipase. In addition to its application in gene screening, we have employed the dye to locate lipase quickly in fractions collected from chromatography columns, and to identify lipolytic protein bands on nondenaturing electrophoresis gels, as shown in Figure 2.

Following overnight incubation of rhodamine B-olive oil plates containing λ-infected *E. coli*, no fluorescence could be detected among the lytic plaques formed in the bacterial lawn. However, after additional days of incubation a few fluorescent

plaques were noted. Analysis of one of these indicated the presence of an approximately 1.3 kilobase DNA insert that was subcloned into the *Eco*RI site of the expression plasmid pUC8–2, generating plasmid pUC8-2.14 (Haas et al., 1991). The nucleotide sequences of both strands of the entire cDNA insert were determined. The fragment consisted of 1287 base pairs, had a G + C content of 45 %, and possessed a consensus polyadenylation sequence. Furthermore, it hybridized well to total *R. delemar* poly(A)$^+$ RNA, strongly suggesting its origin in that organism.

Of the six possible reading frames in the cloned cDNA, only one contained an open reading frame of sufficient length to encode a protein in excess of 30 kDa, the mass of the lipase as determined by biochemical studies. The actual lipase-encoding region within this reading frame was identified by reference to the known *N*-terminal amino acid sequence of authentic lipase. A region of the gene sequence was identified whose predicted complementary amino acid sequence was identical to that of the first 28 amino-terminal amino acids of the pure lipase. In fact, the predicted amino acid sequence agreed completely with that of the purified fungal enzyme not only for residues 1–28 for which conclusive assignments were possible from amino acid sequence data, but also for residues 29–40 for which 'best guess' sequence estimates could be made from the protein sequencing data. This identity between the amino acid sequence predicted by the cDNA and that of the lipase located the *N*-terminal end of the lipase-encoding region within the cloned cDNA. A single strong translation termination site downstream of this site established the size of the mature lipase as 269 amino acids. The molecular mass of the resulting predicted polypeptide was 29.6 kDa, in agreement with the value determined biochemically for the purified authentic fungal enzyme.

Analysis of the DNA sequence indicated that the lipase is initially synthesized as a preproenzyme, consisting not only of the 269-amino acid mature enzyme, but also of a 97-amino acid propeptide fused to its amino terminus, and a 26-amino acid export signal peptide at the amino terminus of the propeptide. Since *E. coli* lacks the necessary proteases for processing fungal maturation signals, one would expect to be able to detect these precursors in *E. coli* extracts. Accordingly, polypeptides with molecular weights of 39.5 and 42.1 kDa, corresponding to the predicted sizes of the corresponding pro- and prepro-lipases, were detected by immunoblotting of electrophoretic gels containing whole-cell lysates of *E. coli* expressing the cloned lipase gene.

Analysis of the amino acid sequence predicted from the lipase gene sequence indicated the presence of the pentapeptide Glycine (Gly)-His-Ser-Leucine (Leu)-Gly. This is of the general type Gly-X-Ser-X-Gly noted to be highly conserved among lipases (Brenner, 1988). This sequence constitutes a portion of the catalytic triad of lipases, the Ser being the primary catalytic residue (Brady et al., 1990). Other than the His and Asp residues that complete the catalytic triad, the pentapeptide is the sole highly conserved sequence common to lipolytic enzymes.

However, analysis of the gene and protein sequences made it clear that lipases from related organisms, as well as from organisms that are less closely related, can share substantial homology. The high levels of structural homology between the Rd and Rm lipases (above) were reflected, though perhaps not as strongly, in their nucleic acid and amino acid sequences. The sequences of the coding portions of the genes for the Rd and Rm lipases were 56 % identical overall. The degrees of

DNA sequence identity within the signal, propeptide, and mature lipase regions were 47 %, 53 % and 59 %, respectively. Maximum alignment of the amino acid sequences of the Rd and Rm lipases was obtained by the introduction of only one gap, a single amino acid long, in each sequence. Upon such alignment, homologies are substantial. Considering both identical and functionally equivalent residues, the predicted sequences are 29 % and 68 % homologous in the pre/pro and mature domains, respectively. The predicted mature lipases from both organisms contain 269 amino acids. In the halves of the molecules containing the active site pentapeptide, sequence conservation is substantially higher, with 76 % of the amino acids being identical or functionally equivalent.

Consistent with the proposal that many *Rhizopus* isolates are actually the same organism (Schipper, 1984), nearly complete homologies, and some identities, were observed between the amino acid sequences of the Rd lipase and those produced by other members of the genus *Rhizopus* (Kujimiya et al., 1992; Uyttenbroeck et al., 1993; Beer et al., 1996). As further sequence and structural information became available, the existence of a family of related enzymes including not only *Rhizopus* and *Rhizomucor* lipases but also those produced by *Humicola lanuginosa* and *Penicillium camembertii* became apparent (Derewenda et al., 1994a,b).

4.6 Subcloning and regulated overexpression of the lipase gene

The initial Rd lipase gene clone, though expressed by its *E. coli* host, did not produce significant amounts of lipase. Furthermore, due to processing inefficiencies on the part of the host, lipase molecules that did accumulate consisted predominantly of larger, immature forms. To overcome these deficiencies and to generate recombinant lipase comparable to that produced by the authentic fungal host, the isolated cDNA was further engineered (Joerger and Haas, 1993). Site-directed mutagenesis was employed to introduce a unique restriction endonuclease recognition site and a translation initiation site just upstream of the sequence for the mature lipase. Thus, a gene encoding the direct production of the mature lipase, containing neither signal peptide nor propeptide, was produced. Similarly, a cDNA that encoded the prolipase, without an attendant export signal peptide, was engineered. This was a construct of considerable interest since it was unclear whether the prolipase was enzymatically active, and whether it exhibited biochemical properties comparable to those of the mature lipase.

During work with the cloned Rd lipase cDNA it became apparent that expression of the gene had a negative effect on bacterial hosts. In some cases, plasmids containing the lipase gene displayed unexpectedly low transformation frequencies, exhibited elevated instabilities, and reduced the viabilities of their hosts (W. Baker and R. Joerger, unpublished observations). Cell lines containing the gene sometimes had visually distinct morphologies. That a lipase could be damaging to its host if produced and held within the cell was not unexpected, given that these enzymes are known to be active on phospholipids (Laboureur and Labrousse, 1964; Slotboom et al., 1970), the major lipid component of the bacterial inner membrane. Thus,

in subcloning the lipase genes into expression plasmids, especially those that had high copy numbers, extra precautions were taken to ensure tight regulation of expression. DNAs for the pro- and mature lipase were cloned into a tightly regulated expression system employing *E. coli* BL21 (DE3) as host and the plasmid pET11-d as vector. This cloning system (Studier et al., 1990) contains dual levels of control of gene expression. Cloned DNAs are placed under the regulatory control of the bacteriophage T7 gene 10 promoter. This promoter is recognized by T7 RNA polymerase synthesized by the bacterial host. Synthesis of this polymerase is itself also regulated, and is under the control of a *lac* promoter. In addition, the cloned lipase gene is under control of the *lac* repressor/operator system. Only by employing this tightly regulated system was it possible to obtain stable cell lines that grew well in the absence of inducer, and also synthesized high levels of lipase or prolipase upon induction. Using these strains, prolipase levels of 9–15 % of total protein, and mature lipase levels of 15–21 % of total protein, were reached following induction.

At these high expression levels, the lipases were not soluble in the cytoplasm. Insoluble particles (inclusion bodies) formed and were recovered by centrifugation following chemical lysis of the cells. Active enzyme was produced by dissolving the inclusion bodies in 8 M urea and diluting the solution 20-fold into the redox system 1 mM cystine/5 mM cysteine. The active enzymes produced in this manner were purified according to the protocol developed for the fungal enzyme. The resulting pure mature lipase had a specific activity comparable to that of authentic fungal enzyme. The prolipase also assumed an enzymatically active configuration upon renaturation, and was purified to yield a preparation with a specific activity comparable to that of the mature lipase. Therefore, the propeptide fragment does not function as an inhibitor of lipolytic activity prior to maturation. Since *E. coli* is generally unable to glycosylate proteins, the recovery of fully active lipase again indicates that glycosylation is not a prerequisite to enzymatic activity. Using this expression route, it was possible to increase the yields of pure enzyme more than 100-fold compared to those obtained using *R. delemar* as the enzyme source.

These efforts provided the first supplies of prolipase. The enzyme displayed a specific activity and optimal pH similar to those of the mature enzyme. In the presence of substrate, it demonstrated the same thermolability as the mature enzyme, both being quickly inactivated above 30 °C. However, in the absence of substrate the prolipase was markedly thermostable, retaining full activity after exposure to temperatures as high as 70 °C. In contrast, mature lipase was quickly inactivated above 40 °C. Apparently, in the absence of a substrate interface the propeptide delays the onset of thermal denaturation, or promotes rapid renaturation of the enzyme. Another difference between the two enzymes was that the prolipase was less toxic to its host than the mature lipase. Thus, it was possible to directly detect and quantitate lipolytic activity in bacteria. This greatly facilitated the subsequent directed mutagenesis of the lipase gene, since it provided a means of readily producing sufficient enzyme for screening and simple characterization without the necessity of isolating and renaturing inclusion bodies. The availability of prolipase also allowed the first crystallization and investigation of its three dimensional structure (Swenson et al., 1994).

4.7 Probing the role of structure-function relationships in substrate selectivity

Identification of the relationship between primary amino acid sequence and enzyme activity offers the opportunity to optimize enzyme performance through directed mutagenesis. An understanding of the structural basis of substrate selectivity could facilitate the production of a suite of customized lipases displaying desired substrate specificities. Such enzymes could be useful as applied catalysts in applications such as the harvesting of selected fatty acids from heterogeneous glycerides, or the transfer of such fatty acids to acceptor molecules. In effect, mutagenesis represents an alternative or adjunct to the screening of natural isolates for new enzyme activities. For these reasons, site-directed mutagenesis, coupled with computer-assisted molecular modeling, was employed to explore structure-function relationships in the *Rhizopus-Rhizomucor* family of enzymes, and to generate new, potentially useful, lipases.

Much of this work was conducted using the prolipase gene, rather than the lipase gene, because its reduced host toxicity, in conjunction with high activity, facilitated direct screening of bacterial populations to identify potential mutants. The structural model of the Rd enzyme was not available at the time this work was begun. However, given the high degree of homology between the Rd and Rm lipases, the available structure of the latter enzyme was used as a model. The Rd lipase structure was used to guide these studies when it subsequently became available.

In both the Rd and Rm lipases, the substrate binding site is a shallow trough along the surface of the enzyme, with the Ser-His-Asp catalytic triad at one end (Figure 4). The side chains of the amino acids forming the walls of this trough are largely hydrophobic, and in some cases are highly conserved among the 1,3-specific lipases. It was postulated that these amino acids were vital to the binding of the acyl chain of fatty acid substrates, or the access of the fatty acids to the active site, and that mutations at these sites could interfere with substrate binding and enzyme activity. Site-directed mutagenesis was employed to make all possible amino acid substitutions at individual locations along the substrate binding trough. The mutant DNAs were introduced into bacteria and the substrate ranges of the resulting lipases were examined, with the goal of generating and recovering enzymes that possessed marked fatty acid chain length specificity. Rhodamine screening media containing either triolein (18 : 1 fatty acid glyceride), tricaprylin (8 : 0) or tributyrin (4 : 0) were employed to identify changes in fatty acid selectivity. Within a factor of 2, the wild-type lipase hydrolyzed long-, medium-, and short-chain fatty acid glyceride esters at the same rates. Mutant lipases were isolated that exhibited between 2- and 12-fold enhancements in their relative activities toward medium- or short-chain fatty acids (Klein et al., 1997a). DNA sequence analysis was employed to identify the amino acid substitutions in these enzymes.

In these initial studies, single mutations were introduced into the lipase gene. The recovery and characterization of modified enzymes allowed the identification of amino acid positions involved in determining the fatty acid substrate specificity of the enzyme. In subsequent work (Klein et al., 1997b), these single mutations were combined to produce double-mutant lipases. In addition, molecular dynamics

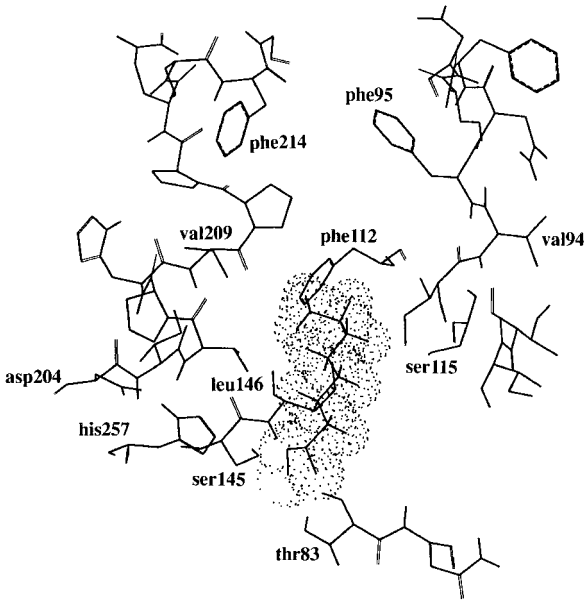

Figure 4. Structural model of the active site region of *Rhizopus delemar* lipase, based on X-ray crystallography and computer-assisted molecular modeling. In this view, the *sn*-3 fatty acyl side chain of tricaprylin is shown (depicted as a dotted van der Waals surface), while the remainder of the triacylglyceride is not displayed. The catalytic residues Ser145, Asp204, and His257 are labeled, along with Thr83, which forms the oxyanion hole that stabilizes the catalytic intermediate. Selected residues lining the acyl binding and catalytic sites are shown. Residues forming part of the acyl binding site that were targeted for mutagenesis (Joerger and Haas, 1994; Klein et al., 1997a,b) are numbered. Modeling of the docking of substrate into the active site was conducted by energy minimization and molecular dynamics simulation.

simulations were conducted to identify alterations in amino acid sequence that might impact substrate specificity. Appropriate mutants were then constructed by directed mutagenesis. Strategies for altering chain length specificity included the introduction of bulky hydrophobic residues at discrete positions in the acyl binding groove, the creation of salt bridges by introducing polar or charged residues across the groove from one another, and the replacement of hydrophobic amino acids with polar ones.

Studies with single mutants had identified two sites, at positions 112 and 209 (Figure 4), located roughly across the binding groove from one another and midway down its length, where amino acid sequence changes affected substrate selectivity (Joerger and Haas, 1994). In each of these mutants the introduced amino acid was a tryptophan (Trp). In each case, mutation increased the relative activity toward short-chain substrates relative to long-chain ones. Molecular dynamics simulations suggested that the Trp might project across the acyl binding groove, preventing access by fatty acids longer than about four carbons in length. It was postulated that if Trp residues were introduced simultaneously at both sites within the same enzyme, geometric or steric constraints might potentiate the effects of the individual mutations on substrate selectivity. Such was subsequently found to be the case, with the double-mutant lipase displaying a short-chain selectivity as much as 40 times greater than

that of its single-mutant progenitors (Klein et al., 1997a). However, not surprisingly, the introduction of these large amino acid side chains into the substrate binding trough also significantly reduced the overall activity of the enzyme.

Molecular modeling indicated that the introduction of a salt bridge across the substrate binding trough slightly farther from the catalytic center might allow access of mid-chain fatty acids while blocking the entry of longer ones. When the corresponding double mutant was created, a three fold increase in activity toward mid-chain fatty acids, compared to that toward long-chain substrates, was observed (Klein et al., 1997a).

One of the most interesting mutant lipases produced during this work was a double mutant wherein a phenylalanine on one side of the binding site (Phe95) was replaced by Asp, while a valine on the other side (Val206) was replaced by a threonine (Klein et al., 1997b). The activity of the wild-type enzyme toward medium chain length fatty acids was about 1.5 times that toward long-chain fatty acids. Singly, each mutation increased activity toward mid-chain fatty acids to 4-to 6-fold greater than that toward long-chain ones. In the double mutant this preference increased to more than 7-fold, roughly an addition of the effects of the individual mutations. Also, whereas the single mutations approximately doubled the activity of the enzyme toward mid-chain fatty acids relative to that toward short chains, the activity of the double mutant toward mid-chain fatty acids was about 100-fold greater than that toward short-chain fatty acids.

Quite unexpectedly, the double mutant also displayed a very sharp and striking dependence of activity upon pH (Klein et al., 1997b). At pH 7.0 the enzyme displayed strong activity and strong selectivity toward mid-chain fatty acids. At, and below, pH 6.5 activity toward these substrates fell, and that toward short-chain substrates increased. At pH 7.5 the enzyme was virtually inactive against all substrates. Thus, the enzyme displayed striking substrate selectivity, but only under a narrow range of conditions. It is unclear whether this behavior is caused by pH-mediated polarization or ionization of the Asp introduced by mutation.

These experiments began the precise correlation of structure with substrate specificity in the lipases, emphasized the importance of choice of assay conditions when screening mutant libraries for activity and specificity, and indicated reaction conditions under which unique substrate selectivity could be achieved. Considerable additional work could be conducted in these areas, since the role of enzyme structure in determining substrate selectivity in the hydrolytic mode has only roughly been identified, and its impact on substrate selectivity in ester synthesis reactions remains unexplored.

4.8 Perspective

It is difficult to convey to those coming recently to the study of lipases just how little was known about these enzymes a mere 15 years ago. At that time, enzyme supplies were limited, many concepts of the structure and function of lipases were largely speculative, and their molecular genetics was unknown. Since then, substantial efforts in numerous laboratories, including the work described here, have built the

solid base of knowledge that makes lipases one of today's best characterized and most frequently employed biocatalysts. Additional work along the lines described here could further define the modes of action of these enzymes, and result in the production of custom catalysts specifically optimized for any of a range of desired biochemical reactions.

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5 Molecular Basis of Specificity and Stereoselectivity of Microbial Lipases toward Triacylglycerols

Jürgen Pleiss

5.1 Biochemical properties

Microbial lipases are widely used to catalyze hydrolysis, alcoholysis, esterification and transesterification of triacylglyerols and analogs, thereby taking advantage of their catalytic efficiency, fatty acid specificity, regio- and stereoselectivity (for reviews, see Faber, 1997; Schmid and Verger, 1998; Bornscheuer and Kazlauskas, 1999). Lipases are long-chain triglyceride-hydrolyzing enzymes, thereby being distinguished from esterases which hydrolyze soluble esters. They have a higher activity toward insoluble substrates than toward the same substrates in monomeric form, an effect which has been termed 'interfacial activation' (Sarda and Desnuelle, 1958). Lipase activity, specificity and selectivity depend upon reaction conditions such as the quality of the substrate interface (Verger, 1997), immobilization support (Adlercreutz, 1991), solvent (water or organic solvent for hydrolysis or esterification reactions, respectively), and water activity in organic solvent (Halling, 1994). However, experimental set-ups which measure competitive factors α in mixtures of different fatty acid chains in organic solvent (Deleuze et al., 1987; Rangheard et al., 1989; Borgdorf and Warwel, 1999) suppress these secondary effects. Thus, the competitive factor α which corresponds to the ratio of the specificity constants k_{cat}/K_m is independent of reaction conditions.

All lipases convert esters of medium (C_4) to long chain (C_{18}) saturated fatty acids. Some of them efficiently hydrolyze fatty acid esters as long as C_{22}, such as lipase from *Rhizomucor miehei* (Rangheard et al., 1992; Kirk et al., 1992). Of special interest is the *cis* (Δ-9) specificity of *Geotrichum candidum* lipase I (Phillips et al., 1995; Borgdorf and Warwel, 1999).

All lipases hydrolyze triacylglycerols at the *sn*-1/3 position; hydrolysis at the *sn*-2 position is catalyzed only by four microbial lipases (*Candida antarctica* lipase A, *Geotrichum candidum* lipase I, *Penicillium simplicissimum* lipase, and *Candida rugosa* lipase). Regioselectivity and *sn*-1/3 stereoselectivity were measured using monomolecular films of di- and triacylgycerol analogues (Ransac et al., 1990), prochiral triacylglycerol emulsions (Rogalska et al., 1993; Stadler et al., 1995; Kovac et al., 1996) and optically pure diacylglycerols (Rogalska et al., 1995). Three lipases were highly stereoselective toward trioctanoin: namely, lipases from *Pseudomonas* sp. and *Pseudomonas aeruginosa* (*sn*-1 selective), and *Candida antarctica* lipase B (*sn*-3 selective). All other lipases show medium or low selectivity. Stereopreference was similar toward both tri- and diacylglycerol substrates, and generally did not depend on the type of substrate. Only for *Candida antarctica* lipase B and *Geotri-*

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chum candidum lipase II, was reversal of stereopreference observed when triolein was used as substrate instead of trioctanoin. Instead, stereoselectivity depends strongly on the quality of the interface, which was investigated by varying the surface pressure in monomolecular films.

To identify further the selectivity-determining factors in substrate-lipase interaction, synthetic *sn*-2 derivatives have been investigated, with the functional ester group replaced by ether, benzylether, amide or phenyl residues (Paltauf and Wagner, 1976; Stadler et al., 1995; Kovac et al., 1996). In both hydrolysis and esterification reactions, stereoselectivity depends strongly on the structure of these substituents.

5.2 Structure information

Since 1990, when the first experimental lipase structures became available (Winkler et al., 1990; Brady et al., 1990), the interaction of lipases with substrate-analogous inhibitors was investigated to understand the structural basis of catalytic activity, substrate specificity and stereoselectivity, and to identify crucial residues. Although their sequences show no overall similarity, all microbial lipases are members of the α/β hydrolase fold family (Brzozowski et al., 1991; Derewenda et al., 1992; Martinez et al., 1992; Ollis et al., 1992; Grochulski et al., 1994; Uppenberg et al., 1995; Schrag et al., 1997). The α/β hydrolase fold consists of a central hydrophobic eight-stranded β-sheet packed between two layers of amphiphilic α-helices. α/β Hydrolases have a common catalytic mechanism of ester hydrolysis, which consists of five subsequent steps (Cygler et al., 1994): after binding of the ester substrate, a first tetrahedral intermediate is formed by nucleophilic attack of the catalytic serine, with the oxyanion stabilized by two or three hydrogen bonds, the so-called oxyanion hole. The ester bond is cleaved and the alcohol moiety leaves the enzyme. In a last step, the acyl enzyme is hydrolyzed. The nucleophilic attack by the catalytic serine is mediated by the catalytic histidine and aspartic (or glutamic) acid. While the geometry of the catalytic machinery is highly conserved, size and shape of the substrate binding site vary considerably. In most lipases, a mobile element covers the catalytic site in the inactive form of the lipase. This 'lid' consists of one or two short α-helices linked to the body of the lipase by flexible structure elements. In the open, active form of the lipase, the lid moves away and makes the binding site accessible to the substrate.

At the time of this publication, structures of 11 microbial lipases have been published in the Protein Data Bank PDB (Bernstein et al., 1977). They can be grouped in five homologous families:

1. *Candida antarctica* lipase B (PDB entries 1TCA, 1TCB, 1TCC, 1LBS, 1LBT);
2. *Pseudomonas* lipases: *P. cepacia* (recently reclassified as *Burkholderia cepacia*) (1LIP, 3LIP, 1OIL), *P. glumae* (1CVL, 1TAH);
3. Filamentous fungi lipases: *Rhizomucor miehei* (1TGL, 3TGL, 4TGL, 5TGL), *Rhizopus* (1LGY, 1TIC), *Humicola lanuginosa* (1TIB), *Penicillium camembertii* (1TIA)

4. Other yeast lipases: *Candida rugosa* (1CRL,1LPM, 1LPN, 1LPO, 1LPP, 1LPS, 1TRH), *Geotrichum candidum* (1THG), and the structurally similar *Candida cylindracea* cholesterol esterase (1CLE)
5. *Fusarium solani* cutinase (1AGY, 1CEX,1CUS, 2CUT, 1OXM, 1XZK, 1XZL, 1XZM, and 31 entries on mutants).

For each family, at least one structure of a complex with a substrate-analogous phosphonate, phosphate or sulfonate inhibitor is available. Comparison of open and closed structures revealed the conformational changes which occur upon binding of a substrate. For lipases of families 2, 3, and 4, the most prominent conformational change is the opening of the lid; *Fusarium solani* cutinase seems not to have a lid, while for *Candida antarctica* lipase B a closed structure has not yet been crystallized, and thus a lid could not yet be assigned. In addition, the conformation of the oxyanion hole differs between closed and open form in *Pseudomonas* and *Candida rugosa* lipases, while the oxyanion hole is pre-formed in filamentous fungi lipases. Apart from these conformational changes upon activation of the lipase, binding of a triacylglycerol analogous inhibitor induces only minor changes of side chain conformation, as concluded from open structures with and without inhibitor for *F. solani* cutinase and *P. cepacia* lipase. This is different from binding of the two enantiomers of a chiral menthyl ester analog to *C. rugosa* lipase: conformational changes induced by binding have been attributed to be the molecular reason for the high stereoselectivity toward secondary alcohols (Cygler et al., 1994).

In all lipases, the substrate binding site is located inside a deep, elliptical pocket on top of the central β-sheet. Shape of the binding sites and binding of the scissile fatty acid differ among the lipases. They have been assigned to three classes (Pleiss et al., 1998): (1) lipases with a hydrophobic, crevice-like binding site located near the protein surface (lipases from *Rhizomucor* and *Rhizopus*); (2) lipases with a funnel-like binding site (lipases from *Candida antarctica*, *Pseudomonas* and cutinase); and (3) lipases with a tunnel-like binding site (lipases from *Candida rugosa* and *Geotrichum candidum*).

Only for two microbial lipases, *Pseudomonas cepacia* lipase and *Fusarium solani* cutinase, has the binding of triacylglyerol analogous inhibitors yet been studied. For *Pseudomonas* lipase, the structure has been solved of a complex with one enantiomer of a chiral substrate analogous inhibitors, (R_C,S_P)-1,2-dioctylcarbamoylglycero-3-*O*-*p*-nitrophenyl octylphosphonate, covalently bound to the catalytic serine (Lang et al., 1998). The inhibitor adopted a bent tuning fork conformation. Four binding pockets for the triacylglycerol analogue were detected: the oxyanion hole, two hydrophobic pockets and one more hydrophilic pocket, which accommodate the three substituents of the inhibitor. Interaction with substrate is dominated by Van der Waals interactions; in addition, a hydrogen bond to the carbonyl oxygen of the *sn*-2 chain contributes to fixation of the position of the inhibitor. Since the S_C enantiomer was not experimentally resolved, the interaction between this less preferred enantiomer and the lipase was modeled. Clashes between a carbonyl oxygen with the C-terminal neighbor L287 of the catalytic histidine and I290 were identified as determinants of stereoselectivity. In a complex of *Fusarium solani* cutinase with (*R*)-1,2-dibutyl-carbamoylglycero-3-*O*-*p*-nitrophenylbutyl-phosphonate (Longhi et al., 1997), the inhibitor also bound in a fork-like shape, with the *sn*-3 chain binding to a small

pocket at the bottom of the active site crevice, and the two dibutyl-carbamoyl chains pointing towards the surface of the protein. It was concluded that the size of the *sn*-3 binding pocket would be responsible for the preference of cutinase for short-chain fatty acids at *sn*-3 position (Mannesse et al., 1995). The chain length specificity of cutinase for the *sn*-1 and *sn*-2 chain, however, could not be explained.

5.3 Modeling and engineering of fatty acid specificity

Using molecular modeling and subsequent verification by site-directed mutagenesis, the relationships between fatty acid chain length specificity and shape or physicochemical properties of binding sites have been investigated. Interesting properties of lipases are specificities toward unsaturated fatty acid chains and length of saturated chains.

Geotrichum candidum lipase I (GCL I) has been shown to have a unique preference for long-chain *cis* (Δ-9) unsaturated acyl chains. This preference is lacking in the highly homologous lipase II of the same organism. By comparing sequence and specificity of hybrids between these two lipases, amino acids were identified which mediate recognition of unsaturated acyl chains (Holmquist et al., 1997). Crucial residues are located at the entrance of the active site and at the bottom of the active site cavity of GCL I. Replacing four residues of GCL I by the corresponding residues from GCL II led to a specificity profile similar to that of GCL II. The reverse mutations in GCL II only partially recovered *cis* (Δ-9) specificity, however.

The family of filamentous fungi lipases has been most extensively investigated for structure-function relationship of chain length specificity. Based on the X-ray structure of a complex of *Rhizomucor miehei* lipase with *n*-hexylphosphonate ethyl ester (Brzozowski et al., 1991), binding of the scissile fatty acid of triacylglycerol substrates have been modeled for *Rhizopus, Rhizomucor miehei* and *Humicola lanuginosa* lipase (Vasel et al., 1993; Lawson et al., 1994; Norin et al., 1994; Klein et al., 1997; Pleiss et al., 1998). The binding pocket of *Rhizomucor miehei* lipase (RML) is a shallow bowl with a long axis of 18 Å, and a width of 4.5 Å at its base, and 6 Å at the protein surface (Figure 1). The scissile fatty acid up to C_8 binds to a cleft at the bottom of the binding pocket. Its bottom is formed by side chains of residues P177, H108 and the catalytic S144. It is lined by V205 and D91 on its left- and right-hand side, respectively. From C_8 to C_{10}, the fatty acid chain rises at the wall of the binding pocket and enters a hydrophobic crevice up to C_{18} (length 9.5 Å), which runs parallel to the surface of the protein. Its width of 5.5 Å at the bottom gives the fatty acid room for movement. Its bottom is formed by residues P209 and P210, its left- and right-hand wall by L208 and F94/F213, respectively.

Location and properties of the scissile fatty acid binding site has been supported by creating mutants of lipases from *Rhizopus* and *Humicola lanuginosa* with altered fatty acid chain length profile (Joerger and Haas, 1994; Atomi et al., 1996; Martinelle et al., 1996; Klein et al., 1997; see also Chapter 4). Experimental effects of mutations in the scissile fatty acid binding site of *Rhizopus delemar* and *Rhizopus oryzae* lipases (Table 1) can be explained by the position of the residue and its interaction with the fatty acid chain (Figure 2). V205 lines the cleft at the bottom of the

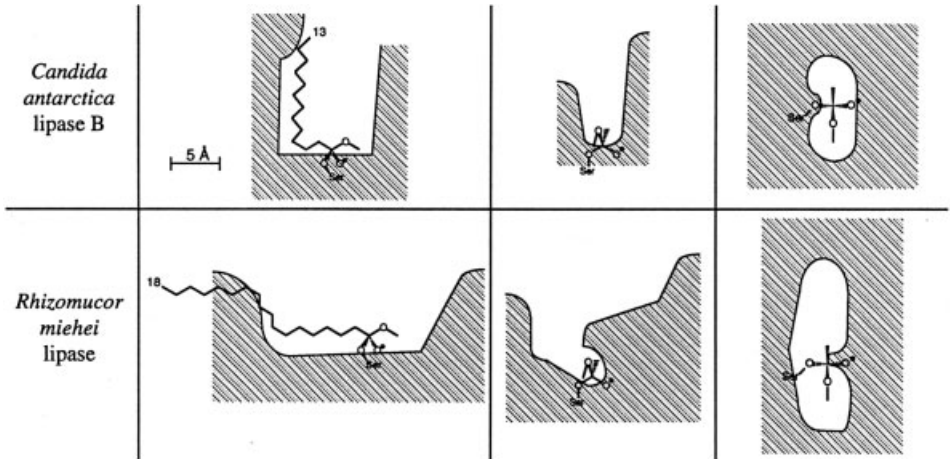

Figure 1. Comparison of the shape of binding pockets of *Rhizomucor miehei* lipase (RML) and *Candida antarctica* lipase B (CALB) (Pleiss et al., 1998). Three perpendicular cross-sections through the proteins are shown. A model of the fatty acid is displayed; a number indicates the length of the longest fatty acid which completely binds inside the binding pocket.

Table 1. Mutations in the scissile fatty acid binding site of lipases from *Rhizopus delemar* and *Rhizopus oryzae*, and their effect on chain length specificity (Pleiss et al., 1998). Equivalent residues in the homologous RML (see Figure 2) are given in parentheses.

Source	Mutation	Structural effect of the mutation	Experimental observation
R. oryzae	F95Y (F94)	Blocks binding at C_{12}	60 % (30 %) increase of caproic acid methyl ester relative to oleic (stearic) acid
R. delemar	F95D (F94)	Hydrophilic at C_{12}	2- fold decrease of hydrolysis
R. delemar	F95D (F94) F214R (F213)	Right wall of hydrophobic crevice becomes hydrophilic (C_{10}–C_{16})	3-fold increase of tricaprylin relative to triolein
R. delemar	F112W (F111)	Blocks binding at C_6	50 % increase of tributyrin relative to triolein
R. delemar	F112Q (F111)	Hydrophilic at C_6	no activity
R. delemar	V206T (V205)	Hydrophilic at C_4	10–20 % activity
R. delemar	V209W (L208)	Blocks binding at C_{10}	2-fold increase of tributyrin relative to triolein
R. delemar	V209W (L208) F112W (F111)	Blocks binding at C_{10} and C_6	80-fold increase of tributyrin relative to tricaprylin; no triolein hydrolysis
R. oryzae	F214Y (F213)	Blocks binding at C_{16}	20 % increase of caproic acid methyl ester relative to oleic and stearic acid

binding pocket and is positioned near C_4 of the scissile fatty acid chain. Replacing it by the hydrophilic threonine decreases activity 5- to 10-fold. Replacing F111, which is near to C_6, by tryptophan increases the relative specificity for short-chain triglycerides. L208 and F94 line the hydrophobic crevice near C_{10} and C_{12}. Replacing them by bulkier and more hydrophilic residues decreases activity and increases relative specificity for fatty acids of short and medium chain length. F213 is located at the end of the hydrophobic crevice near C_{16}. Replacing it by the more bulky and hydrophilic tyrosine reduces relative specificity for oleic and stearic acid. As a strategy to predict mutants with specificity for chain lengths below a given cut-off, binding of longer chains can be inhibited by increasing size and polarity of a residue positioned at the ω-end of the longest chain to be accepted. Binding of substrates of different chain length might also influence flexibility of the lipase, as it has been concluded from molecular dynamics simulations of RML with and without inhibitor (Peters et al., 1997). Opening of the lid and binding of fatty acid analogs substantially reduced fluctuations of solvent-exposed loops, thus making the lipase more rigid.

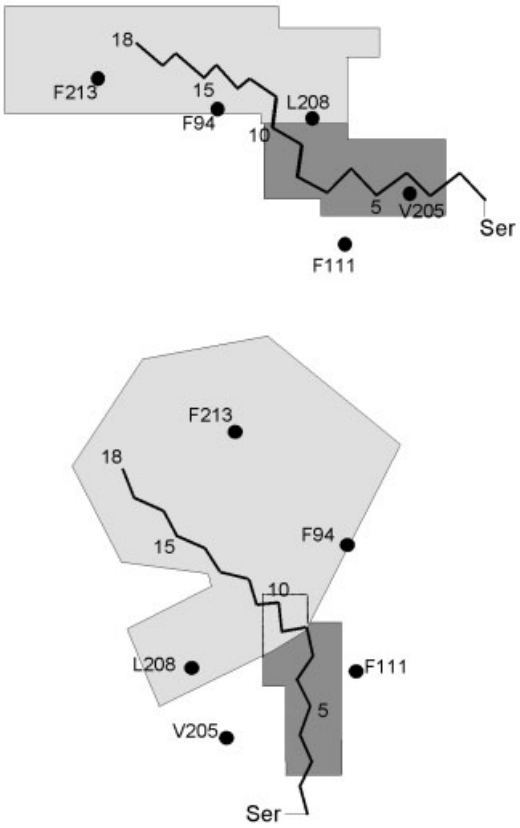

Figure 2. Two perpendicular schematic views of the *Rhizomucor* lipase binding site, and a modeled scissile fatty acid chain of length C_{18} (Pleiss et al., 1998). The narrow bottom of the binding pocket and a hydrophobic patch are shaded dark and light gray, respectively, as calculated by GRID (Goodford, 1985). The position of five residues (see Table 1) which have been shown to mediate chain length specificity in the homologous RDL and ROL are marked as dots.

Candida antarctica lipase B (CALB) is a member of the funnel-like lipases (Pleiss et al., 1998). Its binding pocket is an elliptical, narrow funnel of 9.5 × 4.5 Å (see Figure 1). Up to C_4, the scissile fatty acid binds to a cleft at the hydrophilic bottom of the funnel, which is formed by D134 and the catalytic S105. It is lined by T138, I189, and V190 on its left-hand side, by Q157 and the oxyanion hole residue T40 on its right-hand side. At the end of this cleft, near Q157, the fatty acid kinks sharply and follows the left-hand wall. Up to C_7, the binding site is a narrow cleft, from C_7 to C_{13} it becomes smooth and hydrophobic. This hydrophobic binding site is formed by V154, I285, L144 and V149. Its width decreases with the distance from the active site with its minimum at C_{13} of the fatty acid chain. Here, at 13.5 Å above the bottom, it reaches the end of the funnel. Compared to the long, well-defined hydrophobic crevice of RML, the scissile fatty acid binding site of CALB is relatively short (C_{13}) and has a small hydrophobic area located at the wall of the binding funnel. Based on shape and physico-chemical properties of the binding pocket, chain length preference of CALB is expected to be shifted towards shorter chains compared to RML. Under experimental conditions, where the scissile fatty acid binding site is the primary determinant for chain length specificity (Rangheard et al., 1992), CALB has indeed high activity for short and medium chain length fatty acids and decreasing activity for long-chain fatty acids (Figure 3), while RML has relatively low activity for short-chain fatty acids, but increasing activity for longer-chain fatty acids (Rangheard et al., 1992; Kirk et al., 1992). If, however, assays are used which involve activity at a water-substrate interface, chain length dependency may change drastically, as it has been shown for the hydrolysis of triglycerides by RML (Berger and Schneider, 1991). In addition to the scissile fatty acid binding site, other structural elements will play a role in mediating chain length specificity, such as the alcohol binding site and the lid. W89 in *Humicola lanuginosa* lipase is part of the lid and does not contribute to the scissile fatty acid binding site. However, mutating W89 resulted in a change of the chain length profile (Martinelle et al., 1996), which has been assigned to effects on binding the acyl chain of the substrate.

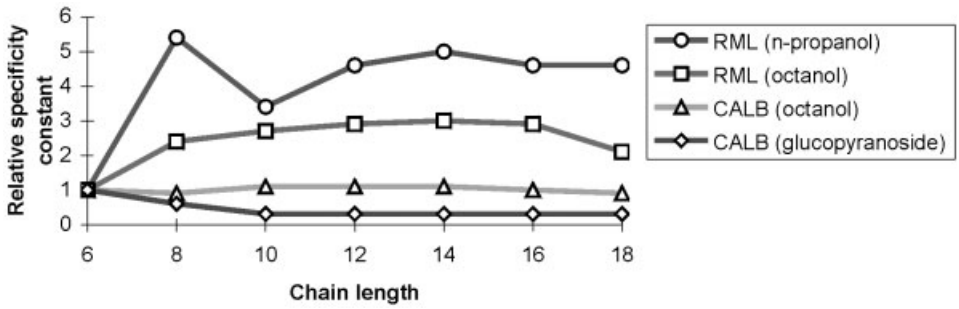

Figure 3. Relative specificity constants k_{cat}/K_m of RML and CALB (normalized to C_6) for fatty acid chains of varying length (Pleiss et al., 1998); RML: alcoholysis of monoester by *n*-propanol (Rangheard et al., 1992) and esterification of *n*-octanol in hexane (Kirk et al., 1992); CALB: esterification of *n*-octanol in hexane (Kirk et al., 1992) and esterification of ethyl D-glucopyranoside (Kirk et al., 1992).

5.4 Modeling and engineering of stereoselectivity

To date, there is no general rule to explain the *sn*-1(3) stereopreference of all lipases toward triacylglycerol and *sn*-2 substituted analogs. It has been shown experimentally that stereopreference and stereoselectivity toward triacylglycerol and analogs depend on both substrate structure and the type of lipase used as catalyst (Paltauf and Wagner, 1976; Ransac et al., 1990; Rogalska et al., 1993; 1995; Stadler et al., 1995; Kovac et al., 1996). This is in contrast to lipase stereopreference toward chiral secondary alcohols, where all lipases prefer the same enantiomer. A rule to predict the preferred enantiomer has been proposed which is based exclusively on structure of the secondary alcohol (Kazlauskas et al., 1991). Generalization of this rule to primary alcohols was restricted to alcohols without oxygen bound at the stereocenter (Weissfloch and Kazlauskas, 1995); thus, it is not applicable to triacylglycerols. For *Mucorales* lipases, a simple method to explain and predict stereoselectivity toward this type of substrates has been proposed (Haalck et al., 1997; Holzwarth et al., 1997; Scheib et al., 1998; 1999). It is based on docking of the substrate to the binding site of *Rhizopus oryzae* lipase (ROL) or RML, and uses molecular dynamics simulations to relax both substrate and protein side chains. Two hydrophobic patches in the binding site of ROL and RML were identified to which the acyl chains of the substrate were assumed to bind: the deep *hydrophobic crevice* consists of the amino acids T83, A89, I93, F95, F112, L146, P178, V206, V209, P210, and F216 (numbering in ROL), and the shallow *hydrophobic dent* which comprises I205, T252, L254, and L258. Each substrate was docked in two orientations: in *sn*-1 orientation, where the *sn*-1 fatty

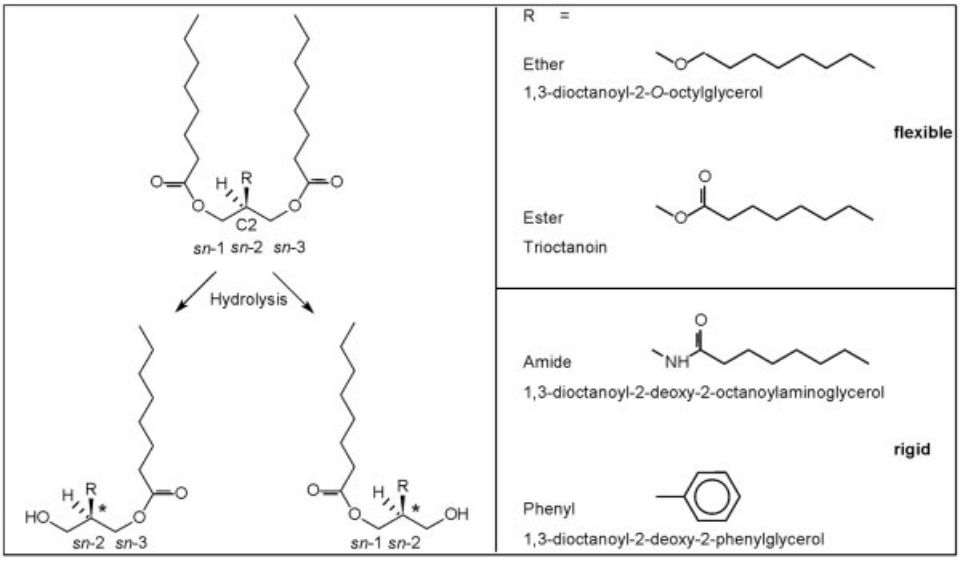

Figure 4. Left: stereoselective hydrolysis of triacylglycerols and analogs to *sn*-1,2- or *sn*-2,3-diacylglycerols and analogs (Scheib et al., 1998). Right: *sn*-2 substituents attached to the (pro-) chiral C2 of glycerol with trivial (top) and systematic (bottom) substrate names. Ether and ester substrates are classified as 'flexible', amide and phenyl substrates as 'rigid'.

acid chain binds to the hydrophobic crevice and is, subsequently, cleaved off; and in *sn*-3 orientation, where the *sn*-3 fatty acid chain binds to the hydrophobic crevice. The hydrophobic dent is assumed to bind the *sn*-2 substituent.

In order to evaluate the influence of flexibility, size and polarity of the *sn*-2 substituent on stereoselectivity, triacylglycerol was compared to the more flexible ether derivative and to the more bulky amide and phenyl derivatives (Figure 4). The suggested model explained why ROL hydrolyzes substrates with an ester or ether group at *sn*-2 preferably in *sn*-1 position, while the amide and phenyl substrates were hydrolyzed in *sn*-3. It also explained why RML, although it is homologous to ROL, hydrolyzes all substrates in *sn*-1.

The catalytic H257 and L258 in ROL and RML form a binding pocket in the hydrophobic dent. All substrates bind with the *sn*-2 substituent close to C_2 of glycerol between the side chains of these two residues, the *His gap.* All substrates interacted sterically with the side chain of L258 in *sn*-1 orientation, but less in *sn*-3 orientation. It was concluded that this steric interaction governs stereoselectivity: for *rigid* and bulky substrates, the *sn*-1 orientation is less favorable than for *flexible* substrates. In *sn*-3 orientation, flexible and rigid substrates adjusted better to the *His gap*, by a conformational change of the glycerol backbone. This adaptation can be directly evaluated by measuring torsion $Ø_{O3-C3}$ around the bond between the hydrolyzed ester oxygen and glycerol C_3 of the substrate in *sn*-3 orientation. It was shown that this torsion angle discriminates between rigid and flexible triradylglycerols: it was 160° to 170° for flexible substrates, but about 120° for rigid substrates (Table 2). For both *Mucorales* lipases, the value of this torsion angle correlated well with experimentally determined stereoselectivity (Figure 5).

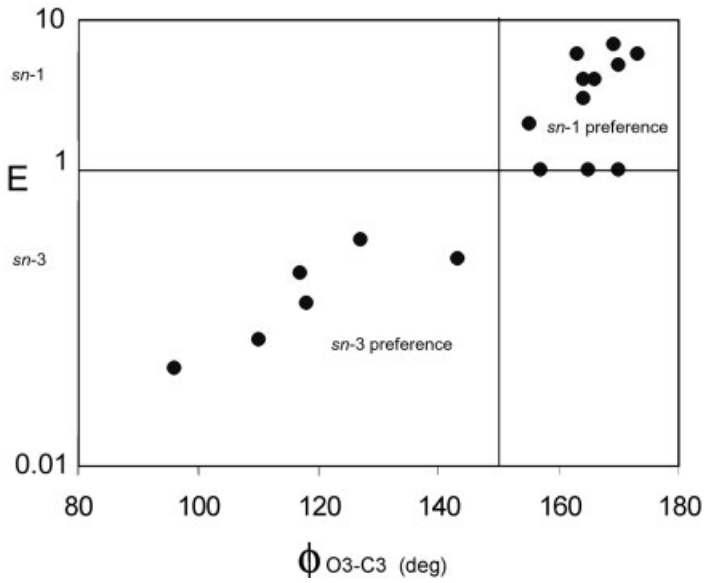

Figure 5. Correlation of experimental E values and torsion angle $Ø_{O3-C3;}$ for *sn*-1 preference, data are directly taken from Table 2; for *sn*-3 preference, reciprocal values of E values are displayed; horizontal and vertical bars divided the data points in two regions: $E \geq 1$ (*sn*-1 preference)/($Ø_{O3-C3} < 150°$) and $E < 1$ (*sn*-3 preference)/($Ø_{O3-C3} < 150°$).

Table 2. Stereopreferences of lipases from *Rhizopus* and *Rhizomucor*, and *Rhizopus* lipase mutants L258X (X = A, S, F); stereopreferences are predicted from Φ_{O3-C3} and compared to experimentally determined stereopreferences and stereoselectivities (enantiomeric excess, ee, and enantioselectivity, E) of hydrolysis. (From Scheib et al., 1999.)

ROL	Φ_{O3-C3} (°)	**Predicted stereopreference**	**Experimentally determined stereopreference**	**ee[1] (%)**	**E[2]**
Wild-type					
Ether substrate	164	*sn*-1	*sn*-1	61 (± 2)	4
Ester substrate	170	*sn*-1	*sn*-1	19 (± 5)	1
Amide substrate	117	*sn*-3	*sn*-3	63 (± 6)	5
Phenyl substrate	118	*sn*-3	*sn*-3	77 (± 3)	8
L258A					
Ether substrate	n.d.	n.d.	*sn*-1	50 (± 5)	3
Ester substrate	157	*sn*-1	*sn*-1	16 (± 4)	1
Amide substrate	127	*sn*-3	*sn*-3	52 (± 4)	3
Phenyl substrate	170	*sn*-1	*sn*-1	68 (± 5)	5
L258S					
Ether substrate	n.d.	n.d.	*sn*-1	65 (± 4)	5
Ester substrate	165	*sn*-1	*sn*-1	14 (± 3)	1
Amide substrate	143	*sn*-3	*sn*-3	60 (± 3)	4
Phenyl substrate	164	*sn*-1	*sn*-1	53 (± 2)	3
L258F					
Ether substrate	n.d.	n.d.	*sn*-1	63 (± 5)	5
Ester substrate	155	*sn*-1	*sn*-1	23 (± 3)	2
Amide substrate	110	*sn*-3	*sn*-3	86 (± 5)	14
Phenyl substrate	96	*sn*-3	*sn*-3	91 (± 2)	22

RML	Φ_{O3-C3}(°)	**Predicted stereopreference**	**Experimentally determined stereopreference**	ee[1] (%)	E[2]
Ether substrate	163	*sn*-1	*sn*-1	69 (± 4)	6
Ester substrate	169	*sn*-1	*sn*-1	73 (± 3)	7
Amid substrate	166	*sn*-1	*sn*-1	56 (± 2)	4
Phenyl substrate	173	*sn*-1	*sn*-1	68 (± 2)	6

[1] $ee = \frac{[A]-[B]}{[A]+[B]} \times 100$, if [A] > [B] (n = 12)

[2] $E = \frac{\ln(1-c(1+ee_P))}{\ln(1-c(1-ee_P))}$, if conversion c < 10 %

n.d.: not determined.

The side chain of L258 was identified as the major factor to discriminate between *sn*-1 and *sn*-3 orientation. To verify and apply this model, the size of this residue was changed by mutating it to the smaller alanine, the more polar serine, and the bulky phenylalanine. As predicted by the model, decreasing its size decreased repulsion of bulky *sn*-2 substituents, thus allowing the substrate to move into the His gap and shifting stereoselectivity toward *sn*-1. This effect is most prominent for the bulky and rigid phenyl substituent. For this substrate, a switch in stereopreference from *sn*-3 to *sn*-1 occurred. For the rigid but smaller amide substrate and for the flexible ester, geometry and stereoselectivity did not change considerably compared to wild-type ROL. For all substrates, enantiomeric excess values of L258A and L258S are similar. Therefore, the stereoselectivity-determining interaction was assumed to be steric rather than polar.

Increasing the size of residue 258 by replacing leucine by phenylalanine had an even more dramatic effect on the geometry of binding and thus of stereoselectivity. Since rigid substrates are pushed out of the gap in *sn*-1 orientation, *sn*-3 stereoselectivity is considerably increased. Flexible substrates are still accommodated in the gap. Hence, only moderate changes of stereoselectivity towards flexible substrates were observed.

While the sequences of ROL and RML are highly similar, and notably L258 is conserved, the stereoselectivity of both enzymes is different: in contrast to ROL, RML hydrolyzes flexible and rigid substrates in *sn*-1. The only nonconservative difference in the substrate binding site-A89 in ROL, W88 in RML-has been shown not to play a role in stereoselectivity: replacing A89 in ROL by tryptophan did not affect stereoselectivity (Scheib et al., 1999). Therefore, it is not obvious which residues mediate stereoselectivity. Using protein modeling and engineering, a second layer of residues was identified which are relevant for function (Beer et al., 1996). Although these residues are not in contact with the substrate, they stabilize the catalytic machinery by a hydrogen network. The authors postulated that H144 in ROL plays a key role in the positioning of the active site H257. Replacing E265 by aspartate resulted in a breakdown of the active site geometry, supposedly due to a new H-bond between H144 and the carboxyl group of D265. Also, packing of the aspartate side chain close to H144 should increase steric hindrance between both residues. These second layer residues also seemed to play a role in stereoselectivity. As a major difference between ROL and RML, triacylglycerol substrates were pushed out of the *His gap* in RML relative to ROL. This is caused by interaction with G266, which is located close to C_2 of glycerol in a short loop, the *G-elbow* loop (Figure 6). While G266 is conserved in both enzymes, the G-elbow loop is shifted toward the binding site in RML as compared to ROL. The neighboring residues to G266 vary in Mucorales lipases. Its *N*-terminal neighbor (T265 in RML, E265 in ROL) interacts with H143 or H144 in RML and ROL, respectively. Because of packing restraints, the side chain of E265 points away from H144 in ROL. In RML, however, T265 is small enough to orient its side chain toward H143 forming a hydrogen bond. Hence, C_α of T265 in RML is located closer to H143 than E265 in ROL, thus shifting the G-elbow loop toward the substrate binding site. Since the substrate binds less deeply in RML, the effect on stereoselectivity is similar to increasing the size of the *His gap*: flexible and rigid substrates bind with a similar geometry and stereoselectivity shifts toward *sn*-1. Thus, the observed differences

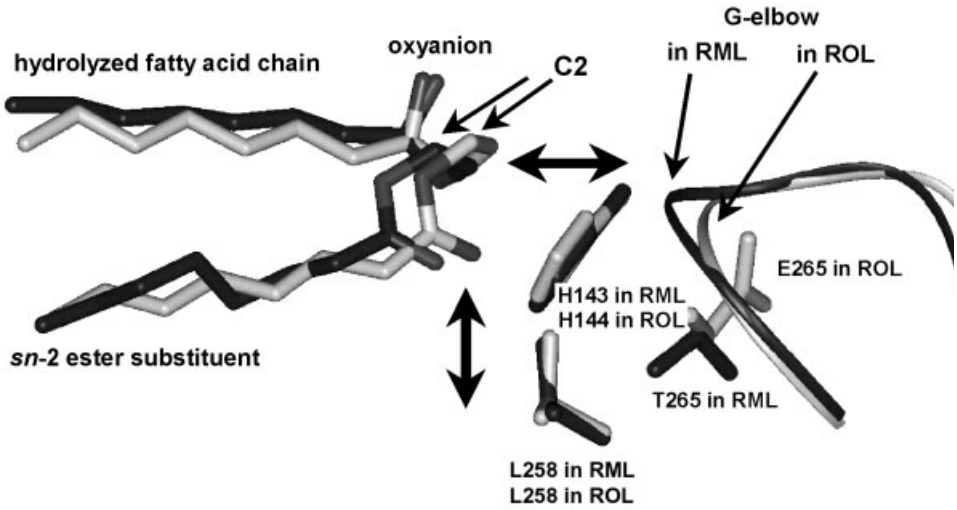

Figure 6. Ester substrate in *sn*-1 orientation in *Rhizopus oryzae* lipase (ROL, light gray) and *Rhizomucor miehei* lipase (RML, dark gray) (Scheib et al., 1999). The position of the G266 varies in both lipases ($C_\alpha - C_\alpha$ distance = 1.3 Å) due to the different position of E265 in ROL and T265 in RML, respectively (horizontal arrow). Therefore, in ROL, the substrate binds deeper into the binding site than in RML ($C2_{RML} - C2_{ROL} = 0.9$ Å). Hence, steric interactions between the *sn*-2 substituent and L258 are more pronounced in ROL rather than RML (vertical arrow). (See color plate, page XVIII).

in stereoselectivity result from residues which do not directly contact the substrate, but have an indirect impact on the binding of triradylglycerols into the substrate binding site.

Indirect, long-ranging effects are playing a role in all cases where residues far away from the binding site mediate substrate recognition. They interact either by changing the structure of the lipase and thus the shape of the binding site, by changing the physico-chemical properties of the protein, by changing its dynamics and the flexibility of the binding site, or via long-range electrostatic interactions with the substrate. While the role of residues directly interacting with substrate are fairly well understood, the role of more distant residues is mostly obscure. Despite the successes in engineering substrate binding sites, folding of a protein and the contribution of individual residues to overall structure and dynamics remains an open question.

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6 Lipase-Catalyzed Synthesis of Regioisomerically Pure Mono- and Diglycerides

B. Aha, M. Berger, B. Jakob, G. Machmüller, C. Waldinger and M.P. Schneider

6.1 Introduction

Agricultural crops such as oil seeds and numerous cereals represent a considerable reservoir of useful and low-cost raw materials such as lipids (fats and oils, phospholipids etc.), proteins and carbohydrates.

By selective combination of their molecular constituents (i.e. fatty acids, glycerol, oligopeptides, amino acids and saccharides) – using both chemical and biocatalytic methods – a wide variety of surface active materials can be prepared, all of which are highly biodegradable as a result of their molecular structures (Figure 1).

Lipases are well-established biocatalysts for the enantio- and regioselective formation and hydrolysis of ester bonds in a wide variety of natural and unnatural substrates. They therefore seemed ideally suited also for the bioconversion of the above-mentioned plant materials and the formation of combination products with surface active properties such as partial (mono-)glycerides, N-acylated amino acids and protein hydrolysates as well as sugar esters (Figure 2).

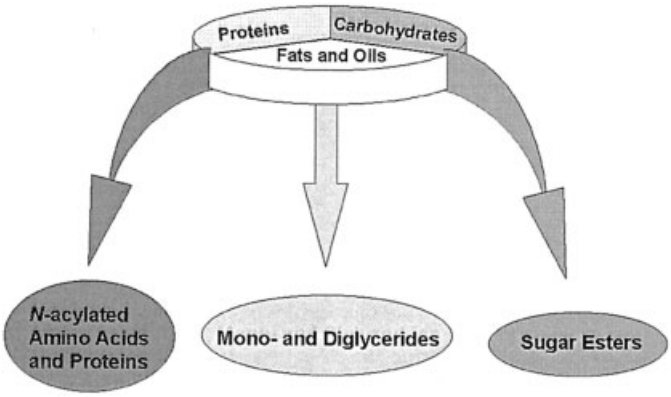

Figure 1. Surface-active compounds from plant materials.

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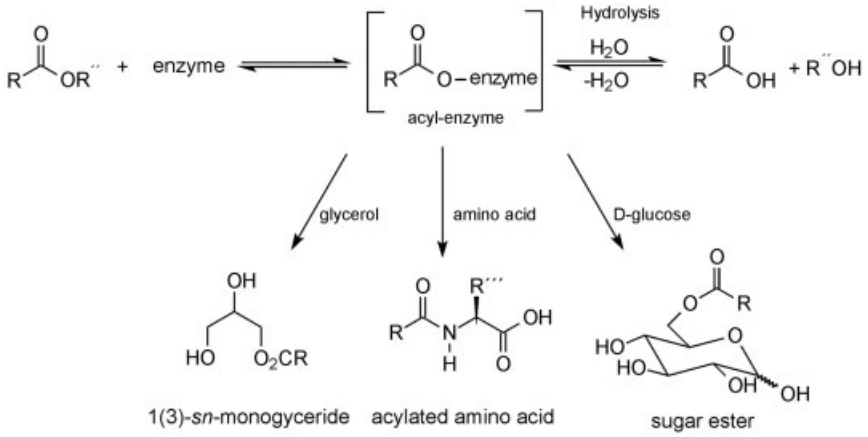


Figure 2. Surface-active compounds via enzymatic acyl transfer.

6.2 Partial glycerides

Mixtures of long-chain monoglycerides and other partial glycerides of varying composition and constitution are widely employed as non-ionic surfactants (emulsifiers) in the processing of foods and related applications (Sonntag, 1982a; Porter, 1991; Lie Ken Jie et al., 1997; Gunstone, 1999). They also serve as starting materials for the preparation of numerous conjugates, e.g. with citric, lactic, tartaric and acetic acid (Aebi et al., 1978).

Isomeric mixtures of such 'monoglycerides' are classically produced by alcoholysis of the corresponding triacylglycerols with two equivalents of glycerol in the presence of metal catalysts at temperatures of 210–240 °C (Lauridsen, 1976; Sonntag, 1982b) (Figure 3). The resulting mixtures contain only ca. 50–60 % of the desired monoglycerides as isomeric mixtures together with isomeric diglycerides, triglycerides and free fatty acids.

Due to the high temperatures employed, these materials are usually colored and not free of odor. 'Monoglycerides' of higher chemical (not isomeric!) purity (> 90 %) can only be obtained by cost- and energy-intensive molecular distillation of these crude mixtures.

6.3 Biotechnological routes to mono- and diglycerides

Enzymes catalyze a wide variety of organic reactions under mild conditions and frequently in a highly chemo-, regio- and stereoselective manner (Schmid and Verger, 1998; Bornscheuer and Kazlauskas, 1999; Gunstone, 1999). This is particularly true for numerous lipases which are available from a wide variety of commercial sources. Consequently, the use of biocatalysts for the preparation of partial glycer-

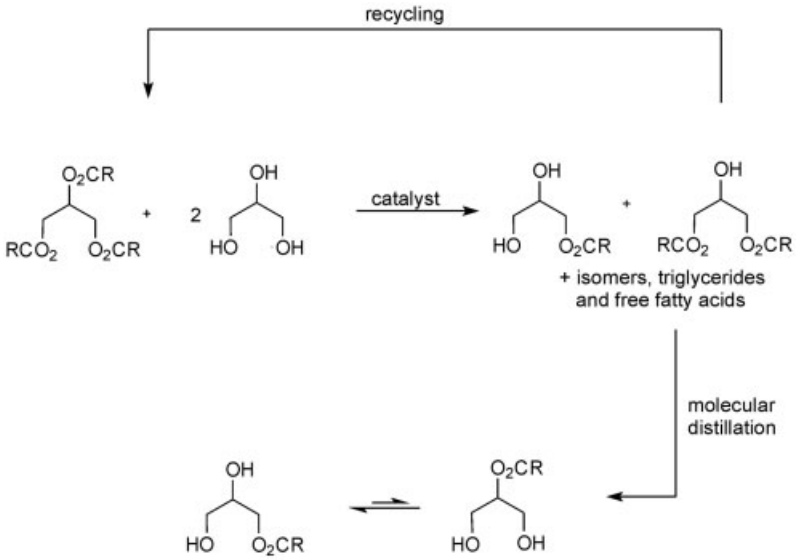


Figure 3. Technical synthesis of 'monoglycerides'.

ides could provide numerous advantages as compared to the above-described, conventional methods such as increased selectivity, higher product purity and quality, energy conservation, and the omission of toxic catalysts (Figure 4).

It is not surprising therefore, that a number of research groups became recently engaged in the synthesis of monoglycerides by lipase-catalyzed reactions (Holmberg and Osterberg, 1988; Weiss, 1990; Hayes and Gulari, 1991; Schneider and Berger, 1992) and the corresponding modification of natural fats and oils. Frequently, however, mixtures of partial glycerides and their isomers are observed in these cases. In this chapter, only selective transformations leading to isomerically pure mono- and diglycerides of defined chemical structures are addressed. Furthermore, we are concentrating here on methods that lead to the title compounds directly from glycerol and various acyl donors, i.e. without additional derivatizations or the use of perma-

- **Starting materials: natural fats and oils**
- **Natural biocatalysts: immobilised and recycled**
- **Non-toxic solvents: recycled**
- **Reaction conditions: mild, neutral pH**
- **Energy consumption: low, room temperature or slightly above**
- **Selectivities: high**
- **Products: natural**
- **Properties: pure isomers, colorless, odorless**

Figure 4. Biotechnological routes to regioisomerically pure mono- and diglycerides: advantages.

nent or temporary protection groups. In this sense, two general routes to the desired molecules are available in principle (Figure 5):

- hydrolysis/alcoholysis of triglycerides; and
- esterification of glycerol;

For stoichiometric reasons it is obvious that only the alcoholysis of triglycerides with glycerol or the direct esterification of glycerol can produce the desired molecules in high yields.

Thus, under carefully controlled conditions and at very low temperatures, the glycerolysis of triglycerides was reported to lead to high yields of these molecules – again as mixtures of regioisomers (McNeill and Yamane, 1991; McNeill et al., 1991). Rapid acyl migrations (Schmid and Verger, 1998; Gunstone, 1999), dominant under aqueous or protic conditions have in fact so far prevented the enzymatic preparation of isomerically pure 1(3)-*sn*-monoacylglycerols.

It was found recently that 1(3)-*sn*-monoacylglycerols are quite stable towards acyl group migrations in aprotic solvents with low water content ($< 2\ \%$) (Robbins and Nicholson, 1987; Berger and Schneider, 1991). Based on this observation, it was felt that the synthesis of isomerically pure 1(3)-*sn*-monoacylglycerols could be achieved by direct enzyme-catalyzed esterifications in such solvents.

Unfortunately, glycerol is immiscible with nonpolar organic solvents, and all earlier attempts at its direct enzymatic esterification in these media have been unsuccessful (Figure 6).

It was found that this basic problem could easily be overcome by prior adsorption of glycerol onto a solid support. Presumably this process creates an artificial liquid – liquid interphase on the support surface, thus creating conditions which are generally thought to be involved in lipase-catalyzed transformations of glycerides, e.g. natural fats and oils.

Typically, glycerol and the corresponding inert solid support (silica gel, active carbon etc.) are mechanically mixed until free-flowing 'dry' powders are obtained

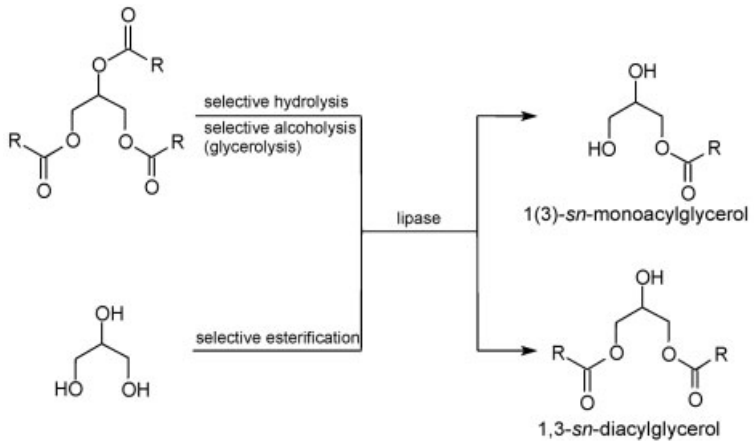


Figure 5. Possible enzymatic routes to isomerically pure mono- and diglycerides.

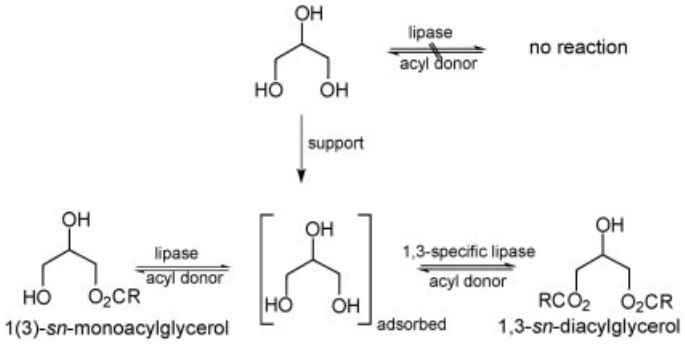


Figure 6. Enzymatic esterification of glycerol in organic media.

which contain up to 50 % (w/w) of glycerol. The new compositions of matter obtained in this way behave completely different from glycerol itself. Suspensions of these materials in nonpolar organic solvents (e.g. *n*-hexane or *t*-BuOMe) react as if the glycerol were dissolved homogeneously in these media. They are treated with the corresponding acyl donor (saturated or unsaturated free fatty acid, fatty acid methyl ester, fatty acid vinyl ester, triglycerides or natural fats and oils) and a variety of lipases (Berger et al., 1992; Berger and Schneider, 1992; Schneider and Berger, 1992; 1993; Berger, 1993; Waldinger and Schneider, 1996). The acyl transfer reactions proceed under these conditions smoothly and without problems, their progress can be monitored conveniently by thin-layer chromatography (TLC).

On completion of the esterification, both the enzyme and the solid support can be removed by simple filtration or centrifugation. After evaporation of the solvent, the resulting glycerides were isolated in high yields and purified further, preferably by recrystallization.

It is interesting to note that our proprietary method (Berger and Schneider, 1991; 1992; Schneider and Berger, 1992; 1993), i.e., the immobilization of glycerol onto a solid support – the key to the solubility problem – and subsequent enzymatic esterification is now used by several other researchers (Charlemange and Legoy, 1995; Kwon et al., 1995; Castillio et al., 1997, Selmi et al., 1997; Stamatis et al., 1998; Elfman-Borjesson and Härröd, 1999), a clear indication for the major breakthrough which was achieved in the direct enzymatic acylation of glycerol.

6.4 Synthesis of regioisomerically pure 1,3-*sn*-diglycerides

Using a molar ratio of glycerol and acyl donor of 1 : 2, numerous isomerically pure 1,3-*sn*-diacylglycerols were prepared in very high yields and purities. Examples for preparations of this type are given in Figure 7 (using fatty acids), Figure 8 (using fatty acid methyl esters) and Figure 9 (using fatty acid vinyl esters).

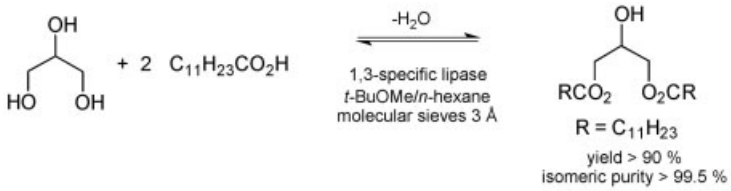


Figure 7. 1,3-Diglycerides from fatty acids.

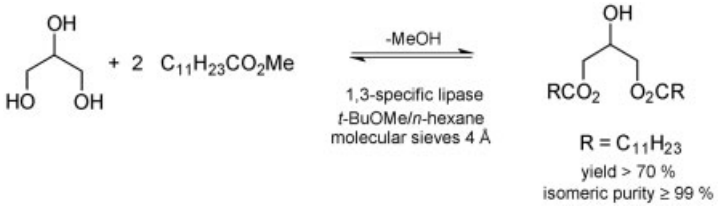


Figure 8. 1,3-Diglycerides from fatty acid methyl esters.

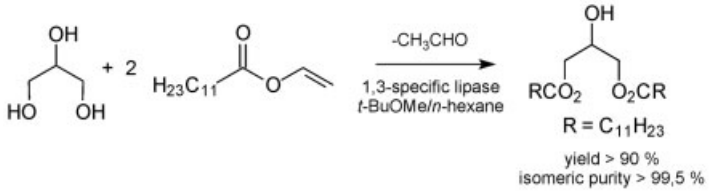


Figure 9. 1,3-Diglycerides from fatty acid vinyl esters.

As is clear from Table 1, the method is well suited for the preparation of isomerically pure 1,3-*sn*-diacylglycerols with fatty acid residues of widely variable chain length and also degree of unsaturation.

Due to the simplicity of the purification process by recrystallization, the method is best suited for the preparation of saturated 1,3-*sn*-diacylglycerols of moderate (C_8) to long (C_{18}) chain lengths.

Isomerically pure 1,3-*sn*-diglycerides proved to be interesting building blocks for numerous synthetic applications, most of them related to selective modifications of the hydroxy group in the 2- position of these molecules. In this respect, we acknowledge the collaboration in this area with the group of Ulbrich-Hofmann (see Chapter 12) (Haftendorn and Ulbrich-Hofmann, 1995; Ulbrich-Hofmann et al., 1998; Haftendorn et al., 2000). Investigations have shown that the 2-hydroxy group of the 1,3-*sn*-diglycerides can be:

- replaced by an amino group (Berger, 1993; Haftendorn and Ulbrich-Hofmann, 1995);
- converted into a phosphate group and isomeric phospholipids with an phosphoethanolamine function at the 2-position (Haftendorn and Ulbrich-Hofmann, 1995);

Table 1. Regioisomerically pure 1,3-*sn*-diglycerides.

Product	Enzyme	Yield (%)	Purity (%)
1,3-*sn*-diacetin	A	75	94
1,3-*sn*-divalerin	A	75	93
1,3-*sn*-dicaproin	A	77	93
1,3-*sn*-dicaprylin	B	86	98
1,3-*sn*-dicaprin	B	84	99
1,3-*sn*-didec-9-enoin	B	84	99
1,3-*sn*-didecanoin	B	84	98
1,3-*sn*-didec-10-enoin	B	85	99
1,3-*sn*-dilaurin	B	85	> 99
1,3-*sn*-dilaurin/myristin	B	72	99
1,3-*sn*-ditridecanoin	B	83	99
1,3-*sn*-dimyristin	B	82	98
1,3-*sn*-dipentadecanoin	B	84	> 99
1,3-*sn*-dipalmitin	B	80	99
1,3-*sn*-distearin	B	81	99
1,3-*sn*-diolein	C	70	98
1,3-*sn*-dilinolein	E	70	97
1,3-*sn*-dierucain	D	71	96
1,3-*sn*-di-(*S*)-corioloin	E	65	98
1,3-*sn*-di-(*R*)-ricinolin	E	61	97
1,3-*sn*-di-(12-(*R*)-hydroxy)stearin	E	52	96

A = lipase from *Rhizopus delemar*
B = lipase from *Mucor miehei*
C = lipase from *Chromobacterium viscosum*
D = lipase from *Penicillium roquefortii*
E = lipase from *Rhizopus niveus*

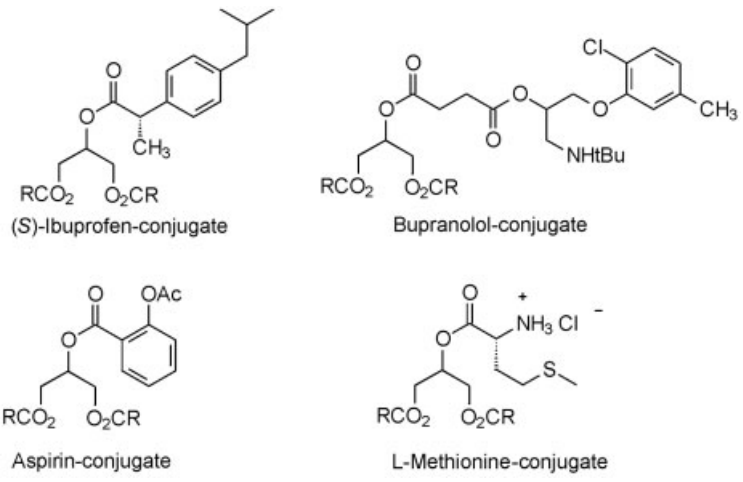


Figure 10. Modified 1,3-diglycerides.

- modified with succinic acid (Berger and Schneider, 1993); or
- modified with amino acids (Aha, 1999; Berger, 1993; Berger and Schneider, 1993).

Furthermore, the 1,3-*sn*-diglycerides constitute attractive coupling partners for the synthesis of numerous conjugates aimed at the lipid modification of pharmaceuticals, e.g., aspirin, (*S*)-ibuprofen, β-blockers (Figure 10) or the preparation of various reagents for the lipid modification of proteins (Berger, 1993; Berger and Schneider, 1993).

6.5 Enzyme-catalyzed synthesis of isomerically pure 1(3)-monoglycerides

Two basically different strategies can be employed for the synthesis of isomerically pure 1(3)-*sn*-monoglycerides:

- Strategy 1: The use of permanent or temporary protection groups of two of the three hydroxy groups of glycerol, followed by the enzymatic acylation and subsequent removal of the protection groups.
- Strategy 2: Enzymatic acylation of glycerol itself, leading directly to the desired molecules.

Examples for the employment of temporary protection groups according to Strategy 1 can be found in the literature describing the enzymatic esterification of isopropylidene glycerol (Figure 11) (Bornscheuer, 1995; Bornscheuer and Yamane, 1995; Hess et al., 1995). Alternatively, the glycerol esters of phenylboronic acid (Figure 12) may be used for temporary protection (Steffen et al., 1992; 1995; Papendorf et al., 1995).

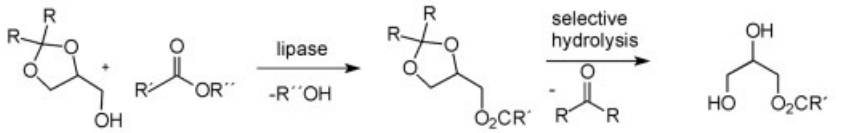


Figure 11. Esterification of isopropylidene glycerol.

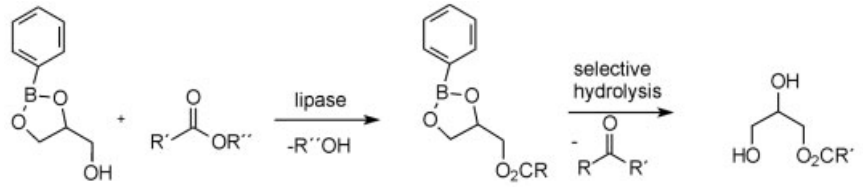
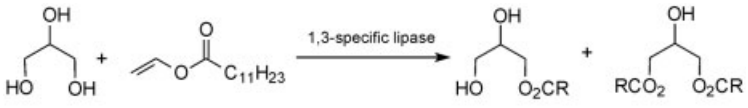


Figure 12. Acylation of glycerol esters from phenylboronic acid.

In view of the additional steps required for the introduction of the protection groups, their removal and finally their product purification, it was decided to study in detail the possibilities for using Strategy 2, i.e., the direct enzymatic acylation of glycerol.

In principle, it should be straightforward to extend the above-described method to the production of regioisomerically pure 1(3)-*sn*-monoacylglycerols. Unfortunately, however, under these conditions the products were produced in only low to moderate yields. Initially, and in view of the high regioselectivities displayed by many lipases with regard to the primary positions of glycerides, it seemed possible to increase the yield of 1(3)-*sn*-monoacylglycerols simply by employing an excess of glycerol, and an increase in yield to 60–70 % was indeed achieved in this manner (Figure 13).

Nonetheless, even with a 10- fold excess of glycerol, the content of 1(3)-*sn*-monoacylglycerols in the reaction mixture did not exceed 70 %. Clearly, the primarily produced monoacylglycerols are excellent substrates for the lipase catalysts and are rapidly converted into the corresponding 1,3-*sn*-diacylglycerols. Another disadvantage is that no triacylglycerols, i.e. natural fats and oils can be employed as acyl donors because in this way, mixtures of regioisomeric monoglycerides would be produced automatically. It became apparent that a practical and useful method for the preparation of regioisomerically pure 1(3)-*sn*-monoacylglycerols had to fulfil the requirements outlined in Figure 14.



R = $C_{11}H_{23}$

ratio	conversion (%)	yield (%)	conversion (%)
1 : 1	30	(12)	70
2 : 1	45	(24)	55
5 : 1	70	(50)	30
10 : 1	65	(45)	35

Figure 13. Approaches to 1(3)-*sn*-monoglycerides.

- **Stoichiometric ratios of reactants**
- **Fatty acid as acyl donors**
- **Triglycerides as acyl donors**
- **Natural fats and oils as acyl donors**
- **No or non-toxic organic solvents**
- **Preparation in useful quantities**
- **High yields**
- **No purification steps**
- **Potential for continuous process**

Figure 14. Isomerically pure 1(3)-*sn*-monoacylglycerols: process requirements.

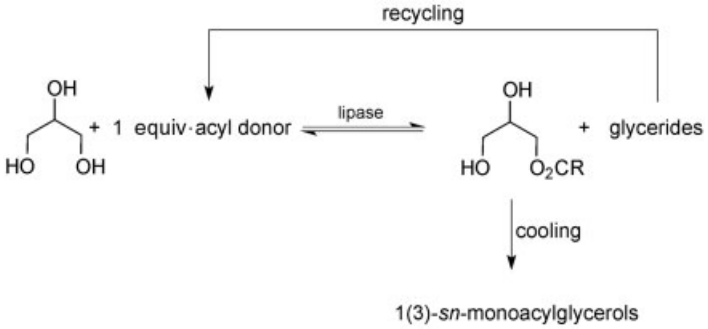


Figure 15. Regioisomerically pure 1(3)-*sn*-monoglycerides: synthetic process.

In order to achieve quantitative conversions of all employed reactants, but without the need for excess glycerol, two major problems had to be resolved:

1. A simple method was required for the continuous separation of the desired products from the reaction mixtures.
2. Simultaneously, an effective recycling method for all undesirable materials was needed, including 2-monoacyl-glycerols, diacylglycerols and triacylglycerols.

Both goals were achieved simply by employing a compartmental separation of the two steps of the process – synthesis and isolation (Figure 15). The enzymatic esterification is carried out in the reactor vessel with stoichiometric amounts of glycerol and the corresponding acyl donor under the desired reaction conditions. The solution of the reaction mixture obtained in this way is circulated into a second vessel (termed

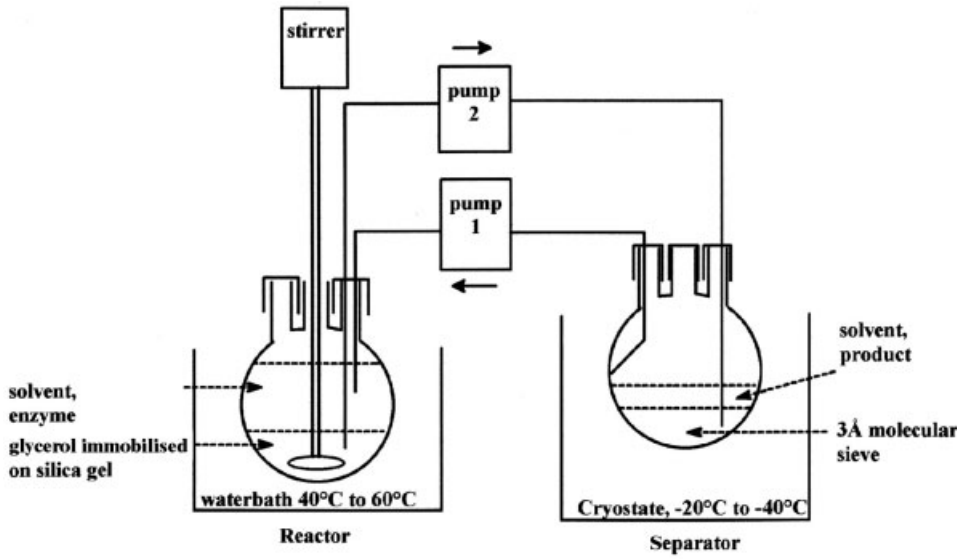


Figure 16. Synthesis of monoglycerides: the apparatus.

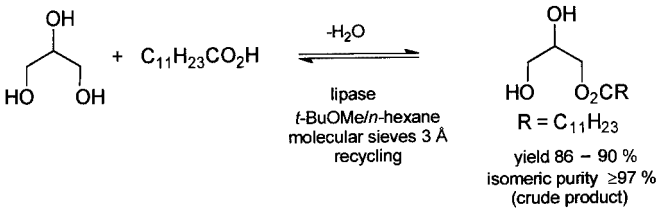


Figure 17. Regioisomerically pure 1(3)-*sn*-monoglycerides by lipase-catalyzed esterification of glycerol.

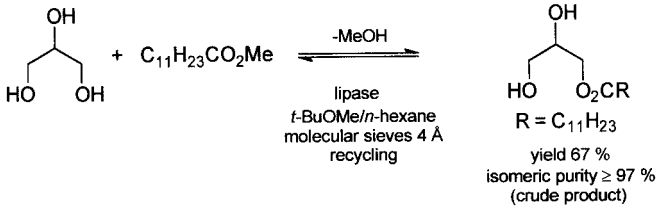


Figure 18. Regioisomerically pure 1(3)-*sn*-monoglycerides by lipase-catalyzed transesterification of glycerol.

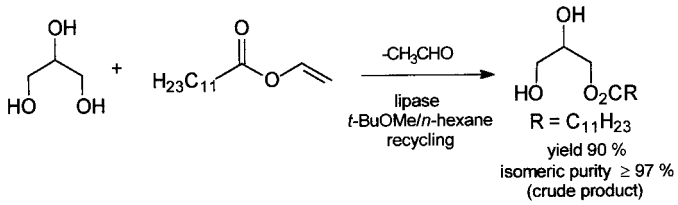


Figure 19. Regioisomerically pure 1(3)-*sn*-monoglycerides by lipase-catalyzed irreversible transesterification of glycerol.

the separator) in which the desired monoacylglycerols are separated at lower temperatures.

A schematic representation of the equipment used in this process is shown in Figure 16. Crucial to the success of this method is the choice of the solvent or solvent mixture in which the desired, more polar 1(3)-*sn*-monoacylglycerols are less soluble than all of the other products, at least at the low temperatures employed. Thus, while the desired products precipitate in the cooled separator, all other products (as well as unreacted acyl donors) remain in solution and are fed back to the reactor, which contains both the enzyme and the glycerol on the solid support. In this way, high yields of monoglycerides may be obtained, using a variety of acyl donors.

The success of the method was demonstrated with numerous acyl donors, such as free fatty acids, fatty acid methyl esters and vinyl esters (Figures 17 – 19). Synthetic triglycerides and natural fats and oils may also be used (see below). Moreover, the method is not limited to natural fatty acids, as unnatural fatty acids may also be used.

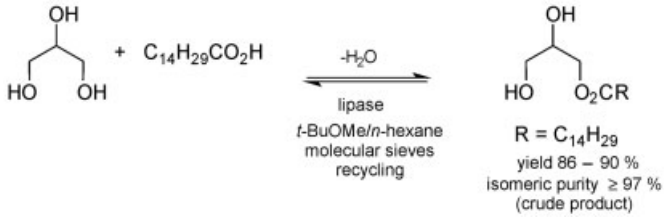


Figure 20. Synthesis of Monopentadecanoin.

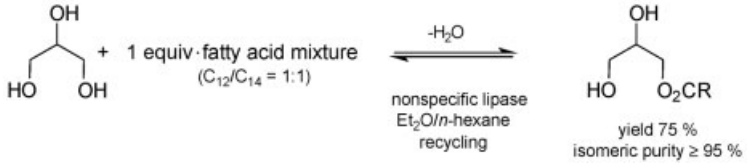


Figure 21. Monoglycerides from technical fatty acid mixtures.

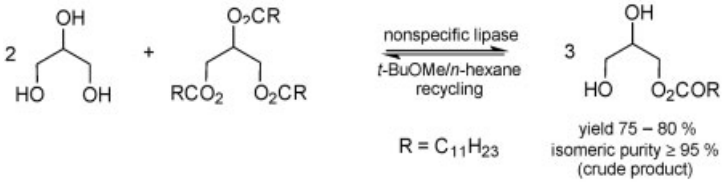


Figure 22. Monoglycerides by glycerolysis of triglycerides.

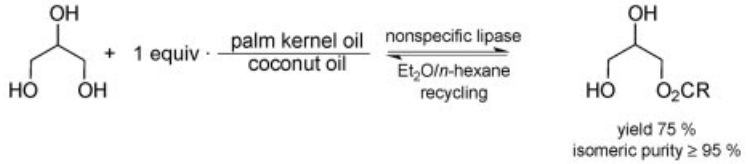


Figure 23. Monoglycerides by glycerolysis of native oils.

The synthesis of monopentadecanoin, a product for cosmetic applications is shown schematically in Figure 20. Of importance from a practical point of view is the fact that unpurified technical fatty acid mixtures can be used directly (Figure 21), leading to the corresponding monoglycerides in high yields and excellent chemical purities.

It is interesting to note that for the conversion of triglycerides (Figure 22) and natural fats and oils into regioisomerically pure 1(3)-monoglycerides, nonspecific lipases are best suited. Clearly, all acyl groups should be usable by the biocatalysts employed, also those in the *sn*-2-positions of triglycerides.

The conversion of natural coconut oil or palm kernel oil leads to mixtures of the corresponding monoglycerides in high yields and purities, without the need for

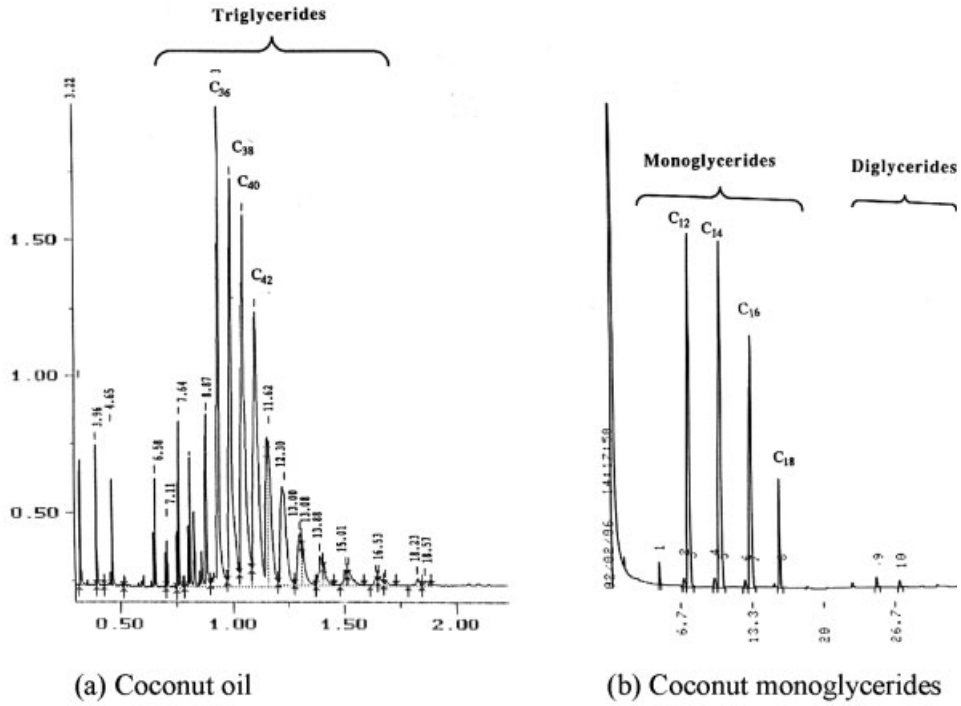


Figure 24. Gas chromatograms of coconut oil and coconut monoglycerides.

further purification steps (Figure 23). The natural distribution of fatty acids, shown as triglycerides in the gas chromatogram of the starting oil (Figure 24a) is converted exclusively into the corresponding mixture of isomerically pure 1(3)-*sn*-monoacylglycerols (Figure 24b), with only traces of the corresponding isomers or diglycerides. A selection of 1(3)-*sn*-monoacylglycerols prepared in this way is listed in Table 2.

6.6 Monoglyceride sulfates

Isomerically pure monoglycerides prepared using the above-described procedure are excellent precursors for monoglyceride sulfates, another class of skin-friendly surfactants (Zeidler, 1986). Using SO_3/pyridine – the normal laboratory method – a complete series of these products was prepared, in high chemical purities (Figure 25).

Table 2. Regioisomerically pure 1(3)-*sn*-monoglycerides.

Product	Enzyme	Yield (%)	Purity (%)
1(3)-*sn*-monovalerin	A	57	93
1(3)-*sn*-(5′-brom)-monovalerin	A	52	95
1(3)-*sn*-(6′-brom)-monocaproin	A	54	95
1(3)-*sn*-monoaprylin	B	60	91
1(3)-*sn*-monocaprin	C	90	97
1(3)-*sn*-monodec-9-enoin	C	90	96
1(3)-*sn*-monoundecanoin	C	89	96
1(3)-*sn*-monoundec-10-enoin	C	88	96
1(3)-*sn*-mono-(10′-oxo)undecanoin	B	60	91
1(3)-*sn*-monolaurin	C	90	98
1(3)-*sn*-mono-(12′-hydroxy)-dodec-9-enoin	B	42	95
1(3)-*sn*-monotridecanoin	C	91	97
1(3)-*sn*-monomyristin	C	91	96
1(3)-*sn*-mono-(13′-oxo)-tetradecanoin	B	52	92
1(3)-*sn*-monopentadecanoin	C	91	96
1(3)-*sn*-monopalmitin	C	90	95
1(3)-*sn*-monotearin	C	88	96
1(3)-*sn*-monoolein	D	65	99
1(3)-*sn*-monolinolein	F	79	96
1(3)-*sn*-monoerucain	F	71	98
1(3)-*sn*-mono-(*S*)-corioloin	E	70	95
1(3)-*sn*-mono-(*R*)-ricinolin	E	66	96
1(3)-*sn*-mono-(12-(*R*)-hydroxy)-stearin	E	56	98

A = lipase from *Rhizopus delemar*
B = lipase from *Mucor miehei* (Lipozyme)
C = lipase from *Pseudomonas* sp.
D = lipase from *Chromobacterium viscosum*
E = lipase from *Penicillium roquefortii*
F = lipase from *Rhizopus niveus*

6.7 Conclusion

The above-described procedures for the synthesis of regioisomerically pure mono- and diglycerides provide ready access to these materials, and in good yields. By using stoichiometric quantities of the starting materials, products of high chemical and isomeric purity are obtained by a novel proprietary process, and without the need of additional purification steps.

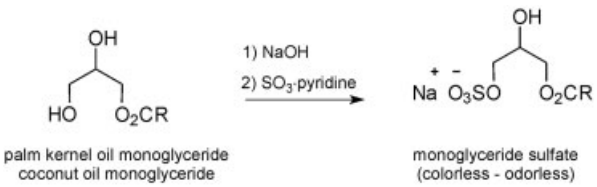


Figure 25. Monoglyceride sulfates from monoglycerides.

A wide variety of acyl donors can be employed, including saturated, unsaturated and functionalized fatty acids, fatty acid methyl esters, fatty acid vinyl esters, triglycerides and also natural fats and oils (coconut oil, palm kernel oil, and others) with their natural distribution of fatty acids.

All products are completely colorless and odorless, and are therefore ideally suited for applications in food technology, cosmetic formulations and for pharmaceutical applications. From a regulatory point of view, it is important to mention that the mono- and diglycerides obtained from natural fatty acids or fats and oils via the action of natural biocatalysts in nontoxic solvents could be termed 'natural' in every respect. The approval of government bodies for the use of these materials should therefore pose fewer problems than previously encountered.

6.8 References

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7 Lipase-Catalyzed Peroxy Fatty Acid Generation and Lipid Oxidation

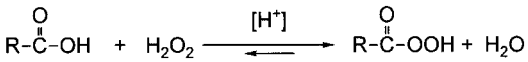
Mark Rüsch gen. Klaas and Siegfried Warwel

7.1 Introduction

Beside their industrial application for bleaching and as disinfectants, peroxy acids are important oxidants in chemical industry as well as in organic laboratory synthesis. Examples for these oxidations are C = C-epoxidation, Baeyer – Villiger-oxidations, hydroxylation of aromatic rings and the oxidation of amines.

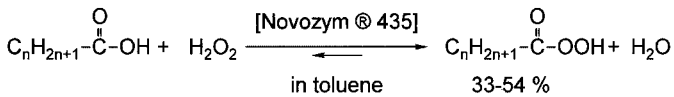
For all these reactions only a few peroxy acids, that are either easily generated in situ (performic, peracetic and trifluoroperacetic acid), or that are reasonably stable and can thus be stored (for example: mcpba, *m*-chloro perbenzoic acid; mmpp, monoperoxy phthalic acid magnesium salt), are regularly applied. *MCpba* is still the most often used peroxy acid in organic laboratory synthesis, although it is potentially explosive (Heaney, 1993) and quite expensive. Peroxy fatty acids have barely been used because until now no convenient synthetic route was known.

The standard method for the preparation of peroxy acids is the reaction of carboxylic acids with hydrogen peroxide catalyzed by a strong acid:



The larger the organic group R, the more stable is the resulting peroxy acid. On the other hand, a larger group R also means that the reaction conditions for peroxy acid generation (temperature, amount and acidity of the catalyst) have to be more severe. Therefore, this synthetic method is not suitable for sensitive or long-chain substrates. More elaborate procedures for the synthesis of particular peroxy acids (Folli et al., 1968) are too expensive and tedious for a widespread use. In conclusion, there is an urgent need for a new simple and versatile method for peroxy acid generation.

Such a new method was first developed by Björkling, Kirk and colleagues, a group working at the Danish world-leading enzyme producer, Novo Nordisk A/S. They found that even-numbered middle-chain C_6–C_{14} fatty acids can be converted with hydrogen peroxide to the corresponding peroxy acids with yields of 33–54 % (Björkling at al., 1990) when catalyzed by Novozym® 435, an immobilized lipase B from *Candida antarctica*:



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These workers also used these peracids *in situ* for epoxidation (Björkling et al., 1990; 1992; Godtfredsen et al., 1990). In the meantime, we investigated extensively the potential of lipase-mediated catalysis for the generation of a variety of peroxy fatty acids, together with their *in situ* application for oxidation reactions in oleochemistry.

