

Enzyme catalysed production of phospholipids with modified fatty acid profile

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Enzyme Catalyzed Production of
Phospholipids with Modified
Fatty Acid Profile

Anders Falk Vikbjerg
PhD Thesis

November 2006

BioCentrum-DTU
Technical University of Denmark

Preface

The present thesis entitled: "Enzyme Catalyzed Production of Phospholipids with Modified Fatty Acid Profile" is part of the requirements for obtaining a PhD degree at the Technical University of Denmark (DTU).

The work in this study was mainly performed at BioCentrum-DTU under supervision of Associate Professor Xuebing Xu (Main supervisor) and Associate Professor Huiling Mu. Part of the experimental work was performed in collaboration with Department of Chemical Engineering under supervision of Associate Professor Gunnar Jonsson, and LiPlasome Pharma A/S under supervision of their Director of Chemical Research Thomas Andresen and Chief Executive Officer Kent Jørgensen.

The project was financed by the Danish Technological Research Council for a period from 2003 to 2006. Honored student award partially supported the participation at the 2006 American Oil Chemists' Society (AOCS) annual meeting and expo in St. Louis, Missouri, USA.

November, 2006
Lyngby, Denmark



Anders Falk Vikbjerg

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I also wish to thank all present and past members in the “Lipid group” for good companionship.

Last, but not least, I would like to thank my wife Benedikte and our two daughters Claudia and Nicole, for their love and support during this work.

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Summary

This project is mainly a study on the enzyme catalyzed production of phospholipids with modified fatty acid profile (structured phospholipids). Besides production of structured phospholipids, membrane purification of structured phospholipids, and properties of selected structured phospholipids in emulsions and liposome formulations were also studied.

Replacements of existing fatty acids in natural soybean phospholipids with others not natural occurring, were done by acidolysis using different commercial microbial lipases and porcine pancreatic phospholipase A₂ (PLA₂). Lipases were used for modification of *sn*-1 positioned fatty acids of the phospholipids, whereas PLA₂ was used for modification of the *sn*-2 positioned fatty acids. Reactions were performed in both packed-bed and batch reactors with or without the presence of organic solvents. Effects of different reaction parameters, on primary- and side reactions, were examined for various reaction systems. TLC-FID method was developed during this work to assist the evaluation of product and byproduct formations.

The incorporation of desired fatty acids into phospholipid and recovery in batch reactors was affected by enzyme load, reaction time, reaction temperature, water content, substrate molar ratio and solvent amount. Influence of temperature and substrate ratio seemed to depend on the particular reaction system. In solvent systems using immobilized *Thermomyces lanuginosa* lipase incorporation of desired fatty acid increased with increased temperature (35-55°) and substrate molar ratio (3-15 mol/mol), whereas in solvent free system using immobilized *Rhizomucor miehei* lipase, incorporation decreased with increase of these parameters in similar range. During PLA₂ catalyzed acidolysis reaction, substrate ratio showed no effect on incorporation or yield, and maximum incorporation was observed at 45 °C. Individually, water content showed no effect on the incorporation in