7.2 Lipase-catalyzed peroxy acid generation

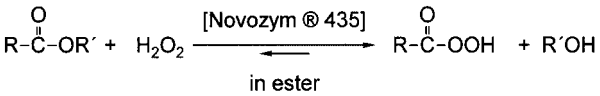
7.2.1 Generation from fatty acids

While examining the substrate range of Novo's peroxy acid synthesis (Rüsch gen. Klaas and Warwel, 1997), it was found that all unbranched fatty acids with four to 22 carbon atoms were good substrates for Novozym® 435-catalyzed peroxy acid synthesis. Beside these fatty acids, however, only very few carboxylic acids (e.g. 2-phenyl acetic acid) produced peroxy acids in good yield. The only α-branched carboxylic acid which yielded any peroxy acid at all was *i*-butyric acid; other branched or benzoic acids were completely inactive.

These results were not completely surprising. The lipase B from *Candida antarctica*, which was used for most of our experiments, is known to possess a notably narrow entrance to the active site (Uppenberg et al., 1994). An obvious solution to our substrate restrictions would thus have been to use another lipase. In total, about 30 lipases were investigated; although most lipases had a certain activity for peroxy acid generation from fatty acids and H_2O_2, stability was mostly – and in contrast to Novozym® 435-very poor. Hence, Novozym® 435 was identified as the enzyme of choice for any preparative application, the substrate range being enhanced by modifying the reaction conditions.

7.2.2 Generation from carboxylic acids esters

Kirk et al. (1994) referred earlier to the possibility of perhydrolysis by H_2O_2 analogously to ester hydrolysis, but did not carry out any investigations in that respect. However, we were successful in developing a useful protocol for lipase-catalyzed ester perhydrolysis by H_2O_2:



Perhydrolysis proceeds especially well if the ester is applied as the solvent, i.e. in a large excess, as it then has certain advantages over the lipase-catalyzed generation of peroxy acids from free fatty acids. In most cases, perhydrolysis works with common 35 % aqueous H_2O_2, whereas for the conversion of free fatty acids 60 % H_2O_2 is used preferably. Considering that 60 % H_2O_2 cannot be obtained from fine chemicals providers such as Merck or Aldrich, the possibility of using 35 % H_2O_2 makes

this method much more attractive to the organic chemist. It should be mentioned here that the chemical industry may on occasion prefer to use 60 % or even 70 % H_2O_2 in order to avoid 'buying and transporting large amounts of water'.

Of even more importance is the broad substrate range of perhydrolysis, as illustrated by the epoxidation of 1-octene with α-substituted carboxylic acids/acid esters (Rüsch gen. Klaas and Warwel, 1997). Epoxidation with these sources of peroxy acids proceeded only by perhydrolysis; however, even with perhydrolysis the bulkiest branching accepted by Novozym® 435 is a single α-methyl group, but not an α-ethyl or an α,α-dimethyl group. Nevertheless, this substrate range clears the way for a biocatalytic route to chiral peroxy acids (Warwel and Rüsch gen. Klaas, 2000).

Novozym® 435-catalyzed perhydrolysis of ethyl acetate or methyl acetate leads to peroxy acetic acid; thus treatment of olefins with Novozym® 435/35 % H_2O_2/ethyl acetate is a highly convenient general method for olefin epoxidation (Rüsch gen. Klaas and Warwel, 1998b). In contrast, the conversion of acetic acid with H_2O_2 is a very poor method, as shown recently (albeit unintentionally) by Jarvie et al. (1999). Other methods of obtaining peracetic acid by biocatalysis are mechanistically interesting, but of little synthetic value (Picard et al., 1997).

Other more unusual peroxy acids, such as peroxy acrylic acid, peroxy citric and peroxy lactic acid, as well as derivatives of peroxy carbonic acid (Rüsch gen. Klaas and Warwel, 1999b) can also be prepared by perhydrolysis; peroxy acrylic and peroxy carbonic acid are also useful in oleochemistry (see Section 8.3.3).

7.3 Lipase-mediated lipid oxidations

Peroxy acids, which are generated by one of the methods described earlier, may be used for various oxidation reactions such as C = C-epoxidation, Baeyer – Villiger oxidations (Lemoult et al., 1995; Pchelka et al., 1998), the oxidation of aldehydes to carboxylic acids, and a variety of oxidative conversions of trialkyl silyl ethers (Rüsch gen. Klaas et al., 1999). Of these chemo-enzymatic oxidations, epoxidation is the most important. Chemo-enzymatic epoxidation of simple olefins has been described for the first time by Björkling et al. (1990), and the mechanism is shown in Figure 1.

As mentioned earlier, a similar system can be based on perhydrolyis, and it was found later that perhydrolysis of dialkyl carbonates leads to a peroxy acid, that is a superior oxidant for acid-sensitive substrates (Rüsch gen. Klaas and Warwel, 1999a).

Apart from the oxidation of simple olefins, biocatalytic peroxy acid formation is extremely useful for epoxidations in oleochemistry, because the carboxyl function necessary for peracid generation and the C = C bond are often conveniently situated in one molecule.

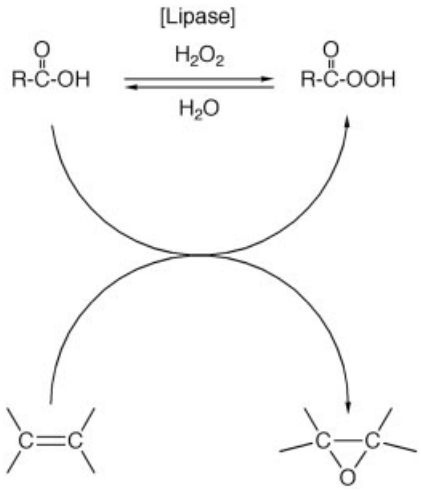


Figure 1. The mechanism of chemo-enzymatic epoxidation (Björkling et al., 1990).

7.3.1 'Self'-epoxidation of unsaturated fatty acids

If an unsaturated fatty acid is treated with H_2O_2 in the presence of Novozym® 435, 'self'-epoxidation occurs (Warwel and Rüsch gen. Klaas, 1995). The principle of this reaction is shown in Figure 2, using oleic acid as an example.

First, the unsaturated fatty acid is converted to an unsaturated peroxy fatty acid, and only this step is catalyzed by the lipase. Subsequently, the unsaturated peroxy

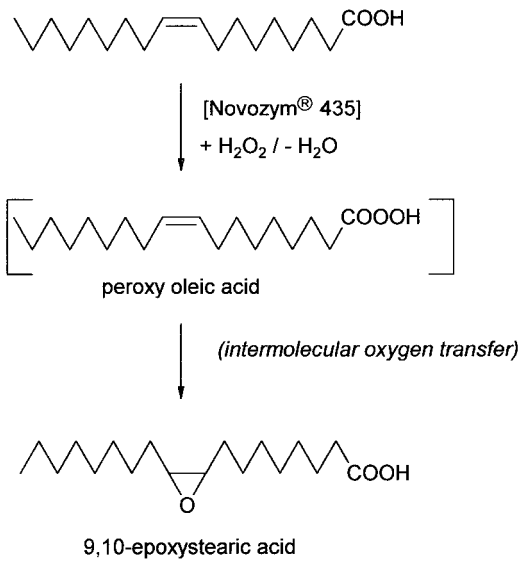


Figure 2. Chemo-enzymatic 'self'-epoxidation of unsaturated fatty acids by Novozym® 435/H_2O_2.

Table 1. Chemo-enzymatic 'self'-epoxidation of unsaturated fatty acids by Novozym® 435/H_2O_2.

No.	Substrate	Product	Yield (GC) (mol%)
1–1	9-decenoic acid	9,10-epoxy decanoic acid	72
1–2	10-undecenoic acid	10,11-epoxy undecanoic acid	77
1–3	13-tetradecenoic acid	13,14-epoxy tetradecanoic acid	78
1–4	oleic acid	*cis*-9,10-epoxy stearic acid	72/88[1]
1–5	elaidic acid	*trans*-9,10-epoxy stearic acid	82
1–6	petroselinic acid	6,7-epoxy stearic acid	77
1–7	linoleic acid	9,10–12,13-diepoxy stearic acid	83
1–8	linoleic acid	9,10-epoxy octadec-12-enoic acid 12,13- epoxy octadec-9-enoic acid	91[2]
1–9	ricinoleic acid	9,10-epoxy-12-hydroxy stearic acid	95

[1] Preparative scale; isolated yield.
[2] Excess linoleic acid.
C = C/H_2O_2 (60 %) = 1 : 1.5; 0.05 mol C = C/g enzyme.
16 h at room temperature (72 h at 40 °C for terminal C = C) in toluene.

acid epoxidizes 'itself' in an uncatalyzed Prileshajev epoxidation (hence 'chemo-enzymatic'). In the cases we examined the reaction is definitely intermolecular; however, for certain C = C-bond positions an intramolecular reaction is likely (compare Corey et al., 1979).

Terminal as well as internal unsaturated fatty acids were epoxidized with good yields (see Table 1) and excellent selectivity ($\geq$ 98 %). Neither the position and configuration of the C = C bond influence the results, nor is the secondary hydroxyl group in ricinoleic acid involved. In the case of linoleic acid it is possible to obtain selectively the diepoxide or the isomeric mono-epoxides by simply changing the C = C/H_2O_2 ratio.

In addition to our own results, the method has been applied successfully by Frykmann and Isbell (1997) for the epoxidation of meadowfoam fatty acids.

The heterogeneous biocatalyst can be recovered by a simple filtration after the reaction, and surprisingly, it can be reused for multiple runs with little loss of activity. Using only 200 mg of Novozym® 435 (enzyme immobilized on carrier), we were able to produce about 40 g of 9,10-epoxystearic acid, which is equal to a turnover of about 200 000 mol product per mol catalyst. Based on this observation, the construction of a fixed-bed reactor for continuous operation is now in progress.

7.3.2 'Self'-epoxidation of unsaturated triglycerides and fatty acid esters

Based on perhydrolysis, a similar method for the epoxidation of unsaturated fatty acid esters is also feasible, but there is a particular complication. If a triglyceride containing unsaturated fatty acids reacts with hydrogen peroxide/Novozym® 435 (Figure 3), peroxy acids will be formed, which will epoxidize C = C bonds. The resulting reaction mixture contains epoxidized triglycerides and some epoxidized free fatty acids, but unfortunately also some epoxidized mono- and diglycerides, which are barely separable from the product.

It was found (Rüsch gen. Klaas and Warwel, 1996), that the generation of mono- and diglycerides is completely suppressed if a small amount of free fatty acids (5 mol%) is added at the beginning of the reaction. Again, perhydrolysis occurs, but the resulting free hydroxyl groups are immediately re-esterified by the excess of free

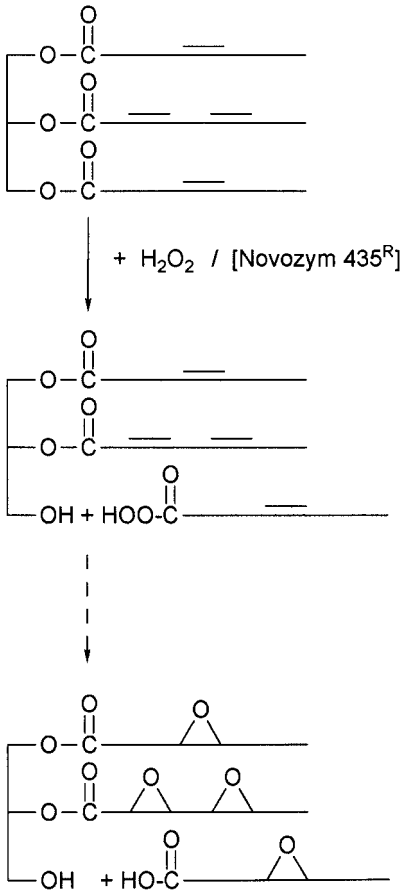


Figure 3. Chemo-enzymatic 'self'-epoxidation of unsaturated plant oils by Novozym® 435/H_2O_2 without addition of free fatty acids.

fatty acids (Figure 4). In this case the reaction mixture consists only of epoxidized triglycerides and epoxidized free fatty acids, and in contrast to mono- and diglycerides the free fatty acids can be easily removed afterwards, e.g. by an alkaline washing. Of course, this procedure involves some interesterification; to avoid any change in fatty acid distribution, the free fatty acids should be of the same composition as the triglyceride used.

Several plant oils were epoxidized this way (Table 2) with yields of 88–96 % and a selectivity of 84–100 %. Even in the case of highly unsaturated linseed oil, very good yields are obtained and the selectivity remains high; thus, without any further work-up the product has superior quality compared to commercial samples.

Using controlled partial epoxidation, it is possible to prepare substitutes that mimic the properties of natural epoxy oils such as *vernonia* oil (Rüsch gen. Klaas and Warwel, 1999b). These are not yet available commercially, but may have some interesting applications as a reactive diluent for paints. Rapeseed fatty acid methyl-

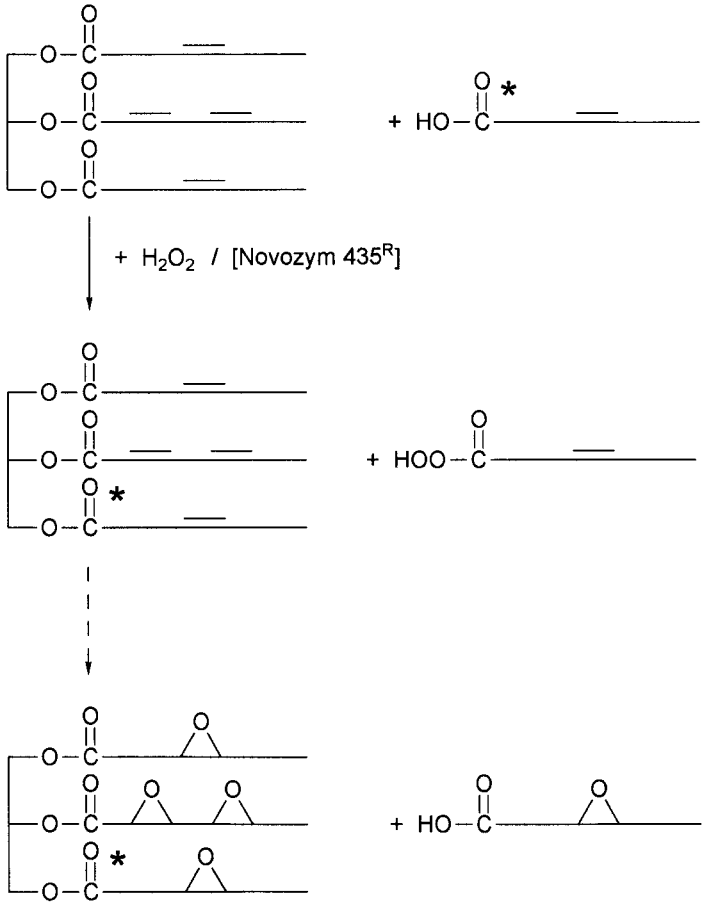


Figure 4. Chemo-enzymatic 'self'-epoxidation of unsaturated plant oils by Novozym® 435/H_2O_2 with addition of free fatty acids.

Table 2. Chemo-enzymatic 'self'-epoxidation of plant oils by Novozym® 435/H_2O_2.

No.	Plant oil	Conversion[1]	Selectivity[2]	Oxirane oxygen[3]
2–1	rapeseed	99	92	5.3
2–2	sunflower	88	100	5.8
2–3	soybean	99	94	7.1
2–4	linseed	98	98	9.9
2–5	castor	95	95	4.5
2–6	sunflower + linseed	43[4]	95	3.8
2–7	soybean + linseed	51[4]	84	4.0

[1] Related to iodine value.
[2] Epoxide formed per C = C-bond converted.
[3] Percentage of oxirane oxygen in weight % according to titration and elemental analysis.
[4] Deliberate restriction of conversion to mimic vernonia oil.
C = C:H_2O_2 (35 %) = 1 : 1.5; 0.05 C = C/g Novozym® 435; 16--72 h at room temperature in toluene; addition of 5 mol% free fatty acid.

esters (RME, 'biodiesel') was epoxidized using the same procedure (Warwel et al., 1996); in this case, the composition of the epoxidized fatty acid esters also closely matched the composition of the biodiesel.

7.3.3 Epoxidation of unsaturated fatty alcohols and their derivatives

Unsaturated fatty alcohols may also be epoxidized by lipase-catalyzed perhydrolysis (Rüsch gen. Klaas and Warwel, 1998b). Generally, peroxy acid formation is not the only lipase-catalyzed reaction to occur and interestingly, the outcome of the reaction depends on the ester applied for peroxy acid generation (Figure 5).

Fatty acid esters such as butyric acid ethylester react to epoxy alkanol acylates in a three-step – one-pot reaction. In the first step, the unsaturated alcohol is esterified, after which the resulting unsaturated ester is then epoxidized. The nature of these consecutive reactions is shown clearly in Figure 6. Both the alcohol and the ester components can be varied widely (Tables 3 and 4), and even epoxy acrylates can be made directly, implying the intermediate formation of peroxy acrylic acid.

The reaction has a completely different outcome using the perhydrolysis of diethyl carbonate. Epoxidation by *in situ* formation of a peroxy carbonic acid derivative proceeds smoothly, but the corresponding acid is not stable and cannot esterify the alcohols; therefore, the product (with a selectivity of $\geq$ 98 %) is the epoxidized alcohol.

In an extension of these syntheses, we explored lipase-mediated conversion of unsaturated fatty alcohol trialkyl silyl ethers and identified a range of synthetically useful one-pot – multi-step reactions (Figure 7) (Rüsch gen. Klaas et al., 1999).

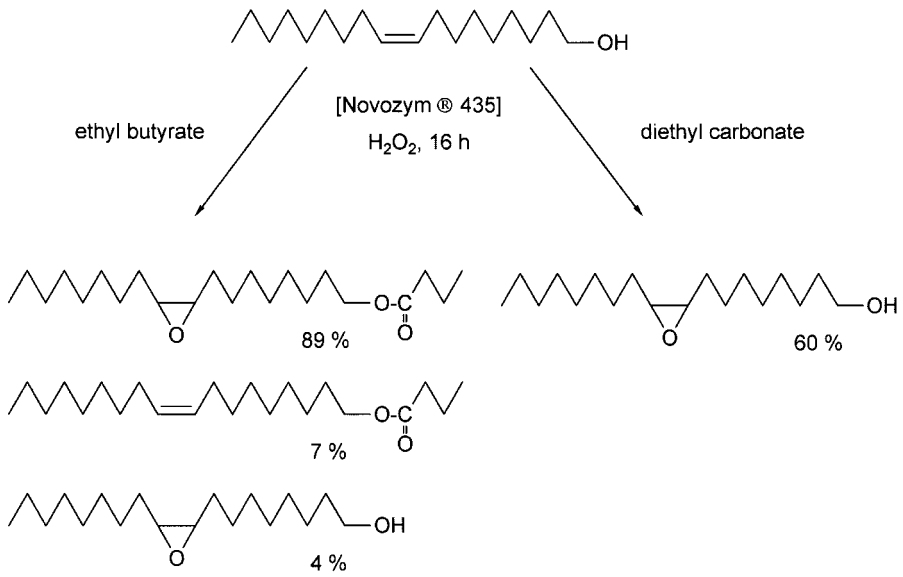


Figure 5. Chemo-enzymatic epoxidation of unsaturated alcohols by Novozym® 435/H_2O_2 using carboxylic acid esters or dialkyl carbonates as the peroxy acid source.

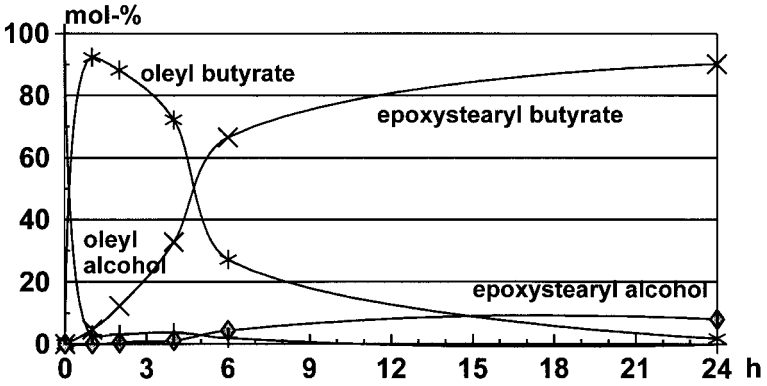


Figure 6. Three-step – one-pot chemo-enzymatic synthesis of 9,10-epoxy stearyl butyrate; product distribution plotted versus reaction time.

Table 3. Three-step – one-pot synthesis of epoxyalkanolbutyrates from unsaturated alcohols with butyric acid ethylester/H_2O_2/Novozym® 435.

No.	Substrate	Yield (GC) of: 9,10-epoxystearyl alcohol (%)	oleyl acylate (%)	9,10-epoxystearyl acylate (%)
3–1	9-octadecen-1-ol	4	7	89
3–2	10-undecen-1-ol	7	7	71
3–3	5-hexen-1-ol	Not estimated	19	66
3–4	2-propen-1-ol	17	46	32

5 mmol alcohol; 7.5 mmol H_2O_2 (60 %); 100 mg Novozym® 435.
10 ml butyric acid ethylester; 22 h at 20–40 °C

Table 4. Three-step – one-pot synthesis of 9,10-epoxystearylacylates from oleyl alcohol with carboxylic acid ethylesters/H_2O_2/Novozym® 435.

No.	Substrate	Yield (GC) of: 9,10-epoxystearyl alcohol (%)	oleyl acylate (%)	9,10-epoxystearyl acylate (%)
4–1	*n*-butyric acid EE	4	7	89
4–2	*i*-butyric acid EE	3	3	80
4–3	decanoic acid EE	–	3	76
4–4	acrylic acid EE	18	4	47

5 mmol oleyl alcohol, 7.5 mmol H_2O_2 (60 %), 100 mg Novozym® 435.
10 ml carboxylic acid ethylester, 22 h at 20 °C.

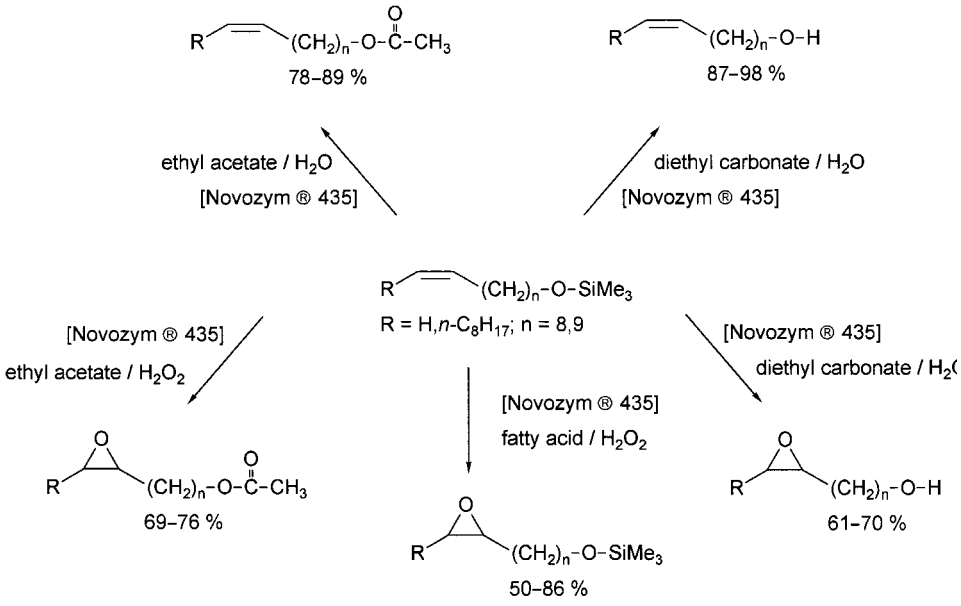


Figure 7. Chemo-enzymatic conversion of unsaturated trimethyl silyl ethers.

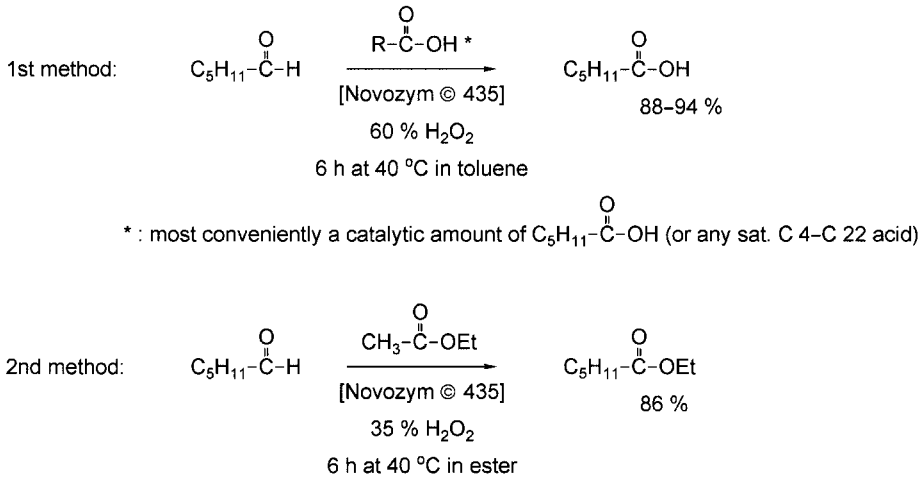


Figure 8. Two methods for chemo-enzymatic aldehyde oxidation.

7.3.4 Oxidation of fatty aldehydes

Autooxidation of aldehydes to carboxylic acid esters by molecular oxygen proceeds via peroxy acids. Therefore, fatty aldehydes can be oxidized quantitatively to fatty acids (or directly to fatty acid esters) with high selectivity by Novozym® 435 and H_2O_2 (Figure 8). Again, two methods can be used; the oxidant can be generated from a free carboxylic acid, or from a carboxylic acid ester. In contrast to epoxidation, the first method is extremely convenient for aldehyde oxidation. By addition of a catalytic amount of the desired carboxylic acid to the aldehyde the reaction can be started, and because of the growing amount of acid in the reaction mixture becomes autocatalytic. When the reaction is complete, there is only a single organic substance in the product. The second method does not yield the carboxylic acid but, in a two-step – one-pot reaction, the carboxylic acid ester, because the intermediate acid is esterified by the excess of ethyl acetate.

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8 Production of Functional Lipids Containing Polyunsaturated Fatty Acids with Lipase

Yuji Shimada, Akio Sugihara and Yoshio Tominaga

8.1 Introduction

Polyunsaturated fatty acids (PUFAs) have various physiological functions, and are used as pharmaceutical substances, in cosmetics, food materials, and health foods. For example, the ethyl ester of eicosapentaenoic acid (20 : 5n-3; EPA) has been used in the treatment of arteriosclerosis and hyperlipemia since 1990 in Japan. Docosahexaenoic acid (22 : 6n-3; DHA) plays a role in the prevention of a number of human diseases, including cardiovascular disease (Kromhout et al., 1985), inflammation (Lee et al., 1985), and cancer (Bravo et al., 1991; Stillwell et al., 1993). DHA has also been reported to show an important function in the brain (Uauy and De Andraca, 1995) and retina (Nuringer et al., 1984), and to accelerate the growth of preterm infants (Carlson et al., 1993; Lanting et al., 1994). For this reason, tuna oil containing DHA has been used as a food material, an ingredient of infant formulas, and a health food. Arachidonic acid (20 : 4n-6; AA) is a precursor of local hormones (prostaglandin, thromboxanes, and leukotrienes) involved in the AA cascade (Minno et al., 1987), and accelerates the growth of preterm infants, as does DHA (Carlson et al., 1993; Lanting et al., 1994). An AA-rich oil produced by a microorganism has begun to be used in a formula for preterm infants in Europe since 1998. γ-Linolenic acid (18 : 3n-6; GLA) is a precursor of AA, and is effective for atopic eczema (Bordoni, et al., 1988; Fiocchi et al., 1994) and rheumatoid arthritis (Jantti et al., 1989; Zurier et al., 1996). Thus, borage oil rich in GLA is used as an ingredient of infant formulas and a health food.

The first generation of PUFA-containing oils were natural oils themselves, but the triglyceride structure and fatty acid composition were not always the most suitable for humans. Thus improvement of oils has been strongly desired, and the second generation of PUFA oils has emerged. The second generation oils include single cell oils produced from microorganisms and oils modified by lipases. Lipases catalyze not only hydrolysis, but also esterification and transesterification, and are very useful for the modification of natural oils. In this chapter, we describe the improvement of PUFA-containing oils with lipases, especially the production of PUFA-rich oils and highly absorbable structured lipids.

8.2 Production of PUFA-rich oils by selective hydrolysis

Oils containing higher concentrations of PUFA have the following advantages: (i) greater physiological effects can be expected from even a small amount of intake; (ii)

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when it is used as an ingredient of various kinds of food, the processing procedures will be easier; and (iii) it can be used as a more effective starting material for the purification of PUFA. PUFA-rich oil has been traditionally produced by winterization. However, the maximum DHA content is 30 % at most according to the method in the industrial production of DHA-rich oil from tuna oil. Meanwhile, application of lipase can enrich PUFA more efficiently. In general, lipases act on PUFA very weakly (Shimada et al., 1995a,b; 1998a), and PUFA can be enriched in the glyceride fraction by hydrolysis of PUFA-containing oil with the enzymes. This reaction, termed selective hydrolysis, is applicable to enrichment of the other PUFAs, AA and GLA. DHA- and GLA-rich oils produced by the method are commercialized as health foods.

8.2.1 Production of DHA-rich oil from tuna oil

Lipases from *Candida rugosa* (Tanaka et al., 1992; Shimada et al., 1994), *Geotrichum candidum* (Shimada et al., 1994), and *Penicillium abeanum* (Sugihara et al., 1996) have highly hydrolyzing ability, but act on DHA very weakly. Thus, these lipases are suitable for the production of DHA-rich oil from tuna oil. In particular, *Candida* lipase (Lipase-OF; Meito Sangyo, Aichi, Japan) is available industrially, and is useful for the production of DHA-rich oil. We investigated several factors affecting the reaction, and determined the following reaction conditions: a mixture of tuna oil, 50 % water, and 200 units (U) g^{-1} reaction mixture was incubated at 35 °C with stirring (Shimada et al., 1994). One unit of lipase is the amount that liberates 1 μmol of fatty acid per minute from olive oil. Figure 1 shows a typical time course of the selective hydrolysis under these conditions. Hydrolysis proceeded rapidly until 4 h, and gradually thereafter. The contents of palmitic, palmitoleic, and oleic acids in glycerides decreased rapidly, and the stearic acid content decreased after a 1–h lag. The EPA content decreased gradually after an increase within the first hour. The content of DHA increased with increasing hydrolysis extent, and reached 48 wt% after 24 h. The time course showed that the lipase activity on the constituent fatty acids was in the order: palmitoleic, oleic acids > palmitic acid > stearic acid > EPA > DHA. In addition, the glyceride fraction after the 24-h hydrolysis contained 88 wt% triglycerides, 10 wt% diglycerides, and 2 wt% monoglycerides.

When tuna oil was hydrolyzed with *Candida* lipase in a mixture containing 50 % water, the DHA content in glycerides depended only on the hydrolysis extent. Tuna oil was hydrolyzed at 35 °C for 24 h with various amounts of the lipase, and the correlation between the hydrolysis extent and the DHA content in glycerides is shown in Figure 2. The DHA content was raised from 23 to 45 wt% in a 78 % yield at 60 % hydrolysis, and to 50 wt% at 70 % hydrolysis in a 70 % yield. Using the selective hydrolysis, 45–50 wt% DHA oil has been commercialized as a health food in Japan since 1994.

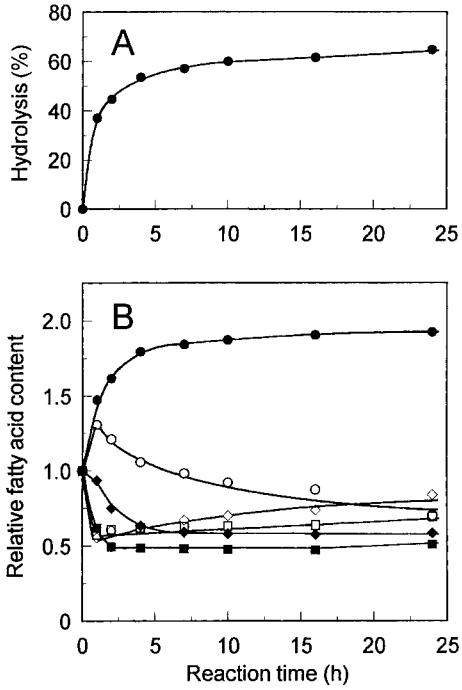


Figure 1. Time course of selective hydrolysis of tuna oil with *Candida rugosa* lipase. (A) Extent of hydrolysis. (B) Main fatty acid contents in undigested glyceride fraction. The fatty acid contents in glycerides were expressed relative to those in the original tuna oil. ■, Palmitic acid (the initial; content, 18.2 %); ◇, palmitoleic acid (4.6 %); ◆, stearic acid (4.4 %); □, oleic acid (18.5 %); ○, EPA (6.9 %); ●, DHA (24.4 %).

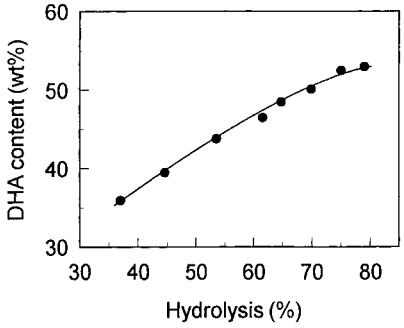


Figure 2. Correlation between hydrolysis extent of tuna oil and DHA content in undigested glycerides. Tuna oil was hydrolyzed at 35 °C for 24 h in a mixture containing 50 % water and with 20 to 2000 U g^{-1} mixture of *Candida rugosa* lipase.

8.2.2 Production of GLA-rich oil from borage oil

Candida lipase acts on GLA as weakly as on DHA, and GLA-rich oil can be produced by selective hydrolysis of borage oil (Syed Rahmatullah et al., 1994; Shimada et al., 1998b). Borage oil (initial GLA content, 22 wt%) was hydrolyzed at 35 °C for 15 min to 24 h with 100 U g^{-1} reaction mixture of *Candida* lipase. Figure 3 shows the correlation between the hydrolysis extent and the GLA content in glycerides. Unlike enrichment of DHA in selective hydrolysis of tuna oil (see Figure 2), the GLA content was not raised over 46 % at above 65 % hydrolysis (Figure 3). The reaction conditions for the efficient enrichment of GLA were as follows: a mixture of borage oil, 50 % water, 20 U g^{-1} mixture of *Candida* lipase was incubated at 35 °C for 16–24 h with stirring. The enzyme amount was one-tenth of that for the hydrolysis of tuna oil, showing that borage oil was a better substrate for the lipase than tuna oil.

8.2.3 Production of AA-rich oil from AA-containing single cell oil

Any oils and fats from plants and animals do not contain high content of AA. Yamada et al. (1988) found that *Mortierella* accumulated AA-containing triglycerides in the mycelia, and the single cell oil containing 25 % to 40 % AA has been produced industrially (Shinmen et al., 1989; Higashiyama et al., 1998). Because *Candida* lipase also acted on AA very weakly, we attempted to enrich AA in glycerides by selective hydrolysis of 25 % AA-containing oil (TGA25; Suntory, Osaka, Japan). A mixture of TGA25 oil, 50 % water, and 90 U g^{-1} mixture of *Candida* lipase was incubated at 35 °C for 16 h with stirring. The resulting glycerides contained 50 % AA at 52 % hydrolysis. To further increase the AA content, glycerides were extracted from the reaction mixture with *n*-hexane, and hydrolyzed again under the same conditions. The extents of the second and third hydrolyses reached 18 % and 17 %, respectively, and the three-time repetitions raised the AA content to 60 % with a 75 % recovery (Table 1) (Shimada et al., 1995b).

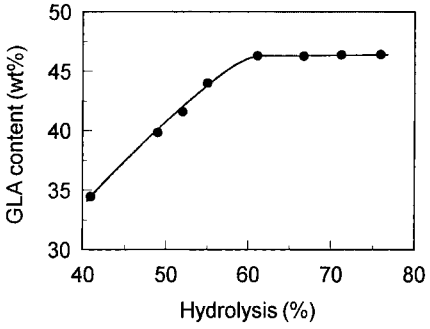


Figure 3. Correlation between hydrolysis extent of borage oil and GLA content in undigested glycerides. Borage oil was hydrolyzed at 35 °C for 0.25 to 24 h in a mixture containing 50 % water and with 100 U g^{-1} mixture of *Candida rugosa* lipase.

Table 1. Main fatty acid content in glycerides obtained by repeated selective hydrolysis of TGA25 oil with *Candida rugosa* lipase.

Reaction	Hydrolysis %	Fatty acid content (wt%)						AA Recovery[1] (%)
		16 : 0	18 : 0	18 : 1	18 : 2	18 : 3[2]	20 : 4	
None[3]	–	13.4	6.3	14.8	21.3	3.2	24.9	100
First	52.4	5.7	3.6	6.7	9.6	4.7	49.5	94.6
Second	18.1	3.8	3.2	4.0	5.5	4.9	54.3	87.2
Third	17.2	2.4	2.3	2.5	3.3	5.2	59.7	75.0

[1] Recovery of the initial arachidonic acid (AA) content of the original TGA25 oil. The recovery was calculated by assuming that all glycerides were recovered by the extraction with *n*-hexane.
[2] γ-Linolenic acid.
[3] Fatty acid composition of the original TGA25 oil.

TGA40 oil (AA content, 40 %; Suntory) was hydrolyzed at 35 °C for 20 h in a mixture containing 50 % water and 15 U g^{-1} of *Candida* lipase. Although the enzyme amount was one-sixth of that in hydrolysis of TGA25 oil, the AA content in glycerides could be raised to 57 wt% at 35 % hydrolysis, and the loss of AA was only 9 % of the initial content (Shimada et al., 1999a). This result shows that the oil containing higher concentration of desired PUFA is useful for the increase in the PUFA content in glycerides.

8.2.4 Separation of PUFA-rich oil from the reaction mixture

Production of a PUFA-rich oil is completed by the removal of free fatty acids (FFAs) from the reaction mixture. From the viewpoint of industrial purification, the extraction of glycerides with *n*-hexane is not suitable because of the risk of explosion and high cost. The reaction mixture contained only glycerides and FFAs, and the main components of the glycerides were triglycerides. The molecular weights of triglycerides and FFAs were completely different; ca. 900 Da and 290 Da, respectively. Thus, molecular distillation should be effective for removal of FFAs.

An attempt was made to purify GLA-rich oil from borage oil hydrolyzate (Table 2) (Shimada et al., 1998b). Borage oil (7 kg) was hydrolyzed at 35 °C for 24 h in a mixture containing 50 % water and 20 U g^{-1} mixture of lipase using a 30-L reactor with stirring at 200 rpm. The GLA content in glycerides was raised to 46 wt% at 61 % hydrolysis. The dehydrated oil layer was applied to film distillation apparatus, and distillation was performed at 150 °C and 0.05 mmHg. Most of the FFAs were recovered in distillate 1-1, but the acid value of the residue was still high at 40. Hence, the second cycle of distillation was performed after increasing the temperature to 160 °C, and 2.3 kg of the residue (acid value, 10) was obtained. To increase further the GLA content, the glycerides were hydrolyzed again under the same conditions using a 10-L reactor. As a result, the GLA content was raised to 55 wt% at 20 % hydrolysis. The distillation of the oil layer at 155 °C and 0.04

Table 2. Large-scale purification of GLA-rich oil from reaction mixture obtained by selective hydrolysis of borage oil with *Candida* lipase.

Procedure	Weight (kg)	Acid value	Amount of glycerides[1] (kg)	GLA Content[2] (wt%)	GLA Recovery[2] (%)
Original oil	7.00	n.d.	7.00	22.2	100
Hydrolysis (1)	6.29	122	2.45	45.7	71.8
Film distillation					
Distillate 1–1	3.26	197	0.05	n.t.	–
Distillate 1–2	0.67	147	0.18	n.t.	–
Residue 1–2	2.28	10	2.17	46.4	64.8
Hydrolysis (2)	2.12	49	1.60	55.3	56.9
Film distillation					
Distillate 2–1	0.52	176	0.06	n.t.	–
Residue 2–1	1.54	4	1.50	55.7	53.8

[1] Calculated by assuming that the acid value of free fatty acids originating from borage oil is 200.
[2] The content and recovery of GLA in the glyceride fraction.
n.t., not tested; n.d., not determined.

mmHg gave 0.5 kg of distillate (acid value, 176) and 1.5 kg of residue (acid value, 4; GLA content, 56 wt%). The first and second distillations recovered 89 % and 94 % of glycerides, respectively. This result showed that the film distillation was very effective for removal of FFA in the large-scale production of PUFA-rich oil.

8.2.5 Reaction kinetics of selective hydrolysis

The glycerides obtained by selective hydrolyses of PUFA-containing oils contained more than 80 wt% of triglycerides, in spite of over 50 % hydrolysis. To clarify this phenomenon, we investigated the reactions occurring concomitantly with hydrolysis. The results showed that PUFAs were esterified not with glycerol but PUFA partial glycerides even in a mixture containing 50 % water, and that interesterification between partial glycerides also occurred. In addition, hydrolysis activities on PUFA glycerides were in the order of mono-PUFA > di-PUFA > tri-PUFA, and hydrolyses of PUFA partial glycerides generated tri-PUFA. On the basis of these results, we concluded that selective hydrolysis proceeded as follows: (i) The ester bonds of fatty acids other than PUFA were hydrolyzed in the early stage of reaction, and PUFA-containing partial glycerides were generated. (ii) Parallel to the hydrolysis of the resulting partial glycerides, esterification occurred between partial glycerides and FFAs including PUFAs, and interesterification also occurred between partial glycerides. (iii) The hydrolysis of esters of non-PUFAs was faster than those of

PUFAs. In addition, the activities on PUFA glycerides was in the order of mono- > di- > triglycerides, and tri-PUFA was scarcely hydrolyzed. These specificities of the lipase resulted in the accumulation of PUFA-rich glycerides (mainly triglycerides) in the reaction mixture (Shimada et al., 1995c).

We also investigated which fatty acids were enriched in glycerides by selective hydrolysis. As shown in Figure 1, selective hydrolysis of PUFA-containing oil released the constituent fatty acids from glycerol backbone according to the fatty acid specificity of lipase. In the reaction, stearic acid was not enriched in the glyceride fraction. However, hydrolysis of cocoa fat substitute (SOS fat; 1,3-stearoyl-2-oleoylglycerol; purity, 69 %) with *Candida* lipase efficiently enriched stearic acid in glycerides (Shimada et al., 1995b). SOS fat contains stearic and oleic acids as main components and palmitic and linoleic acids as minor ones. Because stearic acid was the poorest substrate of the lipase, the fatty acid was enriched in the glycerides.

The GLA content was not raised more than 46 wt% in the selective hydrolysis of borage oil with *Candida* lipase, even though the hydrolysis extent increased (Figure 3). However, the GLA content was raised by repeating the hydrolysis after the removal of FFAs from the reaction mixture (Table 2). We also performed the hydrolysis of triglycerides containing 22 %, 35 %, and 45 % GLA. The release rate of total fatty acids became slow with increasing GLA content in the substrate, but the release rate of GLA did not depend on the GLA content. These results showed that the increase of the GLA content in glycerides was disturbed by FFAs accumulated in the reaction mixture, and by the structure of the triglyceride (Shimada et al., 1998b). The continuous removal of FFAs from the reaction mixture should improve the efficiency of the PUFA enrichment.

8.3 Production of highly absorbable structured lipid containing PUFA and medium-chain fatty acid

8.3.1 Structured lipid

In general, natural oils and fats contain saturated or mono-unsaturated fatty acids at the 1,3-positions and highly unsaturated fatty acids at the 2-position. However, the distribution of fatty acids along the glycerol backbone is not specified, and natural oils and fats are not always the best for humans with respect to their structure and physical properties. Thus, modification by chemical and/or biochemical procedures has been attempted to improve their quality. These modified lipids are structured lipids in a wide sense. Meanwhile, structured lipids in a narrow sense are triglycerides having particular fatty acids at specific positions of glycerol. Structured lipids in a wide sense include single cell oil produced by microorganisms and PUFA-rich oil, as described earlier. Functional lipids composed only of specific fatty acids are also structured lipids in a wide sense. Several low-calorie oils of this group have been commercialized; e.g., triglycerides with behenic acid and medium-chain fatty acids (Caprenin), triglycerides with short-chain fatty acids and stearic acid (Salatrim), and triglycerides with medium- and long-chain fatty acids. Structured lipids in a narrow

sense have superior properties compared to natural oils and fats. For example, a triglyceride with stearic or behenic acid at 1,3-positions and oleic acid at 2-position (SOS or BOB) is used as cocoa butter equivalent, and triglyceride with oleic acid at 1,3-positions and palmitic acid at 2-position triglyceride (OPO) is used in an infant formula. Because triglycerides with medium-chain fatty acids at 1,3-positions and long-chain fatty acids at 2-position (MLM-type) are hydrolyzed to 2-monoglyceride and fatty acids faster than natural oils and fats with long-chain fatty acids (LLL-type), MLM-type structured lipids are absorbed extensively into the intestinal mucosa (Ikeda et al., 1991; Christensen et al., 1995). Therefore, MLM-type structured lipids containing functional fatty acids are expected as nutrition for patients with maldigestion and malabsorption of lipids, and as high-value added health foods. We attempted to produce MLM-type structured lipids containing PUFAs and essential fatty acids by acidolysis of natural oils with caprylic acid (8 : 0, CA) using immobilized *Rhizopus delemar* lipase in an organic solvent-free system, and to purify the structured lipids from the reaction mixture by molecular distillation.

8.3.2 Preparation of immobilized lipase

Acidolysis of natural oils with CA must be performed in a system without water in order to repress simultaneous hydrolysis. When a soluble lipase is used in the system, the enzyme will not disperse well because of aggregation. This problem can be solved by use of immobilized lipase. In this study, we selected a 1,3-positional specific *R. delemar* lipase (Ta-Lipase; Tanabe Seiyaku, Osaka, Japan) which is used as a flavor enzyme for the production of bread, and attempted to immobilize the lipase on a ceramic carrier (SM-10; NGK Insulator, Aichi, Japan) (Shimada et al., 1996a).

After the ceramic carrier (50 g) was suspended in 200mL of 10 % *Rhizopus* lipase solution, 600 mL cold acetone (– 80 °C) was added gradually with stirring gently, and the precipitate dried under reduced pressure. Approximately 90 % of lipase was immobilized on the carrier, but the lipase scarcely showed acidolysis activity in a reaction mixture without water. A reaction mixture containing 2 % water efficiently activated the lipase. Thus, the first reaction was performed at 30 °C for 48 h in a mixture of tuna oil/CA (1 : 1, w/w), 4 % immobilized lipase, and 2 % water with shaking at 130 oscillations/min (Table 3). As a result, 31 mol% CA was incorporated into glycerides, though 18 % partial glycerides were also generated by simultaneous hydrolysis. To repress the hydrolysis, the subsequent reactions were conducted by transferring the immobilized lipase to a fresh oil/CA substrate mixture without addition of water. The CA amount in glycerides was slightly increased in the second reaction, and the generation of partial glycerides was reduced to below 1 % in the third reaction. Therefore, immobilized lipase that had been treated twice (first with adding water; second without adding water) was employed for the production of a MLM-type structured lipid containing DHA.

Table 3. Activation of immobilized *Rhizopus* lipase on acidolysis of tuna oil with caprylic acid (CA).

Reaction cycle	Incorporation of CA (mol %)	Glyceride composition (wt%)		
		Triglyceride	Diglyceride	Monoglyceride
First	31.1	82	15	3
Second	35.8	96	3	1
Third	36.8	99	1	n.d.
Fourth	35.9	99	1	n.d.

n.d., not determined.

8.3.3 Production of a structured lipid containing DHA and CA

Tuna oil was acidolyzed with CA to produce a MLM-type structured lipid containing DHA (Shimada et al., 1996a). Several factors affecting the acidolysis were investigated, and the reaction conditions determined as follows: a reaction mixture of tuna oil/CA (1 : 2, w/w) and 4 % immobilized *Rhizopus* lipase was incubated at 30 °C with shaking. Figure 4 shows a typical time course of the reaction. The contents of palmitic and oleic acids in triglycerides rapidly decreased, and attained a constant value after 20 h. The EPA content gradually decreased and reached a constant value after 40 h. The DHA content scarcely changed during the reaction. The change of the fatty acid contents in glycerides showed that the reaction proceeded according to the fatty acid specificity of *Rhizopus* lipase. Furthermore, the decrease in these fatty acid contents corresponded to the increase in the CA content, showing that the fatty acids in tuna oil were exchanged for CA.

Rhizopus lipase acted on DHA only very weakly in acidolysis of tuna oil, acted on it weakly in esterification of FFAs originating from tuna oil with long-chain fatty

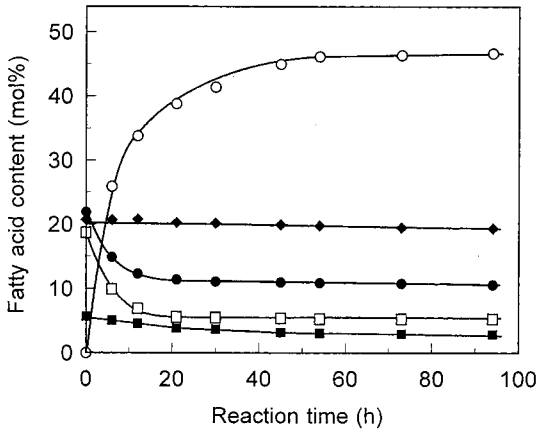


Figure 4. Time course of acidolysis of tuna oil with CA using immobilized *Rhizopus delemar* lipase. ○, CA; ●, palmitic acid; □, oleic acid; ■, EPA; ◆, DHA.

alcohol (Shimada et al., 1997a,b), and acted on it moderately in hydrolysis of tuna oil (Shimada et al., 1995b). These results showed that the fatty acid specificity of a lipase changed slightly in different reaction system. Thus it is important to evaluate the fatty acid specificity under the reaction conditions employed in order to outline the lipase characteristics to maximal effect.

Regiospecific analysis of the transesterified oil was carried out by Grignard degradation with allyl magnesium bromide, followed by isolation and analysis of the 1,3-diglyceride fraction (Shimada et al., 1996a). Table 4 shows the fatty acid compositions of the 1(3)- and 2-positions in the original tuna oil and transesterified oil obtained by acidolysis of tuna oil with CA. The fatty acid compositions at the 2-positions in the oils before and after the reaction were almost the same, and CA was incorporated mainly at the 1,3-positions. It was confirmed from these results that only fatty acids at 1(3)-position in tuna oil were exchanged for CA by *Rhizopus* lipase.

Triglycerides in the original tuna oil and transesterified oil were analyzed by HPLC (Figure 5) (Shimada et al., 1996a). Most triglycerides in tuna oil were eluted from the octadecyl silica (ODS) column after 25-min retention. In contrast, triglycerides in the transesterified oil were eluted before 25 min, and found to be new components. These results showed that all the transesterified oil contained one or two CA(s) at 1(3)-position(s) of the triglycerides. Main peaks were collected and their fatty acid compositions analyzed. The structure of each peak was estimated on the basis of molar ratio of the fatty acids, and is shown in Figure 5. The desired 1,3-caproyl-2-docosahexaenoyl-glycerol (8D8) was contained in peak I, and was eluted together with 1,3-caproyl-2-eicosapentaenoyl-glycerol (8E8).

As shown in Figure 4, acidolysis of tuna oil with CA reached the steady state after 40 h. Hence, the acidolysis was repeated by transferring immobilized lipase to a fresh tuna oil/CA substrate mixture every 2 days (Table 5) (Shimada et al., 1996a). The CA content in triglycerides was 42.5 mol% in the first cycle of the reaction, and immobilized lipase could be used during 15 cycles (30 days) without a significant decrease in the CA content. The CA content gradually decreased after prolonged use, and reached 31 mol% after the 20th reaction. The CA incorporation was, however, recovered to the original level by extending the reaction period of the 21st reaction to 4 days. The 22nd reaction was performed for 2 days in a mixture containing 2 % water, but the incorporation of CA was not recovered. These facts suggested that the decline

Table 4. Fatty acid composition of 1(3)- and 2-positions in tuna oil and transesterified oil obtained by acidolysis.

Oil	Position	Fatty acid composition (mol%)							
		8:0	**16:0**	**16:1**	**18:0**	**18:1**	**20:4**	**20:5**	**22:6**
Original tuna oil	1,3	n.d.	14.7	4.9	5.0	15.4	2.0	3.5	8.8
	2	n.d.	7.2	0.8	0.4	3.3	0.7	2.2	11.9
Transesterified oil	1,3	41.9	3.7	1.1	0.7	2.5	0.7	1.6	7.8
	2	0.5	7.1	1.1	0.4	3.2	0.6	2.1	12.4

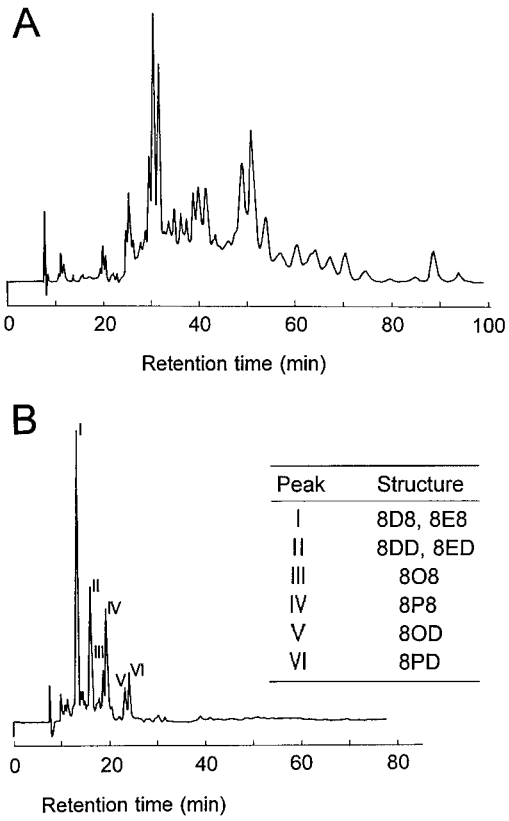


Figure 5. Triglyceride components of original tuna oil (A) and transesterified oil (B). HPLC analysis was performed on two ODS columns (4.6 · 150 mm). The sample was eluted with a mixture of acetone/acetonitrile (1 : 1, v/v) at 40 °C and a flow rate of 0.4 mL min^{-1}. Tricaprylin was eluted at 11.8 min under these conditions. 8, CA; D, DHA; E, EPA, O, oleic acid; P, palmitic acid.

Table 5. Continual batch acidolysis of tuna oil with caprylic acid (CA) using *Rhizopus* lipase.

Cycle number	Fatty acid composition in glycerides (mol%)				
	8 : 0	**16 : 0**	**18 : 1**	**20 : 5**	**22 : 6**
1	42.5	10.8	5.3	3.4	22.8
5	42.9	10.4	5.1	3.8	22.7
10	42.9	10.6	5.3	3.9	21.9
15	40.5	11.3	5.6	3.4	22.9
20	30.7	14.9	8.7	4.8	22.6
21	42.4	10.6	5.3	3.8	22.2

Reactions except for the 21st cycle were performed for 2 days, and the 21st reaction was done for 4 days.

in activity was due to the inactivation of the lipase or its removal from the ceramic carrier, and not to the release of water bound to the immobilized lipase.

8.3.4 Production of structured lipids containing linolenic, α-linolenic acids and CA

When safflower and linseed oils were acidolyzed with CA under the conditions similar to those in the acidolysis of tuna oil with CA, the structured lipids containing linoleic and α-linolenic acids were also produced efficiently (Shimada et al., 1996b). *Rhizopus* lipase acted on safflower and linseed oils more strongly than on tuna oil, and approximately 70 % of the fatty acids at the 1,3-positions of the oils were exchanged for CA after 15 h (the CA content in triglycerides was 47 mol%). To investigate the stability of immobilized lipase, acidolysis was repeated by transferring the immobilized lipase to a fresh substrate mixture every 2 days. In the reactions of safflower and linseed oils, the enzyme could be used for 45 and 55 cycles, respectively. These results showed that our reaction system was very effective for the production of MLM-type structured lipids from natural oils. In this study, we used *Rhizopus* lipase immobilized on the ceramic carrier. Commercial *Rhizomucor miehei* lipase (Lipozyme IM60; Novo Nordisk, Bagsvaerd, Denmark) is a 1,3-positional-specific lipase, and has been used by many researchers. MLM-type structured lipids were efficiently produced under the same conditions using the lipase, and this enzyme had an advantage that pretreatment in a reaction mixture with a small amount of water was not necessary for its activation.

8.3.5 Continuous production of a structured lipid containing GLA and CA by fixed-bed reactor

A batch reaction with immobilized *Rhizopus* lipase was effective to produce MLM-type structured lipids from natural oils and CA. A reactor with an impeller has been generally used for industrial batch reaction, but has the risk that the carrier of immobilized enzyme is destroyed by mechanical agitation. Because the drawback can be avoided by flow reaction with a fixed-bed reactor, we attempted the production of MLM-type structured lipid containing GLA by a continuous flow reaction (Shimada et al., 1999b). The scheme of the reactor used in this study is shown in Figure 6.

Immobilized *Rhizopus* lipase required the pretreatment in a mixture containing a small amount of water for its activation. Thus, immobilized lipase (8 g) was suspended in water-saturated substrate mixture of borage oil/CA (1 : 2, w/w; water content, 1.18 %), and then packed into a column (15 × 62 mm). A substrate mixture saturated with water (100 mL) was fed into the column at 30 °C and a flow rate of 4.5 mL h^{-1} (4.1 g h^{-1}) for the activation of lipase, and the mixture without adding water (water content, 350–500 ppm) was then fed under the same conditions (Figure 7). As a result, the incorporation of CA into triglycerides reached 54 mol%, and the content of partial glycerides in the glyceride fraction was below 1.5 wt%. The CA

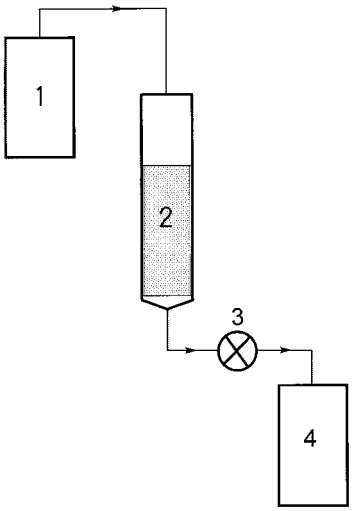


Figure 6. Fixed-bed bioreactor for production of GLA-containing MLM-type structured lipid. 1, Storage vessel of substrate (300 mL); 2, glass column packed with 8 g immobilized *Rhizopus delemar* lipase (15 · 62 mm); 3 peristaltic pump; 4, receiver of eluted reaction mixture (500 mL).

content incorporated into triglycerides did not change for 60 days, and then gradually decreased. The CA content in glycerides was decreased to 40 mol% after 100 days, but returned to the initial level after the flow rate was reduced to 3.1 mL h^{-1}. This result showed that the immobilized lipase could be used for at least 100 days. The daily amount of substrate converted to structured lipid by 1 g of the lipase was 13.5 mL g^{-1} per day. To compare the efficiency in the flow reaction with that in the batch

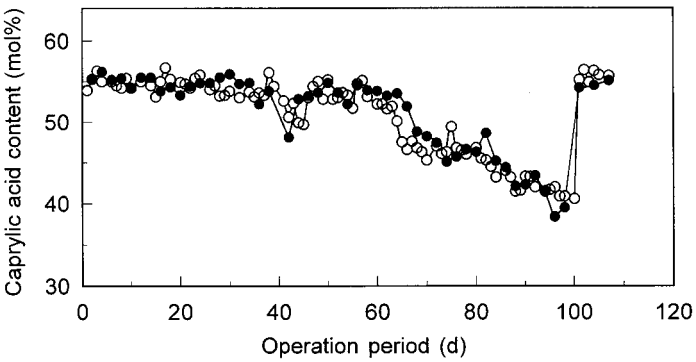


Figure 7. Stability of immobilized *Rhizopus* lipase in a continuous flow and a continual batch reactions. ●, Continuous flow reaction. A substrate mixture (borage oil/CA = 1 : 2, w/w) was fed into a column packed with 8 g immobilized lipase (15 · 62 mm) at 30 °C and 4.5 mL h^{-1} flow rate. After 100 days, the flow rate was reduced to 3.1 mL h^{-1}. ●, Continual batch reaction. A mixture of 13.5 mL borage oil/CA (1 : 2, w/w) and 0.5 g immobilized lipase was incubated at 30 °C for 2 days with shaking. The reaction was repeated by transferring the lipase into a fresh substrate mixture. After 100 days, the reaction period was extended to 3 days.

reaction, the reaction was performed batchwise at 30 °C in a mixture of 13.5 mL borage oil/CA (1 : 2, w/w) and 0.5 g immobilized lipase with shaking. The reaction was repeated by transferring the lipase to a fresh substrate mixture every 2 days; the daily amount of substrate converted to structured lipid by 1 g of the enzyme was the same as that of the flow reaction. As shown in Figure 7, the efficiency of the flow reaction completely agreed with that of the batch reaction. From the industrial viewpoint, the flow reaction with fixed-bed reactor has the following advantages. (i) The carrier of immobilized enzyme is not destroyed even if the lipase is continuously used for more than 100 days. (ii) The procedure of continuous flow reaction is easier than that of batch reaction. (iii) The period of contact of substrates with the lipase is shorter: flow reaction (retention time), 1.3 h; batch reaction, 48 h. The shorter reaction time is advantageous to the processing of an oil containing unstable PUFAs.

The reaction mixture contains triglycerides and FFAs, the molecular weights of which are different. Thus, taking advantage of the differences in their boiling points, an attempt was made to purify triglycerides by film distillation (Shimada et al., 1999b). The reaction mixture that eluted from the above column after 30–50 days was collected, and 1 kg of the mixture was used for the distillation. The distillation was performed by measuring the acid value of the residue as a guide. When the distillation was performed at 130 °C and 0.2 mmHg, 644 g was obtained as the first distillate, the acid value being 369. Because the acid value of the residue was 54, the distillation temperature was increased to 170 °C. As a result, 52 g was separated as the second distillate (acid value 202), but the acid value of the residue was still 26. Further distillation at 195 °C brought the third residue to 2.6 with 24.8 % of recovery. The third residue contained 98.7 % triglycerides, 0.5 % diglycerides, and 0.8 % FFAs. This result showed that film distillation removed FFAs very effectively from the reaction mixture.

8.4 Production of a structured lipid containing AA and palmitic acid

Human milk contains 20–25 % of palmitic acid, and about 70 % of the fatty acid is esterified to the 2-position of triglycerides (Breckenridge et al., 1969; Martin et al., 1993). Pancreatic lipase hydrolyzes dietary oils and fats to FFAs and 2-monoglycerides, and the absorption efficiency of free palmitic acid is relatively low compared with that of free unsaturated fatty acids (Jensen et al., 1986). This result supports that fat absorption is higher in infants fed fats with PA at 2-position of triglycerides than 1,3-position (Innis et al., 1994). It has been hypothesized from these facts that the high absorption efficiency of human milk fat is the result of specific positioning of PA at the 2-position of the triglyceride moiety (Fomon et al., 1970; Innis et al., 1994). Thus, we attempted to synthesize 1,3-arachidonoyl-2-palmitoyl-glycerol (designated APA) in high purity as a material for absorption studies (Shimada et al., 2000). Tripalmitin (palmitic acid, 90 %; stearic acid, 8 %) and AA (91 %) was used as a starting material. Immobilized *Rhizopus* lipase was used after the pretreatment in a substrate mixture containing 2 % water as described above. The acidolysis of tripalmitin with AA was conducted as follows: a mixture of 10 g AA/tripalmitin (1 : 5, w/w) and

0.7 g immobilized lipase was incubated at 40 °C with shaking. Figure 8 shows a typical time course of the change of fatty acid contents in the glyceride fraction. The palmitic and stearic acid contents in glycerides rapidly decreased until 10 h, and gradually thereafter. The increase in AA content in glycerides was completely associated with the decrease in the palmitic and stearic acid contents. The AA contents after 24-and 34-h reactions reached 59 and 63 mol%, respectively.

Glycerides obtained from a 24-h reaction were analyzed by high-performance liquid chromatography (HPLC) (Figure 9). The components eluted between 12 and 14 min were diglycerides, as shown by thin-layer chromatography, and the components eluted after 18 min were triglycerides. The structure of each triglyceride was estimated from the fatty acid composition, and are shown in Figure 9. The contents of AAA, APA, ASA, APP, and APS were calculated to be 7.3, 75.9, 3.1, 12.4, and 1.3 wt% from their peak areas, respectively. High purity of APA could be obtained by preparative HPLC (yield of HPLC, 56 %).

Acidolysis of tripalmitin with AA produced 7 wt% of triarachidonin as a byproduct. Because the positional specificity of *Rhizopus* lipase was very strict as described below, it was strongly suggested that triarachidonin was not generated by enzymatic exchange of fatty acids at the 2-position for AA. The presence of 4.6 wt% of diglycerides showed that hydrolysis occurred concomitantly with the acidolysis. In addition, concomitant esterification could be expected to occur during the reaction. Therefore, triarachidonin may be generated via the following processes: a diglyceride with AA at the 1(3)-position and palmitic acid at the 2-position is generated by hydrolysis, and the palmitic acid migrates spontaneously to the 3(1)-position. Palmitic acid at 3(1)-position is acidolyzed with AA, and diarachidonin is generated. After AA at the 1(3)-position migrated to the 2-position, triarachidonin is synthesized by esterification of free AA to the 1(3)-position. It was actually confirmed that diarachidonin was present in the reaction mixture, because the peak

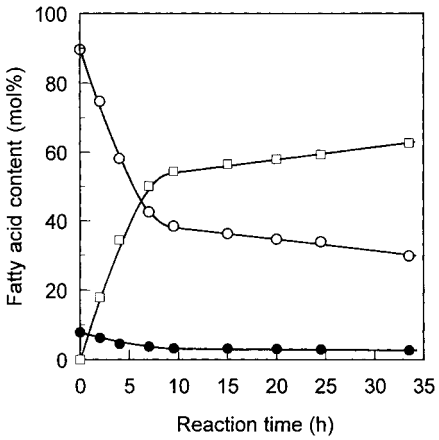
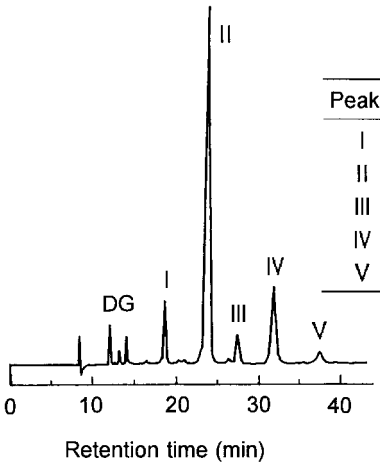


Figure 8. Time course of acidolysis of tripalmitin with AA. A mixture of 30 g AA/tripalmitin (5 : 1, w/w) and 2.1 g immobilized *Rhizopus delemar* lipase was incubated at 40 °C with shaking. ○, Palmitic acid content in glycerides; ●, stearic acid content; □, AA content.



Peak	Structure
I	AAA
II	APA
III	ASA
IV	APP
V	APS

Figure 9. Triglyceride components of transesterified oil obtained by acidolysis of tripalmitin with AA for 24 h. The conditions of analysis were the same as those in Figure 2. A, Arachidonic acid; P, palmitic acid; S, stearic acid.

eluted at 12 min by HPLC analysis contained 95 mol% of AA (Figure 9). This fact strongly supported the hypothesis.

8.5 Application to regiospecific analysis

Regiospecific analysis of triglycerides in natural oils has been conducted by two methods. One is the method of analyzing the fatty acid composition of 1,3-diglycerides or 2-monoglycerides obtained by Grignard degradation (Becker et al., 1993), which was used in this study (Table 4). The other is the method of analyzing the fatty acid composition of FFA obtained by the hydrolysis of an oil with a free 1,3-positional specific lipase (Luddy et al., 1964; Iwai et al., 1980). When this enzymatic method is used, the products in the early stage of the reaction must be analyzed because the esterification and transesterification occur simultaneously during the hydrolysis. However, the constituent fatty acids are liberated from the oil according to the fatty acid specificity of the lipase. Thus, the erroneous result is obtained that the fatty acids, on which the lipase acts strongly, exist at the 1(3)-position.

In our reaction system for the production of MLM-type structured lipid at 30 °C, the simultaneous hydrolysis and nonenzymatic acyl migration did not occur, and fatty acids at 1,3-positions of natural oils were efficiently exchanged for CA (see Tables 3 and 4 and Figure 5). To exchange all fatty acids at 1,3-positions for CA, the glycerides obtained by the acidolysis were recovered by *n*-hexane extraction, and subjected to the reaction three times in total under the same conditions. As a result, the CA contents in transesterified oils obtained from substrate oils except for tuna oil reached 66 mol%, although the CA content in transesterified oil derived from tuna oil was 60 %. Figure 10 shows the results of HPLC analyses of the transester-

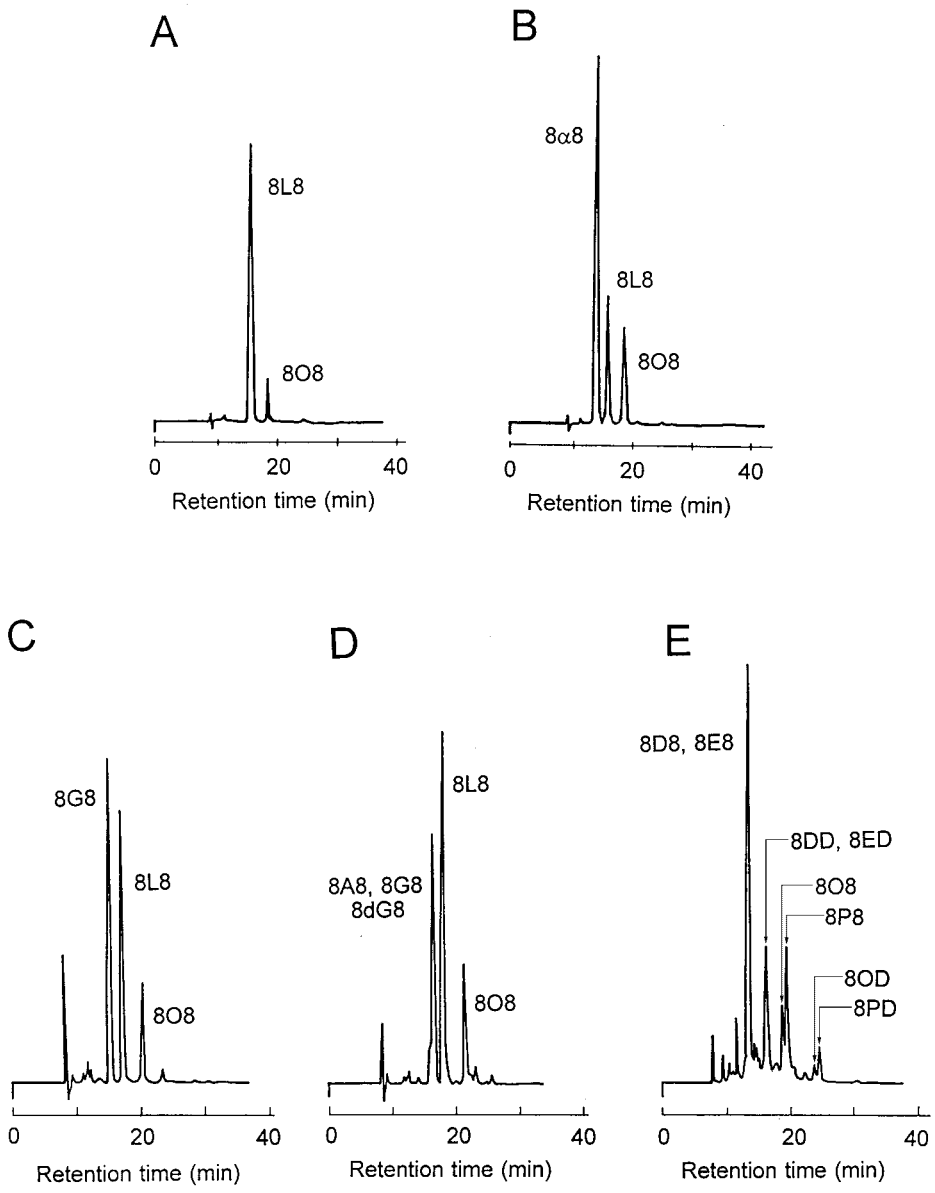


Figure 10. HPLC analysis of transesterified oils obtained by three-time repeated acidolyses of safflower oil (A), linseed oil (B), borage oil (C), TGA25 oil (D), and tuna oil (E) with CA. The conditions of analysis were the same as those in Figure 2. 8, Caprylic acid; L, linoleic acid; O, oleic acid; α, α-linolenic acid; G, GLA; dG, dihomo-γ-linolenic acid; A, AA; E, EPA; D, DHA.

ified oils obtained by acidolyses of safflower, linseed, borage, TGA25 and tuna oils. The results showed that three-time acidolyses of oils except tuna oil exchanged all fatty acids at 1,3-positions for CA. In addition, no generation of tricaprylin or partial glycerides confirmed that simultaneous hydrolysis and non-enzymatic acyl migration scarcely occurred. Therefore, the fatty acid composition at 2-position can be determined from the fatty acid composition of the transesterified oil obtained by three-times repeated reaction (Shimada et al., 1997c). However, because *Rhizopus* lipase acted on DHA only very weakly, all DHA at 1,3-positions of tuna oil were not exchanged for CA. If a 1,3-positional-specific lipase were to be available which acted on all fatty acids strongly, it would be a good catalyst for the enzymatic regiospecific analysis.

8.6 Conclusion

The studies on lipid-related compounds have been delayed compared with those on protein and carbohydrate, because it is difficult to handle those which are insoluble in water and also to determine their structures by microanalysis. However, biotechnology which was developed mainly during the 1980s has been introduced to the field of the oil and fat industry, and a new technology – lipids engineering – has evolved rapidly. In this chapter, we have described the production of PUFA-rich oils and highly absorbable structured lipids from the viewpoint of high-value added oils as foods. Lipases are available for other industrial fields. For example, the enzyme made it possible to purify PUFA from natural oil (Shimada et al., 1997a,d,e; 1998b–e; 1999a), and to convert vegetable oils efficiently to biodiesel fuel (fatty acid methylesters) – a technique which has attracted considerable attention with increasing environmental consciousness (Nelson et al., 1996; Shimada et al., 1999c). In future, we expect further applications of lipases to the improvement of oils and fats, syntheses of useful esters, and to other areas of industrial processing.

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9 Lipase-Catalyzed Synthesis of Structured Triacylglycerols Containing Polyunsaturated Fatty Acids: Monitoring the Reaction and Increasing the Yield

Tsuneo Yamane

9.1 Introduction

Structured triacylglycerols (sTAGs) are in the broadest sense defined as any triacylglycerols (TAGs) that have been restructured to change positions of fatty acids (FAs) and modified to change the FA compositions from the native state (Akoh, 1995). Natural edible fats and oils are simply mixtures of a number of TAGs that are different in terms of both FA species and their distribution along the glycerol backbone. In contrast to natural edible lipids, sTAGs are TAGs modified either chemically or enzymatically in either the type of FA or the position of the FAs. In a less broad sense, sTAGs are structured lipids (SLs) containing mixtures of either short-chain fatty acids, or medium-chain fatty acids (MCFA), or both, and long-chain fatty acids, in the same glycerol molecule. A number of studies have been carried out for the synthesis of sTAGs having (medium chain)-(long chain)-(medium chain)-type FAs (Christophe, 1998). These SLs are claimed to provide fewer metabolizable calories per gram than do traditional fats and oils, and to be efficient food sources for patients with pancreatic insufficiency and other forms of malabsorption. Cocoa-butter substitute, which consists predominantly of stearoyl-oleoyl-stearoylglycerol (SOS) or more generally saturated fatty acid-unsaturated fatty acid-saturated fatty acid type TAG (SUS), and 'Betapol' manufactured by Unilever, which has the structure of oleoyl-palmitoyl-oleoylglycerol (OPO), are included in this category of sTAGs.

In recent years, the concept of sTAGs has been extended to such that sTAGs are designer TAGs with desired fatty acids in terms of their structures and positions as 'nutraceuticals, functional food, or pharmaceuticals' to target the optimal nutrition, better metabolic conditions, and specific diseases. Along this trend, in the strictest sense, the term sTAG is given to a TAG with a particular FA at a specific position of glycerol hydroxyl moieties. The strictest definition of sTAG is applied in this review article, and is led to its classification shown in Table 1 (Iwasaki and Yamane, 2000), i.e. sTAG is either monoacid sTAG, diacid sTAG, or triacid sTAG. Note that in Table 1, FAs are shown in the order of their positions located at *sn*-1, *sn*-2 or *sn*-3 of the glycerol backbone. Thus AAB is not identical to BAA, but they are enantiomers. AAB (or BAA) type diacid sTAGs and all triacid sTAGs are identical to chiral TAGs (Villeneuve et al., 1994). This nomenclature is applied throughout this review.

sTAGs containing polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) or arachidonic acid (AA), have become of

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Table 1. Classification of structured triacylglycerols (sTAG).

No. of different FA	Type	Chirality	Stereoisomer
Monoacid-	AAA	Achiral	
Diacid-	ABA	Achiral	
	AAB, BAA	Chiral	Enantiomers
Triacid-	ABC, CBA	Chiral	Enantiomers
	BCA, ACB	Chiral	Enantiomers
	CAB, BAC	Chiral	Enantiomers

great interest because of various pharmacological effects of these FAs. These include several health benefits on cardiovascular diseases, immune disorders and inflammation, renal disorders, allergies, diabetes, cancer, etc. (Akoh, 1995; Gill and Valivety, 1997a,b). These FAs may be also essential for brain and retina development in humans.

Among sTAGs containing PUFA, sTAGs containing one molecule PUFA and two molecules of medium-chain fatty acids are very noticeable. Several studies have been performed for the synthesis of sTAG containing PUFA at specific sites of the glycerol backbone (Lee and Akoh, 1996; Shimada et al., 1996a; 1999; Soumanou et al., 1997). The absorption of PUFA into the body depends upon the position of PUFA along the glycerol backbone, i.e. at *sn*-1 (or 3) or *sn*-2 position (Christensen et al., 1995). sTAG containing PUFA at *sn*-2 position and MCFA at *sn*-1 and–3 positions can be hydrolyzed into 2–monoacylglycerol (2-MAG) containing PUFA and FA by pancreatic lipase and are efficiently absorbed into intestinal mucosa cells in normal adults. It is to be noted that mammalian pancreatic lipases hydrolyze the ester linkages at the *sn*-1 and *sn*-3 positions with a preference for MCFA over long-chain ones (Battino et al., 1967; Yang et al., 1990). Therefore, for dietary supplement for adult health, sTAG containing PUFA at *sn*-2 position and MCFA at the *sn*-1 and *sn*-3 positions may be suitable. On the other hand, due to the antiatherogenic, antineoplastic, and anti-inflammatory effects of the n-3 PUFAs, their intake is important for newborns for eicosanoid synthesis as well as for normal neonatal brain nervous development and cell membrane structure. Although PUFA are essential to the neonate for both normal growth and metabolism, neonatal intestinal function is immature, resulting in reduced levels of pancreatic lipase and bile acid salts. In addition, pancreatic lipase does not hydrolyze ester bonds containing long-chain n-3 PUFA (Iverson et al., 1992). Therefore, in the case of the newborn, PUFA absorption by pancreatic lipase is not feasible. However, for neonatal adsorption of PUFA, there is an alternative mechanism. PUFA are released from gastric digestion, and gastric lipase exhibits stereospecificity for the α-position of TAG and hydrolyzes the *sn*-3 position twice as fast as the *sn*-1 position (Iverson et al., 1992; Harmosh and Harmosh, 1996). Therefore, for PUFA therapy for neonates, sTAG containing PUFA at *sn*-1 (or -3) position and MCFA at the other sites may be suitable.

sTAG can be synthesized either chemically or enzymatically. However, an enzymatic synthesis of sTAG is more advantageous over a chemical process with regard to several aspects. Enzymes are generally specific, giving rise to less or no bypro-

ducts, and exhibit catalytic action under mild conditions. Enzymatic reactions have another advantage for the synthesis of sTAG containing PUFA because PUFAs are very unstable. They are prone to be easily isomerized, oxidized and polymerized. These properties necessitate the use of as mild conditions as possible, especially oxygen-free conditions.

A monograph dealing with sTAG was published two years ago, which covers most important aspects of sTAG such as technology, metabolism, medical and clinical uses as well as of specific food applications (Christophe, 1998). In this chapter, based on these perspectives, recent advances on the lipase-catalyzed synthesis of sTAG containing PUFA will be summarized, with a special emphasis of both monitoring the reaction and increasing the yield that we have recently studied (Han et al., 1999a,b; Iwasaki et al., 1999; Rosu et al., 1999a,b; Han and Yamane, 1999; Iwasaki and Yamane, 2000).

9.2 Monitoring the reaction

For the production of a targeted sTAG, it is essential to know which types of TAG are formed, and how many FAs are incorporated at a specific hydroxyl position of glycerol. When the intention is to synthesize two types of diacid sTAG containing PUFA, i.e. ABA and AAB (or BAA) types (Table 1) where A = MCFA and B = PUFA the ABA and AAB (or BAA) type sTAGs must be separated and determined by a suitable analytical method. Moreover, when the intention is to produce pure (EPA)C_8C_8 (a AAB-type sTAG) by lipase-catalyzed transesterification between eicosapentaenoic acid ethyl ester (EPAEE) and tricaprylin by the following Equation 1,

$$\text{EPAEE} + \text{Tricaprylin} \rightarrow (\text{EPA})C_8C_8 + C_8\text{EE} \quad (1)$$

a number of chemical species may appear during the reaction, including the two substrates, the targeted sTAG, its positional isomers [C_8(EPA)C_8] and inevitable byproduct (C_8EE). TAG containing two or three moles of EPA that may occur by further replacement of EPAEE, and hydrolytic byproducts that may appear from any esters. Table 2 lists in the first row almost all possible lipid species that may appear in the course of the reaction according to Equation (1). It is highly preferable to detect these species as well as the targeted sTAG. However, separation of more than 11 chemical species in one analysis is not an easy task, and requires an advanced analytical technique.

Several methods have been reported for the determination of the positional distribution of acyl groups in TAG, including enzymatic hydrolysis (Luddy et al., 1963; Foglia et al., 1995), or chemical degradation using Grignard reagent (Becker et al., 1993; Ando et al., 1996), followed by analysis of mono- and di-acylglycerol (MAG and DAG) products by chromatographic techniques, ^{13}C-NMR (Gunstone, 1991; Bergana and Lee, 1996), and silver-ion liquid chromatography (Dobson et al., 1995).

Table 2. Peak identification for the transesterification reaction between EPAEE and tricaprylin by silver-ion HPLC.

Peak no.	Chemical species	Possible isomers	Molecular weight	Number of double bonds
1	Tricaprylin, caprylic acid ethyl ester			
2	Caprylic acid, dicaprylin			
3	Dioctanoyleicosapentaenoylglycerol (TAG-A)	C_8(EPA)C_8 (TAG-A3)	629	5
4	Dioctanoyleicosapentaenoylglycerol (TAG-A)	(EPA)C_8C_8 (TAG-A1) C_8C_8(EPA) (TAG-A2)	629	5
5	Eicosapentaenoic acid ethyl ester (EPAEE)		331	5
6	Eicosapentaenoyloctanoyl diacylglycerol (DAG-A)	(EPA)C_8(OH), C_8(EPA)(OH) (OH)(EPA)C_8, (OH)C_8(EPA) (EPA)(OH)C_8, C_8(OH)(EPA)	503	5
7	Eicosapentaenoic acid (EPA)		303	5
8	Dieicosapentanoyloctanoyl triacylglycerol (TAG-B)	(EPA)C_8(EPA), (EPA)(EPA)C_8, C_8(EPA)(EPA)	787	10
9	Dieicosapentaenoyl diacylglycerol (DAG-B)	(EPA)(EPA)(OH), (EPA)(OH)(EPA), (OH)(EPA)(EPA)	661	10
10	Trieicosapentaenoylglycerol (TAG-C)	(EPA)(EPA)(EPA)	945	15

9.2.1 Enzymatic hydrolysis followed by analysis of both MAG and DAG

This method utilizes the high 1,3-regioselectivity of pancreatic lipase. Partial hydrolysis of TAG by porcine pancreatic lipase gives rise to 2-MAG, which is then separated by TLC. However, this method has two drawbacks. First, partial lipase hydrolysis of TAG may cause acyl migration, resulting in erroneous identification of acyl positional distribution. Second, the lipase method is not reliable for a TAG that contains significant amounts of short- and/or medium-chain fatty acids and very long-chain PUFA (Battino et al., 1967; Yang et al., 1990), because it hydrolyzes short- and medium-chain fatty acid esters preferentially and the ester bond of PUFA is resistant to its cleavage.

9.2.2 Chemical degradation using Grignard reagent

This method uses allyl magnesium bromide to partially deacylate the TAG. From the resulting mixture, four different classes of partial acylglycerols (2-MAG, 1,2-DAG, 2,3-DAG and 1,3-DAG) and unreacted TAG are isolated on a boric acid-impregnated thin-layer chromatography (TLC) plate. The FA composition of each acylglycerol is then analyzed as described in Section 9.2.1. In the chemical degradation analysis, analytical errors may occur readily, and the method time-consuming and labor-intensive.

9.2.3 ^{13}C-NMR

This technique is very useful and precise for the analysis of the positional distribution of PUFA in TAG, but requires an expensive instrument, and also rather large amounts of samples ($>$ 5 mg) which should be as pure as possible.

9.2.4 Silver-ion HPLC

None of the methods described in Sections 92.1–9.2.3 tell us what types of molecular species of TAG are present. Unless each molecular species is separated, only the positional distributions of FAs are determined. In order to determine the composition of a sample and its positional distribution of PUFA, the sample must be separated and purified into each molecular species using a chromatographic technique, after which the purified species must be analyzed by enzymatic, chemical or NMR methods. Accordingly, an easy, simple, and accurate method is needed to determine simultaneously both the molecular species composition of sTAG and the positional distribution of FAs of the reaction mixture.

Silver-ion chromatography is a technique that utilizes the property of silver ions to form reversible polar complexes with double bonds in organic molecules such as unsaturated lipids. This technique enables the separation of unsaturated species according to the number, geometric configuration and position of the double bond. Some researchers have demonstrated the separation of positional isomers of TAG that contain FAs having the same number of double bonds (Christie, 1988; Jeffrey, 1991; Adlof, 1995; Dobson et al., 1995). However, no separation of isomeric TAG containing PUFA was reported. In the author's laboratory, we have succeeded in the separation of (EPA)C_8C_8 [and/or C_8C_8(EPA)] and C_8(EPA)C_8 by using 2–propanol as a modifier in hexane-acetonitrile-based mobile phase for silver-ion HPLC (Han et al., 1999a; Iwasaki et al., 1999). In silver-ion HPLC with spectrophotometric detection at 206 nm, a hexane-acetonitrile-based mobile phase is generally used. However, the mobile phase has a solubility limit of acetonitrile in hexane (Adlof, 1994). 2-Propanol can serve as a third solvent, ensuring good solubility of acetonitrile in hexane. Figure 1A and B show the silver-ion HPLC charts of reaction mixtures produced by a 1,3-specific lipase (Lipozyme^{TM}) and a non-specific lipase

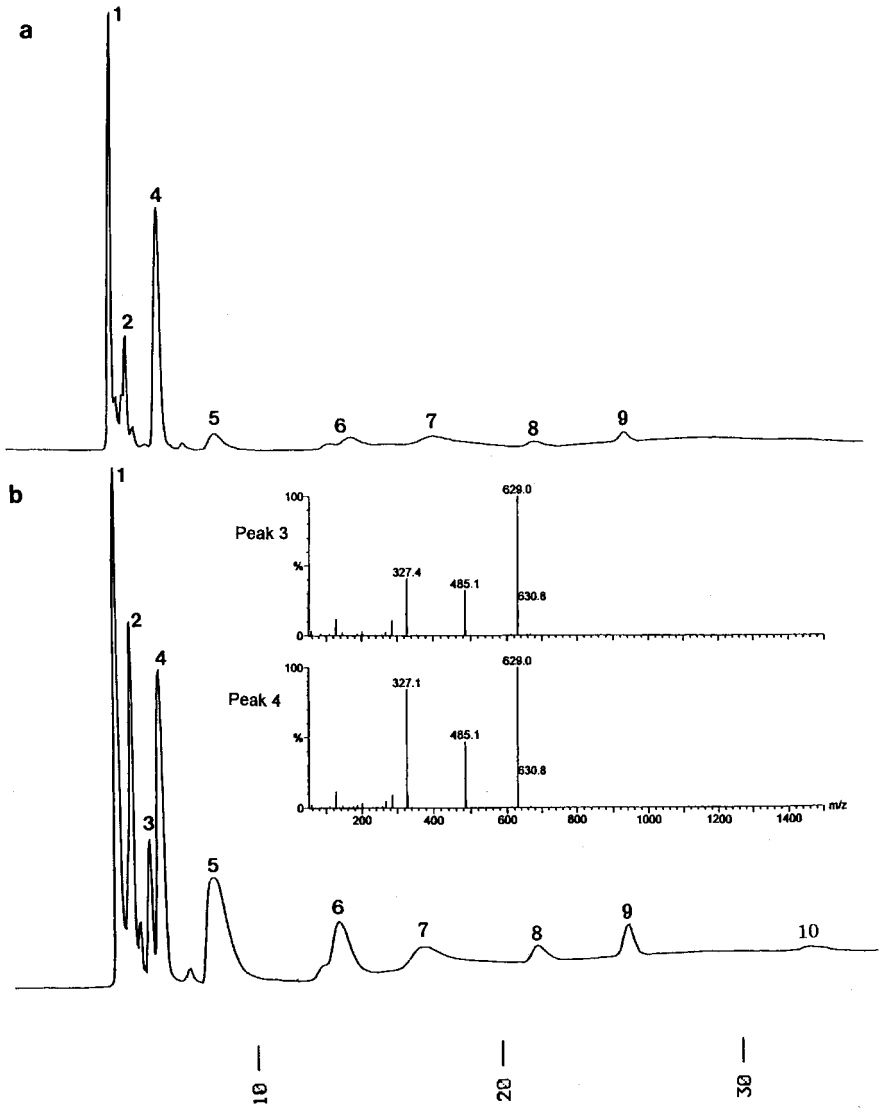

Figure 1. Silver-ion HPLC chromatogram of the 12−h reaction mixture [see Equation (1)] by two different lipases (Han et al., 1999a). (a) Reaction mixture by Lipozyme; (b) Reaction mixture by Liposam. Peak number is identical to that shown in Table 2. Molecular ion and fragmentation of peaks 3 and 4 analyzed by HPLC-APCI/MS are also shown.

(Liposam^{TM}), respectively from the interesterification between tricaprylin and EPAEE after a 12-h reaction time. A solvent gradient program was applied (for details see Han et al., 1999a) which allowed the separation of TAG containing EPA. This depends on the number of EPA molecules incorporated in the glycerol backbone and their isomeric distribution. The molecular weight of each peak was determined by HPLC followed by (atmospheric-pressure chemical ionization)-mass spectroscopy (APCI/MS) assay (as shown in Table 2), together with their peak number. The compounds which were eluted as peaks 3 and 4 had the same molecular mass; thereby the two compounds were isomers of TAG-A. This identification was further confirmed by the observation of fragment ions analyzed by HPLC-APCI/MS, as shown in Figure 1. The identification of peaks 3 and 4 as TAG-A3 and TAG-A1 (and/or TAG-A2) (see Table 2), respectively, was made on the regioselectivities of the enzymes used. Only peak 4 appeared in the reaction using 1,3–specific lipase, whereas peaks 3 and 4 were observed utilizing the non-specific lipase. Thus it was concluded that peak 4 corresponds to TAG-A (TAG-A1 and/or TAG-A2) containing EPA at *sn*-1 (or 3)-position, and peak 3 shows the *sn*-2 positional isomer (TAG-A3).

The gradient of hexane/2-propanol/acetonitrile as mobile phase was also successfully applied to monitor the transesterification reaction between docosahexaenoic acid ethyl ester (DHAEE) and tricaprylin [Equation (2)] (as shown in Figure 2), although the acetonitrile content of solvent A and the mobile phase gradient program were slightly different (Iwasaki et al., 1999).

$$\text{DHAEE} + C_8C_8C_8 \rightarrow (\text{DHA})C_8C_8 + C_8\text{EE} \qquad (2)$$

Again, only peak 4 appeared with 1,3-specific lipase and both peaks 3 and 4 were obtained with non-specific lipase. Each peak identification is shown in Table 3.

The following points should be noted with regard to the silver ion HPLC for Equations (1) and (2):

- Separation of positional isomers is critically affected by the acetonitrile content of solvent A so that it must be optimized for each pair of sTAG.
- One isomer with an unsaturated FA at *sn*-2 position elutes faster than the other with unsaturated FA at *sn*-1 or *sn*-3 position.
- $(\text{DHA})C_8C_8$ elutes later than $(\text{EPA})C_8C_8$.
- The stereoisomers, TAG-A1 and TAG-A2 (see Table 2), and $(\text{DHA})C_8C_8$ and $C_8C_8(\text{DHA})$ (Table 2) cannot be separated. The positional isomers, TAG-B (Table 1), and [$(\text{DHA})C_8(\text{DHA})$, $(\text{DHA})(\text{DHA})C_8$ and $C_8(\text{DHA})(\text{DHA})$] (Table 3), cannot be separated, either.

Silver-ion HPLC was proven to be useful in the analysis of the TAG positional composition of more complicated mixtures. The lipase-catalyzed acidolysis of a single cell oil (SCO) of high DHA (and docosapentaenoic acid, DPA) content with caprylic acid (C_8) was also investigated (Iwasaki et al., 1999) according to Equation (3).

$$\text{SCO} + C_8 \rightarrow \text{TAG containing one DHA (and DPA) and two } C_8 + \text{Others} \qquad (3)$$

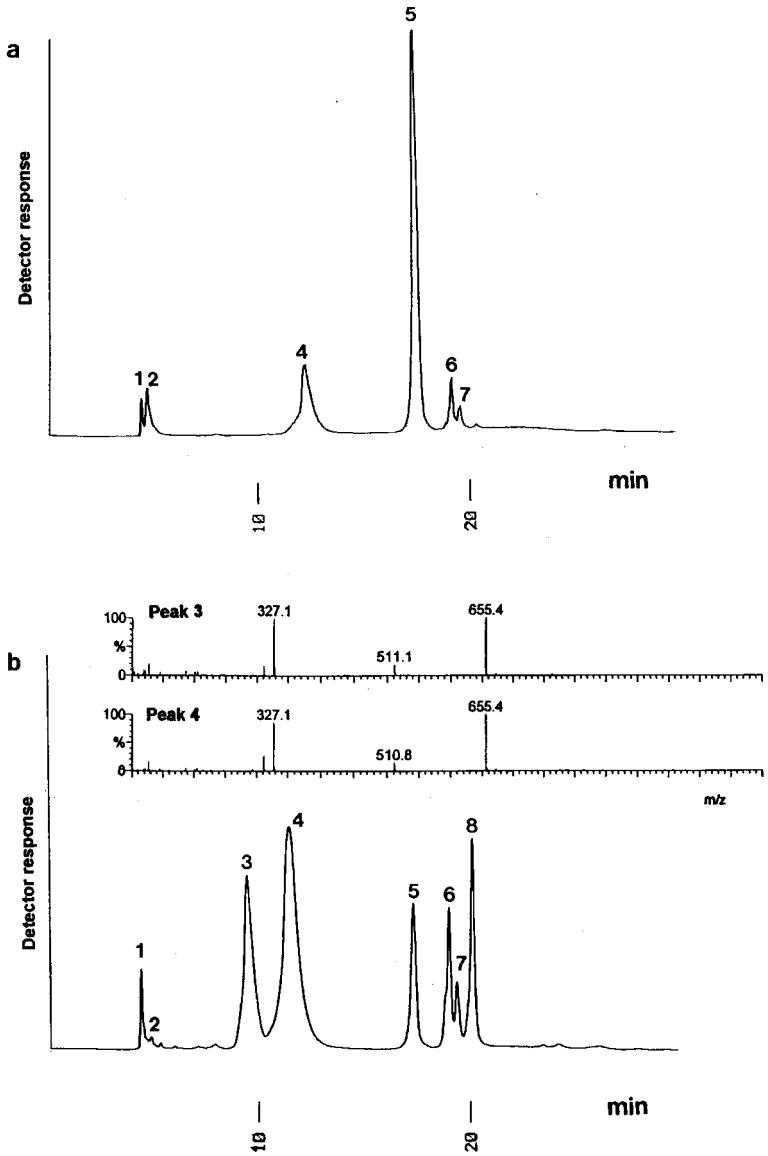

Figure 2. Silver-ion HPLC chromatogram of transesterification shown in Equation (2) by (a) 1,3-specific lipase (Lipozyme) and (b) by non-specific lipase (Liposam) (Han et al., 1999b). Mass spectra of the isomers of dioctanoyldocosahexaenoic are also shown. Each peak identification is shown in Table 3.

Table 3. Peak identification for the transesterification reaction between DHAEE and tricaprylin by silver-ion HPLC.

Peak no.	Chemical species	Possible isomers	Molecular weight	Number of double bonds
1	Tricaprylin, caprylic acid ethyl ester			
2	Caprylic acid, dicaprylin			
3	Dioctanoyldocosahexaenoylglycerol	C_8(DHA)C_8	655	6
4	Dioctanoyldocosahexaenoylglycerol	(DHA)C_8C_8, C_8C_8(DHA)	655	6
5	Doicosahexaenoic acid ethyl ester (DHAEE)		357	6
6	Docosahexaenoyloctanoyl diacylglycerol	(DHA)C_8(OH), C_8(DHA)(OH) (OH)(DHA)C_8, (OH)C_8(DHA) (DHA)(OH)C_8, C_8(OH)(DHA)	503	6
7	Docosahexaenoic acid (DHA)		329	6
8	Didocosahexanoyloctanoyl triacylglycerol	(DHA)C_8(DHA), (DHA)(DHA)C_8, C_8(DHA)(DHA)	839	12

The FA composition of SCO was 4.2 mol% myristic acid (MA), 2.5 mol% pentadecanoic acid, 46.3 mol% palmitic acid (PA), 1.3 mol% stearic acid (SA), 10.2 % DPA, and 35.5 mol% DHA. The TAG fraction of the reaction mixture was subjected to silver-ion HPLC. In the spectrophotometric detection at 206 nm, the sensitivity of each TAG species depends mainly on its double bond number. Therefore, the detector shows very weak responses for all saturated TAG species. The detector can only estimate the ratio of the desired TAG to their positional isomers that contain the same number of double bonds because the detector's response is assumed to have the same value for each isomer. The ratio of the positional isomers can be estimated from the corresponding peak areas. Most major peaks, amounting to 15 – 17 in number, could be identified. The desired sTAGs, C_8(DHA)C_8 and C_8(DPA)C_8 could be separated from their positional isomer, C_8C_8(DHA) [and (DHA)C_8C_8], C_8C_8(DPA) [and (DPA)C_8C_8], respectively.

9.2.5 Chiral HPLC

Silver-ion HPLC cannot separate stereoisomers, i.e. AAB and BAA types of sTAG. In order to determine the enantiomeric excess of the chiral diacid sTAGs, a stereospecific analysis of the product must be carried out. The basis of the stereospecific analysis of TAG is the generation of the enantiomeric MAGs and DAGs from the parent TAG by Grignard reagent. These are then converted while maintaining their chiral information into diastereomeric derivatives that allow measurement of fluor-

escence at a particular wavelength. Subsequent analysis by a chiral HPLC results in the separation of *sn*-1,2 and *sn*-2,3 diastereomers (Itabashi and Takagi, 1987; Takagi and Ando, 1990; Christie et al., 1991; Uzawa et al., 1993; Kim et al., 1994; Chandler et al., 1998). In the case of sTAG containing PUFA, it may be unnecessary for enantiomeric DAGs to be converted into the diastereomeric derivatives because the DAGs have an absorption at 206 nm; thus they can be directly detected spectrophotometrically.

9.2.6 High-temperature gas chromatography (HTGC)

For analyzing the reaction mixture of sTAG synthesis by gas chromatography (GC), precaution should be taken against instability of PUFA at elevated temperature. On-column injection (Traitler, 1988) is the best technique for transferring a sample con-

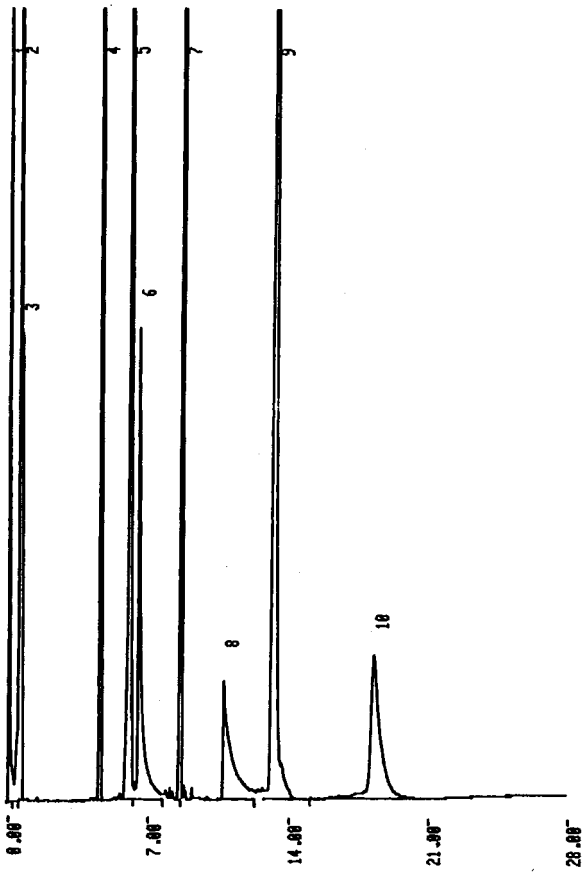

Figure 3. Gas chromatogram of the reaction mixture [see Equation (1)] (Han et al., 1999a). Peak 1, solvent; 2, C8EE; 3, caprylic acid; 4, internal standard (*n*-eicosane); 5, EPAEE; 6, dicaprylin; 7, tricaprylin; 8, DAG; 9, TAG-A; 10, TAG-B.

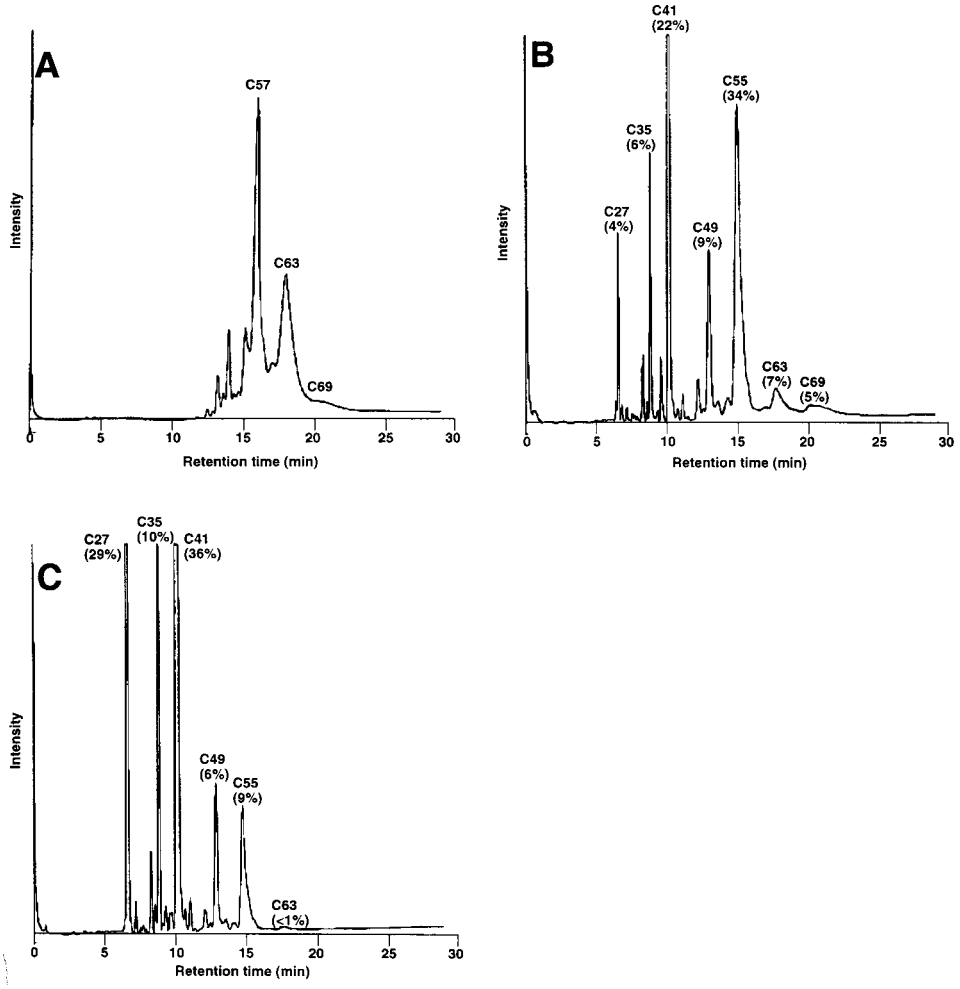

Figure 4. Gas chromatograms of the TAG fraction [see Equation (3)] (Iwasaki et al., 1999). (A) From single cell oil (SCO). (B) From the reaction by *Rhizomucor miehei* lipase. (C) From the reaction by *Pseudomonas* lipase. The numbers of carbon atoms for the major peaks are indicated after the symbol C. For (B) and (C), the contents of TAG species (% peak area) are shown in parentheses.

taining PUFA onto a capillary column in order to avoid sample deterioration before entering the column. Careful programming of the column temperature is required to achieve a good peak separation. For quantitative analysis, *n*-eicosane can be used as an internal standard. Figure 3 shows a GC chromatogram of the reaction mixture shown in Equation (1) using a non-regiospecific lipase (Novozyme^{TM}) (Han et al., 1999a). Changes in the content of TAG-A during the time course of the reaction can be conveniently monitored by GC analysis.

In HTGC analysis of a TAG fraction obtained from the reaction shown in Equation (3), TAG species are separated depending on their carbon numbers; the composition

of TAG species can then be calculated from the peak areas. The TAG fraction of the original SCO and acidolyzed oil were analyzed by HTGC (an example is shown in Figure 4) (Iwasaki et al., 1999). The peaks were identified by comparing their retention times to those of authentic TAGs.

9.2.7 TLC/FID

Although thin-layer chromatography/flame ionization detector (TLC/FID) (IatroScan®) is a very convenient analytical instrument, its application to the monitoring of the time course of the synthesis of sTAG containing PUFA is limited to the detection of the decrease in the pure substrate contents of the reaction mixture and the formation of partial acylglycerols (byproducts). However, separation within a class of partial acylglycerols or TAGs is not possible.

9.3 Increasing the yield

As is the case with most enzymatic reactions, the performance of enzymatic synthesis of pure sTAG containing PUFA depends on many factors involving type of reaction, enzyme and its immobilization, temperature, water content, composition of substrates, physical properties of the substrates, reaction time, mode of operation, etc. Some of these factors are briefly mentioned in the following text, together with examples of reactions yielding pure AAB- and ABA-types of sTAG, both of which contain PUFA.

9.3.1 Choice of enzyme and its immobilization

Enzymes involved in sTAG synthesis are exclusively lipases. Careful exploitation of positional (regional) specificity, FA specificity and stereospecificity of lipases can provide a maximum yield of the desired sTAG. To date, most researchers have utilized fungal lipases such as those from *Rhizomucor miehei, Rhizopus delemar, Rhizopus javanicus*, and *Rhizopus niveus*, which are 1,3-specific and hence are effective to synthesize ABA type sTAG. As for non-regiospecific lipases, a number of microbial lipases are commercially available such as those from *Candida* sp. (*C. antarctica* and *C. rugosa*) and those from *Pseudomonas* sp. (*Ps. fragi, Ps. cepacia, Ps. glumae* and *Ps. fluorescens*). Some lipases also exhibit stereospecificity, e.g. *Rhizomucor miehei* and *Carica papaya* latex lipases have been claimed to show *sn*-1 and *sn*-3 preferences, respectively, in interesterification reactions (Villeneuve et al., 1997; Chandler et al., 1998), although they are not absolute. There is a potential that, in future, it may be possible to synthesize a chiral sTAG by using such a stereospecific lipase.

Immobilization of lipases provides some benefits; i.e. increased stability, ease of recovery and re-use of the enzyme, thereby reducing the production cost. *Rhizomucor miehei, Candida antarctica* and *Pseudomonas* sp. lipases are available commercially in immobilized forms. Chandler et al. (1998) prepared immobilized lipase using macroporous polypropylene particles and Shimada et al. (1996a,b; 1999) immobilized *Rhizopus delemar* lipase onto porous ceramic particles for their studies on enzymatic sTAG production. The author's group reported the effective immobilization of fungal and bacterial lipases on fine $CaCO_3$ powder (Rosu et al., 1997; 1998a,b). $CaCO_3$ powder is used commercially as a food additive, so it is a very cheap and safe material. The enzymes were effectively immobilized by physical adsorption, which is an easy method of immobilization. Due to tight adsorption, leakage of the enzymes was negligible in neat liquid organic substrate, which resulted in the product being perfectly free from contamination with the protein. The immobilized lipases were reusable on many occasions (Rosu et al., 1997).

9.3.2 Solvent-free systems

Since the emergence of 'nonaqueous enzymology', it has been recognized (though not explicitly) that all enzymatic reactions in organic media have been classified into two systems: solvent systems and solvent-free systems. In the former system, the substrate(s), is dissolved in an inert liquid organic solvent. The solvent does not participate in the reaction in any respects, but provides an environment in which the dissolved substrate(s) is consumed by the enzymatic action. By contrast, in the latter system, no organic compounds (except enzyme or immobilized enzyme) other than the substrate(s) exist in a bioreactor. In other words, the bioreactor is occupied with substrate(s) only. In some cases, the reaction system is composed of two or more substrates, one of which exists in a large excess (much higher than the stoichiometric molar ratio). In such a case, the excess substrate also works as bulk solvent for the second substrate. This case is sometimes called 'reaction-in-neat'.

Solvent-free systems have a number of merits over solvent systems if they work successfully, including very high volumetric productivity, avoidance of enzyme inactivation by the solvent, and preference for safety in food industry. The solvent-free system also offers a better factory environment, without the need for explosion-free equipment, and the absence of the solvent is highly desirable for the health of workers engaged in bioprocessing. One possible disadvantage of using the solvent-free system, even if it is feasible, may be longer reaction times as compared to the solvent counterpart, and the enzyme may be inactivated due to the longer duration of the reaction. It should be noted, however, that a longer reaction time is quite reasonable if one considers the fact that in the solvent-free system greater absolute amounts of substrate(s) exist in the bioreactor volume than in the solvent system. Volumetric productivity [(kg product formed) $\cdot$ (liter of reactor volume)$^{-1}\cdot$h^{-1}] of the solvent-free system may be higher than that of the solvent system if they are compared on the basis of the same volume of the reaction mixture and the same amount of the enzyme used. The solvent-free system can be implemented not only in a monophasic system, but also in a biphasic system, as exemplified by the author's group (Rosu et

al., 1997; 1998a,b; 1999a). Public perception about the advantages of solvent-free biotransformations might also bring about a shift from early R&D experiments using organic solvents to industrial implementation of solvent-free bioprocesses. This also holds true for the production of sTAG containing PUFA (Shimada et al., 1996b; 1999). All experiments about lipase-catalyzed sTAG formation described by the author's group have been performed in solvent-free systems (Han and Yamane, 1999; Han et al., 1999a,b; Iwasaki et al., 1999; Rosu et al., 1999a,b).

9.3.3 Increasing the yield in a microaqueous system

A reaction scheme of the lipase-catalyzed synthesis of sTAG containing PUFA can be generally formulated as shown in Equations (4) and (5):

$$\text{S-S'} + \text{S"} \underset{\leftarrow}{\longrightarrow} \text{sTAG} + \text{S'} \tag{4}$$

$$\text{S} + \text{S"} \underset{\leftarrow}{\longrightarrow} \text{sTAG} + \text{H}_2\text{O} \tag{5}$$

Equation (4) is a transesterification, which is further subdivided into acidolysis, alcoholysis and interesterification depending on acid, alcohol or ester serving as S", respectively (Yamane, 1987). Equation (5) represents an esterification between an acid and an alcohol, liberating H_2O. There are several strategies that may be successfully applied to the reactions shown in Equations (4) and (5) in order to increase the yield of sTAG.

Substrate ratio

In Equations (4) and (5), one of the substrates is a PUFA or a PUFA derivative (e.g. ethyl ester). Pure PUFAs are relatively expensive, and so should be used as the 'limiting substrate' to achieve a total conversion of the substrate, while an excess molar amount of the other substrate (e.g. a triglyceride) often results in good yields due to a favored equilibrium.

Thermodynamic shift

Because both reactions in Equations (4) and (5) are reversible, the yield of the targeted sTAG increases as the byproducts S' or H_2O are removed from the reaction mixture, by further shifting of the equilibrium ('thermodynamic shift'). The principle is straightforward, and can be always applied to any (bio-)chemical reaction. Methods to achieve this include winterization (Yamane et al., 1993) when the solubility of S' becomes low at reduced temperature, N_2 gas bubbling or vacuum (reduced pressure) when S' is volatile or has a low boiling point [e.g. ethanol in Equation (4)] or when H_2O [Equation (5)] is removed by dehydration using activated molecular sieves, etc. Tautomerization of vinyl alcohol is another way of eliminating S'

from the reaction system (Bornscheuer and Yamane, 1995). When an industrial-scale production is concerned, a combination of a packed-bed reactor, a substrate reservoir, and a vacuum apparatus may be more realistic than a large stirred-tank reactor operated under reduced pressure (Yoshida et al., 1997).

Side reactions (hydrolysis and acyl migration)

Notable side reactions that are concomitant with Equation (4) are hydrolysis and acyl migration. The former depends naturally on the water content. Excess amounts of water always decrease the final yield of the desired product due to hydrolysis of the desired ester. Acyl migration which is confirmed by the formation of 1,3-DAG, may (more or less) not be inevitable. This depends on a number of factors such as water content, reaction temperature, enzyme load, reaction time and substrate ratio (Xu et al., 1998a; 1999). Xu et al. (1998b,c) studied the lipase-catalyzed interesterifications of fish oil with caprylic acid, and of medium-chain TAG with sunflower oil, in a solvent-free system in pilot batch and continuous operations. In a pilot batch production 0.22 – 1.37 % h^{-1} acyl migration occurred in the former operation, whereas in the latter reaction acyl migration was further reduced 4-fold at a similar extent of incorporation (see also Chapter 11).

Water content

In applying the effect of the thermodynamic shift, however, special precautions must be taken against trace amounts of H_2O existing in the reaction mixture (Yamane, 1987, 1988; Yamane et al., 1998). In a microaqueous solvent-free system, water plays two roles: (i) it is necessary to maintain the catalytic activity of the lipase; and (ii) it promotes hydrolysis, an unfavorable side reaction [Equation (4)]. It has been well documented that the water level in the reaction system critically affects the performance of enzymatic reactions in organic media. Water has a profound influence on both yield and rate of reactions. Essential water or bound-water that is actually in equilibrium with water in the bulk solution must be retained to keep both activity and stability of the enzyme molecule. However, an excess amount of water always reduces the final yield of the targeted sTAG due to hydrolysis that results in the formation of byproduct(s). When dry N_2 gas bubbling or vacuum is applied for removing S' [Equation (4)], it may also remove water that is essential for the enzyme's catalytic activity. As depicted in Equation (5), the liberated water should be removed, but essential water should be retained. The effect of water on the reaction performance is usually controlled by adjusting the thermodynamic water activity (a_w) of the reaction components and the enzyme. The crucial role of trace amounts of water in solvent-free biotransformations makes it a major factor in 'microaqueous organic media' systems (Yamane, 1987; Yamane, 1988; Yamane et al., 1998).

9.4 Examples of syntheses of pure sTAG containing PUFA

Among a number of reactions to produce sTAGs, only reactions involving PUFA biotransformations are discussed here. These are classified into two types: (i) reactions involving natural edible oils (vegetable oil or fish oil); and (ii) reactions between chemically defined pure TAG and pure FA or FA esters. Reports dealing with the former are summarized in Table 4. In the following only reactions between chemically defined pure TAG and pure FA or FA esters are discussed.

9.4.1 AAB-type sTAG

Han *et al.* have recently studied the reaction in a solvent-free system (Han et al., 1999a,b; Han and Yamane, 1999) [Equation (6)]:

$$\text{EPAEE} + C_8C_8C_8 \rightarrow (\text{EPA})C_8C_8 \text{ [and/or } C_8C_8(\text{EPA})] + C_8\text{EE} \qquad (6)$$

Three microbial lipases (fungal, yeast and bacterial) were tested for the formation of the pure targeted product (Han et al., 1999a), and a fungal lipase (*Rhizomucor miehei*) was proven to give almost pure AAB type sTAG. An investigation of the molar ratio of EPAEE to tricaprylin in the range of 1 : 1 to 1 : 5, the ratio of 1 : 3 gave the highest yield (ca. 90 %). The influence of the temperature was also studied and showed that the selectivity of the lipase seemed to decrease slightly at higher temperatures. Also, the initial a_w affected the yield significantly. A vacuum was applied to shift the reaction equilibrium toward synthesis reaction by removing C_8EE. While applying vacuum, the water level of the reaction system was controlled to the optimal level by addition of suitable amounts of water at predetermined time intervals. Intermittent periodical addition of suitable amounts of water made the reaction faster and enabled a higher final yield (Figure 5) (Han and Yamane, 1999). Using a_w control, the yield of the targeted sTAG reached 90 % after 16 h.

Table 4. Synthesis of sTAG containing PUFA from natural edible oils.

Substrates	Enzyme source	Immobilization	Reference
Tuna oil + caprylic acid	*Rhizopus delemar*	Ceramic carrier	Shimada et al., 1996a
Borage oil + caprylic acid	*Rhizopus delemar*	Ceramic carrier	Shimada et al., 1999
Single cell oil + caprylic acid	*Rhizomucor miehei*	Macroporous ionic resin	Iwasaki et al., 1999
Fish oil + tricaprylin	*Candida antarctica*	?	Lee and Akoh, 1998

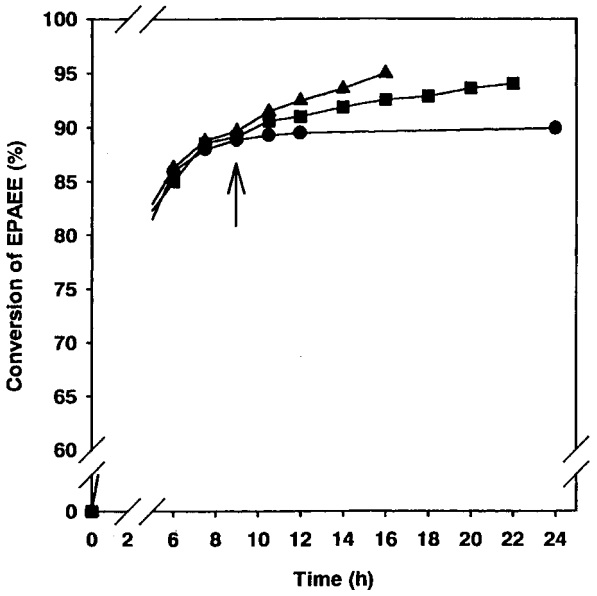

Figure 5. Effect of water activity (a_w) control during vacuum application on molar yield of C_8C_8(EPA) [and/or (EPA)C_8C_8] (Han and Yamane, 1999). The reaction was performed without vacuum at normal pressure in a closed vessel (●), reaction under 3 mmHg pressure applied at 9 h (indicated with a vertical arrow) thereafter with no a_w control (■), and reaction under 3 mmHg applied at 9 h (indicated with a vertical arrow) thereafter with a_w control at 0.07–0.17 (▲).

9.4.2 ABA-type sTAG

Pure ABA-type sTAG can be produced by several methods, including a two-step reaction (Soumanou et al., 1998; Schmid et al., 1998) as shown in Equations (7) and (8).

$$\text{TAG} + \text{Alcohol} \rightarrow \text{2-MAG} + \text{FA Esters} \quad (7)$$

$$\text{2-MAG} + \text{FA} \rightarrow \text{ABA-type sTAG} + H_2O \quad (8)$$

The first step [Equation (7)] is an alcoholysis (ethanolysis) of TAG (triolein, trilinolein, peanut oil or fish oil) in an organic solvent (e.g. methyl-*t*-butyl ether or acetone) using a 1,3-regiospecific lipase. 2-MAG was obtained in up to 80–90 % yield at $>$ 95 % purity by crystallization. The second step [Equation (8)] is an esterification of these 2-MAG which in hexane, again with 1,3-regiospecific lipase, gave almost pure ABA-type sTAG. For example, Betapol (OPO) was obtained in $>$ 78 % yield and 96 % purity.

The author's group reported a chemo-enzymatic synthesis of C_8(EPA)C_8, as shown in Equations (9) and (10) (Rosu et al., 1999a).

$$\text{Glycerol} + C_8 \rightarrow \text{1,3-Dicaprylin} + H_2O \quad (9)$$

$$\text{1,3-Dicaprylin} + \text{EPA} \rightarrow C_8(\text{EPA})C_8 + H_2O \quad (10)$$

With stoichiometric ratios of the two substrates in a solvent-free system and water removal by vacuum, 98 % of 1,3-dicaprylin was obtained by the esterification [Equation (9)] under optimal conditions. 1,3-Dicaprylin was then subjected to the second chemical esterification [Equation (10)] by using 1,1'-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) in dry chloroform. The yield after purification by silica gel column chromatography was 42 %, and the purity of TAG was 98 %, of which 90 % was $C_8(EPA)C_8$. Thus, the chemo-enzymatic process was unsatisfactory. 1,3-DAG can be also produced by chemical esterification of 1,3-dihydroxyacetone with DC in the presence of DMAP followed by reduction by sodium borohydride (Awe et al., 1989; Baba et al., 1994). The use of expensive, xenobiotic catalysts and low yields are disadvantages of chemical and chemo-enzymatic methods. Therefore, a higher yield of esterification to meet the industrial demand may not be expected unless a more efficient catalyst is found.

More recently, the author's group has developed a novel two-step enzymatic process that seems more promising as shown in Equations (11) and (12) (Rosu et al., 2000):

$$\text{Glycerol} + 3\text{EPAEE} \rightarrow (\text{EPA})(\text{EPA})(\text{EPA}) + C_2H_5OH \quad (11)$$

$$(\text{EPA})(\text{EPA})(\text{EPA}) + C_8\text{EE} \rightarrow C_8(\text{EPA})C_8 + 2\text{EPAEE} \quad (12)$$

The first step [Equation (11)] is an esterification in a solvent-free system with non-regiospecific lipase. When immobilized *Candida antarctica* lipase (Novozyme^{TM}) was used under optimal conditions in appropriately reduced pressure, over 90 % yield of the targeted product was achieved from stoichiometric ratios of the substrates. The reaction mixture from step (11) was then subjected to the second step [Equation (12)] without any purification after separation of the immobilized enzyme. The second step [12] is again an interesterification in a solvent-free system with 1,3 – regiospecific lipase (*Rhizomucor miehei*, Lipozyme^{TM}). When excess molar ratio of C_8EE over tri-EPA (100/1) was used, ca. 100 % of the yield of the targeted product was obtained (Figure 6). The unreacted C_8EE and the byproduct (free caprylic acid, EPAEE and free EPA) can be removed fractionally by short-pass distillation from the acylglycerol fraction which contains more than 90 % of $C_8(EPA)C_8$. It is to be noted that although molar incorporation of EPA into the glycerol backbone is only one from three moles in a single cycle of the reaction, the two remaining moles can be reused in the first step [Equation (11)] of the next cycle of the reaction so that all the EPAEE is eventually converted to the desired sTAG. The advantages of this process are that: (i) no organic solvent is used; (ii) isolation and purification of the intermediates is not necessary; and (iii) the liberated EPAEE (and small amounts of free EPA) and remaining caprylic acid ethyl ester are reusable. Figure 7A and B depict the typical time courses of reactions shown in Equations (11) and (12), respectively (Rosu et al., 2000).

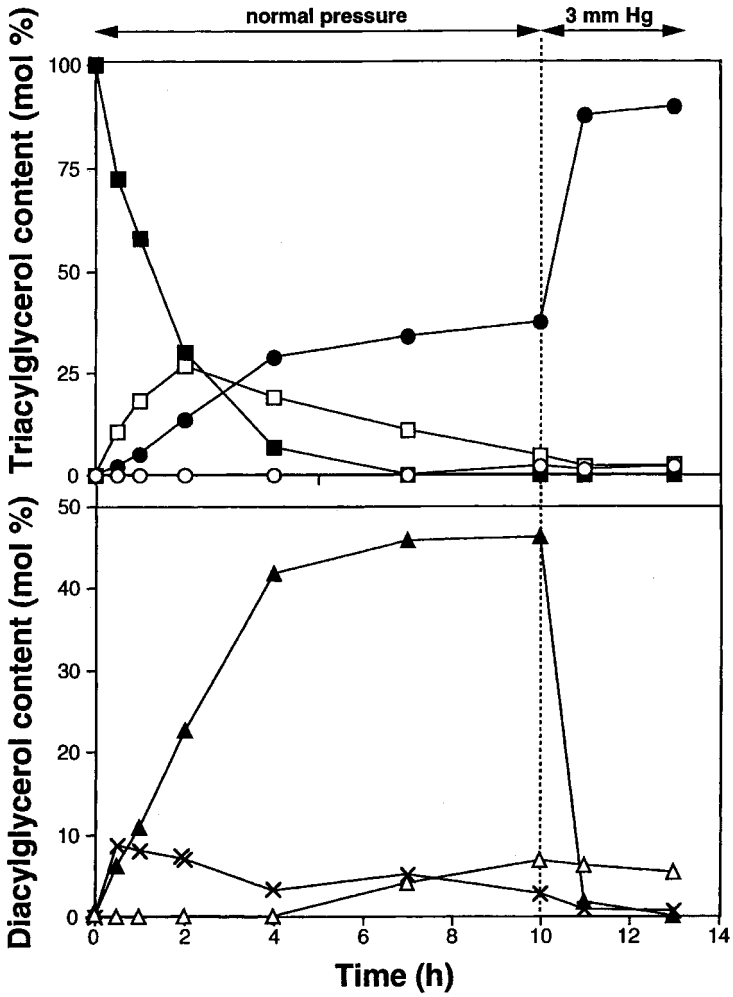

Figure 6. Time course of the reaction shown in Equation (12) with 1,3–specific lipase (Rosu et al., 1999). The molar ratio of tri-EPA/C_8EE was 1/100. The reaction was performed with 2 % initial water content at normal pressure for 10 h, followed by 3 h at 3 mmHg. TAG content [•, C_8(EPA)C_8; ■, (EPA)(EPA)(EPA); □, C_8(EPA)(EPA) and/or (EPA)(EPA)C_8; and ○, $C_8C_8C_8$]. DAG content [△, C_8C_8OH; ▲, C_8(EPA)OH; and ×, (EPA)(EPA)OH]. Note that no MAGs were detected.

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10 Enrichment of Lipids with EPA and DHA by Lipase

Gudmundur G. Haraldsson

10.1 Introduction

The long-chain n-3 type polyunsaturated fatty acids (PUFA) are characteristic of marine fat (Ackman, 1989) and commonly occur in triacylglycerols (TG) (Hölmer, 1989) and phospholipids (PL) (Vaskovsky, 1989) of fish. They originate in the lipids of photosynthetic microalgae that constitute phytoplankton and accumulate through the food chain in fish, which are unable to biosynthesize them (Sargent et al., 1995). Microalgae of marine origin is another important source of n-3 PUFA which is being utilized to an increasing extent (Henderson, 1999). The most ubiquitous of the n-3 fatty acids in fish are *cis*-5,8,11,14,17-eicosapentaenoic acid (EPA) and *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA). Fish oil is virtually pure TG comprising more than 50 different fatty acids (Ackman, 1982). The chain length ranges from C_{14} to C_{24} of varying degree of unsaturation, from saturated to polyunsaturated. Of the polyunsaturated fatty acids EPA and DHA usually account for between 5 % and 15 % each, depending on type of fish species, with the total n-3 content usually varying between 15 % and 25 %. Usually in fish the mid-position of the glycerol moiety is more enriched with n-3 PUFA, DHA in particular. Fish PL are generally much more highly enriched with EPA and especially DHA, 20 % to 50 % of each depending on the PL type. The total PL content of fish usually remains between 1 % and 1.5 % as based on total fish wet weight, roughly an order of magnitude lower than the TG (Haraldsson et al., 1993a).

The beneficial health effects of marine fat are now well established and almost exclusively attributed to the n-3 PUFA, EPA and DHA in particular (Stansby, 1990; Nettleton, 1995). There was a boost in the 1980s when it became evident that the n-3 PUFA might offer beneficial effects on the heart and cardiovascular diseases. This was initiated by reports from Danish scientists based on epidemiological studies on the Greenland Eskimos with a special emphasis on EPA. During the 1980s, scientists dedicated their interest almost exclusively to the beneficial effects of EPA on heart diseases and various inflammatory diseases. Consequently, concentrates aiming at as high EPA content as possible were under a high demand by the pharmaceutical industry as well as the health food industry as food supplements, with less emphasis on DHA. This was soon to change, and recently, there has been a dramatic shift in interest among scientists toward DHA, and concentrates of high DHA content are now under even a stronger demand. During the 1990s, it became DHA and its beneficial effects on pregnancy, infants and brain and nervous system development that occupied the attention of scientists (Jumpsen and Clandinin, 1995; Haumann, 1997).

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This has resulted in strong demands for their concentrates by the pharmaceutical industry, as well as the health food industry, as food supplements. In this chapter, the involvement and use of lipase in the n-3 field will be discussed. First, their application will be put in perspective with recent development and demand for various concentration forms of EPA and DHA and how they have found use in concentrating EPA and DHA in fish oil. Also, how they can be utilized to prepare structurally labeled lipids and finally, their role in the synthesis of various lipid forms containing EPA and DHA. More details are provided in the divided (though by no means exhaustive) review sessions, where our own contributions in the field are discussed in more detail and put into context with other related work.

10.2 Recent developments and role of lipase in the n-3 field

Concentrates of EPA and DHA first appeared on the market in the early 1980s. Max EPA was the first dietary n-3 supplement product on the market containing 18 % EPA and 12 % DHA in the natural TG form (Seven Seas, 1994). It has been widely used for various clinical studies for more than 15 years. TG up to the level of 30 % EPA+DHA can be prepared directly from fish oils without splitting the fat by a careful selection of fish oils and various methods such as winterization, molecular distillation and solvent crystallization (Ackman, 1988; Breivik and Dahl, 1992). Concentration beyond that level on the TG form is difficult, as it requires a cleavage of the fatty acids off the acylglycerols, either as free acids or monoesters. Various physical methods and combination of methods are available for concentrating EPA and DHA once released. Molecular distillation, supercritical fluid extraction and urea complexation can be used to concentrate them to the 50–85 % level. Concentration beyond the 90 % level and separation and purification of EPA and DHA requires more specific methods based on HPLC (Breivik et al., 1997). A whole range of monoester concentrates of EPA and DHA are now commercially available, usually as ethyl esters, some of which have been registered as drugs in various countries.

Resynthesis of TG highly enriched with EPA and DHA from the concentrates is by no means easy by traditional chemical esterification methods based on Lewis acid or alkoxide catalysis. The highly labile n-3 PUFA are very sensitive against the rather drastic conditions offered by these traditional methods. The all-*cis* n-3 framework makes them extremely prone to oxidation, *cis-trans* isomerization, double-bond migration, or polymerization. Despite that, some European companies have recently launched onto the market a whole variety of TG concentrates comprising 50–70 % EPA+DHA. These products usually constitute a mixture of roughly 55 % TG, 35–40 % diacylglycerols (DG) and 5–10 % monoacylglycerols (MG), which apparently reflects some compromise between efficiency and lability in their production.

Recently, lipases have been introduced to the n-3 field to solve these problems (Haraldsson and Hjaltason, 1992). These enzymes offer a high efficiency and mildness, and their application in organic media is now firmly established (Bornscheuer

and Kazlauskas, 1999). They offer TG concentrates of EPA and DHA of a whole range of composition including 100 % EPA or DHA, of high purity, highly efficiently, and in excellent yields. Neither chemicals nor organic solvents are required; thus these processes are highly feasible from industrialization as well as environmental hazard points of view. Nowadays, numerous industrial companies in the field are realizing the advantages and potential offered by the lipases, and some of them have marketed – or are about to market – TG concentrates of EPA and DHA. It is interesting to know that the Japanese authorities have not allowed concentrated ethyl esters or chemically modified n-3 concentrates to be sold as health food, only concentrated TG produced by lipases having been allowed for general sale.

Lipases can also be employed to enrich various other lipid classes with n-3 fatty acids, including PL and ether lipids (EL) of the 1-*O*-alkyl-2,3-diacyl-*sn*-glycerol type, which are characteristic of the fat in shark liver oil. PL highly enriched with EPA and DHA of the type occurring in fish are highly interesting and will certainly offer many interesting possibilities to the food supplement and pharmaceutical field. There is no doubt that there will soon be a strong demand for such PL. Lipases can also be used to provide such lipids that are structurally labeled with EPA and/or DHA into the *sn*-2 or *sn*-3 positions of PL or EL.

Lipases have also found applicability in concentrating EPA and DHA in fish oils. This application is based on their fatty acid selectivity, which can be utilized in concentrating EPA and DHA by kinetic resolution. Concentration levels of 50–70 % EPA+DHA can easily be obtained in high recovery directly from fish oil by lipase-catalyzed hydrolysis or alcoholysis reactions. Higher levels are obtained with free acids or monoesters, and separation of EPA and DHA is also offered by lipases which discriminate between EPA and DHA. Enrichment levels above 90 % can be obtained in two-step enzyme processes in high recoveries, and it is anticipated that lipases may be used to purify EPA and DHA toward high purity levels. This is also highly important from an industrialization point of view, as some of these processes do not require solvents or chemicals.

Currently, much research effort is being placed on the application of lipases for producing positionally labeled or structured lipids. Again, this is based on lipase selectivity, but in this case by their regioselectivity towards their natural acylglycerol substrates. 1,3-Regioselective lipases are lipases that act with a high preference or exclusively at the primary alcoholic end-positions of the TG, and have found an enormous use in this field. This is of great importance in the n-3 field, and TG enriched with EPA or DHA in the *sn*-2 position or the *sn*-1 and *sn*-3 positions will be offered for clinical and nutritional purposes. The potential in this field is clearly very high.

A different type of structured lipids, enantiostructured TG, may also find interest in the future. Their production is based on lipase enantioselectivity towards either the *sn*-1 or the *sn*-3 position of TG. Reports already exist in the literature of lipases displaying this type of selectivity towards TG (Villeneuve et al., 1995; Chandler et al., 1998), but this needs to be amplified further to offer some realistic advantages in the near future. There is little doubt that the resulting chiral structured lipids may offer various interesting aspects to the n-3 lipid field.

Finally, lipases can be employed to provide chiral precursors of glycerol derivatives as building blocks for asymmetric synthesis of the more complex PL and EL,

including ether-type PL and plasmalogens. This again is based on lipase enantioselectivity. There are several reports in the literature of kinetic resolution of racemates or biotransformations involving prochiral glycerol derivatives. Again, lipases can be used to introduce EPA or DHA at a later stage in the synthesis.

From what has been discussed above it is clear that the possibilities of employing lipases in the field of lipids and n-3 PUFA are virtually endless. In what follows in this chapter the application of lipases to produce various lipid classes highly enriched with EPA and DHA including TG, EL and PL will be described. The application of lipases to concentrate EPA and DHA from fish oil will also be described. Finally, the application of lipases to produce structured lipids containing EPA and DHA will be discussed briefly.

10.3 Enrichment of triacylglycerols with EPA and DHA

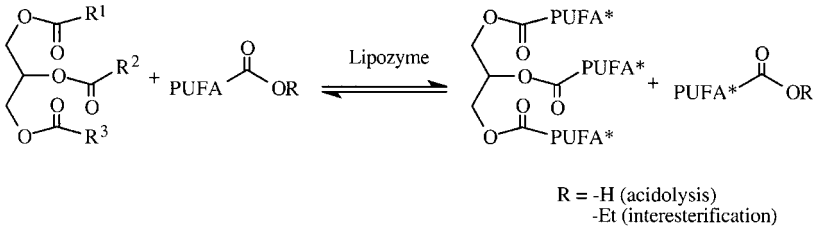
This Section is divided into two parts. The first part deals with incorporation of EPA and DHA into fish oil by lipase-catalyzed transesterification reactions. The second part involves the direct esterification of glycerol with PUFA as free acids to obtain TG of identical composition to the PUFA being used by a different lipase. That methodology also offers homogeneous TG of 100 % EPA and 100 % DHA.

10.3.1 Enrichment of cod liver oil with EPA and DHA

It is relatively easy to concentrate EPA and DHA up to high levels as free fatty acids or monoesters. However, the natural form of these fatty acids in fish oil is TG, and the major challenge was to produce natural TG highly enriched with EPA and DHA, far beyond the 30 % level mentioned earlier. A highly successful solution to that problem was based on treating cod liver oil with free fatty acid or monoester concentrates of EPA and DHA in the presence of lipase to effect fatty acid exchange between the natural TG and the concentrates (Haraldsson et al., 1989). From the marketing point of view it was also important that cod liver oil already had a long history (decades) on the market as a vitamin A and D supplement (Hjaltason, 1989), and that the product could be claimed as being derived directly from cod liver oil.

Lipozyme, the immobilized 1,3-regiospecific lipase from the fungus *Mucor (Rhizomucor) miehei* (available commercially from Novo Nordisk in Denmark), was employed to effect transesterification reactions of cod liver oil with concentrates of EPA and DHA. The cod liver oil comprised approximately 9–10 % each of EPA and DHA, and TG highly enriched with n-3 PUFA was accomplished, of high purity and in virtually quantitative yields. Interesterification and acidolysis reactions with ethyl ester and free fatty acid concentrates, respectively, both comprising 85 % EPA + DHA content, resulted in TG containing 60–65 % EPA+DHA and well over 70 % total n-3 PUFA. At that time this represented by far the highest EPA- and DHA-enriched TG product available. Both reactions were conducted in the absence of any solvent, using 10 % dosage of lipase, as based on the weight of fat, at

60–65 °C, the lipase preparation constituting 10 % water. A 3-fold excess of free fatty acids or ethyl esters was used, as based on number of molar equivalents of esters present in the TG. The reactions are demonstrated in Scheme 1.

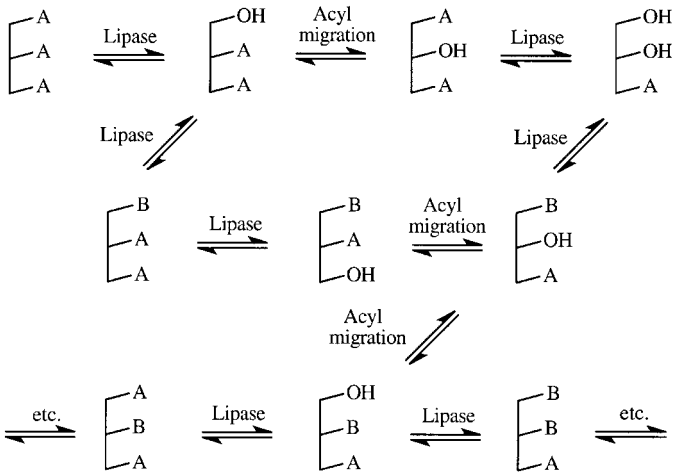


Scheme 1. Lipozyme-catalyzed enrichment of cod liver oil with PUFA by acidolysis (R = H) or interesterification (R = Et). PUFA* refers to equilibrium composition, but R1, R2 and R3 to the initial fatty acid composition of individual positions of cod liver oil, sn-1, sn-2 and sn-3, respectively.

In both reactions the lipase displayed a significantly higher activity toward EPA than DHA, and the interesterification reaction took place considerably faster than the acidolysis reaction. Some hydrolysis side reaction was observed, especially for the interesterification reaction, but this could be reduced considerably by lowering the water content of the lipase (Haraldsson et al., 1993b).

Despite the 1,3-regiospecificity of the lipase, the mid-position became enriched to an equal extent to the end-positions. Intramolecular nonenzyme-promoted acyl-migration processes (Kodali et al., 1990) were responsible for this, as was established by investigating the fatty acid composition of individual positions of the acylglycerols when the reactions proceeded (Haraldsson and Almarsson, 1991). This means that, at equilibrium, the fatty acid composition of the TG was reflected by a weighted average of the initial composition of the cod liver oil TG and the concentrates. This is exactly what was wanted, since the aim was to enrich the fish oil to the maximum levels. In order to allow that, prolonged reaction time was required because the acyl-migration processes were considerably slower than the lipase-promoted processes and rate-limiting for the equilibrium. In order to obtain an equilibrium for the interesterification, a 24-h reaction was needed, whereas the acidolysis reaction required 48 h.

Scheme 2 shows a simplified proposal of the transesterification reactions. This is a multi-component system involving more than 50 different fatty acids, TG, DG, MG as well as PUFA either as free acids or ethyl esters. Each component has its own distinguished relationship to the biocatalyst. The processes must be initiated by lipase-promoted hydrolysis at the end-positions to produce 1(3),2-DG, which can either undergo an acyl migration to form a more stable 1,3-DG or a much faster lipase-catalyzed esterification to reform a PUFA-enriched TG. Once formed, the 1,3-DG can undergo a second hydrolysis to form 1(3)-MG, which in turn can undergo PUFA acylation at the end-position to form a PUFA enriched 1,3-DG, followed by a subsequent PUFA acyl migration to form a 1(3),2-DG with PUFA located at the mid-position. This continues until an equilibrium has been reached with an identical fatty acid composition of each constituent and individual positions of the acylgly-



Scheme 2. A simplified proposed pathway for the transesterification reactions of triacylglycerols with free acids or monoester.

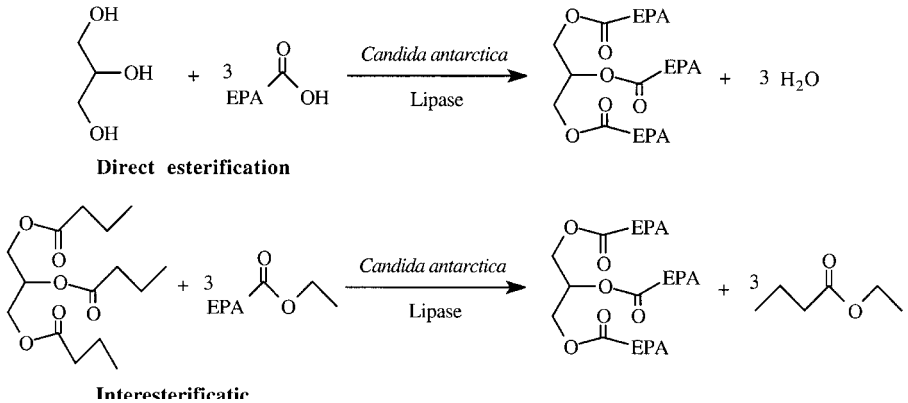
cerols. In addition, the lipase may be expected to act directly at the mid-position of the acylglycerols, despite its regioselectivity, presumably much more slowly. It is assumed that the more protic conditions associated with the acidolysis reaction may enhance the acyl-migration processes in that case, although the excessive amounts of free acids present will slow the hydrolysis reactions and at the same time retard incorporation into the mid-position.

10.3.2 Homogeneous triacylglycerols of EPA and DHA

Using the methodology described above, the EPA and DHA fatty acid composition of the TG product was determined by a weighted average of the initial fatty acid composition of the cod liver oil TG and the composition of the n-3 concentrates. Thus, in order to obtain elevated levels of EPA and DHA into an ordinary fish oil such as cod liver oil, excessive amounts of concentrates were needed. To avoid that limitation and to obtain TG of composition identical to the concentrates being used, a modified procedure based on a direct esterification of free fatty acids with glycerol was developed (Haraldsson et al., 1993c; 1995). This also opened the possibility of synthesizing TG homogeneous with either EPA or DHA, i.e. 100 % EPA or DHA. This was considered our synthetic challenge and primary goal – the crown on the TG issue in terms of EPA and DHA enrichment.

A different lipase, this time an immobilized nonregiospecific yeast lipase from *Candida antarctica* [also available commercially from Novo Nordisk in Denmark (SP 382); has now been replaced by Novozyme], was highly efficient in generating TG of both 100 % EPA and 100 % DHA content. This was accomplished by a direct esterification of glycerol with stoichiometric amount of pure EPA and DHA, without any solvent, by stirring at 65 °C under vacuum, with a 10 % dosage of the immobilized lipase, as based on the weight of substrates. The co-produced water was

condensed into a liquid nitrogen-cooled trap during the progress of the reaction, thus driving the reaction to completion (Scheme 3).



Scheme 3. Direct esterification of glycerol with EPA (top) and interesterification of tributyrin with EPA ethyl ester (bottom) using Candida antarctica lipase.

The resulting TG, homogeneous with either EPA or DHA, were afforded in near-quantitative yields of excellent purity. High-field ^{1}H- and ^{13}C-NMR spectroscopy was found extremely valuable as a probe to monitor the progress of the reactions. It also enabled us to follow the incorporation of EPA and DHA into glycerol to form the various intermediary acylglycerols participating in the direct esterification process, the 1- and 2-MG, 1,2- and 1,3-DG and the TG. The progress of the reactions is illustrated graphically in Figures 1 and 2, respectively for EPA and DHA, as based on mol% incorporation of fatty acid equivalents into the acylglycerols. As can be seen in Figures 1 and 2, EPA reacted considerably more rapidly than DHA. In both cases the reaction mixture was dominated by 1,3-DG during the first few hours of the reaction. At that stage both 1,2-DG and 1-MG reached a maximum

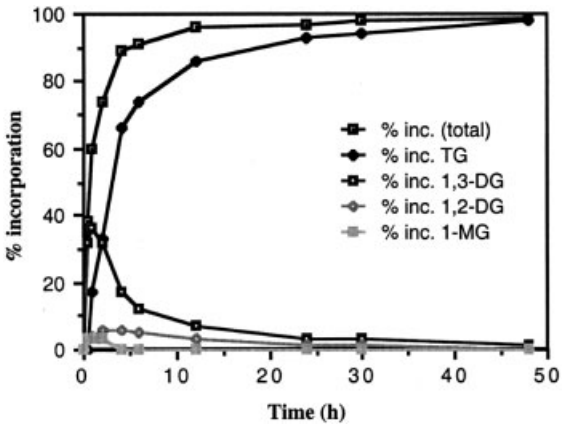


Figure 1. Progress of the direct esterification of glycerol with EPA.

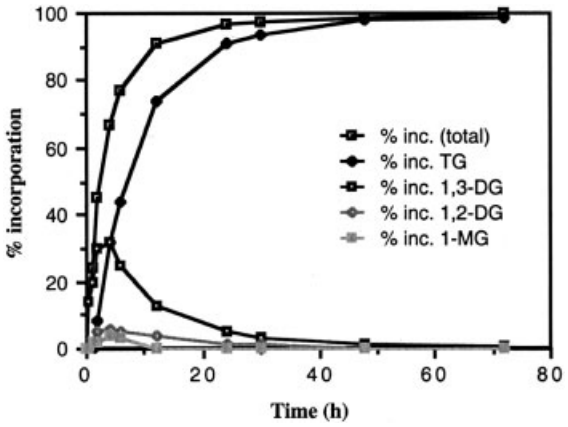
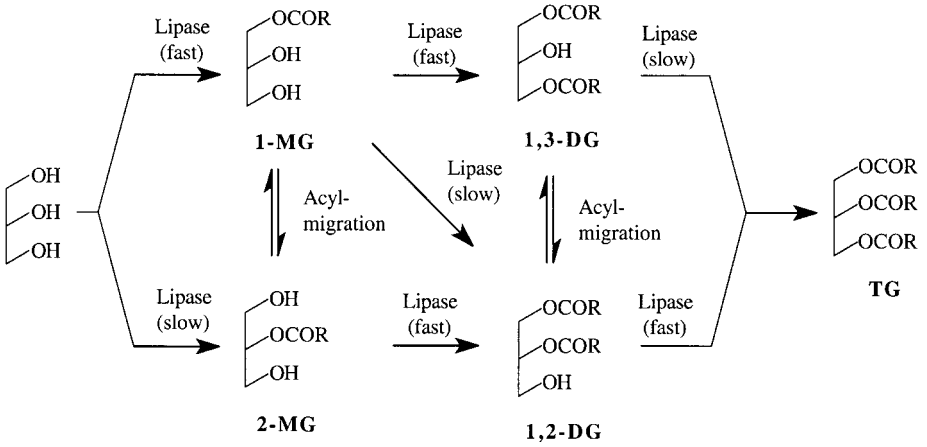


Figure 2. Progress of the direct esterification of glycerol with DHA.

at considerably lower levels, but 2-MG was never detected. Both reactions were virtually completed after 48 h.

The results implicate that the nonregioselective lipase acted considerably faster at the end-positions than the mid-position of the glycerol moiety. This is in accordance with the general assumption that lipases normally display a strong preference for primary rather than secondary alcoholic substrates due to increased bulkiness of the secondary alcohols, rendering them less nucleophilic (Haraldsson, 1992). In order to form TG from 1,3-DG either the nonregioselective lipase will have to act directly at the mid-position, or alternatively, an intramolecular acyl migration of the 1,3-DG into 1,2-DG (or 2,3-DG) must take place together with a subsequent acylation of the resulting 1,2-DG. Both the enzymatic acylation of the mid-position and the acyl migration are believed to be considerably slower than the enzymatic acylation at the end-positions. Again, the acyl-migration processes may be playing



Scheme 4. A proposed reaction pathway for direct esterification of glycerol with free acid (RCOOH) by Candica antarctica lipase.

an important role during the process. Based on the above results a proposed pathway for the rather complicated reactions is illustrated in Scheme 4.

Interesterification reaction involving tributyrin and pure EPA and DHA as ethyl esters under identical conditions offered similar products (Haraldsson et al., 1993c; 1995). This reaction is also illustrated in Scheme 3. Similar treatment of glycerol with concentrated ethyl esters of EPA and DHA was observed to be inferior to the above-mentioned processes in terms of extent of conversion (Haraldsson et al., 1992). Finally, it is evident that the immobilized *Candida antarctica* lipase is superior to other lipases in terms of the direct esterification reaction between glycerol and free fatty acids, as can be witnessed by several literature reports (Haraldsson et al., 1992; Li and Ward, 1993a; He and Shahidi, 1997).

It was also demonstrated that this methodology can be used to provide TG of a composition identical to any composition of the starting free acids (Haraldsson et al., 1992). A similar procedure (which is on the point of industrialization in Norway) is designed to produce pure TG that are highly enriched with EPA and DHA. TG of higher than 90 % purity were obtained when a mixture of MG, DG and TG obtained by hydrolysis of tuna oil with the *Candida rugosa* lipase and thus enriched with DHA was treated with Lipozyme and free fatty acids (McNeill et al., 1996; Moore and McNeill, 1996). It is most probable that this procedure has already been scaled-up and industrialized in Europe for providing DHA concentrates as TG for infant formula.

10.4 Ether lipids highly enriched with EPA and DHA

Nonpolar glyceryl ether lipids of the 1-*O*-alkyl-2,3-diacyl-*sn*-glycerol type are major constituents in the liver oils of various species of elasmobranch fish such as dogfish and shark (Kayama and Mankura, 1998; Sargent, 1989). They have been claimed to display various beneficial effects on human health (Mangold and Paltauf, 1983). The 1-*O*-alkyl-*sn*-glycerols are highly valuable compounds, bearing a strong resemblance to the well-known platelet activating factors, and can be prepared from the unsaponifiable fraction of dogfish and shark liver oil. There are three main fatty alcohol constituents present in the 1-*O*-alkyl moiety of the glyceryl ethers, $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$, the last being the most abundant. They correspond to chimyl, batyl and selachyl alcohols, respectively, named after their sources in the liver oils of chimeras, sharks and rays of the Chimaeroidei, Batoidei and Selachoidei families. Their structure is illustrated in Figure 3. Very recently, we have demonstrated that lipase can be used to resolve kinetically racemic mixtures of these alcohols (Haraldsson et al., 1999).

In order possibly to combine the claimed beneficial effects of EL and fish oil, EL were treated with EPA and DHA by the lipase catalysis procedures already described (Haraldsson and Thorarensen, 1994). The EL were isolated in a pure state, as was established by high-field NMR, from shark liver oil concentrates using preparative HPLC. EPA and DHA made up only 0.4 and 2.5 % of the initial fatty acid composition of the EL acyl counterparts, respectively. Pure 1-*O*-alkyl-*sn*-glycerols were obtained from the EL by sodium methoxide-catalyzed methanolysis.

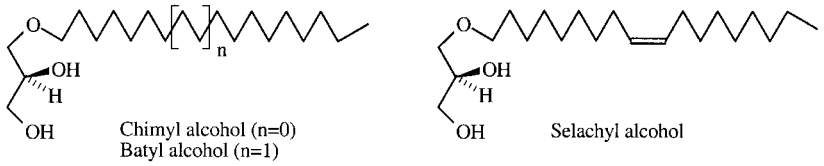
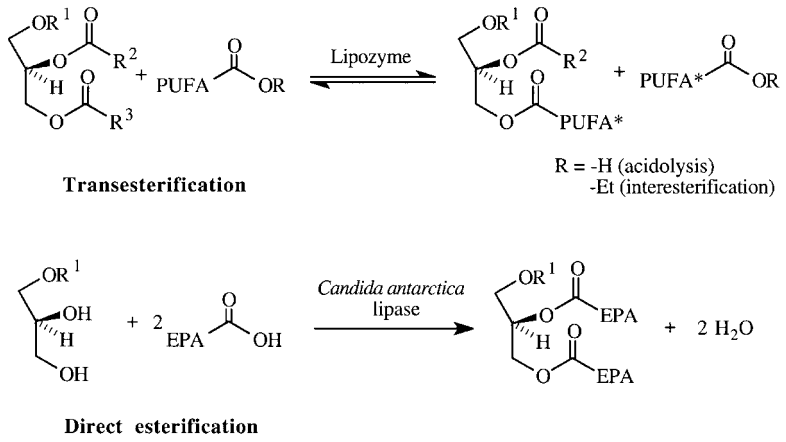


Figure 3. The chemical structure of chimyl, batyl and selachyl alcohols.



Scheme 5. Lipozyme-catalyzed enrichment of 1-0-alkyl-2, 3-diacyl-sn-glycerols by transesterofocatopm reactopms (top) and direct esterification of 1-0-alkyl-sn-glycerols with EPA using CAndida antarctica lipase (bottom). R1 refers to the 1-0-alkyl group composition. FOr further abbreviations see Scheme 1.

EL highly enriched with either EPA or DHA or both EPA and DHA were obtained by transesterification reactions with concentrates of EPA and DHA, using Lipozyme in a manner identical to that described previously for cod liver oil. Direct esterification of the 1-*O*-alkyl-*sn*-glycerols with EPA and DHA resulted in homogeneous EL, when using the *Candida antarctica* lipase. These reactions are demonstrated in Scheme 5 for EPA. It was interesting to notice that the EL were apparently far less prone to acyl migrations as compared to the TG under the transesterification conditions. This is presumably related to the fact that only one of the primary end-positions is now available for the lipase.

10.5 Phospholipids highly enriched with EPA and DHA

PL are major constituents of cell membranes, and play essential roles in the biochemistry and physiology of cell functions (Mead et al., 1986). PL in fish and marine species are highly enriched with the long-chain n-3 type PUFA, 40–50 % content of EPA and DHA not being uncommon in some PL classes in fish (Haraldsson et al., 1993a). The n-3 PUFA presumably play significant roles in adjusting the membrane

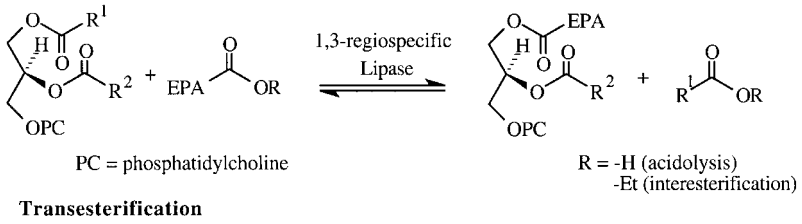
integrity and functions at the lower temperatures, by adding to the membrane fluidity and mobility as a result of their higher unsaturation. Among the PL, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are by far the most abundant in fish flesh, especially the former, which commonly comprises 50–60 % of the total PL content.

PL of vegetable and plant origin are highly enriched with n-6 fatty acids and commonly used as health supplements. Purified PL of fish origin highly enriched with n-3 PUFA, on the other hand, are not available on the market at all. They are by no means readily obtainable, since tedious extraction procedures from fish are required (Haraldsson et al. 1993a). We therefore decided to attempt the preparation of such PL highly enriched with EPA and DHA from the more readily available plant or animal lecithins (Haraldsson and Thorarensen, 1995; 1999).

Attempts to prepare PL enriched with n-3 PUFA by various esterification reactions involving lipases (Totani and Hara, 1991; Mutua and Akoh, 1993) and phospholipases (Na et al., 1990; Mutua and Akoh, 1993; Härröd and Elfman, 1995) have usually resulted in low yields as a result of predominant hydrolysis side reactions, and the incorporation of the n-3 PUFA did not reach any elevated levels. Takahashi and co-workers (Hosokawa et al.,1995a) have succeeded in enhancing the incorporation of EPA into soy PC while at the same time suppressing the hydrolysis side reaction in a lipase-catalyzed acidolysis with Lipozyme. This was accomplished by using propylene glycol as a water-mimic in hexane as a solvent, the claimed PC recovery being 80 %. Thus, a far lower water content was required for the catalyst to perform. The authors assumed that the EPA incorporation took place explicitly at the *sn*-1 position and that 80 % of the theoretical incorporation level was reached, which corresponds to about 35 % EPA content in the resulting PC. These authors have also reported on the preparation of structured PC highly enriched with EPA and DHA at the *sn*-2 position (Hosokawa et al., 1995b). This was accomplished by a direct esterification of 2-lysophosphatidylcholine (2-LPC) from soya with EPA and DHA using porcine phospholipase A_2 in glycerol as a solvent with formamide as a water-mimic. The molar yields were apparently far lower or close to 40 %.

In our own contribution to this field preliminary studies were conducted on pure diacyl-*sn*-glycero-3-phosphatidylcholine from egg yolk in lipase-catalyzed interesterification reactions with concentrates comprising 55 % EPA and 30 % DHA as free acids (acidolysis) and ethyl esters (interesterification), according to Scheme 6 (simplified, only shown for EPA). Reaction conditions almost identical to those previously described for cod liver oil TG and shark liver oil EL were applied. As could be anticipated for lipase, the rate of the reactions involving the PC possessing the zwitterionic head groups, was much lower when compared to their native TG. Accordingly, much higher quantities of lipase were required, up to 5- to 10-fold that used previously, which again resulted in high extent of hydrolysis side reaction. Lipozyme was far more efficient than the *Candida antarctica* lipase and became the lipase of choice for further studies.

The interesterification reaction was found to proceed at a comparable rate to the acidolysis reaction, and similar incorporation levels of EPA and DHA were obtained. As before, EPA was a considerably better substrate than DHA. It was decided to look further into the acidolysis reaction, since the water-associated hydrolysis side reactions were largely suppressed by the excessive amounts of free fatty acids present in



Scheme 6. Lipozyme-catalyzed transeserification reactions of PC. For abbreviations see Scheme 1.

that case. Highest levels of incorporation were obtained when employing 100 % dosage of lipase, when PC containing 32 % EPA and 16 % DHA were accomplished after 72–h reaction time, presumably as a mixture of PC and LPC.

More detailed information about the nature of the acidolysis reaction was offered by ^{31}P-NMR spectroscopy analysis with pure dipalmitoyl-*sn*-glycero-3-phosphatidylcholine and 98 % pure EPA as free acid. The results revealed that optimal results in terms of activity were obtained with lipase containing 5 % water, which not only resulted in the highest incorporation levels of EPA into both PC and LPC, but also in highest extent of hydrolysis side reaction. This demonstrates rather well the complex role played by water in terms of compromising the lipase activity, hydrolysis side reactions, reaction rate and extent of incorporation under the microaqueous reaction conditions.

Further experiments were therefore conducted with lipase containing 5 % water. Maximum incorporation of EPA was obtained with 100 % dosage of lipase, 58 % into PC and 70 % into LPC. Under that condition, the extent of hydrolysis reached 39 %, as based on number of moles of ester equivalents present at the initial PC, with 39 %

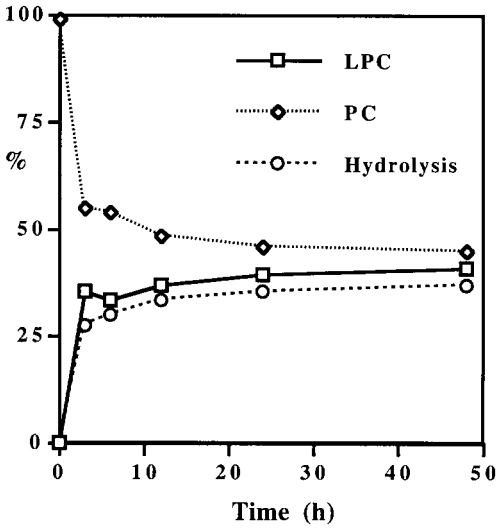


Figure 4. Extent of hydrolysis and changes in PC and LPC composition during the progress of the acidolysis reaction with 70 % lipase dosage.

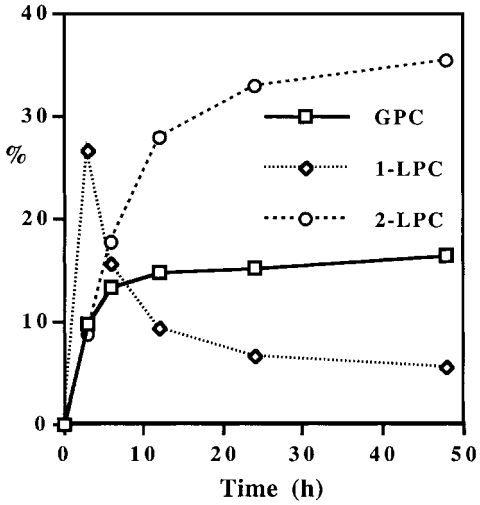
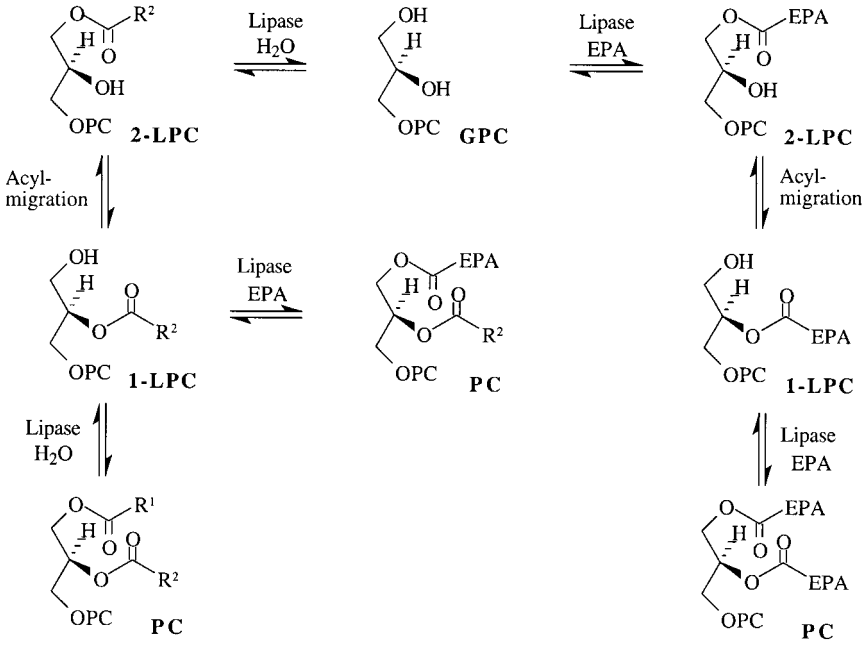


Figure 5. Composition of various PL intermediates during the progress of the acidolysis reaction with 70 % lipase dosage.

PC, 44 % LPC and 17 % GPC present in the product mixture, based on moles of phospholipids, according to the ^{31}P-NMR studies.

Additional results are exhibited in Figures 4 and 5, which demonstrate the power of the ^{31}P-NMR technique in this field. Figure 4 shows the extent of hydrolysis as well as changes of PC and LPC during the progress of the reaction. From these results, it is evident that major changes in composition occurred already during the first 3 h of the reaction in terms of extent of hydrolysis and PC and LPC incorporation. More detailed insight into the progress of the reaction is provided in Figure 5. At the beginning of the reaction 1-LPC was the predominant hydrolysis product. After 3 h, 27 % 1-LPC, 9 % 2-LPC, 10 % *sn*-glycerol-3-phosphatidylcholine (GPC) and 55 % PC were present (molar basis). After 6 h, 1-LPC and 2-LPC had leveled out at 16 % content, but after 48 h an equilibrium was reached with 2-LPC now dominating in the hydrolysis product mixture at 36 % content, with only 6 % 1-LPC, 15 % GPC and 45 % PC present in the reaction mixture.

Based on these findings, the mechanism displayed in Scheme 7 was proposed for the lipase-catalyzed acidolysis of PC with free fatty acids. The proposed mechanism explains how PC of higher than 50 % EPA content can be formed with both the *sn*-1 and *sn*-2 positions involved, as well as the participation of all the intermediates implied by the ^{31}P-NMR spectroscopy analysis. According to that mechanism, the lipase utilizes water to bring about hydrolysis at the *sn*-1 position of the starting PC to produce 1-LPC. That intermediate can undergo either re-esterification to form EPA-enriched PC or a much slower acyl migration to form 2-LPC. The lipase can easily hydrolyze that intermediate to form GPC. Esterification at the *sn*-1 position of GPC with EPA by the aid of the lipase, followed by acyl migration to form EPA-enriched 2-LPC and finally lipase-catalyzed esterification with EPA, leads to the formation of the highly EPA-enriched PC. Thus, the postulated mechanism can be used to rationalize the reaction system behavior: EPA-enriched PC and (presum-



Scheme 7. A proposed simplified reaction pathway for lipase-catalyzed acidolysis of PC with EPA. For abbreviations see Scheme 1.

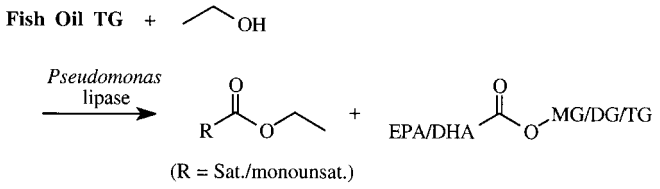
ably) EPA-deficient 1-LPC dominate in the reaction mixture during the early stages of the reaction, whereas at the later stage highly EPA-enriched 2-LPC and PC become dominant.

10.6 EPA and DHA concentrates from fish oils by lipase

Based on their fatty acid selectivity and discrimination against n-3 PUFA, lipases can be used as an alternative means of concentrating EPA and DHA in fish oils by kinetic resolution. There are numerous reports in the literature describing the application of lipase to concentrate EPA and DHA in fish oils (Haraldsson et al., 1997). The reactions involved include hydrolysis and alcoholysis of TG and monoesters, direct esterification of free acids with alcohols, and various transesterification reactions. In this respect lipases can be divided into three categories: (i) those which display no or very low activity toward fish oils or fish oil fatty acids as substrates; (ii) those discriminating against n-3 fatty acids and can be used to concentrate EPA and DHA together; and (iii) those which offer a strong discrimination between EPA and DHA, usually in favor of EPA. The lipases belonging to the third group can be used to concentrate EPA and/or DHA individually.

The lipases belonging to the first group clearly offer very little application in the fish oil field. The lipases in the second group act very well on the bulk of saturated and monounsaturated fatty acids present in the fish oil, leaving EPA and DHA largely

unaffected. This enables the preparation of concentrates of EPA plus DHA. *Pseudomonas* lipases belong to this category, as has been demonstrated by Haraldsson and co-workers (Haraldsson et al., 1997). Two commercially available *Pseudomonas* lipases were observed to afford a concentrate of approximately 50 % EPA+DHA in very high recovery (80–90 %) and highly efficiently without a solvent, since simply a 2-fold stoichiometric amount of ethanol was required. This demonstrates that lipases can be used as a valid alternative to conventional physical methods such as molecular distillation. One of the main advantages was the considerable reduction of bulkiness of the process, since no organic solvent was required and the ethyl esters produced were directly distilled off by short-path distillation from the residual acylglycerol mixture of EPA and DHA (Scheme 8).



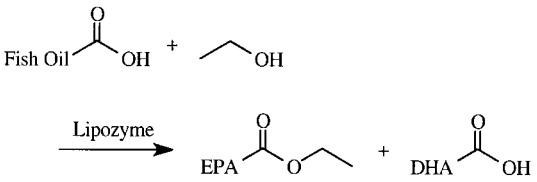
Scheme 8. Ethanolysis of fish oul by Pseudomonas lipase.

Geotrichum candidum lipase also belongs to this category of lipases not discriminating much between EPA and DHA. That lipase was used to concentrate EPA together with DHA in tuna oil by hydrolysis reaction up to the 50 % levels, similarly with high recoveries of EPA and DHA, by Shimada and co-workers (Shimada et al., 1994; 1995). It is of interest that the residual acylglyceride mixture from the reaction comprised TG to a large extent (85 %) which was related to selectivity of that lipase favoring MG and DG rather than TG. This lipase displayed a somewhat lower activity toward DHA than EPA, which is the usual behavior of lipases. An exception to that behavior was observed from the *Pseudomonas* lipases mentioned above, which displayed higher activity toward DHA than EPA (Haraldsson et al., 1997) (see also Chapter 8).

Lipases belonging to the third class displaying moderate to strong discrimination between EPA and DHA, all in favor of EPA, include *Candida rugosa* lipase (formerly named *Candida cylindracea*), *Rhizopus delemar* lipase, and the fungal lipase from *Rhizomucor miehei*. Hydrolysis of tuna oil with the *Candida rugosa* lipase afforded an acylglycerol mixture highly enriched with DHA, up toward the 50 % level (Hoshino et al., 1990; Tanaka et al., 1992; McNeill et al., 1996; Moore and McNeill, 1996). With that lipase there are indications that TG molecules in fish oil containing DHA may be resistant to the lipase (Tanaka et al., 1992). Shimada and co-workers have demonstrated that the *Rhizopus delemar* lipase can be used to highly enrich free acids from tuna oil with DHA by a direct esterification with a long-chain alcohol (Shimada et al., 1997a,b). The residual free acids contained 73 % DHA in very high recovery (84 %), and no solvent was required. A second esterification afforded further purification of DHA up to almost 90 % in 71 % overall recovery.

Similar levels of DHA enrichment were obtained by Haraldsson and Kristinsson from tuna oil free acids in a direct esterification reaction with ethanol in the absence

of a solvent using Lipozyme (Haraldsson and Kristinsson, 1998) (Scheme 9). By a similar methodology they were able to purify EPA free of DHA to above the 90 % purity levels from a free fatty acid concentrate of EPA in high recoveries. These examples demonstrate that enrichment levels well beyond the urea crystallization method can be obtained highly efficiently by lipase. The fact that an immobilized lipase can be reused 20 to 40 times or more, with little or no deterioration of the lipase, suggests that the application of lipase in the field of fish oils is a highly feasible choice from the industrial point of view.



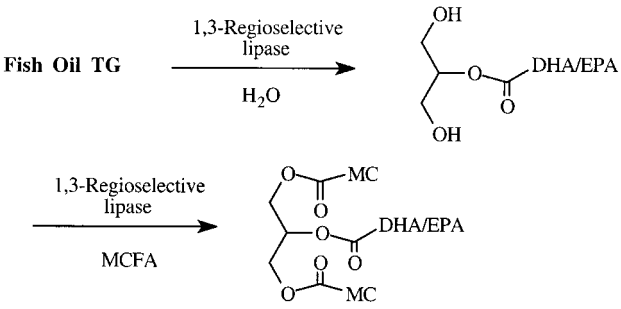
Scheme 9. Direct esterification of fish oil free acids with ethanol by lipase.

10.7 Structured lipids containing EPA and DHA

Lipases have also found important application in producing structurally labeled TG (Gunstone, 1998) with the n-3 PUFA located either at the mid-position or the end-positions. This is based on using 1,3-regiospecific lipases for transesterification or hydrolysis of TG with the fatty acids in the *sn*-2 position remaining almost intact in the acylglycerol products. That usually is dependent upon the reaction time and the slower acyl-migration processes must be avoided or kept to a minimum. From a nutritional point of view, structured lipids containing n-3 PUFA at the mid-position with medium-chain fatty acid (MCFA) at the end-position have recently received most attention.

Shimada and co-workers reported on the production of structured lipids containing DHA in the *sn*-2 position by exchanging fatty acids at the 1- and 3-positions of tuna oil for caprylic acid using an immobilized 1,3-regiospecific *Rhizopus delemar* lipase (Shimada et al., 1996). Yamane's group reported on the lipase-catalyzed acidolysis of single-cell oil containing DHA and docosapentaenoic acid (DPA) with caprylic acid (Iwasaki et al., 1999). They were aiming at structured lipids containing caprylic acid at the *sn*-1 and *sn*-3 positions and DHA or DPA at the *sn*-2 positions of the product. Similarly, Xu and co-workers treated fish oils with caprylic acid using Lipozyme as a biocatalyst in a solvent-free system in a pilot-scale production (Xu et al., 1998).

Schmid and co-workers have approached the synthesis of structured TG containing EPA and DHA in the mid-position differently by proposing a two-step strategy (Schmid et al., 1998). Their strategy is based on the generation of 2-MG highly enriched with n-3 PUFA. They were produced in high yield and excellent purity from fish oil TG, which were subjected to an acidolysis reaction in an organic solvent, catalyzed by 1,3-regiospecific lipases. Subsequent esterification of the 2-MG with fatty acids is supposed to result in structured TG highly enriched with n-3 PUFA located in the *sn*-2 position. This approach is illustrated in Scheme 10.



Scheme 10. A two-step strategy to generate structured TG comprising EPA/DHA at the sn-2 position and medium-chain fatty acids (MCFA) at the sn-1 and sn-3 positions.

Akoh and co-workers have treated various types of TG with n-3 PUFA in lipase-catalyzed transesterification reactions. These include incorporation of n-3 fatty acids into vegetable oils (Huang and Akoh, 1994), melon seed oil (Huang et al., 1994), trilinolein (Akoh et al., 1995), evening primrose oil (Akoh et al., 1996), borage oil (Akoh and Moussata, 1998), and TG of medium-chain fatty acids, trilaurin, tricaprin and tricaprylin (Lee and Akoh, 1996; 1998). In all cases it appears that the lipases used (1,3-regiospecific *Rhizomucor miehei* and nonspecific *Candida antarctica* lipases) acted preferably at the 1,3-positions. This resulted in high incorporation levels of the n-3 fatty acids into these positions, but high levels of n-3 fatty acids were also incorporated into the *sn*-2 position, although lower. Other groups have also reported on the incorporation of n-3 PUFA into various TG using lipase including borage oil (Ju et al., 1998) and vegetable oils (Li and Ward, 1993b).

10.8 Concluding remarks

In this chapter the possibilities and advantages offered by lipases to the enrichment of lipids with n-3 PUFA, EPA and DHA in particular, have been discussed. Lipases already play an important role in the production of highly enriched concentrates of EPA and DHA in the natural TG form. There are examples of industrial-scale production involving lipase of such products of high quality and purity, which are believed to be safer for human consumption than corresponding products made chemically. The application of lipases to concentrate EPA and DHA up to high purity levels has also been demonstrated. It is believed that such processes may also soon be industrialized and will replace traditional physical methods used to concentrate and purify EPA and DHA on a large scale.

From an industrial point of view, there are several important advantages offered by lipases. They can be used without organic solvents, and this reduces the bulkiness of such processes to a large extent. This is also important from the environmental and explosion hazard points of view. The mildness offered by lipase is also very important, not only in terms of energy cost (many lipase-promoted reactions can be run at room temperature), but also from the quality point of view, as the n-3 PUFA are

extremely labile. Lipases are relatively inexpensive and efficient biocatalysts, and can be supplied in an immobilized form that results in high productivity as they can be re-used tens of time, with little deterioration. It is anticipated that the production of structured lipids containing n-3 PUFA, together with medium-chain fatty acids in labeled positions, will soon commence on an industrial scale. Lastly, it is expected that, in the future, lipases will also find important uses in the asymmetric synthesis of chiral lipids, including EL and PL containing EPA and DHA. Such compounds should offer a variety of nutritional, health and medical benefits to mankind.

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11 Modification of Oils and Fats by Lipase-Catalyzed Interesterification: Aspects of Process Engineering

Xuebing Xu

Abbreviations and symbols*

CBE	cocoa butter equivalents
DAG	diacylglycerols
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
FFA	free fatty acids
HMF	human milk fat
L	long-chain fatty acids
LCFA	long-chain fatty acids (C_{14}-C_{22})
LLL	triacylglycerols containing only long-chain fatty acids
LMM	triacylglycerols containing one long-chain fatty acid and two medium-chain fatty acids
sn-LML	triacylglycerols with LCFA at the *sn*-1,3 positions and MCFA at the *sn*-2 position of the glycerol backbone, similar for *sn*-LLM, *sn*-MLM, etc.
M	medium-chain fatty acids
MAG	monoacylglycerols
MCFA	medium-chain fatty acids (C_8-C_{12})
MCT	medium-chain triacylglycerols
MMM	triacylglycerols containing only medium-chain fatty acids
PBR	packed-bed reactor
PUFA	polyunsaturated fatty acids
RSM	response surface methodology
SL	structured lipids
SSL	specific structured lipids
TAG	triacylglycerols
STR	stirred-tank reactor
X (Y)	unknown fatty acids
XXX (YYY)	any triacylglycerols only containing fatty acid X (Y)

* Other symbols and uncommon abbreviations used in equations, formula, and figures are explained in the places where they appear or in the figure legends.

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11.1 Introduction

Enzymatic modification of fats and oils has long been of great interest (Yokozeki et al., 1982; Macrae and Hammond, 1985). The idea was initiated in the early 1980s for the production of cocoa butter equivalents (CBE), which were patented individually by two companies, Unilever (Coleman and Macrae, 1980) and Fuji Oil (Matsuo et al., 1980). Extensive progress and increasing interest continued until the late 1980s, at which time major advances in fundamental microaqueous enzymology had been achieved. One of the great advances was the elucidation of lipase structures through the study of protein engineering (Brady et al., 1990), and another was the development of enzymatic catalysis in microaqueous media (Zaks and Klibanov, 1984). It is established now that lipase, if optimally applied, works as well in microaqueous media as in an aqueous system (Klibanov, 1997). Thermodynamic predictions for biocatalysis and functions of water activity in organic media were also extensively investigated in relation to this nonaqueous enzymology (Halling, 1994). The wide applications of lipase technology in the lipids industry require stable, pure, and specific commercial lipases to be available at low cost. The major progress in genetic production has made this requirement possible.

Most lipase applications in the lipid field lie in lipid hydrolysis, synthesis and modification, and these have been reviewed and discussed recently (Mukherjee, 1990; Eigtved, 1992; Akoh, 1996; Bornscheuer and Kazlauskas, 1999). As yet, the applications have concentrated on value-added products such as CBE, human milk fat (HMF) substitutes, structured lipids (SL), and polyunsaturated fatty acid (PUFA)-enriched products. Other applications such as monoacylglycerols, diacylglycerols, and other esters, and margarine fats have also been widely investigated. The typical reactions for the production of these materials include hydrolysis, esterification, and interesterification (acidolysis, alcoholysis, and ester – ester exchange). The typical reactions for the lipase applications in lipid modifications are included in Figure 1.

A large number of reviews have been published focusing on lipase-catalyzed hydrolysis and esterification, including the general state of the art (Yahya et al., 1998; Gandhi, 1997; Lortie, 1997; Eigtved et al., 1988), kinetics (Malcata et al., 1992; Jansen et al., 1996), processes and bioreactors (Buhler and Wandrey, 1987; Yamane, 1987; Hirano, 1988; Kloosterman et al., 1988; Linfield, 1988; Malcata et al., 1990; Balcao et al., 1996b; Vaidya, 1996), media (Bozeix et al., 1992), and products (Bornscheuer, 1995). Therefore, this chapter focuses on enzymatic interesterification including ester – ester exchange, acidolysis, and alcoholysis.

The fundamental enzyme technology has been well established throughout textbooks (Gacesa and Hubble, 1987; Chaplin and Bucke, 1990), monographs (Wingard et al., 1976; Kennedy, 1987), and review papers (Pitcher, 1978; Buchholz, 1982). It is not the intention of this chapter to re-describe some of these details; rather, the chapter focuses on several engineering aspects of the lipase-catalyzed interesterification for the modification of oils and fats, i.e., special features of the reaction or microaqueous systems, applications of fundamental enzyme technology in lipase-catalyzed reactions, etc. Although a list of reviews has been published since the early 1980s (as mentioned earlier), a discussion on engineering has not yet been published on the modification of oils and fats by lipase-catalyzed interesterification.

(1) Cocoa butter equivalents (sn-1,3 regiospecific lipases)

sn-POP + St (-EE) ⇌ sn-POSt/StOP + sn-StOSt + P (-EE)

sn-POP + sn-StOSt ⇌ sn-POSt/StOP

OOO + St (-EE) ⇌ sn-StOO/OOSt + sn-StOSt + O (-EE)

(2) Breast milkfat substitutes (sn-1,3 regiospecific lipases)

PPP + O ⇌ sn-POO/OOP + sn-OPO + P

Butter oil + PUFA ⇌ HMF substitutes + X

(3) Specific structured lipids (sn-1,3 regiospecific lipases)

LLL + M ⇌ sn-MLL/LLM + sn-MLM + L

MMM + L ⇌ sn-LMM/MML + sn-LML + M

(4) PUFA-enriched products (fatty acids selective lipases)

Fish oil ⇌ DHA (EPA and DPA)-enriched products + X

Borage oil ⇌ GLA-enriched products + X

HEAR ⇌ 22:1-enriched products + X

(5) Partial acylglycerols (regio- or fatty acids-specific lipases)

XXX ⇌ HOXX + HOXOH + X (hydrolysis)

XXX + Gly ⇌ HOXX + HOXOH (glycerolysis)

(6) Other esters (regio- or fatty acids-specific lipases)

Sugar + X ⇌ sugar esters + H_2O

RCOOH + R*OH ⇌ RCOOR* + H_2O

(7) Margarine fats (specific or nonspecific lipases)

XXX + YYY ⇌ sn-XXY/YXX/XYX + sn-YYX/XYY/YXY

Figure 1. Reaction schemes for the production of different products using different lipases. Fatty acids: P, palmitic; O, oleic; St, stearic; L, long-chain fatty acids; M, medium-chain fatty acids; X and Y, fatty acids; PUFA, polyunsaturated fatty acids; DHA, docosahexaenoic; EPA, eicosapentaenoic; DPA, docosapentaenoic; and 22 : 1, erucic. Other abbreviations: EE, ethyl ester; HMF, human (breast) milk fat; Gly, glycerol; *sn*, stereospecific numbering; and R or R*, carbon-hydrogen groups.

11.2 Lipase-catalyzed interesterification: compositional profiles at reaction equilibrium

11.2.1 Ester – ester exchange between triacylglycerols with nonspecific lipases

Ester – ester exchanges between triacylglycerols by chemical methods have been used industrially for the modification of oils and fats to produce margarine fats or structured lipids. With the increasing interest in enzyme technology and potential benefits therefrom, lipase-catalyzed ester – ester exchange has been also studied for various purposes. The technology is currently under evaluation both technologically and commercially by many groups and companies.

The reaction between triacylglycerols catalyzed by nonspecific lipases proceeds toward randomization provided that the nonspecific lipases have equal specificity towards the three positions of the glycerol backbone, and have equal preference towards different fatty acids. The products are, therefore, randomized at the reaction equilibrium. The triacylglycerol profiles of the products can be calculated from their fatty acid composition according to the rule of randomization (Sonntag, 1982). If the enzymatic *randomization* is performed between two triacylglycerols: LLL and MMM, the reaction will result in eight triacylglycerols or isomers. The reaction is illustrated in Figure 2. Three isomers of LLM (*sn*-LML, *sn*-LLM and *sn*-MLL) or LMM (*sn*-LMM, *sn*-MML, and *sn*-MLM) each are usually combined into one species. Therefore, the four species of the products under different substrate molar ratios (SR_m, between LLL and MMM) can be calculated theoretically according to the following equations:

$$LLL\ (mol\ \%) = \frac{100}{(SR_m + 1)^3} \tag{1}$$

$$LLM\ (mol\ \%) = \frac{300 \cdot SR_m}{(SR_m + 1)^3} \tag{2}$$

$$LMM\ (mol\ \%) = \frac{300 \cdot (SR_m)^2}{(SR_m + 1)^3} \tag{3}$$

$$MMM\ (mol\ \%) = \frac{300 \cdot (SR_m)^3}{(SR_m + 1)^3} \tag{4}$$

Chemical ester – ester exchange proceeds according to the random theory with little exception in practice, if the reaction is successfully conducted and completed. However, enzymatic ester – ester exchange depends very much on whether the assumption of absolute nonspecificity is held on the lipase in question. Some lipases may have slight preferences towards certain fatty acids, or the lipase may not be absolutely nonspecific to the three positions. Therefore, the calculation from these equations can be used only as a guideline for the experimental design and reaction control.

The ester – ester exchange between triacylglycerols with *regiospecific lipases* will produce similar species to that catalyzed by nonspecific lipases. However, the content of each species in the products might be different from nonspecific lipases because the reaction catalyzed by regiospecific lipases proceeds in a different way in which fatty acids in the *sn*-2 position do not exchange with other fatty acids, but remain in the same place. Studies have shown that this was virtually true in practice with regiospecific (*Rhizomucor miehei*) and nonspecific (*Candida antarctica*) lipases in a reaction between cottonseed oil and coconut oil (Mohamed et al., 1993).

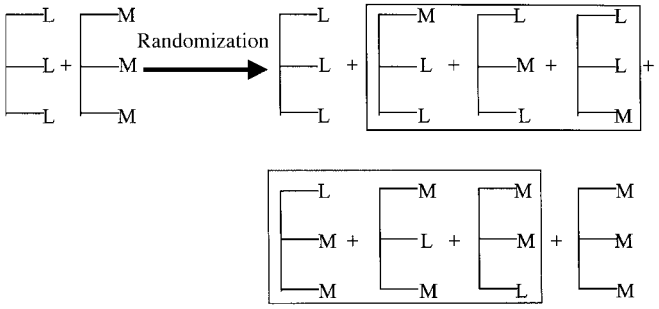

Figure 2. Reaction schemes of enzymatic randomization by ester – ester exchange between two triacylglycerols (LLL and MMM). The triacylglycerol species in each of the rectangles have the same proportions if the lipases have the same specificity towards different fatty acids and the same preferences to the three positions of the glycerol backbone.

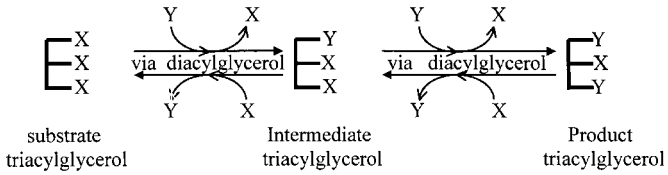

Figure 3. Reaction scheme of lipase-catalyzed acidolysis between a triacylglycerol (XXX) and an acyl donor (Y).

11.2.2 Acidolysis between triacylglycerols and free fatty acids with regiospecific lipases

The reactions between triacylglycerols and fatty acids with *regiospecific lipases* can be depicted as shown in Figure 3. This reaction has been used for the production of CBE, Betapol, and other specific structured lipids (SSL). For a reaction between a triacylglycerol (XXX) and a fatty acid (Y), there will be an equilibrium between the three main triacylglycerol species: XXX, *sn*-YXX/XXY, and *sn*-YXY in the main reaction scheme. Since the lipases are *sn*-1,3-specific, there will be no other isomers formed theoretically. Provided that the lipases have the same specificity towards both X and Y and *sn*-1 and *sn*-3 positions and that no side reactions occur, the content of the three main species after equilibrium will only depend on the substrate molar ratios and can be calculated theoretically. The following equations can be used for the calculation:

$$XXX\ (mol\ \%) = \frac{400}{(2 + S_r)^2} \tag{5}$$

$$YXX/XXY\ (mol\ \%) = \frac{200 \cdot S_r}{(2 + S_r)^2} \tag{6}$$

$$YXY\ (mol\ \%) = \frac{100 \cdot (S_r)^2}{(2 + S_r)^2} \tag{7}$$

where S_r is the substrate molar ratio (Y/XXX). In practice, it should be borne in mind that lipases are not equally specific towards different fatty acids and *sn*-1 and *sn*-3 positions, and side reactions, usually, cannot be completely avoided. Therefore, above calculations can be used only as a theoretical guide and cannot replace practical studies or parameter optimizations. The equilibrium or *maximum incorporation* of acyl donors can also be calculated at a certain substrate molar ratio. The equation is given below:

$$Inc_{\max} = \frac{200}{3}\ \frac{S_r}{S_r + 2} = \frac{200}{3}\ \frac{1}{1 + 2S_r'} \tag{8}$$

where $S_r' = 1/S_r$, i.e., the molar ratio between oil and acyl donors.

11.2.3 Alcoholysis between triacylglycerols and glycerol with nonspecific lipases

Alcoholysis between triacylglycerols and glycerol is also called *glycerolysis*, which is one of the major methods for the production of partial acylglycerols. Lipase-catalyzed glycerolysis has been studied for the production of monoacylglycerols due to the inherent advantages such as mild temperature and better preservation of sensitive essential fatty acids. The reaction between triacylglycerols and glycerol catalyzed by nonspecific lipases will proceed toward randomization if the same premises of nonspecific lipases are assumed, as described in Section 12.2.1. The composition of the products can therefore be calculated from the molar ratio between triacylglycerols and glycerol according to the rule of *randomization*. If one triacylglycerol (LLL) is reacted with one molar glycerol (Gly), the reaction mixture will consist of eight compounds in equal concentration (12.5 mol%) provided hydrolysis is negligible. The reaction is illustrated in Figure 4. Three diacylglycerol (DAG) or monoacylglycerol (MAG) isomers are usually combined into one species. Therefore, the four species of the products under different substrate molar ratios (1:n, between LLL and Gly) can be calculated theoretically according to the following equations:

$$LLL\ (mol\ \%) = \frac{100}{(1 + n)^3} \tag{9}$$

$$DAG\ (mol\ \%) = \frac{300 \cdot n}{(1 + n)^3} \tag{10}$$

$$MAG\ (mol\ \%) = \frac{300 \cdot n^2}{(1 + n)^3} \tag{11}$$

$$Gly\ (mol\ \%) = \frac{100 \cdot n^3}{(1+n)^3} \tag{12}$$

The relationship between the four species and the substrate molar ratio (n) can be depicted in Figure 5 to illustrate the tendency. It can be seen that the highest content of *monoacylglycerols* can be obtained at the substrate molar ratio (n) of 2 with the content of 45 % at reaction equilibrium. Further improvements of the yield of monoacylglycerols have to resort to other techniques such as solid-liquid glycerolysis or *in situ* separations, which was summarized in a review by Bornscheuer (1995). These calculations can be used as a guide for process design or experimental set-up. The calculation is based on the reaction at equilibrium and those assumptions as the premise, such as absolute nonspecificity of lipases and no preferences toward certain fatty acids. Therefore, again calculation from these equations cannot be used without limitations.

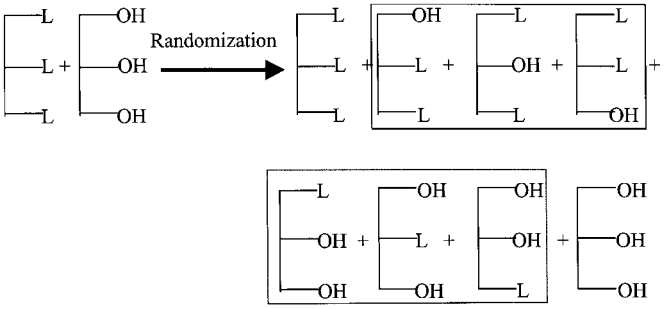

Figure 4. Reaction schemes of enzymatic randomization by alcoholysis between a triacylglycerol (LLL) and a glycerol (Gly). The acylglycerol species in each of the rectangles have the same proportions if the lipase has the same preference to the three positions of the glycerol backbone.

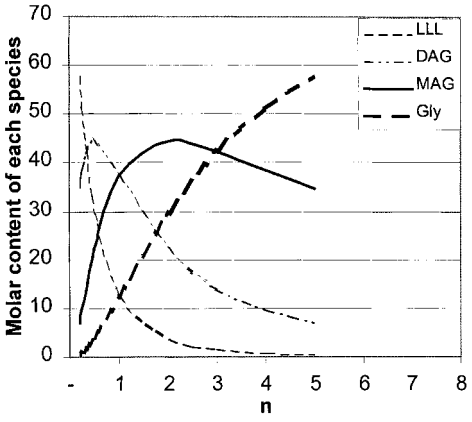

Figure 5. The relationships between substrate molar ratio (n) and the contents of acylglycerol species at equilibrium during reactions catalyzed by nonspecific lipases, which are assumed to have the same preference to the three positions. LLL, triacylglycerol; DAG, diacylglycerol; MAG, monoacylglycerol; Gly, glycerol.

11.3 Kinetics of lipase-catalyzed interesterification

Lipase-catalyzed interesterification most likely takes place in the sequential hydrolysis – esterification hypothesis via a ping-pong bi-bi mechanism. Hydrolysis is often treated as a single-substrate reaction system that can be explained by the Michaelis – Menten equation (Miller et al., 1991). In the microaqueous reaction system, the content of water is limited and, therefore, must be considered to be one of the substrates in the kinetic description. Consequently, both hydrolysis and esterification steps can be treated as two-substrate systems, which can be described as a ping-pong bi-bi mechanism. The initial reaction rate can be expressed in the form as:

$$V = \frac{V_{max} C_A C_B}{K_A C_A + K_B C_B + C_A C_B} \tag{13}$$

where C_A and C_B are concentrations of substrates A and B, respectively, K_A and K_B are reaction constants for A and B, respectively, and V_{max} is the maximum reaction rate.

To account for the effects of all chemical species participating in the interesterification between triacylglycerols and free fatty acids throughout the entire reaction, the models were derived from the sequential hydrolysis and esterification hypothesis and the model therefrom was further simplified by experiments (Reyes and Hill, 1994). The disappearance rate of fatty acid acyl donors, which equals to the incorporation rate into the substrate triacylglycerols, was described as:

$$-r = \frac{(V_R C_{FAD} C_{DAG} - V_M C_W C_{ST}) E_T}{(1 + K_I C_{DAG})(C_{FAD} + C_{RFA})} \tag{14}$$

where V_R, V_M, and K_I are the combined reaction rate constants, C is concentration, FAD is fatty acid acyl donor, DAG is diacylglycerol, W is water, ST is newly formed triacylglycerol, RFA is released fatty acid, and E_T is the total enzyme concentration. As can be seen from the model, the contents of water and diacylglycerols were included in the equation. The simulation work using the equation provided that: (i) there was a limit beyond which increasing the initial water content produced no further increase in the initial rate of the reaction; (ii) an increase in the initial DAG concentration produced a concomitant increase in the rate of the interesterification; (iii) the free fatty acids inhibited the hydrolysis reaction; and (iv) there was a limit beyond which increasing the initial content of substrate triacylglycerols produced no significant increase in the rate of either the hydrolysis or the esterification reactions.

Balcao and Malcata (1996) derived a single rate equation in a general form for all type of interesterifications including hydrolysis and esterification. However, the model was so complicated and too many constants were involved in the equation that it is difficult to use it in practice. Kinetic models based on ping-pong bi-bi mechanisms or the educated simplifications thereof have been increasingly utilized. Kyotani et al. (1988a) demonstrated that the models derived from the acylglycerol-enzyme complex hypothesis were best to fit the experimental results in

a batch solvent system. The hypothesis consists of four steps. The lipase forms a TAG·E complex in the first step, followed by a DAG·E complex and fatty acid in the second step. The DAG·E complex is then combined with the other fatty acid to turn into the other TAG·E complex. The last stage, in which the new TAG·E complex dissociates, completes the reaction. The steps can be summarized as:

$$TAG_1 + E \rightleftharpoons TAG_1 \cdot E \rightleftharpoons DAG_1 \cdot E + FA_1$$

$$DAG_1 \cdot E + FA_2 \rightleftharpoons TAG_2 \cdot E \rightleftharpoons TAG_2 + E$$

Under water content control of the system by spontaneous regulation during the reaction in each experiment, the rate constants for each step, after a series of studies, were found to be affected by the water content in the system (Kyotani et al., 1988b).

It is tempting to develop simple kinetic models for practical applications with fewer rate constants. This often needs to consider overall performance of the reaction instead of the reaction mechanism. When studied by Xu et al. (1988a), the apparent incorporation of acyl donors was found to have a relationship with reaction time in form of the Michaelis – Menten equation (Equation 15).

$$\mathrm{Inc} = \frac{\mathrm{Inc}_{max} t}{K_i + t} \tag{15}$$

The $\mathrm{Inc}_{\max}$, equivalent to $V_{\max}$, was confirmed to be equal to the incorporation under equilibrium at certain substrate molar ratios, which can be calculated directly from the substrate molar ratio [Equation (8)]. Basheer et al. (1995a,b) derived equations for both ester – ester exchange and acidolysis in a solvent system. The concentration change of each species in the reaction mixture as the function of time was established for both reactions. The models fitted the experimental data well, and only one constant existed in the models. For the comparison of reaction rates under different conditions and for the screening of lipases for enzymatic interesterification, the following simple model is much useful:

$$r = \frac{1}{t} \ln \frac{Inc_{max} - Inc_0}{Inc_{max} - Inc_t} \tag{16}$$

where t is the reaction time or residence time, $\mathrm{Inc}_{\max}$, Inc_0, and Inc_t are the maximum incorporation of acyl donors, acyl incorporation at time 0 and t. The enzyme activity can be compared by calculating rV/W where V is the volume of the substrate liquid in the reactor and W is the weight of enzyme in the reactor.

11.4 Immobilized lipases

Lipases are normally used in an immobilized form in industry because reuse or continuous use of the lipase is made possible, and the separation of the lipase easy. The stability of the lipase is often increased by immobilization. The advantages of the various types of available enzyme reactors can also be more readily exploited by using immobilized lipases, especially the use of packed-bed reactors. However, there are some shortcomings of immobilization. Some enzyme activity may be permanently lost during immobilization, due to irreversible denaturation generated during the immobilization procedure, especially if this is achieved chemically. Immobilization is an additional operation, and adds cost to a process; the immobilized biocatalyst particles also always occupy a larger volume in reactors than do the equivalent amounts of free enzyme due to the presence of the carrier. The effect of immobilization on the activity in general can be expressed as 'effectiveness factor' (η). This represents the activity of the immobilized enzyme divided by the enzyme activity of an equivalent quantity of free enzyme assayed under the same conditions. An effectiveness factor of 1.0 indicates good reaction control, with no appreciable reduction in the activity of the enzymes by immobilization or diffusional restrictions. Values less than 1.0 give an indication of the amount of activity lost during immobilization, plus the extent of diffusion limitation on enzyme activity. Four main factors have been identified which modify the intrinsic properties of enzymes, either during or after immobilization:

1. Conformational effects are caused by the chemical modification of the enzyme protein during immobilization. These effects can be especially serious on the enzyme activity when amino acid residuals, which form part of the active site or are important in maintaining the tertiary structure of the enzyme, are involved.
2. Steric effects occur because some of the enzyme molecules are often immobilized in a position that the active site is relatively inaccessible to substrate molecules.
3. Microenvironmental effects occur because of the difference of hydrophobicity and electric charges that cause partitioning effects between different components in the reaction mixture towards the enzyme support. For example, hydrophilic substrates will be selectively attracted to the surface and pores of hydrophilic support. Similarly positively charged substrates will be attracted into negatively charged supports, giving a local high substrate concentration and low pH inside the support.
4. Lastly, diffusional restrictions occur because the substrate must diffuse to the immobilized enzyme before reaction can take place. Diffusional limitations on the activity of immobilized enzymes are of the greatest importance because they considerably affect the design of enzyme reactors and process operations (Gacesa and Hubble, 1987; Prenosil et al., 1987; Chaplin and Bucke, 1990).

The *external diffusional limitations* are due to the restricted rate of diffusion of substrate in the thin film of poorly mixed fluid surrounding each enzyme particle. External diffusional restrictions can be decreased by increasing the degree of agitation in stirred-tank reactors or the flow rate in packed-bed reactors, or by using less vis-

cous substrates. The substrate diffusion across the thin film from the bulk mixture to the surface of the support follows Fick's law. For a system where the substrate diffusion coefficient is independent of substrate concentration, the mass transfer rate at steady state will be equal to the reaction rate:

$$k_L a(S_b - S_S) = \frac{V_{max} S_S}{K_m + S_S} \tag{17}$$

where k_L is the substrate mass transfer coefficient, a is the external surface area, S_b and S_s are the bulk and surface substrate concentrations, V_{max} is the maximum reaction rate, and K_m is the Michaelis constant. When the left-hand term is smaller than the right-hand term, the reaction rate is decided by the substrate diffusion. In such a situation, external diffusional limitations occur. k_L relates to the *Sherwood number* which, in turn, corresponds with Reynolds number and Schmidt number. These numbers can be calculated at certain experimental conditions and, therefore, k_L can be determined for stirred-tank reactors as well as packed-bed reactors. For packed-bed reactors with the commercial immobilized lipase, Lipozyme IM, it was reported that the external diffusional limitations could be neglected when the linear flow rate of the substrate was higher than 5×10^{-5} m s^{-1} in a packed-bed reactor for a reaction of ester – ester exchange between triolein and trimyristin (Jung and Bauer, 1992). Fig-

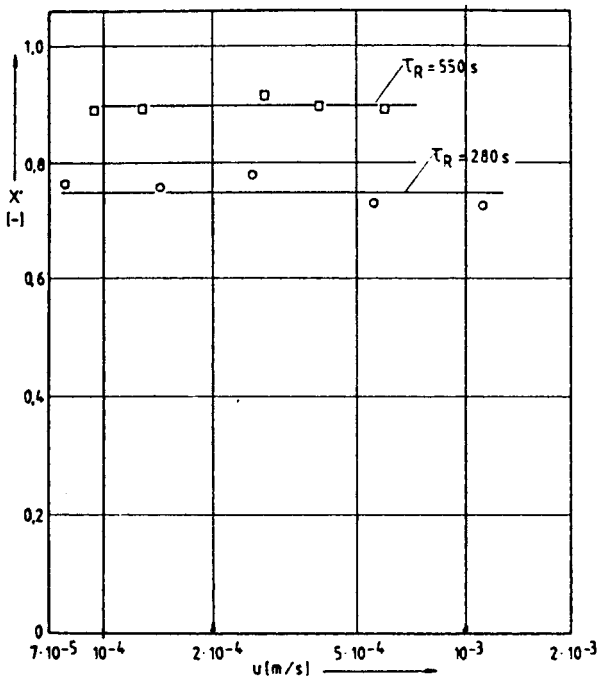

Figure 6. Influence of linear flow rate u (m s^{-1}) on the packed-bed conversion (X') for constant residence time (τ_R) under the constant particle diameter and pore size, for the Lipozyme IM-catalyzed ester – ester exchange between triolein and trimyristin. (From Luck et al., 1988.)

ure 6 illustrates the conversion (X ′) under same residence time (τ_R) and various linear flow rates higher than $5 \cdot 10^{-5}$ m s^{-1} (u).

Internal diffusional limitations are more crucial for immobilized enzymes. Substrate mass transfer within the enzyme particles is limited because of the small size and tortuosity of the pores in the enzyme support. It relates to the particle radius (r), porosity of the particle (ε), and the tortuosity of the pores (τ), which are described by a inclusive factor, the *Thiele modulus* (ϕ), as follows:

$$\phi = \frac{r}{3}\sqrt{\frac{V_{max}\pi}{K_m D\varepsilon}} \tag{18}$$

where V_{max} is the maximum reaction rate, K_m is the Michaelis constant, and D is the substrate diffusion coefficient in free solution. Effectiveness factor (η), with regard to internal mass transfer limitations, directly relates to the Thiele modulus (ϕ) and bulk substrate concentration relative to K_m (S_b/K_m). The less ϕ and larger S_b/K_m, the higher η will be obtained and less internal mass transfer limitations will be caused. This effect is illustrated in Figure 7 (Luck et al., 1988; Abraham, 1988). The s/K_m in the figure is equivalent to the S_b/K_m in the text. Internal diffusional limitations can be recognized if the activity of an immobilized enzyme is increased when it is crushed, i.e. when the length of the diffusion pathway is reduced. Internal diffusional limitations can be minimized by using a low molecular weight substrate, a high substrate concentration, a low biocatalyst concentration, and small highly porous enzyme particles in which the pores are as large, nontortuous, and interconnected as possible, or by immobilizing the enzyme only to the outside surface of the support. Thus, there is a compromise whether forming a very active immobilized preparation with a low effectiveness factor or a less active preparation with a higher effectiveness factor. In fact, the effectiveness factor will vary throughout the support particles, being higher near the surface and lower near the center of the particles. For Lipozyme IM, the internal diffusional limitations did occur as studied by Jung and Bauer (1992) and

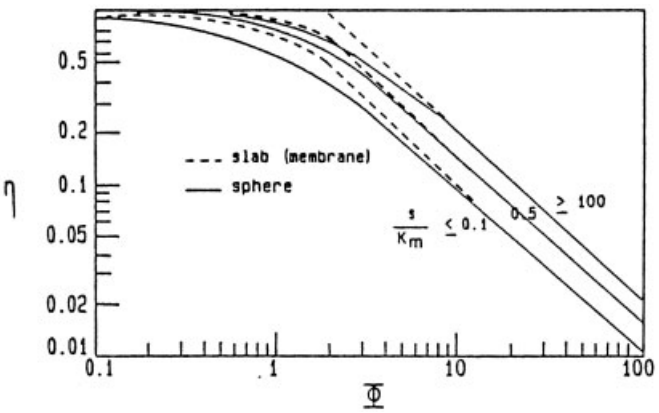

Figure 7. The relationship between internal effectiveness factor (η) and the Thiele modulus (Φ) for values of s/K_m and two geometries of enzyme particles. (From Abraham, 1988.)

were also observed in the lipase-catalyzed acidolysis (Xu et al., 1998b). In a system of the lipase-catalyzed acidolysis between an oil and a medium-chain fatty acid, for example fish oil and caprylic acid, the molecular weight of the oil is 5- to 6-fold that of the free fatty acid, and the diffusion coefficient of the oil will be much smaller than that of the acid. This is certainly one of the reasons leading to the longer reaction time needed to reach equilibrium when higher substrate molar ratios between fatty acids and substrate oil is used (Xu et al., 1998b).

11.5 Stability and reusability of lipases

The stability of an enzyme depends on the characteristic nature of the enzyme and the conditions under which it is used. The factors that stabilize and inactivate enzymes are not well understood. Immobilization can influence the operational stability; both increases and decreases in stability having been observed previously. Generally the activity of enzymes may be reduced due to immobilization; however, the stability is often enhanced by immobilization because the tertiary structure and/or conformation of the enzymes can be held in active forms by enzyme-support linkage. For the *irreversible thermal deactivation* and in the absence of diffusional restrictions, enzyme activity (A) decays exponentially with time $A = A_0 \exp(-k_d t)$, where k_d is the deactivation coefficient, t is time, and A_0 is the initial activity. For Lipozyme IM, a first-order model was proposed by Hansen and Eigtved (1987) and Posorske et al. (1988).

Fouling is one of the drawbacks of packed-bed reactors. Fouling causes a loss in the activity of immobilized enzyme by deposition onto the surface of enzyme particles of colloidal materials or polar compounds that exist in the substrate; this prevents access of the substrate to the enzyme. The extent of fouling depends on the presence, amount, and nature of the fouling materials and on the location of the enzyme in/on the support materials; fouling is accentuated when viscous substrates are used. In the system of lipase-catalyzed lipid modifications, the stability of lipases is affected by lipid quality. It was found that minor compounds in oils and fats, such as lipid hydroperoxides, phospholipids, emulsifiers, chlorophyll, carotenoids, lipid polymers, heavy metal ions and even some antioxidants, had effects on the stability of lipases (Xu et al., 1998c). It has also been shown that highly unsaturated oils and fats as substrates reduce the lifetime of the enzymes used (Hansen and Eigtved, 1987; Posorske et al., 1988). Therefore, high-quality starting oils and fats and careful protection of lipid oxidation during processing are necessary requirements to ensure a longer stability of lipases. However, the refining of oils and fats is also costly, and so a compromise must be found between the quality of lipids and the stability of lipases.

11.6 Bioreactors for the lipase-catalyzed interesterification

Enzyme reactors are often classified by operation (batch or continuous) and mixing performance (from well mixed to plug flow). The often referred to stirred-tank reactor (STR) can be operated in batch or continuous mode, and is often treated as the well-mixed reactors in which the molecules are maintained in a constant state of agitation. A packed-bed reactor is a type of reactor for continuous operation which can be treated as plug flow in the ideal state, meaning that no back-mixing occurs. In batch well-mixed reactors, the composition of the reactants varies during the course of the reaction but is constant throughout the reactor; in contrast, in plug-flow reactors the composition of the reactants is time-invariant, and varies only along the length of the reactor. Thus, in plug-flow reactors, the effectiveness factor will be high near the inlet and low near the outlet, due to the decrease in substrate concentration as the reactants pass through the reactor, whereas in batch reactors the effectiveness factor varies with the time of the reaction because the substrate concentration decreases with time (Gacesa and Hubble, 1987; Prenosil et al., 1987; Chaplin and Bucke, 1990). In practice, despite careful design and operation, the ideal states can be only approached. For example in packed-bed reactors with Lipozyme IM, more than 2-fold calculated residence time (ideal state) was needed to utilize the original reactants after a new substrate had been fed in at the inlet, indicating the wide actual residence time distribution (Xu et al., 1998b).

11.6.1 Batch stirred-tank reactors

Batch reactors are a versatile and traditional form of reactors that are still useful in certain applications and product development trials, especially for infrequent operations. Michaelis – Menten kinetics can be used as a basis for a kinetic description of batch reactors, after integration with regard to time to give total conversion of substrate achieved. For an uninhibited, irreversible reaction employing a single enzyme in a batch reactor under isothermal conditions, the equation that describes the performance is:

$$XS - K_m \ln(1 - X) = \frac{kE\ t}{V} \tag{19}$$

where X is the proportion of substrate converted, S is the initial substrate concentration, K_{m} is the Michaelis constant, kE is the maximum activity of the total enzyme in the reactor, t is the reaction time, and V is the volume of the substrate mixture. The characteristic behavior of the lipase-catalyzed acidolysis between oils and free fatty acids was previously reported (Xu et al., 1998a). The incorporation of acyl donors (Inc) in the batch reactor can be described in the form of the Michaelis – Menten equation as given in Equation (15). $\mathrm{Inc}_{\mathrm{max}}$ in the equation is likely only in relation to substrate molar ratios, but K_{i} is mostly influenced by water content and temperature (Xu et al., 1998a).

Most previous work on the lipase applications in lipid modification has been conducted in batch reactors, including those tubes or flasks with shaking or magnetic stirring. Most of these studies were conducted in solvent systems and/or in milligram/gram levels. One kilogram and up to 20-kg-scale studies were also performed in solvent-free systems (Xu et al., 1998a). It was found that acyl migration could not be avoided in batch reactors, especially in large-scale systems. The optimization with response surface methodology (RSM) was conducted for the production of structured lipids in batch reactors in the solvent-free system. The undesired acyl migration could be reduced to lower levels in optimal conditions (Xu et al., 1998d). However, a certain degree of compromise was required to obtain both high acyl incorporation and low acyl migration (Xu et al., 1998e). The cause of strong acyl migration in batch reactors is mostly due to the longer reaction time needed for a certain degree of acyl incorporation as the ratio between enzyme and substrate is generally low. The breakdown of enzyme particles by stirring could be another important reason for the high level of acyl migration, because most enzyme carriers catalyze acyl migration (Freeman and Morton, 1966; Fureby, 1995). This is one of the particular drawbacks when using batch reactors for the production of specific structured lipids among other common features such as batch operation, high volume, enzyme loss, extra filtration, etc.

11.6.2 Packed-bed reactors

Packed-bed reactors (PBR) are the most frequently used reactors in commercial-scale operations for immobilized enzymes. They are best used continuously and on a large scale so as to minimize labor costs and overheads, and to facilitate control, resulting in more reproducible product quality compared to batch processes. PBR require relatively low power input and have the lowest reactor volume because the highest ratio of enzyme to substrate is maintained in the enzyme bed. This system will result in the highest reaction rate and the least reaction time needed to reach a certain extent of conversion. For an ideal PBR, the performance of the reactor can be described as:

$$XS - K_m \ln(1 - X) = \frac{k\ E\ t_R}{V\varepsilon} = \frac{kE}{V_F} \tag{20}$$

where X is the proportion of substrate converted, S is the initial substrate concentration, K_m is the Michaelis constant, kE is the maximum activity of the total enzyme in the reactor, t_R is the residence time, V is the volume of the packed-bed, ε is the voidage of the bed, and ν_F is the volumetric flow rate. The characteristic behavior of the lipase-catalyzed acidolysis between oils and fatty acids in PBR was studied previously (Mu et al., 1998; Xu et al., 1998b).

One important experimental parameter is the term S/K_m, the *dimensionless substrate concentration*, which is usually defined as β. This term is especially useful when considering to make a process design and experimental set-up. The term is derived from the Michaelis – Menten equation as follows:

$$v = \frac{V_{max}S}{K_m + S} = \frac{V_{max}\frac{S}{K_m}}{1 + \frac{S}{K_m}} = \frac{V_{max}\beta}{1 + \beta} \tag{21}$$

At low levels of S/K_m or β where a high degree of conversion can be obtained, or a low substrate concentration is used, the reaction rate is essentially first order. Here the reaction time required to give a particular degree of conversion is directly proportional to S/K_m or β. However, at high values of S/K_m or β where the reaction is essentially zero order, the time needed to reach a given degree of conversion is independent of S/K_m or β. In industrially practicable processes, high degrees of conversion of substrate to product are usually required and, therefore, the time required for a comparatively small increase in conversion is critically dependent on the prevailing value of S/K_m or β. For the production of SSL in packed-bed reactors and using solvent-free systems, the term S/K_m is related directly to the substrate molar ratio. It was found that the reaction time needed for a half-maximum incorporation of acyl donors increased rapidly with the increase of substrate molar ratio (Xu et al., 1998b).

In PBR, there is an important relationship between the flow rate through the reactor (ν_F) and the degree of conversion (X). The increase of ν_F will lead to the decrease of X [Equation (20)]. It was observed previously that the incorporation of caprylic acid was decreased with the increase of flow rate in the reaction between canola oil and caprylic acid (Xu et al., 2000). However, the activity of immobilized enzymes is increased by the increase of flow rate. The phenomenon can be reflected by the increase of incorporation per unit time, which leads to the higher productivity of the reactor.

One of the special features of PBR is the pressure drop through the bed. This can be described by theoretical equations or statistical models. Excessive pressure drop often leads to the compression of biocatalyst particles due to friction between the fluids and the support particles, or due to partial blockage of the column bed by particulate materials. Compaction of the packed bed is especially likely when small or irregularly shaped or packed particles are used in long columns over a long period. The pressures generated cause deformation or fracture of the enzyme particles, and result in the gradual decrease in the void volume. Compaction can be minimized by using relatively large, incompressible, smooth, spherical, and evenly packed particles, or sectionalized columns, or by reducing the length-to-diameter ratio of the column. The set-up of two flexible layers in the ends of the packed bed, with glass wool for example, was also shown to be effective for the reduction of pressure drop (Xu et al., 1998b).

Nonideal flow of PBR is caused by back-mixing, convection, or the creation of stagnant regions in the reactor, for instance by channeling. Back-mixing of reactants in packed-bed reactors arises because the presence of solid particles in the column, causes elements of the flowing fluid to mix, and because the different fluid elements take different routes through the reactor. Thus, different fluid elements have different residence times in the reactor, giving an opportunity for different extents of reaction to take place. Nonideal flow can be minimized by the use of even-sized, smooth, spherical, evenly packed support particles and by the absence of accumulated solids or gases in the column. Back-mixing certainly affects the yield of the reaction and the

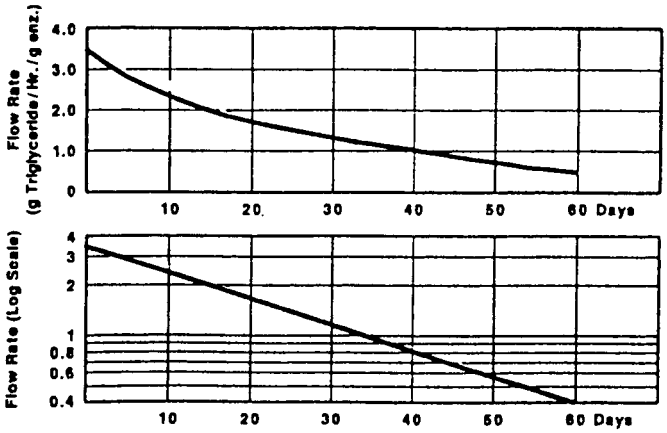

Figure 8. The relationship between flow rate and operation time for a constant incorporation of lauric acid into soybean oil in a packed-bed reactor with Lipozyme IM as the biocatalyst. The experiment was conducted at 60 °C and the flow rate was adjusted to maintain the incorporation of 20 % under the substrate weight ratio of 5 : 2 (soybean oil: lauric acid). (From Posorske et al., 1988.)

degree of acyl migration because some reactants may have shorter reaction time and some may have longer time, which has close correlation with both the product yield and the degree of acyl migration.

Flow rate or *residence time* is the most crucial factor for the reactions in PBR. When enzyme activity is gradually reduced, the flow rate must be regulated to maintain the desired reaction progress. The experimental relationship between flow rate and activity was observed in previous studies (Hansen and Eigtved, 1987; Posorske et al., 1988) (Figure 8). Other factors such as temperature, water content, and substrate molar ratio are also important. Temperature not only affects the lipase activity but also affects the mass transfer. Water content was not crucial in the short-term running of the reactor (Mu et al., 1998), but for long-term operations it affected the enzyme stability (Xu et al., 1998b).

Theoretical and practical comparisons have been conducted widely in literature between STR and PBR in terms of productivity, efficiency, and other characteristics (Gacesa and Hubble, 1987; Prenosil et al., 1987; Chaplin and Bucke, 1990). For the production of regiospecific products, such as Betapol, CBE, SSL, etc., one more merit attributed to PBR can be added. Acyl migration in the PBR was found to be extremely low compared to that in batch STR (Xu et al., 1998b); as a consequence, acyl migration can be neglected in the design of PBR and process optimization.

11.6.3 Membrane reactors

Membranes are thin barriers that can perform various degrees of separation using differences in concentration, pressure, and electrical potential gradients between the two compartments they separate. Membrane separations are achieved as a result of combined actions among membrane, extractant, and molecules to be separated.

Membrane reactors are defined as the reaction systems in which membranes are implemented. Membrane reactors have a very handy *in situ* separation capability lacking in other types of reactors. Combining various membrane separations and enzymatic reactions can generate many membrane reactor systems. The selective removal of inhibitory byproducts better utilizes enzyme catalytic activity, and leads to high productivity of the reactor. For example, in the lipase-catalyzed acidolysis between medium-chain triacylglycerols (MCT) and long-chain polyunsaturated fatty acids (PUFA), the released medium-chain fatty acids (MCFA) can be selectively removed from the system, and this will change the equilibrium of the reversible reaction and force the balance towards the products. The other characteristics of membrane reactors include phase separation in the presence of solvent that makes biphasic reactions possible and allows for an easy extraction; this has led to the study of extractive bioconversions.

Many reports have been published regarding the application of membrane reactors to different reaction systems, especially the applications in water phase, such as the hydrolysis of protein, starch, cellulose, and other macromolecules. These applications have been reviewed (Chang and Furusaki, 1991; Prazeres and Cabral, 1994; Kragl, 1996). The application of membrane reactors in lipid processing is still in its infancy, but many reports have focused on using membranes as the separation media (Snape and Nakajima, 1996). Membrane reactors for enzyme-catalyzed lipid reaction systems have been mainly used in the hydrolysis of oils and fats (Hoq et al., 1985; Prond et al., 1988; Taylor and Craig, 1991; Garcia et al., 1992; Goto et al., 1992; Cuperus et al., 1993; Prazeres et al., 1993) and the esterification between alcohol and fatty acids (van der Padt et al., 1990; 1992). In few cases of lipase-catalyzed interesterification, membranes were used to retain the lipases when the reaction mixture was circulated (Basheer et al., 1995c) or as the carrier for lipase immobilization (Balcao and Malcata, 1998). *In situ* separations in the membrane-implemented diffusion cell or in the ultrafiltration reactor have been performed during the enzymatic acidolysis between MCT and EPA/DHA concentrates (X. Xu et al., unpublished results) and the membrane selectivity of MCFA over EPA or DHA has been observed. A further incorporation of EPA and DHA into MCT in both membrane reactors has been obtained beyond the reaction equilibrium level defined when no membrane was installed (Xu et al., 2000).

Many primary factors affect the separations and unit operations used in bioprocessing, including molecule size, diffusivity, volatility, solubility, surface activity, and hydrophobicity (Chang and Furusaki, 1991). Bioseparation can be successful only if membrane, extractant, and molecules of interest are interacted in harmony. Ultrafiltration and reverse osmosis are modulated by pressure, whereas dialysis and membrane extraction are carried out using the concentration differences. In many cases membrane separations are based on a single separation. However, there is a possibility of using two or more separation mechanisms for membrane separa-

tions. In these cases membrane separations will be more complicated, but the separation factor will be larger than using single separation mechanisms. In a membrane diffusion cell, a concentration gradient was used as the driving force for the separation of medium-chain fatty acids. In an ultrafiltration reactor (X. Xu et al., unpublished results), pressure was used as the driving force.

Membranes are the key factor for a promising membrane reactor, especially in lipid reaction systems. Commercial membranes are produced mainly for water systems. The selection of membranes for lipophilic systems is the important step in obtaining a potentially successful application. Membrane stability is, of course, the important factor for lipophilic systems, both for practical and economical considerations. It is often difficult to find a membrane that possesses high selectivity, acceptable flux, and long stability in the lipid separation system. This may be one of the major reasons for the slow progress of membrane applications in lipid processing, as reviewed elsewhere (Snape and Nakajima, 1996; Parmentier and Fanni, 1999).

The kinetics of membrane reactors for lipase-catalyzed acidolysis depends on the difference of reaction rate and transport rate. Normally, the transport is a limiting step of the system, whereas the reaction rate depends on the transport rate. The highest productivity can be obtained at the steady state where the reaction and transport rates are identical:

$$\frac{V_{max}S}{K_m + S} = k_c \Delta C + k_P \Delta P \tag{22}$$

where V_{max} is the maximum reaction rate, S is the substrate concentration, K_m is the Michaelis constant, k_c and k_p are the constants, ΔC is the concentration gradient, and ΔP is the pressure gradient. The kinetics of membrane reactors depends much on the reactor configurations and operations (Mulder, 1996). When the enzyme is immobilized on the membrane wall, as for hollow-fiber membrane reactors, the modeling of the membrane reactor is much more complicated.

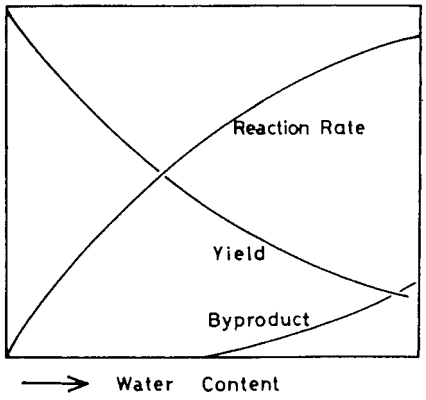

Figure 9. Effect of water content on the interesterification conducted in a microaqueous system for the modification of oils and fats. (From Yamane, 1987.)

11.7 Control of water content/activity

The control of water content or water activity is a key factor of lipase-catalyzed interesterification in the microaqueous biocatalysis system. As discussed in Section 12.3, water not only maintains the activity of lipases but also participates in the interesterification. The optimal control of water content affects the reaction rate, product yield, product selectivity, and operational stability. A general profile of effects of water content on the first two variables is shown in Figure 9. This shows that at lower water content, the yield of the product may be high, but the rate of reaction may be lower. In contrast, at higher water content the rate of reaction becomes higher, but the yield drops (Yamane, 1987). However, there will be an optimal water content for the reaction from the engineering point of view. Water content in the reaction system is directly related to the content of diacylglycerols and the degree of acyl migration (Xu et al., 1998d; 1999). On the other hand, water control during the operation of the process is very important for the control of lipase stability, as demonstrated previously in long-term operations in packed-bed reactors (Xu et al., 1998b).

The control of water content during lipase-catalyzed interesterification lies in the removal of water from, and addition to, the system. Removal of water can be performed by vacuum, addition of molecular sieve, or by circulating the liquid through a silica gel bed (Kyotani et al., 1988b), or bubbling the liquid with inert gas for laboratory operations. The addition of water to lipases can be conducted by spraying water directly onto the lipase and conditioning for a certain time, or by on-line injection and mixing for batch and continuous bioreactors. In laboratory experiments, the water content control for continuous operation in packed-bed reactors was conducted by mixing the water in substrate (Xu et al., 1998b) or substrate passing through a water-saturated silica gel bed (Hansen and Eigtved, 1987). For monitoring and automatic controls of water content, a water sensor connected to the reaction system is required.

Water activity is a dynamic parameter for an enzymatic reaction system. Lipases were shown to have similar water activity profiles in different reactions (Wehtje and Adlercreutz, 1997). In another study, it was found that reaction rate with suspended Lipozyme IM showed similar dependence on water activity in different organic solvents (Valivety et al., 1992). The control of water activity for a lipase-catalyzed reaction system is, however, practically difficult due to mass transfer limitations of water vapor diffusion, especially for continuous operations in packed-bed reactors. Halling (1994) studied the theoretical background and practical control of water activity in enzymatic reactions. Water activity in laboratory experiments is usually regulated by saturated salt solutions or salt pairs (Halling, 1994). The control of water activity is normally performed before reaction takes place, as control during the reaction is much more difficult for a number of reasons. Vaidya (1996) described several types of reactor configurations for the continuous enzymatic esterification with *in situ* water activity control. Wehtje et al. (1993) also described a method in which a silicone tubing was used to control the water activity of the reaction mixture continuously during biocatalysis in organic media. However, these systems are rather limited to small-scale operations or to certain applications. A practical system for water activity control requires further investigation for large-scale bio-

reactors or continuous systems for the modification of oils and fats by lipase-catalyzed interesterification.

11.8 Processes for the modification of oils and fats via enzymatic interesterification

Most studies for the modification of oils and fats by lipase-catalyzed interesterification have used STR or simple glass vessels for the reactions. PBR were shown to be more promising for industrial developments with immobilized lipases. Fuji Oil used a STR for the large-scale production of CBE by lipase-catalyzed interesterification (Hashimoto, 1993; Owusu-Ansah, 1994). The schematic arrangement of the process is shown in Figure 10. As may be seen from the scheme, STR was used for the reaction system and hexane was used as the medium. Ethyl stearate served as the acyl donor to reduce the melting point and boiling point. Ethyl stearate also has higher solubility in hexane than stearic acid. Therefore, the viscosity of the system will be lower and the distillation temperature can be reduced for purification.

Unilever have developed a two-stage process for the production of CBE and Betapol (Figure 11) (Quinlan and Moore, 1993; Rozendaal and Macrae, 1997). The substrate passed the first packed enzyme bed and the first stage product was purified by distillation to remove the free fatty acids. The purified product was further mixed

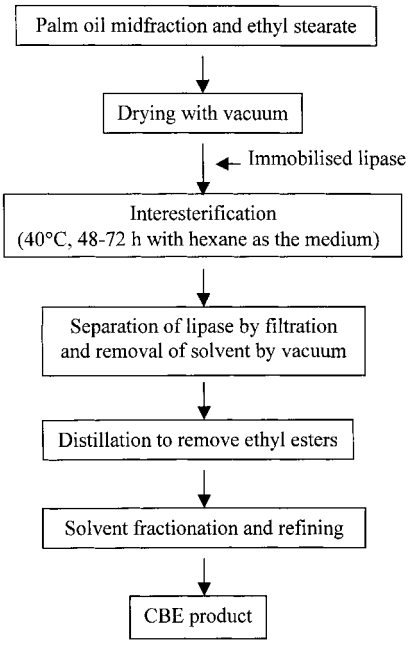

Figure 10. Process scheme for the production of cocoa butter equivalents (CBE) via lipase-catalyzed interesterification in a stirred-tank reactor. (Adapted from Owusu-Ansah, 1994.)

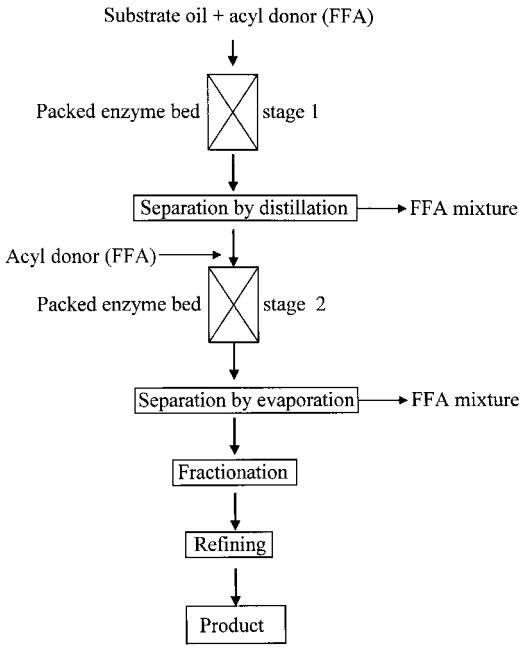

Figure 11. Industrial process for the production of cocoa butter equivalents (CBE) and Betapol via lipase-catalyzed interesterification using packed-bed reactors. For CBE production, high oleic sunflower oil was used as the substrate oil and stearic acid as the acyl donor. For Betapol production, palm stearin was used as the substrate oil and high oleic sunflower oil fatty acids as the acyl donors. (Adapted from Quinlan and Moore, 1993; Rozendaal and Macrae, 1997.)

with acyl donors and passes to the second stage packed-bed reactor. The second stage product was purified by distillation and further fractionated to separate original substrate oils or diacylglycerols formed. A final refining, including bleaching and deodorization, was conducted to make the product suitable for edible purposes.

Another two-stage process was developed on laboratory scale for the production of structured triacylglycerols; alcoholysis was the first step, and esterification the second step (Schmid et al., 1998; Soumanou et al., 1998a,b; 1999). The first step was a reaction between substrate oils and ethanol catalyzed by a lipase to produce monoacylglycerols in the solvent system. The reaction mixture in the solvent was crystallized and fractionated at low temperature. The monoacylglycerols were then separated from the system and further reacted with required fatty acids in the solvent medium. This process was reported to be suitable for the production of high-purity product. Monoacylglycerols, however, are very unstable even at room temperature. Acyl migration might occur and lead to the formation of different isomers (byproducts), especially when the process is scaled-up as the heat transfer and mass transfer would be different from those in flasks. Therefore, careful reaction control might be required and precise parameters should be obtained through process optimization.

For the production of SSL in large scale for nutritional applications, a process using the PBR was built in the pilot plant with the capacity of 10 kg per day

(Xu et al., 1998b). The process contained a substrate tank including heating and stirring, a product tank equipped with cooling system, a gear or metering pump, flow and pressure controls, and a packed enzyme bed containing a jacket. The water content control was assisted by water addition to the feeding tank, stirring, and temperature control. It was found that the steady production could be obtained at optimal conditions. Short path distillation was optimized for the downstream purification of specific structured lipids. A one-stage reaction was usually used because 65–75 % incorporation at *sn*-1,3 positions could be obtained. When higher incorporation of acyl donors is necessary, two stages could be conducted, though this of course reduced the productivity of the process.

For a membrane-applied process, membranes have been used as a lipase carrier (Balcao et al., 1996a; Balcao and Malcata, 1998), lipase filter (Basheer et al., 1995c), free fatty acid separation medium (Keurentjes, 1991; Schroen, 1995), and reaction and separation system (X. Xu et al., unpublished results). So far, both high flux and high selectivity (rejection) of membranes are impossible to achieve for the separation of free fatty acids from triacylglycerols (Snape and Nakajima, 1996). However, in a specifically defined application, fluxes could be made compatible with the reaction systems if the membrane reactors were to be properly designed and operated.

11.9 References

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Phospholipases

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12 Phospholipases Used in Lipid Transformations

Renate Ulbrich-Hofmann

Abbreviations

AOT	*bis*-(2-ethylhexyl)sodium sulfosuccinate
GPI	glycosylphosphatidylinositol
HEPES	*N*-(2-hydroxyethyl)-piperazine-*N*'-ethanesulfonic acid
Lyso-PC	lyso-phosphatidylcholine
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PLA_1	phospholipase A_1
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PS	phosphatidylserine
SDS	sodium dodecylsulfate
Tris	tris(hydroxymethyl)aminomethane

12.1 Classification of phospholipases and their substrates

Phospholipases are a group of enzymes whose natural function consists of the hydrolysis of phospholipids. This class of compounds represent the main components of biological membranes. Phospholipids also occur in large amounts in plant seeds and chicken eggs. Some phospholipid members are biological signal molecules; phospholipases, therefore, effect the metabolism, construction, and reorganization of biological membranes, and are involved in signal cascades. The molecular feature of phospholipids is the diester structure of phosphoric acid in which one alcoholic moiety (X) is polar and the other (Y) is nonpolar (Figure 1). The third acidic function of the phosphoric acid is ionized at physiological pH. Most natural phospholipids are glycerophospholipids (phosphatides), where the nonpolar alcoholic component is a 1,2-diacyl-*sn*-glycerol (Figure 1). The esterification to phosphoric acid is in *sn*-3 position of the glycerol moiety. The notation *sn* refers to the 'stereospecific numbering' adopted by IUPAC-IUB (IUPAC-IUB Commission on Biochemical Nomenclature, 1978a,b). As polar alcoholic components, charged or noncharged compounds such as choline, ethanolamine (both positively charged), serine (zwitterionic), inositol and glycerol (both noncharged) occur, yielding either neutral or negatively charged phospholipids. Inositol can be further modified by one or more

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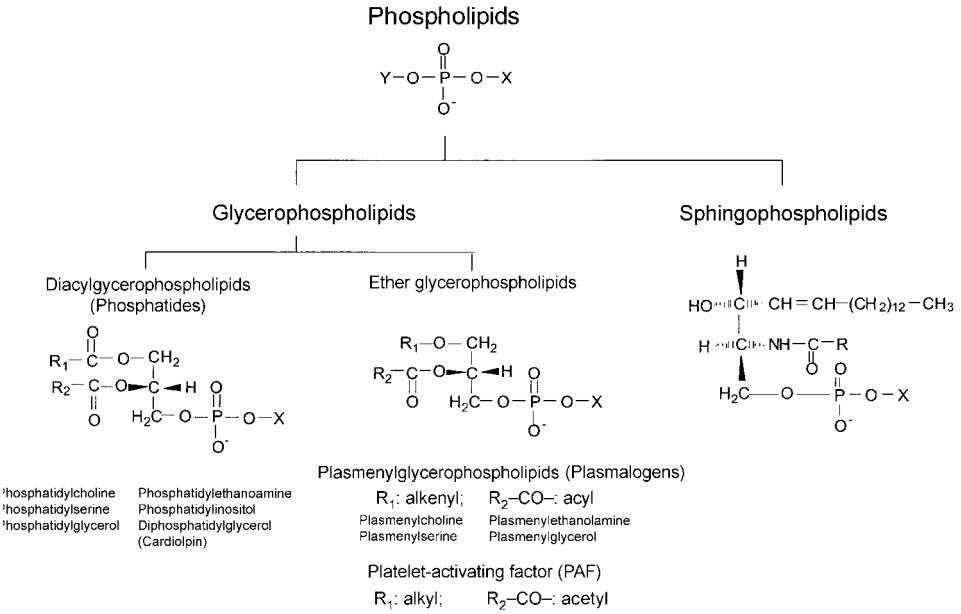

Figure 1. Naturally occurring phospholipids.

phosphate moieties or glycan structures. The diacyl moieties are formed by fatty acids, the composition of which in natural products is heterogeneous with respect to their chain lengths ($C_{12}-C_{22}$) and degrees of unsaturation. There are mostly saturated fatty acyl moieties in the *sn*-1 position and unsaturated fatty acyl moieties in the *sn*-2 position. The composition of the fatty acids depends on the source of the phospholipids with marked preferences. Thus, fatty acids of phosphatidylcholine (PC) from soybean contain 15.2 % palmitic, 7.4 % oleic, 6.4 % linolenic, 2.8 % stearic, 67.7 % linoleic and 0.5 % other acids, whereas the fatty acid composition of phosphatidylcholine (PC) from egg yolk consists of 38.5 % palmitic, 31.7 % oleic, 11.8 % linoleic, 9.2 % stearic, 5,2 % docosahexaenoic, 1.7 % arachidonic, and 1.8 % other acids (Gunstone, 1996). The *sn*-2 carbon is a chiral center. The structure of the naturally occurring phospholipids is equivalent to that of L-glycerol-3-phosphate. Glycerophospholipids with only one acyl moiety are called lysophospholipids. In 1-lysophospholipids, the acyl moiety in *sn*-1 position is lacking, in 2-lysophospholipids the *sn*-2 position is free.

Other naturally occurring compounds belonging to the glycerophospholipids (Figure 1) are ether glycerophospholipids such as plasmenylglycerophospholipids (plasmalogens) and the platelet-activating factor (PAF). They are also derived from the glycerophosphoric acid ester, but in comparison with the diacylglycerophospholipids they contain an alkenyl or an alkyl moiety instead of the acyl moiety in the *sn*-1 position which is ether-linked to the glycerol backbone. Finally, the sphingophospholipids are also based on phosphoric acid diesters with a long-chain amino alcohol such as sphingosine or *N*-acylsphingosine (ceramide) as nonpolar compo-

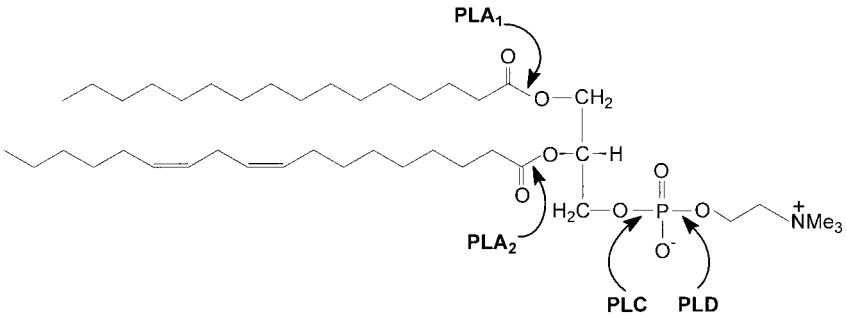

Figure 2. Action of different phospholipases on 1,2-diacyl-*sn*-glycerophosphocholine.

nent. The most important sphingophospholipid is sphingomyelin, bearing ceramide and choline as ester components of phosphoric acid.

Corresponding to the structure of the diacylglycerophospholipids, several phospholipases are known which differ in their specificity according to the position of the bond attacked in the phospholipid molecule. As shown in Figure 2, phospholipase A_1 (PLA_1) catalyzes the hydrolysis of the ester bond in *sn*-1 position, and phospholipase A_2 (PLA_2) that in *sn*-2 position. Phospholipase C (PLC) catalyzes the hydrolysis of the phosphodiester bond at the glycerol side, and phospholipase D (PLD) that at the polar side. Some special types of these enzymes possess an additional specificity concerning the polar alcoholic component, such as the phosphatidylinositol (PI)-specific PLC (PI-PLC) or the glycosylphosphatidylinositol (GPI)-specific PLD. The latter cleaves the GPI anchor with which some proteins are covalently connected and inserted into the plasma membrane. Phospholipases hydrolyzing glycerophospholipids in both the *sn*-1 and the *sn*-2 position are termed phospholipase B (Saito et al., 1991; Oishi et al., 1996; 1999; Merckel et al., 1999). Other related enzymes are lysophospholipases specific for the hydrolysis of 2-lysophospholipids or 1-lysophospholipids (Karasawa and Nojima, 1991; Wang et al., 1997; reviewed in Waite, 1987). Finally, sphingomyelinase is to be regarded as a phospholipase similar to PLC, hydrolyzing sphingomyelin to *N*-acylsphingosine and choline phosphate (Ikezawa et al., 1978; Levade and Jaffrézou, 1999).

In addition to the hydrolytic activity, some phospholipases catalyze transesterification reactions. Best-known for this property is PLD (see Sections 12.3 and 12.4), although the physiological function of this activity has not yet been elucidated (Yu et al., 1996).

12.2 Sources of phospholipases

Corresponding to their importance in the metabolism of phospholipids, phospholipases are widespread among prokaryotes and eukaryotes. In recent years, intensive research into phospholipases, particularly into PLA_2, PLC, and PLD, has been commenced since their significance in several cell regulating processes such as signal

transduction, membrane trafficking, etc., has been realized (reviewed in Tischfield, 1997; Balsinde and Dennis, 1997; Rhee and Bae, 1997; Exton, 1997; Leslie, 1997). In biocatalysis, selected phospholipases (PLA_2, PLD) have been applied for several decades (see Section 12.5.). Stimulated by the progress in enzyme technology, the interest in the application of phospholipases has also increased in recent years, and a further impetus is expected due to the present strong progress in gene technology.

12.2.1 Phospholipase A_1

Enzymes possessing PLA_1 activity have been described as intracellular, membrane-bound or soluble enzymes from rat liver lysosomes (Waite et al., 1981), bovine testis (Higgs et al., 1998), *Aspergillus oryzae* (Watanabe et al., 1999), *E. coli* (Scandella and Kornberg, 1971; Nakagawa et al., 1991), *Corticium centrifugum* (Uehara et al., 1979), hornet venom (Soldatova et al., 1993), tobacco hornworm (Nieder and Law, 1983) and other sources (references in Van den Bosch, 1980) as well as extracellular forms from *Tetrahymena pyriformis* (Arai et al., 1986) or *Tetrahymena thermophila* (Guberman et al., 1999). Hitherto, PLA_1, however, has been poorly studied systematically and scarcely applied in biocatalysis (see Section 12.5.).

12.2.2 Phospholipase A_2

PLA_2 is probably the best investigated lipolytic enzyme, mostly studied from animal sources. There is a wide spectrum of different PLA_2s comprising high molecular weight intracellular (Ca^{2+}-dependent and Ca^{2+}-independent) and low molecular weight extracellular or secretory enzymes (Dennis, 1994; 1997). Intracellular PLA_2s are found in almost every mammalian cell (Van den Bosch, 1980) where they play manifold roles in membrane metabolism and in the release of arachidonic acid acting as precursor for the biosynthesis of prostaglandins and leukotrienes. Mammalian pancreas and venoms from snakes and bees are the richest sources for secretory PLA_2s, which have preferably been used for biocatalytic application (Table 1). Although no recombinant PLA_2 is available commercially, the expression of PLA_2s from porcine pancreas (Verheij and de Haas, 1991; Janssen et al., 1999), bovine pancreas (Noel and Tsai, 1989; Deng et al., 1990; Zhu et al., 1995), snake venom (Kelley et al., 1992; Chang et al., 1996) and bee venom (Dudler et al., 1992; Annand et al., 1996) in *E. coli* was successful. PLA_2 from porcine pancreas has also been expressed in *Saccharomyces cerevisiae* (Bekkers et al., 1991).

Although *Streptomyces* (Verma et al., 1980; Suzuki and Sugiyama, 1993) and *Aspergillus* spp. (Winter et al., 1998) are known to contain PLA_2, microbial sources of PLA_2 have taken an inferior part in basic as well as in applied research, probably because of the good accessibility and high yields of the animal enzymes. PLA_2 is also reported to occur in plants (Kawakita et al., 1993; Kim et al, 1994; May et al., 1998; Stahl et al, 1998), the state of research on this enzyme, however, is still in its early stages.

Table 1. Sources of phospholipases used in biocatalysis.

Phospholipase (EC number)	Source	Molecular mass (kDa)	pI	pH optimum	Reference
PLA_2 (3.1.1.4)	Porcine pancreas	13.9	7.4	7.9–8.4	De Haas et al., 1968
	Bovine pancreas	14.0	6.4; 7.6	8.5; 8.0	Dutilh et al., 1975
	Cobra venom (*Naja naja naja)*	13.4	5.1	7–9	Reynolds and Dennis,1991
	Bee venom (*Apis mellifica*)	19.0	10.5	8.0	Shipolini et al., 1971
PLC (3.1.4.3)	*Bacillus cereus*	23.0		6.6–8.0	Little, 1981
	Clostridium perfringens	43.0		5.4	Takahashi et al., 1981; Krug and Kent, 1984
PI-PLC (3.1.4.10)	*Bacillus cereus*	29.0	5.4	7.2–7.5	Ikezawa and Taguchi, 1981
PLD (3.1.4.4)	Cabbage	87.0	4.7	5.5–5.7 (at 40 mM $CaCl_2$)	Lambrecht and Ulbrich-Hofmann, 1992; Lambrecht and Ulbrich-Hofmann, 1993
		90.2	4.7	5.5 (at 45 mM $CaCl_2$); 7.5 (at 10 mM $CaCl_2$)	Abousalham et al., 1993
		91.8 (PLD1) 92.1 (PLD2)			Pannenberg et al., 1998
	Streptomyces chromofuscus	50–57	5.1	8	Imamura and Horiuti, 1979
	Streptomyces hachijoensis	16	8.6	7.5	Okawa and Yamaguchi, 1975
	Streptomyces lydicus	56	7.4	6	Shimbo et al., 1990
	Streptomyces sp. (Asali Kasai; Sigma)	46	4.2	5.5	Asali Kasei Enzymes catalog, T-39, 1992
	Streptomyces antibioticus	64.0	6.5	5.5	Shimbo et al., 1993
	Streptomyces PMF	53.864	9.1	4–6	Carrea et al., 1995
	Streptomyces PM43	54.147	9.1	6–7	Carrea et al., 1995
	Streptomyces PMR	52.672	9.2	4–7	Carrea et al., 1997b
	Streptoverticillium cinnamoneum	18.2	8.44	8.5	Streikuviene et al., 1986
		53.879		6.0	Ogino et al., 1999

12.2.3 Phospholipase C

The primary interest in PLCs originates from their great importance in cell function and signaling. Besides PLC with the preference for PC as substrate, the highly specific PI-PLC has attracted much attention (Rhee and Bae, 1997). *Bacillus cereus* proved to be an appropriate organism from which to obtain both types of enzymes with similar properties as those of mammals. The nonspecific PLC can be overexpressed in *E. coli* (Tan et al., 1997). Biocatalytic studies also preferably use PLCs from *Bacillus cereus* (Table 1). In addition, *Clostridium perfringens* (Krug and Kent, 1984; Tso and Siebel, 1989), *Achromobacter xylosoxidans* (Kostadinova et al., 1991), *Pseudomonas fluorescens* (Ivanov et al., 1996) and other microorganisms (reviewed in Titball, 1993) have been used as producers of PLC.

12.2.4 Phospholipase D

In contrast to the phospholipases mentioned so far, PLD was used as potent biocatalyst (see Section 12.5), long before its importance in mammalian cell signaling was realized. Its occurrence not only in plants such as carrots, cabbage, peanuts, castor beans, cotton-seeds, but also in microorganisms and mammals has long been known (reviewed in Heller, 1978; Dennis, 1983; Waite, 1987). At present, a vehement progress in knowledge on the physiological function and regulation of PLD in plants, microorganisms and above all human tissues can be observed (reviewed in Daniel et al., 1999; Exton, 1998; 1999; Frohmann et al., 1999; Houle and Bourgoin, 1999; Jones et al., 1999; Liscovitch et al., 1999; Pappan and Wang, 1999; Rudge and Engebrecht, 1999; Venable and Obeid, 1999). In biocatalysis, however, the traditional sources of cabbage and *Streptomyces* strains have maintained their dominant position to date (Table 1). The great number of studies on cloning, sequencing and expression of PLDs from different species (see Section 1.3.3) undoubtedly will promote the utilization of PLDs from other sources. The optimization of enzymes for biotechnological purposes by site-directed mutagenesis or directed evolution (Arnold, 1998; Bornscheuer, 1998) is expected.

12.3 Molecular structure and mechanism of phospholipases used as biocatalysts

The state of research into phospholipases interesting for biocatalyses is rather different. While the tertiary structures and catalytic mechanisms of PLA_2 from several extracellular sources have been thoroughly investigated and the crystal structures of two PLCs have also been elucidated, no tertiary structure of a PLD has been made available so far.

12.3.1 Phospholipase A_2

In contrast to intracellular PLA_2s – the structural information on which is poor (reviewed in Ackermann and Dennis, 1995) – amino acid sequences of more than 150 PLA_2 enzymes of the extracellular type are known (reviewed in Danse et al., 1997). PLA_2s from pancreas and snake venoms are small proteins of high homology and occur in multiple forms. All secretory PLA_2s have molecular masses between 13 and 15 kDa, require Ca^{2+} ions in the millimolar concentration range for optimum activity and possess – apart from very few exceptions – seven disulfide bridges, this probably being the reason for their high stability towards denaturing influences. Reduction of the disulfide bridges results in loss of enzyme activity which can be restored after reoxidation (Van Scharrenburg et al., 1980). Despite its remarkable stability against denaturation, the structure of PLA_2 seems to be very sensitive to point mutations, as revealed in a study of PLA_2 from bovine pancreas (Yuan et al., 1999b). Based on their primary structures, PLA_2s can be classified into four groups (reviewed in Kini, 1997). For instance, group I and group II enzymes, differ in the position of a disulfide bridge and in the length of the C-terminal chain. Mammalian pancreatic PLA_2s belong to the group I consisting of 115 – 120 amino acid residues and seven disulfide bridges. They are synthesized as zymogens and are activated by proteolysis. Figure 3 shows the crystal structure of PLA_2 from bovine pancreas, elucidated by Dijkstra et al. (1981), as an example. It contains three major and two minor α-helical segments (approximately 50 % α-helix content), a double-stranded antiparallel β-sheet (approximately 30 % β-sheet content), and a calcium-binding loop. The crystal structures of PLA_2s from porcine pancreas (Dijkstra et al.,

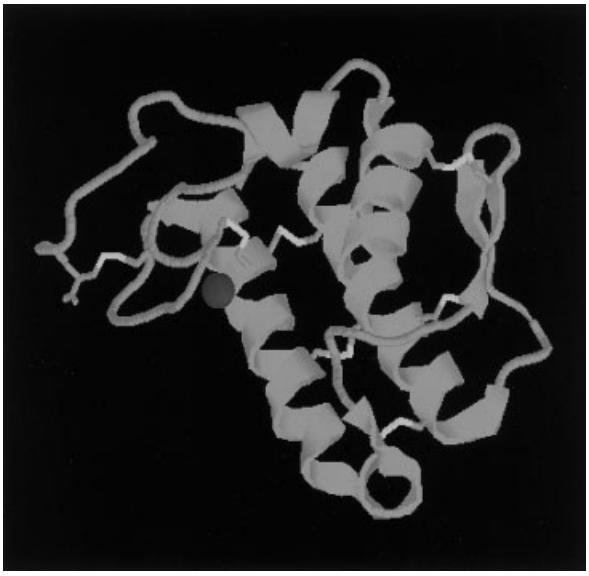

Figure 3. Tertiary structure of PLA_2 from bovine pancreas with seven disulfide bridges and one Ca^{2+} ion. The structure was taken from the Brookhaven Protein Data Bank, file 1BP2, and drawn by RasMol. (See color plate, page XIX).

1983), or from snake venom such as *Crotalus atrox* (Keith et al., 1981; Brunie et al., 1985), *Naja naja atra* (Scott et al., 1990b) or *Agkistrodon halys* pallas (Wang et al., 1996), are very similar. Some enzymes show a tendency to form dimers or higher oligomers (Fremont et al., 1993). PLA_2 from bee venom, belonging to group III, has little homology with the pancreatic and snake enzymes, but significant similarity in the three-dimensional structure and in the catalytic mechanism (Scott et al., 1990a). Several of the bee venom isoforms are *N*-glycosylated (Hollander et al., 1993), but without significant influence on the kinetic properties of the enzyme (Dudler et al., 1992).

The active sites of secretory PLA_2s are composed of a hydrophobic channel with an Asp-His catalytic dyad. The catalytic action of PLA_2 has probable similarities to that of serine proteases, but does not proceed via an acyl enzyme intermediate. Rather, it utilizes the His residue in the active site, assisted by an Asp residue, to polarize a bound H_2O, which then attacks the carbonyl group (Figure 4). The Ca^{2+} ion, located in the conserved Ca^{2+}-binding loop, may stabilize the transition state (Yu and Dennis, 1991a).

Much effort has been directed to mapping the interfacial binding site important for the kinetic phenomenon of interfacial activation (see Section 1.4.2). For pancreatic PLA_2 it has been proposed to be the flat external surface that surrounds the active-site slot, including cationic and hydrophobic residues of the *N*-terminus, the C-terminus and some other residues (Scott, 1997; Yuan and Tsai, 1999). By electron paramagnetic resonance spectroscopy, Lin et al. (1998) defined the interfacial binding surface of bee venom PLA_2 as a patch of hydrophobic residues. For PLA_2 from pig (Van den Berg et al., 1995b) and beef (Yuan et al., 1999a), the structures in solution have also been determined by multidimensional NMR spectroscopy, revealing flexibility of several structural regions, e.g., the *N*-terminus, playing a role in interfacial activation.

Because of the great importance of interfacial activation of PLA_2s an evaluation of substrate specificity is very difficult (see Section 12.4.3). Absolute specificity, however, is found for the *sn*-2 position of the substrate, corresponding to the naturally occurring enantiomeric form of phospholipids. The various secretory PLA_2s show somewhat different preferences with respect to the polar head groups (Rogers et al., 1996), which can be modified by site-directed mutagenesis (Bhat et al., 1993; Beiboer et al., 1995), as well as with respect to the chain length (Lewis et al., 1990) and the saturation degree (Bayburt et al., 1993) of the fatty acyl chains. Although PLA_2 is

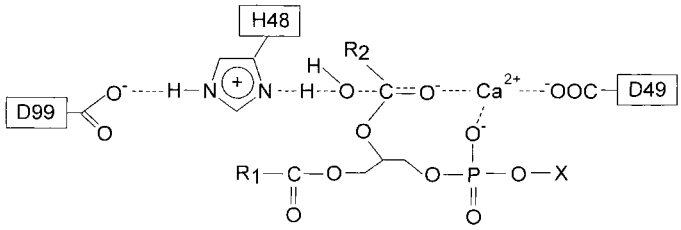

Figure 4. Interactions of the catalytic diad D99–H48 with the substrate and Ca^{2+} in secretory PLA_2s. (Mechanism adapted from to Yu and Dennis, 1991.)

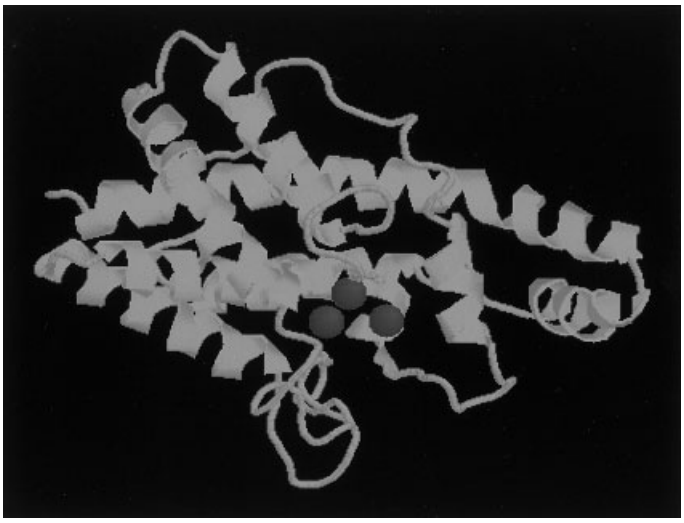

Figure 5. Tertiary structure of PLC from *Bacillus cereus* with three Zn^{2+} ions. The structure was taken from the Brookhaven Protein Data Bank, file 1AH7, and drawn by RasMol. (See color plate, page XIX).

considered an acyl-esterase, a thio-esterase activity has also been demonstrated for PLA_2 from porcine pancreas if compounds with an acyl-thioester bond in the *sn*-2 position of glycerophospholipids were used as substrate (Yu and Dennis, 1991b). A certain activity has also been reported towards nonphospholipid thioesters such as palmitoyl-CoA or palmitoyl-acyl carrier protein (Nocito et al., 1996). Compounds where the *sn*-2 ester has been replaced by an amide, a phosphonate, a difluoromethyl ketone or a sulfonamide, respectively, act as inhibitors of PLA_2 (references in Gelb et al., 1995). In addition, 2,3-diacyl-*sn*-glycero-3-phosphocholines (Bonsen et al., 1972), fatty acid amides, phosphate diesters, substituted *N*,*N*-*bis*-carboxymethyl anilines and other compounds (references in Gelb et al., 1995) are inhibitors of PLA_2. More information on the molecular structure and mechanism of PLA_2s can be found in recent reviews (Dennis, 1994; 1997; Scott and Sigler, 1994; Verheij and Dijkstra, 1994; Gelb et al., 1995; Roberts, 1996; Scott, 1997; Tischfield, 1997; Yuan and Tsai, 1999).

12.3.2 Phospholipase C

Bacterial PLCs are usually small metalloenzymes. PLC from *Bacillus cereus* (see Table 1) is a monomeric extracellular enzyme containing 245 amino acid residues. Cloning and sequencing of its gene have revealed a 24-residue signal peptide and a 14-residue propeptide (Johansen et al., 1988). The crystal structure (Hough et al., 1989) indicates that 66 % of the amino acid residues form ten α-helical regions folded into a single, tightly packed domain, while the remaining amino acid residues are present as loops (Figure 5). The enzyme contains three zinc ions forming a metal

cluster on the inner surface of a deep cleft in the molecule. The metal cluster is a part of the active site and attracts the phosphate group of the substrate molecule. According to molecular modeling (Byberg et al., 1992), the substrate is bound via co-ordination of two phosphate oxygens to the three metal ions and co-ordination of the ester carbonyl from the C(2) fatty acid to one Zn^{2+}. In analogy with other phosphodiesterases, the reaction is suggested to proceed by an in-line attack of an activated water molecule on the phosphodiester, followed by collapse of the resulting penta-coordinate intermediate to give 1,2-diacylglycerol and the phosphorylated head group with inversion of stereochemistry at phosphorus. From studies by site-directed mutagenesis it has been concluded that Asp55 likely serves as the general base in the catalysis (Martin and Hergenrother, 1998a).

Although the enzyme does not contain disulfide bonds, it is extremely stable, even in the presence of 8 M urea at 40 °C (Little, 1978), but loses its stability when the Zn^{2+} ions are removed. In guanidine hydrochloride the enzyme can be unfolded and refolded reversibly by the removal and addition of Zn^{2+} (Little and Johansen, 1979). As concluded from temperature factors for the protein backbone and structural studies of complexes between PLC and various inhibitors, substrate analogs and reaction products, the molecule contains a highly stable inner core and is hardly influenced by ligand binding (reviewed in Hough and Hansen, 1994). At the molecular surface acidic, basic and neutral hydrophilic residues are distributed uniformly with the exception of two loop regions adjacent to the active site. These regions form a nonpolar surface and may influence substrate – enzyme interactions prior to binding in the active site. Studies by site-directed mutagenesis showed that Glu4 belonging to a highly flexible region flanking the active site is important for substrate binding, but not for catalysis (Tan and Roberts, 1998).

PLC from *Bacillus cereus* is termed a broad-spectrum enzyme. In addition to its preferred substrate PC, it also hydrolyzes phosphatidylserine (PS) or phosphatidylethanolamine (PE), but with lower activity. In analyzing the substrate specificity of PLC, Massing and Eibl (1994) found that the negative charge at the phosphate group must be balanced by a positive charge of the head group at suitable distance to fulfil the substrate requirements of the enzyme. Good substrates for PLC from *B. cereus* have an ester bond at position *sn*-2 and an ester or ether bond at the *sn*-1 position of the glycerol, while an ether bond at the *sn*-2 position completely abolishes the ability to act as substrate.

Interestingly, crystal structures of PI-PLCs do not resemble those of PC-specific PLC or other phospholipases. They consist of a single domain folded as a $(\alpha\beta)_8$-barrel and are metal-independent. The catalytic mechanism is similar to that of ribonucleases (reviews in Katan, 1998; Griffith and Ryan, 1999).

12.3.3 Phospholipase D

The reason for the restricted information on the molecular properties of PLDs is probably due to the difficulty in obtaining the enzyme in highly pure or crystallized form. Highly purified PLDs from plants have been prepared from peanut seeds (Heller et al., 1974), citrus callus (Witt et al., 1987), cabbage (Table 1), soybean

(Abousalham et al., 1995), and castor bean (Wang et al., 1993a). Purified microbial PLDs allowing the determination of molecular data were obtained from several *Streptomyces* and *Streptoverticillium* strains (Table 1). Recently, the crystallization of a PLD from *Streptomyces antibioticus* has been achieved successfully (Suzuki et al., 1999). Highly purified mammalian PLDs were obtained from porcine lung (Okamura and Yamashita, 1994), murine (Jenco et al., 1998) and human tissues (Hammond et al., 1997).

Most types of PLD require Ca^{2+} ions for activity, the optimum concentrations being extremely high (20–100 mM) in case of plant enzymes (Heller, 1978). The pH optimum is strongly dependent on the Ca^{2+} concentration. For PLD from soybean, the pH changes from 7.5 at 10 mM $CaCl_2$ to pH 5.5 at 40 mM $CaCl_2$ (Abousalham et al., 1995). PLDs are capable of hydrolyzing and transesterifying a broad range of phospholipids including PC, phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylserine (PS), phosphatidylinositides (PI), lyso-PC, cardiolipin, and plasmalogens (Heller, 1978), with preferences depending on the enzyme source and isoform. With respect to the *sn*-2 position of phosphatides, catalysis is stereospecific. PLD from cabbage catalyzes the cleavage of the naturally occurring PC form much faster than that of its enantiomeric form. This stereospecificity, however, is completely lost if the 2-acyl group in the substrate is replaced by an alkyl group (Bugaut et al., 1985). Alkylphosphate esters such as the antitumor agent hexadecylphosphocholine are also substrates for PLD (Dittrich et al., 1996; 1998). The degree of unsaturation influences the substrate specificity of PLD (Abousalham, 1997). In addition to water, a wide range of alcohols can serve as phosphatidyl acceptors (see Section 1.5.4). Anionic amphiphiles such as sodium dodecylsulfate (SDS), triphosphoinositide, monocetylphosphate (Dawson and Hamington, 1967), phosphatidic acid (Jung et al., 1989), 1,3-diacylglycero-2-sulfate or 1,3-diacylglycero-2-phosphate (Dittrich et al., 1998) were found to act as activators of PLD from cabbage, while neutral lipids such as Triton X-100 (Okawa and Yamaguchi, 1975), cholesterol and 1,2-diacylglycerols were reported to activate PLD from *Streptomyces chromofuscus* (Yamamoto et al., 1993; 1995). Phosphatidic acid or other charged lipids with a free phosphate group are also activators of this enzyme (Geng et al., 1998). The activation effect was attributed to a Ca^{2+}-mediated interaction of the compound with PLD at an allosteric site located in the C-terminal region of the enzyme (Geng et al., 1999). Choline, ethanolamine and protamine sulfate (Dawson and Hamington, 1967), 1,3-diacylglycero-2-phosphocholines (Dittrich et al., 1998; Haftendorn et al., 2000), lysophosphatidylethanolamine (Ryu et al., 1997), alkylphosphate esters (Dittrich et al., 1996; 1998) or aluminum fluoride (Li and Fleming, 1999) proved to be inhibitors of PLD. Irreversible inhibition was obtained by 4-chloromercuribenzoate (Yang et al., 1967), diethylpyrocarbonate (Lee et al., 1989; Secundo et al., 1996) or 4-bromophenacylbromide (Lee et al., 1989). Reviews on plant, microbial and mammalian PLDs can be found in Waite (1987).

A remarkable advance in the knowledge on PLDs has started by the intense research into the molecular genetics of PLD. PLD genes have been cloned and expressed from *Streptomyces chromofuscus* (Yoshioka et al., 1990), *Streptomyces acidomyceticus* (Hasegawa et al., 1992), *Streptomyces antibioticus* (Iwasaki et al., 1994), *Corynebacterium pseudotuberculosis* (Hodgson et al., 1992; McNamara

et al., 1995), *Ricinus communis* L. (Wang et al., 1994; Xu et al., 1997), and *Corynebacterium ulcerans* (McNamara et al., 1995). For *Arabidopsis*, four types of PLD were identified (Pappan et al., 1997a,b; 1998; Qin et al., 1997, 1999). Two of these (PLDβ and PLDγ) are activated by phosphatidylinositol 4,5-bisphosphate similarly to several mammalian PLD isoforms. Two forms of PLD were cloned and expressed also from man (Hammond et al., 1995; Lopez et al., 1998), mouse (Colley et al., 1997a,b) and rat (Park et al., 1997; Kodaki and Yamashita, 1997). From rice and maize, cDNAs coding for PLD were isolated and analyzed (Ueki et al., 1995). In cabbage, two isoforms of PLD were identified on the basis of their cDNAs (Pannenberg et al., 1998) with 85 % (PLD1) and 83 % (PLD2) amino acid sequence identity to PLDα of *Arabidopsis thaliana*. Both isoforms contain eight Cys residues. Recently, Kim et al. (1999) have described the cloning and expression of a PLD from cabbage, which corresponds to the sequence of PLD2. In *Saccharomyces cerevisiae* an open-reading frame on the chromosome XI was identified encoding a protein with PLD activity (Waksman et al., 1996). Corresponding deletion mutants showed that in fact this sequence is responsible for the formation of PLD in yeast (Ella et al., 1996; Waksman et al., 1996) and resulted in finding a second structurally unrelated type of PLD in this organism (Waksman et al., 1997). This form might correspond to the PLD described by Mayr et al. (1996). A PLD gene was also cloned from *Candida albicans* (Kanoh et al., 1998), showing highest homology to PLD from *Saccharomyces cerevisiae*.

On the basis of the numerous available DNA sequences coding for PLD, several structural features and evolutionary ancestors of PLDs could be derived (Ponting and Kerr, 1996; Sung et al., 1997; reviewed in Morris et al., 1996; Wang, 1997; Wakelam et al., 1997; Waite, 1999). Although homology between plant, yeast and human genes of PLD is low, four homologous regions termed as regions I, II, III, and IV can be defined. PLDs from *Streptomyces* have homologies with short parts of the regions I, II, and IV, whereas region III is absent in the enzyme from these species. Sequence homologies have been found also with cardiolipin synthases and phosphatidylserine synthases, which both catalyze a reaction including also the transfer of a phosphatidyl residue. Several more proteins can be assigned to this family of enzymes, also termed as PLD superfamily, e.g., several endonucleases, poxvirus envelope proteins or the murine toxin from *Yersinia pestis*. The hallmark of all these proteins is the $HXKX_4DX_6G(G/S)$ motif containing conserved His, Lys, Asp, Gly and/or Ser residues. This motif, however, is not absolutely conserved but allows small variations. All enzymes with this so-called HKD motif catalyze the cleavage of phosphodiester bonds. Therefore, this motif is suggested to participate in the active site. For endonuclease from *Salmonella typhimurium* (Gottlin et al., 1998), human PLD (Sung et al., 1997) and the murine toxin from *Yersinia pestis* (Rudolph et al., 1999), studies by site-directed mutagenesis proved His and Lys residues of the HKD motif as being essential for activity. As yet, all PLDs analyzed contain the HKD motif in duplicate, which are shown for selected enzymes from plants and microorganisms in Figure 6.

For PLD from rat brain, deletion mutants provided direct evidence that both HKD motifs are necessary for enzyme activity (Xie et al., 1998). Recently, the crystal structure of a recombinant form of the corresponding endonuclease from *Salmonella typhimurium* has been determined (Stuckey and Dixon, 1999). This is the first

Bovine PKC	GLSDPYVKLKL	(19)	NPRWDESF		
Arabidopsis PLDα	GETRLYATIDL	(15)	NPKWYESF	HQKIVVVDSEMPSRGGSEMRRI	HTKMMIVDDEYIIIGS
Arabidopsis PLDβ	ITSDPYVSVSV	(16)	NPVWMQHF	HQKNVIVDADAGGNRRKI	HSKGMVVDDEYVVIGS
Arabidopsis PLDγ	ITSDPYVTVSI	(15)	NPVWIEHF	HEKTVIVDSEAAQNRRKI	HSKGMVVDDEFVLIGS
Arabidopsis PLDγ2	FTSDPYVTVSI	(16)	ILCGMPAF	HQKTMIVDAEAAQNRRKI	HSKGMVVDDEFVLIGS
Cabbage PLD1	GETQLYATIDL	(16)	NPKWYESF	HQKIVVVDSEMPSRGGSQMRRI	HTKMMIVDDEYIIIGS
Cabbage PLD2	GETQLYATIDL	(16)	NPKWYESF	HQKIVVVDSEVPSQGGGSEMRRI	HSKMMIVDDEYIIVGS
Castor bean PLD	GVSKLYATIDL	(16)	NPRWYESF	HQKIVVVDSAMPNGDSQRRRI	HTKMMIVDDEYIIIGS
Cowpea PLD	GVTKLYATIDL	(16)	NPKWNESF	HQKIVVVDSALPGGGGSDKRRI	HTKMMIVDDEYIIIGS
Craterostigma PLD1	GTPKIYASIDL	(16)	NPRWYESF	HQKIIVVDSDLPSGGSDKRRI	HAKMMIVDDEYIIIGS
Craterostigma PLD2	GTPKIYASIDL	(16)	NPRWYESF	HQKIIVVDSDLPSGGSDKRRI	HAKLMIVDDEYIIIGS
Maize PLD	GATKIYATVDL	(16)	NPRWYESF	HQKIVVVDHEMPNQGSQQRRI	HTKMMIVDDEYIIIGS
Pimpinella PLD	GTPKIYASIDL	(16)	NPKWNESF	HQKIVVVDSEMPTSGSENRRV	HAKMMIVDDEYIIIGS
Rice PLD1	GATKVYSTIDL	(16)	NPRWYESF	HQKIVVVDHELPNQGSQQRRI	HTKMMIVDDEYIIIGS
Rice PLD2	GATRLYATIDL	(16)	NPRWYEVF	HQKTVIVDHDMPVPRGGGSRRI	HSKMMIVDDEYIIVGS
Tobacco PLD	GTPAIYATVDL	(16)	NPRWYESF	HQKIVVVDSELPSGESEKRRI	HSKMMIVDDEYIIVGS
Upland cotton PLD	ITSDPYVTIAV	(15)	NPVWMQHF	HQKTVIVDADAGNNHRKI	HSKGMIVDDEFIIVGS
Candida albicans PLD				HEKLCIIDHTYAFLGGIDL	HAKTMIVDDRSVIIGS
Saccharomyces cerevisiae PLD				HEKFVVIDETFAFIGGTDL	HAKILIADDRRCIIGS
Streptomyces antibioticus PLD	ADGKPYALHHK	(16)	YPAWLQDF	HSKLLVVDGKTAITGGING	HHKLVSVDDSAFYIGS
Streptoverticillium cinnamoneum PLD	ADGHPYALHHK	(16)	YPSWLQDF	HSKLLVVDGESAVTGGINS	HHKLVAVDSSAFNIGS

Figure 6. HKD motifs and sections of the C2 domain in PLDs from plants and microorganisms. Amino acid sequences are taken from the NCBI Genbank. From the C2 domain the sequences of the β3 and β5 sheets are shown and compared with the well-known sequence of the C2 domain in protein kinase C (PKC). The numbers in parentheses indicate the number of amino acids between β3 and β5. (See color plate, page XX).

tertiary structure of a PLD family member, and conclusions to common structural features of these enzymes have been derived. The crystal structure suggests that residues from two HKD-motifs form a single active site. The conserved His residue of one motif acts as a nucleophile in the catalytic mechanism, forming a phosphoenzyme intermediate, whereas the His residue of the other motif probably functions as a general acid in the cleavage of the phosphodiester bond. Moreover, the structure of the endonuclease suggests that the conserved Lys residues are involved in phosphate binding. The suggested reaction mechanism includes a phosphatidyl – enzyme intermediate, and is in accordance with previous stereochemical analyses of substrates and products of cabbage PLD proving that catalysis by PLD proceeds with retention of configuration at the phosphorus (Bruzik and Tsai, 1984). From studies on the hydrolysis of PC by PLD with $H_2{}^{18}O$ (Holbroock et al., 1991) it was concluded that the P–O bond rather than the O–C bond of the choline phosphate moiety is cleaved. Recent studies by site-directed mutagenesis and recombinant protein fragments of PLD from *Streptomyces antibioticus* (Iwasaki et al., 1999) also resulted in the conclusion that the two HKD motifs in PLD are not functionally equivalent. The His residue acting as catalytic nucleophile and binding to the phosphatidyl group of the substrate is located on the *C*-terminal half of PLD, whereas the N-terminal half did not contain such a nucleophile. The proposed reaction course is demonstrated in Figure 7. The first step involves the formation of the phosphatidyl – enzyme intermediate, and requires an in-line nucleophilic attack on the phosphorus atom of the substrate by H442 and protonation of the leaving alcohol moiety by H168 as a general acid, while the second step requires deprotonation of a water molecule by the deprotonated H168 as a general base and another in-line nucleophilic attack on the phosphorus atom by the deprotonated water. In difference, studies on chemical protein modification of PLD from *Streptomyces* sp. (Secundo et al., 1996) suggest that Lys but not His residues are essential for activity of the enzyme.

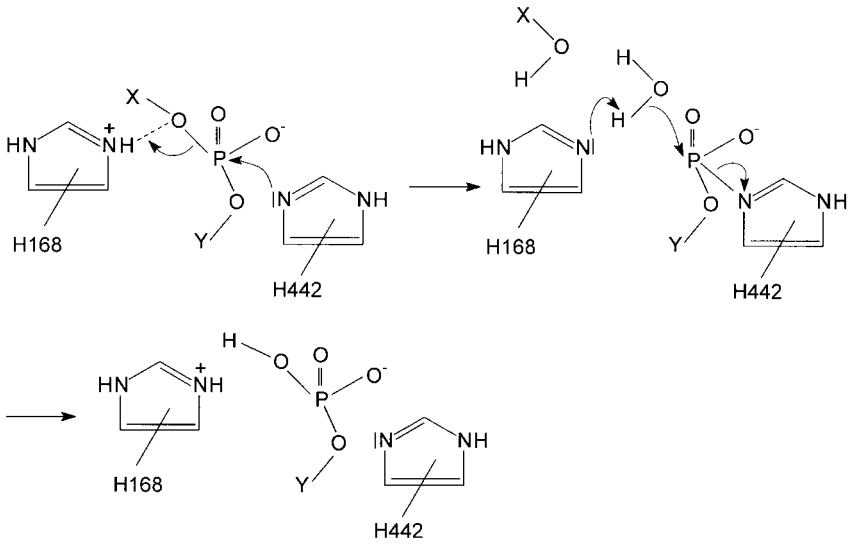

Figure 7. Reaction course of the hydrolysis of phospholipids by PLD. (Mechanism as suggested by Iwasaki et al., 1999, for PLD from *Streptomyces antibioticus*).

Besides the catalytic motifs, PLDs show some other typical structural features. PLDs from plants possess a C2 domain in the *N*-terminal region (Ponting and Parker, 1996; reviewed in Rizo and Südhof, 1998). This domain occurs in more than 100 proteins, as well as in PLCs and protein kinases C. In some of these proteins the C2 domain possesses Ca^{2+}-binding properties and mediates phospholipid binding. The C2 domain comprises approximately 130 residues forming structures of an eight-stranded antiparallel β-sandwich (reviewed in Nalefski and Falke, 1996). A part of the C_2 domains assigned to the β3- and β5-sheets of selected PLDs is shown in Figure 6. A phosphoinositide binding motif mediating activation of these enzymes has been identified in some mammalian and yeast PLD isoenzymes (Sciorra et al., 1999).

12.4 Kinetic particularities of phospholipases and their consequences in lipid transformation

Although a number of membrane-bound phospholipases exist, those enzymes commonly used in biocatalysis are water-soluble. Their substrates, however, are mostly water-insoluble and tend to form supramolecular structures.

12.4.1 Suprastructures of phospholipids

Above their critical micellar concentration (cmc), which is in the range of 10^{-10} M, phospholipids aggregate into a number of polymorphic phases (reviewed in Walde et al., 1990; Chopineau et al., 1998). Some of the most important aggregates are shown schematically in Figure 8. The monolayer is formed at the water – air interface, while micelles, bilayers, uni- or multilamellar vesicles (liposomes) and hexagonal H_{II}-phases arise in the aqueous phase. The type of suprastructure arising depends on the physico-chemical structure of the phospholipid, the medium including ions and additional surfactants, as well as the conditions of preparation (e.g., ultrasonic treatment). An important parameter for the type of aggregation is the ratio between polar and nonpolar moieties in the phospholipid molecule. Cylindrically shaped molecules (Figure 9A) such as in PC tend to form bilayers, while cone-shaped molecules with a dominating hydrophilic moiety (Figure 9B) (such as lyso-PC) form micelles, and molecules shaped like an inverted truncated cone and with a dominating hydrophobic region (such as phosphatidylethanolamine) (Figure 9C) prefer to form hexagonal structures. In organic solvents, reverse micelles can be produced (Figure 8) which, in their interior, are able to host water and hydrophilic compounds such as salts and even proteins. The phospholipid aggregates change as their surroundings are modified. In particular, additional surfactants such as SDS or Triton X-100 belong to effective solubilizers of phospholipids (reviewed in Lichtenberg et al., 1983; Lasch, 1995). They promote the formation of micellar structures, where they are incorporated to give mixed micelles (or mixed reverse micelles). In addition to these lyotropic phase transitions, which are mediated by the medium, phospho-

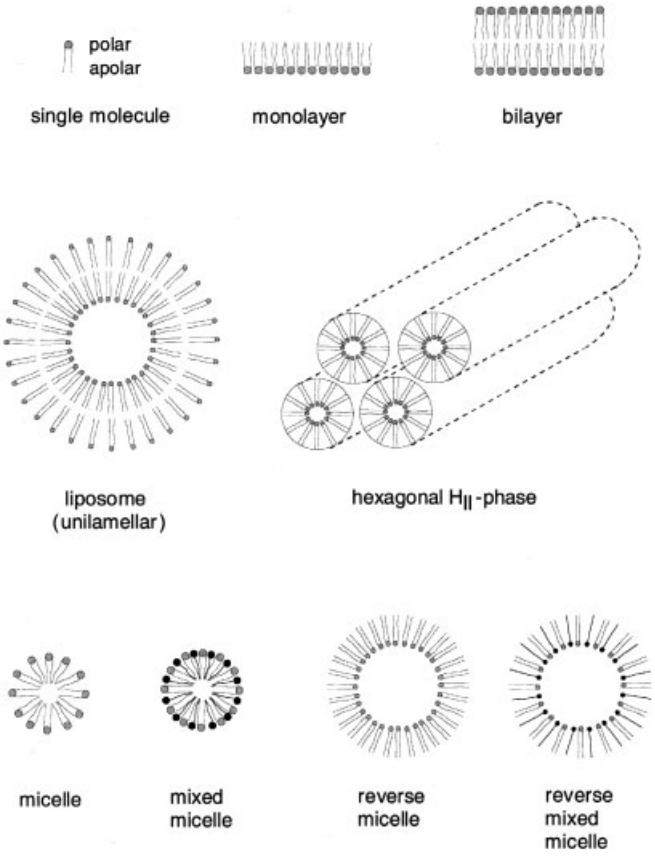

Figure 8. Supramolecular structures of phospholipids.

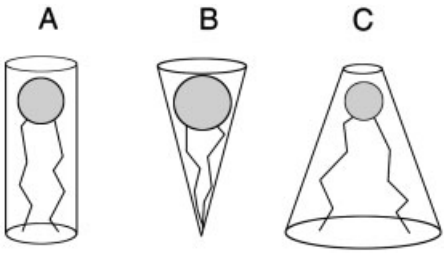

Figure 9. Geometry of phospholipids. (A) cylinder; (B) cone; (C) inverted truncated cone.

lipids can also undergo thermotropic phase changes reflecting the fluidity of the fatty acid chains.

While in vivo phospholipases attack phospholipid clusters of biological membranes, phospholipid substrates in vitro are prepared as liposomes or micelles, often under addition of synthetic surfactants promoting the solubilization of the phospholipids and thus providing uniform aggregate structures.

12.4.2 Interfacial activation of phospholipases

As is generally valid for lipolytic enzymes, phospholipases are more active towards their substrates in aggregated than in monomolecular forms. The molecular mechanisms of this so-called interfacial activation has been the subject of controversial debate, mainly performed on secretory PLA_2 (reviewed in Scott and Sigler, 1994; Gelb et al., 1995). In essence, there are two conceptions that are not mutually exclusive and possibly even both effective. The 'substrate model' attributes the activation by interfaces to the orientation, conformation or hydration states of the phospholipid molecules in the aggregates, facilitating substrate diffusion from the interfacial binding surface to the active site (Scott et al., 1990b). According to this model, activation must not be connected with conformational changes of the enzyme structure. It is supported by comparison of crystal structures of PLA_2 in the absence and presence of substrate analogs, uncovering no significant structural modifications of the enzyme (reviewed in Scott and Sigler, 1994). In contrast, the 'enzyme model' is based on studies by NMR, fluorescence and also crystallographic measurements indicating some conformational changes at lipid binding (reviewed in Scott and Sigler, 1994; Kilby et al., 1995; Van den Berg et al., 1995a). Regions that are flexible in the free enzyme, such as the *N*-terminal region and a surface loop become more ordered in the presence of a substrate analog.

Several approaches have attempted to model the interface-activated kinetics. Verger et al. (1973) generally formulated the principle of enzyme activation by interfaces according to Equation (1), where the binding to the interface converts the enzyme E to its activated form E*. This binds a substrate molecule and forms the product P in a normal catalytic way. The model has been elaborated for a reaction of PLA_2 at a monolayer.

$$E \rightleftarrows E^* \overset{+S}{\rightleftarrows} E^*S \rightarrow E^*P \rightleftarrows E^* + P \tag{1}$$

The 'surface dilution model' (Deems et al, 1975; Dennis, 1994) includes one activating substrate molecule into the reaction equation [Equation (2)]. It is based on the assumption that the water-soluble enzyme (E) first binds to one phospholipid molecule (S), constrained in an interface, to form ES. A second phospholipid molecule is then bound in the catalytic site and forms the Michaelis complex ESS, from which P is formed via the complex ESP.

$$E + S \rightleftarrows ES \overset{+S}{\rightleftarrows} ESS \rightarrow ESP \rightleftarrows ES + P \tag{2}$$

This model has been derived from kinetic data of the action of cobra venom PLA_2 in mixed micelles with the nonionic surfactant Triton X-100.

Both models yield mathematically equivalent kinetics, and are appropriate to fit the experimental results. On special substrates forming large anionic vesicles, Jain and Berg (1989) analyzed whether PLA_2 remains associated with the interface while hydrolyzing successive phospholipid substrate molecules ('scooting mode') or whether it dissociates from the interface after each catalytic event ('hopping mode'). They found a preference for the scooting mode with this substrate.

The phenomenon of interfacial activation can also be observed with other phospholipases such as PLC (Hough and Hansen, 1994) and PLD (Allgyer and Wells, 1979; Nakagaki and Yamamoto, 1984; Lambrecht et al., 1992) where it has been, however, less thoroughly analyzed hitherto. As studied on the catalysis of PLC or PLD on monolayers, surface pressure (Quarles and Dawson, 1969; Goodman et al., 1996) and charge (Chen and Barton, 1971; Kondo et al., 1994b) of the monolayer strongly influence the activation effect.

Another interesting feature in the action of many phospholipases that is related to the phenomenon of interfacial activation is the lag-burst behavior in the initial phase of the catalyzed reaction. In difference to most enzymes with soluble substrates, the initial rates are not the maximum rates. In the case of phospholipases, maximum rates are reached only after a latency period. Studies of this phenomenon on PLA_2 (Callisen and Talmon, 1998), PLC (Basáñez et al., 1996; Ruiz-Argüello et al., 1998), and PLD (Dorovska-Taran et al., 1996) supported the hypothesis that full enzyme activity requires some kind of membrane surface irregularities or defects, originating from the products of the initial molecular turnovers. Therefore, phospholipase activity appears to be controlled by the overall geometry and composition of the phospholipid aggregates.

12.4.3 Consequences of kinetic particularities to characterization of phospholipases

Since phospholipases preferentially hydrolyze substrates in aggregated form, their activities depend not only on the substrate and enzyme concentrations but also on the physical state of the substrate, i.e., on the organization and dynamics of the interphase where catalysis occurs. One of the most important prerequisites for reasonable kinetic measurements is the reproducible preparation of substrate aggregates. Preferably uniform aggregates such as monolayers, micelles formed from short-chain phospholipids, liposomes, or mixed micelles formed from phospholipids and additional surfactants are used. Classical kinetic concepts are not valid for phospholipases, although kinetic analyses by the Michaelis – Menten approach or similar models are often used. These yield apparent parameters which can be applied only for the comparison of relative affinities and maximum velocities of different enzymes under exactly the same conditions of substrate preparation. Therefore, comparisons of results obtained in different laboratories are of restricted value, and most findings on substrate specificities cannot be generalized. Furthermore, the structure of the substrate aggregates will change during the reaction due to the arising products

which modify the aggregate structure either by remaining in the particles or by being released. Therefore, in biocatalytic processes changes of the catalytic constants in course of the reaction must be taken into account. Finally, in the evaluation of activators or inhibitors of phospholipases, it is difficult to differentiate between effects caused by molecular interactions of the effector with the phospholipase and those resulting from modifications of the morphology and charge of the phospholipid interphase.

12.4.4 Catalysis in the presence of organic solvents

In biocatalytic applications of phospholipases (see Section 12.5.), organic solvents are often involved in the reaction systems. In general, reactions are performed in shaken emulsion systems, where a nonpolar organic solvent such as diethyl ether or ethyl acetate containing the dissolved phospholipid is mixed with an aqueous phase containing the enzyme in buffer solution (see Section 12.5). In such systems, the peculiarities of the kinetics of phospholipases have scarcely been regarded. Indeed, the influence of solvents on the aggregate structures enhance the complexity of the system. Moreover, an additional reaction partner is often introduced, e.g., an acceptor alcohol in transphosphatidylation by PLD, which may partition between the aqueous phase, the organic phase and the aggregates. In addition, due to the heterogeneity of the reaction system, diffusion processes may become relevant. Finally, denaturation of the enzymes by the organic solvents must be taken into consideration. As a consequence, most studies on the reactions of phospholipases in the presence of organic solvents barely allow general conclusions.

In a study using D- and L-serine as substrates in the transphosphatidylation by a PLD from *Streptomyces* sp., the following ranking of solvents in emulsion systems was reported: ethyl acetate > diethyl ether > benzene > chloroform > toluene (Juneja et al., 1989a). In contrast, in the reaction of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine with 4-methoxyphenol catalyzed by a PLD from *Streptomyces* sp. rates depended on the solvent in the sequence of benzene > dichloromethane > toluene > diethyl ether > ethyl acetate (Takami et al., 1994a), whereas a ranking of dichloromethane > diethyl ether > ethyl acetate > benzene was found for the formation of 1,2-dipalmitoyl-*sn*-glycero-3-kojic acid by this enzyme (Takami et al., 1994b). The phosphatidyl glucose production from PC by PLD from *Actinomadura* sp. was high in an emulsion system containing diethyl ether, benzene, toluene, and dichloromethane, whereas the yield was low in chloroform and no reaction was observed in hexane (Kokusho et al., 1993).

In the transphosphatidylation reaction between PC and 3-dimethylamino-1-propanol by PLD from *Streptomyces* PMF in water – ethyl acetate emulsion systems, Carrea et al. (1997a) found an interfacial saturation by the enzyme and concluded that only PLD located at the interface of the water – organic phase is active. By measuring the enzymatic hydrolysis of PC monolayers formed at the polarized nitrobenzene/water interphase by peanut PLD, Kondo et al. (1992; 1994) showed that the rate of hydrolysis depends markedly on the potential drop across the interphase. Hirche et al. (1997a) and Hirche and Ulbrich-Hofmann (1999), in analyzing

solvent effects in hydrolysis and transphosphatidylation of PC by PLD from cabbage and *Streptomyces* spp., postulated that the interfacial pressure being correlated with the package density of the PC aggregates was the main regulator of enzyme activity. Therefore, PLD catalysis in emulsion systems seems to be governed by similar interfacial mechanisms of recognition and activation as in aqueous systems. The physico-chemical properties of the organic solvent, together with the structure of the acceptor alcohol, decisively influence the ratio of transphosphatidylation to hydrolysis rates, with the same tendencies for different PLDs (Hirche and Ulbrich-Hofmann, 2000). Small amounts of aliphatic alcohols are able to activate PLD in hexane systems which are almost inert without alcohols. An activation by water-miscible organic solvents, which correlated with the solvent polarity, was also observed with PI-PC (Wu and Roberts, 1997).

Both PLA_2 (Misiorowski and Wells, 1974; Rakhimov et al., 1986; Wu and Lin, 1994; Morgado et al., 1995) and PLD (Subramani et al., 1996) have been described to be active also in water-poor organic solvents such as diethyl ether or isooctane, where they are entrapped in the interior of reverse micelles formed by PC and additional surfactants. Modification of the properties of phospholipases can be also reached by modification with methoxypolyethylene glycol (Matsuyama et al., 1991), lipid-coating (Okahata et al., 1995) or immobilization (see Chapter 13).

12.5 Examples of application

Phospholipids and their partial hydrolysis products, the lysophospholipids, have numerous applications in the food, cosmetic, pharmaceutical, and other industrial branches (Cevc, 1993). Due to their amphiphilic nature, phospholipids are good emulsifiers or mediators of solubility, besides being distinguished by their good biocompatibility and biodegradability. Their tendency to form supramolecular structures (see Section 12.4.1) allows them to be used as vesicles for the inclusion and the transport of drugs and other substances. Recently, cationic phospholipids in the form of liposomes have attracted much attention as transfection vectors in gene therapy (Yanagihara et al., 1995). Finally, some phospholipids or phospholipid analogs (phosphatidylnucleosides, plasmalogens, alkylphosphate esters) themselves are biologically active compounds, acting as antitumor agents (Houlihan et al., 1995) and lung surfactants (references in Namba, 1993). The structural variations of natural phospholipids allows their physico-chemical properties to be varied as emulsifiers or vesicle materials, as well as in the design of new potential drugs.

For transformation of natural phospholipids, or for selected steps in the synthetic production of phospholipids, phospholipases are valuable tools because of their regio- and stereoselective catalysis. In addition to phospholipases, esterases (lipases), phosphatases and kinases are also used for this purpose. The synthetic applications of phospholipases are summarized in the following examples. Reviews on phospholipases as catalysts can also be found in Mukherjee (1990), Kötting and Eibl (1994), D'Arrigo and Servi (1997) and Servi (1999).

12.5.1 Phospholipase A_1

Although several reports exist on hydrolysis reactions catalyzed by PLA_1 (Fauvel et al., 1984; Kucera et al., 1988; Mustranta et al., 1994; 1995), the enzyme has not yet obtained marked significance in enzymatic syntheses. One reason for this is the instability of the hydrolysis products. 1-Lysophosphatides formed by PLA_1 are subjected to rapid acyl transfer, yielding the more stable 2-lysophospholipids, and therefore are not of great practical interest. The second reason is that transesterification of phospholipids is accomplished also by several lipases, e.g. from *Rhizopus delemar* (Brockerhoff et al., 1974), *Rhizopus delemar* (Haas et al., 1993), *Mucor javanicus* (Morimoto et al., 1993), which are better available and more thoroughly investigated.

12.5.2 Phospholipase A_2

Secretory PLA_2s, mainly from pancreatic sources, are well-known as industrial catalysts in the production of 2-lysophospholipids from natural or synthetic PC (references in Mustranta et al., 1994). Lysophospholipids are important emulsifiers in the food and pharmaceutical industries. In recent years increasing efforts have been made to use PLA_2 for reacylation or transacylation of phospholipids in the *sn*-2 position (Figure 10 and Table 2). In fact, reacylation was found more successful than transacylation. Yields of acylation products starting from lyso-PC have been improved from 7 % in early reports (Pernas et al., 1990) to 60 % in the more recent study by Egger et al. (1997), in which success was based on careful control of water activity in the reaction mixture.

Application of PLA_2 has been envisaged also in medicine. In the treatment of hypercholesterolemia, an extracorporeal shunt system was designed which con-

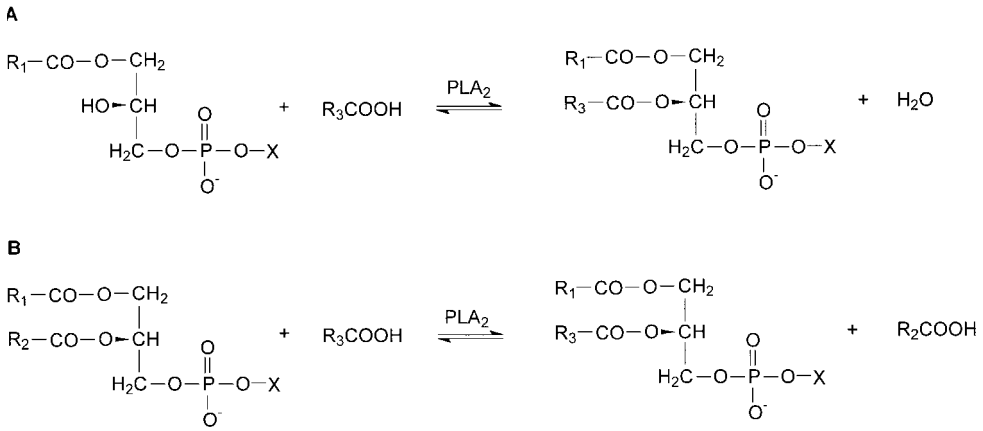

Figure 10. Modification of phospholipids by PLA_2. (A) Introduction of fatty acids into lyso-glycerophospholipids. (B) Exchange of fatty acids in glycerophospholipids.

Table 2. Fatty acids introduced by PLA_2 into glycerophospholipids.

Fatty acid introduced	Educt	Source of PLA_2	Solvent system	Reference
[^{3}H]-Oleic acid	lyso-PC (egg yolk)	*Naja naja* and bee venom, porcine and bovine pancreas, *Streptomyces violoceoruber*	toluene, benzene	Pernas et al., 1990
Eicosapentanoic ($C_{20:5}$) and docosahexanoic ($C_{22:6}$) acids	lyso-PC	porcine pancreas	buffer/isooctane, AOT[1] (microemulsion)	Na et al., 1990
Saturated and unsaturated fatty acids	glycerophos-pholipids	bee or snake venom, porcine pancreas (PLA_2 immobilized)	hexane, heptane, petroleum ether, chlorinated hydrocarbons	Pedersen, 1991
Palmitic acid	lyso-PC	cobra venom (*Naja naja)*	chloroform (1 % water)	Lin et al., 1993
Saturated and unsaturated fatty acids	PC (egg yolk) lyso-PC (egg yolk) and others	porcine pancreas	chloroform and other	Mingarro et al., 1994
Polyunsaturated fatty acids	lyso-PC (egg yolk)	porcine pancreas (PLA_2 immobilized)	isooctane, ethanol	Lilja-Hallberg and Härröd, 1994; Lilja-Hallberg and Härröd, 1995
Polyunsaturated fatty acids	lyso-PC (egg yolk)	porcine pancreas (PLA_2 immobilized)	isooctane, CO_2 propanol (in a high-pressure reactor, under controlled water activity)	Härröd and Elfman, 1995
Eicosapentanoic ($C_{20:5}$) and docosahexanoic ($C_{22:6}$) acids	lyso-PC (soybean)	porcine pancreas	glycerol, formamide	Hosokawa et al., 1995
Oleic acid and other fatty acids	lyso-PC (egg yolk)	porcine pancreas (PLA_2 immobilized)	toluene (under controlled water activity)	Egger et al., 1997

[1] AOT, *bis*(2-ethylhexyl)-sulfosuccinate.

tained PLA_2 immobilized onto Sepharose (Labeque et al., 1993). This approach is based on the decrease of plasma cholesterol by degradation of phospholipids in low-density lipoproteins (LDL). An advanced model of this reactor is the plasma separator reactor which combines plasma separation and enzymatic conversion of phospholipids in LDL (Shefer et al., 1993). With the same aim of plasma clearance from LDL particles, PLA_2 from cobra venom was also immobilized to chitosan beads in a circulating packed-bed reactor (Chen and Chen, 1998).

12.5.3 Phospholipase C

Because of its high stereospecificity, PLC can be used to obtain enantiomerically pure 1,2-diacyl glycerols (Anthonsen et al., 1999) by hydrolysis of corresponding glycerophospholipids. These compounds are important in biomedical research because of their function in signal cascades. They can also serve as substrates for the production of synthetic phospholipids with natural configuration. Furthermore, hydrolysis catalyzed by PLC can be applied for the resolution of racemic phospholipid mixtures. Thus, PAF and other enantiomerically pure phospholipids were prepared starting from 1-*O*-alkyl-2-acetyl-*rac*-glycerol or 1,2-diacyl-*rac*-glycerols. After chemical conversion into the phosphocholine derivatives and subsequent treatment with PLC, the phospholipids with unnatural configuration are separated from 1-*O*-alkyl-2-acetyl-*sn*-glycerol or the 1,2-diacyl-*sn*-glycerols (Kötting and Eibl, 1994).

The enzymatic cleavage of the phosphate ester bond between the glycerol moiety and phosphocholine in glycerophospholipids by PLC may also be used in the production of organic phosphates such as glycerol-3-phosphate or dihydroxyacetone phosphate (D'Arrigo et al., 1995). Takami and Suzuki (1994) produced dihydroxyacetone phosphate by PLC-catalyzed cleavage of phosphatidyldihydroxyacetone, obtained from PC by PLD (Table 3). The formation of 1,3-cyclic glycerophosphate has been reported by the action of PLC from *Bacillus cereus* on phosphatidylglycerol (Shinitzky et al., 1993).

A PLC-catalyzed transfer reaction was first described by Kanfer and Spielvogel (1975), in which ^{14}C sphingomyelin was formed by the transfer of the phosphorylcholine moiety of PC to *N*-(^{14}C)-oleoyl-sphingosine. A series of interesting transesterification reactions have recently been described by means of bacterial PI-PC. Starting from inositol 1,2-cyclic phosphate, which was obtained by PI-PLC-catalyzed cleavage from soybean phosphatidylinositol, diverse *O*-alkyl inositol 1-phosphates were synthesized (Figure 11). The type of alkyl moieties ranged from simple methyl via mannityl to peptidyl groups (Bruzik et al., 1996).

Moreover, there are reports on the involvement of PLC in chemo-enzymatic multistep syntheses such as the synthesis of phosphatidylinositol bearing a polyunsaturated acyl group, where PLC from *Bacillus cereus* is used to remove the phosphorylcholine moiety from 2-docosahexaenoyl-1-stearoyl-*sn*-glycerophosphocholine before chemical phosphorylation and coupling with *myo*-inositol (Baba et al., 1996).

Table 3. Head groups introduced by PLD into 1,2-diacyl-*sn*-glycerophospholipids.

Head group introduced	Educt[1]	Source of PLD	Solvent system	Reference
Aliphatic primary alcohols	PC (egg yolk)	Cabbage	acetate buffer/SDS	Dawson, 1967
	PC	Savoy cabbage	acetate buffer/ diethyl ether	Yang et al., 1967
	PC (egg yolk), DLPC, DMPC, DPPC and other	Cabbage	acetate buffer/ diethyl ether	Eibl and Kovatchev, 1981
	DMPC, DPPC	Cabbage	acetate buffer/ diethyl ether	Sale et al., 1989
	PC (egg yolk)	*Streptomyces* sp. (PLD lipid-coated)	acetate buffer/ benzene	Okahata et al., 1995
Unsaturated propanols	PC (egg yolk)	Cabbage	acetate buffer/ diethyl ether	Kovatchev and Eibl, 1978
Halogenethanols	PC (egg yolk)	Cabbage	acetate buffer/ diethyl ether	Kovatchev and Eibl, 1978
3,3,-Dimethyl-1-butanol	DHPC	*Streptomyces* sp.	acetate buffer/ chloroform	Martin and Hergenrother, 1998b
Aliphatic secondary alcohols (Figure 13A)	PC	*Streptomyces* sp.	acetate buffer/ ethyl acetate or methylene chloride	D'Arrigo et al., 1994
Ethylene glycol	PC (egg yolk)	Cabbage	acetate buffer/SDS	Dawson, 1967
Higher alkandiols	PC (egg yolk)	Cabbage	acetate buffer/ diethyl ether	Kovatchev and Eibl, 1978
Glycerol	PC (egg yolk)	Cabbage	acetate buffer/SDS	Dawson, 1967
	PC	Savoy cabbage	acetate buffer/ diethyl ether	Yang et al., 1967
	PC	Cabbage	acetate buffer/ diisopropyl ether or other (in a microporous membrane reactor)	Lee et al., 1985
	PC	Cabbage	acetate buffer/ diethyl ether (micelles or emulsion systems)	Juneja et al., 1987a
	PC	Cabbage (PLD immobilized)	acetate buffer/ diethyl ether	Juneja et al., 1987b
Dihydroxyacetone	DPPC	*Streptomyces* sp.	acetate buffer/ ethyl acetate	Takami and Suzuki, 1994

Table 3. continued.

Head group introduced	Educt[1]	Source of PLD	Solvent system	Reference
(*R*)- and (*S*)-isopropylideneglycerol (Figure 13B)	PC	*Streptomyces* sp.	acetate buffer/ methylene chloride	D'Arrigo et al., 1996a
Phosphatidylglycerol	PG (^{3}H-labelled)	Cabbage	acetate buffer/ diethyl ether	Stanacev et al., 1973
	PG	*Streptomyces* sp.	acetate buffer/ methylene chloride	D'Arrigo et al., 1996a
Cyclic nonaromatic alcohols (Figure 13C)	PC	*Streptomyces* sp.	acetate buffer/ ethyl acetate, methylene chloride	D'Arrigo et al., 1994; D'Arrigo et al., 1996b
Ethanolamine	PC	Savoy cabbage	acetate buffer/ diethyl ether	Yang et al., 1967
	PC (egg yolk)	Cabbage	acetate buffer/SDS	Dawson, 1967
	DMPC, DPPC	Cabbage	Acetate buffer/ diethyl ether	Smith et al., 1978
	PC	Cabbage, *Streptomyces chromofuscus*, *Streptomyces prunicolor*, and other *Streptomyces* spp.,	acetate or phosphate buffer/ethyl acetate, diethyl ether or other	Juneja et al., 1988
	PC (egg yolk)	*Streptomyces mediocidicus*, *Streptoverticillium cinnamoneum*, *Streptoverticillium hachijoense*	buffer/diethyl ether	Nakajima et al., 1994
	PC (soybean)	*Streptomyces* sp.	acetate buffer/ methylene chloride	D'Arrigo et al., 1996b
Choline	PE in lecithin from soybean and egg yolk	*Streptomyces* spp., cabbage	acetate buffer/ ethyl acetate	Juneja et al., 1989b
D- and *L*-Serine	PC (egg yolk), DMPC, DEPC	Savoy cabbage	acetate buffer/ diethyl ether	Comfurius and Zwaal, 1977

Table 3. continued.

Head group introduced	Educt[1]	Source of PLD	Solvent system	Reference
	PC	Cabbage, *Streptomyces chromofuscus*, *Streptomyces prunicolor* and other *Streptomyces* spp. (PLD immobilized)	acetate or phosphate buffer/ethyl acetate, diethyl ether or other	Juneja et al., 1989a
	PC (egg yolk)	*Streptomyces* sp. (PLD lipid-coated)	acetate buffer/ benzene	Okahata et al., 1995
	PC (soybean)	*Streptomyces prunicolor*	phosphate buffer/ ethyl acetate	Sakai et al., 1996
	disulfide-modified PC	*Streptomyces chromofuscus*	acetate buffer/ diethyl ether	Diaz et al., 1998
myo-Inositol	PC (egg yolk)	Spinach	acetate buffer/ diethyl ether	Mandal et al., 1980
Nucleosides	DPPC, DSPC, DOPC	*Streptomyces* sp.	acetate buffer/ chloroform	Shuto et al., 1987; Shuto et al., 1988b; Shuto et al., 1995; Shuto et al., 1996
	DMPC	*Streptomyces* sp.	acetate buffer/ chloroform	Wang et al., 1993b
Saccharides	PC (egg yolk), PC (soybean), PE, PG, DMPC, DPPC, DHePC, SM	*Actinomadura* sp.	aqueous salt solution/diethyl ether or other	Kokusho et al., 1993
	DOPC	*Streptomyces* sp.	acetate buffer/ chloroform	Wang et al., 1993b
Azasugars (Figure 13D)	DOPC	*Streptomyces* sp.	acetate buffer/ chloroform	Wang et al., 1993b
Peptides	DOPC	*Streptomyces* sp.	acetate buffer/ chloroform	Wang et al., 1993b
	PC (egg yolk)	*Streptomyces* sp. (PLD lipid-coated)	acetate buffer/ benzene	Okahata et al., 1995
L-Ascorbic acid	PC (egg yolk), DMPC	*Streptomyces lydicus* and other	acetate buffer/ diethyl ether	Nagao et al., 1991
Arbutin (Figure 13E)	DPPC	*Streptomyces* sp.	acetate buffer/ethyl acetate, diethyl ether or other	Takami et al., 1994b
Koji acid (Figure 13F)	DPPC	*Streptomyces* sp.	acetate buffer/ ethyl acetate, diethyl ether or other	Takami et al., 1994b

Table 3. continued.

Head group introduced	Educt[1]	Source of PLD	Solvent system	Reference
2,5,7,8-Tetramethyl-6-hydroxy-2-(hydroxy-ethyl)chroman (Figure 13G)	PC (egg)	*Streptomyces lydicus*	buffer/diethyl ether	Koga et al., 1994
Diverse aromatic compounds (Figure 13H)	DPPC	*Streptomyces* sp.	acetate buffer/ benzene or other	Takami et al., 1994a
4-Methoxyphenol	DPPC	*Streptomyces* sp. (PLD immobilized)	acetate buffer/ benzene	Takami and Suzuki, 1995
Arsenocholine (Figure 13I)	DOPC	Cabbage, *Streptomyces* sp.	acetate buffer/ diethyl ether	Hirche et al., 1997b
N-heterocyclic alcohols	PC	*Streptomyces* sp.	acetate buffer/ethyl acetate, methylene chloride	D'Arrigo et al., 1996b
(Figure 13J)	DOPC	Cabbage, *Streptomyces* sp.	acetate buffer/ diethyl ether	Hirche et al., 1997b

[1] DEPC, 1,2-dielaidoyl-*sn*-glycero-3-phosphocholine; DHPC, 1,2-dihexanoyl-*sn*-glycero-3-phosphatidylcholine; DHePC, dihexadecyl-*sn*-phosphatidylcholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; SM, sphingomyelin.

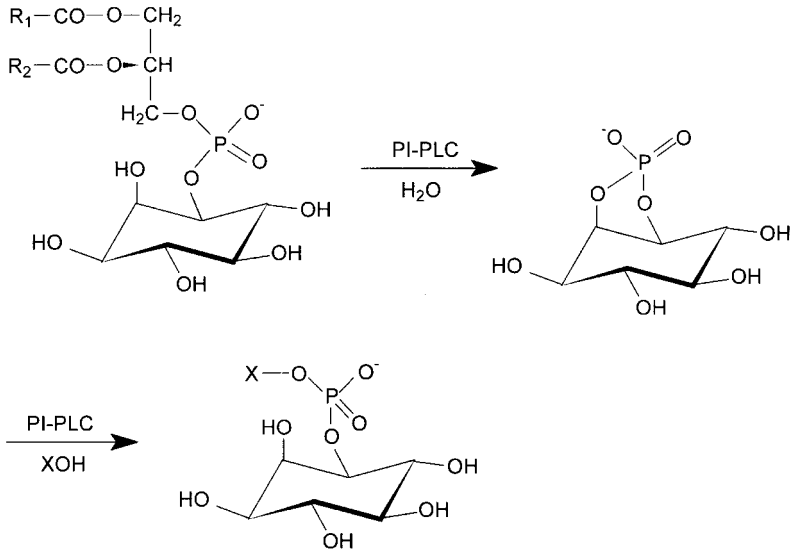

Figure 11. Synthesis of *O*-alkyl inositol 1-phosphates by PI–PLC. XOH: alkanols, alkandiols, glycerol, sugar alcohols, aminoalcohols, serine, uridine, peptides, pentaethylene glycol.

12.5.4 Phospholipase D

PLD has been the most used phospholipase hitherto, mainly due to its relatively strong propensity to catalyze transesterification at the terminal phosphate ester bond of phospholipids. This transphosphatidylation is in competition to hydrolysis (Figure 12), but in contrast to transesterification reactions by other types of phospholipases, some PLDs favor transphosphatidylation over hydrolysis and allow the synthesis of new phospholipids in high yield. The syntheses of diacylglycerophospholipids by PLD are reviewed in Table 3, while selected structural formulae of head groups introduced are shown in Figure 13. PG, PS or PE are also known to be produced by transphosphatidylation with PLD in industrial scale. While in earlier reports PLD from cabbage was mostly used, more recently PLDs from *Streptomyces* strains have been preferred for synthetic purposes (Table 3). The observed transphosphatidylation activities are very different where – according to the kinetic particularities of PLDs described in Section 12.4 – the source of the enzyme, the reaction media and the acceptor alcohols seem to be interdependent, and the derivation of general rules is difficult. In fact, PLDs from some *Streptomyces* strains seem to be predestined for transesterification of glycerophospholipids whereas PLDs from other strains, e.g., from *Streptomyces chromofuscus* fail in this function. PLD from cabbage has an intermediate position in this series. Water and alcohol strongly compete for the phosphatidyl moiety, and the formation of phosphatidic acid has to be tolerated in most reactions.

While all conversions listed in Table 3 start with natural or synthetic PC, Table 4 summarizes PLD-catalyzed transesterification of compounds not containing the diacylglycerol backbone. These examples show that alkyl moieties attached to glycerol by an ether bond, as well as 3-fold unsaturated fatty acids in the substrate molecule, are accepted. Interestingly, even alkylphosphocholines lacking the glycerol back-

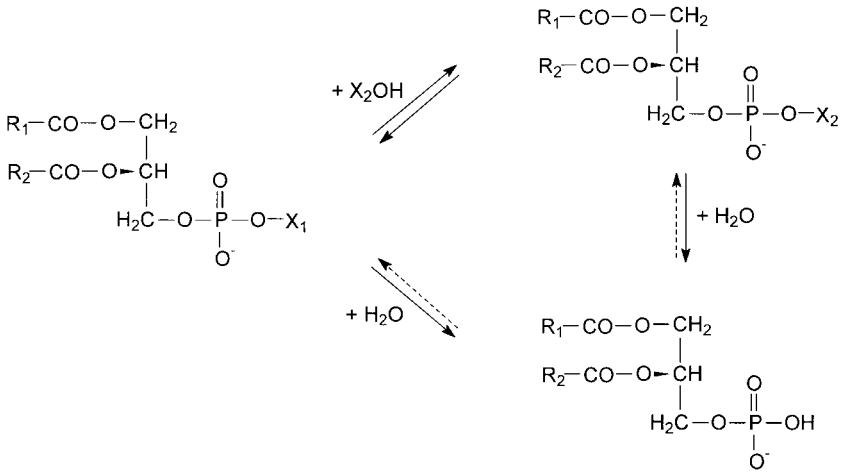

Figure 12. Hydrolysis and transphosphatidylation of glycerophospholipids by PLD. X_2OH: acceptor alcohol

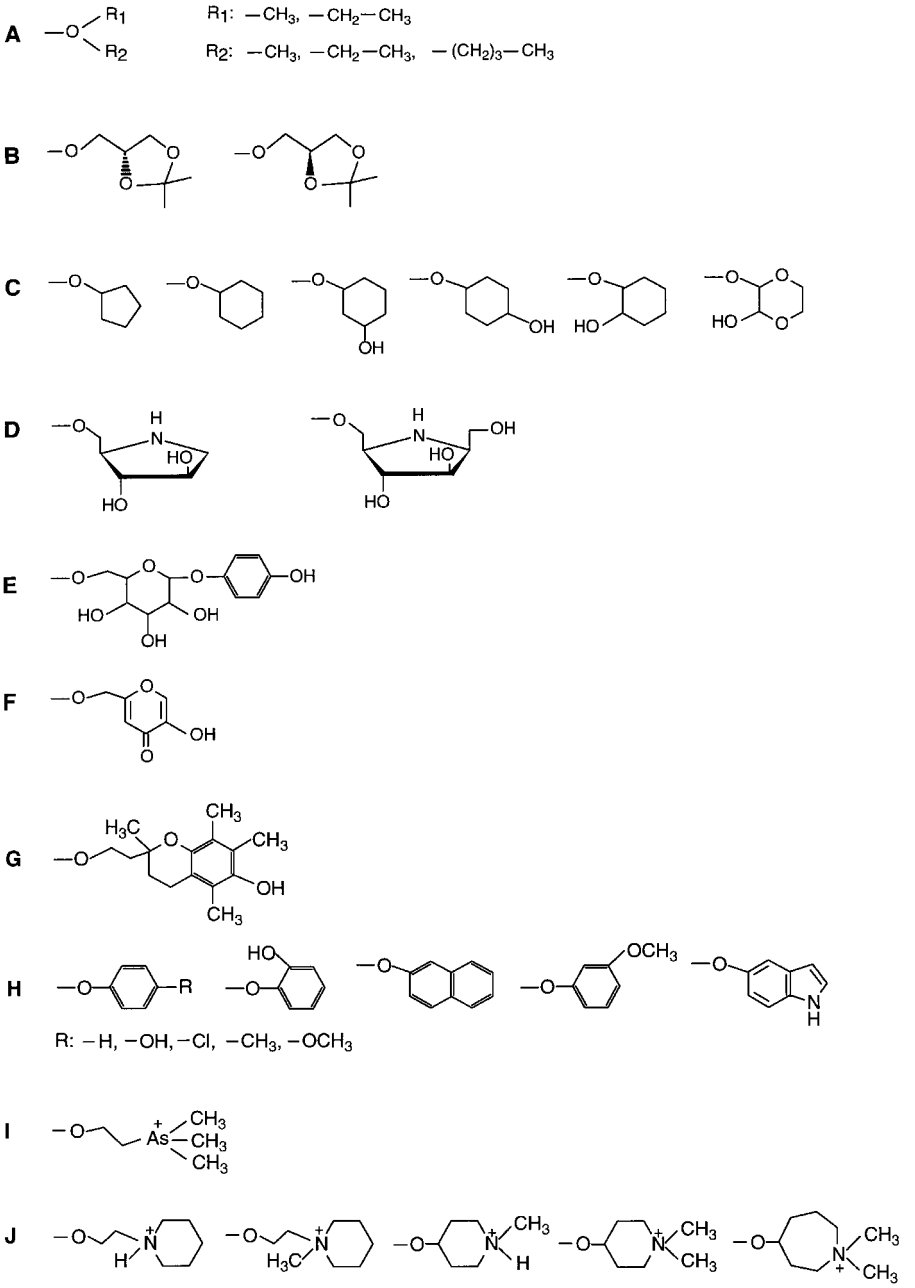

Figure 13. Head groups introduced by PLD into glycerophospholipids. (Illustrations to Table 3).

Table 4. Head groups introduced by PLD into phospholipid structures different from PC.

Educt	Head group introduced	Source of PLD	Solvent system	Reference
Plasmenylcholine	Ethanolamine	*Streptomyces chromofuscus*	Tris buffer	Wolf and Gross, 1985
^{14}C-labelled ethanolamine plasmalogen	Dimethylethanolamine	cabbage	HEPES buffer/ diethyl ether	Achterberg et al., 1986
^{14}C-labelled ethanolamine plasmalogen	Choline	cabbage	HEPES buffer/ diethyl ether	Achterberg et al., 1986
PAF-acether	Saccharides	*Streptomyces* sp.	acetate buffer/ diethyl ether	Testet-Lamant et al., 1992
1-*O*-Alkyl-2-acetyl-*sn*-glyceryl-3-phosphoryl-choline	Ethanol, ethanolamine, *N*-monomethylethanolamine, *N*,*N*-dimethylethanolamine	cabbage	acetate buffer/ diethyl ether	Satouchi et al., 1981
1,2-Dioctadecynoyl-*sn*-glycerophosphocholine	Ethanolamine, L-serine, glycerol	*Streptomyces antibioticus*, *Streptomyces* sp., cabbage, peanut	buffer/chloroform	Pisch et al., 1997
O-alkyl-glycerophosphoric ester analogs	L-serine	cabbage	acetate buffer/ diethyl ether/ chloroform	Brachwitz et al., 1990
Alkylphosphocholines	Nucleosides	*Streptomyces* sp.	acetate buffer/ chloroform	Shuto et al., 1988a
Hexadecylphosphocholine	L-serine	cabbage	acetate buffer/ diethyl ether/ chloroform	Brachwitz et al., 1989
Hexadecylphosphonocholine	L-serine	cabbage	acetate buffer/ diethyl ether/ chloroform	Brachwitz et al., 1989; Brachwitz et al., 1997
Hexa- and octadecylphosphocholine	Piperidine derivatives	cabbage, *Streptomyces chromofuscus*	acetate buffer/ hexane/1-octanol	Aurich et al., 1997

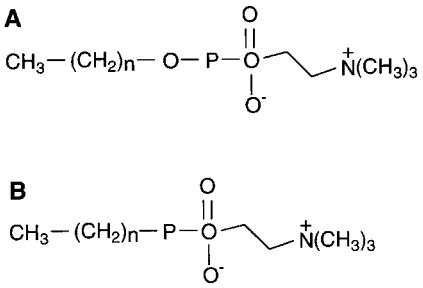

Figure 14. Chemical structures of alkylphosphocholines (A) and alkylphosphonocholines (B).

bone (Figure 14A) and – still more surprisingly – alkylphosphonocholines (Figure 14B) can be transesterified by means of PLD. These compounds possess antitumor properties and are therefore important drugs (Eibl et al., 1992; Stekar et al., 1993). The preference of certain PLDs for transesterification observed in the conversion of PC, however, cannot be applied to the reactions of alkylphosphate esters (Aurich and Ulbrich-Hofmann, unpublished results). Here, PLD from cabbage or *Streptomyces chromofuscus* was unambiguously more appropriate for transesterification than PLD from *Streptomyces* sp. (Sigma).

As seen from Table 3 and Figure 13, the spectrum of chemical structures of alcohols which can be introduced as head group is wide, and ranges from small aliphatic alcohols to multi-ring systems. In general, primary alcohols are better acceptors than secondary ones, whereas no tertiary alcohol is known to be introduced. D'Arrigo et al. (1994; 1996b) demonstrated that PLD from *Streptomyces* sp. is appropriate to introduce also secondary alcohols into phospholipids, whereas PLD from cabbage has long been considered to be incapable of accepting secondary alcohols (Kötting and Eibl, 1994). However, Hirche et al. (1997b) and Aurich et al. (1997) have demonstrated recently with *N*-heterocyclic alcohols that PLD from cabbage is also suitable to transfer secondary alcohols to phosphatidyl or alkylphosphoryl moieties. A comparison of the ratios of the initial rates of transphosphatidylation and hydrolysis for PLDs from *Streptomyces* sp. and cabbage reveals that the selectivity for primary alcohols is greater for PLD from *Streptomyces* sp. than for PLD from cabbage (Hirche and Ulbrich-Hofmann, 2000). In all cases studied so far the formation of transphosphatidylation products is kinetically controlled, because most products are again hydrolyzed as time advances (see Figure 12).

In addition to the examples given in Tables 3 and 4, some special applications have been reported. Thus, PLD from *Streptomyces chromofuscus* has been used to convert lysophospholipids to cyclic lysophosphatidic acid (Friedman et al., 1996). In the reaction sequence for the synthesis of *ara*-CDP-1,2-diacylglycerides, PLD from *Streptomyces chromofuscus* has been used for the catalysis of an intermediate step, including the removal of choline from 1,2-diacyl-*sn*-glycero-3-phosphocholine (Rebecci et al., 1993). Transphosphatidylation by PLD from *Streptomyces* sp. was also applied in the synthesis route of optically active hydroperoxides of phospholipids (Yoneda et al., 1992).

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13 Preparation and Application of Immobilized Phospholipases

Peter Grunwald

This chapter provides a short introduction into the field of phospholipids and phospholipases, followed by a brief survey on immobilization techniques, suitable carrier materials and some fundamental aspects of the kinetics of immobilized biocatalysts. The section about immobilized phospholipases deals mainly with the immobilization of phospholipase A_2 (PLA_2) and phospholipase D, but also includes some data on lipases exhibiting phospholipase A_1 activity.

13.1 Introduction

Nowadays, the immobilization of enzymes, which means transferring them from a water-soluble to a water-insoluble state, is a well-established biotechnological method, and the application of immobilized biocatalysts has gained increasing significance in fields such as medicine, pharmacy, analytical chemistry, organic synthesis, food and environmental technology. The main advantages of using immobilized enzymes are that they are re-usable – an essential prerequisite for economic production if the applied enzymes are expensive (e.g., phospholipase C and phospholipase D) – and that the products obtained are not spoiled by catalytically active protein since immobilized biocatalysts can be separated easily from the reaction mixture by filtration or centrifugation. Furthermore, they can be used in different types of reactors for continuous production, as well as in flow injection analysis (FIA) devices. Immobilization techniques also offer the opportunity of obtaining information about the biocatalyst itself. They have been employed to identify whether single subunits of an oligomeric enzyme are catalytically active, or in the study of the mechanisms of protein refolding (Chan, 1976; Mosbach 1980; Martinek and Mozhaev, 1985). Ferreira et al. (1994) immobilized the dimeric *Crotalus atrox* venom PLA_2 by single-subunit attachment to a support. From the finding that the catalysts obtained exhibited comparatively low specific activities, these workers concluded that only the dimeric form of this PLA_2 was the fully active form. Further examples for this type of application are provided in connection with the immobilization of phospholipases (see Section 13.4).

13.2 Phospholipids and phospholipases

Phospholipids – together with other polar lipids – are vital constituents of membranes in animal and vegetable organisms. All the natural representatives can be

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derived from *sn*-glycerophosphates. Fatty acid residues are attached to C_1 or C_2 or both C-atoms of the glycerol backbone that may vary considerably with respect to the degree of saturation and chain length. The individual species of the glycerophospholipids differ in the nature of the alcohol esterified to the phosphate group at C_3. In case of the amino alcohol choline, the correct name of the resulting compound is 1,2-diacyl-*sn*-glycero-3-phosphatidylcholine, normally abbreviated to phosphatidylcholine (PC). Other important phospholipids are phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylglycerol (PG), and phosphatidic acid (PA). The trivial term lecithin is the name for a mixture containing neutral lipids, polar lipids, and carbohydrates and which is used as a food additive in Europe and the USA (Schneider, 1997a).

Although phospholipids occur in most membranes of living organisms, vegetable oil seeds (soybean, rape seed, sunflower seed) are mainly used for the recovery of phospholipid mixtures, produced as byproducts from vegetable oil refining processes. Egg yolk and brain are occasional sources for phospholipids used for pharmaceutical and cosmetic preparations. The composition of the phospholipid mixtures, as well as that of the fatty acid residues attached to the glycerol backbone of the phospholipid molecules, are characteristic of the respective source.

Naturally occurring phospholipid mixtures are used directly as crude lecithins, or after processing with methods such as solvent fractionation, deoiling, and chromatographic purification. They may also be modified chemically (hydroxylation, hydrogenation, acetylation) or by using enzymes, and a multitude of possible applications results from this. Due to their amphiphilic nature, phospholipids are used as emulsifying agents and surfactants in the food industry, as ingredients in cosmetics (lyposomes) or for the formulation of pharmaceutical products (drug release). Clinical studies have produced evidence for a cholesterol-lowering potential of PC, and PS has proved to be an efficacious drug in the treatment of age-related cognitive deterioration. A comprehensive survey of the applications of phospholipids has been published by Schneider (1997b).

Glycerophospholipids are substrates for *phospholipases* which are capable of modifying them by attacking the molecules at definite points. Phospholipase A_1 (phosphatidylcholine 1-acyl-hydrolase, EC 3.1.1.32; PLA_1) and phospholipase A_2 (phosphatidylcholine 2-acyl-hydrolase; PLA_2) catalyze the hydrolysis of the carboxyl ester groups at positions *sn*-1 and *sn*-2, respectively, leading to the corresponding lyso-phospholipids. Two phospholipase C species (EC 3.1.4.3 and EC 3.1.4.10) catalyzing the cleavage of the phosphate ester bond at C_3 to form diacyl glycerols and organic phosphate compounds are known – PLC (EC 3.1.4.10) accepts phosphatidylinositol as substrate. In the presence of phospholipase D (EC 3.1.4.4), the ester bond between the phosphate residue and the terminal polar head group is hydrolyzed to form a phosphatidic acid (PA).

Depending on the source, PLA_1 and PLA_2 may also show a tendency to catalyze a transesterification reaction, though hydrolysis reactions are normally more efficient. In contrast, phospholipase D (PLD) is unique with respect to its ability to catalyze transphosphatidylation reactions. This polar head group exchange was the basis for the synthesis of many new phospholipids in the recent past (Bornscheuer and Kazlauskas, 1999). In this connection it must be taken into account that – depending on the reaction conditions and on the source of the applied PLD – this reaction competes

with the hydrolysis of the substrate molecules to PA. Furthermore, the product obtained by the transfer reaction principally is also a suitable substrate for PLD, so that the product formation is kinetically controlled. To summarize, the catalytic potential of phospholipases allows several different strategies for the synthesis of new phospholipids or phospholipid mixtures with altered composition (Table 1; Figure 1). Starting from the inexpensive crude lecithin, PLA_2 is used to manufacture lyso-phospholipids on an industrial scale. In the presence of PLD, either PA or phospholipids with modified head groups are obtained. PLA_1 and PLA_2 convert natural PC to glycerophosphorylcholine, from which PCs with defined acyl residues are prepared by chemical reacylation. Further treatment with PLD in the presence of an alcohol acceptor again considerably enhances the number of interesting and high-value compounds available, due to the wide substrate specificity of PLD. Examples have recently been published by Ulbrich-Hofman et al. (1998), Hirche et al. (1997) and Pisch et al. (1997).

The *reaction behavior* of phospholipases and their catalysis mechanisms – as understood so far – is quite complex. Depending on their concentration, phospholipids form different types of aggregates in water. One consequence is an uncertainty with regard to the full substrate specificity of phospholipases. Furthermore, the kinetics of these enzymes is markedly affected by the aggregation state. Normally, the rate of phospholipase-catalyzed reactions decreases significantly when the substrate concentration falls below the critical micellar concentration (CMC) value where the phospholipid molecules exist in the reaction mixture as monomers (Pieterson et al., 1974). A model for the binding mechanism of PLA_2 to micellar structures (with the consequence of interfacial activation) and the chemistry of catalysis was developed by Scott et al. (1991) on the basis of results from high-resolution X-ray structures of PLA_2/inhibitor complexes (Chinese cobra *Naja naja* venom and bee-venom PLA_2).

The catalytic activity of many phospholipases increases after the addition of surfactants such as sodium dodecylsulfate (SDS) or Triton X-100 to the reaction mixture, as these form mixed micelles with the substrate, though this may not be valid for all enzymes. In the case of PLA_1 for example, Triton X-100 enhances the activity towards all substrates with the exception of PE, which clearly forms mixed micelles whose physical structure has an inhibitory effect. Most phospholipases require metal ions (mainly Ca^{2+}) in order to develop activity, but again differences exist with respect to the amount and type of this cofactor, according to the enzyme source.

Table 1. Some examples for well-known, current applications of native and immobilized phospholipases.

Enzyme	Reaction	Product/properties
Phospholipase A_2	Hydrolysis	Lysophospholipids/enhanced emulsifying capacity
Phospholipase D	Hydrolysis	Phosphatidic acid/masking taste of bitter food ingredients
	Transesterification	Products such as PG, PS/enhanced emulsion capacity and temperature stability, therapeutic applications (PS)

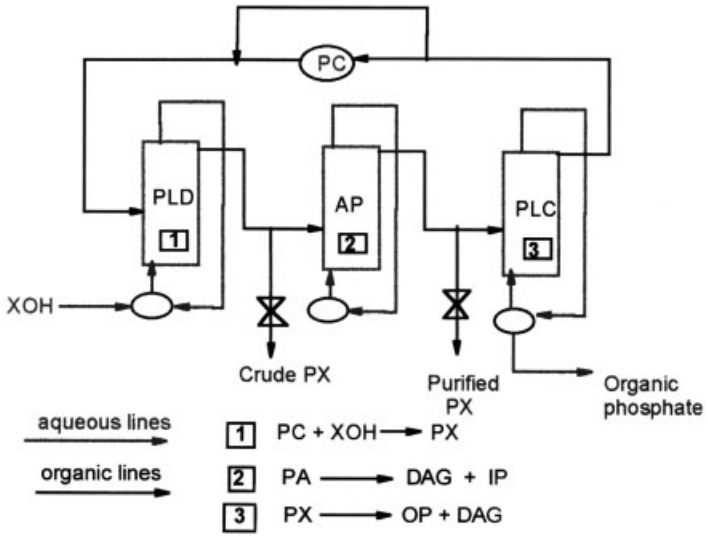


Figure 1. A scheme for the continuous production of polar head group-modified phospholipids (1), their purification by PA-removal (2), and hydrolysis to the corresponding organic phosphate (3), as proposed by Servi (1999). The three enzymes PLD, alkaline phosphatase, and PLC are immobilized in hollow-fiber hydrophobic membrane reactors.

Another important parameter is the polarity of the reaction mixture, which can be adjusted by the addition of water-soluble organic solvents. It has been shown that the presence of ethanol stimulates the activity towards the attack of PC by PLA_2 (Grunwald et al., 1995). A preincubation of PLA_2 in organic solvents leads to a change in the K_m value, and the activation energy of PLA_2, and in the case of preincubation in 1-butanol, the activity of this enzyme is markedly increased (Remus and Grunwald, 1995).

13.3 Immobilization of biocatalysts

Although immobilized biocatalysts are defined as enzymatically active materials which are transformed artificially into a catalytically active and insoluble mode, they have their counterparts in living organisms because many enzymes are associated with cellular material. *In vivo*, the kinetics of such enzymes are influenced by parameters such as the composition of the microenvironment where they act, by conformational changes due to binding, or by mass transfer. Hence they may play an important role in the complex control mechanisms within living cells, similar to that of allosteric enzymes. Accordingly, the behavior of enzymes that are immobilized by fixation to the surface of a carrier, or by entrapment within a polymer matrix, depends on the reaction conditions and the way in which immobilization is carried out. Indeed, by using a specific choice of carrier material, the apparent properties of the enzymes can be altered.

13.3.1 Immobilization techniques

The immobilization of single enzymes for one-step reactions or whole cells for more complex reactions (for example, the production of ethanol in the presence of yeast cells) can be performed in different ways:

- Fixation onto the surface of a water-insoluble carrier (adsorption, adherence, binding via ionic or metal complex interaction, covalent binding).
- Entrapment within microspheres (Chang, 1998), hollow-fiber modules, enzyme membrane reactors, etc., where the biocatalysts are still dissolved but prevented from direct contact with the bulk solution by semipermeable membranes.
- Entrapment within a gel matrix; this method is used preferentially for the immobilization of cells (Guiselly, 1989; Rehm and Omar, 1993).
- Crosslinking of enzyme molecules with bifunctional reagents to an insoluble 'macromolecule'; in this process, no carrier material is needed.

If an enzyme acts in organic medium it may be regarded as immobilized in so far as it is insoluble and thus easy to separate from the reaction mixture for re-use. In addition, several features that result from the immobilization of an enzyme to a solid support, including diffusion limitation, conformational changes, or reduced molecular flexibility (see Section 13.3.3) are also observed to a greater or lesser extent for enzymes applied in organic media (Klibanov, 1997). Therefore, and due to the fact that the presence of organic solvents is closely linked to phospholipase activity, some examples of phospholipase-catalyzed reactions in near-water-free organic solvents are included in Sections 13.4.1 and 13.4.2.

Immobilization by adsorption is normally a very mild and simple method, and the residual activity of the adsorbed biocatalyst will remain high. However, due to the

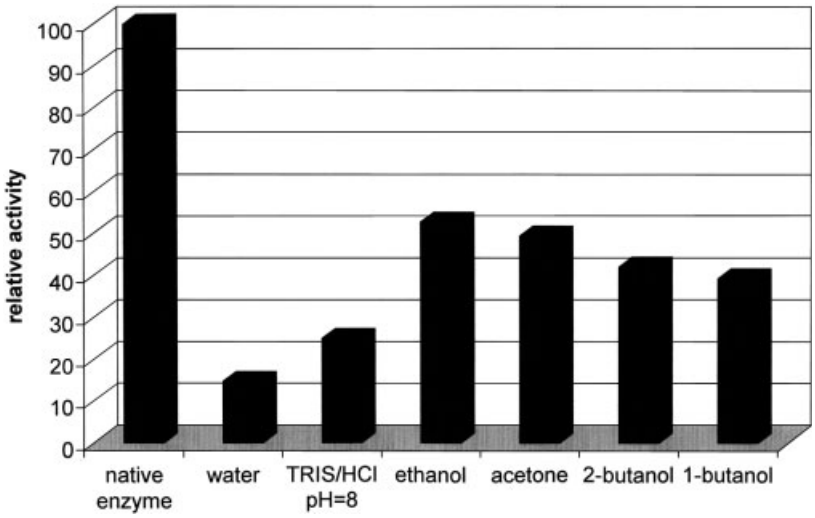


Figure 2. Immobilization of phospholipase A_2 by adsorption to Cab-osil® M-5 (Fluka) from aqueous solutions and different organic solvents.

weak interaction forces the application of enzymes immobilized in this way often suffers from a considerable protein leakage, limiting their repeated use. The loss of protein can be reduced by additional crosslinking. Exceptions to this behavior are known however, and some companies offer immobilized enzymes. For example, an immobilized triacylglycerol hydrolase (EC 3.1.1.3) which catalyzes esterification and interesterification reactions has been obtained by adsorption of the enzyme onto macroporous anion-exchange resins (Lipozyme® RM; Novo Nordisk). Some examples of the adsorptive immobilization of PLA_2 to a SiO_2-matrix from different reaction media are shown in Figure 2. No significant protein release was observed after washing with water; however, the enzyme was removed in time from the carrier during PC-hydrolysis (see Section 13.4.1).

By contrast, *covalent binding* leads to tight fixation of a protein molecule, but this is often accompanied by a heavy loss of catalytic activity. The carrier surface must bear functional groups in order to enable covalent linkage. The activity of the immobilized catalyst obtained is influenced by many experimental parameters that must be optimized; these include the concentration of functional groups, the reaction time between the enzyme and the chemically modified carrier, the reaction temperature, the pH, ionic strength, and polarity of the reaction milieu, the amount of enzyme present during the immobilization procedure, the porosity of the carrier material and its particle size (see Sections 13.3.2 and 13.3.3). In many cases the additional insertion of a spacer (the chain length of which must also be optimized) between the enzyme molecule and the carrier surface improves the catalytic activity significantly (Manecke et al., 1979).

Glutaraldehyde is a bifunctional compound that is often used to bind the enzyme to a carrier with terminal amino groups on its surface. The coupling reaction occurs predominantly via amino groups of the biocatalyst, and this can result in low activities of the bound enzyme when the corresponding amino acids are essential for the catalytic process, as has been shown for example with the immobilization of PLA_2 (see Section 13.4.1). Other bifunctional reagents applied for this purpose are carboxylic acid dichlorides or carbodiimides. As in these cases side reactions are possible, it may be advantageous to use heterobifunctional spacers with one functional group modified for enzyme binding after it has been bound to the surface. Carriers are available commercially which are activated with epoxy residues (Table 2) and allow direct coupling of the biocatalyst by the formation of highly stable C–S–, C–N– or C–O– bonds. One disadvantage of these materials is that in some cases the reaction time required to reach high enzyme loading and sufficient activity (see corresponding product information), together with a high storage stability, is very long (Tischler and Wedekind, 1999).

Immobilization may also be achieved by means of biologically active compounds. Solomon et al. (1987) prepared highly active immobilized enzymes by binding them via corresponding monoclonal antibodies to a suitable carrier. Farooqi et al. (1997) recently reported a method which uses the carbohydrate recognition site of lectins to bind glycoproteins such as glucose oxidase. Interesting biocatalysts were also obtained by co-immobilization of enzymes and cells or cell fragments, the aim being to combine different catalytic properties (Hartmeier, 1983; Hartmeier et al., 1987). A significant progress in the field of fine chemicals production in the presence of enzymes was the development of enzyme membrane reactors with simultaneous co-

factor regeneration. The cofactor molecules, e.g., NADH are prevented from penetrating the membrane by enlarging their molecular weight via attachment to polyethylene glycol (Kula and Wandrey, 1987; Wandrey, 1987). Immobilization methods for lipases and reactor configurations used for the modification of fats and oils are reviewed by Malcata et al. (1990).

13.3.2 Carrier materials

Among the *inorganic carrier materials*, SiO_2 should be mentioned especially, as this can be manufactured as spherical beads of uniform diameter and pore size (controlled pore glass) (Figure 3). The surface of these particles can be activated by reaction with organosilicon compounds such as 3-aminopropyltriethoxysilane, followed by the immobilization via glutaraldehyde or other bifunctional reagents (Janasek and Spohn, 1998). Functionalized porous SiO_2 has often been used for the covalent fixation of phospholipases (see Sections 2.4.1 and 2.4.2). Further inorganic materials applied for immobilization include metal oxides and hydroxides of Al, Zr, Ti, etc. and, as shown recently, mesoporous phases (Gimon-Kinsel, 1998).

Organic carriers must to subdivided into natural and synthetic polymers. Materials based on hydrophilic polysaccharides such as cellulose, crosslinked dextrans or agarose belong to the first group, and are modified for example with BrCN or 2-(diethylamino)-ethanol (DEAE-cellulose). Other naturally occurring polysaccharides mainly applied to the immobilization of micro-organisms include Na-alginate, which is transferred from a sol to a mechanically stable gel through the exchange of Na^+ ions by Ca^{2+} ions (Klein et al., 1983), κ-carrageenan (ter Meer, 1984), or chitosan (Ziehr et al., 1987), the primary amino groups of which can also be used for coupling reactions. Shinonaga et al. (1996) successfully applied crosslinked chitosan beads (Fuji Spinning Co.) as a porous support to immobilize

Table 2. A selection of commercially available carrier materials and their properties, used for enzyme immobilization.

Carrier	Active group/ Concentration ($\mu mol\ g^{-1}$)	Surface size ($m^2\ g^{-1}$)	Pore diameter (nm)	Particle size (μm)
Eupergit C	epoxy/>600	(–)	200[1]	150
Eupergit C250L	epoxy/>200	(–)	1000[1]	250
VA-Biosynth	epoxy/300	140	30	50–2000
Deloxan DAP III	-NH-R-NH_2/(–)	400	25 ...100[2]	400
Trisoperl	-NH_2/80–330	30–120	31–126	100–200
Fractogel (EMD)	epoxy/1500	(–)	80	40–90
	azlacton/100	(–)	80	40–90

[1] Exclusion range (kDa).
2 Maximum of pore size distribution.

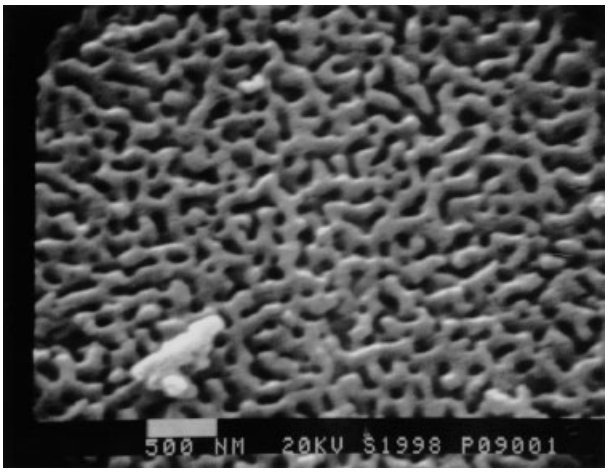


Figure 3. Scanning electron micrograph of the surface of a porous glass bead TRISOPERL (length of bar: 500 nm). The surface is modified with active groups for covalent binding of biocatalysts. (Illustration courtesy of Schuller GmbH, Germany.)

cells of *Streptomyces lydicus* for the continuous production of PLD in an air-lift reactor.

The synthesis of polymers with a wide variety of properties offers the opportunity to prepare supports adapted to the special requirements of a given biocatalyst. Polyacrylate, polyamide, polystyrene, etc. are polymers often applied for enzyme immobilization by physical or chemical binding. Co-polymers of methacrylamide with *N*,*N'*-methylene-*bis*-acrylamide and allylglycidyl ether, or those prepared from vinylacetate and divinylethylene urea, both of which are activated by epoxy groups (Table 2; Figure 4) have been successfully used for the direct covalent binding of industrially important enzymes such as penicillin G amidase (Burg et al., 1988; Kolarz et al., 1990) and have found widespread application in immobilization practices.

To summarize, a carrier should have the following properties: a hydrophilic surface if the reaction is to be carried out in aqueous solution; functional groups for

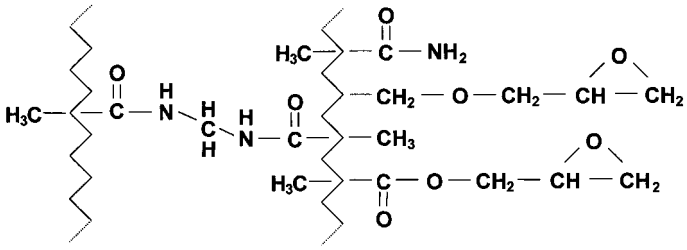


Figure 4. A scheme of the chemical structure of Eupergit (Röhm), a porous co-polymer of *N*,*N*'-methylene-*bis*-(methacrylamide), glycidyl methacrylate, allyl glycidyl ether, and methacrylamide with oxirane groups for covalent enzyme coupling.

covalent binding; a surface area of about 100 $m^2 g^{-1}$ in order to guarantee a sufficient protein capacity with a pore size of approximately 100 nm (depending on the size of the protein molecule); and mechanical and chemical stability. A selection of commercially available carrier materials, together with some of their properties is listed in Table 2. Guidelines for the characterization of immobilized biocatalysts have been elucidated by the Working Party on Immobilized Biocatalysts within The European Federation of Biotechnology, and are illustrated by an article concerning the immobilization of *Nitrosomonas europaea* in Ca-alginate (van Ginkel et al., 1983).

13.3.3 Kinetics of immobilized enzymes

The attachment of an enzyme to the surface of a water-insoluble carrier or an entrapment of the biocatalyst within a polymer matrix is often accompanied by a change of its kinetic behavior which may be classified as follows.

Conformational changes

These are often caused by a covalent binding of an enzyme to a support. As a consequence of rigid fixation, the enzyme molecules may lose their ability to undergo the necessary conformational changes during the interaction with the substrate within the region of the active site. An immobilization via amino or carboxylic groups is comparable with a chemical modification of the amino acids of the enzyme. A short distance between the enzyme molecules and the carrier – and especially an orientation of its active site towards the surface after the binding process – aggravates or even prevents access of the substrate and/or effectors to the enzyme. A high protein loading can also create steric hindrance. Amino acids in, or near to, the active site may be involved in the immobilization reaction. All these constraints may lead to changes in the kinetic parameters of the bound enzyme compared to the corresponding data of the biocatalyst in solution.

Partitioning effects

As the reaction medium and the insoluble carrier are two different phases, then depletion as well as enrichment of substrate, buffer constituents, etc. in one of the phases is possible. As a result, the immediate surrounding – the microenvironment – of the enzyme on the surface of the carrier material is different from that of the bulk solution. This means that the enzyme-specific data calculated with concentrations measured in the solution are apparent values and must (if possible) be corrected by the corresponding partitioning coefficients. Especially if the carrier is a polymeric matrix (e.g. an ion-exchange resin), this partitioning effect causes an increase or a decrease of the pH-optimum and the situation becomes more complex when the substrate and/or the products are charged. In addition to ionic partitioning, hydrophobic as well as specific interactions between a given solute and the matrix, are also observed.

Diffusion limitation

An important quality criterion of a carrier applied to enzyme immobilization is its protein capacity. Thus, porous materials are mainly used for this purpose and the kinetics of biocatalysts immobilized to such carriers by adsorption or covalent binding or by entrapment within a polymer matrix is often influenced by diffusion effects. Such effects are subdivisible into two types: the *external* and the *internal* diffusion barrier. The theoretical treatment of this type of heterogeneous catalysis profited greatly from the large amount of knowledge acquired in connection with research on catalysts for chemical process engineering. Important parameters such as the Damköhler number Φ^2, the Thiele modulus φ ($\varphi = (\Phi^2)^{0.5}$), and effectiveness factor η (Damköhler, 1937; Thiele, 1939) characterize the mass transfer in technical as well as in natural catalyst systems. The effectiveness factor is expressed by $\eta = r_i/r_0$ in which r_i and r_0 are the measured reaction rate in the presence of the immobilized biocatalyst, and the reaction rate in solution for equal enzyme concentrations and reaction conditions. The external diffusion limitation arises from the fact that a particle in solution is surrounded by the so-called unstirred Nernst layer with the thickness δ through which the substrate molecules have to penetrate by diffusion towards the particle surface. As a consequence of the resulting concentration gradient, the immobilized catalyst will not be faced with the same substrate concentration as it would be in solution under the same conditions; hence the measured reaction rates will be different. As δ depends on the relative movement of the catalyst-containing particles to that of the reaction medium, the mass transfer resistance can be reduced by enhancing the stirring rate in a continuous-stirred tank reactor (or the bulk liquid flow rate in a packed-bed reactor).

If the diffusion coefficient for the substrate molecules in solution (D_0) is higher than that within the porous support (D_{eff}), a concentration gradient is formed so that the substrate concentration decreases with increasing distance from the surface of the particle, and the enzyme-catalyzed reaction slows down. In case of strong internal diffusion limitation and a large particle diameter 2 R the substrate may no longer reach the enzyme molecules in the region of the particle center. Mathematical treatment of the substrate diffusion process (using Fick's second law) and the catalytic reaction running in parallel (under the assumption that the Michaelis – Menten mechanism is valid, and that the system is at steady state) leads to an expression containing the Damköhler number $\Phi^2 = L^2 \cdot V'_{max}/(D_{eff} \cdot K'_m)$ where V'_{max} and K'_m are the maximum reaction rate (or density of biocatalyst) and the Michaelis – Menten constant within the matrix, and L is the thickness of a membrane. In case of a spherical bead with the diameter $2R$, L is replaced by $R/3$. The Damköhler number is representing the relation of a characteristic reaction rate V'_{max}/K'_m to a characteristic diffusion rate D_{eff}/L^{-2}. The larger Φ^2 (or φ) the steeper the substrate concentration decreases within the catalyst bead. The equation also contains information of how to diminish the mass transfer resistance and thus also of how to increase η. Figure 5a and b show to what extent the activity of an immobilized enzyme depends on the particle size and on the protein loading (Grunwald, 2000). Apart from the particle size and biocatalyst loading test an existing diffusion limitation can be recognized by an enhanced K_m value and a diminished activation energy E_A due to the fact that diffusion-controlled processes merely reveal activation energies between 5 and

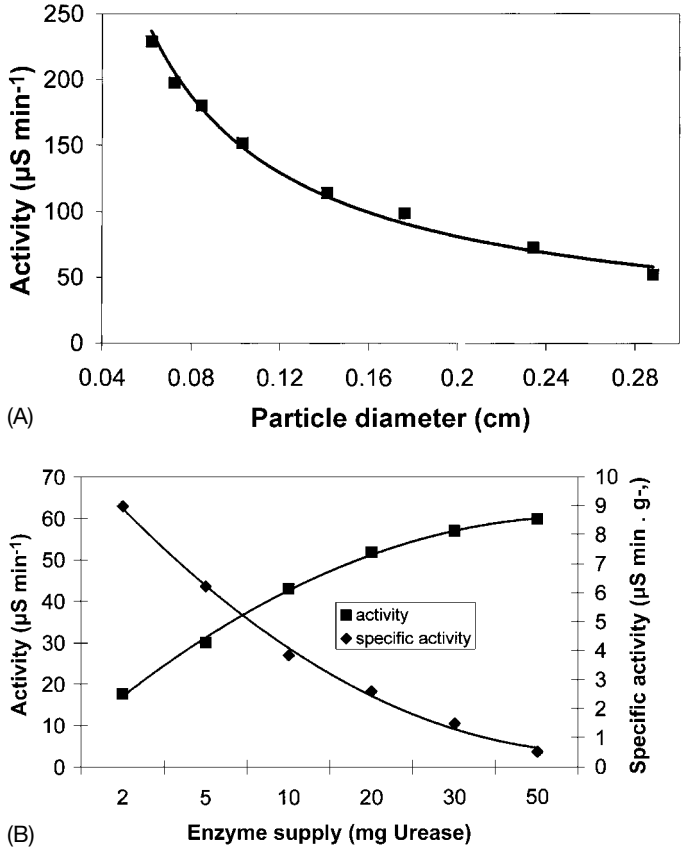


Figure 5. (A) The activity of urease immobilized by entrapment within spherical Ca-alginate beads versus particle diameter. The activity is inversely proportional to the particle size. (B) The activity of urease entrapped within Ca-alginate increases with increasing amount of enzyme supply. However, due to diffusion limitation the specific activity decreases markedly.

10 kJ mol^{-1} (Buchholz and Rüth, 1976; Lasch, 1979). More detailed reports on this theme have been published by Bisswanger (1994), Lasch (1979) and Korus and O'Driscoll (1974), and with many practical examples by Buchholz and Kasche (1997).

13.4 Immobilization of phospholipases

Compared to corresponding publications on lipases, the number of reports dealing with the immobilization of phospholipases is small. Research has mainly been carried out based on aspects of the application of immobilized phospholipases for analytical purposes. Other contributions attend to the possibility of the immobilization

Table 3. Activities of soluble and immobilized PLA_2. (Data from Lombardo and Dennis, 1986).

Substrate	Triton X-100	Specific activities (µmol/(min · g))		
		soluble PLA_2	immobilized PLA_2	(%)
Dipalmitoyl-PC (5 mM)	20 mM	1120	19	1.70
Dipalmitoyl-PE (5 mM)	20 mM	13	5.3	40.8
Diheptanoyl-PC (0.8 mM)	–	86	77	87.5
Diheptanoyl-PC (3.2 mM)	–	3400	280	8.24
Diheptanoyl-PE (0.2 mM)	–	81	25	30.9
Diheptanoyl-PE (0.2 mM)	2 mM	71	25	32.5

of phospholipases as such. Here, the influence of experimental parameters (described in Sections 13.3.1 – 13.3.3) on the properties of the carrier-bound enzymes and their optimization with respect to high residual catalytic activity are investigated. From the different immobilization methods described in Section 13.3.1, it was clear that adsorption and covalent attachment were mainly used, and that the carrier materials most frequently applied were porous glass and synthetic organic polymers. Until now, immobilized phospholipases have not been used in industrial processes, as for example the conversion of lecithin to lysolecithin in the presence of PLA_2, nor in connection with the phospholipase-catalyzed synthesis of new phospholipids on a preparative scale. By far the most investigations in this field have been carried out with the immobilization of PLA_2.

13.4.1 Immobilization of phospholipase A_2

Fundamental research on this topic has been carried out by Dennis and co-workers. In an early publication, they described the immobilization of a cobra venom PLA_2 on porous glass beads by diazo coupling (Adamich et al., 1978). The products obtained had residual activities of just 1 %. A few years later, Lombardo and Dennis (1985; 1986) reported on studies carried out with PLA_2 from the same source (Indian cobra; *Naja naja naja*), but attached covalently to an agarose gel via tresyl chloride. The protein binding yield was up to 87 %. For test reactions, substrates such as dipalmitoyl-PC, diheptanoyl-PC and the corresponding PE-derivatives were used. A selection of data from these publications is listed in Table 3; these show that the activity of immobilized PLA_2 towards dipalmitoyl-PC/Triton X-100 micelles again is just 1 – 2 % of the specific activity of the soluble (native) PLA_2. The exchange of dipalmitoyl-PC by dipalmitoyl-PE leads to significantly lower activities, but a reduced activity difference between the immobilized and the native species. Transition from the monomeric to the micellar state of diheptanoyl-PC causes a 40-fold increase in activity when the reaction is catalyzed by soluble PLA_2. In the presence of immobilized PLA_2, the reaction rate is merely enhanced by a factor of 3.5. Triton X-100 does not

affect the activity of either form of the enzyme in case of low diheptanoyl-PE concentrations. The data obtained with diheptanoyl-PC suggest that the behavior of immobilized and soluble PLA_2 against monomeric phospholipids is similar; however, the activity on micellar substrate differs by more than one order of magnitude. This tremendous increase in PLA_2 activity towards substrate molecules organized into lipid – water interfaces was shown previously by Verhey et al. (1981). An analysis of the kinetic data for substrate concentration above the CMC-value revealed that the apparent K_m values for native and immobilized PLA_2 were similar, whereas V_{max} for the soluble PLA_2 was about 10-fold higher.

The poor residual activities found for immobilized PLA_2 (between 1 and 2 %) raises the question of whether this is due to a mass transfer resistance produced by the carrier matrix, to partitioning effects, or to the applied coupling method itself (see Section 13.3.3). Ferreira et al. (1993) were the first to repeat the experiments of Lombardo and Dennis. However, in contrast to the latter, they covalently bound a cobra venom enzyme *Naja naja kaouthia* PLA_2 to *N*-hydroxysuccinimide-activated agarose (Biorad) with enzyme fixation primarily through ε-amino groups and to diaminodipropylamine-derivatized agarose (Pierce) by use of a carbodiimide where enzyme binding occurs through carboxylic residues. The results of Lombardo and Dennis could be confirmed in so far as the binding of PLA_2 via succinimide was accompanied by a strong activity loss. Both succinimide and tresyl chloride use the ε-amino groups of lysine residues to attach the enzyme to the carrier surface. By contrast, with the carbodiimide method PLA_2-preparations with an activity retention of 50 % were obtained, clearly indicating that the chemistry of enzyme binding is crucial in this case. A pH shift of the microenvironment could be excluded as the activity – pH profiles for both types of PLA_2 were nearly identical. A very important finding was that the carbodiimide-bound PLA_2 exhibited interfacial recognition in the presence of short-chain phospholipid micelles, as is characteristic for the soluble form. From this it can be deduced further that a substrate-induced aggregation of PLA_2 molecules to dimers – or higher-order aggregates – obviously is not linked to the interfacial activation observed at concentrations around the CMC-value. A corresponding hypothesis discussed (controversially) for a long time was formed on the basis of chemical modification of PLA_2 (Roberts et al., 1977) and other experiments (van den Bosch, 1982). These results also showed that such experiments may contribute to the enlightenment of theoretical questions such as the mechanism of enzymatic catalysis.

It can be demonstrated simply by adsorption experiments that the activity of covalently bound PLA_2 is mainly effected by the type of chemistry used. Adsorption of PLA_2 (porcine pancreas; Lecitase, from Novo) to a highly dispersive silicon oxide (Cab-osil M-5, Fluka) with a surface size of 200 m^2g^{-1} yielded catalytically active products (see Figure 2). The residual activities of PLA_2 adsorbed from an aqueous or from a Tris/HCl buffer solution (pH 9) were 15 % and 26 %, respectively. Lowering the polarity of the reaction milieu by addition of water-miscible organic solvents such as ethanol led to an increase in the activity of adsorbed PLA_2 to above 50 % (unpublished results). However, these preparations were not suited for continuous use because of the weak binding forces between carrier surface and enzyme. The fact that the temperature behavior of adsorbed PLA_2 is the same as found for the soluble enzyme with respect to the activation energy and the temperature optimum is

an indication for the adsorption of PLA_2 in its native state, and for the absence of diffusion limitation.

The activities of native and immobilized PLA_2 as a function of temperature are compared in Figure 6 (Remus and Grunwald, 1995). In this case, the enzyme was bound chemically to VA-Epoxy Biosynth (Riedel-de Haën), a polymeric carrier the surface of which is preactivated by epoxy groups (see Table 2), which means that the enzyme should be bound predominantly by ε-amino residues of PLA_2. The results also showed that this type of binding is unfavorable. The activity retention was 4 % at room temperature, but reached the activity of soluble PLA_2 for 25 °C at 70 °C due to a markedly enhanced temperature stability of the biocatalyst caused by its attachment to the carrier material which partly compensated for the strong activity loss. The high activation energy measured for the PC-hydrolysis in presence of PLA_2 immobilized via the epoxy-groups of the carrier proved that diffusion limitation could not be the reason for the low activities. Furthermore, the pH-profiles for both, the activity of the native as well as that of the immobilized PLA_2 were similar, which showed that partitioning effects could be neglected (see Section 13.3.3).

The amino acids that may be involved principally in the binding of PLA_2 from porcine pancreas via functional groups or spacers such as epoxy, glutaraldehyde, are four arginine, nine lysine, and three histidine residues, and the *N*-terminal alanine. Some of these amino acids are important for substrate binding (Lys62, Lys56), either as part of the active site (Arg43, His48) or for the interfacial recognition site. Avoiding enzyme binding through these amino acid residues is most likely the reason for the successful immobilization experiments of Ferreira et al. (1993), though the amino acid sequence of the cobra venom PLA_2 is not identical with that of porcine pancreatic PLA_2. Our results for the immobilization of this enzyme (porcine pancreatic PLA_2) are shown in Figure 7A and B. We used macroporous organofunctional polysiloxanes of the Deloxan-type (Degussa-Hüls), with a specific surface

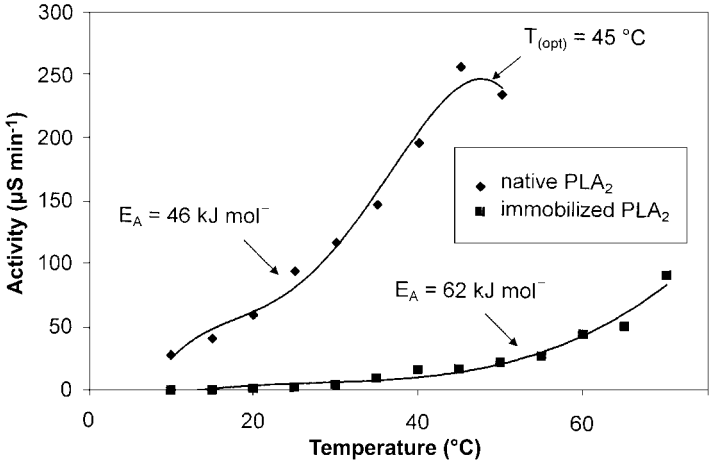
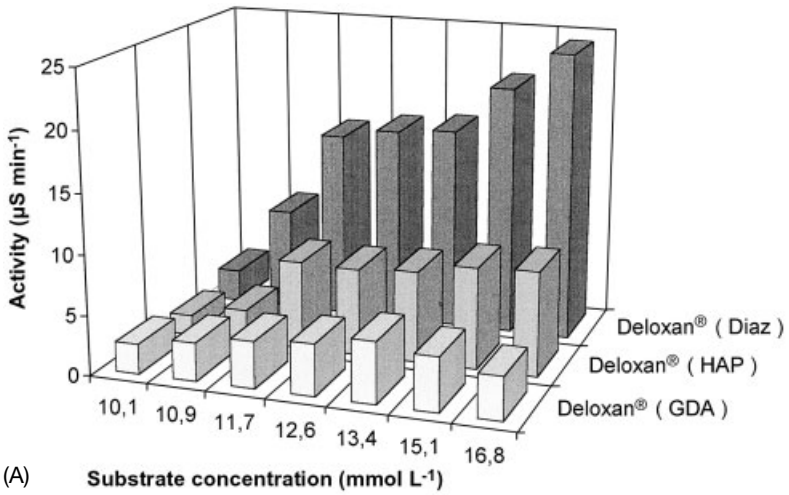


Figure 6. The temperature course of the activity of native (◆) PLA_2 and PLA_2 covalently attached to VA-Epoxy Biosynth (Riedel-de Haën) via an epoxy spacer (■). The immobilized enzyme exhibits a markedly increased temperature stability.

area of about 400 $m^2 g^{-1}$ bearing ethylenediamine groups on their surface (Deloxan DAP III; Table 2) suitable for operating in aqueous as well as in organic media. The standard immobilization procedure is enzyme fixation via glutaraldehyde, or by a carbodiimide. We chose the glutaraldehyde method, and also immobilization by azo coupling. For the latter, the ethylenediamine residues of the polysiloxane support were reacted with *p*-nitrobenzoylchloride, forming a peptide bond. After the reduction of the $–NO_2–$ group to an $–NH_2–$ group, followed by diazotation, the PLA_2 was bound to the Deloxan surface by a strong azo linkage. Furthermore,



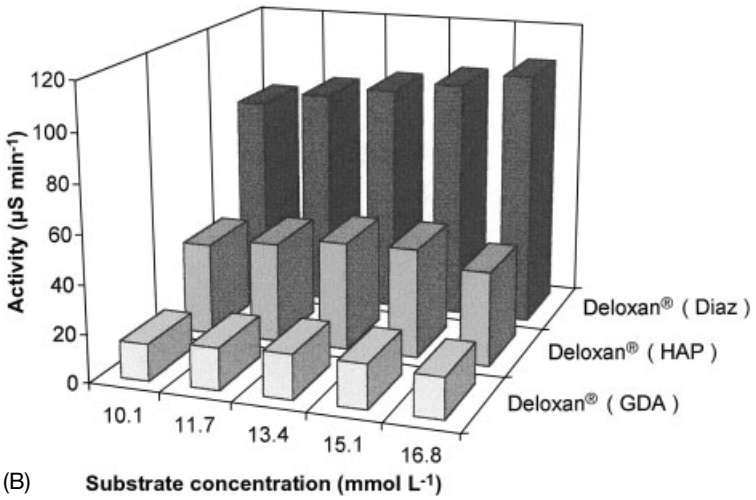


Figure 7. (A) The activity of PLA_2 immobilized to Deloxan carriers (Degussa) by adsorption (HAP) and by covalent binding (GDA, Diaz) as a function of the substrate concentration. The reaction medium contained 20 % (v/v) ethanol. (B) If ethanol in the reaction mixture is exchanged for the same amount of ethyl acetate, the activity increases by more than a factor of 4.

the adsorption of PLA_2 from aqueous solutions to a polysiloxane carrier with a hydrophobic surface (Deloxan HAP) was examined. The hydrolysis of PC was performed in the presence of SDS (Texapon K12; Henkel) in a solution containing 4 mM $CaCl_2$, 20 vol% of an organic solvent, and Tris/HCl to adjusting the pH to 9. The data in Figure 7A reconfirm that the immobilization of PLA_2 by including ε-amino residues in this process when GDA is taken as a spacer, leads to low residual activities. The activity values increase for preparations obtained by adsorption, and are the highest for PLA_2 bound covalently via the azo linkage. The sensitivity of the hydrolysis rate to the design of the reaction medium becomes obvious from the results shown in Figure 7B. As a consequence of the exchange of ethanol by ethyl acetate, the catalytic activities are raised by a factor of 4 to 5, independently of the type of binding. The PLA_2-Deloxan catalysts revealed residual activities of above 70 % in the case of ethanol/water, and more than 85 % when the PC-hydrolysis was carried out in the ethyl acetate/water mixture, compared to the native enzyme under corresponding reaction conditions. PLA_2 immobilized by diazotation can be stored at 4 °C for several months without considerable loss of activity. The high operational stability of these products has been proven by repeated use in a column reactor, so that in principle these biocatalysts could be applied for the continuous production of lysolecithins, or in a FIA device for the determination of PC concentrations (Grunwald, unpublished results). As reported by Maderoy et al. (1995), PLA_2 from bee venom could be also successfully immobilized through adsorption to the weakly acid cation-exchange resin carboxymethyl Sephadex (CM-Sephadex, Serva). The activity retention was above 80 % and the operational stability – obviously due to additional electrostatic interactions between the enzyme and the surface of the support – was demonstrated by eight successive applications without activity loss. The activity proved to be strongly dependent on the amount of enzyme bound. Indeed, it decreased from 115 μmol $(mg \cdot min)^{-1}$ for 15 mg fixed enzyme per g carrier to 10 μmol $(mg \cdot min)^{-1}$ when 45 mg g^{-1} were attached to the support. This was the result of increasing steric hindrance and/or diffusion limitation with increasing enzyme loading. Compared to the soluble enzyme, the pH/activity profile was considerably broadened. Similar results were obtained by Lambrecht and Ulbrich-Hofmann (1993) for PLD immobilized to octyl-Sepharose.

Shen and Cho (1995) found that the acylation of Lys7 and Lys10 of *Agkistrodon piscivorus piscivorus* PLA_2 as well as of *Naja naja naja* PLA_2 prior to their covalent immobilization to beaded carbonyldiimidazol-activated crosslinked agarose (Pierce) yielded high activities compared to those of nonacylated PLA_2 towards defined substrates such as large unilamellar vesicles of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine. This holds true also for the hydrolysis of phospholipids on the surface of low-density lipoproteins, a point which is of interest in connection with the application of immobilized PLA_2 in an extracorporeal shunt for the treatment of hypercholesterolemia (Labeque et al., 1993). A detailed report of this procedure, including the synthesis of 4-nitro-3-octanoyloxybenzoic acid, the Lys7 and Lys10 acylation of PLA_2, and its immobilization was published recently (Cho and Shen, 1999). These findings again corroborate the importance of the chemistry of enzyme binding for the resulting catalytic activity, and also indicate the importance of these lysine residues for the catalytic action of the two venom PLA_2s.

A special type of application of immobilization techniques is that of enzyme purification by affinity chromatography. Bernal and Pidgeon (1996) used so-called immobilized artificial membranes (IAMs) for the enrichment of PLA_2 from *Crotalus atrox*. IAMs (Qiu et al., 1994) are prepared by immobilizing an ω-carboxyphosphatidylcholine ligand on silica propylamine surfaces. The ligand density corresponds approximately to that of a monolayer. Remaining amino groups are end-capped with decanoic and propionic anhydride. An interesting aspect of this investigation is that IAMs containing the glycerol backbone, as well as those lacking it, were used. It transpired that these differences were not important for the purification process, which suggests that for the interaction of the PLA_2-surface with the 'membrane' the polar head group of the substrate is the decisive factor. This example demonstrates once more that immobilization can be a useful tool to enlighten theoretical questions – in this case the mechanism of enzyme/substrate interaction. Moreover, it was possible – starting from a crude venom protein mixture with 4.5 % PLA_2 – to enrich the enzyme content up to 70 % with this technique.

Mustranta et al. (1994) investigated the *transesterificatio*n of synthetic dimyristoyl-PC with oleic acid catalyzed by commercially available lipase preparations from *Aspergillus niger* (immobilized by adsorption on diatomaceous earth) and *Rhizomucor miehei* (bound to an anion-exchange resin (Lypozyme; Novo) and its dependence on different reaction conditions. The enzymes exhibited lipase and phospholipase activity, both being considerably higher in the *Aspergillus niger* lipase. Nevertheless, the *Rhizomucor miehei* enzyme was more active in transesterification and 35 % of modified PC was the maximum yield. The reactions were carried out in solvent-free medium (oleic acid) and in toluene. The optimum water content related to the obtained amount of oleyl myristoyl-PC was 0.5 % and 1 % in case of *Aspergillus niger* and *Rhizomucor miehei* in toluene, and 1 % and 2.5 %, respectively in the solvent-free reaction mixture. On the basis of the experience that transesterification occurs mainly at the 1-position of phospholipids, and that no dioleyl-PC was found, it can be assumed that the production of 1-oleyl 2-myristoyl-PC was catalyzed by lipase. A further example of research in the field of transesterification of phospholipids with lipase preparations was published by Hara and Nakashima (1996), who used eight different acetone-dried *Rhizopus* species. The cells were immobilized on biomass support particles (BSP, HR-40, Bridgestone) where they formed a dense film. Compared with the immobilization of extracellular lipase, this procedure is advantageous as the cultivation of the lipase producer and its adhesion onto the BSP-surface occurs simultaneously (Nakashima et al., 1988). With the incorporation of heptanoic acid into PC it could be shown that most of the immobilized *Rhizopus* species exceeded the transesterification potential of commercially available immobilized lipases (Lipozyme IM20 and IM60 from Novo). BSP of polyurethane foam have also proven to be useful carriers for the immobilization of *Streptoverticillium cinnamoneum*, with the aim of producing extracellular PLD (Fukuda et al., 1998). The work of Haas et al. (1995) also dealt with the investigation of lipases concerning their ability to hydrolyze phospholipids apart from triglycerides. With respect to this, commercially available, immobilized lipases were tested. For example, the data obtained with Lipozyme IM20 showed that triglycerides in water-saturated hexane as solvent were hydrolyzed completely within 20 h at

37 °C, whereas the degree of PE- and PC- hydrolysis was about 50 % after 60 h under the same conditions.

Since the early 1980s many reports have been published about the catalytic activity of hydrolytic enzymes in (nearly) water-free organic solvents of low polarity. Pernas et al. (1990) showed that extracellular PLA_2 (from *Naja naja* venom, bee venom, porcine and bovine pancreas and *Streptomyces violaceoruber*) is capable of catalyzing the esterification of lyso-PC with [^{3}H]-oleic acid. They made use of the findings of Zaks and Klibanov (1988) that enzymes incubated in a buffer solution at a pH value necessary for optimum activity (in this case a Tris/HCl-buffer of pH 9 containing 10 mMol $CaCl_2$) prior to lyophilization exhibit the so-called pH-memory effect. The synthesis was carried out in solvents such as benzene, toluene and chloroform, which did not affect the activity of the PLA_2. The highest degree of transesterification was 6.5 % obtained with *Naja naja* PLA_2. Adding methanol to the reaction mixture causes a suppression of the PC synthesis, which gives rise to the assumption (stated earlier by others) that the small amount of water left after lyophilization is essential for the catalytic effect, and that this water is removed from the microenvironment of the enzyme in the presence of small amounts of polar solvents. Lin et al. (1993) also investigated the behavior of PLA_2 in apolar organic solvents such as chloroform with low water content. With regard to the hydrolysis of 1,2 dimyristoyl-*sn*-glycerophosphocholine (DMPC), they found that bee-venom PLA_2 showed the highest activity followed by *Naja naja* and *Naja mocambique* venom PLA_2, whereas pancreatic PLA_2 yielded only poor activities. A small amount of methanol added to chloroform with 1.7 % water increased the rate of the bee venom enzyme-catalyzed hydrolysis by a factor of 4, in contrast to the normally observed effect of this solvent on phospolipase activity. This is possibly due to the fact that removal of residual water from the microenvironment of the enzyme takes time, and that the positive influence of methanol on the solubility of the substrate is initially predominant. Similar findings were reported by Campanella et al. (1998a) in connection with the use of immobilized PLD for analytical purposes (see Section 13.4.2). The course of hydrolysis rate as a function of temperature had a minimum at $T = 45$ °C due to conformational changes of DMPC in 2-position, as proved by NMR spectroscopy. Furthermore, the authors described a novel route for a venom PLA_2-catalyzed transacylation of *L*-lyso-PC with long-chain acyl donors. The best results (37 % yield) were obtained with palmitic anhydride in chloroform-water (100/1, v/v) at 37 °C. Härröd and Elfman (1995) developed a high-pressure reaction unit for the synthesis of new phospholipids in isooctane, carbon dioxide and propane as solvents. The products were obtained by the reaction of lyso-PC with polyunsaturated fatty acids (PUFA) of fish oil origin (mainly consisting of eicosapentaenoic and docosahexaenoic acids) and PLA_2 from porcine pancreatic glands immobilized to the polysiloxane matrix Deloxan as catalyst. Optimum results with a yield of 25 % were achieved in a solvent mixture of 91 % PUFA and 9 % propane.

13.4.2 Immobilization of phospholipase D

Possible applications of immobilized PLD are the synthesis of new phospholipids via transphosphatidylation, the analytical area, and the processing of crude phospholipid mixtures by enrichment of their PA content (see Table 1), which improves the emulsifying properties and the temperature stability. However, as yet the use of enzymatically modified food additives in Europe is restricted to hydrolyzed products due to the European food regulations (E 322).

The first extensive study of transphosphatidylation reactions catalyzed by immobilized PLD from white cabbage was published by Tobback et al. (1988). They investigated many different carrier materials with respect to their suitability for the attachment of PLD by maintaining the enzymatic activity, including Celite, alumina oxide, active carbon (adsorptive binding of PLD), octyl-Sepharose, porous glass (covalent binding of PLD), DEAE-Sephadex (ionic binding of PLD), Ca^{2+}-alginate, and polyvinylalcohol (entrapment of PLD). The quality of the biocatalysts obtained was tested by their ability to convert dioleoyl-PC into dioleoyl-PE. The best results were obtained with PLD bound to porous glass and to DEAE-Sephadex in reaction mixtures containing diethylether or *n*-propylacetate.

Reuter (1997) immobilized PLD from white cabbage on SiO_2 (Cab-osil M5) to test the usability of the products obtained for the enrichment of PA in phospholipids and phospholipid mixtures. For this purpose, the SiO_2 carrier was first derivatized with 3-aminopropyltriethoxysilane or with 3-glycidoxypropyltrimethoxysilane. PLD was either bound directly or by additional bifunctional spacers such as glutaraldehyde, dimethyladipimidate dihydrochloride (DMAD), decanoic acid dichloride (DAD) or by an azo linkage, respectively. The standard reaction solution used

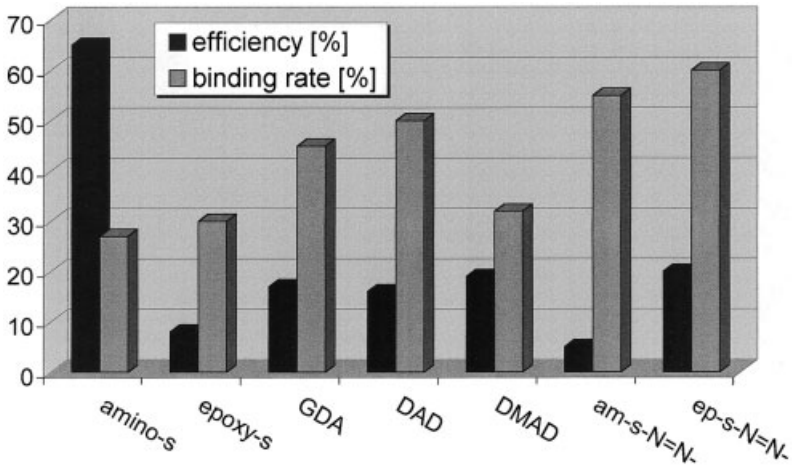


Figure 8. Residual activity and binding rate for phospholipase D immobilized to SiO_2 (Cab-osil® M-5) via different (bifunctional) spacers: 3 -- aminopropyltriethoxysilane (amino-s), (3-glycidoxypropyl)trimethoxysilane (epoxy-s), amino-s and glutaraldehyde (GDA), amino-s and decanoic acid dichloride (DAD), amino-s and dimethyladipimidate dihydrochloride (DMAD), amino-s and epoxy-s containing an additional azo linkage (am-s-N = N-, ep-s-N = N-).

for the determination of PLD-activity contained 0.625 mM PC, 15 mM SDS, 0.3 M sodium acetate buffer (pH 5.1), and 40 mM $CaCl_2$. A comparison of some of the different biocatalysts with respect to their residual activity and protein binding yield is shown in Figure 8. The highest activities were retained when PLD was attached to SiO_2 functionalized with 3-aminopropyltriethoxy silane. Although the type of binding had to be an ionic or adsorptive one the release of PLD was not observed. The other synthesis variants exhibit only low activities under these reaction conditions; however, those catalysts prepared with long-chain spacers such as DMAD and DAD showed residual activities above 100 % with increasing substrate concentrations. An explanation for this finding cannot yet be given, apart from the fact that it seems reasonable to suppose that binding to the carrier surface via a longer-chain favors flexibility of the enzyme structure as well as accessibility of its active site for the micelles. This is also confirmed in immobilization experiments performed by Reuter (1997) with the Fractogel EMD Azlacton carrier (Merck KGaA), which led to highly satisfactory results concerning the residual activity of PLD. Fractogels (Table 2) are synthetic, methacrylate-based, beaded polymers. Long, linear, polymer chains – so-called 'tentacles' – are bound covalently to the matrix, bearing functional ligands for an attachment of biomolecules with minimized steric hindrance. Furthermore, immobilized PLD with high efficacy resulted from covalent binding to carriers such as Deloxan DAP III (with GDA as spacer), and from adsorptive fixation to the hydrophobic surface of Deloxan HAP. However, it must be mentioned that the two quality criteria, 'storage stability' and 'operational stability' were not fulfilled sufficiently, which is probably due to the well-known poor stability of PLD from white cabbage. For example, the catalysts obtained with the tentacle polymer or with functionalized SiO_2, where PLD was bound via decanoic acid dichloride could be used repeatedly on five occasions without activity loss. Thereafter, the activity decreased continuously. Nevertheless, it should be possible to employ these immobilization techniques and carrier materials for the synthesis of highly active PLD preparations with long-term stability using more stable PLDs from microbial sources that are currently available.

A special type of PLD-immobilization was presented by Okahata et al. (1995), who prepared lipid-coated PLD by mixing an aqueous PLD-solution with dialkyl amphiphiles dissolved in acetone (Figure 9). The precipitate obtained, which had a protein content of about 7 % after lyophilization, was used for the transphosphatidylation of egg PC with, for example, *n*-butanol. The reactions were performed in biphasic systems consisting of benzene and an acetate buffer. Because of its lipid-coating, the enzyme – together with the substrate – was soluble in the organic phase, whereas the choline moiety was released into the aqueous phase. The ratio of reaction rates of lipid-coated PLD from *Streptomyces* sp. and native PLD was found to be about 300. Among the lipids used for PLD-coating, anionic lipid molecules turned out to be most suitable with respect to the residual activity. As the actual reaction is carried out in a homogeneous phase, its kinetics can be described by Michaelis – Menten mechanisms over the entire concentration range of the two substrates PC and the acceptor alcohols. From an analysis of the kinetic data the authors concluded that PC is bound first to the enzyme before this intermediate reacts with the nucleophile. Furthermore, it could be shown that the substrate specificity was not affected by lipid coating. The general applicability of lipid-coated PLD for the synthesis of

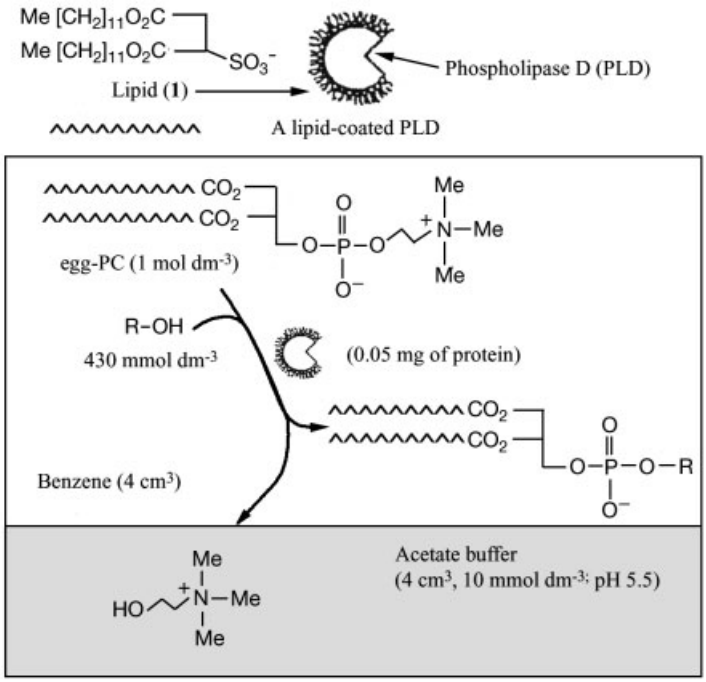


Figure 9. A schematic illustration of a lipid-coated PLD and transphosphatidylation of egg PC with alcohol in benzene solution in the presence of aqueous buffer (Okahata et al., 1995).

new phospholipids was demonstrated by preparing corresponding compounds where the choline residue was exchanged by glucose (68 %), galactose (75 %), deoxyadenosine (75 %), thymine (70 %), etc. on the gram scale.

Lambrecht and Ulbrich-Hofmann (1992) used the fact that PLD is bound preferentially by adsorption to carriers with a hydrophobic surface, and that the binding efficiency is markedly increased in presence of Ca^{2+} ions for the purification of PLD from white cabbage leaves. The authors compared these findings with the Ca^{2+} requirement of the enzyme necessary to develop catalytic activity towards phospholipid micelles. From the solution of a crude protein mixture containing 50 mM $CaCl_2$, PLD was adsorbed onto octyl-Sepharose CL-4B (Pharmacia) and could be eluted selectively through the removal of Ca^{2+} ions with EDTA. The yield of PLD was 85–90 %, and the purification factor 103. These results were used by the same authors for an adsorptive immobilization of PLD from white cabbage to different carrier materials with long-chain anchor groups (octadecyl, octyl, etc.) mediated by calcium ions. The catalytic activity of the adsorbed PLD turned out to be dependent on both the Ca^{2+} concentration during the binding process and the nature of the carrier. To obtain maximum activity, 10 mM $CaCl_2$ was required in the case of octadecyl-Si 40 Rp 18 (Serva), but 40 mM $CaCl_2$ for octyl-Sepharose CL-4B. Other findings of this investigation were that the storage stability increased markedly (PLD adsorbed to octyl-Sepharose lost 30 % activity within 14 days when stored at 8 °C in an acetate buffer, pH 7, whereas the native enzyme was completely inactive after 4

days' storage under the same conditions), and a considerable broadening of the pH-optimum compared to the soluble PLD resulting in near-equal activities in pH range between 5 and 7 (Lambrecht and Ulbrich-Hofmann, 1993).

A similar stabilization effect on PLD by attaching the enzyme to a solid support was observed by Takami and Suzuki (1995) when they bound PLD from *Streptomyces* sp. to different cation-exchange resins. In contrast to strong acid types with sulfonic acid exchange groups mainly catalyzing the hydrolysis to PA, weak acid types such as Amberlite IRC-50 proved to be excellent supports with respect to the activity towards the transphosphatidylation of 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine to 4-methoxyphenol in non-polar solvents. The yield of 1,2-dipalmitoyl-3-*sn*-phosphatidyl-4-methoxyphenol (DPP-PMP) was 45 % after 20 h and the amount of the corresponding PA below 2 %. The immobilization was performed by simply adding 4 units of PLD in 10 µl of a 0.2 M acetate buffer (pH 5.6) to a stirred suspension of 50 mg Amberlite IRC-50 in 1 mL benzene. After repeated stirring, and sonication until the benzene phase became clear, the immobilized biocatalyst could be used immediately after removal of the solvent. As the resin absorbs water up to 50 % of its own weight, all the enzyme, together with the aqueous buffer solution, can be assumed to be entrapped within the porous matrix of the carrier. Appropriate solvents for the synthesis of DPP-PMP were benzene, toluene, and methylene chloride. In the presence of water-soluble organic solvents, no catalytic activity with respect to DPP-PMP formation was observed because of the reasons already discussed. Under comparable conditions (0.1 % acetate buffer, 1 mL benzene) native PLD was inactive. An increase in the amount of buffer of up to 5 % of the reaction volume enhanced the production rate of DPP-PMP.

With respect to an economical large-scale production of PG and PS, the use of immobilized PLD is of special interest. Earlier investigations into this topic with PLD attached to octyl-Sepharose CL-4B revealed biocatalysts with a low operational stability (Juneja et al., 1987; 1988). Recently, Wang and co-workers (1997) tested different carrier materials (and immobilization procedures) for the immobilization of PLD from *Pseudomonas* sp., such as Amberlite XAD-2, controlled pore glass (CPG), polyethyleneimine (PEI)-cellulose, and calcium alginate-enveloped PEI-glutaraldehyde. Surprisingly, the latter component turned out to be the most suitable for the synthesis of PG by head group exchange of refined soybean lecithin (40 % PC, 31.2 % PE, 17.6 % PI, and 10.1 % PA). The optimum composition of the support was obtained with 1.39 % calcium alginate, 7.78 % PEI, and 1.22 % glutaraldehyde. The optimum reaction parameters with respect to the yield of PG (which was maximally 85 %) were a reaction temperature of 25 °C to 30 °C, a diethyl ether to water ratio of between 1.5 and 2.5, and a pH of 8.2, which is astonishing in so far as normally Ca-alginate itself is not stable under alkaline conditions. As far as the operational stability is concerned, PLD immobilized to the Ca-alginate/PEI/glutaraldehyde system could be used for 15 repeated batches without significant loss of activity, after which the degree of conversion declined significantly to about 10 % after 40 repeated batch operations. Similar observations concerning the long-term stability of the biocatalyst were made in an earlier investigation by Lee et al. (1985) who used PLD from cabbage in a microporous membrane reactor for the continuous production of PG.

Because of the increasing interest in using PLDs as catalysts for the synthesis of new phospholipids (D'Arrigo and Servi, 1997; Servi, 1999), or in connection with the biological importance of PLD, several methods for the determination of PLD activity with different detection methods have been published in the recent past. These are based on head group release by hydrolysis of [^{3}H]-methylcholine labeled PC, on transphosphatidylation with [^{3}H]-acyl-PC or [^{32}P]-PC, and on the change in the amount of charged particles during the course of reaction (conductivity measurements) (for a review of their advantages and drawbacks, see Morris et al., 1997). Becker et al. (1997) developed a flow injection analysis (FIA) where the choline produced in presence of PC and PLD is oxidized by choline oxidase (EC 1.1.3.17). The simultaneously released hydrogen peroxide is monitored by chemiluminescence detection (see below). The viability of this method has been demonstrated by the characterization of PLDs from three different microbial sources with respect to their kinetic data. The detection limit for choline is given with 75 pmol min^{-1}.

As already pointed out for PLA_2 the activity of PLD also depends strongly on parameters such as co-factor concentration or the ratio of surfactants (SDS, Triton X) to substrate concentration – in other words, the physical and chemical properties of the interface. Furthermore, it should be considered that many PLD-catalyzed syntheses are carried out in organic solvents which had been taken into account by Aurich et al. (1999), who presented a simple, sensitive, and exact method for the determination of PLD activity in emulsion systems with transphosphatidylation of PC in presence of 1-butanol and PC dissolved in dichloromethane, and sodium acetate buffer containing the PLD (*Streptomyces* sp.). For the detection of products arising from both the hydrolysis, as well as the transphosphatidylation reaction, HPTLC proved to be a most suitable method.

The majority of the well-known analytical methods can be applied for the determination of PLD activity, and also for the analysis of phospholipid concentrations. However, a major disadvantage of all these procedures is wasting of the often very expensive enzymes applied. This problem can be overcome by using the involved biocatalysts in immobilized state, as described by Masoom et al. (1990). Controlled pore glass (Fluka) derivatized with 3-aminopropyl triethoxysilane was used for the covalent binding of enzymes through glutaraldehyde as spacer. The authors established two different flow injection systems. The first utilized a column containing immobilized PLC that was connected with a column filled with alkaline phosphatase (EC 3.1.3.1) and choline oxidase co-immobilized on the SiO_2 support. The second approach made use of immobilized PLD and choline oxidase in two different columns. The amount of H_2O_2 developed through the action of choline oxidase was determined amperometrically. The detection limits for PC in phospholipid mixtures were 1 nmol and 10 nmol, respectively and the time required for one quantitation including calibration with standard PC-solutions is 15 min.

Yaqoob et al. (1997) developed a flow injection procedure for the determination of glycerol-3-phosphate (GP) and glycerolphosphorylcholine (GPC). A reliable method for measuring GPC concentrations is important in connection with clinical investigations involving epididymal secretion. In their study, the authors reported a flow injection analysis with chemiluminescence detection. Based on the following scheme:

$$\text{glycerophosphorylcholine} + H_2O \xrightarrow{PLD} \text{glycerol-3-phosphate} + \text{choline} \quad (1)$$

$$\text{glycerol-3-phosphate} \xrightarrow{\text{G-3-PO}} \text{dihydroxyacetonephosphate} + H_2O_2 \quad (2)$$

$$2\ H_2O_2 + \text{luminol} + H_2O_2 + OH^- \xrightarrow{Co^{2+}} h\nu + \text{3-aminophthalate} + N_2 + 3\ H_2O \quad (3)$$

they used FIA-equipment with two mini-columns containing immobilized PLD and immobilized glycerol-3-phosphate oxidase (G-3-PO). The H_2O_2 produced [see Equation (2)] reacts in a subsequent step with luminol at pH >7, and in the presence of Co^{2+} as catalyst under the production of chemiluminescence. The enzymes were bound covalently to CPG (Sigma Chemicals Co.) functionalized with 3-aminopropyltriethoxysilane via glutaraldehyde. The amount of enzyme bound was > 90 %, and the mini-columns could be used for 300 h during a 3-month period with intermediate storage at 4 °C and without deterioration of the enzymatic activity. Optimum reaction conditions with respect to a maximum chemiluminescence yield were a luminol and a Co^{2+} concentration of 10^{-5} M, a pH of 6 (maximum formation of H_2O_2) and a flow rate of 0.7 mL min^{-1}. The detection limits for GP and GPC are $5 \cdot 10^{-7}$ M and 10^{-6} M, respectively, and the sample throughput 40 per hour for each analyte.

The development of a bi-enzymatic organic phase electrode by Campanella et al. (1998a) is based on the poor solubility of lecithin and lecithin-containing products, as it functions in organic solvents and allows the determination of phospholipids directly after solubilization. Again, the two enzymes PLD (*Streptomyces chromofuscus*) and choline oxidase were used in this biosensor immobilized to κ-carrageenan. This polysaccharide forms coils at higher temperatures. By cooling, the coils are transferred to helices that aggregate to a gel (thermo-reversible gelation). Therefore, the preparation of the immobilized enzymes is as follows: A solution of 0.2 g of κ-carrageenan in 10 mL of water is first heated slightly and then poured onto a Petri dish. After cooling and drying, a small disk is cut from the gel, and 25 μL of the enzyme solution (100 U of PLD and 500 U of choline oxidase) is applied to its surface. A second disk is then pressed on the top of the first, in order to obtain the enzyme membrane; this is then used in a gas diffusion electrode with amperometric detection (Clark electrode). The best results with respect to both the solubility of the substrate and the response and lifetime of the biosensor where found when a water-saturated mixture of chloroform/hexane (50 % v/v) was applied. A further increase in solubility of dietetic or pharmaceutical products containing phospholipids, as well as in sensitivity, was observed in the presence of 1 % methanol. However, in this case the life time of the electrode was reduced from 11 days to 3 days. The range of linearity was 2.1 mg l^{-1} to 42.4 mg l^{-1} (in the absence of methanol) and 1.1 mg l^{-1} to 66.1 mg l^{-1}). The detection limits were 1.05 mg l^{-1} and 0.55 mg l^{-1}, respectively. In another paper, Campanella et al. (1998b) compared this analytical device with a similar technique where the two enzymes were simply sandwiched between the gas-permeable membrane and the dialysis membrane after hydration with a small amount of 0.1 M glycine buffer (pH 8.5). Both methods were used to determine lecithin concentrations in several food samples. The electrode with the membrane-entrapped enzymes had a response time of 10 min, which was twice

that found for the electrode working with the biocatalysts bound by adsorption to κ-carrageenan. The linearity ranges were 34 mg l^{-1} to 169 mg l^{-1} and 2.1 mg l^{-1} to 42.4 mg l^{-1}, and the minimum detection limits 16.5 mg l^{-1} and 1.05 mg l^{-1}, respectively.

13.5 Summary and outlook

Phospholipases have a vast potential in terms of their application. In the field of foodstuffs, PLA_2 is of great importance as a biocatalyst for the modification of lecithin mixtures on an industrial scale into the corresponding lyso-compounds by hydrolysis of the acyl chain in *sn*-2 position. Furthermore, *sn*-2-lyso-PC can be used as a starting material in esterification reactions for the synthesis of phospholipids with a new acyl chain composition. PLA_1, though not yet available commercially, may be substituted by a number of lipases, again opening up new routes of synthesis. Interesting and actual examples are the lipase-catalyzed incorporation of PUFAs into naturally occurring phospholipids in the presence of fish oil as substrate for the manufacture of diets, or the use of immobilized PLA_2 in the treatment of hypercholesterolemia. Among the known phospholipases, PLD is the most versatile with respect to substrate specificity and ability to catalyze transphosphatidylation reactions. Apart from its application in the large-scale production of valuable, albeit rarely occurring phospholipids such as PG and PS, a multitude of new natural and unnatural lipids can be prepared with PLD (both in combination with other phospholipases and with lipases which show phospholipase activity) by the PLD-catalyzed polar head group exchange, with possible applications in the fields of food additives, pharmaceuticals, and cosmetics.

Although the application of phospholipases in their immobilized state offers many advantages, at present these enzymes are employed mainly in solution. As the problems concerning the immobilization of phospholipases – and especially of PLA_2 – have been overcome, and in the knowledge that stable phospholipases (for example PLDs) of bacterial and other origin have been found in the recent past, it should be expected that the preparation and application of immobilized phospholipases will be the subject of much greater attention in the future. Servi (1999) proposed the production of organic phosphate and diacylglycerol using PLD, alkaline phosphatase and PLC immobilized in hollow-fiber membrane bioreactors (see Figure 1). Several similar arrangements of different phospholipases immobilized in such reactors or bound to suitable solid supports and used in fixed-bed reactors are conceivable for the continuous synthesis of defined phospholipids. In addition, with the modular construction of such a small plant it should be possible to meet the requirements for the production of a particular phospholipid simply by exchanging one or more phospholipase-containing units.

The current situation in this field may be characterized by the fact that problems concerning the immobilization of phospholipases, which itself results in highly active catalysts, can be regarded as being solved. In contrast, with very few exceptions immobilized phospholipases exhibit low long-term stabilities, and this is especially true for phospholipase D. In the future, a major point of emphasis will be placed on the synthesis of immobilized phospholipases as being suitable for repeated use over a

long period of time. In this way, they can be used long-term in either the economic production of phospholipids, or for analytical purposes in FIA devices.

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14 Enzymatic Conversions of Glycerophospholipids

Patrick Adlercreutz

14.1 Introduction

Glycerophospholipids, which are present in almost all living organisms, constitute the main components of many biological membranes. These membranes are selectively permeable barriers around cells, and around organelles inside cells. However, glycerophospholipids also have many other biological functions, for example as part of cell signaling cascades.

Glycerophospholipids are useful for many applications, mainly due to their surface activity. For example, they are used as emulsifiers in food and cosmetics. Furthermore, glycerophospholipids constitute a key constituent of liposomes which are used in cosmetics and as drug carriers. A general formula of glycerophospholipids, together with the enzymes which can hydrolyze the different ester bonds in the molecules, is shown in Figure 1. As will be shown later in the chapter, most of those enzymes can be used to make ester bonds in the same positions.

14.2 Synthetic strategies

When glycerophospholipids are isolated from biological materials, the crude extracts normally contain a complex mixture of molecular species containing different fatty acids and different polar groups. Separation according to polar group can often be carried out rather easily, and this is sufficient for many applications. However, the separation of molecules differing in their fatty acid content is much more difficult

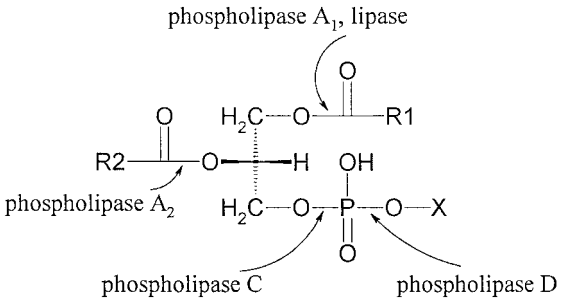

Figure 1. Glycerophospholipid molecule and the enzymes which can be used to break or form the different ester bonds. R1 and R2 are alkyl groups of fatty acids, and X depends on the class of glycerophospholipid; in phosphatidylcholine X = $(CH_2)_2N^+$ $(CH_3)_3$.

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due to the large number of possible combinations and the small differences in the properties of the different molecular species. When completely homogeneous preparations (identical molecules) of glycerophospholipids are needed, synthesis is a more convenient route than is isolation from biological material. The synthesis can be started either from simple building blocks, such as glycerol, fatty acids, etc., or from a mixture of natural glycerophospholipids. In the latter case, the part of the molecule which is identical with the target substance is retained (for example the glycerol backbone with the chiral center), while the other parts are exchanged. Another important type of lipid modification is the introduction of fatty acids which are not present in the natural lipid. There is for example considerable interest in the incorporation of polyunsaturated fatty acids from fish oils and other sources into glycerophospholipids.

This chapter will concentrate on the enzymatic methods for glycerophospholipid synthesis. In addition, a number of chemical methods are available, but these are beyond the scope of this text (for a review, see Paltauf and Hermetter, 1994). In some cases, it is beneficial to use a combination of chemical and enzymatic steps.

When enzymes are used to exchange fatty acids in glycerophospholipids, the exchange reaction can be carried out either as a two-step process, which is hydrolysis

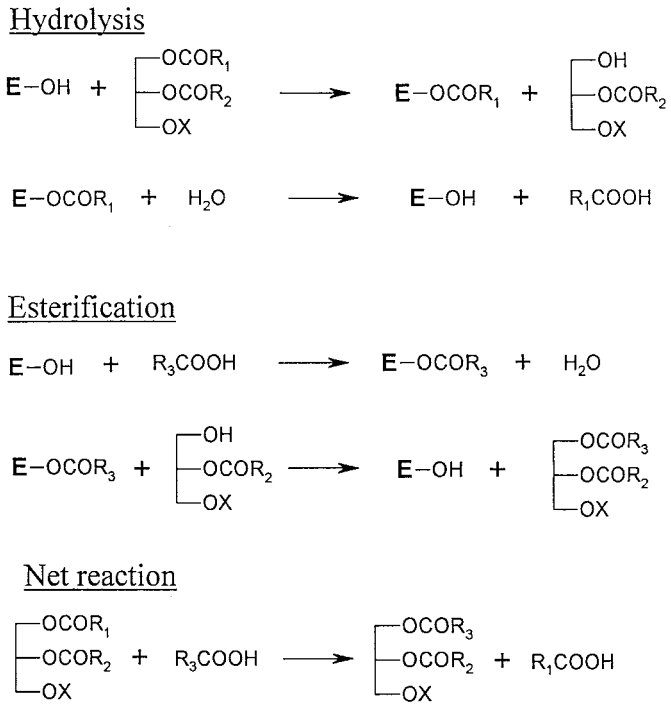

Figure 2. Two-step fatty acid exchange. The example shows the exchange of the fatty acid in the *sn*-1 position of glycerophospholipids using hydrolysis followed by re-esterification with a new fatty acid. Both steps are catalyzed by a lipase. Normally, there is a separation step between the two biocatalytic steps. E-OH is the lipase with the hydroxyl group of the active site serine.

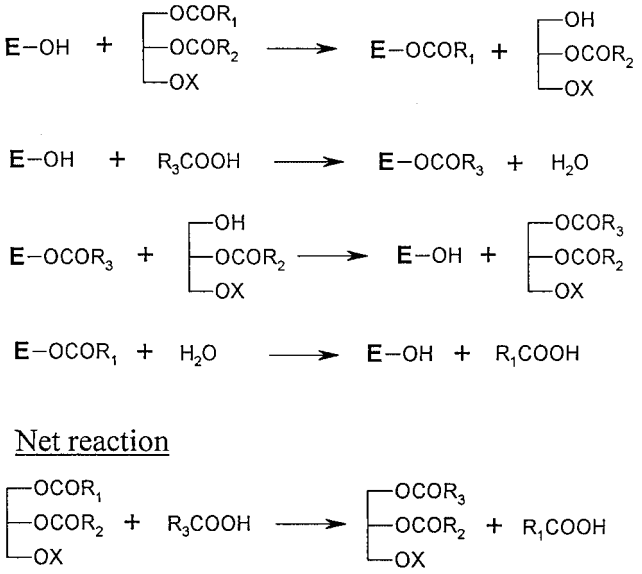

Figure 3. One-step fatty acid exchange. The example shows the exchange of the fatty acid in the *sn*-1 position of glycerophospholipids using an acidolysis reaction. E-OH is the lipase with the hydroxyl group of the active site serine.

followed by esterification (Figure 2), or as a one-step process of transesterification (Figure 3). In fact, the two approaches involve the same steps in the reaction sequence and provide the same net reaction (Figures 2 and 3). Division of the exchange reaction into two steps makes the process more laborious to perform. However, the moiety hydrolyzed off in the first step can be effectively removed in a separation step and thus does not compete with the moiety to be incorporated; hence, purer products can be obtained.

It should be noted that in the one-step approach, acyl donors other than free acids can also be used, for example esters.

Before going into detail about different synthetic possibilities, we will discuss spontaneous acyl migration in lipids. It is important to be aware of this reaction as it is involved in most of the synthetic applications discussed later in the chapter.

14.3 Acyl migration

One of the main advantages of enzymes as catalysts is their selectivity. The enzymes shown in Figure 1 are selective to act in the positions of the molecule indicated by the arrows. In order fully to take advantage of this selectivity it is important that exchange reactions between the different positions do not occur in an uncontrolled manner.

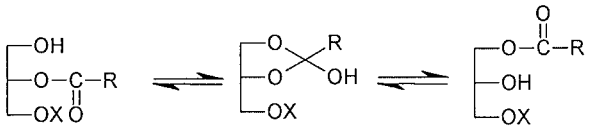

Figure 4. Acyl migration in lyso-glycerophospholipids.

When the fatty acid exchange reactions involve intermediates with free hydroxyl groups adjacent to acyl groups, there is a risk for acyl migration in these molecules (Figure 4). Figures 2 and 3 show that this can be expected both in the two-step and one-step approaches. In most cases, the acyl migration reaction destroys the selectivity of the process and leads to unwanted side products. However, in some cases one can make use of the acyl migration to simplify the reaction schemes (see below). In any case, it is important to be aware of the risk of acyl migration and in most cases it should be suppressed as much as possible.

Acyl migration can be catalyzed by several substances which are present in the reaction mixtures. Migration is catalyzed by acids and bases, but supports used for enzyme immobilization, salts and possibly even charged groups on the enzyme surface may also catalyze the reaction (Millqvist Fureby et al., 1996). In order to minimize acyl migration, potential catalysts should be eliminated as far as possible and the amount of enzyme increased to shorten the reaction time and hence the available time for acyl migration to occur. Problems may arise if substances present in the enzyme preparation catalyze acyl migration; in such cases, the enzyme loading in the preparation should be increased rather than the amount of preparation, and modifications attempted of the composition of the preparation.

On occasion, acyl migration has been inhibited successfully using borates (Morimoto et al., 1993). Reaction products in which acyl migration can be expected should be stored at low temperature.

14.4 Hydrolysis and alcoholysis reactions for the removal of fatty acids from lipids

Good methods exist for the removal of fatty acids from glycerophospholipid molecules (Figure 5). If both fatty acids are to be removed, chemical hydrolysis using tetrabutylammonium hydroxide as catalyst works very well (Brockerhoff and Yurkowski, 1965). An enzymatic method to remove both fatty acids of phosphatidylcholine has been reported (Inada, 1996) in which *Candida rugosa* lipase solubilized in organic solvents by covalent coupling to polyethylene glycol was used as catalyst in water-saturated benzene. Using this approach, the yield of glycerophosphorylcholine was 99 %.

The selective removal of one of the fatty acids is difficult to accomplish with chemical methods, and the use of enzymes provides a better alternative. Hydrolytic removal of the fatty acid in the *sn*-1 position may be carried out with a phospholipase A_1, though for most applications lipases are used. It has been shown that many li-

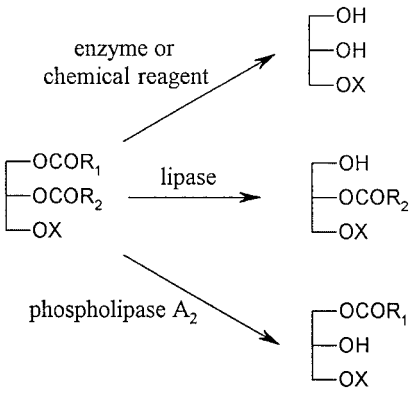

Figure 5. Non-selective or regioselective removal of fatty acids from glycerophospholipids. The reactions used can be either hydrolysis or alcoholysis reactions.

pases which are selective for the 1- and 3-positions in triglycerides are selective for the *sn*-1 position of glycerophospholipids. As several cheap (mostly of microbial origin), easy-to-handle lipases are available, they constitute a practical synthetic tool. The hydrolysis of phosphatidylcholine can be carried out in aqueous solution, the reaction rate being improved by the addition of a surfactant such as Triton X-100 (Morimoto et al., 1993) so that mixed micelles are formed. In order to obtain the 2-acyl lysophosphatidylcholine in a pure form it was necessary to use a boric acid-borax buffer to suppress acyl migration.

The hydrolysis of glycerophospholipids can also be carried out in organic solvents. Immobilized *Rhizomucor miehei* lipase (Lipozyme) was used to catalyze the hydrolysis of phosphatidylcholine in different organic solvents (Haas et al., 1993), the best results being obtained in relatively polar solvents such as butanone.

Quantitative removal of the fatty acid in the *sn*-1 position has been reported using lipases from *Rhizomucor* or *Rhizopus* in a microemulsion (Hara et al., 1997). To prepare the microemulsion, Aerosol OT (the sodium salt of dioctylsulfosuccinate) was used as surfactant and isooctane as organic phase. Microemulsions using the same components were used as the reaction medium to remove the fatty acid in the *sn*-2 position of phosphatidylcholine using porcine pancreatic phospholipase A_2 (Morgado et al., 1995). Almost 100 % conversion was achieved in 10 min. It should be noted that during hydrolysis in microemulsions, phosphatidylcholine acts both as a substrate and as a surfactant active in the formation of the microemulsion. As the reaction proceeds, phosphatidylcholine is converted to lysophosphatidylcholine, and so the behavior of the microemulsion may change, especially if high substrate concentrations are used.

The phospholipase A_2-catalyzed hydrolysis of phospholipids is used in industrial processes. For example, in the processing of vegetable oils it is important to remove the phospholipids from the oil. As it is easier to remove lysophospholipids than fully acylated phospholipids, it is worthwhile carrying out a phospholipase A_2-catalyzed hydrolysis (Buchold, 1993), the lysophospholipids being extracted into an aqueous phase after the reaction has been completed.

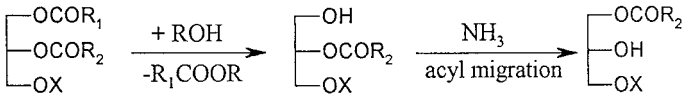

Figure 6. Lipase-catalyzed alcoholysis of phosphatidylcholine. The lipase produces the 2-acyl-lysophosphatidylcholine. In order to obtain the other regioisomer, 1-acyl-lysophosphatidylcholine, acyl migration was induced by ammonia vapor. (From Sarney et al., 1994.)

In the above-mentioned examples, the regioselective removal of fatty acids was achieved using hydrolysis reactions, but alcoholysis reactions may be used for the same purpose. For example, in the synthesis of lysophospholipids, alcoholysis in pure alcohol (ethanol or other short-chain alcohols) was carried out using *Rhizomucor miehei* lipase as catalyst (Sarney et al., 1994). The reaction was regioselective for the *sn*-1 position, and a conversion of more than 98 % was achieved. In order to prepare the other regioisomer, 1-acyl-lysophosphatidylcholine, acyl migration was induced by ammonia vapors (Sarney et al., 1994) (Figure 6).

14.5 Esterification reactions for the incorporation of fatty acids into lipids

14.5.1 Lipase-catalyzed esterification

Esterification of glycerophosphate

Glycerophosphate constitutes a basic building block for glycerophospholipids. For the synthesis of the natural isomers, pure *sn*-3-glycerophosphate should be used as substrate unless the enzymatic reactions are stereoselective enough to involve only the natural enantiomer. When an enantiomerically pure product is not needed, racemic glycerophosphate may be used as starting material.

Glycerophosphate is a highly polar compound, and the reaction to couple it enzymatically to hydrophobic fatty acids is not straightforward. The reaction was first reported using Lipozyme in a solvent-free system with free fatty acid as acyl donor. The monoacylation product, lysophosphatidic acid (LPA), was obtained in moderate yield (32 %) and small amounts of the diacylation product, phosphatidic acid, were also formed (Han and Rhee, 1995). The yield of LPA (containing caproic acid) was increased to 45 % when the water activity of the solvent-free reaction mixture was maintained at 0.18 by the introduction of a suitable salt hydrate pair in the headspace of the reactor (Han and Rhee, 1998).

The use of fatty acid vinyl esters as acyl donors has proven quite favorable in the acylation of glycerophosphate using *Rhizopus arrhizus* lipase as catalyst (Virto et al., 1999). Vinyl esters are frequently used in transesterification reactions to make them virtually irreversible. The vinyl alcohol formed as reaction product spontaneously tautomerizes to acetaldehyde, thereby shifting the equilibrium position; hence it is possible to achieve very high conversions. Vinyl laurate which was used for acyla-

tion of glycerophosphate, is a liquid at room temperature and can thus function both as substrate and solvent in the reaction. The best results were obtained without any additional solvent. Vinyl esters are activated substrates, and their hydrolysis is an important side reaction. In the case of glycerophosphate acylation, the water content in the reactors was low at the start of the reaction and, due to hydrolysis of the vinyl ester, was decreased to very low values. It has been shown previously that lipases, in contrast to most other types of enzymes, can demonstrate activity at very low water activity (Valivety et al., 1992). Even under these very dry conditions, the lipase can continue to function, and because of the low water activity the equilibrium is in favor of the acylated products.

The *Rhizopus arrhizus* lipase is specific for the 1-position in glycerophospholipids; thus the first acylation product was 1-acyl-*rac*-glycero-3-phosphate (lysophosphatidic acid, Figure 7) (Virto et al., 1999). This would have been the sole product if acyl migration had not occurred. However, due to acyl migration the initial product isomerized to 2-acyl-*rac*-glycero-3-phosphate. Only relatively low amounts of this product are expected to be formed since lipids with the acyl group on a primary hydroxyl of the glycerol backbone are usually thermodynamically more favored. However, 2-acyl-*rac*-glycero-3-phosphate is a substrate of the lipase and can be acylated to form 1,2-diacyl-*rac*-glycero-3-phosphate (phosphatidic acid; Figure 7). Depending on the reaction conditions, the main product was either lysophosphatidic acid or phosphatidic acid. Reactions were carried out in atmospheres with fixed water activity in order to control the water activity in the reaction mixtures. Such control was ineffective during the period when hydrolysis of the vinyl ester occurred at a high rate. During this period, water activity control was too slow, and water activity in the reaction mixture was very low. However, towards the end of the reactions, water activity control influenced the product distribution. A low water activity favored a relatively high amount of phosphatidic acid (due to relatively fast acyl migration and equilibrium position in favor of acylated products). A practical

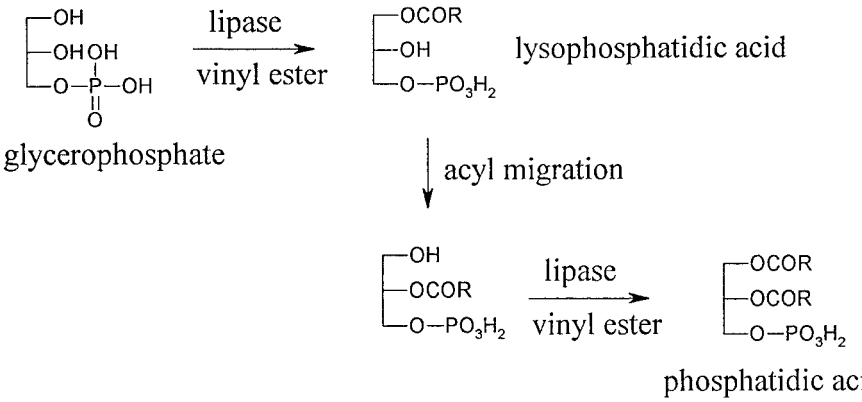

Figure 7. Acylation of D,L-glycero-3-phosphate with vinyl esters in a reaction catalyzed by *Rhizopus arrhizus* lipase. Due to limited acyl migration in the product of the first reaction step (lysophosphatidic acid, 1-acyl-*rac*-glycero-3-phosphate), a second acylation reaction could occur yielding phosphatidic acid. (From Virto et al., 1999.)

Table 1. Lipase-catalyzed acylation of glycerophosphate and glycerophosphorylcholine.

Enzyme	Solvent	Acyl donor	Product[1]	Yield[2]	Comments	Reference
Rhizomucor miehei lipase[3]	No	Fatty acid	LPA	32 % (isolated)		Han and Rhee, 1995
Rhizomucor miehei lipase[3]	No	Fatty acid	LPA	45 %	Water activity control	Han and Rhee, 1998
Rhizopus arrhizus lipase on polypropylene	No	Vinyl esters	LPA	> 90 %	Product precipitation	Virto et al, 1999
Rhizomucor miehei lipase[3]	CH_2Cl_2	Acid anhydride	LPC	71 % (isolated)		Mazur et al., 1991
Rhizomucor miehei lipase[3]	No	Fatty acid	LPC	36 %	Water activity control	Han and Rhee, 1998
Candida antarctica lipase B[4]	*t*-BuOH	Vinyl esters	LPC	> 95 %	Product precipitation	Virto and Adlercreutz, 2000a

[1] LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine.
[2] Analytical yields unless otherwise stated.
[3] Lipozyme, commercially available immobilized preparation.
[4] Novozyme 435, commercially available immobilized preparation.

means of obtaining very high yields of 1-acyl-*rac*-glycero-3-phosphate (LPA1) was to carry out the reaction at a temperature below the melting point of this product. Precipitation of LPA1 occurred in the reaction mixture, and close to quantitative yields were obtained with a number of different fatty acids. The temperature required to achieve good results depended on the chain length and degree of unsaturation of the fatty acid.

The reaction conditions and yields in the published methods for enzymatic LPA synthesis are summarized in Table 1.

Acylation of glycerophosphorylcholine and related substances

Acid anhydrides are useful acyl donors in some lipase-catalyzed reactions. A high yield (71 %) of lysophosphatidylcholine (LPC) was obtained in the acylation of glycerophosphorylcholine using Lipozyme as catalyst and methylene chloride as solvent. Glycerophosphorylcholine was not dissolved in the solvent, but acylation of the substrate suspension proved to be quite effective (Mazur et al., 1991). The reaction conditions and yields in the published methods for enzymatic LPC synthesis are summarized in Table 1.

Glycerophosphorylcholine was acylated using vinyl esters (Virto and Adlercreutz, 2000a) in a manner similar to the acylation of glycerophosphate described above. In this case, *Candida antarctica* lipase B was shown to be a suitable catalyst. It was possible to carry out the reaction in a solvent-free system, but higher yields were obtained when a small amount of *t*-butanol (about the same weight as the sub-

Table 2. Phospholipase A_2-catalyzed acylation of lysophospholipids.

Enzyme	Solvent	Acyl donor	Product[1]	Yield[2]	Comments	Reference
Naja naja venom	Toluene	Oleic acid	PC	6.5 %		Pernas et al., 1990
Pancreatic	Microemulsion (isooctane)	PUFA	PC	6 %		Na et al., 1990
Pancreatic	Benzene	Oleic acid	PC	35 %		Mingarro et al., 1994
Pancreatic	Isooctane	PUFA	PC	21 %		Lilja-Hallberg and Härröd, 1995
Pancreatic	Propane	PUFA	PC	25 %	High-pressure reactor	Härröd and Elfman, 1995
Pancreatic	Glycerol	PUFA	PC	40 %	Formamide added as water mimic	Hosokawa et al., 1995b
Pancreatic	Glycerol	PUFA	PE	27 %		Hosokawa et al., 1995a
Pancreatic on XAD-8	Toluene	Fatty acids	PC	60 %	Water activity control	Egger et al., 1997

[1] PC, phosphatidylcholine; PE, phosphatidylethanolamine.
[2] Analytical yields.
PUFA, polyunsaturated fatty acids.

strates) was used as solvent. At temperatures favoring the precipitation of the monoacylated products, LPC, high yields were obtained. The formation of the diacylated product, phosphatidylcholine (PC), was less favored than the formation of phosphatidic acid in the synthesis discussed above; this might be due to the substrate preferences of the enzymes used. Another possible reason is that acyl migration which is a necessary step for the diacylation might have been slower in LPC than in LPA.

Free fatty acids can also be used as acyl donors in this type of reaction. A solvent-free esterification of glycerophosphorylcholine with capric acid catalyzed by Lipozyme resulted in a LPC yield of 36 % when the water activity was controlled to 0.60 by the use of a pair of salt hydrates (Han and Rhee, 1998). When glycerophosphorylethanolamine was used as substrate in a similar reaction, the optimal water activity was 0.37 and the corresponding LPE yield was 23 % (Han and Rhee, 1998). When lauric acid was used in the *Candida antarctica* lipase B-catalyzed acylation of glycerophosphorylcholine, a yield of about 50 % was obtained (Virto and Adlercreutz, 2000a).

14.5.2 Phospholipase A_2-catalyzed esterification

The phospholipase A_2-catalyzed esterification of lysophospholipids, especially of LPC, has been the subject of several studies. Reaction conditions and yields are summarized in Table 2, but in the early studies, low yields (6–7 %) were obtained (Pernas et al., 1990). In most of the published methods, the aim has been to introduce polyunsaturated fatty acids into the glycerophospholipid (Na et al., 1990). Isooctane was found to be a good solvent for esterification of LPC (Lilja-Hallberg and Härröd, 1995), while a high fatty acid concentration and a low water content were found to favor the esterification reaction, as would be expected from the law of mass action. The highest yield was 21 %. Reaction temperatures of up to 80 °C were attempted, but when these were highest nonenzymatic esterification occurred in addition to degradation of the polyunsaturated fatty acids.

A high yield in the phospholipase A_2-catalyzed esterification (35 %) has been reported in anhydrous benzene (Mingarro et al., 1994). The reaction has also been attempted in high-pressure reactors (Härröd and Elfman, 1995); the best yield (25 %) was obtained using propane as solvent, though it was possible also to use carbon dioxide.

One means of achieving a low water activity is to use a water-miscible solvent with a low water content. Using this approach with glycerol as solvent, a yield of 27 % was obtained for the esterification of lysophosphatidylethanolamine with polyunsaturated fatty acids (Hosokawa et al., 1995a).

Recently, the pancreatic phospholipase A_2-catalyzed esterification of LPC with fatty acids was studied in some detail (Egger et al., 1997). The reactions were carried out under controlled water activity with toluene as solvent and immobilized pancreatic phospholipase A_2 as catalyst. A water activity of at least 0.22 was needed for the reaction to occur, and although the reaction rate was found to increase with increasing water activity, the final yield decreased with increasing water activity, as expected. In order to achieve a high yield in a reasonable time, an approach with stepwise changes in water activity was used. The water activity was initially high to provide a high reaction rate, but was then decreased. At each water activity, the reaction was continued until equilibrium was approached. Finally, when the water activity was reduced from 0.22 to 0.11, the reaction stopped after some time because the resulting water activity was too low for that enzyme (Egger et al., 1997). The yield obtained (60 %) corresponded to the equilibrium position at the lowest water activity at which the enzyme was active. If enzymes which are active at still lower water activities can be found, the yield can be further improved.

14.5.3 Possibilities for enzymatic esterification of glycerophospholipids

Starting from a fully deacylated phospholipid, several hypothetically possible methods exist of using enzymatic esterification reactions to prepare acylated lipids (Figure 8). The lipases are normally selective for esterification in the *sn*-1 position, and phospholipase A_2 for the *sn*-2 position. However, if the reactions are carried out

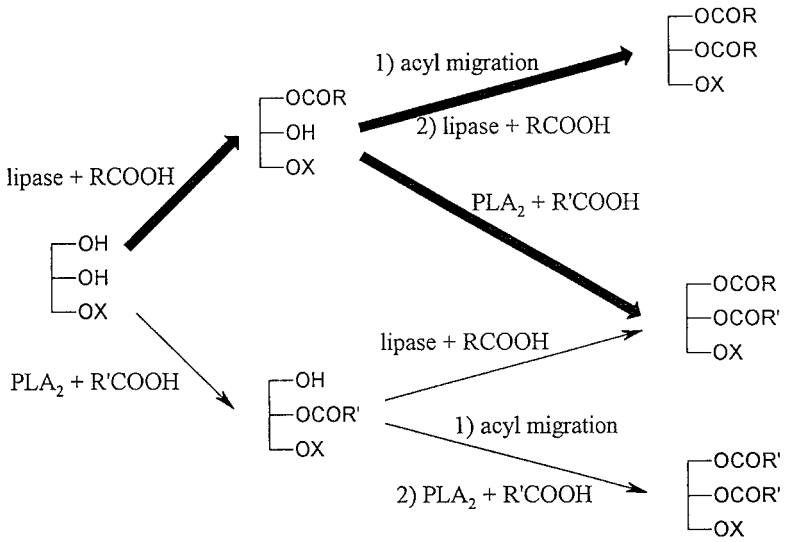

Figure 8. Theoretically possible ways to acylate glycerophospholipids. The reactions which have been found to work reasonably well (at least for one class of glycerophospholipids) are marked with bold arrows, and other reactions with normal arrows. The final products are glycerophospholipids with either the same fatty acid in both positions or with two different fatty acids introduced in a regioselective manner. PLA_2, phospholipase A_2.

under conditions which induce acyl migration, fully acylated lipids can be prepared. As mentioned earlier, the lipase-catalyzed synthesis of phosphatidic acid from glycerophosphate and of phosphatidylcholine from glycerophosphorylcholine have been reported (Virto and Adlercreutz, 1999a; Virto et al., 1999). The corresponding reaction catalyzed by phospholipase A_2 has not yet been reported, but it should be possible to carry out. The acyl migration step should be more favorable in this case as the 1-acyl-lysolipid is more stable than the 2-acyl-lysolipid. The syntheses using just one of the enzymes and acyl migration yield products with the same fatty acid in both positions. In order to produce glycerolipids with one specific fatty acid in the *sn*-1 position and another in the *sn*-2 position, the two enzymes should be used separately (Figure 8).

14.6 Lipase-catalyzed transesterification reactions

Lipases are useful for the exchange of fatty acids in the *sn*-1 position of glycerophospholipids using the one-step transesterification approach. In the earliest attempts, the fatty acid and the phospholipid were dispersed as micelles in aqueous buffer, but the yields were moderate (14–23 % yield with 18 % incorporation of the new fatty acid) (Brockerhoff et al., 1976). When the diacylated glycerophospholipid is the desired product, a main point is to limit the extent of lysophospholipid forma-

tion. Some lysophospholipid must be formed (see Figure 3), but in the final product there should not be too much of this material. Compared to reactions involving fatty acid exchange in triacylglycerols, the equilibria are much less favorable for glycerophospholipids. Thus, under similar conditions (water activity, fatty acid concentration, etc.) the product mixture contains much more partially deacylated compounds in the case of phospholipids than in the case of triacylglycerols. Different reaction systems containing only low amounts of water have been evaluated for this kind of reactions (Adlercreutz, 1994). Immobilization of the lipase on a porous support and suspending this enzyme preparation in the reaction mixture has been used successfully in a number of studies. Using *Rhizopus arrhizus* lipase immobilized on polypropylene as catalyst and toluene as solvent, a yield of 60 % of modified phosphatidylcholine was obtained when the water activity was controlled at a low level of 0.11 (Svensson et al., 1992). In this case, virtually complete exchange of the fatty acid in the *sn*-1 position was achieved.

In the incorporation of extra eicosapentaenoic and docosahexaenoic acids (EPA and DHA) into fish oil, it was found useful to carry out the reaction in a mixture of the reactants with just a small addition of a polar solvent, such as formamide, ethylene glycol or propylene glycol. The function of the polar solvent was to act as a 'water mimic' and thus activate the enzyme while still maintaining a low water activity in the reaction mixture (Hosokawa et al., 1995b).

14.7 One-step exchange of fatty acids using phospholipase A_2 as catalyst

In the reactions catalyzed by pancreatic phospholipase A_2, no acyl-enzyme is formed, and therefore transesterification reactions according to Figure 3 are not possible. On the other hand, if reaction conditions can be found under which both hydrolysis and esterification occur simultaneously, fatty acids can be exchanged in an one-step process. In order to obtain a good yield of the fully acylated lipid, it is necessary to use a relatively low water activity, or the equilibrium mixture will contain too much lysolipid. A one-step fatty acid exchange in glycerophospholipids has been carried out using pancreatic phospholipase A_2 as catalyst (Hosokawa et al., 1998), an incorporation of 35 % of polyunsaturated fatty acids and a yield of 18 % being achieved using glycerol with low water content as solvent (Hosokawa et al., 1998).

14.8 Reactions catalyzed by phospholipase C and D

Phospholipase C has been used to a relatively low degree for synthetic purposes. In the reported applications, the enzyme has been used to catalyze hydrolysis; starting from natural glycerophospholipids, the hydrolysis products were chiral diacylglycerol and phosphorylated alcohols, which may be of considerable interest (Anthonsen et al., 1999).

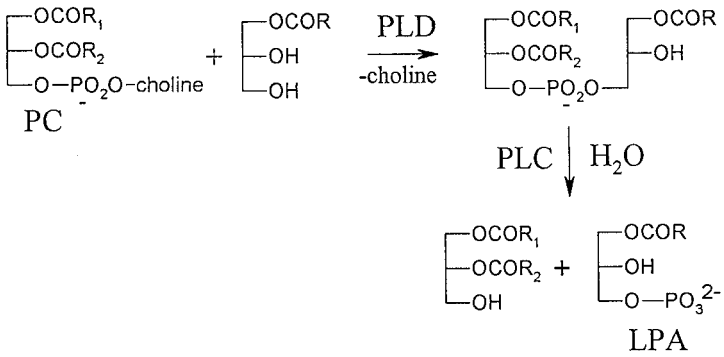

Figure 9. Example of the combined use of phospholipases C and D (PLC, PLD) to produce phosphorylated compounds, in this case lysophosphatidic acid (LPA). PC, phosphatidylcholine. (From Virto and Adlercreutz, 2000b.)

Phospholipase D functions well in both hydrolysis and transphosphatidylation reactions. In the latter, alcohols occurring naturally in glycerophospholipids and other alcohols can be incorporated. Some of the phospholipase D enzymes have relatively broad substrate specificity, and the kinetics are often favor the transphosphatidylation reaction. However, it is beyond the scope of this text to describe all the reactions possible (for a recent review, see Servi, 1999; see also Chapters on Phospholipases).

On occasion, it is beneficial to use a combination of phospholipases C and D. A glycerophospholipid such as phosphatidylcholine can thus be used to phosphorylate an alcohol. In the first step, the alcohol to be phosphorylated replaces the choline in the polar group of the phospholipid by a phospholipase D-catalyzed transphosphatidylation (Figure 9). In the next step, phospholipase C cleaves the phosphoester bond on the other side of the phosphate, producing a diacylglycerol and the phosphorylated alcohol (Virto and Adlercreutz, 2000b) (Figure 9).

Table 3. List of the enzymatic glycerophospholipid conversions which have been shown to work reasonably well.

Enzyme	Reactions
Lipase	H, E, T
Phospholipase A_2	H, E
Phospholipase C	H
Phospholipase D	H, T

H, hydrolysis; E, esterification; T, transesterification.

14.9 Possible exchange reactions in glycerophospholipids

Reactions catalyzed by lipases and phospholipases which have reported to work reasonably well are listed in Table 3. By using lipases, hydrolysis, esterification and transesterification can each be carried out in the *sn*-1 position. The hydrolysis reaction can give practically quantitative yields, while the esterification and transesterification reactions give reasonable yields (up to about 60–70 %) if suitable reaction conditions are used. With all the different phospholipases available, hydrolysis can provide essentially quantitative yields. Pancreatic phospholipase A_2 functions reasonably well for esterification, but cannot catalyze transesterification. However, it has been shown possible to use this enzyme to exchange the fatty acid in the *sn*-2 position in a one-step reaction. In this case, hydrolysis and re-esterification with the new fatty acid occur in parallel.

With regard to the incorporation of new polar groups, the only reaction reported to work well is the transphosphatidylation catalyzed by phospholipase D.

In summary, by using the three enzymes of lipase, phospholipase A_2 and phospholipase D, the different regions of the glycerophospholipid molecule that one might wish to exchange, can indeed be exchanged. Thus, it is possible – by starting with a natural glycerophospholipid mixture – to create a tailor-made and well-defined product.

14.10 Conclusions

Enzymes can be used to convert a natural glycerophospholipid mixture to a well-defined product having specified fatty acids in the *sn*-1 and *sn*-2 positions, together with a specified, natural or unnatural, polar group. The enzymes used to catalyze these reactions include lipases, phospholipase A_2 and phospholipase D. The exchange reactions may often be carried out either using a two-step combination of hydrolysis and esterification with an intermediate separation step to remove the moiety to be replaced, or as a one-step reaction. In the latter case, an excess of the moiety to be incorporated is required as there is a competition between this moiety and that to be replaced.

14.11 References

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Lipoxygenases

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15 Application of Lipoxygenases and Related Enzymes for the Preparation of Oxygenated Lipids

Ivo Feussner and Hartmut Kühn

15.1 Introduction

Lipoxygenases (linoleate: oxygen oxidoreductases; EC 1.13.11.12; LOXs) constitute a family of lipid-peroxidizing enzymes which are widely distributed in the plant and animal kingdom (Brash, 1999). They contain one nonheme iron per mole enzyme and catalyze the regio- and stereoselective dioxygenation of polyenoic fatty acids forming *S*-configurated hydroperoxy derivatives (Rosahl, 1996). In mammals, LOXs are classified into 5-, 8-, 12-, and 15-LOXs according to their positional specificity of arachidonic acid (AA) oxygenation (Yamamoto, 1992). This classification is straightforward and commonly used, but suffers from several disadvantages which may lead to confusions among scientists not working in the field. Since AA is either not present in higher plants or is a minor constituent of storage lipids, plant LOXs are classified into 9- and 13-LOXs with respect to their positional specificity of linoleic acid (LA) oxygenation (Gardner, 1991). For many years plant and animal LOXs have been considered to oxygenate mainly free polyenoic fatty acids forming oxygenated derivatives which may exhibit biological activities (Ford-Hutchinson et al., 1994; Kühn, 1996; Rosahl, 1996). However, more recent studies suggested that certain plant and animal LOX-isoforms are capable of oxygenating also ester lipid substrates, such as phospholipids (Brash et al., 1987; Murray and Brash, 1988), triacylglycerols (Feussner et al., 1995; 1998) and cholesterol esters (Belkner et al., 1991). Even more complex lipid–protein assemblies such as biomembranes (Kühn et al., 1990a; Maccarrone et al., 1994) and lipoproteins (Belkner et al., 1993; Upston et al., 1996) were metabolized by LOXs.

Oxygenation of naturally occurring polyunsaturated fatty acids may proceed enzymatically and nonenzymatically. Both reactions lead to the formation of hydroperoxy fatty acids, but there are important differences between the two processes (Kühn and Thiele, 1999). The most important difference is the specificity of the product pattern. Nonenzymatic lipid peroxidation leads to an unspecific product mixture consisting of various positional and optical isomers. These product isomers can be separated by a combination of reverse-phase, straight-phase and chiral-phase high performance liquid chromatography (HPLC). These separation procedures are rather laborious and require special equipment such as HPLC devices and suitable columns. In contrast, during LOX reaction polyenoic fatty acids are usually oxygenated to one specific product isomer which exhibits a high degree of optical purity. Thus, for large-scale preparation of structurally well-defined oxygenated polyenoic fatty acids, LOX isoenzymes exhibiting different positional specificities may be used. However, for the time being it is not possible to prepare all hydro(pero)xy

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fatty acid isomers enzymatically since the diversity of LOX positional specificity is not sufficient. For instance, currently it is not possible to prepare (5*R*)-hydroperoxy eicosatetraenoic acid ((5*R*)-HPETE)) since no LOX with a (5*R*)-specificity has been discovered so far. It may even be the case that such a LOX is not available in nature. In this case it may be possible to create such enzymes by site-directed mutagenesis. In order to do this, one has to identify the structural basis of LOX specificity so that certain amino acids can be targeted by mutagenesis.

A number of reviews have been published to date discussing the diversity of LOX-derived products formed in biological systems (Feussner and Wasternack, 1998). However, the use of isolated LOXs in organic solvents and their immobilization for biotechnological applications is less well investigated. The interested reader is referred to reviews by Gardner (1996; 1997) and Piazza (1996). This chapter will mainly describe our recent advances in creating LOX mutants which exhibit an altered product specificity.

15.2 LOXs are versatile catalysts

LOXs are multifunctional enzymes, which catalyze at least three different types of reactions: (i) dioxygenation of lipid substrates (dioxygenase reaction); (ii) secondary conversion of hydroperoxy lipids (hydroperoxidase reaction); and (iii) formation of epoxy leukotrienes (leukotriene synthase reaction).

15.2.1 Dioxygenase reaction

Although much effort has been put into the investigation of the dioxygenase reaction, its detailed mechanism remains a matter of discussion (Glickman and Klinman, 1996a,b; Hwang and Grissom, 1996; Nelson, 1996; Sloane, 1996; Prigge et al., 1997). Kinetic isotope effects appear to indicate that the rate-limiting step of the overall reaction is the stereoselective hydrogen removal from a doubly allylic methylene (Glickman and Klinman, 1996b). LOXs contain one nonheme iron per mole enzyme which is catalytically active and undergoes redox shuttling (Figure 1) (De Groot et al., 1975). From natural sources a LOXs is usually prepared in its ferrous (Fe(II)) ground state which is catalytically inactive and requires activation to start the catalytic cycle. This activation can be achieved by the reaction with small amounts of hydroperoxides. Such peroxides oxidize the inactive ferrous ground state enzyme to an active ferric form (Fe(III)-LOX) (Figure 1, step a). After binding of the substrate, a hydrogen is abstracted from a doubly allylic methylene group, resulting in reduction of Fe(III) and the formation of a carbon centered pentadienenyl radical (Figure 1, step b). Under certain conditions, such as reduced oxygen tension or in the presence of excess substrate, this radical may dissociate from the active site (Figure 1, step f), leading to the formation of unspecific oxygenation products. Alternatively, it is assumed that for some LOXs the substrate may not perfectly be aligned so that an unspecific oxygenation may be possible. Such an imperfect fit in the active site and

the resulting mobility of the substrate during catalysis might be designed specifically to promote release of free radical intermediates leading to undirected lipid peroxidation (Brash, 1999). Under aerobic conditions molecular oxygen reacts with the radical to form a hydroperoxy radical (Figure 1, step c). Subsequently, the Fe(II) will be oxidized to Fe(III) (Figure 1, step d) and the hydroperoxide anion is protonated. The final reaction product dissociates from the catalytically active Fe(III)-LOX; this concludes the catalytic cycle and the next substrate molecule can be bound.

According to an alternative model (Corey and Nagata, 1987), an electrophilic addition of Fe(III) to C_1 of the (1*Z*,4*Z*)-pentadiene system may occur, forming an organoiron-intermediate. This process is followed by a stereoselective abstraction of a proton from the bisallylic methylene. Then molecular dioxygen reacts with the bisallylic organoiron-intermediate via δ-bond insertion forming the (1*S*,2*E*,4*Z*)-1-hydroperoxy-2,4-pentadiene and the Fe(III)-LOX. In the organoiron model, regio- and stereoselectivity of dioxygen insertion is controlled by the Fe(III)-C_1 bond. In contrast, steric factors controlling the binding of the carbon-centered fatty acid radical at the enzyme and/or the geometry of the diffusion path of dioxygen appear to be of relevance for the radical mechanism. It should be stressed that both the radical and the organoiron mechanism may explain most of the me-

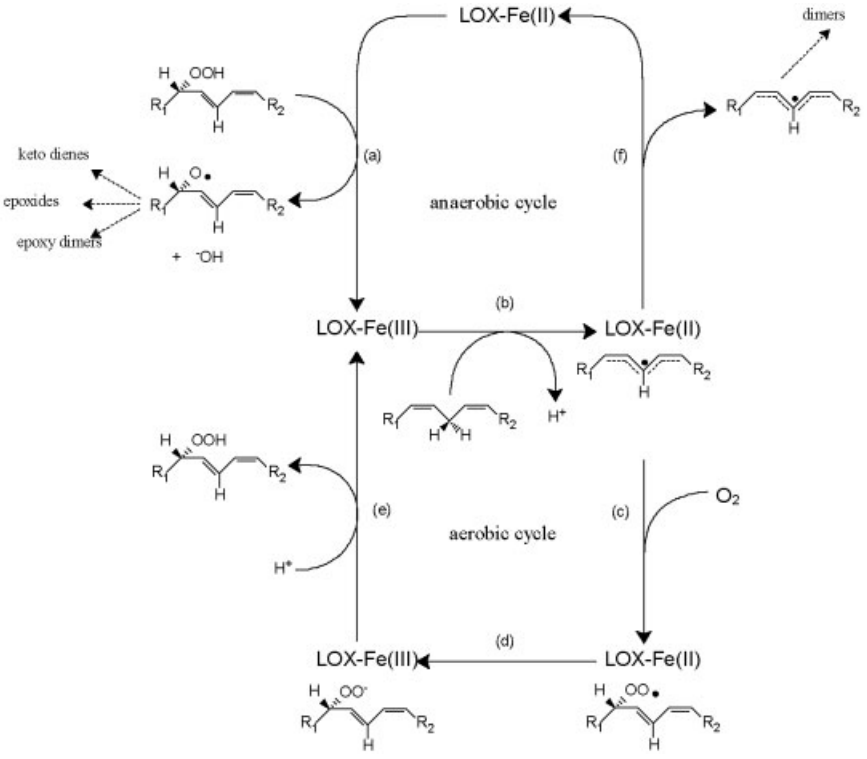

Figure 1. Catalytic cycle of LOXs according to De Groot et al. (1975) and Hilbers et al. (1995).

chanistic data available, and it is rather difficult to prove or disprove either of these models experimentally. A key intermediate in the radical mechanism, the carbon-centered alkyl radical has apparently been detected by EPR (Pistorius et al., 1976). In contrast, there is no direct experimental evidence for the formation of the organoiron intermediate. However, experimental data are available which suggest the formation of organoiron complexes between LOXs and hydroperoxy fatty acids (Nelson et al., 1994).

In addition to the naturally occurring polyenoic fatty acids containing (1*Z*,4*Z*)-pentadienyl systems, LOXs may also oxygenate unusual substrates such as allylic ketones. For instance, the keto derivative of ricinolic acid may be metabolized into a conjugated unsaturated diketo fatty acid or may be cleaved into the corresponding ω-keto C13–fatty acid (Kühn et al., 1991a). Furthermore, oxygenated polyenoic fatty acids which still contain doubly allylic methylenes such as 12- or 15-HETE can be converted to double-oxygenation products (Bild et al., 1977; van Os et al., 1981; Schwarz et al., 1998).

15.2.2 Hydroperoxidase activity

Under certain reaction conditions such as reduced oxygen pressure and in the presence of a reductant, which reduces the ferric enzyme back to its ferrous state, LOX may catalyze the degradation of hydroperoxy lipids to an array of secondary products. Polyenoic fatty acids are preferred as reductant, but other hydrophobic reducing agents such as guaiacol may also be used. The hydroperoxidase reaction is initiated by a homolytic cleavage of the peroxy bond forming alkoxy and hydroxyl-radicals. These radical intermediate may react with other components in the assay system so that a variety of secondary products may be formed (Figure 1, anaerobic cycle) (Garssen et al., 1971; De Groot et al., 1973; Kühn et al., 1991b).

15.2.3 Leukotriene synthase activity

Hydroperoxy fatty acids which still contain bisallylic methylenes can be converted by LOXs to epoxy leukotriene derivatives. For instance, 5-HPETE can be converted by purified 5/8-LOXs to 5,6-epoxy leukotriene A_4 ((5*S*,6*S*,7*E*,9*E*,11*Z*)-5,6-epoxy-7,9,11-eicosatrienoic acid) (Shimizu et al., 1984). Similarly, 15-HPETE may be transformed by 12/15-LOXs to the corresponding 14,15-leukotriene A_4 (Bryant et al., 1985). The leukotriene synthase activity of LOXs may be regarded as combination of oxygenase and hydroperoxidase activity. Hydroperoxy fatty acids which still contain at least one bisallylic methylene are substrates for leukotriene formation and the reaction involves both, stereoselective removal of a hydrogen from a bisallylic methylene and homolytic cleavage of the hydroperoxy group. The biradical formed via these reactions is stabilized during epoxide formation. Epoxy leukotrienes are rather unstable compounds and rapidly undergo epoxide hydrolysis at acidic pH. The resulting diols are frequently used as indicators for the leukotriene synthase activity of LOXs.

15.3 The structural bases of the positional specificity of LOXs and alteration of the positional specificity by modifying the substrate or by site-directed mutagenesis

The positional specificity of LOXs is a result of two more or less independent catalytic processes.

1. Regio- and stereospecific hydrogen removal: with substrate fatty acids containing several doubly allylic methylenes such as linolenic acid (LeA), AA or eicosapentaenoic acid hydrogen abstraction from two, three or four doubly allylic methylenes, respectively, is possible. The positional specificity of the enzyme determines which of these bisallylic methylenes will be attacked.
2. Regio- and stereospecific oxygen insertion: when hydrogen is abstracted from a certain doubly allylic methylene, molecular oxygen can be introduced either at the [+2] or at the [-2] position (Figure 2). Thus, a fatty acid containing three doubly allylic methylenes such as AA can be oxygenated by a LOX to 6 regio-isomeric hydroperoxy derivatives (HPETEs), namely 15- and 11-HPETE (originating from C_{13} hydrogen removal), 12- and 8-HPETE (C_{10} hydrogen removal) and 9- and 5-HPETE (C_7 hydrogen removal).

In the early days of LOX research, before structural data on this enzyme family became available, the mechanistic reasons for the positional specificity were investigated by targeted modification of LOX substrates. Experiments with AA isomers (positional isomerism of the double bonds) suggested that the positional specificity of LOXs may not be an absolute enzyme property, but may depend on the orientation of the substrate molecule at the active site of the enzyme (Hamberg and Samuelsson, 1967; Kühn et al., 1990b). The experimental data indicated that, for oxygenation of polyenoic fatty acids by the LOXs from rabbit reticulocytes and soybeans, the distance of the bisallylic methylene from the methyl end of the fatty acid is important. Furthermore, it was concluded that arachidonate 12-LOXs may have a deeper substrate-binding pocket than 15-LOXs which makes an optimal substrate orientation for 12-lipoxygenation more likely (Figure 3A, shown for LA).

After sequence data of various LOXs became available, the mechanistic reasons for the positional specificity were explored by targeted alteration of the enzymes. Multiple sequence alignments of various LOXs with different positional specificities suggested critical amino acids in mammalian 12- and 15-LOXs which may be considered as primary determinants of the positional specificity (Sloane et al., 1991; Borngräber et al., 1996). When the space-filling M419 or F353 of the human and/or rabbit reticulocyte-type 15-LOXs are mutated to smaller residues, the substrate fatty acids may slide in farther into the substrate-binding pocket which favored arachidonate 12-lipoxygenation (Sloane et al., 1991; Borngräber et al., 1996). When the substrate binds deeper, the bisallylic methylene at C_{10} of AA approaches the catalytically active nonheme iron, whereas C_{13} is dislocated. In these experiments the site of hydrogen abstraction was altered by site-directed mutagenesis, but the direction of the [+2] radical rearrangement remained unchanged.

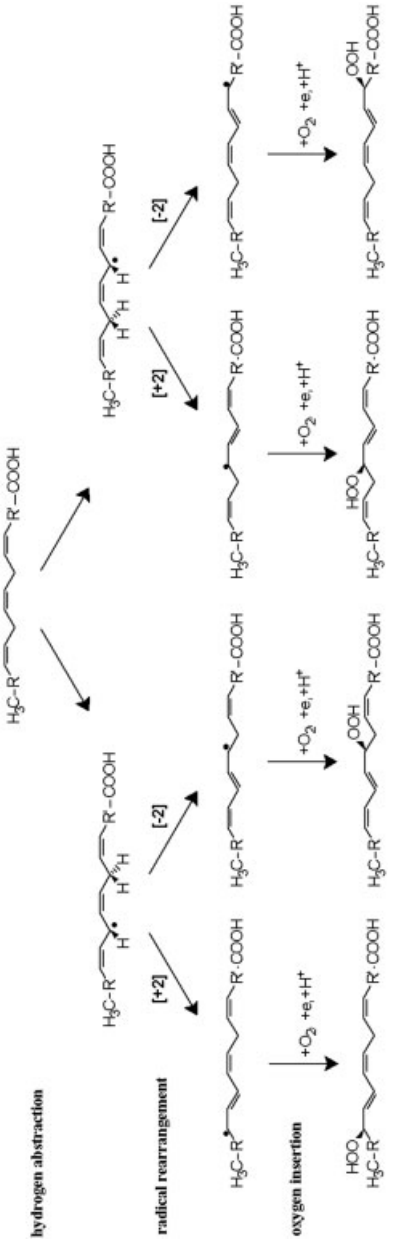

Figure 2. Radical mechanism of the LOX reaction.

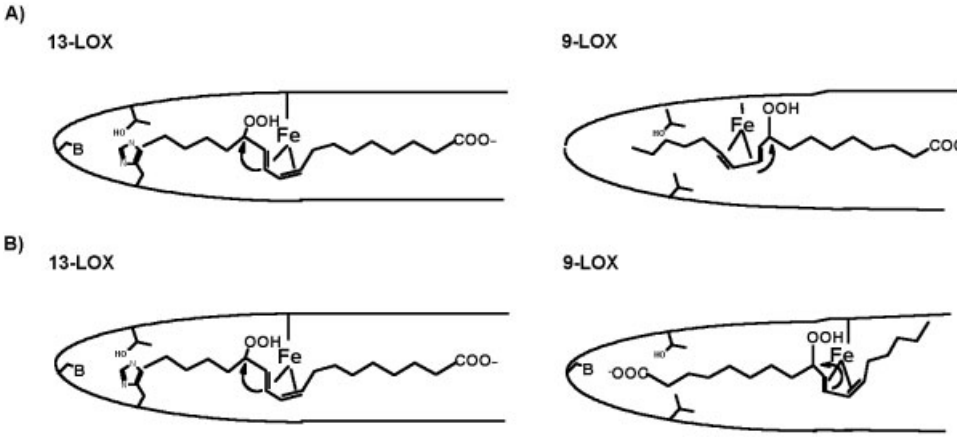

Figure 3. Comparison of the two different models explaining the positional specificity of LOXs. (A) Straight substrate alignment at the active site of LOXs for the space-related theory. (B) Orientation-dependent theory: straight- and inverse-substrate orientation at the active site of LOXs.

Earlier attempts to alter the direction of radical rearrangement during LOX reaction did not lead to active LOX mutants. However, recently a linoleate 13-LOX from cucumber seedlings was completely converted to a 9-lipoxygenating species by mutation of a single amino acid (Hornung et al., 1999).

For the time being there is no comprehensive theory which explains the positional specificity of various LOX isoforms. However, there are two hypotheses which may rationalize the effects of substrate binding on the oxygenation specificity. The space-related hypothesis was developed for mammalian LOXs after the X-ray structures of plant and mammalian LOXs became available (Minor et al., 1996; Skrzypczak-Jankun et al., 1997; Gillmor et al., 1998). It postulates that the volume of the substrate-binding pocket may be decisive for the positional specificity of the LOX reaction. Mammalian arachidonate 15-LOXs have a smaller substrate-binding cavity, and since fatty acids may penetrate the active site with their methyl terminus first they are dioxygenated close to this end of the molecule (Figure 3A, left-hand side). In contrast, more space appears to be available in the substrate-binding cleft of 12-LOXs. In that case, the fatty acid substrate may slide in farther into the substrate-binding pocket and thus, it is oxygenated closer to the carboxy terminus (Figure 3A, right-hand side). For 5-LOXs an even deeper active site was postulated so that the doubly allylic C-7 approaches the nonheme iron. This model explains the different sites of hydrogen abstraction, but the reversal of direction of radical rearrangement is hard to fit into this concept. The second hypothesis to explain the positional specificity of LOXs is the orientation-related theory. This concept postulates that substrate fatty acids are inversely bound at the active site of 5- and 15-LOXs (Egmond et al., 1973; Kühn et al., 1986; Gardner, 1989; Lehmann, 1994; Prigge et al., 1998) (Figure 3B). For linoleate 15-lipoxygenation the substrate appears to penetrate the active site with its methyl terminus, favoring a [+2] radical rearrangement (Figure 3B, left-hand side). In contrast, when the substrate slides into the active site with its carboxy terminus a [-2] radical rearrangement would

be favored (Figure 3B, right-hand side). In addition, the stereochemistry of the LOX reaction can be explained perfectly with this concept. However, an inverse head-to-tail substrate orientation should be inhibited by an energy barrier associated with burying the polar carboxylate in the hydrophobic environment of the substrate-binding cage (Browner et al., 1998; Gillmor et al., 1998). This energy barrier would be reduced strongly, if a polar amino acid were to be located at the surface of the active site (residue B in Figure 3B). Similarly, demasking of a polar residue as consequence of site-directed mutagenesis may also reduce this energy barrier.

15.3.1 Alteration of the positional specificity by site-directed mutagenesis of LOX preferring C_{18} fatty acids as substrates

In the past, site-directed mutagenesis studies have mainly been carried out with mammalian LOXs. In these experiments two regions have been identified in the primary structure containing sequence determinants for the positional specificity. An alignment of these determinants within selected plant LOXs is shown in Table 1. Amino acids aligning with the Sloane-determinants (Sloane et al., 1991) are highly conserved among plant LOXs, irrespective of their positional specificity. In contrast, there is an amino acid heterogeneity among plant LOXs at the position which aligns with P353 of the rabbit reticulocyte 15-LOX (Borngräber-determinants) (Borngräber et al., 1996). For a more comprehensive understanding of the mechanistic reasons for the positional specificity of LA oxygenation by plant LOXs, structural modeling of the active site of plant LOXs was carried out. For this purpose the X-ray coordinates of soybean LOXs-1 and -3 were utilized (Boyington et al., 1993; Minor et al., 1996; Skrzypczak-Jankun et al., 1997). These modeling studies revealed that an arginine residue is localized in the vicinity of the putative substrate-binding pocket of plant LOXs which may interact with the carboxy group of the substrates when there is an inverse head-to-tail substrate orientation (Figure 3B, residue B). Moreover, in wild-type plant LOXs there is a phenylalanine or a histidine located at the position, which aligns with the second Sloane-determinants of mammalian LOXs (Table 1).

These amino acids may shield the positive charge of the arginine and thus, the substrate may penetrate the active site with its methyl end since there is no counterpart to neutralize the charge of the carboxy group (Hornung et al., 1999). Mutagenesis studies of the Sloane-determinants with the lipid body LOX of cucumber seedlings indicate for the first time the possibility to convert a plant LOX catalyzing a [+2] radical rearrangement to a LOX catalyzing a [-2] rearrangement (Hornung et al., 1999) (Figure 4). In this case a single point mutation (H608V) converted the wild-type linoleate 13-LOX to a 9-lipoxygenating mutant species. From these results it is hypothesized that the exchange of the bulky phenylalanine to a less space-filling valine may inverse the orientation of the substrate at the active site (Figure 3B). However, when more complex substrates such as methyl linoleate (LAMe), α-LeA, γ-LeA or trilinolein (TL, see below) were used the situation becomes more complex, and more structural determinants within the active site may be required for a perfect alignment of the lipid substrate (Table 2).

Table 1. Alignment of amino acid residues possibly determining the positional specificity of plant and mammalian LOXs.

Enzyme	Acc.-No.	Amino acid residues (Position of amino acid residues)[1]	Amino acid residues (Position of amino acid residue)[2]
13-LOX			
Cucumber lipid body LOX	X92890	Thr/His (607/608)	Val (542)
Soybean seed LOX-1	P08170	Thr/Phe (556/557)	Ser (491)
Potato LOX-H1	X96405	Ser/Phe (614/615)	Ser (551)
Arabidopsis LOX-2	P38418	Cys/Phe (611/612)	Ala (548)
Rabbit reticulocyte LOX	P12530	Ile/Met (418/419)	Phe (353)
9-LOX			
Potato tuber LOX	P37831	Thr/Val (579/580)	Val (514)
Tobacco elicitor-ind. LOX	X84040	Thr/Val (580/581)	Ser (515)
Arabidopsis LOX-1	Q06327	Thr/Val (577/578)	Ser (522)
Barley grain LOX-A	L35931	Thr/Val (574/575)	Ser (511)
Human LOX	P09917	Ala/Asn (424/425)	Phe (359)

[1] According to Sloane et al. (1991).
[2] According to Borngräber et al. (1996).
Acc. no., accession number.

To investigate the role of other sequence determinants for the positional specificity of plant LOXs, we point-mutated the Borngräber-determinant of the cucumber lipid body LOX (V542F). Introduction of the space-filling phenylalanine in addition to the already existing bulky H608 led to an enzyme species which exhibited a so far unknown positional specificity (Figure 5). The V542F mutant converted γ-LeA mainly to (6*S*,7*E*,9*Z*,12*Z*)-6-hydroperoxy-7,9,12-octadecatrienoic acid. In contrast, the wild-type enzyme produced mainly the corresponding (13*S*)-hydroperoxy derivative. Here again, the direction of radical rearrangement was inverted by the mutation.

The lipid body LOX from cucumber seedlings and the LOX-2 from barley grains are capable of oxidizing unpolar lipids (Holtman et al., 1997; Feussner et al., 1997a). When expressed in *E. coli* or under *in vivo* conditions the cucumber lipid body LOX oxygenates all three LA moieties of trilinolein (Figure 6, Cc-16-LOX) (Feussner et al., 1998). At the pH-optimum of soybean LOX-1, TL is oxygenated only to 28 % of the amount converted by the lipid body LOX (Figure 6, Gm-LOX versus Cs-lb-LOX). Moreover, TL oxygenation by soybean LOX-1 lead mainly to mono-hydroperoxy derivatives (Figure 6, peak 1), whereas oxygenation by lipid body LOX lead to a tri-hydroperoxy derivative (Figure 6, peak 3). Since triacylglycerols do not contain free carboxylic groups, no major differences are expected when the patterns of oxygenation products of the wild-type lipid body LOX and of their linoleate 9-lipoxygenating mutants were compared. Indeed, the wild-type enzyme and all 9-lipoxygenating mutants exhibited a trilinoleate 13-LOX activity (Hornung et al., 1999). In addition, the rates of TL oxygenation by the 9-lipoxygenating mutants were comparable to those measured for other plant LOXs with a substrate preference against free polyenoic fatty acids. Moreover, TL oxygenation by the mutant enzymes mainly led to mono-hydroperoxy derivatives (Hornung et al., 1999).

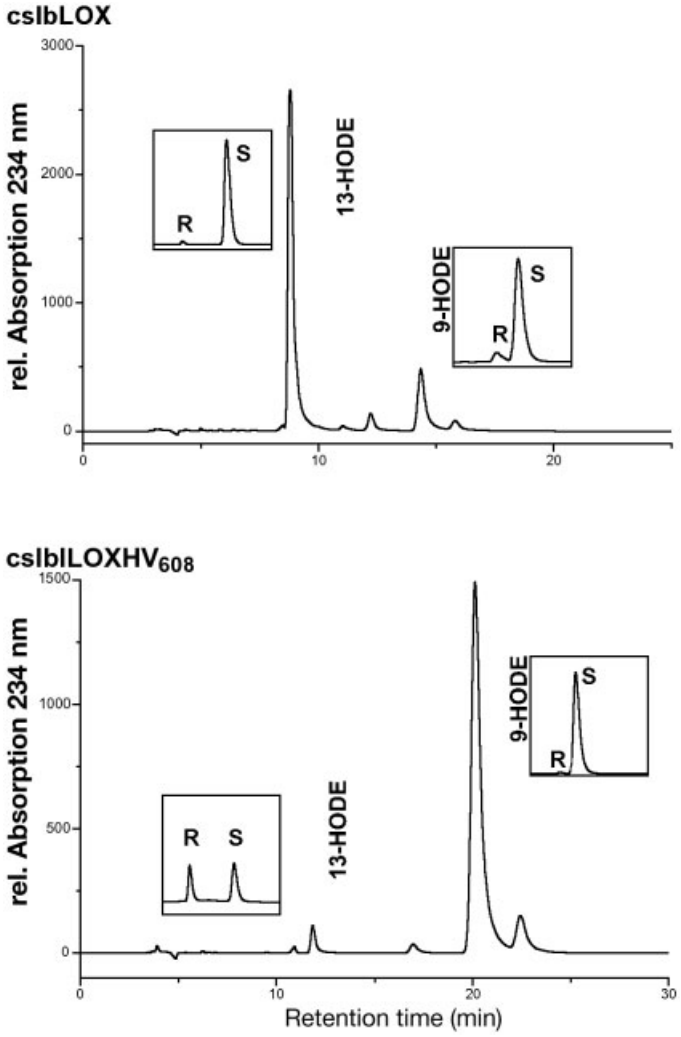

Figure 4. Straight-phase HPLC analysis of hydroxy polyenoic fatty acids formed from LA by the cucumber lipid body LOX and its H608V mutant according to Hornung et al. (1999). The separation of the enantiomers on chiral-phase HPLC is shown in the insets.

Table 2. Products formed from the reaction of wild-type lipid body LOX and of its mutant with LA, LAMe, α-LeA or γ-LeA.

	Products obtained with LA	**Products obtained with LAMe**	**Products obtained with α-LeA**	**Products obtained with γ-LeA**
Mutants	Positional isomers 13 : 9	Positional isomers 13 : 9	Positional isomers 13 : 16 : 12 : 9	Positional isomers 13 : 10 : 9 : 6
	Major enantiomer[1]	Major enantiomer[1]	Major enantiomer[1]	Major enantiomer[1]
Wild-type LOX	84 : 16 S : S	84 : 16 S : S	61 : 2 : 19 : 18 S : rac : rac : s	58 : 2 : 13 : 27 S : rac : S : S
$H_{608}V$	5 : 95 rac : S	5 : 95 rac : S	8 : 3 : 14 : 75 rac : rac : rac : S	19 : 6 : 61 : 14 rac : rac : S : rac
$H_{608}M$	21 : 79 S : S	21 : 79 S : S	18 : 3 : 17 : 62 S : rac : rac : S	23 : 6 : 47 : 24 s : rac : S : s
$T_{607}I$	45 : 55 S : S	45 : 55 S : S	32 : 5 : 17 : 46 S : rac : rac : S	27 : 7 : 23 : 43 s : rac : S : S
$T_{607}LH_{608}V$	11 : 89 s : S	11 : 89 s : S	13 : 12 : 40 : 35 s : s : rac : S	37 : 14 : 15 : 34 S : rac : rac : S
$T_{607}LH_{608}M$	18 : 82 S : S	18 : 82 S : S	18 : 4 : 53 : 26 rac : rac : S : S	–
$V_{542}F$	41 : 59 S : S	–	28 : 8 : 36 : 28 S : rac : rac : rac	25 : 7 : 14 : 54 S : rac : rac : S

[1] S > 80% to *S*-Enantiomer; s = 70–80% *S*-Enantiomer; rac = 50–70% *S*-Enantiomer, racemic.

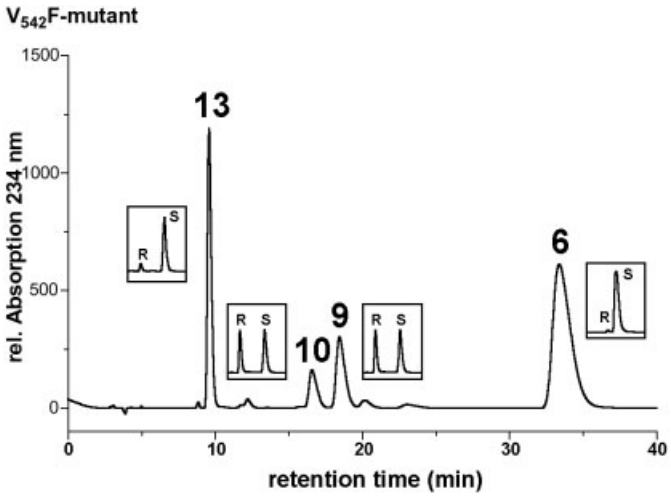

Figure 5. Straight-phase HPLC analysis of hydroxy polyenoic fatty acids formed from r-LeA by the V542F mutant of cucumber lipid body LOX. The separation of the enantiomers on chiral-phase HPLC is shown in the insets.

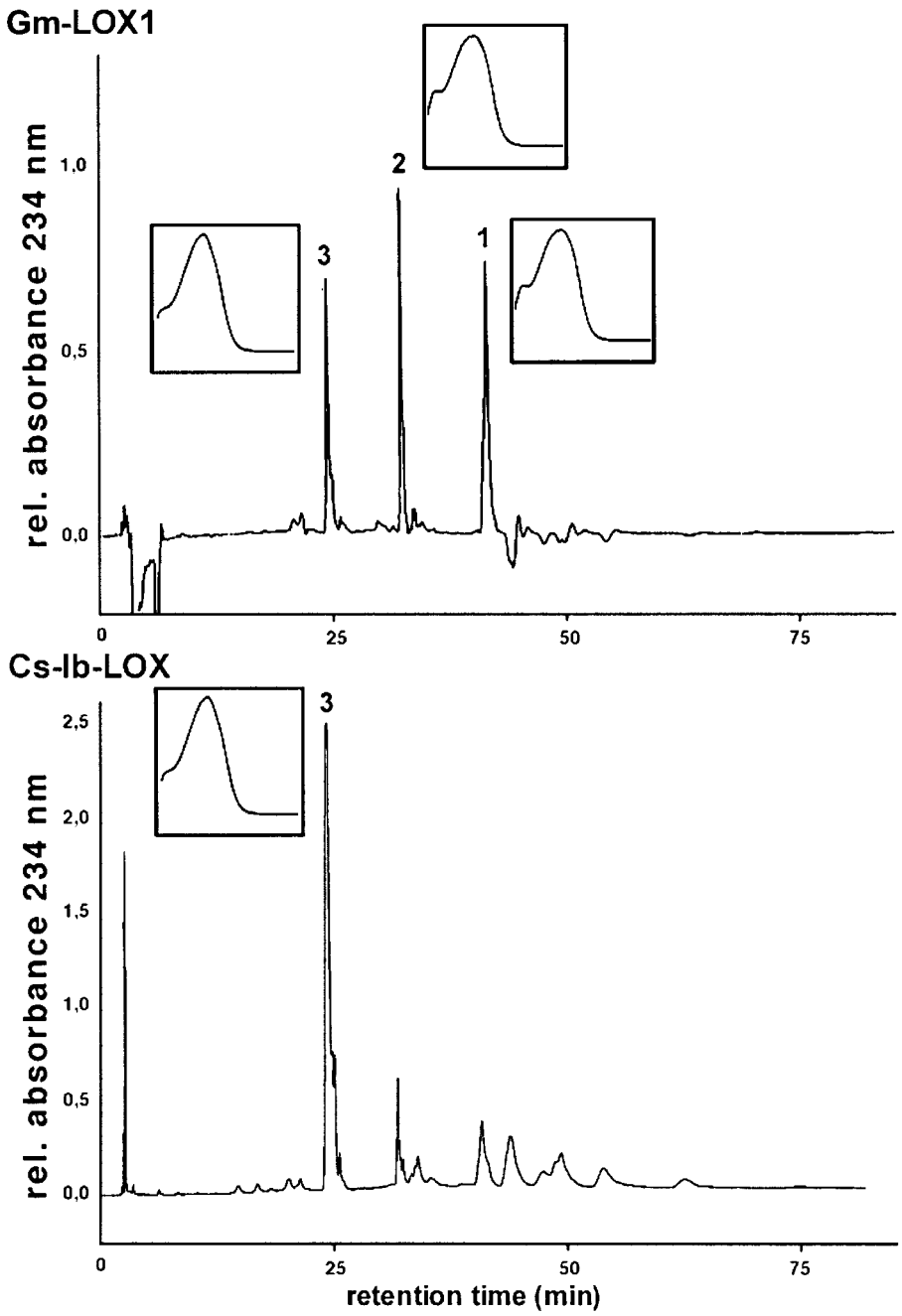

Figure 6. Reversed-phase HPLC analysis of oxidized TL derivatives formed by LOX-1 from soybean (GM-LOX1) and by the cucumber lipid body LOX (Cs-lb-LOX) according to Feussner et al. (1998). The insets show the corresponding UV spectra.

15.3.2 Specificity of mammalian LOXs

In contrast to plant cells, most mammalian tissues contain large amounts of AA and in various animal cells – such as polymorphonuclear leukocytes, monocytes or macrophages – this fatty acid is even the major polyenoic fatty acid. Since AA is the major substrate for the formation of prostaglandins and leukotrienes, research has been focused in the past on the oxidative metabolism of this fatty acid. However, in several mammalian cells and tissues and also in extracellular lipids (e.g., plasma lipoproteins), LA is more abundant than AA. Thus, the oxidative metabolism of LA via the LOX pathway may also lead to bioactive compounds, which may have been underestimated so far. In fact, (13*S*,9*Z*,11*E*)-13-hydroxy-9,11-octadecadienoic acid, the major oxygenation product of LA via the 15-LOX pathway has been shown to exhibit interesting biological activities (Kühn, 1996). Nevertheless, for the time being AA metabolism remains in the center of eicosanoid research, although investigations of the metabolic fate of other polyunsaturated fatty acids may have become increasingly important during the past few years.

According to the currently used nomenclature, mammalian LOXs are categorized with respect to their positional specificity of AA oxygenation into 5-LOXs, 8-LOXs, 12-LOXs, and 15-LOXs (Funk, 1996; Brash, 1999; Kühn and Thiele, 1999). Although this nomenclature is straightforward and commonly accepted, it suffers from several disadvantages, which may lead to confusion among scientist not working in the field. The major disadvantage of this nomenclature is that it is based on a single enzyme property and does not consider other structural and functional enzyme characteristics. Moreover, the positional specificity of LOXs is not an absolute enzyme property but depends strongly on the way that the enzyme interacts with the substrate. This interaction is of course influenced by a variety of factors such as substrate concentration (Kühn et al., 1990a), the physico-chemical state of the substrate (Began et al., 1999), pH (Gardner, 1989) or temperature, but may also depend on the structures of both, enzyme and substrate. If this hypothesis is correct, it may be possible to alter the positional specificity by targeted modification of the substrate and/or by site-directed mutagenesis of critical amino acids involved in positioning the fatty acid substrate at the active site.

Alteration of positional specificity with C_{20} fatty acid substrates of mammalian and plant LOXs

Since site-directed mutagenesis requires detailed sequence information on various LOX isoforms, the problem of enzyme/substrate interaction was initially approached by targeted substrate modification. In 1967, Hamberg and Samuelsson investigated the structural reasons for the positional specificity of the soybean LOX reaction using different polyenoic fatty acids. They concluded that the distance of the bis-allylic methylene where hydrogen abstraction takes place from the methyl end of the substrate molecule appeared to be important (Hamberg and Samuelsson, 1967). Similar results were later on obtained with the rabbit 15-LOX (Kühn et al., 1990b). From these data a topological model of enzyme substrate interaction was developed suggesting that polyenoic fatty acids may penetrate the active site

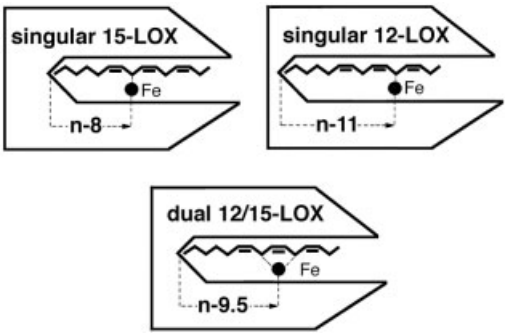

Figure 7. Binding of polyenoic fatty acids at LOXs exhibiting a singular or dual positional specificity. The solid circles represent the hydrogen acceptor of the enzyme (nonheme iron) and the 'horse-shoe-like' structure symbolizes the hydrophobic substrate-binding pocket of the enzyme. The estimated distance of the hydrogen acceptor from the bottom of the hydrophobic pocket is given in methyl groups.

of 12/15-LOXs with the methyl end (Kühn et al., 1986; Lehmann, 1994). According to this model (Figure 7) AA is oxygenated at C_{15} when the substrate is aligned in such a way that the double allylic methylene C_{13} is localized in close proximity to the hydrogen acceptor (nonheme iron). In contrast, AA 12-oxygenation may result when the substrate fatty acid penetrates somewhat deeper into the binding pocket to approach the bisallylic C_{10} to the iron (Figure 7). In other words, a shallow substrate-binding pocket may favor AA 15-oxygenation, whereas a deeper cleft may lead to 12-HETE formation. If the depth of the binding cleft is in between, an enzyme with a dual positional specificity results (Figure 7).

In order to obtain additional evidence indicating the importance of substrate structure for the positional specificity, the oxygenation of 15-HETE by the LOXs from soybeans (van Os et al., 1981) and rabbits (Schwarz et al., 1998) was investigated. This substrate was converted by the rabbit enzyme mainly to (14*R*,15*S*)-DiH(P)ETE. In contrast, the soybean enzyme formed a mixture of (5*S*,15*S*)- and (8*S*,15*S*)-DiH(P)ETE. Most interestingly, after methylation the rabbit LOX mainly catalyzed 5-lipoxygenation, and the share of (5*S*,15*S*)-DiH(P)ETE formed by the soybean enzyme was strongly increased (Schwarz et al., 1998). These data indicated that with 15-HETE as substrate the arachidonate 15-LOXs from rabbit reticulocytes and soybeans constitute 5-LOXs. An inverse head to tail orientation of the substrate (Figure 8) may be discussed as a mechanistic reason for this unexpected product pattern, and additional experimental evidence exists supporting this hypothesis:

- Methylation of 15-HETE strongly stimulated its oxygenation rate. This result may be explained by the fact that methylation increases the hydrophobicity of the carboxy terminus, reducing the energy barrier associated with burying the carboxylate group in the hydrophobic substrate-binding pocket. In this context it is of particular interest that the oxygenation rates of the substrates which are oxygenated at C-15 (AA, 5-HETE, 8-HETE) were not augmented upon methylation, but were even impaired (Schwarz et al., 1998).

- Introduction of the bulky and hydrophilic glycerol moiety at the carboxy-terminus reversed the effects of methylation. This may be explained by the fact that the glycerol moiety prevents an inverse substrate orientation.
- F353L and I418A mutation in the rabbit 15-LOX led to a strong increase in the formation of (8*S*,15*S*)-DiH(P)ETE during 15-HETE oxygenation. Similarly, the R403L mutant of the rabbit enzyme which catalyzed 12- and 15-lipoxygenation of AA, oxygenated 15-HETE to (8*S*,15*S*)-DiH(P)ETE (Schwarz et al., 1998). However, this product was formed only in small amounts by the wild-type enzyme.
- Site-directed mutagenesis of R403 (which was supposed to interact with the carboxylate group of the substrate) to an uncharged leucine favors an inverse substrate orientation, as indicated by an increased (5*S*,15*S*)-DiH(P)ETE+(8*S*,15*S*)-DiH(P)ETE to (14*R*,15*S*)-DiH(P)ETE ratio (Schwarz et al., 1998).

These findings suggest the ability of 15-LOXs to tolerate both the methyl end and the carboxy terminus of fatty acid substrates in the substrate-binding pocket. With a defined substrate there may be a binding equilibrium between the AA-like orientation (methyl terminus slides into the binding pocket) and the inverse alignment (carboxylate group penetrates into the pocket). This equilibrium may be influenced by functional groups on either ends of the fatty acid substrates, and by the enzyme isoform. With polyenoic fatty acids the methyl end of the fatty acid slides into the binding pocket. However, introduction of an OH-group at C_{15} or C_{12} may shift the equilibrium towards an inverse orientation, although the formation of (14*R*,15*S*)-DiH(P)ETE from 15-HETE by the rabbit enzyme suggested that a large share of the substrate appears to be bound in an AA-like way. Methylation of the carboxylate group of 15-HETE shifted the equilibrium further towards an inverse orientation. In contrast, introduction of a bulky and polar glycerol residue may shift the equilibrium back, favoring an AA-like orientation. It should be stressed that the lack of

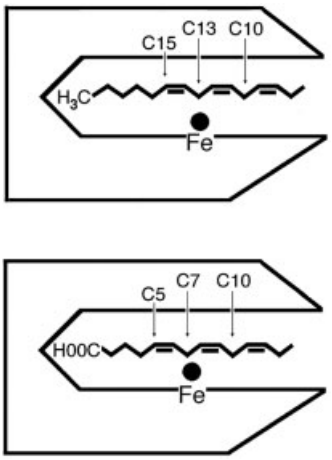

Figure 8. Straight- and inverse orientation of polyenoic fatty acid substrates at the active site of the rabbit 15-LOX. The solid circles represent the hydrogen acceptor of the enzyme (nonheme iron) and the 'horse-shoe-like' structure symbolizes the hydrophobic substrate-binding pocket of the enzyme.

(14*R*,15*S*)-DiH(P)ETE by the soybean LOXs does not exclude an AA-like substrate orientation. It may be possible that only the inversely aligned substrate is oxygenated by the enzyme. If the hydrogen abstraction is sterically hindered, for instance by a large distance of the doubly allylic methylene from the nonheme iron, the share of 15-HETE which is bound in an AA-like way, may not be oxygenated although it was bound at the active site. Thus, the lack of (14*R*,15*S*)-DiH(P)ETE formation by the soybean LOXs does not exclude that a share of the substrate may be bound in an AA-like manner. Similarly, the formation of (5*S*,15*S*)-DiH(P)ETE from 15-HETE methyl ester by the reticulocyte enzyme does not exclude an AA-like substrate alignment, because abortive substrate binding may interfere with product formation.

Alteration of positional specificity by targeted site-directed mutagenesis

After sequence information on various mammalian LOXs became available (Dixon et al., 1988; Fleming et al., 1989; Funk et al., 1990; Yoshimoto et al., 1990), scientists began to investigate LOX/substrate interaction by site-directed mutagenesis. In order to identify suitable targets for site-directed mutagenesis, multiple sequence alignments of various LOXs were required which would provide information on conserved sequence differences between various LOX subfamilies.

Conversion of arachidonate 15-LOX to 12-lipoxygenating enzyme species

In 1991, Sloane and colleagues carried out a multiple alignment of 12- and 15-LOX sequences and found four conserved differences between the two families (Sloane et al., 1991). These amino acids of the human 15-LOX were mutated to their counterparts present in 12-LOXs. An enzyme species resulted which converted AA to 12- and 15-HETE in almost equal amounts. Separate mutation of these four amino acids indicated that the alterations in the product pattern were due exclusively to M419V exchange (Sloane et al., 1991). In a follow-up study (Sloane et al., 1995), the authors carried out simultaneous mutations of I418 and M419 by changing them to the residues present in the bovine and porcine 12-LOX. In doing this, the human 15-LOX was converted completely to a 12-lipoxygenating species (12-/15-HETE ratio 20 : 1). These data indicated that I418 and M419 may constitute sequence determinants for the positional specificity of the human 15-LOX. Later experiments with the rabbit reticulocyte 15-LOX (Kühn et al., unpublished data), with the human platelet 12-LOXs (Chen and Funk, 1993), and with the porcine leukocyte 12-LOX (Suzuki et al., 1994) confirmed this conclusion. However, a similar strategy was not successful to alter the positional specificity of the leukocyte-type 12-LOXs from rats (Watanabe and Haeggstrom, 1993) and mice (Kühn et al., unpublished data). Thus, there must be differences in the mechanism of the positional specificity between the porcine and the murine leukocyte-type 12-LOXs. In order to obtain more detailed information about these differences, we created chimeric LOX species combining cDNA fragments of the rabbit reticulocyte 15-LOX cDNA with pieces of the two above-mentioned enzymes (Borngräber et al., 1996). As first step, a chimeric LOX was created in which a large amino acid fragment (301 amino acids) of the rabbit reticulocyte 15-LOX was inserted into the porcine leukocyte-type 15-

LOX. The exchanged region contained the amino acids I418 and M419, which had been previously characterized as primary determinants for the positional specificity of the human 15-LOX (Sloane et al., 1995). The chimeric enzyme converted AA mainly to 15-HETE, indicating that important sequence determinants for the positional specificity were present in the fragment inserted. Using the inverse strategy (insertion of a comparable fragment of the porcine 12-LOX into the rabbit 15-LOX), the rabbit 15-LOX was converted to a 12-lipoxygating species. A similar effect was seen when a comparable piece of the murine enzyme was inserted into the rabbit LOX. Next, the size of the inserted fragment was gradually reduced; the results obtained suggested that additional sequence determinants, which are different from those described before (I418 and F419 of the human 15-LOX), appear to be important for the positional specificity of the murine 12-LOX. These determinants must be localized in an 86-amino acid fragment (amino acids 270 to 355) of the murine leukocyte-type 12-LOX (Borngräber et al., 1996). Modeling the enzyme/substrate interaction of LOX using the X-ray coordinates for the soybean enzyme we found that only the seven C-terminal amino acids of this peptide fragment may line the substrate-binding cavity (positions 349 – 355). Alignment of this short amino acid sequence revealed just one conserved amino acid exchange (F353L) among the LOXs of interest. In the leukocyte-type 12-LOXs of mice and rats this amino acid is a small leucine, whereas a bulky phenylalanine is located at this position in the human and rabbit 15-LOXs and in the porcine leukocyte-type 12-LOX. In order to test the hypothesis that amino acid 353 may constitute a sequence determinant for the positional specificity, site-directed mutagenesis was carried out. Indeed, we found that the F353L mutation converted the rabbit enzyme to a 12-lipoxygenating species (12-/15-HETE ratio of 2 : 1). These data suggested that the amino acids at positions 353, 418, and 419 form the bottom of the substrate-binding cleft (Figure 9) and the following conclusions were drawn:

- Mammalian LOXs containing a small amino acid at position 353 are 12-LOX, independent of the size of the residues 418 and 419 (rat and mouse leukocyte-type 12-LOX).
- When a space-filling amino acid is localized at position 353, the residues 418 and 419 become important for the positional specificity.
- When bulky amino acids are localized at positions 353, 418 and 419, 15-lipoxygenation is favored (human and rabbit 15-LOX). However, when a bulky residue at position 353 is combined with a less space-filling amino acid at positions 418 and 419, AA is oxygenated at C-12 (porcine leukocyte-type 12-LOX).

After the crystal structure of various plant and mammalian LOXs became available (Boyington et al., 1993; Minor et al., 1996; Skrzypczak-Jankun et al., 1997; Gillmor et al., 1998), the model of the substrate-binding pocket in mammalian LOXs was substantially improved. In fact, it was calculated that the volume of the substrate-binding pocket of 12-LOXs is about 6 % bigger than that of 15-LOXs (Skrzypczak-Jankun et al., 1997; Browner et al., 1998). Interestingly, 5-LOX have an even larger substrate-binding pocket (20 % larger than 15-LOXs). Moreover, the X-ray coordinates predicted that I593 may be involved in defining the size and shape of the substrate-binding cleft. To test this hypothesis, site-directed

mutagenesis of I593 (I593A) was carried out and the mutant enzyme converted AA to 12- and 15-HETE in a ratio of about 1 : 1 (Borngräber et al., 1999). To compare the relative importance of the sequence determinants, we mutated each of them to a small alanine (ala-scan) or to a space-filling phenylalanine (phe-scan) so that the volume of the pocket was either increased or decreased. The results of these experiments suggested that enlargement or alteration in packing density in the substrate-binding pocket of the rabbit 15-LOX increased the share of 12-LOX products, whereas a smaller active site appeared to favor 15-lipoxygenation (Borngräber et al., 1999). Moreover, we found that the sequence determinants functionally interact with each other. Alteration in the positional specificity was observed when a space-filling amino acid was mutated to a smaller residue, but this was reversed when a large amino acid was introduced at another critical position (Borngräber et al., 1999). A schematic view of the topology of substrate binding at the active site of reticulocyte-type 15-LOXs is shown in Figure 9.

In order to go beyond a 15-LOX/12-LOX exchange and to convert the 15-LOX to an 8- or even a 5-lipoxygenating mutant, it was attempted to increase the volume of the substrate-binding site as much as possible by creating double, triple, and quadruple mutants. Double mutations, such as F353A+I418A or F353L+I593A were well tolerated and the mutant enzyme converted AA to 12-HETE. However, after triple alanine mutation (F353A+I418A+I593A), the enzyme exhibited a residual activity of only about 15 %. Here again, 12-HETE was detected as major product. This result may suggest that the pocket became too large to align properly with the fatty acid substrate. In addition, several other multiple mutants were created aimed at increasing the volume of the substrate-binding pocket, but unfortunately, all of them turned out to be catalytically inactive. Thus, one may conclude that excessive re-engineering of the LOX active site may not be possible without a loss in functionality (Borngräber et al., 1999).

So far, all studies on the enzyme/substrate interaction and on the positional specificity were carried out with free fatty acids as substrates. In contrast, the reticulocyte 15-LOXs are capable of oxygenating more complex substrates (Murray and Brash, 1988; Kühn et al., 1990a) and there is experimental evidence suggesting that membrane phospholipids and lipoprotein cholesterol esters may be the preferred natural substrates (Kühn and Brash, 1990). Since these substrates are much more space-filling than polyenoic fatty acids, it remains to be investigated how they

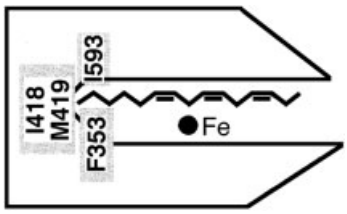

Figure 9. Schematic view of the topology of substrate binding at the active site of reticulocyte-type 15-LOXs. The solid circles represent the hydrogen acceptor of the enzyme (nonheme iron) and the 'horseshoe-like' structure symbolizes the hydrophobic substrate-binding pocket of the enzyme.

are aligned at the active site, and whether the rules established for polyenoic fatty acids are also applicable for complex substrates.

Conversion of the human arachidonate 5-LOX to 8- and 15-lipoxygenating species

As indicated earlier, it is relatively easy to convert a 15-LOX to a 5-lipoxygenating species when the structure of the LOX substrate is altered appropriately. In contrast, previous attempts failed to transform a 12/15-LOXs to a 5-lipoxygenating enzyme by site-directed mutagenesis. When the residues 418 and 419 of the human and the rabbit reticulocyte-type 15-LOX were mutated to the amino acids present in the human 5-LOX, inactive enzyme species resulted.

As indicated before, two theories exist which relate the positional specificity of 5-LOXs to that of 12/15-lipoxygenating enzymes:

1. The space-related theory (Funk and Loll, 1997; Skrzypczak-Jankun et al., 1997; Browner et al., 1998), which suggests that the product specificity of a LOX is determined by the volume of the substrate-binding pocket. Fatty acids are bound at the active site of all LOXs in a conserved AA-like orientation. Arachidonate 12/15-LOXs have a smaller substrate-binding cleft as 5-LOXs and thus may not penetrate as deep into the pocket, as may be the case for 5-LOX. On the other hand, the space available at the active site of 5-LOXs may allow a substrate alignment optimal for 5-lipoxygenation.
2. The orientation-related theory (Kühn et al., 1986; Lehmann, 1994; Funk and Loll, 1997; Prigge et al., 1998): according to this hypothesis 5-lipoxygenation requires an inverse head-to-tail orientation of the substrate so that fatty acid substrates may slide into the binding pocket with the carboxylic group ahead. With this hypothesis the stereochemistry of (5*S*)-lipoxygenation can easily be explained. An inverse substrate orientation would be energetically favored, if polar or even charged amino acids were present at the bottom of the substrate-binding pocket. However, molecular modeling of the enzyme/substrate interaction suggested that the closest potential positive charge in the human 5-LOX would be H354 which is located at a distance of more than 6 Å from the position of the modeled substrate carboxylate group (Browner et al., 1998).

Although there is a substantial body of circumstantial experimental evidence favoring the orientation-related hypothesis, neither of the two theories has been proven experimentally. The orientation hypothesis can only be proven if co-crystallization studies of a 5-LOX with a substrate fatty acid are carried out. The space-hypothesis can be tested by site-directed mutagenesis. As mentioned above, at least all attempts failed to convert a mammalian 15-LOX to a 5-lipoxygenating species. We recently approached this problem with an inverse strategy, attempting to convert the human leukocyte 5-LOX to a 15-lipoxygenating enzyme. In order to decrease the volume of the substrate-binding cage, we mutated the sequence determinants of the 5-LOX to the more space-filling counterparts present at these positions in 15-LOXs. An A424I

or N425M exchange (A424 and N425 of the human 5-LOX align with I418 and M419 of the rabbit and human 15-LOX) transformed the wild-type 5-LOX to enzyme species which produced significant amounts of 8-HPETE (8–15 %) in addition to 5-HPETE. In contrast, an A603I exchange (A603 of the human 5-LOX aligns with I593 of the rabbit enzyme) did not influence the positional specificity. The fourth sequence determinant of the 15-LOX (F353) aligns with F359 in 5-LOXs. In order to reduce the volume of the binding pocket, we mutated F359 to an even more bulky tryptophan. This F359W mutant turned out to be a major 5-LOX with a significant share of 8-HPETE formation (5-/8-HPETE ratio of about 2 : 1). Unfortunately, no 15-HPETE was observed with any of the single mutants. To reduce further the volume of the substrate-binding pocket, we combined the effective single mutations to create double, triple, and even quadruple mutants. When the N425M was combined with a F359W and A424I (F359W+N425M and A424I+N425M) the share of 8-HPETE formation was strongly increased (60–90 %), but no 15-HPETE formation was observed. However, when the F359W was combined with the A424I, a small (5–10 %) but significant share of 15-lipoxygenation was detected. This effect was even more pronounced when the F359W+A424I+N425M triple mutant was constructed. Here, an almost 1 : 1 distribution of 15- and 8-HPETE was found, and both products turned out to be chiral with a strong preponderance of the *S*-isomer. For the rabbit 15-LOX it has been reported that I593A exchange altered the positional specificity (Borngräber et al., 1996), but inverse mutagenesis on the human 5-LOX (A603I) did not have major effects. It might be possible that the loss of the pocket volume achieved by this mutation for 5-LOXs was not strong enough to alter the positional specificity of the wild-type enzyme. However, when this amino acid exchange was performed on the F359W+A424I+N425M triple mutant the product pattern was further shifted towards 15-HPETE formation. In fact, with this quadruple mutant, AA was mainly oxygenated to 15-HPETE (85–95 %), with 8-HPETE being a minor product.

The 15-lipoxygenating quadruple mutant was purified by ATP–agarose chromatography and its basic enzymatic characteristics were determined. Although we altered the positional specificity of AA oxygenation, no major effects were observed on other enzymatic characteristics. Both the wild-type and the mutant enzyme were activated by Ca^{2+}, ATP and phosphatidylcholine, and the substrate specificity with different fatty acids was very similar. Biological membranes and lipoproteins, which are suitable substrates for reticulocyte-type 15-LOXs but not for 5-LOXs, were not oxygenated by the mutant. Although the arachidonate dioxygenase activity of the mutant enzyme was only 30 % of that of the wild-type counterpart, the mutant enzyme exhibited a comparable leukotriene synthase activity with 5-HPETE as substrate. When the wild-type 5-LOX was incubated with 5-HPETE the leukotriene A_4 hydrolysis products were the major compounds formed (80–90 %), and only small amounts (5–20 %) of double oxygenation products were detected. In contrast, with the 15-lipoxygenating quadruple mutant the product mixture was the other way around. With this enzyme the double oxygenation products (5*S*,15*S*)- and (5*S*,12*S*)-DiH(P)ETE were the major reaction products, and leukotriene A_4 was only formed in small amounts (7–15 %). However, since the quadruple mutant converted 5-HPETE much faster than the wild-type enzyme the overall yield of leukotriene A_4 formation was similar with the two enzyme species.

Optimizing arachidonate lipoxygenating species for biotechnological applications

Current knowledge on the structural determinants of the active site of plant and mammalian LOXs was summarized in Section 15.1.3. This information may be helpful to design stable LOX species exhibiting a desired positional specificity for biotechnological application. Moreover, it may be possible to optimize the biochemical properties of LOXs already being used for such purpose. The physiologically most relevant mammalian LOX isoforms are the 5-LOXs, which catalyze the initial steps in leukotriene biosynthesis. Leukotrienes are important mediators of hyperergic and inflammation disease (Samuelsson et al., 1987) and inhibitors are already available as anti-asthmatic drugs. Although mammalian 5-LOXs have been purified from natural and recombinant sources, the purified enzymes are unstable and difficult to handle (Ford-Hutchinson et al., 1994). Moreover, they require a set of essential cofactors such as Ca^{2+}, ATP and phospholipid vesicles for maximal activity. To circumvent these problems several plant arachidonate 5-LOXs have been tested whether they constitute as a suitable model for the physiologically more relevant mammalian enzymes. Among plant 5-LOXs only the potato tuber enzyme converts

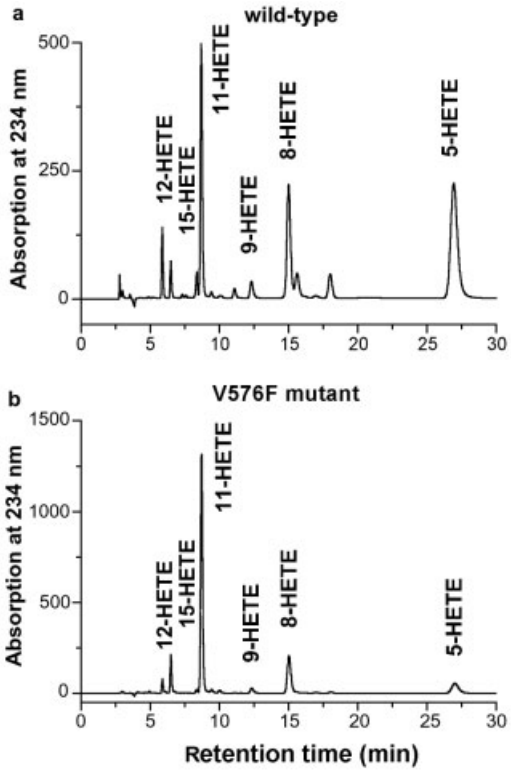

Figure 10. Straight-phase HPLC analysis of hydroxy polyenoic fatty acids formed from AA by the potato tuber LOX and its V576F mutant.

AA with acceptable yields (Reddanna et al., 1990). This enzyme does not require essential co-factors, and is stable over months in suspension without a significant loss in activity when stored at −20 °C (Reddanna et al., 1990). Unfortunately, the positional specificity of this enzyme, as that of other plant LOXs (Feussner and Kühn, 1995), is less stringent than that of the human enzyme. In fact, it converts AA to a mixture of 11-, 8- and 5-HETE in a ratio of 1:1:2 (Figure 10, wild-type). Since the amino acid sequence of the potato tuber LOX is available (Geerts et al., 1994), it should be possible to alter the positional specificity by site-directed mutagenesis to make the product pattern more specific. As one example of our approach we created the V576F mutant of this enzyme (V576 aligns with F353 of the rabbit 15-LOX) and obtained a highly specific product pattern of AA oxygenation (Figure 10). Moreover, this mutation did not affect other biochemical parameters of the enzyme.

15.3.3 Alteration of positional specificity by modifying the physico-chemical state of LOX substrates

The positional specificity of LOXs is not an absolute enzyme property, but depends heavily on how the enzyme interacts with the substrate. This interaction is influenced by a variety of factors such as substrate concentration (Kühn et al., 1990a), the physico-chemical state of the substrate (Began et al., 1999), pH (Gardner, 1989) or temperature (Georgalaki et al., 1998), but may also depend on the structures of both, enzyme and substrate. Since LOX substrates have a limited water solubility, aqueous preparations constitute a complex mixture of monomers, acidic soap dimers and higher molecular structures such as uni- and/or multilaminar micelles or liposomes. In addition, ionic and/or nonionic detergents are frequently used for LOX assays to increase the availability of the substrate, and these detergents even increase the complexity of the substrate suspension (Lopez-Nicolas et al., 1994; 1997a,b; Began et al., 1999). Under physiological conditions, polyenoic fatty acids are bound predominantly to membranes (Glickman and Klinman, 1995), and this association may also impact the reaction characteristics.

It has been shown recently that it is possible to change the positional specificity of the soybean LOX-1-catalyzed reaction by altering the physico-chemical state of the fatty acid substrate (Began et al., 1999). Therefore, polyenoic fatty acids were inserted into phosphatidylcholine micelles with their tail groups buried inside, and the authors showed that these modified fatty acids were better substrates for soybean LOX-1. With Tween 20-solubilized LA the enzyme had an alkaline pH optimum and it exclusively formed the (13*S*)-hydroperoxy derivative (Gardner, 1989). However, with LA or AA inserted into phosphatidylcholine micelles, LOX-1 synthesized exclusively the (9*S*)-hydroperoxy- or (5*S*)-hydroperoxy derivative, respectively, and this transformation was no longer dependent on the pH value. Thus, LOX-1, could utilize polyenoic fatty acids bound to membranes as physiological substrates, and it utilized the carboxylic head group of the fatty acid inserted in the phosphatidylcholine micelles as a recognition site for 9-lipoxygenation. This was confirmed by activity measurements using the fatty acid methyl esters as substrates.

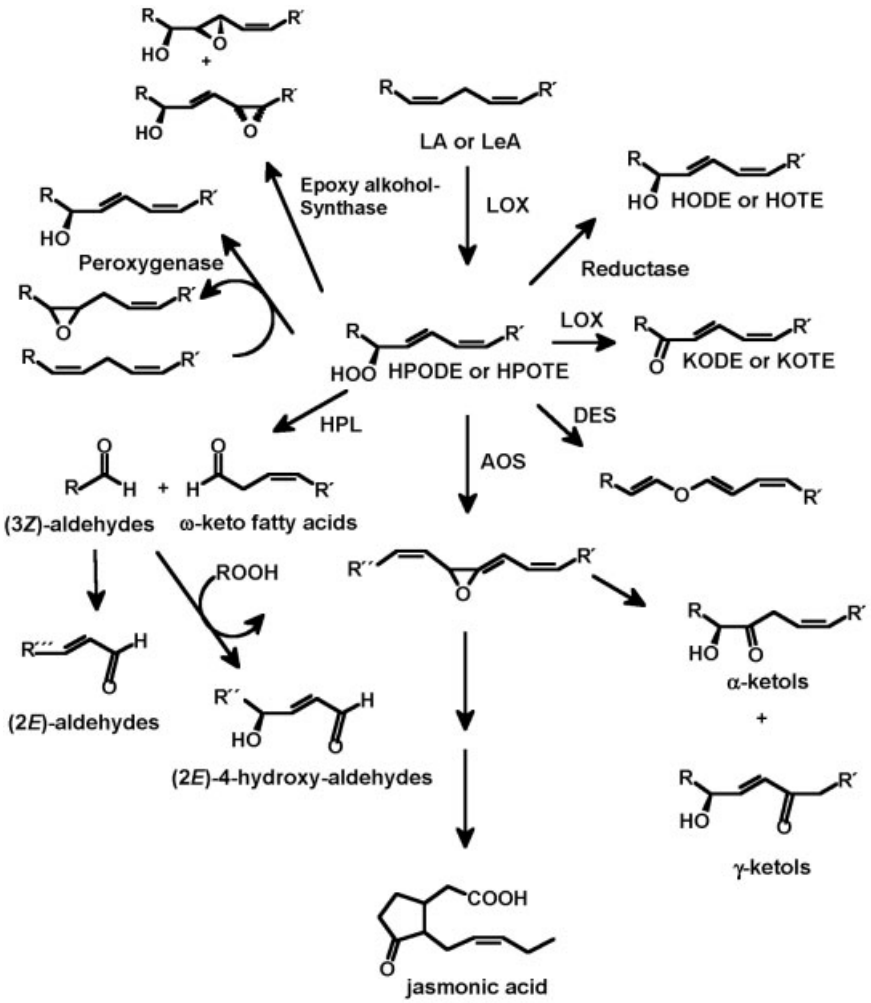

Figure 11. Formation of oxylipins in plants by the LOX pathway.

In addition, it has been shown for soybean LOX-1 that its positional specificity against LA is affected by a pH-dependent mechanism (Gardner, 1989). By contrast, the (13*S*)-hydroperoxy derivative is formed at all pH values, though in the presence of Tween 20 (possibly by shielding the carboxylate group at neutral or acidic pH-values), 9-lipoxygenation was not observed above pH 8.5 and it increased linearly when the pH was shifted towards more acidic values. From these data it was concluded that the (9*S*)-hydroperoxy derivative is formed only from the nonionized carboxylic acid form of LA, and methyl esterification of LA blocked the formation of the (9*S*)-hydroperoxy derivative by LOX-1, but not that of the (13*S*)-hydroperoxide. Hence it was suggested that 9-lipoxygenation occurs only when the fatty acid substrate is aligned in a head-to-tail orientation compared to 13-lipoxygenation (see

Figure 3B) by interacting with a basic residue at the bottom of the substrate-binding cleft (see Figure 3B, residue B).

15.4 Formation of oxylipins with plant enzymes

In plants, LOX-derived hydroperoxy fatty acids are further metabolized via several pathways (Figure 11), leading to a diversity of fatty acid derivatives functionalized by oxygen (Hamberg, 1995; Blee, 1998b). These metabolites are collectively called oxylipins (Blee, 1998b). The different pathways of oxylipin formation are named according to the major enzyme involved, and some of these metabolic routes have already been well characterized:

- The hydroperoxide lyase pathway (HPL), which involves an oxidative cleavage of hydroperoxy fatty acids to short-chain aldehydes and keto fatty acids (C_{12}- or C_9-ω-keto compounds) (Matsui, 1998).
- The allene oxide synthase pathway (AOS), leading to the formation of an unstable allene oxide intermediate, which subsequently is either hydrolyzed nonenzymatically to yield α- or γ-ketols, or is metabolized to the plant hormone jasmonic acid (Wasternack and Parthier, 1997).
- During the peroxygenase pathway (POX), hydroperoxy fatty acids are converted to epoxy-, epoxyhydroxy-, dihydroxy-, or polyhydroxy fatty acids. This conversion of fatty acid hydroperoxides is paralleled by a co-oxidation of unsaturated fatty acids (Blee, 1998a).

In addition to these well-characterized pathways, there are other metabolic routes which include the formation of divinyl ethers (DES) (Grechkin, 1998), the formation of epoxy hydroxy fatty acids by an epoxy alcohol synthase (Hamberg, 1999), and the reduction of hydroperoxy fatty acids to corresponding hydroxy fatty acids (Feussner et al., 1997b).

Unfortunately, our knowledge on the biochemical characteristics of the enzymes involved in these pathways is rather limited. Only some of them (AOS and HPL) have been purified and/or cloned to date (Song and Brash, 1991; Song et al., 1993; Laudert et al., 1996; Matsui, 1998). On the other hand, much experimental data are available as to the chemistry of the reactions involved, and some of them have recently been reviewed (epoxy-, dihydroxy-, epoxyhydroxy- or polyhydroxy fatty acids) (Gardner, 1997). In addition, a few reports described the use of POX for formation of epoxy fatty acids in nonaqueous media (Piazza et al., 1999) and immobilized HPL for the formation ω-keto fatty acids and dicarboxylic fatty acids of odd and even chain length (Nunez et al., 1997a,b; 1999).

15.5 References

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16 Properties and Applications of Lipoxygenases

Gilles Iacazio and Dominique Martini-Iacazio

Abbreviations

13(*S*)-H(P)ODE
(9*Z*,11*E*,13*S*)-13-(hydroperoxy)hydroxy-9,11-octadecadienoic acid
15(*S*)-H(P)ETE
(5*Z*,8*Z*,11*Z*,13*E*,15*S*)-15-(hydroperoxy)hydroxy-5,8,11,13-eicosatetraenoic acid
5(*S*),15(*S*)-diH(P)ETE
(6*E*,8*Z*,11*Z*,13*E*,5*S*,15*S*)-5,15-di(hydroperoxy)hydroxy-6,8,11,13-eicosatetraenoic acid
5(*S*)-H(P)ETE
(6*E*,8*Z*,11*Z*,14*Z*,5*S*)-5-(hydroperoxy)hydroxy-6,8,11,14-eicosatetraenoic acid
8(*S*),15(*S*)-diH(P)ETE
(5*Z*,9*E*,11*Z*,13*E*,8*S*,15*S*)-8,15-di(hydroperoxy)hydroxy-5,9,11,13-eicosatetraenoic acid
9(*S*)-H(P)ODE
(10*E*,12*Z*,9*S*)-9-(hydroperoxy)hydroxy-10,12-octadecadienoic acid
9(*S*)-H(P)OTE
(10*E*,12*Z*,15*Z*,9*S*)-9-(hydroperoxy)hydroxy-10,12,15-octadecatrienoic acid

AA	arachidonic acid
BSLOX	barley seed lipoxygenase
DCC	dicyclohexylcarbodiimide
DHA	docosahexaenoic acid
DMAP	dimethylaminopyridine
EPA	eicosapentaenoic acid
HPOD	hydroperoxide
LA	linoleic acid
LnA	α-linolenic acid
LOX	lipoxygenase
PUFA	polyunsaturated fatty acid
SBLOX-1	soybean lipoxygenase-1
TMSTr	trimethylsilyltriflate
TPP	triphenylphosphine

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16.1 Introduction

Lipoxygenases (LOXs) are nonheme iron dioxygenases (Galliard and Chan, 1980; Vliegenthart and Veldink, 1982; Vick and Zimmerman, 1987; Siedow, 1991; Gardner, 1991; 1996; Yamamoto, 1991; Ford-Hutchinson et al., 1994; Lehman, 1994; Kühn and Thiele, 1995; Piazza, 1996) which catalyze the stereospecific incorporation of dioxygen into the 1,*Z*-4,*Z* pentadienyl system of polyunsaturated fatty acids (PUFAs) to generate optically active conjugated dienic hydroperoxides (Figure 1).

LOXs are ubiquitous eukaryotic enzymes found in yeasts (Shechter and Grossman, 1983), filamentous fungi (Matsuda et al., 1978), macroscopic fungi (Grosch and Wurzenberger, 1984), algae (Hamberg and Gerwick, 1993), bryophytes (Matsui et al., 1991), vascular plants (Siedow, 1991), corals (Bundy et al., 1986), fishes (Hsieh et al., 1988) and mammals (Yamamoto, 1991). Generally speaking, LOXs display a very high regiospecificity (position of oxygenation) and a very high stereospecificity (configuration of the two conjugated double bonds and absolute configuration of the formed asymmetric carbon bearing the hydroperoxide function). LOXs are classified with respect to their regiospecificity using either linoleic acid (LA) with vegetal LOXs or arachidonic acid (AA) with other LOXs. For example soybean LOX isoenzyme-1 (SBLOX-1) is classified as a 13-LOX, whereas potato tuber LOX is classified as a 9-LOX when LA is used as substrate (Figure 2).

It should be noted that despite an opposite regiospecificity, the two enzymes yield both a hydroperoxide (HPOD) of *S* absolute configuration, bearing a 2*E*,4*Z* conjugated dienic system.

With AA as substrate, various positions could be oxygenated depending on the regiospecificity of the enzyme used. The most encountered LOXs are 5-, 8-, 12- and 15-LOXs. It should be stressed that a 13-LOX (LA) will be classified as a 15-LOX (AA), one speaks of a (n-6)-LOX, and that a 9-LOX (LA) will oxygenate AA mainly at the 5 position (5-LOX).

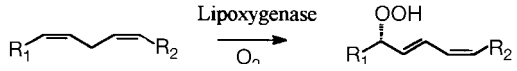


Figure 1. Reaction catalyzed by LOXs.

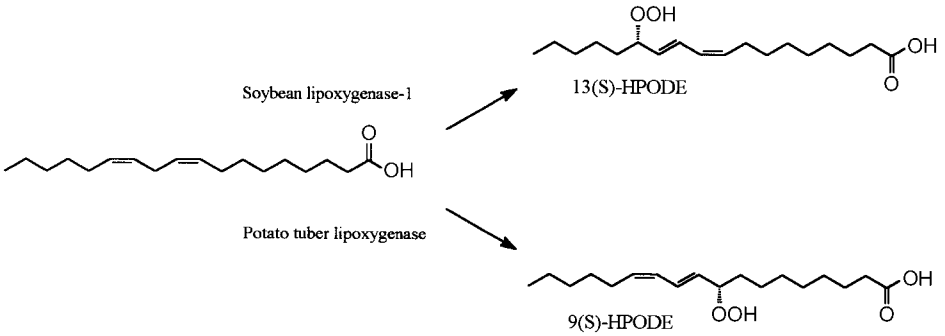


Figure 2. Opposite regiospecificities shown by two plant LOXs with LA as substrate.

PUFA-HPODs are potentially cytotoxic compounds as they can induce radical chain reactions that are deleterious to living organisms. In eukaryotes, PUFA-HPODs are catabolized by various pathways, leading to more stable products. Furthermore, these metabolites generally possess extremely important physiological roles such as jasmonic acid (Hamberg and Gardner, 1992) in plants or leukotrienes (Maycock et al., 1989) and lipoxins (Serhan, 1994) in mammals.

16.2 How to use lipoxygenases

16.2.1 The reaction catalyzed by lipoxygenases

As described in Section 2.1, the reaction catalyzed by LOXs is generally highly specific, leading to near-optically pure PUFA-HPODs. As the total chemical synthesis of such HPODs is an extremely difficult task, lipoxygenation of PUFAs is the most obvious and simple way to obtain access to such compounds. Although this is an example of where enzymatic catalysis has overshadowed chemical synthesis, the catalytic properties of LOXs must nevertheless be taken into account when performing enzymatic synthesis of HPODs. In the following, examples of how to use LOXs on a synthetic scale based on their catalytic properties will be discussed. As only one LOX is available commercially (i.e., SBLOX-1) our knowledge of LOXs is derived mainly from studies dealing with that isoenzyme. It is the consideration of the authors that the conclusions drawn from SBLOX-1 are broadly applicable to other LOXs.

Perhaps one of the most serious drawbacks in the use of LOXs on a preparative scale has been the low solubility of PUFAs in an aqueous environment. Indeed, it has been shown that LA has a critical micellar concentration of $1.5 \cdot 10^{-4}$ M in borate buffer (Lagocki et al., 1976). Above this concentration a strong substrate inhibition has been noted, showing that free fatty acids are the true substrates of LOXs (Galpin and Allen, 1977). The addition of a co-solvent (ethanol) or surfactants (Tween, Triton) has long been used to improve the solubility of the substrate to $5 \cdot 10^{-3}$ M. At this point of the discussion it should also be stressed that the presence of the product of the reaction (i.e., PUFA-HPODs) is necessary in order for the enzyme to be fully active (Jones et al., 1996). Descriptions of how the above-mentioned problems have been solved to allow the enzymatic synthesis of PUFA-HPODs to be conducted on a multigram scale, and at high substrate concentration (up to 0.1 M), will be discussed in the following text.

16.2.2 First examples of synthetic uses of soybean lipoxygenase-1

The enzymatic production of 15(*S*)-HPETE from AA and SBLOX-1 was first studied in 1975, using a buffered system and a substrate concentration of $1.6 \cdot 10^{-3}$ M (1 g in 2 L), together with gentle bubbling of oxygen (Baldwin et al., 1975). After reduction (with $NaBH_4$), 15(*S*)-HETE was obtained in 45–50 % yield. This was the first report

dedicated specifically to the enzymatic synthesis of PUFA-HPODs and, as stated by the authors, this work represented a major improvement since it was the first time that hundreds of milligram of such an HPOD had been synthesized, even if the yields were modest and the substrate concentration quite low.

Seven years later, Laakso described the continuous production of 13(*S*)-HPODE and 15(*S*)-HPETE using immobilized SBLOX-1 covalently coupled to agarose (Laakso, 1982). A buffered solution of substrates (10^{-4} M) was passed through a reactor column filled with the linked enzyme at a flow rate of 11 mL h^{-1}. Productivity of the system was about 0.6 mg of HPOD mL^{-1} of wet gel per hour, and when operating at 25 °C for a period of 125 h, no loss of productivity was observed.

Despite the value of these two examples, lipoxygenation at that time still had problems of substrate solubility, and this led to a need for high reaction volumes. However, following these pioneer studies, various methods have been developed in order to allow the use of higher substrate concentrations.

16.2.3 Oxygen pressure

In, 1990, Iacazio et al. described the use of SBLOX-1 under positive oxygen pressure at high LA concentration, using a Schlenck tube as a reaction vessel (Iacazio et al., 1990). To 10 mL of borate buffer ($Na_2B_4O_7 \cdot 10H_2O$; 0.1M; pH 9.5) were added 280 mg of LA (1 mmol, 10^{-1} M) and 20 mg of enzyme. The temperature was kept at 0 ∼ 4 °C, and the reaction started by pressurization of the reactor (4 bar of pure oxygen) and vigorous stirring of the reactants. At the beginning of the reaction the substrate was mainly undissolved, but as the reaction proceeded, the reaction mixture cleared and was perfectly limpid after 10 min. This indicated a rapid solubilization of the substrate and after 1 h, an 80 % yield of HPOD was achieved. After reduction (using $NaBH_4$) and normal and chiral-phase HPLC analysis, the distribu-

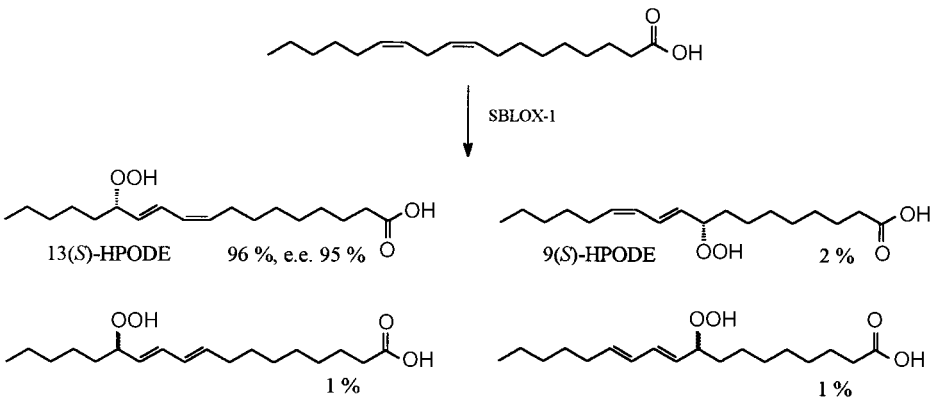
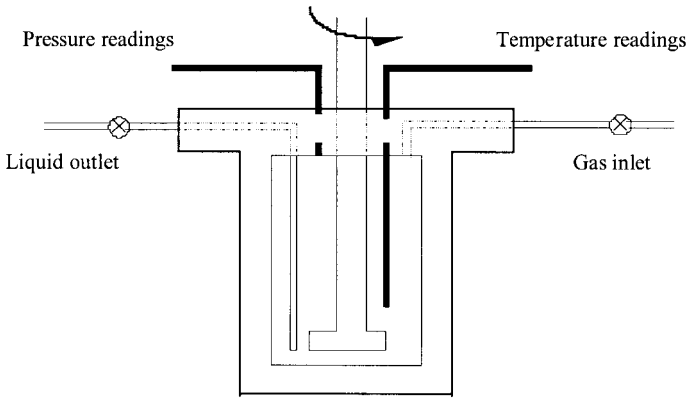


Figure 3. Isomeric distribution of linoleic acid HPODs shown by SBLOX-1 at high substrate concentration under oxygen pressure (Iacazio et al., 1990).

tion of the various isomers was determined (Figure 3), 13(*S*)-HPODE being the major product of the reaction (96 %, 95 % ee).

Four years later the use of a much more sophisticated stainless steel reactor (Scheme 1) was described (Martini et al., 1994). The reactor (MU 4004), which was provided by SOTELEM (Rueil Malmaison, FRANCE), was originally designed as a chemical reactor, and had a working capacity of 100 mL, a working temperature up to 150 °C, and a working pressure range of 0 to 200 bar.



Scheme 1 SOTELEM MU 4004 stainless steel reactor used for lipoxygenation (Martini et al., 1994).

The vessel proved to be particularly suited to carry out lipoxygenation reactions, and allowed detailed studies to be made of the influence of different parameters on the reaction, especially oxygen or air pressure, temperature, pH and substrate and enzyme concentrations. LA was chosen as substrate, the agitation speed was set at maximum (1600 rpm), and 30 mL of borate buffer (0.1 M) was used as solvent.

Each parameter was studied separately, and in each case the kinetics of HPOD formation were followed by UV spectroscopy. At the end of the reaction the HPODs formed were reduced and the distribution of each isomers determined by normal-phase HPLC. The enantiomeric excess of the major product of the reaction (13(*S*)-HPODE) was also determined by chiral-phase HPLC. From this study, the following optimal values were determined: oxygen pressure: 2.5 bar (1, 2.5, 5, 10, 25, 50, 100 bar), temperature 5 °C (5, 15, 25, 35 °C), pH: 11 (7, 8, 9, 10, 11), substrate concentration: 0.1 M (0.05, 0.1, 0.2 M), and enzyme concentration 4 mg mL^{-1} (0.5, 1, 2, 4 mg mL^{-1}). It should also be noted that in each case, the best yields in HPOD was correlated with the highest selectivity in 13(*S*)-HPODE. Thus, it could be concluded that deviation from the optimal catalytic activity of the enzyme resulted in a lower selectivity. All optimal values of each parameters were then grouped in a single experiment. After 30 min of reaction, a maximum yield of 99 % in HPOD (UV determination) was reached, with 13(*S*)-HPODE being the major isomer formed (95.5 %, 98 % ee).

A major feature of these experiments was that only low oxygen pressures were needed to conduct high-yield lipoxygenation. This prompted the authors (Martini et al., 1994) to use a microbial fermentor for the oxygenation of LA on a 100-fold greater scale (0.1 mol). This was applied to the chemo-enzymatic synthesis of a natural product, (+)-coriolic acid (13(*S*)-HODE). A 2-L Biolafitte (St. Germain en Laye, France) fermentor was filled with 950 mL of borate buffer (pH 11) and maintained at 5 °C. LA (28 g, 0.1 mol) dissolved in ethanol (50 mL) was then added, together with 4 g of SBLOX-1. The reaction was started by pressurization of the fermentor (1.5 bar of O_2) and mixing (1000 rpm). The reaction was completed in 30 min (95 % yield), and the HPODs reduced and purified on a medium-pressure chromatography column (silica gel). Depending on the fractionation, (+)-coriolic acid (97 % ee) was obtained nearly pure (99 %) in 54 % yield or as an isomeric mixture (96.5 %) in 78 % overall yield. This final experiment showed that lipoxygenation could be conducted, using readily available vessels, on a 0.1 mol scale for the multigram synthesis of (n-6)-(*S*)-PUFA-HPODs and their corresponding alcohols; moreover, this was a process that could easily be scaled-up.

16.2.4 Oxygen bubbling

As an alternative method to 'under-pressure lipoxygenation', the group of Roberts developed a practical method involving common vessels, such as Erlenmeyer flasks, combined with oxygen bubbling and magnetic agitation in order to perform a correct oxygenation of the reaction mixture (Maguire et al., 1993). By using a 2-L conical flask, over-foaming was avoided, and 208 mg of LA placed in 200 mL of buffer was oxygenated by 17 mg of crude SBLOX-1. After 1 h the reaction was stopped, and the formed HPODs reduced, leading to the formation of (+)-coriolic acid in 75–80 % yield. This method was easy to perform, and allowed the generation of hundred of milligrams of PUFA-HPODs of high purity with low-cost vessels and equipment.

16.2.5 Use of a biphasic system

As PUFAs are poorly soluble in aqueous media, the use of a biphasic system has been tested, the substrate being dissolved in an organic solvent (Drouet et al., 1994).With SBLOX-1 as catalyst and LA as substrate, a 8 : 1 borate buffer:octane ratio was shown to be the most appropriate, and substrate concentrations in the range 10–40 g L^{-1} have been tested. The final yield in HPODs varied from 78 % to 30 % as LA concentration increased. At 10 g L^{-1}, 13(*S*)-HPODE was the major isomer formed (92 %). In this work, the need for strong agitation and oxygen bubbling was also claimed to achieve satisfactory yields in HPODs. As for the previous method, this technique was well suited for the generation of hundred of milligrams of PUFA-HPODs.

Other methods such as microemulsion systems (Piazza, 1992) have been developed to overcome the low solubility of PUFAs in water, but do not offer great benefits over the other methods described in Sections 16.2.3 – 16.2.5.

16.2.6 Soybean lipoxygenase-1 use: some conclusions

It has long been the impression of workers in the field of LOXs that to conduct lipoxygenation, the PUFA substrate must be dissolved in the reaction medium. However, based on studies performed on 'under-pressure lipoxygenation', this is clearly not the case, as LA concentrations as high as 0.1 M could be used. At these concentrations, we are dealing with dispersed 'pieces of soap' rather than with dissolved substrates. Nevertheless, a near-quantitative conversion of the PUFA in HPOD could be achieved, a finding which could be explained as follows. It is necessary to maintain a sufficient quantity of dissolved oxygen in the medium to allow an optimal catalytic activity of the enzyme during the entire course of the reaction, thus lowering substrate inhibition (Berry et al., 1997; 1998). Indeed, it is well known that in the absence of oxygen, SBLOX-1 can catalyze an anaerobic reaction between its substrate and its product. This reaction is thought to generate radicals that are deleterious to the enzyme, leading to its irreversible inactivation. Thus, correct oxygenation of the reaction medium is vital in order to conduct high substrate concentration lipoxygenation. As seen in Sections 16.2.2 and 16.2.3, this can be achieved either by oxygen bubbling or pressurization combined with strong agitation. Hence, while correct oxygenation is maintained, the enzyme will maintain its optimal catalytic activity, with side chain reactions being reduced and product specificity enhanced. Another key to the success of high substrate concentration lipoxygenation is the finding that the hydroperoxides formed act as surfactants, thus solubilizing increasing amounts of substrate as the reaction proceeds. The optimal basic pH (9 – 10) of SBLOX-1 is another advantage, as fatty acids are more soluble as salts in aqueous environments.

High substrate concentration lipoxygenation is now a well-established method for the synthesis of n-6(*S*)-PUFA-HPODs, on a multigram scale using SBLOX-1 as catalyst, as long as a sufficient oxygenation is maintained during the course of the reaction. In the following Sections, the diversity of substrate and product specificities shown by LOXs that render them attractive biocatalysts in the lipid biotechnological field will be discussed further.

16.3 Substrate and product specificities of lipoxygenases

16.3.1 Soybean lipoxygenase-1 (SBLOX-1)

Substrate specificity of SBLOX-1

The publication in 1967 by Hamberg and Samuelsson was a landmark in the field of LOXs (Hamberg and Samuelsson, 1967). Indeed, this work led to a number of important conclusions being made on SBLOX-1 substrate and product specificities, and on the stereochemical outcome of the reaction catalyzed by this enzyme. By testing different natural PUFAs as well as various synthesized unnatural analogs with varying chain lengths and double bond positions, Hamberg and Samuelsson came to the conclusion that "the structural requirement for substrates of SBLOX-1 is the presence of a *cis*,*cis*-1,4-pentadiene group with its methylene group located in position ω-8". This study indicated that such substrates would be oxygenated if the chain length were between C_{18} and C_{22}, and that substrates with an additional double bound located either in ω-3 (LnA) or in ω-9 (γ-linolenic acid) position would be oxygenated by SBLOX-1 as well as heavily unsaturated fatty acids such as AA ($C_{20:4}$), eicosapentaenoic acid (EPA,$C_{20:5}$) and docosahexaenoic acid (DHA,$C_{22:6}$). If a methyl group was branched in ω-6 position, or if the pentadiene system was located either in ω-8 or ω-9 position, then no oxygenation occurred. The authors showed the enzyme to be n-6-specific, and that the resulting asymmetric carbon, bearing the hydroperoxide function, was of the *S* absolute configuration. By using tritium labeling, they also established that an antarafacial relationship existed between the attack of oxygen and the stereospecific removal of the pro *S* hydrogen atom of the methylene group of the pentadienyl system of the substrate.

The oxygenation by SBLOX-1 of various positional isomers of LA, in which the methylene interrupted *Z*,*Z*-pentadienyl system occurs from the 2,5- to the 14,17-position (Figure 4) was studied two years later by Holman et al. (1969). These workers noticed two maxima of activity corresponding to the natural substrate, LA and to the 13*Z*,16*Z* isomer (∼ 50 % of LA activity). It was later shown that the formed 17-HPOD was of the *S* absolute configuration (Egmond et al., 1975). Hence, some flexibility in the position of the pentadienyl system was tolerated by SBLOX-1, a fact confirmed later by Kühn et al. (1990) using a series of AA isomers that possessed a 1*Z*,4*Z*,7*Z*,10*Z*-undecatetraenyl system located at either ω-3, ω-4, ω-5, ω-6 or ω-7 position (Figure 5).

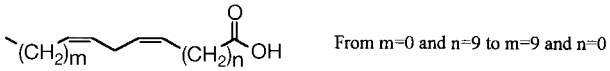


Figure 4. Oxygenation of various positional isomers of linoleic acid catalyzed by SBLOX-1 (Holman et al., 1969).

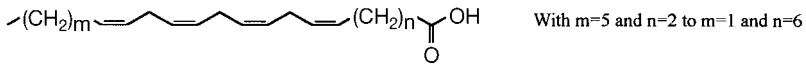


Figure 5. Oxygenation of various positional isomers of AA catalyzed by SBLOX-1 (Kühn et al., 1990).

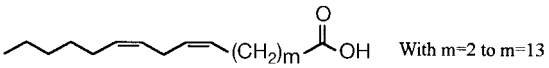


Figure 6. Oxygenation of a series of (ω6Z,ω9Z)-$C_{13} \sim C_{24}$ dienoic acids catalyzed by SBLOX-1 (Matsui et al., 1992).

The two substrates (ω-3 and ω-6) with a pentadienyl system positioned at the ω-6 position were oxygenated by SBLOX-1 at the highest rate, while the other three substrates were oxygenated at 20 % (ω-7), 25 % (ω-5) and 60 % (ω-4) of that of AA (ω-6).

The influence of the chain length of substrates bearing a 1*Z*,4*Z* pentadienyl system located at the ω-6 position was also studied in detail (Matsui et al., 1992) using an entire series of (ω6Z,ω9Z)-$C_{13} \sim C_{24}$ dienoic acids and SBLOX-1 (Figure 6). It was shown that the rates of oxygenation of the C_{18}, C_{19}, C_{20} (maximum) and C_{21} dienoic acids were the highest. For C_{17} and C_{22} dienoic acids, the rates of oxygenation were 60 % of that of LA (C_{18}), while those of C_{16}, C_{23} and C_{24} dienoic acids fell between 20 % and 30 %. For lower chain lengths (C_{15}, C_{14} and C_{13}) acids, the rates of oxygenation were insignificant.

Beside needing a correct positioning of the pentadienyl system around the n-6 position, SBLOX-1 substrates must also possess an anionic ionizable group at one end of the carbon chain. While this requirement is fulfilled in natural substrates by the presence of the carboxylate group, it has been shown that a sulfate group could also be used (Bild et al., 1977a). By contrast, methyl esters (Hatanaka et al., 1984) or triglycerides of PUFA are poor substrates for SBLOX-1, leading to low yields in HPODs and a far less product specificity. Dienols synthesized by the reduction of the carboxylate group of natural PUFAs are also oxygenated at a rate lower of that of the parent compounds (20 % for example for the dienol arising from LA) and with a far less product specificity (Hatanaka et al., 1989). On the other hand, Brash and co-workers have shown that linoleic and arachidonic acids esterified in phosphatidylcholine could be oxygenated by SBLOX-1 ($\sim 20-30$ % of the rate of free acids) with no loss in product specificity (Brash et al., 1987). Finally, it should be noted that anacardic acid (Figure 7), a constituent of cashew nut shells (*Anacardium occidentale* L.) is a substrate of SBLOX-1, with dioxygen being inserted at the ω-4 position (Shoba et al., 1992).

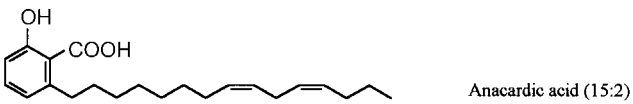


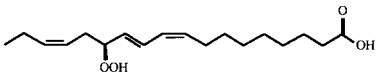
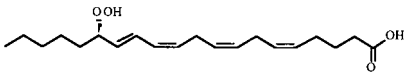
Figure 7. Anacardic acid, a substrate of SBLOX-1 (Shoba et al., 1992).

Product specificity of SBLOX-1 with natural PUFAs

Beside LA and AA being used widely as substrates of LOXs, other PUFAs such as α- and γ-linolenic acids, EPA and DHA have been studied. For example, using the 'under-pressure lipoxygenation' methodology (see Section 2.2.3), the n-6(*S*)-HPODs of α-linolenic and arachidonic acids have been synthesized (Table 1) in near-quantitative manner on a 3 mM scale (0.1 M substrate concentration) using a commercial preparation of SBLOX-1 (Martini et al., 1996a, Martini and Iacazio, 1997).

Porter et al. (1979) have also shown using γ-linolenic acid (3.29 mM) that the corresponding n-6 HPOD is formed exclusively with SBLOX-1 as catalyst (pH 9, 0 °C). When EPA or DHA are used as substrates of SBLOX-1 the same n-6 specificity is observed, but only at high pH (Takagi et al., 1987). Indeed, with these two substrates as well as with AA, pH-dependent double dioxygenations are observed. In 1977, Bild *et al*. were the first to report, using AA, such double dioxygenation catalyzed by SBLOX-1 (Bild et al., 1977b). When the reaction was conducted at high enzyme concentration and at pH 6.8 it was observed that two equivalents of dioxygen were consumed per substrate equivalent, and that the initially formed conjugated dienic monohydroperoxide (UV absorbance maximum at 238 nm) disappeared slowly, leading to a conjugated trienic chromophore (UV absorbance maximum at 260, 269, and 279 nm). The structure of the dihydroperoxide formed was 8,15-dihydroperoxy-(5*Z*,9*E*,11*Z*,13*E*)-eicosatetraenoic acid (8,15-diHPETE).

Table 1. High substrate concentration 'under-pressure lipoxygenation' of α-linolenic and arachidonic acids. (From Martini et al., 1996a; Martini and Iacazio, 1997.)

Substrate	Time (min)	Yield (%)	Product	Enantiomeric excess (%ee) (Abs. conf.)
LnA	10	> 99		> 99 (*S*)
AA	15	99		99 (*S*)

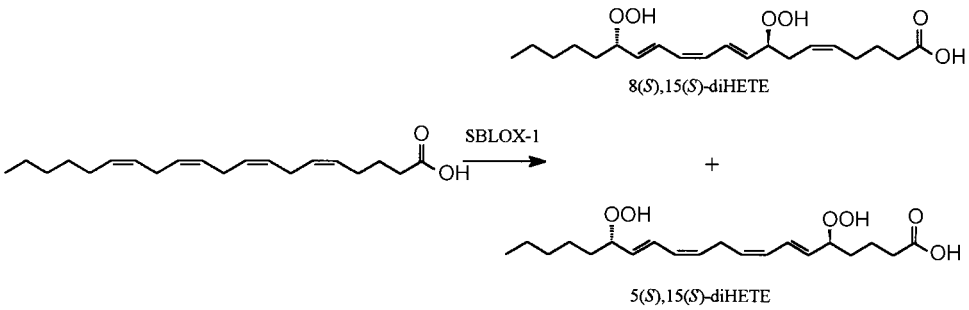


Figure 8. Double dioxygenation catalyzed by SBLOX-1 (Bild et al., 1977b; Van Os et al., 1981).

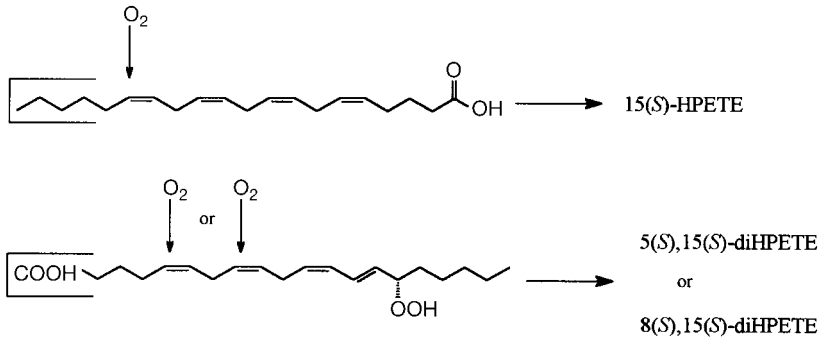


Figure 9. Substrate orientation used to explain the double dioxygenation catalyzed by SBLOX-1 (Gardner, 1991; Lehman, 1994).

Some years later, the Vliegenthart group re-examined the double dioxygenation of AA (Van Os et al., 1981). Along with the already reported formation of 8(*S*),15(*S*)-diHPETE (60 %) (Figure 8), they found another *bis*-hydroperoxide whose structure was established as 5(*S*),15(*S*)-diHPETE (40 %). A classical explanation given for this apparently forbidden double dioxygenation is the fact that at more acidic pH, the carboxylic acid function is at least transiently under its protonated form allowing an inverse orientation of the substrate in the active site of the enzyme leading to oxygenation at position 5 or 8 (Figure 9).

If the reaction is left for longer (2 h), then small amounts of conjugated tetraenic material identified as lipoxin A and B could be detected whose origin is probably a further transformation of 5(*S*),15(*S*)-diHPETE (Sok et al., 1988).

Use of unnatural PUFAs as soybean LOX-1 substrates

Despite its narrow substrate specificity, SBLOX-1 combines numerous advantages prone to seduce synthetic organic chemists (i.e. very high regio-, stereo- and enantiospecificity). Indeed, some authors have prepared unnatural substrates to take advantages of both the specificity of the enzyme and the possibilities of organic synthesis.

For example, in 1989, Zhang and Kyler synthesized a series of methylene interrupted (*Z*,*Z*)-dienic alcohols which upon esterification with adipic acid monomer became a substrate of SBLOX-1 (Zhang and Kyler, 1989). After reduction of the HPODs formed and hydrolysis, various chiral dienols were obtained which represented valuable chiral synthons (Figure 10).

All the tested compounds were substrates of SBLOX-1, the reaction times varying from 1 h (**8**) to 24 h (**7**). The substrate:enzyme ratio (w/w) used ranged from 0.06 (**1**) to 3.0 (**3**), the regiospecificity (**A**:**B**) varied from 99 : 1 (**7**,**8**) to 77 : 23 (**5**), and the enantiomeric excess of the major regioisomer (**A**) from 96 % ee (**2**) to more than 99 % ee (**3**).

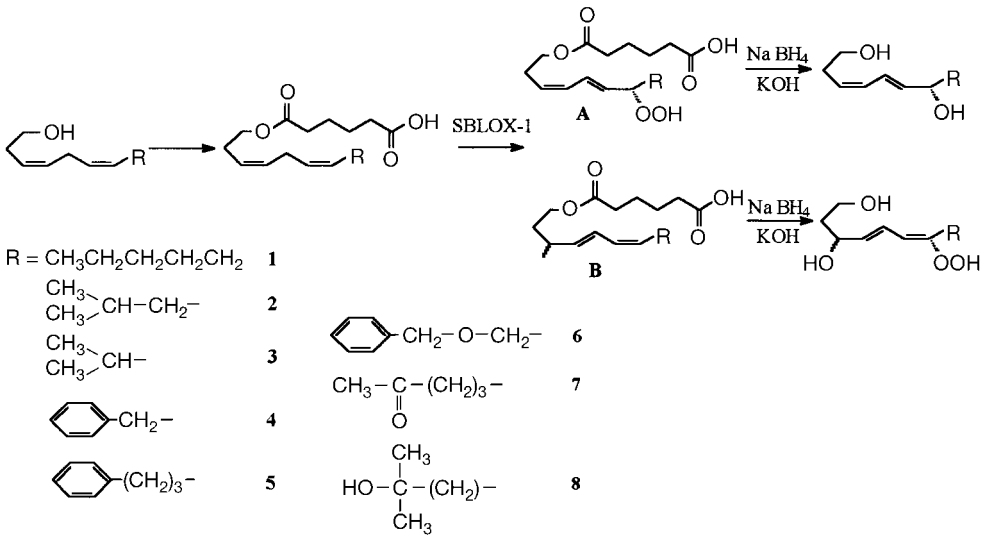


Figure 10. Action of SBLOX-1 on various unnatural substrates constructed from methylene interrupted dienic (*Z,Z*) alcohols and adipic acid (Zhang and Kyler, 1989).

Recently, we have adapted the idea of Zhang and Kyler of using adipate as a prosthetic group, but in contrast to these authors (whose aim was to conduct n-6 lipoxygenation), our goal was to realize a 5-lipoxygenation on the carbon skeleton of AA by using SBLOX-1. Such a 5-lipoxygenation would result from an inverse orientation of the substrate in the active site of the enzyme (Figure 11).

The reverse orientation of AA is clearly disfavored (Figure 11), since both pockets will be filled with a residue of opposite nature. To favor such an inverse orientation, the carboxylic function should be transformed to a more polar one (methyl ester for

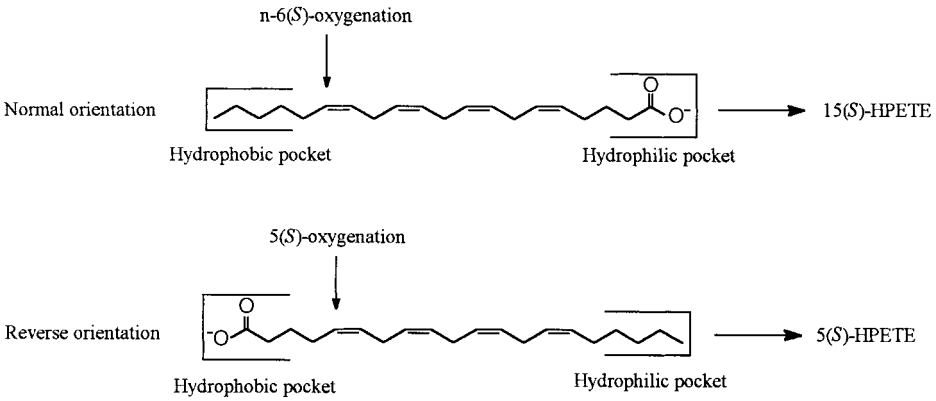


Figure 11. Regiospecificity of SBLOX-1 according to the orientation of the substrate in the active site (Martini et al., 1996a).

example) and a carboxylate ending chain should be place somewhere on the hydrocarbon chain of the substrate. To fulfill these two requirements, we used methyl 15(*S*)-HETE previously synthesized from SBLOX-1 and AA in the normal orientation followed by hydroperoxide reduction and esterification (Martini et al., 1996a). Then, by taking advantage of the 15-hydroxy functionality, we added a succinyl chain (from succinic anhydride) to methyl 15(*S*)-HETE. Thus the newly synthesized substrate **9** should be recognized by SBLOX-1 in such a way that the formal 5-oxygenation of the carbon skeleton of AA will be allowed (Figure 12).

Compound **9** is indeed a substrate of SBLOX-1, but should be used at low substrate concentration (10^{-3} M) since at higher substrate concentrations the 5-hydroperoxide formed is unstable in the reaction medium. Nevertheless, by using a 2-L fermentor, the reaction could be conducted on hundreds of milligram scale, and it was shown that the second dioxygenation performed by SBLOX-1 was essentially diastereoselective. Thus, this work represents a chemo-enzymatic synthesis of the naturally occurring 5(*S*),15(*S*)-diHETE which is obtained in 59 % yields and more than 98 % diastereoisomeric excess (Martini et al., 1996a).

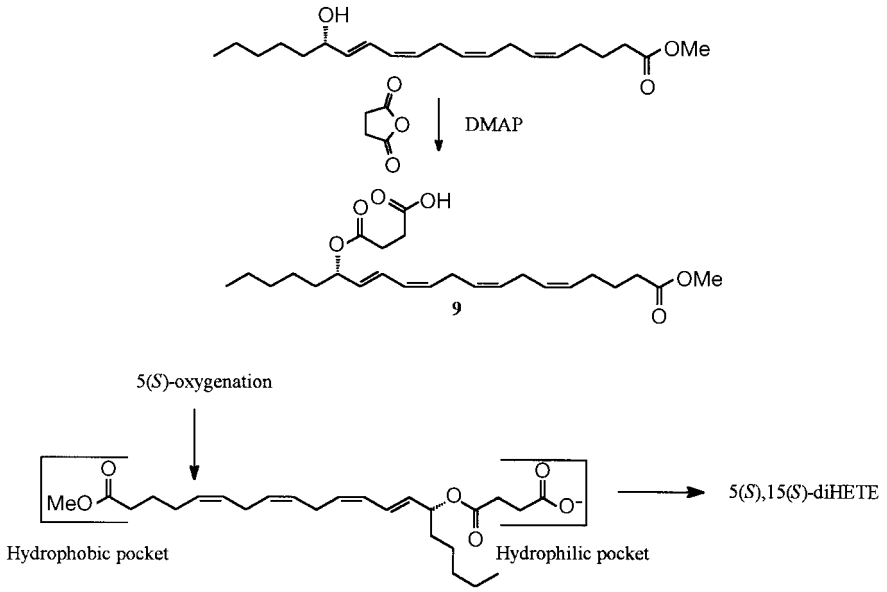


Figure 12. Synthesis of a derivative of AA prompt to be oxygenated at the formal 5 position by SBLOX-1 (Martini et al., 1996a).

16.3.2 Other lipoxygenases

Lipoxygenases 5- or 9-specific

Beside n-6 LOXs with SBLOX-1 as an archetype, 5- or 9-LOXs (depending on whether arachidonic or linoleic acids are used as substrates) are the second most studied LOXs. To obtain access to both 9-HPODE and 5-HPETE, a vegetal source of LOX was the only viable alternative since, to our knowledge, no such LOXs are available commercially at a reasonable price. The most common source of 9-LOX is potato (Mulliez et al., 1987), tomato (Regdel et al., 1994), barley seeds (Van Aarle et al., 1991) and wheat (Kühn et al., 1987), and corn germs (Veldink et al., 1972). With LA as substrate, such vegetal LOXs display a rather high selectivity in 9(*S*)-HPODE formation. However, as in the case of AA, various proportions of 8(*S*)-HPETE and 11(*S*)-HPETE have been detected, as well as 5(*S*)-HPETE (Mulliez et al., 1987; Regdel et al., 1994). Matthew and co-workers reported a rather simple method to produce 9(*S*)-HPODE. The flesh (225 g) of tomatoes and ammonium linoleate were first mixed in 750 mL of 0.1 M acetate buffer pH 5.5 (final substrate concentration 1 mM) and then incubated at 25 °C with 15 s oxygen flushing every 2 min. At the end of the reaction the yield in HPODs was 69 % with a 9- to 13-HPODE ratio of 96 : 4, and a high optical purity for the formed 9(*S*)-HPODE (Matthew et al., 1977).

Following the success of the 'under-pressure lipoxygenation' methodology (see Section 16.2.3), we were interested in using 9-LOXs to generate 9(*S*)-HPODE and 5(*S*)-HPETE to extend our procedure. We anticipated that the critical point of such a synthesis would be the optimum pH of the LOX used. Indeed, unlike SBLOX-1, 9-LOXs have acidic or neutral optimum pHs. A survey of the literature revealed that barley seed LOX combined several advantages, some of which were an optimum pH centered around 7.5, and a good disposability of the vegetal source. In order to reach a sufficient specific activity and to eliminate a hydroperoxide-degrading activity present in the seeds, the partial purification of barley seed LOX (BSLOX) was undertaken according to an existing purification procedure (Van Aarle et al., 1991). This involved only classical methods of purification which could be easily performed, and led to a BSLOX solution of 4$\sim$ 5 U mg^{-1} of protein, a specific activity which was comparable to that of commercially available SBLOX-1. Using the same methodology developed for SBLOX-1, we found the following values to be optimal using LA as substrate: borate buffer 0.1 M, pH 8, oxygen pressure 2.5 bar, temperature 5 °C, enzyme concentration 400 U per 30 mL, and substrate concentration 0.05 M. By using all these optimized values in a single experiment we were able to produce from LA and LnA, 9(*S*)-HPODE and 9(*S*)-HPOTE respectively (Figure 13) in 40 – 50 % yields, with high selectivity (more than 95 %) and very high enantiomeric excess (more than 98 % ee). The unreacted substrate could be recovered at the end of the reaction (Martini et al., 1996b).

Despite the lower substrate concentration used and a lower yield as compared with SBLOX-1, BSLOX is a useful enzyme to generate both 9(*S*)-HPODE and 9(*S*)-HPOTE with high selectivities. Unfortunately, with AA as substrate, very low yields in 5-HPETE were obtained (less than 5 %). Further work is needed to understand this failure, as 5-HPETE is a highly valuable compound, especially in human physiology, being the precursor of leukotrienes.

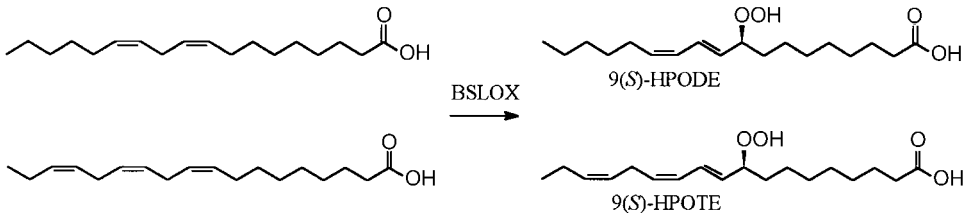


Figure 13. Oxygenation of linoleic and α-linolenic acids by barley seed LOX (Martini et al., 1996b).

With this example it could be broadly concluded that the methodologies developed for the use of SBLOX-1 could be applied to other LOXs, but that a major drawback to perform high substrate concentration lipoxygenation is the optimum pH of the enzyme used, which should be as basic as possible.

Lipoxygenases 8- or 12-specific

In relation to human physiological studies, other LOXs of various specificities such as 8-LOX or 12-LOX versus AA are important enzymes. To the best of our knowledge, and most likely because of the difficulty of collecting sufficient quantities of such enzymes as well as a lack of equivalent in the vegetal field, no work has yet been described that is dedicated specifically to the biotechnological production of 8- and 12-HPETE.

16.3.3 Chemical synthesis of conjugated PUFA hydroperoxides

As an alternative method to the enzymatic generation of PUFA-HPODs, such compounds could be generated chemically through auto-oxidation or photo-oxidation of PUFAs. In these cases, racemic mixtures of all possible HPODs regioisomers are clearly formed. As shown by Porter et al. (1979) in the photo-oxidation of AA, the various regioisomers could be separated (though not totally in some cases) by normal-phase HPLC (order of elution, 12-HPETE, 15- and 14-HPETE, 11-HPETE, 9-HPETE, 8-HPETE and 5- and 6-HPETE). The reaction could be performed on the millimolar scale (450 mg, 1.48 mg mL^{-1}) in an organic solvent, with a yield of HPOD of about 35–40 % (Porter et al., 1979). Although not realized by Porter et al., it should be mentioned that following the progress made in chiral HPLC, it may now be possible to separate enantiomers of regioisomerically pure HPODs. Hence, the combination of chemical methods and HPLC separations might represent an alternative to a bioconversion for the synthesis of small quantities of enantiomerically pure PUFA HPODs when the corresponding LOX is not easily available.

16.4 Applications of PUFA hydroperoxides in fine chemistry

16.4.1 Reduction of PUFA hydroperoxides

In living cells, PUFA-HPODs are potentially toxic compounds which may initiate radical chain reactions. They are detoxified rapidly in both plants (Gardner, 1991) and mammals (Pace-Asciak and Asostra, 1989) by various catabolic pathways, leading to physiologically active molecules such as jasmonic acid, leukotrienes, and lipoxins. They can also be reduced simply to their corresponding alcohols. In certain cases, the formed alcohol accumulates, as in the case of rice suffering from rice blast disease (coriolic acid) (Kato et al., 1984) or in seed oil of *Dimorphotheca aurantiaca* (dimorphecolic acid or 9(*S*)-HODE) (Smith et al., 1960). These two natural compounds, as well as other HPOD-derived alcohols, could be easily synthesized in a two-step chemo-enzymatic procedure. After lipoxygenation, the hydroperoxide could be reduced in the reaction medium (using $NaBH_4$ or $SnCl_2$) or after extraction (for example with diethyl ether) by triphenylphosphine (TPP). In the synthesis of both coriolic acid (Martini et al., 1994) and dimorphecolic acid (Martini et al., 1996b), the latter procedure is preferred since the reaction is clean and easy to perform, and the excess of TPP as well as its formed oxide are easily separated from the alcohol by flash column chromatography over silica.

16.4.2 Lactonization of hydroxy-acids

Coriolic acid, for example, possesses both an alcohol and a carboxylic acid function which might give rise after cyclization to a macrocyclic C_{13} lactone (Figure 14). Roberts and colleagues synthesized such a lactone (Maguire et al., 1991) from coriolic acid in 40 % yield (di-2-pyridyl disulfide, TPP, xylenes, 140 °C, 20 h), the cyclic ester being used further in a Diels-Alder reaction.

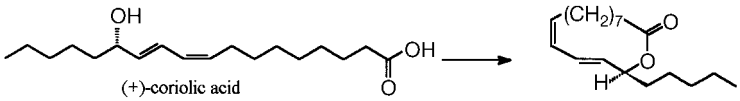


Figure 14. Lactonization of coriolic acid (Maguire et al., 1991).

16.4.3 Protection of the hydroperoxide function and obtaining of chiral synthons

In 1991, Dussault *et al*. reported a method of protection of the hydroperoxide function by acid ketalization with 2-methoxy propene (Dussault et al., 1991) Starting from enzymatically generated 13(*S*)-HPODE followed by HPOD protection, they obtained access to a valuable optically active γ-peroxy-α,β-unsaturated aldehyde (Figure 15) through selective ozonolysis.

This chiral synthon could be further used in olefination through a Wittig reaction. Indeed, various conjugated dienic hydroperoxides such as 15(*S*)-HPETE have been synthesized in this way (Dussault et al., 1991; Dussault and Lee, 1992). In a subsequent paper (Dussault and Lee, 1995) the same team reported, using the same general methodology, a very elegant synthesis of 5(*S*)-HPETE. To conduct this synthesis, synthon **10** was first produced enzymatically, and then used according to the following retrosynthetic strategy (Figure 16).

Substrate **11** was first chemically synthesized (30 % overall yield, seven steps) in order to generate **10** through SBLOX-1 oxygenation and subsequent ozonolysis (Figure 17). Access to 5(*S*)-HPETE was then completed by Wittig olefination and deprotection (34 % yield from **11**, six steps).

At the same time, a Japanese group (Baba et al., 1990) used the same perketal protection strategy to synthesize a structured hydroperoxide phospholipid contain-

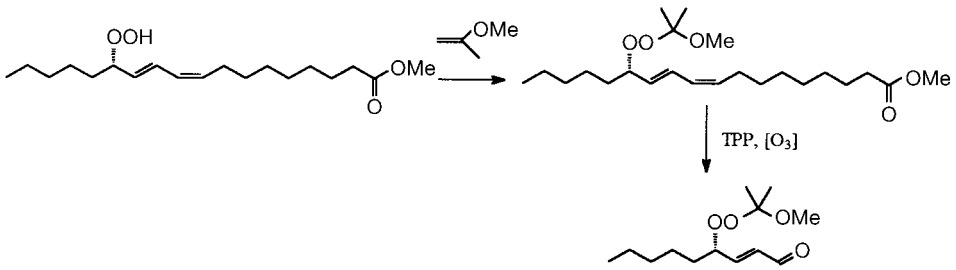


Figure 15. Protection and ozonolysis of methyl 13(*S*)-HPODE (Dussault et al., 1991).

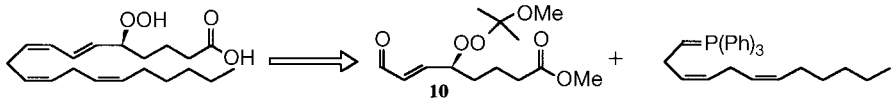


Figure 16. Retrosynthetic strategy for the chemo-enzymatic production of 5(*S*)-HPETE (Dussault and Lee, 1995).

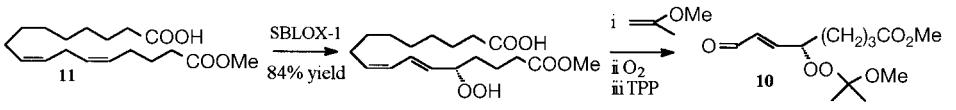


Figure 17. Chemoenzymatic synthesis of chiral synthon **10** (Dussault and Lee, 1995).

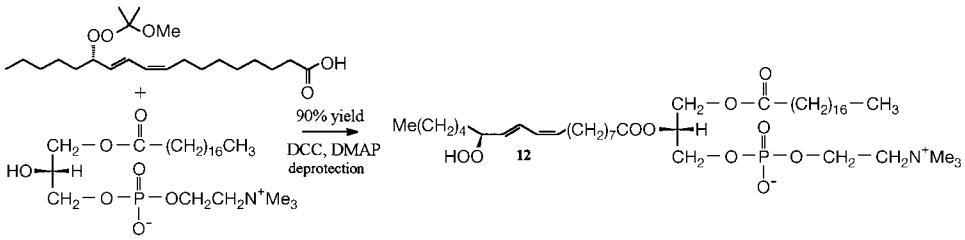


Figure 18. Last steps synthesis of the HPOD phospholipid **12** (Baba et al., 1990).

ing 13(*S*)-HPODE. 1-stearoyl-*sn*-glycero-3 phosphocholine was first generated by a chemo-enzymatic approach involving lipases. The protected HPOD was then ligated through DCC-DMAP methodology which, after deprotection, afforded the target compound **12**: 1 stearoyl-2-[13'(*S*)-hydroperoxy-(9'*Z*,11'*E*)-octadecadienoyl]-*sn*-glycero-3-phosphocholine (Figure 18).

16.4.4 Exploiting the double dioxygenation of arachidonic acid

In 1989, Corey and co-workers (Corey et al., 1989) exploited the SBLOX-1 double dioxygenation of AA to realize a simple synthesis of lipoxin A_4, an important physiologically active eicosanoid of the AA cascade. As seen previously (Section 16.3.1), the double dioxygenation affords after chemical reduction, two dihydroxy

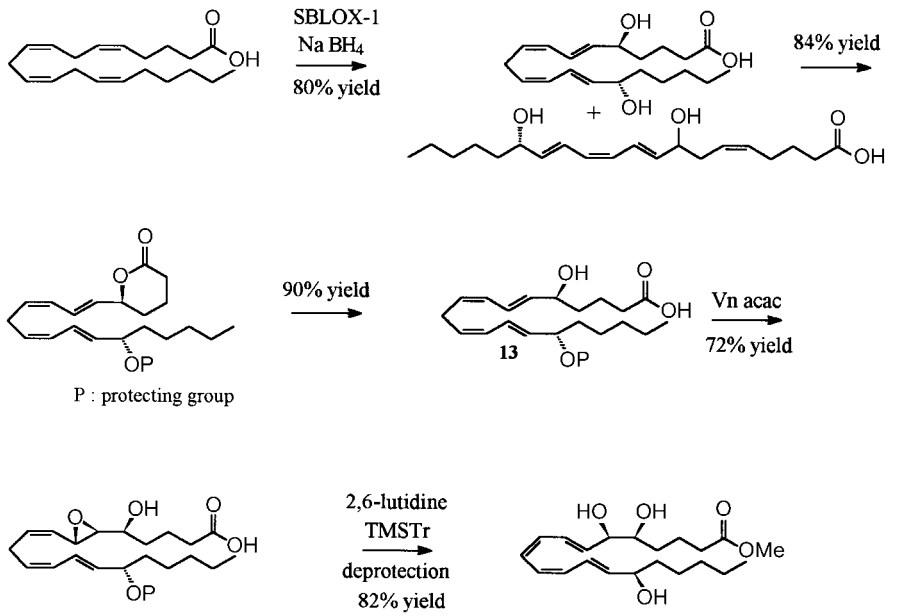


Figure 19. Chemoenzymatic synthesis of lipoxin A_4 (Corey et al., 1989).

derivatives (i.e. 8(*S*),15(*S*)-diHETE and 5(*S*),15(*S*)-diHETE) in a 65 : 35 ratio and in 80 % yield. 5(*S*),15(*S*)-diHETE was then purified from its regioisomer by a lactonization procedure, leaving 8(*S*),15(*S*)-diHETE unreacted. After purification, suitable protection and hydrolysis, compound **13** was available and further epoxidized by vanadyl acetoacetonate (Vn acac), leading to a mixture of two isomers in a 5 : 1 ratio (erythro:threo). The major isomer was then purified over silica, and transformed in conjugated tetraenoic derivative which upon deprotection afforded lipoxin A_4 (Figure 19).

16.4.5 Decomposition of PUFA hydroperoxides by metal salts

Various groups have reported the rearrangement of PUFA-HPODs using various metal salts in α,β-epoxy alcohols. For example, methyl 13(*S*)-HPODE is transformed by vanadium oxyacetylacetonate (Hamberg, 1987) into methyl 11,12-epoxy-13-hydroxy octadecadienoate, the two possible diastereoisomers being formed in equal amounts (Figure 20). The same compounds were obtained with titanium isopropoxide as catalysts (Piazza et al., 1997), the threo isomer being slightly predominant. In a later paper, Piazza et al. used nobium ethoxide as catalyst to obtain access mainly to the erythro isomer (Piazza et al., 1998).

Such transformations are important, since α,β-epoxy alcohols are natural products which result from the catabolism of PUFA-HPODs.

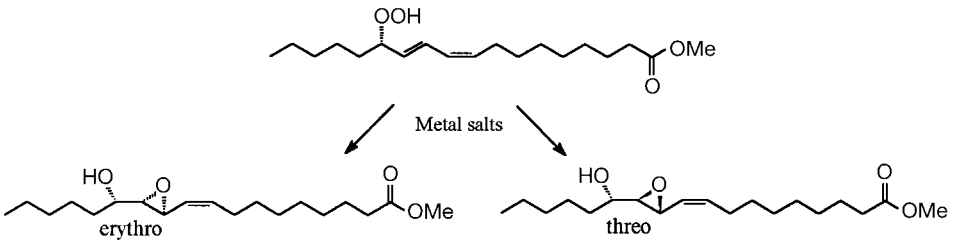


Figure 20. Action of metal salts upon PUFA-HPODs (Hamberg, 1987; Piazza et al., 1997; 1998).

16.5 Conclusions

Despite their quite narrow substrate specificity, LOXs are of interest in the field of organic synthesis because of their generally high regio-, stereo-, and enantioselectivity. This interest lies also in the fact that they catalyze the first step in the hydroperoxide catabolic pathway of PUFAs, leading to compounds of major physiological relevance in both plants and mammals.

From a biotechnological point of view, the use of LOXs is now quite well established. One of the most important points for the successful application of such enzymes is the need to maintain a sufficient quantity of dissolved oxygen in the reaction medium during the entire course of lipoxygenation. This is beneficial in terms of both product yield and product specificity, and also allows the use of high substrate

concentrations. To obtain access to n-6(*S*)-HPODs, SBLOX-1 is the enzyme of choice; this is due not only to the fact that this enzyme is by far the best studied LOX, but also is available commercially and has high specificity. To generate other PUFA-HPODs, the choice of the catalyst will be dictated mainly by its availability as well as by its optimum pH (which should be as high as possible), in order to facilitate substrate dissolution at high concentration. Gene cloning and heterologous expression may, in the future, represent a means of circumventing the low availability of LOXs from sources other than vegetal ones – something which remains a bottleneck to the wider use of such enzymes.

In addition to their use in physiological studies, PUFA-HPODs might also be employed in very various ways in organic syntheses, as exemplified in Section 2.4. Thus, the combination of chemical and enzymatic methods allows access to natural products of the linoleic and arachidonic acids cascades in a much easier manner than does total chemical synthesis. The products derived therefrom could also be used in the biotechnological production of flavors. Indeed, small molecules such as hexanal and hexenal (green note), nonenal and nonadienal (cucumber-like note), octen-1,3-ol (fungal note) are naturally generated by the catabolism of PUFA-HPODs via an enzyme called hydroperoxide-lyase (Grosch and Wurzenberger, 1984; Hatanaka, 1993). Thus, combination of these two enzymes might offer a means of producing such aromas with a natural label. It has also been shown that coriolic acid might be degraded by yeasts (Albrecht et al., 1992), through the β-oxidation pathway, to aromatic lactones such as δ-decalactone (peach aroma). These selected examples support the opinion that the field of LOXs is of major interest from both fundamental and industrial points of view.

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Miscellaneous Enzymes

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17 Enzymatic Synthesis and Modification of Glycolipids

Siegmund Lang, Christoph Syldatk and Udo Rau

17.1 Enzymatic synthesis of glycolipids

The biochemical transfer of sugars or sugar alcohols on lipids such as fatty acids or fatty alcohols may have considerable impact in the industrial production of surfactants, sweeteners, food ingredients (e.g., emulsifiers), and chemical and pharmaceutical intermediates. For instance, sucrose laurates with different degrees of esterification are currently being used in food and personal care formulations. In addition, their antitumoral, antibacterial and insecticidal properties have also been reported, thus indicating their great versatility (Kato and Arima, 1971; Nishikawa et al., 1976; Chortyk et al., 1996).

In the future, enzymatically derived glycolipids should be able to compete with well-known alkyl polyglucosides (APGs; Henkel, Düsseldorf, Germany) which are produced from renewable resources using chemical methods (Hill et al., 1997; von Rybinsky and Hill, 1998). Especially when the production conditions would be non-toxic, despite of higher-priced, biosurfactants could surpass conventional products in more high-value goods.

The use of single enzymes, lipases, proteases and glycosidases, permits the preparation of a wide range of glycolipids which can be designed to possess the desired physico-chemical properties in the final product. In addition, mild reaction conditions and the regioselectivity of sugar acylation or alkylation are advantageous to the biocatalysts. Reviews on this topic have been published by Vulfson (1993), Coulon and Ghoul (1998) and Bevinakatti and Mishra (1999), as well as by Lang and Fischer (1999).

The aim of this chapter is to describe these results in more detail, and to update our most recent findings which have been published during the past two years.

17.2 Lipase- and protease-catalyzed esterification of lipids with sugars and sugar alcohols

Using aqueous buffer systems, lipases (EC 3.1.1.3) are commonly known to hydrolyze triacylglycerols and other fatty acid esters at the lipid–water interface. The reversal reaction, acyl transfer, can be rendered possible through carrying out the biocatalysis in 'enzyme-friendly' organic solvents. In particular, those with log P values > 3 (cyclohexane, *n*-hexane, *n*-octane) favor the formation of ester linkages, minimize enzyme inactivation and enable efficient re-use of the biocatalyst. Another important feature is the water activity (a_w) of the entire reaction sys-

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tem. Bearing in mind that the conformational mobility of the enzyme is dependent on the bound-water content, the most suitable a_w values must be determined for successful reactions. Apart from these (physical) parameters which concern the solvent system, the reactands – fatty acid and sugar – often also need to be modified in order to achieve a better overall yield. For instance, it is known that the use of enol esters, acid anhydrides or activated esters of fatty acids leads to reaction rates 10^2 to 10^3 times higher than the use of free acids (Faber, 1997). With regard to the acylation positions, sugars have commonly been functionalized at primary hydroxyl groups, e.g., in 6-OH of glucose.

In general, the following reaction conditions are known from enzyme-catalyzed glycolipid production methods:

- Use of free and immobilized lipases and proteases
- Use of fatty acids (C_4–C_{22}) and of mono/disaccharides, sugar alcohols and of their respective modified forms
- Use of different organic solvents and of solvent-free systems
- Use of molecular sieves or working at reduced pressure for the removal of by-products (e.g., water or methanol)
- Reactions at 30 °C to 80 °C, for periods of 6 to 72 h

Within this chapter, studies on the esterification of fatty acids with sugars are classified into four types:

1. Lipase-mediated catalysis in organic solvents or solvent-free systems.
2. Lipase-mediated catalysis in the presence of adjuvants.
3. Lipase-mediated catalysis using hydrophobized sugars.
4. Protease-mediated catalysis in organic solvents.

17.2.1 Lipase-mediated catalysis in organic solvents or solvent-free systems

In order to produce defined carbohydrate fatty acid monoesters by lipase-mediated catalysis, the most interesting approach in economic terms would be the direct connection of the free fatty acid (or native triglyceride) with the sugar. Additionally, this should be done in the presence of harmless solvents or under solvent-free conditions. This model reaction is presented in Figure 1. With regard to the lipid reactand solubility, there are no problems in fulfilling these requirements; however, carbohydrates are poorly soluble in the common organic solvents such as *n*-octane or *n*-hexane. Rather, they are soluble in only very few hydrophilic solvents such as dimethylsulfoxide (DMSO), dimethylformamide, or pyridine. After arranging the sugar ester synthesis approaches in accordance with increasing log *P* value, Table 1 shows that some authors have solved this problem by using activated fatty acids in polar organic solvents. Log *P* value is defined as the partitioning of a given solvent between 1-octanol and water in a two-phase system (Laane et al., 1987).

Another approach consists of using intermediate-polarity solvents such as 2-methyl-2-butanol. This allows partial solubilization of both substrates in the reac-

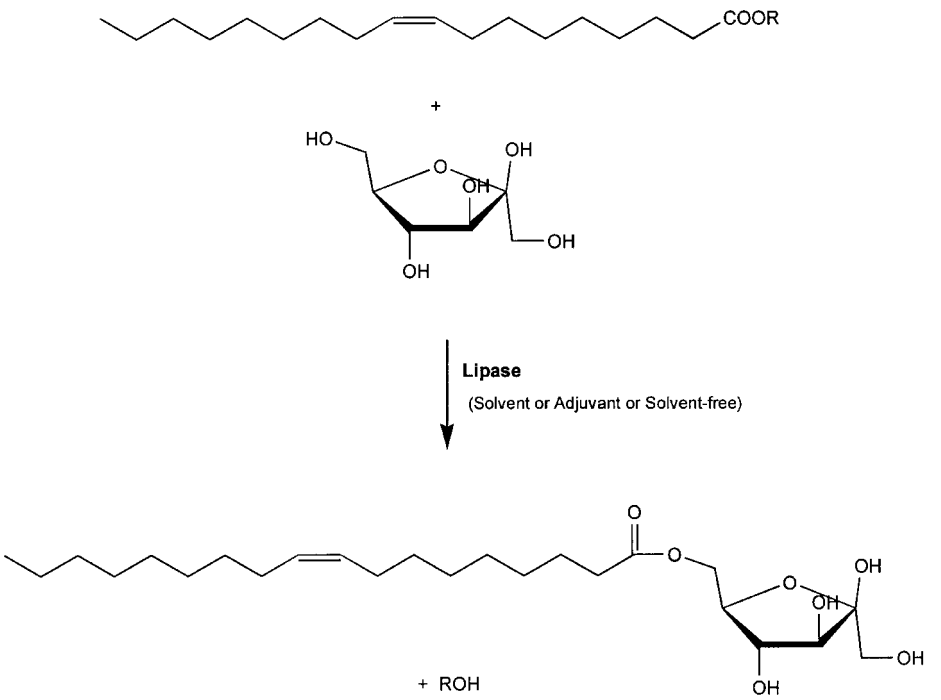

Figure 1. Scheme of the lipase-catalyzed synthesis of fructose-monooleate (main isomer) (e.g., R = H, CH_3, CH = CH_2, diacylglyceryl).

tion medium. Using finally *n*-hexane, supercritical carbon dioxide, or even the fatty acid to be esterified, the sugar substrates should be mainly in a solid state. Investigations carried out since 1986 (Therisod and Klibanov) have shown many approaches to have been performed in this manner. The log *P* values of solvents ranged from – 1.30 to + 6.60. Alternatively, free acids, alkyl-, trichloroethyl-, vinyl- or glyceryl-esters were used for lipid reactands.

Water generated during the esterification was removed by the addition of activated molecular sieves. In the case of vinyl esters of fatty acids, the resulting byproduct vinyl alcohol tautomerizes to the low-boiling-point acetaldehyde. However, one must be cautious since it has been recently reported that several lipases (e.g., from *Candida rugosa* and *Geotrichum candidum*) lost most of their activity when exposed to acetaldehyde (Weber et al., 1995). In comparative studies, Coulon et al. (1995) showed that, in the presence of solvent, the transesterification gave better results than direct esterification. In solvent-free conditions, only direct esterification was available.

In most cases the immobilized lipases of *Candida antarctica* and of *Rhizomucor miehei* (formerly *Mucor miehei*) served as catalysts. Among the carbohydrates studied, fructose was used very frequently. Unfortunately, due to their specific behavior in solution, four isomers (furanose and pyranose forms, α- and β-anomers) of monoacylated fructose were found (e.g., Scheckermann et al., 1995; Jung et al., 1998). In

Table 1. Lipase-catalyzed glycolipid syntheses in a) organic solvents, ranked for their log *P* values, and b) solvent-free systems.

Lipase	Solvent	Log *P*	Lipid reactand	Sugar/Polyol	Reference
Humicola lanuginosa[1]	Dimethylsulfoxide	– 1.30	Vinyl laurate	Sucrose	Plou et al., 1999
Chromobacterium viscosum	Buffer/2-Pyrrolidone	– 0.90	$C_{14:0}$–$C_{20:0}$, $C_{18:1}$	Sorbitol, Fructose, Glucose	Janssen et al., 1991
Candida antarctica B[1]	Acetonitrile	– 0.33	$C_{8:0}$	Glucose	Ljunger et al., 1994
Porcine pancreas[1]	Acetonitrile	– 0.33	$C_{4:0}$, Tributyrin	Fructose	Bagi and Simon, 1999
Porcine pancreas	Pyridine	+ 0.71	Trichloroethyl laurate	Glucose	Therisod and Klibanov, 1986
Porcine pancreas	Pyridine	+ 0.71	Triolein, Corn oil, Peanut oil, Sunflower oil	Ribitol, D-Sorbitol	Chopineau et al., 1988
Alcaligenes sp.	Pyridine	+ 0.71	Divinyl sebacate	Glucose	Kitagawa and Tokiwa, 1998
Byssochlamys fulva	*t*-Butanol	+ 0.80	$C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{18:3}$	Fructose, Glucose; Maltose, Sucrose	Ku and Hang, 1995
Candida antarctica[1]	*t*-Butanol	+ 0.80	Ethyl butanoate, Ethyl laurate	Maltose, Sucrose, Trehalose	Woudenberg-van Oosterom et al., 1996
Candida antarctica B[1], *Rhizomucor miehei*[1]	*t*-Butanol	+ 0.80	$C_{2:0}$–$C_{20:0}$	Glucose, Maltose	Degn et al., 1999
Humicola lanuginosa[1]	2-Methyl-2-butanol/ Dimethylsulfoxide	+ 1.30/–1.30	Vinyl laurate	Sucrose	Ferrer et al., 1999
Rhizomucor miehei[1]	2-Methyl-2-butanol	+ 1.3	$C_{18:0}$	Fructose	Khaled et al., 1991
Rhizomucor miehei[1]	2-Methyl-2-butanol	+ 1.3	$C_{10:0}$–$C_{18:0}$	Fructose	Scheckermann et al., 1995
Candida antarctica B[1]	2-Methyl-2-butanol	+ 1.3	$C_{18:1}$ Methylester	Fructose	Coulon et al. 1995
Candida antarctica B[1]	2-Methyl-2-butanol	+ 1.3	$C_{12:0}$, $C_{18:0}$	Sorbitol, Fructose, Glucose	Ducret et al., 1995
Candida antarctica B[1]	2-Methyl-2-butanol	+ 1.3	$C_{10:0}$, $C_{12:0}$, $C_{18:1}$, $C_{22:1}$	Glucose, Fructose, Xylitol	Ducret et al., 1996
Candida antarctica B[1]	2–Methyl-2-butanol	+ 1.3	$C_{18:1}$ Methylester	Fructose	Jung et al., 1998

Table 1. Lipase-catalyzed glycolipid syntheses in a) organic solvents, ranked for their log *P* values, and b) solvent-free systems.

Lipase	Solvent	Log *P*	Lipid reactand	Sugar/Polyol	Reference
Candida antarctica B[1]	2-Methyl-2-butanol	+ 1.30	$C_{18:1}$ methylester, Rape seed oil	Fructose	Coulon et al., 1999
Pseudomonas sp.	*n*-Hexane, *n*-Octane, *n*-Dodecane	+ 3.5, + 4.5, + 6.6	$C_{16:0}$	Glucose, Galactose, Fructose, Sorbose, Sucrose, Maltose, Lactose	Tsuzuki et al., 1999
Rhizomucor miehei[1]	*n*-Hexane	+ 3.5	$C_{12:0}$, $C_{16:0}$, $C_{18:1}$	Fructose[2], Glucose[2]	Stamatis et al., 1998
Rhizomucor miehei	Supercritical CO_2		$C_{12:0}$, $C_{16:0}$, $C_{18:1}$	Fructose[2], Glucose[2]	Stamatis et al., 1998
Rhizomucor miehei[1]			$C_{8:0}$	Fructose	Guillardeau et al. 1992
Rhizomucor miehei[1]			$C_{18:1}$	Isosorbide, Sorbitol	Mukesh et al., 1993
Candida antarctica B[1]			$C_{18:1}$	Fructose	Coulon et al., 1995
Rhizomucor miehei[1]			$C_{10:0}$	Sucrose	Kim et al., 1998

[1] Immobilized enzymes.
[2] Adsorbed to silica gel

the case of glucose, maltose, trehalose or sucrose, only the primary 6– and/or 6'-positions were acylated. A regioselective acylation of a secondary hydroxyl in sucrose, the 2-OH, was recently reported by Plou et al. (1999). Using vinyl laurate and the lipase of *Humicola lanuginosa* adsorbed to Celite in DMSO, the main product was 2-*O*-lauroylsucrose. However, the authors found also that the same reaction occurred partially in the presence of Celite, Eupergit, and even the simple Na_2PO_4 alone. These chemical acylations must therefore be taken into account in acylations of hydroxyl-containing compounds with enol esters in polar solvents using immobilized enzymes.

Subsequently, an enzymatic fructose transesterification was reported and performed recently in a 2-L pilot-scale reactor (Coulon et al., 1999). After initial studies to remove the strong inhibitors of sugar ester synthesis, water and methanol as byproducts (through raising the temperature, though that may cause stability problems), 2-methyl-2-butanol (b.p. 102 °C) was used as solvent and the pressure was reduced to 200 mbar. Using immobilized *Candida antarctica* lipase as biocatalyst and oleic acid methyl ester as acyl donor, more than 90 % of fructose was acylated, compared to only 50 % acylation at atmospheric pressure. This is explained by the evaporation of the reaction byproduct (methanol), which shifted the equilibrium. After drying over molecular sieves in a bypass, the solvent could be recycled in the reactor. Moreover, synthesis performed with an equimolar mixture of both substrates also gave promising results: although the reaction rate was slower than synthesis performed with an excess of fatty acid, the fructose monooleate concentration was still high, 44 g L^{-1} rather than 56 g L^{-1}. The concentration of residual acyl donor was very low. Downstream processes for the recovery of pure fructose monooleate were simplified in this case. *Candida antarctica* lipase also catalyzed sugar synthesis very efficiently using rapeseed oil as acyl donor.

17.2.2 Lipase-mediated catalysis in the presence of adjuvants

A method for the synthesis of sugar fatty acid esters has been reported which is based on a mainly solid-phase system. The acylation of a solid sugar with a fatty acid was performed via lipase-mediated catalysis in the presence of a very small amount of organic solvent (e.g., acetone, tetrahydrofuran or *t*-butanol) serving mainly as adjuvant (see Table 2). In a typical experiment, the reaction mixture consisted of equimolar (typically 0.5 mmol) free carbohydrate and fatty acid and an organic solvent as adjuvant (in a concentration of 100–300 %, w/w), to maintain a small catalytic liquid phase. In hydrophobic-solvent systems mentioned in the previous chapter, the liquid phase volume was several magnitudes larger. Although water generated during esterification was removed by the addition of activated molecular sieves, this method seemed unsuitable from an economic point of view.

With regard to solvent-free systems, to date, water removal is easier and has been achieved in open test tubes, evacuation *in vacuo*, pervaporation using special membranes, and dry gas bubbling. However, when organic solvents are present in the reaction medium, the exclusive removal of water becomes difficult: low-boiling-point solvents such as acetone, *t*-butanol or 2-methyl-2-butanol would also be re-

Table 2. Lipase-catalyzed glycolipid syntheses in the presence of adjuvants.

Lipase	Adjuvant	Log *P*	Lipid Reactand	Sugar/Polyol	Reference
Candida antarctica B[1]	Acetone or Dioxane	–0.23 –1.10	$C_{12:0}$–$C_{18:0}$	Glucose, Mannose, Galactose, Mannitol, Sorbitol	Cao et al., 1996, Cao et al., 1997
Candida antarctica B[1]	2-Methyl-2-butanol/ *n*-Hexane	+ 1.3/ + 3.5	$C_{12:0}$	Sorbitan	Sarney et al., 1997
Candida antarctica B[1]	Dioxane or Tetra-hydrofuran (or mono/ diglyme)	–1.10 + 0.49	Vinyl- and methylesters of $C_{4:0–C22:0}$ (saturated and unsaturated)	Glucose, Fructose, Sucrose, Ascorbic acid	Aha et al., 1997a; 1997b; 1998; Berger et al., 1998
Candida antarctica B[1]	*t*-Butanol	+ 0.80	Phenylbutyric acid	*n*-Alkyl-glycosides	Otto et al., 1998a
Candida antarctica B[1]	*t*-Butanol	+ 0.80	*n*-Butanol, Cinnamic alcohol	Glucuronic acid	Otto et al., 1998b
Candida antarctica B[1]	*t*-Butanol	+ 0.80	Arylaliphatic acids, Aliphatic acids ($C_{8,10}$)	Glucose, Alkyl-glucoside	Otto et al., 1998c
Candida antarctica B[1]	*t*-Butanol	+ 0.80	Arylaliphatic acids	Glucose	Otto et al., 2000
Candida antarctica B[1]	Ethyl methyl ketone Acetone	–0.80 0.23	$C_{8:0}$, $C_{16:0}$, $C_{18:0}$ Methylesters of $C_{8:0}$, $C_{16:0}$, $C_{18:0}$	Glucose Glucose	Yan et al., 1999

[1] Immobilized enzymes.

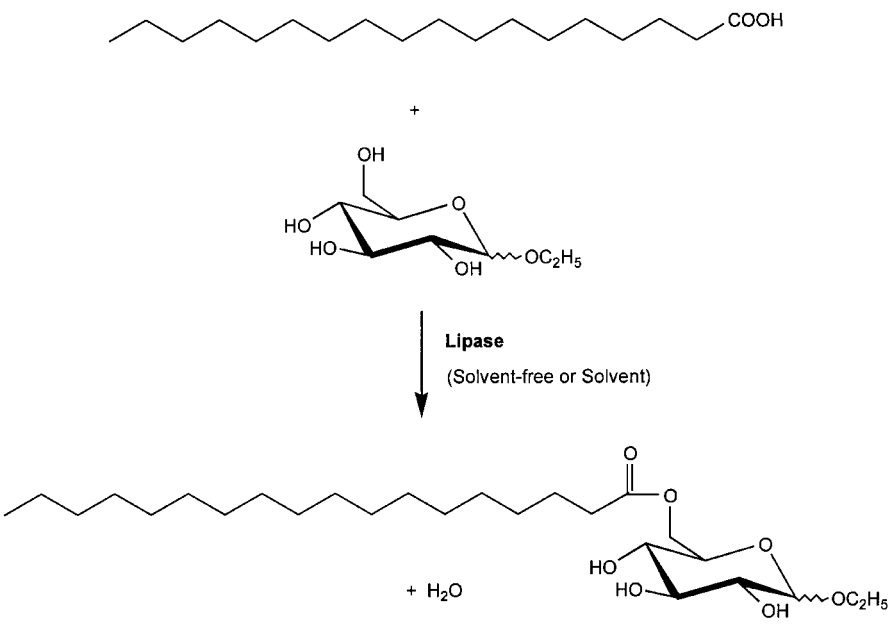

Figure 2. Scheme of the lipase-catalyzed synthesis of ethyl-β-D-glucoside 6–*O*-monooctadecanoate.

moved from the reaction mixture. Recently, a reaction system was reported in which the byproducts from lipase-catalyzed sugar ester synthesis were removed by azeotropic distillation using ethyl methyl ketone (for water) or acetone (for methanol) at 'lipase-friendly' temperatures (Yan et al., 1999). The authors claimed this to be an adjuvant-type process, as only 10 % of the solvent was present in the round-bottomed flask, whereas 90 % was circulating through the condenser and Soxhlet extractor. The authors believed that this method might be developed into a process that is practicable on a large scale.

17.2.3 Lipase-mediated catalysis using hydrophobized sugars

The typical reaction scheme of glycolipid synthesis using alkyl-glycosides for the fatty acid acylation is shown in Figure 2. In order to bypass the problems of low solubility of mono- and disaccharides in nonpolar organic solvents and those of their high melting points, Björkling et al. (1989) first recommended hydrophobization of the carbohydrates prior to lipase-catalyzed acylation. Besides 1-*O*-ethyl-D-glucoside, the synthesis worked well with *n*-propyl, *iso*-propyl, butyl, *iso*-butyl, and even with phenyl glucoside. Fatty acids were in the range of $C_{8:0}$ to $C_{18:0}$ or $C_{18:1}$, and also with $C_{22:1}$. In addition, the reaction was performed at reduced pressure and under solvent-free conditions. Using lipase B from *Candida antarctica*, more than 95 % yield of the 6-*O*-monoester could be obtained. The transfer of the process to pilot plant scale caused no major problems, and a production in 20-kg scale was set

Table 3. Lipase-catalyzed glycolipid syntheses using alkyl glycosides.

Lipase	Solvent	Log *P*	Lipid Reactant	Modified Sugar	Reference
Candida antarctica[1] *Humicola* spec.[1] *Mucor miehei*[1]	–		$C_{8:0}$ $C_{18:0}$, $C_{18:1}$	Ethyl D-glucopyranoside, *i*-Propyl D-glucopyranoside, *n*-Propyl D-glucopyranoside, *n*-Butyl D-glucopyranoside	Björkling et al., 1989; Kirk et al., 1992; Andresen and Kirk, 1995
Candida antarctica[1]	Benzene/Pyridine (2/1, v/v)	+2.00/ +0.71	Methyl oleate	Methyl glucoside, Methyl galactoside, Octyl galactoside	Mutua and Akoh, 1993
Candida antarctica[1]	Benzene/Pyridine (2/1, v/v)	+2.00/ +0.71	Methyl oleate, Eicosapentaenoic acid, Docosahexaenoic acid	Methyl glucoside, Methyl galactoside, Octyl galactoside	Akoh and Mutua, 1994
Candida antarctica[1]	t-Butanol	+0.80	Ethyl propionate, Ethyl decanoate	Methyl- to Dodecyl-glucoside/ fructoside/galactoside	Goede de et al., 1994
Pseudomonas cepacia	Acetonitrile	−0.33	Octanoic acid	Methyl α-D-galactopyranoside	Córdova et al., 1997
Candida antarctica[1]	–		Dodecanoic acid	3-Chloropropyl D-glucopyranoside	Kirk et al., 1998
Porcine pancreas	–		ε-Caprolactone	α,β-Ethyl glucopyranoside	Bisht et al., 1998
Candida antarctica[1]	–		Trimethylene carbonate	α,β-Ethyl glucopyranoside	Bisht et al., 1998
Candida antarctica[1] *Rhizomucor miehei*[1]	2-Methyl-2-butanol; solvent-free	+1.30	Oleic acid	α-D-Butyl glucoside	Bousquet et al., 1999

[1] Immobilized enzymes.

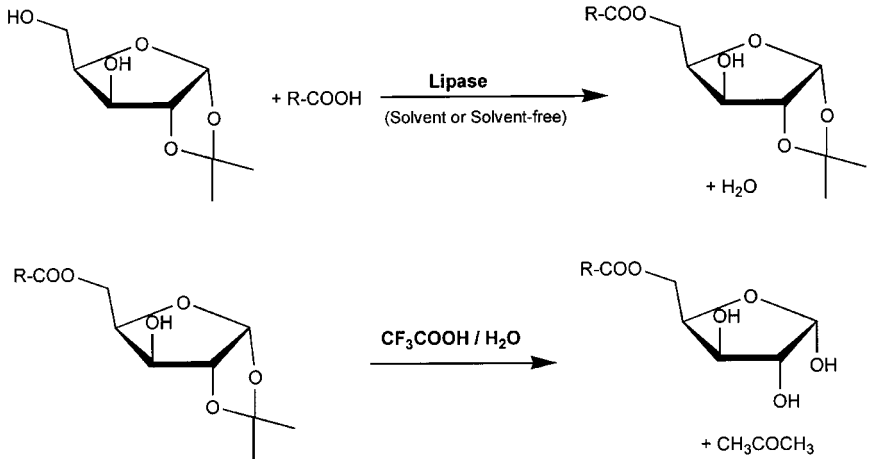

Figure 3. Scheme of the lipase-mediated synthesis of sugar ester from isopropylidene-D-xylofuranose and fatty acid.

up in order to prepare material for application tests (Andresen and Kirk, 1995). One disadvantage for economic purposes could be that selective removal of the protecting group is difficult.

Table 3 provides an overview of studies performed by other groups in this field. Two special reactions deserve mention: a new type of cationic surfactant, 6-*O*-monoester of 3-(trimethylammonio)-propyl-D-glucopyranoside, was prepared in high yields by a chemo-enzymatic synthesis. For instance, to introduce dodecanoic acid into the 6–position of 1-protected glucose, *C. antarctica* lipase B was used successfully in a solvent-free system at 75 °C with 90 % conversion (Kirk et al., 1998). In another study using porcine pancreatic and *C. antarctica* lipase B, the ring-opening polymerization of both ε-caprolactone (ε-CL) and of trimethylene carbonate (TMC) by the multifunctional ethyl glucopyranoside (EGP) was catalyzed and resulted in the formation of EGP-oligo(ε-CL) and EGP-oligo(TMC) conjugates with molecular weights up to 7200 Da.

Besides the 1-*O*-alkyl glycosides, isopropylidene, benzylidene and phenyl(butyl) boronic acid are especially suitable protectional agents for sugars. The advantage is their facile removal from the carbohydrate after successful enzymatic acylation. In contrast to the ester linkage between the fatty acid and the sugar, the acetalic and boronic acid protection groups can be removed selectively by trifluoroacetic acid or mild acetone (or methanol)/water hydrolysis, respectively (Figure 3). Some studies based on these approaches are presented in Table 4. For example, Ward et al. (1997) investigated the *Rhizomucor miehei* lipase-catalyzed synthesis of a sugar ester containing arachidonic acid. When the fatty acid to 1,2–*O*-isopropylidene-D-xylofuranose ratio was 1–2 : 1, a maximum conversion of 84 % was obtained. After downstream processing, the chemical structure was confirmed to be xylose 5-arachidonate. A new chemo-enzymatic approach to the preparation of dimeric (gemini) sugar fatty acid esters has been developed by Gao et al. (1999). First, lipases (*Candida antarctica*, *Rhizomucor miehei*) catalyzed the monoacylation (6- or 6'-OH) of acet-

Table 4. Lipase-catalyzed glycolipid syntheses using complexated sugars.

Lipase	Solvent/Adjuvant	Log *P*	Lipid Reactand	Modified Sugar	Reference
Mucor miehei[1] *Chromob. viscosum*[1] *Pseudomonas* sp.[1]	–		$C_{8:0}$–$C_{18:0}$, $C_{18:1}$; Methyl esters of $C_{8:0}$–$C_{18:0}$, $C_{18:1}$	1,2-*O*-Isopropylidene-D-glucofuranose; 1,2:3,4-Di-*O*-isopropylidene-D-galactopyranose; 1,2-*O*-Isopropylidene-D-xylofuranose	Fregapane et al., 1991
Mucor miehei[1]	–		$C_{12:0}$, $C_{18:0}$, $C_{18:1}$	1,2:3,4-Di-*O*-isopropylidene-D-galactopyranose; 1,2-*O*-Isopropylidene-D-xylofuranose	Fregapane et al., 1994
Mucor miehei[1]	Toluene	+2.50	$C_{8:0}$–$C_{18:0}$, $C_{18:1}$	Lactose tetraacetal; Maltose triacetal	Sarney et al., 1994
Mucor miehei[1] *Candida antarctica*[1]	–		$C_{12:0}$–$C_{18:0}$, $C_{18:1}$, $C_{20:4}$, $C_{22:6}$,	1,2-*O*-Isopropylidene-D-xylofuranose	Ward et al., 1997
Candida antarctica[1]	–		2-Bromotetradecanoic acid	1,2:3,4-di-*O*-isopropylidene-D-galactopyranose	Gao et al., 1999
Mucor miehei[1]	Toluene	+2.50	2-Bromotetradecanoic acid	Lactose tetraacetal	Gao et al., 1999
Pseudomonas cepacia[1]	THF/Toluene (1/4, v/v)	+0.49/ +2.50	Trifluoroethyl butanoate	4,6-*O*-Benzylidene-methyl-α- and β-glucopyranoside	Panza et al., 1993
Pseudomonas cepacia[1]	THF	+0.49	Vinyl acetate	4,6-*O*-Benzylidene-β-D-gluco/galactopyranosides	Gridley et al., 1998
Mucor miehei[1]	*n*-Hexane	+3.50	$C_{18:0}$	Fructose-phenylboronate complex	Schlotterbeck et al., 1993; Scheckermann et al., 1995
Mucor miehei[1]	*n*-Hexane; *n*-Heptane	+3.50 +4.00	$C_{18:0}$	Glucose-phenylboronate or butylboronate complex	Oguntumein et al., 1993
Pseudomonas sp.	*t*-Butanol	+0.80	Vinyl acrylate, $C_{12:0}$, Olive oil, Corn oil	Glucose- or galactose-phenylboronate complex	Ikeda and Klibanov, 1993

[1] Immobilized enzymes.

Table 5. Protease-catalyzed glycolipid syntheses.

Protease	Solvent	Log *P*	Lipid reactand	Sugar	Reference
Protease N	Dimethylformamide	–1.00	Trifluoroethyl caprylate	Sucrose	Carrea et al., 1989
Alcalase	Pyridine	+ 0.71	Vinyl laurate	Sucrose	Soedjak and Spradlin, 1994
Subtilisin Carlsberg[1]	Dimethylformamide	–1.00	Trichloroethyl caprylate	Sucrose	Plou et al., 1995
Subtilisin CLEC	Pyridine	+ 0.71	Vinyl laurate	Sucrose	Polat et al., 1997
Trypsin (Porcine pancreas; modified)	Dimethylformamide	–1.00	$C_{18:1}$	Glucose	Ampon et al., 1991
Streptomyces sp.	Dimethylformamide	–1.00	2,2,2-Trichloroethylbutyrate, Divinyl adipate	Glucose	Shibatani et al., 1997
Streptomyces sp.	Dimethylsulfoxide or dimethylformamide	– 1.30 – 1.00	Divinyl adipate	Glucose, Mannose, Galactose, α-Methyl D-galactose	Kitagawa et al., 1999

[1] Adsorbed to Celite.

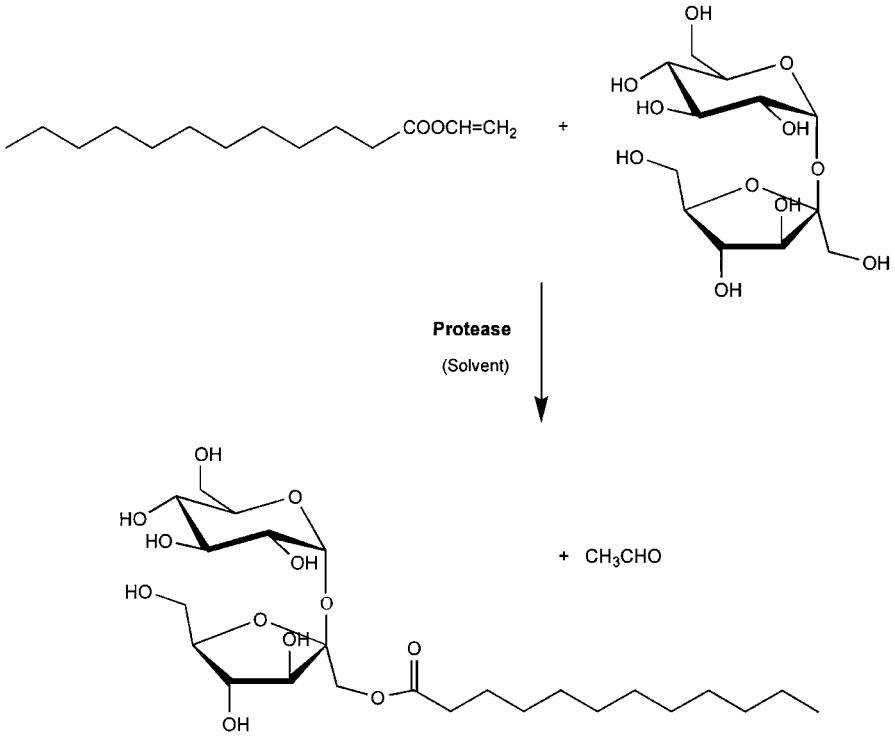

Figure 4. Scheme of the protease-catalyzed synthesis of sucrose-monolaurate.

alyzed galactose or lactose with 2-bromotetradecanoic acid. Second, these products were chemically dimerized with dicarboxylic acids, and after deprotection by trifluoroacetic acid, in the case of 1,2 : 3,4-di-*O*-isopropylidene-D-galactopyranose and lactose tetra-acetal esters, the desired gemini products were obtained in reasonable overall yields. Additionally, a trimeric sugar ester surfactant was prepared in a similar fashion in just one step by reacting 6-*O*-(2-bromotetradecanoic) methyl-α-D-glucoside with 1,3,5-tris (4-carboxy butyloxy)-benzene.

17.2.4 Protease-mediated catalysis in organic solvents

Beside lipases, proteases were also used successfully for the regioselective acylation of sugars and related compounds in anhydrous organic solvents. The most common solvents employed in these processes are dimethylformamide and pyridine (Table 5). As for the regioselectivity of acylation of sucrose, several proteases of the subtilisin family catalyze selectively the acylation of the primary 1'-OH in the fructose ring (Figure 4), which is frequently demonstrated in the literature (Carrea et al., 1989; Soedjak and Spradlin, 1994; Plou et al., 1995; Rich et al, 1995; Polat et al., 1997). With lipases as catalysts, the preferred acylation position is the 6-hydroxyl group

(Rich et al., 1995; Woudenberg et al., 1996; Kim et al., 1998; Ferrer et al., 1999). On the other hand, the acylation position in comparable monosaccharides is the same for both hydrolases, the 6-OH group. Recently, Kitagawa et al. (1999) examined the protease-catalyzed transesterifications between hexoses and divinyladipate. In dimethylformamide hexoses such as D-glucose, D-mannose, D-galactose and α-methyl-D-galactoside were acylated with divinyladipate by alkaline protease from *Streptomyces* sp. to give corresponding 6–*O*-vinyl adipoyl sugars. Surprisingly, when the denaturing co-solvent DMSO was added to the solvent, galactose was selectively acylated at only the C_2 position.

17.3 Glycosidase-catalyzed glycosylation of fatty alcohols with sugars

Alkyl-glycosides have the advantage over sugar fatty acid esters as they are more stable in alkaline conditions. However, in contrast to the above-mentioned lipase-catalyzed processes, their production by suitable enzymes is more problematic at present.

In general, it has been shown that alkyl-glycosides can be synthesized from alcohols and carbohydrates using glycosidases as catalysts. Under physiological conditions, glycosidases hydrolyze glycosidic bonds. The synthesis of alkyl-glycosides can be carried out either by reverse hydrolysis or by transglycosylation reaction involving alcohol as a glycosyl acceptor. The difference between the two means of synthesis depends on the nature of the glycosyl donor. In reverse hydrolysis, the reaction is performed with a monosaccharide (e.g., glucose), while in transglycosylation a glycoside (e.g., disaccharide) is used as the glycosyl donor (Figure 5). The first is an equilibrium-controlled, and the latter a kinetically controlled approach. Generally, the transglycosylation is much faster and leads to higher yields than that of the reverse hydrolysis. Both reactions can be conducted either in monophasic or biphasic systems consisting of: (i) more or less water; and (ii) the liquid alcohol to be glycosylated (in most cases no additional organic solvent is used).

In general, both initial velocity and yield decrease when the carbon chain length of alcohol increases. Only in rare studies were alcohols longer than C_8 suitable lipid reactands. Overviews relating to glycosidase-catalyzed glycolipid syntheses are shown in Tables 6 and 7. The dominant enzyme studied by most authors was the almond β-glucosidase (EC 3.2.1.21). Often, two-phase systems based on aqueous buffer and the alcohol were used. Several studies have been conducted to investigate the effect of operating conditions, mainly water content, on the synthesis reaction. When studying this in detail, Ismail et al. (1999a,b) recently found that in the case of a monophasic system (butanol phase), addition of water led to a significant increase in product concentration. The highest concentration was obtained at 17 % (v/v) of water in a transglycosylation system using lactose, *n*-butanol and the β-galactosidase (EC 3.2.1.23) from *Aspergillus oryzae* for *n*-butylgalactoside synthesis. In the biphasic system, the reaction performance was lower than that in the monophasic system, probably due to the shift of equilibrium towards the hydrolysis and the synthesis of

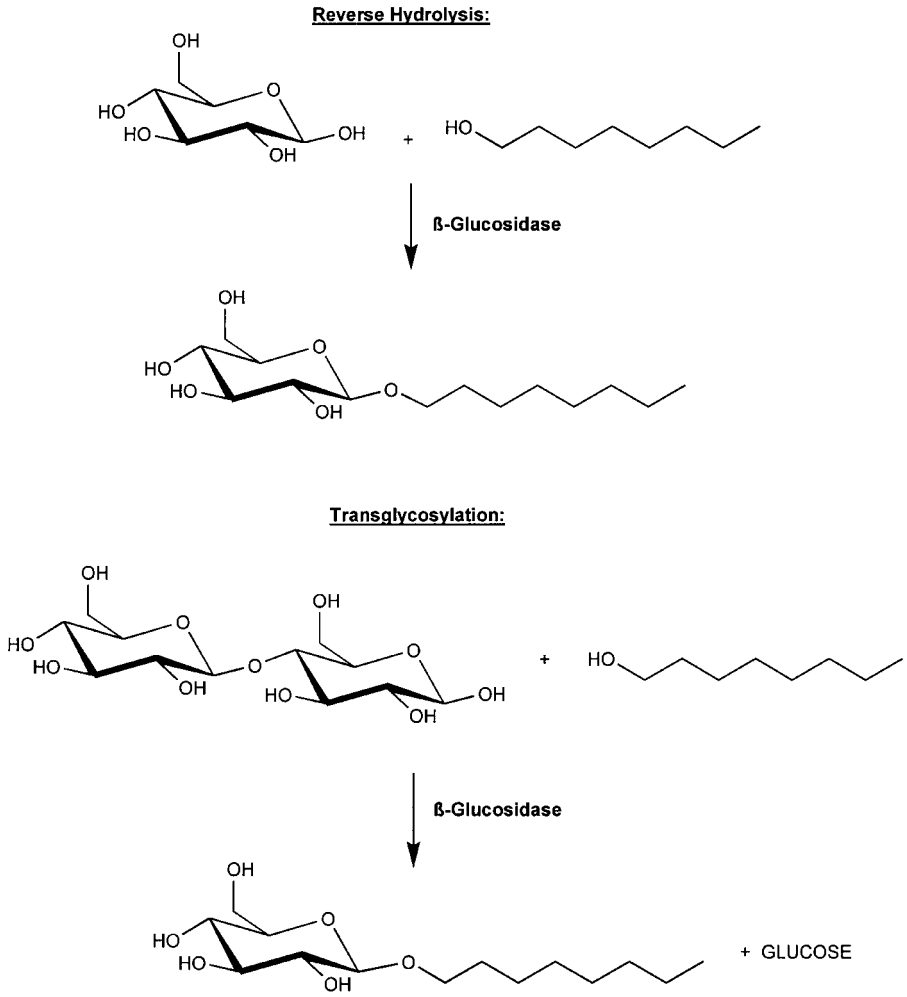

Figure 5. Scheme of two routes for the glycosidase-catalyzed synthesis of 1-*O*-alkyl-glycosides.

oligosaccharide reactions occurring in the aqueous phase. Testing in parallel the reverse hydrolysis method with almond β-glucosidase, glucose and *n*-butanol, in this case the monophasic system (8 – 13 % water content) also seems better than the biphasic approach for the synthesis of *n*-butylglucoside.

Table 6. ß-Glucosidase-catalyzed glycolipid syntheses. Method 1: reverse hydrolysis; method 2: transglycosylation.

ß-Glucosidase	Alcohol	Sugar	Method	Reference
Almond[1]	Benzyl alcohol	Glucose Cellobiose	1 2	Marek et al., 1989
Almond[1]	C_5-C_{12}	Glucose	1	Vulfson et al., 1990a
Almond[1]	C_5-C_8	Methyl-ß-glucoside, Cellobiose, Glucose	2 1	Vulfson et al., 1990b
Trichoderma viride	C_7, C_8	Cellobiose	2	Shinoyama et al., 1991
Almond	C_3, C_6, C_8	Glucose	1	Chahid et al., 1992
Almond	C_8	Glucose	1	Ljunger et al., 1994
Almond	Allyl alcohol, benzyl alcohol	Glucose, galactose	1	Vic and Crout, 1995
Almond	4-Hydroxybutanoic acid-*N*-butylamide	Glucose Cellobiose	1 2	Fischer et al., 1995
Pyrococcus furiosus[1]	4-Hydroxybutanoic acid-*N*-butylamide	Cellobiose	2	Fischer et al., 1996
Almond, *Fusarium oxysporum*	C_3-C_8	Glucose	1	Tsitsimpikou et al., 1996
Aspergillus niger	C_1, C_2	Cellobiose	2	Yan and Liau, 1998
Almond[1]	C_4	Glucose	1	Otto et al., 1998c
Almond[1]	C_6, C_8	Glucose	1	Yi et al., 1998
Almond	2-(4-Methoxybenzyl) -1-cyclohexanol	Glucose/ Phenyl-ß-glucose	1/2	Zarevucka et al., 1999
Almond	C_4	Glucose	1	Ismail et al., 1999b

[1] Immobilized enzymes.

Table 7. Glycosidase-catalyzed glycolipid syntheses. Method 1: reverse hydrolysis; method 2: transglycosylation.

Glycosidase	Alcohol	Sugar	Method	Reference
ß-Galactosidase (*Aspergillus oryzae*)	C_4	Methyl-ß-galactoside	2	Beecher et al., 1990
ß-Galactosidase (*Aspergillus oryzae*)	C_4	Lactose	2	Ismail et al., 1999a
ß-Galactosidase (*Escherichia coli*)	2-(4-Methoxy-benzyl)-1-cyclo-hexanol	Galactose/ Phenyl-ß-D-galactose	1/2	Zarevucka et al., 1999
ß-Mannosidase (*Aspergillus niger*)	C_1_C_8	ß-1,4-Mannobiose	2	Itoh and Kamiyama, 1995
ß-Mannosidase (*Rhizopus niveus*)	C_1_C_4, Benzyl alcohol	ß-1,4-Mannobiose	2	Fujimoto et al., 1997
ß-Xylosidase (*Aspergillus niger*)	C_1–C_8; sec. $C_{3,4,6}$; Benzyl alcohol; Cyclohexanol	Xylobiose	2	Shinoyama et al., 1988; Shinoyama et al., 1991b
Culture supernatant/ *Aureobasidium pullulans*	C_8, C_{10}, sec. C_8, 2-Ethylhexanol	Xylan; Xylotetraose	2	Matsumura et al., 1996
Dried cells/ *Aureobasidium pullulans*	C_8	Xylan	2	Matsumura et al., 1999c
ß-Glycosidase (*Sulfolobus solfataricus*)	C_1_C_8; *sec*. $C_{5,6,7}$; 2-Methyl butanol	Phenyl ß-D-glucoside, phenyl ß-D-galactoside	2	Trincone et al., 1991
α-Transglucosidase (*Talaromyces duponti*)	C_1_C_7	Maltodextrins, maltose	2	Bousquet et al., 1998
exo-ß-D-Glucos-aminidase (*Penicillium funiculosum*)	C_1–C_6; *i*-$C_{3,4}$; Benzyl alcohol	Chitosan	2	Matsumura et al., 1999a,b

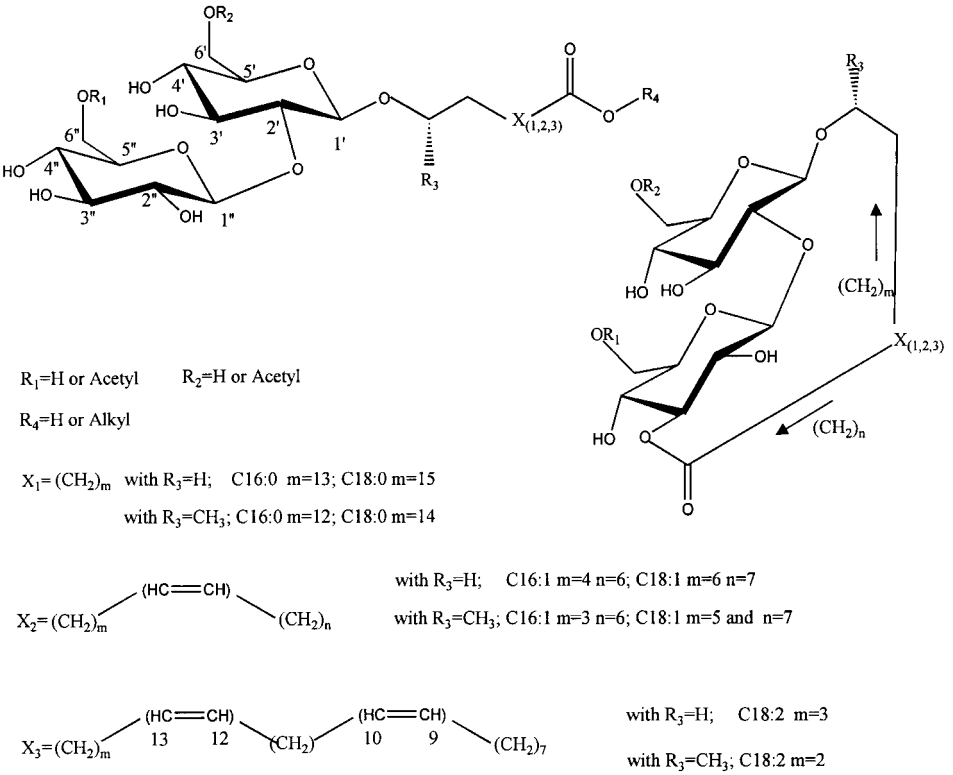

Figure 6. Principal molecular structures of sophorolipids (SL). Top: acidic-SL; Right: lactonic-SL. For example, using glucose and rapeseed oil as substrates, the following sophorolipid is predominantly secreted (see also Table 8): $R_1 = R_2 =$ acetyl, $R_3 =$ methyl, $R_4 =$ H, X_2, m = 6, n = 7, 17-L-([2'-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl]-oxy)-*cis*-9-octadecenoic-1',4"-lactone-6',6"-diacetate.

17.4 Chemically and enzymatically modified sophoroselipids

The yeasts *Candida (Torulopsis) bombicola* ATCC 22214 and *Candida (Torulopsis) apicola* IMET 43747 secrete high amounts of sophorolipids (sophoro (se) lipids) (Table 8). These glycolipids (Figure 6) always contain the disaccharide sophorose glycosidically linked to (un-) saturated [ω]- or [ω-1]- hydroxy C_{18} or C_{16} fatty acids. Examinations have revealed that at least 14 structurally different sophorolipids are produced (Tulloch et al., 1968; Asmer et al., 1988; Weber et al., 1990; Davila et al., 1993).

The native sophorolipids are predominantly acetylated at $C_{6'}$ and $C_{6"}$ (Asmer et al., 1988). Both, acidic-SL and lactonic-SL, are always present in native culture suspensions of *Candida bombicola*, and can be obtained in concentrations up to 422 g L^{-1} (Table 8). If alkanes, fatty acids or triglycerides are used as substrates, then a carboxylic group is always connected to the end of the lipid moi-

Table 8. Batch and feed-batch production of sophorolipids.

Carbon source ($g\ L^{-1}$)	Yield ($g\ L^{-1}$)	$Y_{SL/S}$ ($g\ g^{-1}$)[1]	Productivity ($g\ L^{-1}\ day^{-1}$)	Reference
Glucose (304), rapeseed ethyl ester (184)	320	0.65	38.4	Davila et al., 1992
Glucose (100), canola oil (105)	160	0.78	20.0	Zhou and Kosaric, 1995
Glucose (160), oleic acid (45)	180	0.87	21.6	Rau et al., 1996
Single cell oil (20), rapeseed oil (400)[2]	422	1.0	24.7	Daniel et al., 1998; Daniel, 1999
Glucose (300), rapeseed (colza) ethyl ester (240)	310[3] (565)[4]	0.57	51.8	Marchal et al., 1997
Glucose (350), rapeseed oil (212)	360	0.64	51.2	Rau et al., 1998

[1] $Y_{SL/S}$ = g sophorolipid formed/g substrate consumed.
[2] First stage: Formation of single cell oil on deproteinized whey with *Cryptococcus curvatus*; second stage: Production of sophorolipids.
[3] Anhydrous sophorolipid.
[4] Crude sophorolipid, contains 45 % (w/w) water.

ety. The acidic-SL (Figure 6) is found as major compound in the aqueous supernatant. The acid group is able to form an internal ester linkage with the OH at $C_{4''}$. The resulting lactonic-SL precipitates as solid compound at the bottom of the cultivation vessel. The percentage distribution between acidic- (20–43 % w/w) and lactonic-SL (57–80 % w/w) varies depending on the substrate used (Davila et al., 1994; Manzke, 1999). However, all analyzed cultivations generally reveal a preferred formation of the lactonic compound. The lactonic-SL can be easily transformed into the acidic-SL by alkaline hydrolysis with concomitant deacetylation at $C_{6'}$ and $C_{6''}$. The microbial formation of one particular sophorolipid is not possible. Therefore, it is of interest to modify SL structure chemically or enzymatically.

17.4.1 Chemically modified sophorolipids, chemically modified

The nonionic deacetylated methyl ester SL (Figure 6; $R_1 = R_2 = H$, $R_4 = CH_3$) is easy to prepare by refluxing acidic-SL with methanolic sodium methoxide (Tulloch et al., 1968) or by the addition of methanol and a strong acid. Reacting the methyl ester SL with a desired alcohol in the presence of alkaline agent results in ester exchange. Using this procedure, ethyl-, propyl-, butyl-, hexyl-, octyl-, decyl-, lauryl-, myristyl-, palmityl-, stearyl- and oleyl-ester were synthesized (Inoue et al., 1980). Ishigami (1994) prepared SL-alkyl and -aryl amides deriving from acidic-SL. In this patent, two examples are represented at length by reacting acidic-SL with benzylamine or decylamine in a methylene chloride solvent in the presence of *N*-methyl-2-chloro-pyridinium iodide and tributylamine, resulting a SL-benzylamide and SL-decylamide, respectively (Figure 7). However, all SL-amides synthesized showed no extraordinary reduction in surface tension (Table 9).

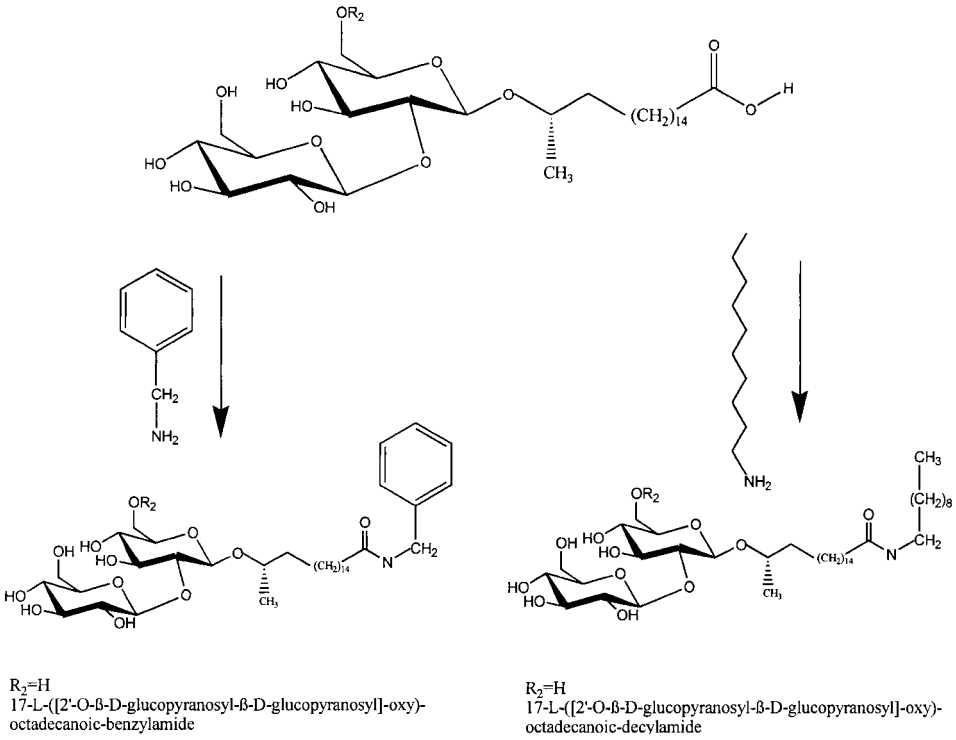

Figure 7. Conversion of mono- or diacetylated 17-L-([2'-O-β-D-glucopyranosyl-β-D-glucopyranosyl]-oxy)-octadecanoic acid into acid amides by the reaction with amine compounds in a methylene chloride solvent in the presence of *N*-methyl-2-chloro-pyridinium iodide and tributylamine (Ishigami, 1994).

Lang et al. (1999) also amidated sophorolipids, but they used lactonic-SL as feedstock. Depending on the *N*-alkylation grade of the amine (branched alkyl chain, dialkylation), the reaction had to be performed differently. In the case of primary *N*-alkylated amines only catalytic amounts of ammonium chloride were necessary for successful conversions. The longer the alkyl chain, the faster was the reaction. The formation of the *N*,*N*-diethyl amide was only possible if diethyl amine was predeprotonized with *n*-butyl lithium.

17.4.2 Enzymatically modified sophorolipids, enzymatically modified

Thus, to date, physiological variables during fermentations have provided routes to the variation of sophorolipid composition, but have not led to well-defined pure compounds. An alternative strategy to the tailoring of sophorolipid structure during the *in vivo* processing step is to develop methods for the regioselective modification of sophorolipids after microbial formation.

Table 9. Surface-active properties of sophorolipids and sophorosides.

Compound	σ (mN m^{-1})	CMC (mg l^{-1})	Reference
Diacetylated 17–OH-$C_{18:1}$ lactonic-SL (Figure 6)	35	40	Rau et al., 1999
Monoacetylated 17-OH-$C_{18:1}$ lactonic-SL	40.4	15	Otto et al., 1999
Deacetylated 17-OH-$C_{18:1}$ acidic-SL	40	100	Rau et al., 1999
Mixture of $C_{18:1}$ and $C_{18:0}$ (Figure 6):			Inoue et al., 1980
Deacetylated 17-OH-C_{18}-methyl ester SL	40	–	
Deacetylated 17-OH-C_{18}-ethyl ester SL	39.5	–	
Deacetylated 17-OH-C_{18}-propyl ester SL	38	–	
Acetylated 1-Dodecyl sophoroside	31	100	Brakemeier et al.,
Deacetylated 1-Dodecyl sophoroside	33	100	1998a,b
Diacetylated 2-dodecyl-sophoroside	30	100	Lang et al., 1999
Acetylated 2-dodecyl-glucoside	40	–	
Acetylated 2-Tetradecyl sophoroside)	31.4	40	
Deacetylated 17-OH-$C_{18:1}$ acidic glucolipid (Figure 9)	40	150	Rau et al., 1999
Deacetylated 17-OH-$C_{18:1}$-diethyl	47	5	Lang et al., 1999
SL amide	45	80	Ishigami, 1994
n-butyl SL amide	44	20	
2-butyl SL amide	63	20	
n-decyl SL amide	60	5	
n-hexyl SL amide	39	–	
Deacetylated 17–OH-$C_{18:0}$-benzyl SL amide (Figure 7)			

σ = Surface tension of water against air at 25 °C.
CMC = critical micelles concentration.
SL = Sophorolipid.

Native sophorolipids are differently acetylated at $C_{6'}$ and $C_{6''}$. Therefore, requirements exist for the formation of sophorolipids with homogeneous acetylation degree. A regioselective enzymatic deacetylation of 17-L-([2'-O-β-D-glucopyranosyl-β-D-glucopyranosyl]-oxy)-*cis*-9-octadecenoic-1',4''-lactone-6',6''-diacetate (Figure 8) leaving the lactone ring intact is reported by de Koster et al. (1995) and Otto et al. (1999). The monoacetylated lactonic-SL was synthesized from the diacetylated one using cutinase from *Fusarium solani f.* sp. *pisi* or immobilized *Candida rugosa* lipase. The substrate (0.3 g) was incubated with 20 units of cutinase at 40 °C for 20 min at pH 9. A yield was not specified. The process using *Candida rugosa* lipase (200 mg) required a prolonged incubation time of 24 h, though 95 % of the substrate (1 g) was converted. Both enzymes released only the acetyl group at $C_{6'}$. Molecular modeling studies showed that the diacetylated substrate fits well into the binding pocket of cutinase, and that $C_{6'}$-OAc is well accessible to the active site of the enzyme. In contrast, the acetyl group at $C_{6''}$ is closed in between nonreducing glucose and fatty acid chain and, thus, is not susceptible to enzymatic hydrolysis. The mono- and diacetylated SL showed only a slight difference in surface activity (Table 9) due to the removal of the exposed $C_{6'}$-OAc substituent and not to a change in conforma-

tion. A complete deacetylation at $C_{6'}$ and $C_{6''}$ under preservation of the lactone ring is also possible by the attack of an immobilized acetylesterase. The immobilization of the acetylesterase prevents side reactions as cleavage of the lactone ring and glycosidic linkages (Asmer et al., 1988).

Recently, Scholz et al. (1998) and Bisht et al. (1999) succeeded in the enzymatic synthesis of well-defined sophorolipid analogs. A careful spectroscopic analysis was performed of all modified SL. They used 17–OH-$C_{18:1}$ sophorolipids as feedstock based on a cultivation with glucose/oleic acid as substrates. The alkaline hydrolysis according to Tulloch et al. (1968) resulted in the deacetylated 17-OH-$C_{18:1}$ sophorolipid acid as sole product. Unfortunately, this product was soluble only in highly polar solvents such as water, dimethylformamide, DMSO or pyridine. The conversion with a mixture of sodium methoxide, ethoxide or butoxide yielded in a sophorolipid ester (Figure 8, top) with enhanced hydrophobic characteristics soluble in anhydrous tetrahydrofuran. Lipase B from *Candida antarctica* (Novozym 435) was

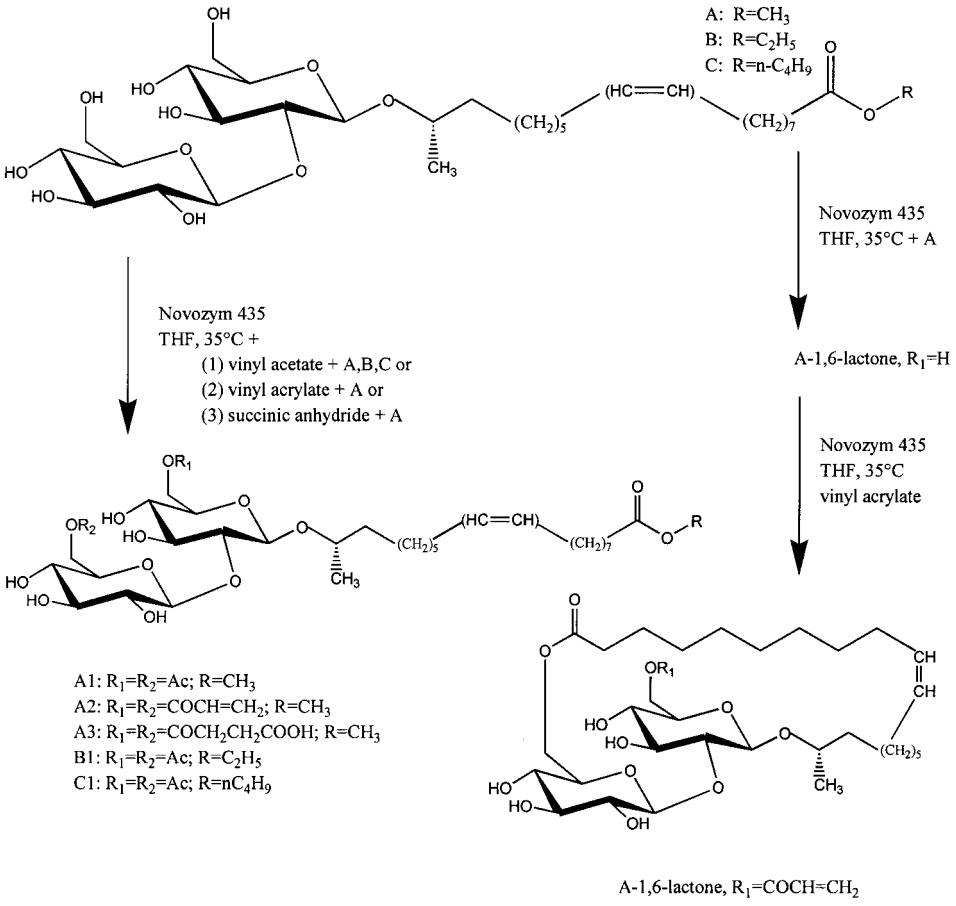

Figure 8. Enzymatic modification of deacetylated 17-OH $C_{18:1}$ ester SL by Novozym 435 lipase. (Redrawn from Bisht et al., 1999.)

used as catalyst for acetylation of this sophorolipid ester at $C_{6'}$ and $C_{6''}$ with vinyl acetate (Figure 8; A1, B1, C1) in dry THF. The methyl ester SL was additionally subjected to further esterification with vinyl acrylate (A2) or succinic anhydride (A3). The mono-acryl derivative of the methyl ester SL was prepared over a novel lactonic intermediate (A-1,6-lactone). This lactone differs in the site at which the sophorose ring is attached to the fatty acid. Unlike the natural sophorolipids (Figure 6), the fatty acid carboxyl carbon C_1 is linked to the $C_{6''}$ hydroxyl, not to the $C_{4''}$ hydroxyl. The synthesis provided a SL analog that contained only one primary hydroxyl group. Hence, this compound is an excellent candidate for the regioselective conversion into the corresponding monoacryl derivative linked only to one remaining primary site. Bisht et al. (1999) envisioned that these SL analogs would lead to a new family of glycolipids that may be important immunomodulators with potential anticancer activity (Scholz et al., 1998; Krivobok et al., 1994; Isoda et al., 1997; Otto et al., 1999). Furthermore, they propose certain compounds like mono- and difunctional acryl sophorolipid derivatives as useful monomers for the preparation of unusual amphiphilic vinyl homo- and co-polymers. Unfortunately, the surface-active properties of these modified sophorosides were not measured.

In spite of the accessibility of sophorolipids to several commercially available lipases, they show stability against at least 13 glycosidases (Otto et al., 1999). These tests were performed to investigate the possibility of applying sophorolipids in detergent formulations. The probable reason for their nonreactivity is, beside steric effects preventing access to the glycosidase active sites, a fast interfacial inactivation of the glycosidases due to the surface active properties of the SL. Unlike lipases, which are reported to be activated at interfaces, glycosidases can be very rapidly denatured in the presence of interfaces (Kazlauskas and Bornscheuer, 1998; Otto et al., 1998c).

Recently, Rau et al. (1999) described a process for the regioselective release of a glucose molecule using glycosidases. First, they converted the diacetylated lactonic sophorolipids, based on glucose and rapeseed oil as substrate, into a homogeneous deacetylated acidic form by alkaline hydrolysis. Extraction of the neutralized SL with *n*-pentanol led to a desalted product. This compound was employed as substrate for the conversion in a screening with 30 glycosidases, of which the two en-

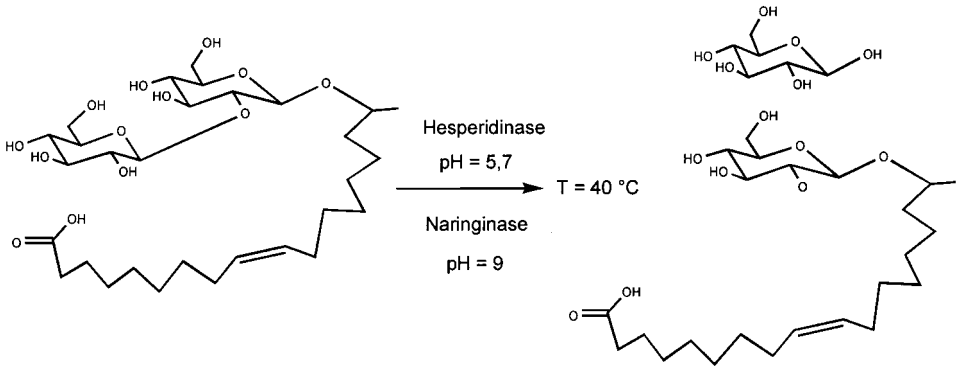

Figure 9. Enzymatic formation of a glucolipid.

zymes hesperidinase (EC 3.2.1.40) at pH 5.7 (yield 80 % w/w) and naringinase (Sigma N1385) at pH 9 were able to release specifically one glucose unit from the disaccharide lipid (Figure 9). The product of this treatment was isolated quantitatively and identified as a novel acidic glucolipid (17-L-(β-D-glucopyranosyl)-oxy-octadec-9-*cis*-enoic acid) by FAB-MS and NMR. The interfacial and surface tension of this glucolipid resembles those of the deacetylated acidic-SL used as feedstock for the enzymatic conversion (Table 9).

17.5 Surfactant properties of enzymatically synthesized and modified glycolipids, surfactant properties

17.5.1 Surfactant properties of enzymatically derived glycolipids

As for the sugar monoesters (see Section 1.1), the following results can be presented. Monosaccharide-based products containing fatty acids of the chain length C_{10} to C_{14} lower the surface tension of water from 72 mN m^{-1} to 30–27 mN m^{-1} (critical micellar concentration = 10^{-5} to 10^{-3} mol L^{-1} at 37 °C) and increase the stability of oil-in-water emulsions (Scheckermann et al., 1995; Ducret et al., 1996; Lang et al., 1997). The sugar esters based on $C_{18:0}$ or $C_{18:1}$, are forming stabilized monolayers in Langmuir film balance experiments. With film pressure values of more than 40 mN m^{-1} at a collapse point of 28 and 32 $Å^2$/molecule, respectively, there are very good conditions for wetting purposes (Lang et al., 1996). These long-chain products are also suitable to stabilize water-in-oil emulsions (Ducret et al., 1996). As for arylaliphatic glucose esters, critical micellar concentrations between 2 and 95 mmol and minimal surface tensions around 35 to 45 mN m^{-1} were determined (Otto et al., 1998a). Ethylglucoside esters of $C_{9:0}$ to $C_{12:0}$ are able to remove cutting oil from the surface of iron grains (Andresen and Kirk, 1995).

The surface-active properties of heptyl-β-D-xyloside were investigated by Shinoyama et al. (1991a). The critical micellar concentration was determined to be 30 mM at approximately 28 mN m^{-1}. The corresponding molecular area at saturation adsorption, the micellar weight and the aggregation number were 43 Å, 20.7 and 78, respectively. This surfactant was found to have a surface activity similar to that of commercially available *n*-octyl-β-D-glucoside.

17.5.2 Surfactant properties of native and modified sophorolipids

Table 9 summarizes surface tensions of native and modified sophorolipids taken from literature. The SL and derivatives reduced the surface tension of water from 72 mN m^{-1} to 30 mN m^{-1} (2-tetradecyl sophoroside) at minimum. In case of the *n*-decyl SL amide the surface tension was only lowered to 63 mN m^{-1}. Unpolar SL (derived from R_f-values of thin layer chromatography) and acetylated SL possess a higher surface activity compared to polar and deacetylated SL. A short-

ening of the hydrocarbon chain also enhances the surface active properties, while amidation or esterification of the carboxyl group showed a negative effect. However, the sophorosides present only minor differences in their surface tension. One explanation could be the spatial arrangement of the molecules at the air – water interface. According to a computer-generated model by de Koster et al. (1995), the classical diacetylated lactonic SL, as well as the acidic SL, should not possess a defined head/tail order, but a polar and unpolar side. The free hydroxyl groups of the disaccharide part, as well as the ester and ether groups, are all at one (the hydrophilic) side of the plane. The outer side of the carbohydrate, together with the fatty acid bridge, forms the hydrophobic part of the molecule. $C_{6'}$-OAc is positioned at the hydrophilic side, whereas $C_{6''}$-OAc is largely surrounded by the disaccharide and the fatty acid parts. This principal structure primary determines the surface-active characteristics of the molecule, and the modifications represented in Table 9 should impinge to only a small extent on the entire molecular arrangement.

17.6 Conclusions

The chemo-enzymatic synthesis of glycolipids using lipases, glycosidases and proteases as well as the enzymatic modification of naturally occurring glycolipids will become important methods for the preparation of new compounds with interesting interfacial and biological activities. Essential advantages of the biocatalysts are mild reaction conditions, their regioselectivities and, in some cases, their stereoselectivities. These methods are well established for preparative use, and may also have an important impact on the industrial production of glycolipids for use as surfactants, sweeteners, food ingredients, cosmetics and pharmaceuticals in future. Drawbacks, which have still to be overcome are low product yields and productivities in case of lipases and esterases; and low enzyme stabilities, low productivities and limited substrate specificities in case of glycosidases. Novel methods such as the lipase-catalyzed solid-phase synthesis, including addition of adjuvants, which can successfully be applied at the preparative scale, may not easily be used on an industrial scale. Potentialities to overcome some of the limitations mentioned above may be the design of ‘tailor-made’ and more stable biocatalysts. For instance, using rational and evolutional protein design as well as development and integration of new reaction processes, e.g. for the continuous removal of water and products in order to obtain higher yields with the shift of reaction equilibrium. The application and re-use of immobilized enzymes might reduce costs. With regard to the modification of naturally occurring glycolipids, the availability of low-priced precursors remains the limiting factor, but this should also be overcome in the future. In spite of these disadvantages, the modification of glycolipids using enzymes often represents the only possibility of synthesizing a specific molecule. However, if a molecule with special characteristics related to a profitable application is synthesizable, then a process for its economic production will be determined.

17.7 References

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18 Fatty Acid Hydroxylations using P450 Monooxygenases

Ulrich Schwaneberg and Uwe T. Bornscheuer

18.1 Introduction

Cytochrome P450 enzymes belong to the class of monooxygenases (E.C. 1.14.x.y). They are widely distributed in nature (Ortiz de Montellano, 1995; Lewis, 1996) and play a key role in various steps of primary and secondary metabolism as well as in detoxification of xenobiotic compounds (Stegeman and Lech, 1991; Goldstein and Faletto, 1993; Bolwell et al., 1994). Structural similarities of P450 enzymes reside in a heme system in the catalytic center which contains – in contrast to other enzymes – a fifth cysteine ligand co-ordinated to the iron atom. This feature is responsible for the characteristic spectral properties of P450 enzymes: the maximum absorption at 450 nm in the differential spectra of carbon monoxide, which gave P450 systems their name (Omura and Sato, 1964).

From a functional point of view, all P450 enzymes catalyze the transfer of molecular oxygen to usually nonactivated aliphatic or aromatic X-H bonds (X: -C, -N, -S) (Goldstein and Faletto, 1993). Furthermore, a remarkable number of P450 enzymes are capable of epoxidizing -C = C- double bonds (Ruettinger and Fulco, 1981; Lewis, 1996). For these oxygenation reactions most P450 enzymes require cofactors such as NADPH or NADH as reduction equivalents.

P450 enzymes play a pivotal role in metabolic pathways such as ergosterol biosynthesis (Berg et al., 1988), the biosynthesis of insect and plant hormones (Durst and O'Keefe, 1995; Feyereisen, 1999), the formation of colors and odors of plants (Holton et al., 1993; Holton, 1995), in aromatisation of androgen to estrogen, and in the metabolism of arachidonic acid to prostaglandins, leukotrienes and thromboxanes (Kupfer and Holm, 1989; Simpson et al., 1997). Furthermore, they are involved in activation and detoxification of xenobiotic compounds. As phase I enzymes (Goldstein and Faletto, 1993), P450 enzymes generate water-insoluble or barely soluble compounds for further metabolism by oxygenation. Subsequent reactions are performed by phase II enzymes such as glutathione transferases, *N*-acetyltransferases or sulfotransferases, which add a further polar group to the initially hydroxylated or epoxidized compounds and thereby make these metabolites water-soluble and bioavailable (Goldstein and Faletto, 1993).

The diversity of P450-catalyzed conversions has a tremendous biotechnological, pharmacological and toxicological potential. This is especially important because many reactions are difficult to perform by chemical means. In the following sections an overview regarding this potential with special emphasis on P450-mediated lipid modifications is provided. Limitations, as well as actual developments and future prospects, are also discussed.

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For other applications of P450s, readers are referred to a number of books and reviews (Juchau, 1990; Gonzalez and Gelboin, 1994; Ortiz de Montellano, 1995; Durst and O'Keefe, 1995; Lewis, 1996; Juchau et al., 1998).

18.2 Hydroxylation of fatty acids, by monooxygenases

Hydroxylated fatty acids have various (potential) applications as polymer building blocks, as intermediates in antibiotic synthesis (Schneider et al., 1998) or in medical fields. In lactonized form they can serve as perfume ingredients.

The hydroxylation of nonactivated -C–H bonds is one of the most useful biotransformations (Johnson, 1978; Kieslich, 1980; Mansuy and Battione, 1989) because the reactivity profile of enzymes in hydroxylations of -C–H bonds declines in the order secondary > tertiary > primary (Faber, 1997), in contrast to the reactivity in chemical radical reactions (tertiary > secondary > primary). Moreover, enzymatic hydroxylations often occur with high regio- and stereoselectivity. On the other hand, they can only compete with chemical syntheses, if the latter are more expensive or not feasible. Several chemical methods for the hydroxylation of fatty acids are well-known. For instance, the formation of ω-hydroxy fatty acids is achieved by oxidation of cyclic ketones using the Baeyer – Villiger reaction followed by alkaline ring opening. Until now, the Baeyer – Villiger oxidation via transition metal catalysts has been limited to cyclobutanone (Phillips and Romao, 1999), cyclopentanone and cyclohexanone and their derivatives (Strukul, 1998; Paneghetti et al., 1999). A more general pathway of synthesis starts from dihalogen alkanes, followed by the conversion to hydroxyhalogen alkanes and a Grignard reaction to introduce the carboxyl group (Becker et al., 1993). Remarkable progress has been achieved in catalytic chemistry in the past few years allowing regioselective hydroxylations of primary nonactivated -C–H bonds. For instance, the use of an aluminophosphate ($AlPO_4$-18) matrix containing Co^{2+} and Mn^{2+} ions and molecular oxygen as oxidant resulted in mainly terminal hydroxylation of *n*-alkanes (Hartmann and Ernst, 2000). For the stereoselective synthesis of hydroxylated fatty acids at in-chain positions only a few laborious pathways – mainly for the ω-1 position of C_{12} to C_{18} fatty acids – were described (Voss and Gerlach, 1983; Villemin et al., 1984). These reports include multistep syntheses and require protecting group chemistry.

Readers who are interested in learning more about the physiological roles of hydroxy fatty acids and their further metabolism are referred to a range of reviews describing for example P450 enzymes involved in the formation of arachidonate metabolites in the regulation of blood pressure (Hercule and Oyekan, 2000; McGiff, 1991, Oyekan et al., 1998; McGiff et al., 1991), in brain biochemistry (Yehuda et al., 1999) and peroxisome profileration (Bocos et al., 1995).

18.3 P450 fundamentals

18.3.1 Classification and nomenclature of Monooxygenases

The P450 superfamily is one of the largest and oldest gene families (Nelson and Strobel, 1988; Nelson et al., 1993). Based on a list of P450 enzymes published by Nelson et al. in 1996, 481 P450 sequences from 74 gene families were reported. Since then, the number of P450s increased tremendously, and currently more than 1000 putative P450 sequences are described (*http://drnelson.utmem.edu/CytochromeP450.html*). For example, the genome project of the plant *Arabidopsis thaliana* has led until the end of December 1999 to the identification of more than 270 new putative P450 genes (*http://drnelson.utmem.edu/Arablinks.html*) (Nelson, 1999). The classification of P450 genes is based on primary sequence homologies. All P450 genes with a protein sequence homology $> 40\,\%$ belong to the same gene family. If the protein sequence homology is $> 55\,\%$, the corresponding P450 genes belong to the same subfamily. To describe a cytochrome P450 gene, it is recommended to use the italicized abbreviation 'CYP' for all P450 genes except for mouse and *Drosophila*, which are represented by italicized 'Cyp'. The CYP abbreviation is followed by an arabic number denoting the family, a letter designating the subfamily (when two or more exist), and an arabic numeral representing the individual gene within the subfamily. For example, *CYP*4A1 represents the *first* gene in the P450 subfamily *A* of the P450 gene family *4*. To illustrate the evolutionary relationships, it was suggested to merge P450 families to so-called P450 clans (Nelson, 1998). According to this classification, the *CYP*51 family was identified to be the likely common ancestor of all P450 enzymes (Nelson, 1999).

Depending on the realization of the electron transfer system (reductase system), P450 enzymes are divided into four classes (Degtyarenko, 1995; Peterson and Graham, 1998):

- Class I: these mainly occur in mitochondrial systems and most bacteria. The electron systems consist of a FAD-domain as reductase and a further iron – sulfur protein.
- Class II: are often located in the endoplasmic reticulum and require only a single protein for the electron transfer, a FAD/FMN-reductase.
- Class III: do not require reduction equivalents. The P450 enzymes directly convert peroxygenated substrates which have already "incorporated" the oxygen.
- Class IV: only one enzyme is known, which receives its electrons directly from NADH.

18.3.2 Postulated reaction cycle of Monooxygenases

The postulated reaction cycle of P450 enzymes is shown in Figure 1 for P450cam from *Pseudomonas putida (*Hedegaard and Gunsalus, 1965; Tyson et al., 1972; Lewis, 1996), which catalyzes the regio- and stereospecific hydroxylation of camphor to 5-exo-hydroxycamphor.

It typifies how enzymatic oxygenation is performed under physiological conditions using molecular oxygen as oxidant. In the inactive and substrate-free form of P450cam (Kaim and Schwederski, 1995) the low-spin Fe^{III} (d^5, S = 0.5) center is six-fold co-ordinated via a porphyrin system, a fifth cysteinate ligand and a water molecule. Often, water molecules in enzymes which are co-ordinated to metal centers represent replacement positions for substrates. In the initial reaction step of the P450cam reaction cycle the water ligand is removed after binding of a camphor molecule near to the heme center **2** (Poulos et al., 1985, 1987). The camphor binding induces a change from the low-spin Fe^{III} configuration to a high-spin Fe^{III}-camphor complex **2** with an iron center far away from the plane porphyrin system (*out of plane*

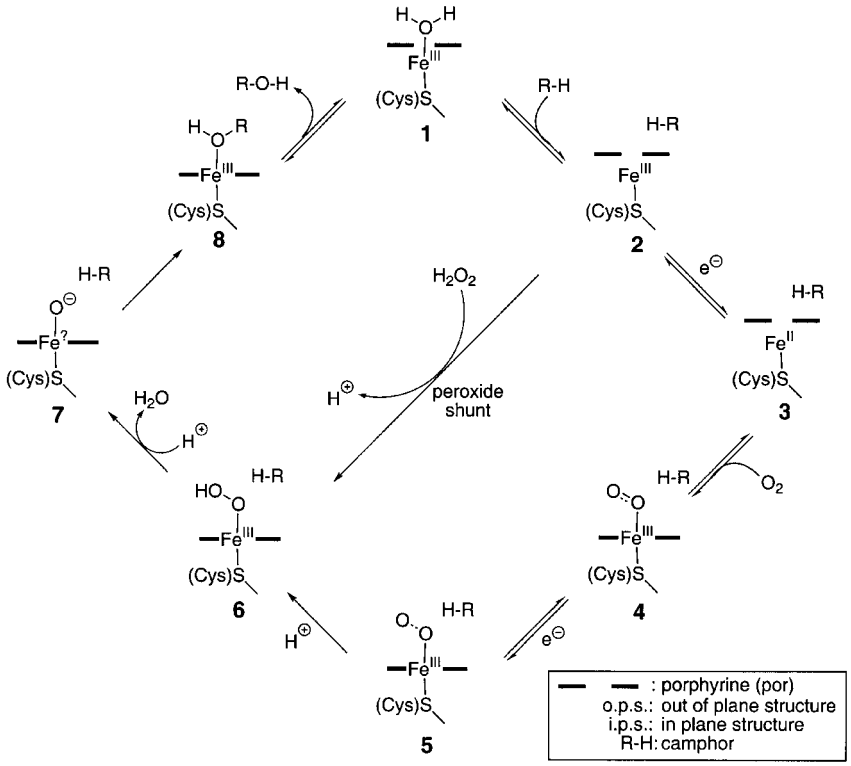

Figure 1. Reaction cycle of P450cam from *Pseudomonas putida*. **1**, P450 aquo complex, low-spin Fe^{III}, o.p.s; **2**, P450 camphor, high-spin Fe^{III}, o.p.s.; **3**, P450 camphor, high-spin Fe^{II}, o.p.s.; **4**, P450 camphor O_2^-, low-spin Fe^{III}, i.p.s.; **5**, P450 camphor O_2^{2-}, low-spin Fe^{III}, i.p.s.; **6**, P450 camphor HO_2^-, low-spin Fe^{III}, i.p.s.; **7**, P450 camphor, activated oxygen, $Fe^?$, i.p.s.; **8**, P450 camphor adduct, likely low-spin, Fe^{IV} with a cation radical porphyrin or Fe^{III} with neutral porphyrin, i.p.s.

structure). The redox potential is thereby increased from – 300 mV to – 173 mV, facilitating the oxidation or direct oxygenation of the iron center via the shunt pathway (**2** → **6**) by strong oxidants such as peroxides, periodate or peracids (Coon et al., 1996; Karuzina and Archakov, 1994; Joo et al., 1999a). Under physiological conditions, a one-electron reduction occurs, resulting in the formation of a high-spin Fe^{II} center (S = 2) **3** (Poulos and Raag, 1992). This configuration with four unpaired electrons is well prepared to bind triplet oxygen. The low-spin dioxy-iron (III) complex **4** was isolated and characterized by cryocrystallography (Schlichting et al., 2000) revealing the iron atom in-plane to the porphyrin system. The next step of the reaction cycle, a further one-electron reduction, produces a very unstable peroxo-iron complex **5**. The structure of the end-on or site-on coordinated dioxygen species is uncharacterized until now (Schlichting et al., 2000). For the cleavage of the iron-bound dioxygen, a two-proton shuttle is realized, involving water molecules and amino acid residues Asp_{251}, Asn_{255} and Thr_{252} (Gerber and Sligar, 1994; Vidakovic et al., 1998) of P450cam in a 'protein-solvent hydrogen-bonding network' (Schlichting et al., 2000). The oxy-ferryl species **7**, produced with simultaneous formation of water, has a rather planar heme, having the iron slightly above the plane and a short iron-to-oxygen distance suggesting an Fe = O bond. The oxidation state of the iron in this species and the electronic state of the heme could not be determined from the crystal structures. The removal of water allows the camphor molecule to move toward the heme, to be hydroxylated via a P450cam – substrate complex (Schlichting et al., 2000) to 5-hydroxycamphor and thereby regenerating the low-spin Fe^{III} **1** center. The postulated rebound mechanism (Ortiz de Montellano, 1995; Filatov et al., 1999) is under debate due to recent mechanistic studies (Toy et al., 1998) using ultrafast radical clocks to probe the presence of a free radical, that suggests a concerted oxene-insertion mechanism.

18.4 Fatty Acid-hydroxylating P450s Monooxygenases

The fatty acid-hydroxylating P450 enzymes can be divided – depending on the hydroxylation products – into terminal and subterminal fatty acid hydroxylases (Figure 2).

The hydroxylation of a nonactivated –C–H bond of a terminal $–CH_3$ group is thermodynamically 3 kcal mol^{-1} (Ortiz de Montellano et al., 1992) inferior to the hydroxylation of a secondary $–CH_2$-group. It requests higher steric demands toward the P450 enzyme with respect to substrate fixation, orientation and control of the hydroxylation cycle. The product profile of terminal and subterminal fatty acid hydroxylases clearly reflects these differences between the P450 species. The cytochrome P450 fatty acid ω-hydroxylases are a distinct family (*CYP*4) (Ortiz de Montellano et al., 1992) of cytochrome P450 enzymes, that exhibit a high preference for the hydroxylation of the terminal $–CH_3$ of saturated and unsaturated fatty acids, including compounds such as prostacyclins, thromboxanes and prostaglandins (Kupfer, 1980). Rather little is known about the mechanism of ω-hydroxylation due to a lack of crystal structures. Nevertheless, it is likely that ω-hydroxylating P450 enzymes achieve their regioselectivity by steric factors through nonbonding

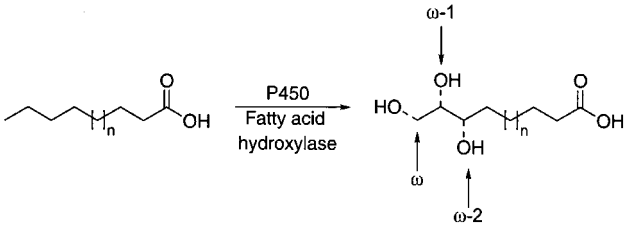

Figure 2. P450-catalyzed hydroxylations of fatty acids occur at different positions.

interactions (Ortiz de Montellano et al., 1992). P450 hydroxylases which preferable produce subterminal hydroxylated fatty acids are generally less specific cytochrome P450 enzymes which usually accept a wider substrate range and thereby generate two or more hydroxylation products (Table 1).

The oxidative reaction does not always stop after monohydroxylation, and further oxidation products such as aldehydes, ketones and carboxylic acids are often formed (Boddupalli et al., 1990, 1992). Interestingly, for P450 BM-3, a fatty acid hydroxylase from *Bacillus megaterium* (Ruettinger, 1989), it was shown that the composition of the initial and further oxidation products depends on the oxygen supply in the reaction solution (Schneider et al., 1999).

A large number of P450 enzymes of the gene families *CYP*4, *CYP*52 and *CYP*102 are known to catalyze fatty acid hydroxylations. Only well-characterized and recombinantly expressed P450s are presented in more detail in Table 1 and the following survey.

18.4.1 The *CYP*102 family

This family consists of only three members of which one – produced by the strain *Bacillus megaterium* – is able to catalyze the hydroxylation of fatty acids with the yet highest reported, turnover numbers for P450 monooxygenases in the range of > 1000 equiv min^{-1}. The corresponding enzyme is named P450 BM-3 and was cloned and functionally expressed in *E. coli* (Narhi and Fulco, 1986, 1987; Ruettinger et al., 1989).

P450 BM-3 (CYP102) is a water-soluble heme enzyme having a molecular weight of 118 kDa. It is a natural fusion protein containing the P450 and reductase part on one polypeptide chain (Narhi and Fulco, 1987; Ruettinger et al., 1989). The heme domain (Ravichandran et al., 1993; Li and Poulos, 1997) and reductase domain (Sevrioukova et al., 1999) were crystallized separately in the presence and/or absence of palmitoleic acid. From the four crystallized P450 enzymes known to date (Poulos et al., 1987; Ravichandran et al., 1993; Hasemann et al., 1994; Park et al., 1997), P450 BM-3 shows the highest sequence homology to eucaryotic P450 systems, and serves as preferred structural model (Burke et al., 1997; Lewis, 1998, 1999; Lewis and Lee-Robichaud, 1998).

Table 1. Survey of P450 enzymes involved in fatty acid modification.

P450-family	Source	Expression system	Chain-length	Hydroxylation position		References
				terminal	subterminal	
CYP2M1	rainbow trout liver	COS-7 & insect cells	C_{12}	–	ω-6	Yang et al., 1998
CYP4	Mammalian				only ω-1	
4Al[a]	Rat, rabbit	Various	C_{12}, C_{14}, C_{16}, $C_{20:4}$	Yes	2.5–50 %	Hoch et al., 2000
4A2[a]	Rat	Various	C_{12}, C_{14}, $C_{20:4}$	Yes	14 –29 %	Hoch et al., 2000
4A3[a]	Rat	Various	C_{12}, C_{14}, $C_{20:4}$	Yes	25 –45 %	Hoch et al., 2000
4A4	Rabbit	COS-1 cells	C_{16}, $C_{20:4}$	Yes	< 20 %	Roman et al., 1993
4A5	Rabbit	Various	C_{12}, C_{16}, C_{20}	Yes	23 –42 %	Hosny et al., 1999
4A6	Rabbit	COS-1 cells	C_{12}, C_{16}, $C_{20:4}$	Yes	8 –27 %	Roman et al., 1993
4A7	Rabbit	Various	C_{10}–C_{22}	Yes	6 –47 %	Sawamura et al., 1993
4A8[a]	Rat	Various	C_{12}, C_{14}, C_{16}	Yes	40 –62 %	Hoch et al., 2000
4A11[a]	Human	Various	C_{12}, C_{14}, C_{16}	Yes	5.9–45 %	Adas et al., 1999; Hoch et al., 2000
4B1	Human, rat, rabbit, mouse	Various	C_8–C_{10}, C_{12}	Yes	< 50 %	Fisher et al., 1998; Zheng et al., 1998
CYP52	*Candida maltosa*					
A3		*S. cerevisiae*	C_{12}, C_{14}, C_{16}	Yes	–	Zimmer et al., 1996
A4		*S. cerevisiae*	C_{12}, C_{14}, C_{16}	Yes	–	Zimmer et al., 1996
A5		*S. cerevisiae*	C_{12}, C_{14}, C_{16}	Yes	< 5 %, ω-1	Zimmer et al., 1996
A9		*S. cerevisiae*	C_{12}, C_{14}, C_{16}	Yes	< 5 %, ω-1	Zimmer et al., 1996
A10		*C. maltosa* CHA1	C_{12}	Yes		Zimmer et al., 1998a
A11		*C. maltosa* CHA1	C_{12}	Yes		Zimmer et al., 1998a
CYP81B1	*Helianthus tuberosus*	*S. cerevisiae* WR, WAT11, WAT21	C_{10}, C_{12}, C_{14}	–	ω-1, ω-3, ω-4	Cabello-Hurtado et al., 1998
CYP86A1	*Arabidopsis thaliana*	*S. cerevisiae*	C_{12}, C_{14}, C_{16}, C_{18}	Yes	–	Benveniste et al., 1998
CYP102	*Bacillus megaterium*	Various *E. coli*	C_{12}–C_{22}	Mutant F87A	ω-1, ω-2, ω-3	Boddupalli et al., 1990; Oliver et al., 1997b

[a] modified *N*-terminus and *C*-terminal His_6-tag

Table 2. Substrate profile of P450 BM-3 wild-type and mutants.

Substrates	Fatty acid chain-length	P450	K_m [μM]	K_{cat}/K_m [$*10^5 M^{-1}*s^{-1}$]	References
Saturated FA		WT	136, 7, 1.4	1.9, 82, 600	Oliver et al., 1997a
	C_{12}, C_{14}, C_{16}	R47E	2000, 18, 4	0.15, 3.9, 30	Oliver et al., 1997a
	C_{12}	F87A	167	1.5	Oliver et al., 1997b
Polyunsaturated FA		WT	n.m.	n.m.	Capdevila et al., 1996
	$C_{20:3}$, $C_{20:4}$, $C_{20:5}$	F87V	n.m.	n.m.	Graham-Lorence, 1997
ω-Oxo-FA	C_{12}, C_{14}, C_{16}, C_{18}	WT	n.m.	n.m.	Davis et al., 1996
p-Nitrophenoxy-FA		WT	n.m.	n.m.	Schwaneberg et al., 1999a
	C_8, C_{10}, C_{11}, C_{12}, C_{15}	F87A	n.m.	n.m.	Schwaneberg et al., 1999a
Trialkylmethylammonium		WT	782, 87, 5	200, 25, 2	Oliver et al., 1997a
	C_{12}, C_{14}, C_{16}	R47E	95, 23, 9	23, 6.6, 0.22	Oliver et al., 1997a

FA, fatty acids; n.m., not measured

A scheme of the binding site is shown in Figure 3, with the amino acids Arg_{47} and Tyr_{51} (which are responsible for the substrate fixation) and Phe_{87} (which influences the regioselectivity of the fatty acid hydroxylation) each being highlighted. The substrate profile of P450 BM-3 is summarized in Table 2.

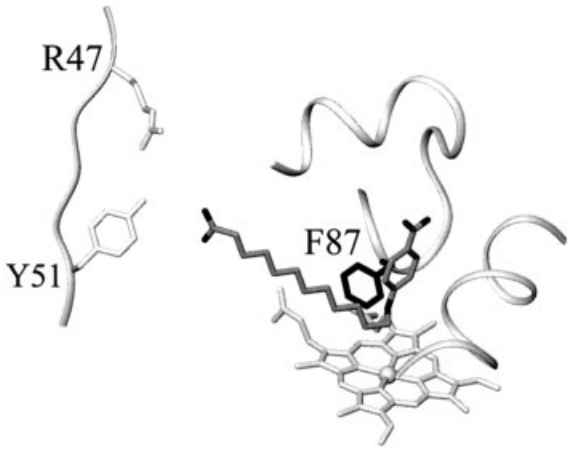

Figure 3. Model of the heme-domain of P450 BM-3 with bound *p*-nitrophenoxy dodecanoic acid (12-pNCA). Amino acids involved in substrate binding (Arg_{47}, Tyr_{51}) and regioselectivity (Phe_{87}) are highlighted.

Table 3. Regioselectivity of P450 BM-3 catalyzed hydroxylations of aliphatic substrates (Miura and Falco, 1975).

Substrate	Chain-length	Distribution of hydroxyl isomers[a] [%]		
		ω-1	ω-2	ω-3
Fatty acid	12	**36**	30	34
	13	17	**65**	18
	14	**44**	28	28
	15	32	**49**	19
	16	31	**48**	21
	17	**49**	35	16
	18	39	**47**	14
Alcohol	12	22	**51**	27
	13	24	**49**	27
	14	30	**44**	26
	15	27	**54**	19
	16	22	**41**	37
Amide	12	21	**45**	34
	14	34	34	32
	15	28	**57**	15
	16	29	**45**	26

[a] major products are highlighted in bold numbers

Fatty acids, alcohols, amides and other compounds which are able to establish hydrogen bonds with Arg_{47} and/or Thr_{51} will be hydroxylated. Esters and nonpolar compounds such as alkanes, which are unable to create these interactions or which cannot path the "hydrophobic mouth" at the beginning of the substrate access channel, are not or only marginally converted by P450 BM-3. Regioselectivity of the subterminal fatty acid hydroxylase P450 BM-3 depends strongly on the chain-length of the fatty acid substrate (Table 3).

Truan and co-workers (Truan et al., 1999) determined the absolute configuration of three primary metabolites obtained from palmitic acid showing high enantiomeric excess for (*R*)-15- and (*R*)-14-hydroxypalmitic acid (both 98 % ee) and somewhat lower selectivity for ω-3 hydroxylation (72 % ee) yielding (*R*)-13-hydroxypalmitic acid. Arachidonic acid is metabolized by P450 BM-3 to nearly enantiomerically pure (*R*)-18-hydroxyeicosatetraenoic acid (80 % of total products) and 14(*S*),15(*R*)-epoxyeicosatrienoic acid (20 % of total products). The arachidonic acid analogs eicosapentaenoic acid and eicosatrienoic acids were converted quantitatively to 17(*S*),18(*R*)-epoxyeicosatetranoic acid or 17-, 18-, and 19-hydroxyeicosatrienoic acid in a ratio of 2.4 : 2.2 : 1 (Capdevila et al., 1996). In addition to hydroxylation, Boddupalli and co-workers observed the formation of ketones such as 14-oxo- and 13-hydroxy-14-oxo-palmitic acid, which was related to a dehydrogenase

activity of P450 BM-3 (Boddupalli et al., 1992). If ω-oxo fatty acids, such as 18-oxooctadecanoic, 16-oxohexadecanoic, 14-oxotetradecanoic and 12-oxododecanoic acids, are used as substrates, the corresponding α,ω-diacids are generated (Davis et al., 1996).

Oliver and co-worker (1997a,b) showed that the amino acid at position 87 modulates the regioselectivity of the fatty acid hydroxylation. A single mutation (Phe87Ala) shifted the regioselectivity for lauric and myristic acid from subterminal to nearly exclusive terminal hydroxylation. An exchange of Phe_{87} to Val converted cytochrome P450 BM-3 into a regio- and stereoselective (14*S*,15*R*)-arachidonic acid epoxygenase (Graham-Lorence, 1997).

Replacement of Arg_{47} with Glu resulted in the ability of this P450 BM-3 mutant to hydroxylate *N*-alkyltrimethylammonium compounds (Table 2), which was explained by an inversion of the substrate binding conditions (Oliver et al., 1997a). P450 BM-3, heterologously expressed in *E. coli*, has been used *in vivo* to produce mixtures of chiral 12-, 13- and 14-hydroxypentadecanoic acid in preparative scale at high optical purity (Schneider et al., 1998). Furthermore, P450 BM-3 and its mutant Phe_{87}Ala can be expressed in gram scale and efficiently purified in a single step for further enzyme-based biotransformation reactions in preparative scale (Schwaneberg et al., 1999b). The pNCA activity assay shown in Figure 4 allows the determination of the P450 BM-3 wild-type and mutant F87A activity without background reaction (Schwaneberg et al., 1999a). After the hydroxylation of the terminal position, an unstable hemiacetal is produced which spontaneously dissociates into the ω-oxo-carboxylic acid and the chromophore *p*-nitrophenolate. The latter can be easily quantified at 410 nm using a spectrophotometer.

The pNCA assay is amenable to automation (Schwaneberg et al., 1999a), and is usable in a high-throughput screening environment to identify P450 BM-3 variants with beneficial mutants to overcome the deficiencies of the P450 BM-3 enzyme with respect to temperature, organic solvent or pH stability.

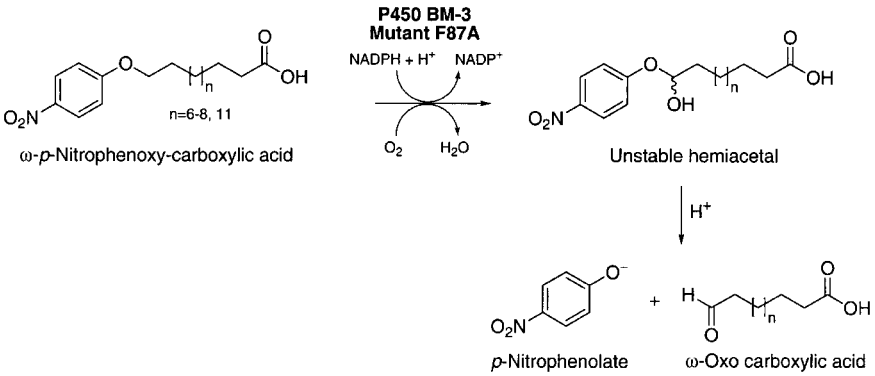

Figure 4. Principle of the colorimetric pNCA assay allowing the determination of the fatty acid hydroxylating activity of P450 BM-3 from *Bacillus megaterium* mutant F87A (Schwaneberg et al., 1999a).

18.4.2 The *CYP*52 family

Members of the *CYP*52 family have been found in *Candida* species such as *C. maltosa* (Schunck et al., 1989; Ohkuma et al., 1995), *C. apicola* (Lottermoser et al., 1996) and *C. tropicalis* (Seghezzi et al., 1992). They catalyze the terminal hydroxylation of *n*-alkanes, which represents the first and rate-limiting step in the alkane degradation pathway, and the ω-hydroxylation of fatty acids (Scheller et al., 1998). Most of these *CYP*52 genes were shown to be inducible by *n*-alkanes, alkenes, hydroxycarboxylic acids and carboxylic acids. In the yeast *Candida maltosa* – the mainly investigated *Candida* species – eight structurally related *CYP*52A genes have been identified (Zimmer et al., 1996). The characterization of these P450s resulted in a phylogenetic tree (Zimmer et al., 1998a) which describes the evolutionary distance among the members of the *CYP*52A family. This is also reflected by the differences in their substrate specificity. For instance, CYP52A3 isoenzymes (P450 Alk1A, P450 Cm1) prefer alkanes, CYP52A4 (P450 Alk3A, P450 Cm2) and CYP52A5 (P450 Alk2A) hydroxylate alkanes and fatty acids in similar extents, whereas CYP52A9 (P450 Alk5A), CYP52A10 and CYP52A11 prefer fatty acids as substrates. The individual substrate specificities are in good agreement to the inducer used for P450 expression (Ohkuma et al., 1995; Zimmer et al., 1996, 1998a).

As shown in Table 4, even the major *n*-alkane-inducible P450 form of *C. maltosa*, CYP52A3, catalyzes the ω-hydroxylation of lauric, myristic, and palmitic and oleic acid in remarkable turnover numbers between 5 and 41 equiv min^{-1}. The upon alkane induction of *C. maltosa* only poorly formed CYP52A10 and CYP52A11 enzymes failed to convert hexadecane, and could not initiate the first step of the alkane degradation pathway. However, lauric acid was found to be converted much more efficiently than palmitic acid by CYP52A10 and CYP52A11 (Zimmer et al., 1998a). Interestingly, all of these P450 enzymes have a carboxylic acid hydroxylation activity and Scheller and co-workers found that a single P450 enzyme, CYP52A3 from *C. maltosa*, catalyzes the complete oxygenation cascade starting from *n*-al-

Table 4. Turnover numbers in the fatty acid hydroxylation activity of P450 enzymes of the *CYP*52A family.

P450	**Turnover** [equiv min^{-1}]						**References**
	LA	**MA**	**PA**	**OA**	**DD**	**HD**	
Alk1A, Cm1	6	13	16	26	44	48	Zimmer et al., 1996
Alk2A	22	23	22	43	26	22	Zimmer et al., 1996
Alk3A	23	41	7	5	26	23	Zimmer et al., 1996
Cm2	40	42	3	2	24	20	Zimmer et al., 1996
Alk5A	33	41	51	81	2	12	Zimmer et al., 1996
CYP52A10	3.6	n.m.	0.2	n.m.	n.m.	< 0.1	Zimmer et al., 1998a
CYP52A11	4.0	n.m.	0.25	n.m.	n.m.	< 0.1	Zimmer et al., 1998a

n.m., not measured; LA, lauric acid; MA, myristic acid; PA, palmitic acid; OA, oleic acid; DD, dodecane; HD, hexadecane

kanes to α,ω-dicarboxylic acids (Scheller et al., 1998). Moreover, they observed that α,ω-dicarboxylic acids act as competitive inhibitors of *n*-alkane binding (Scheller et al., 1998).

The mutual exchange of Val in P450 Cm2 and Leu in P450 Alk3A at position 527 led to a direct transposition of the fatty acid chain-length specificities (Zimmer et al., 1998b), suggesting that amino acids at this site may determine the efficiency of fatty-acid hydroxylation relatively independent of other active-site residues. Moreover, Met to Leu substitutions at the corresponding alignment position in P450 Cm1 (CYP52A3), P450 Alk2A (CYP52A5) and P450 Alk5A (CYP52A9) altered the fatty-acid specificity of these enzymes (Zimmer et al., 1998b). In comparison to the structure of the bacterial P450 BM-3 (CYP102), it was reported by Zimmer et al. (1998b) that the amino acid at position 527 may be to close to the substrate-binding pocket near to the heme of CYP52A enzymes, which hydroxylate fatty acids at the ω-position.

18.4.3 The *CYP*4 family

The literature reporting fatty acid hydroxylations performed by P450 enzymes of the family *CYP*4 is extensive, and would go beyond the scope of this article. In order to present this family adequately, general characteristics of the *CYP*4 family and of a mainly investigated member, P450 4A1, are discussed in the following paragraphs.

*CYP*4 is one of the oldest P450 families and contains 22 subfamilies (Simpson, 1997). CYP4 enzymes are primarily involved in hydroxylation of fatty acids, prostaglandins, leukotrienes and other eicosanoids in mammalian species (Rendic and Di Carlo, 1997). The major fatty acid hydroxylating members of the *CYP4*A subfamily are summarized in Table 1. These enzymes all show strong preference for hydroxylation of the ω-position of fatty acids. Another common feature of many CYP4 enzymes is their inducibility by hypolipidemic agents (Simpson, 1997).

A comparison of the CYP4 enzymes is difficult due to the diversity of experimental systems employed for activity measurements. The CYP4A enzymes have been expressed in different hosts such as liver (Baron et al., 1981), kidney (Imaoka et al., 1993), HepG2 cells (Chaurasia et al., 1995), yeast (Hardwick et al., 1987) or with and without *N*-terminal modifications in *E. coli* (Hoch et al., 2000). Additionally, the conditions for reconstitution of the P450 domain with a reductase part often differ. A further handicap of these membrane-associated P450 enzymes is that no crystal structure has yet been published, which makes the identification of structure function relationships difficult. Homology modeling based on the crystal structures of P450 BM-3 identified some key residues of the CYP4A proteins (Lewis and Lake, 1999). For example, Lewis and Lake (1999) postulated that the ion-pairing to the fatty acid at position Arg_{47} of CYP102 finds its counterpart in CYP4A1 at Lys_{93}, in CYP4A4 at Lys_{90} and in CYP4A11 at Lys_{94}. For CYP4A1, they further suggested that the orientation for the ω-hydroxylation is achieved via a combination of electrostatic and hydrophobic interactions between substrate and enzyme, involving residues Lys_{93} and Arg_{230} necessary for ion-pairing with the carboxyl group of the fatty acid substrate and the residues Val_{118}, Leu_{131}, Leu_{223}, Val_{385} and Leu_{493}, which sup-

port or allow hydrophobic interactions to the fatty acid substrate. The CYP4A1 enzyme, which was first isolated from rat liver (Tamburini et al., 1984), is one of the most investigated and active fatty acid-hydroxylating P450 enzymes of the *CYP*4A family. Inspired by the natural fusion protein P450 BM-3, Fisher and co-workers fused the rat liver NADPH reductase to the rat liver P450 4A1. Subsequent expression of the fusion protein in *E. coli* resulted in a fatty acid hydroxylation activity between 10–30 equiv min^{-1} (Fisher et al., 1992a,b). The activity of this fusion protein was stimulated 10-fold (Chaurasia et al., 1995) and its active site was mapped by using fatty acid analogs with bulky groups at the ω-position and in the middle of the fatty acid chain (Bambal and Hanzlik, 1996a,b). They showed that the P450 4A1 fusion protein can hydroxylate lauric acid analogs bearing bulky groups (e.g., *t*-butyl or *p*-/*m*-tolyloxy) at the ω-position. In contrast, 1,3- or 1,4-phenylene units inserted near the middle of the chain, bind less well and were not hydroxylated (Bambal and Hanzlik, 1996a, b). For this artificial fusion protein, NADPH was successfully replaced by electrochemical reduction via the mediator cobalt(III)sepulchrate resulting in the hydroxylation of lauric acid (Faulkner et al., 1995).

By the use of a reconstituted system with the human P450 reductase, Hoch and coworkers (2000) reported high turnover numbers for rat P450 4A1 with values up to 649 equiv min^{-1}.

As shown in Table 5, a strong preference was also found for lauric acid compared to longer-chain fatty acids. Interestingly, the activity of this rat P450 4A1 depends strongly on the substrate concentration. An increase in the substrate concentration from 100 μM to 300 μM reduces the activity more than 60-fold of this heterologous in *E. coli*-expressed P450 4A1 (Hoch et al., 2000).

For P450s of the *CYP*4A family, the preferred substrates are $C_{12}-C_{20}$ fatty acids. Shorter (C_7-C_{10}) ones are not hydroxylated, or only at a low turnover. These C_7-C_{10} fatty acids are converted by CYP4B1 isoenzymes with turnover numbers between 0.8 and 11 equiv min^{-1} (Fisher et al., 1998). The rabbit P450 4B1 converts not only fatty acids, but also hydroxylates – as do some members of the *CYP*52 family – alkanes such as heptane, octane, nonane and decane at comparable or higher turnover numbers (Fisher et al., 1998).

Table 5. Substrate profile and activity of P450 enzymes of the CYP4A1 enzyme.

Fatty acid	**Turnover** [equiv min^{-1}] (**Fatty acid** [μM])	**Binding constant** K_s
Lauric acid	649 (100)	30
Myristic acid	230 (100)	46
Palmitic acid	60 (100)	n.m.
Arachidonic acid	6 (80)	n.d.
Oleic acid	1.4 (80)	n.d.

n.m., not measured; n.d., not detectable

18.5 Further aspects and future prospects

The information provided in this article, and especially the examples given above, demonstrate that P450 enzymes are indeed a versatile and useful class of enzymes. Industrial processes based on whole-cell hydroxylation steps are already known for example in progesterone modifications (Sallam et al., 1977), 3-α-hydroxyl-5-beta-cholic acid (Kulprecha et al., 1985) or β-hydroxybutyric acid production (Evans et al., 1979). However, the use of isolated and purified P450 enzymes in a preparative-scale synthesis of fine chemicals has to our knowledge not yet been reported. Major limitations in the use of P450 enzymes reside in their difficult handling, low turnover numbers (often between 0.1 and 100 equiv min^{-1}), lack of thermostability (Juchau, 1990; Yamazaki et al., 1997) and high sensitivity against organic solvents (Wade et al., 1972; Erjomin and Metelitza, 1983; Kuhn-Velten, 1997). This is especially true for membrane-bound P450s, which represent the largest population of P450 enzymes, because their activity depends strongly on the reconstitution conditions, and the ratio of P450 to reductase (Scheller et al., 1996), as well as the phospholipid composition used for reconstitution (Blanck et al., 1989; Kisselev et al., 1998). The lack of simple and rapid activity assays required to characterize P450 enzymes or to screen for improved P450 enzyme variants (Moore et al., 1997; Zhao et al., 1998), as well as the need for cofactor recycling, pose further major handicaps on the way to exploiting this class of catalysts for industrial applications.

However, much effort has been undertaken during recent years to overcome these drawbacks. Nowadays, even mammalian P450 enzymes can be expressed after *N*-terminal modification at high levels in *E. coli* (Barnes et al., 1991; Fisher et al., 1992a,b; Gillam et al., 1993; Barnes, 1996) or in yeast expression systems, yielding 10–100 mg P450 per liter of fermenter broth. To render membrane-associated P450 enzymes water-soluble, and to avoid reconstitution experiments, artificial fusion proteins between the P450 domain and the reductase part have been successfully constructed (Murakami et al., 1987; Fisher et al., 1992a,b; Yabusaki, 1995; Chun et al., 1997). A promising method of bypassing the cofactor regeneration of NADPH is, as shown for example for P450 BM-3, by using electrochemical means (Faulkner et al., 1995; Estabrook et al., 1996a,b), or with cobalt(III)sepulchrate, which can be reduced by cheap zinc dust (Schwaneberg et al., unpublished results). Alternatively, the shunt pathway (Figure 1) has been successfully evolved to drive the P450cam catalyst with hydrogen peroxide instead of NADPH (Joo et al., 1999a,b). In addition, the tremendous development of directed evolution methods during the past few years has resulted in powerful tools such as gene-shuffling (Stemmer, 1994) or the StEP-method (Zhao et al., 1998). These tools have been applied successfully to improve a wide range of different enzyme properties such as reactivity, solvent stability (Moore and Arnold, 1996), thermal stability (Giver et al., 1998) and regio- and stereoselectivity (Bornscheuer et al., 1999; Henke and Bornscheuer, 1999; May et al., 2000). It is likely that directed evolution will be applied to P450 enzymes in the near future, resulting in P450 catalysts which are more suitable for industrial applications.

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18.7 References

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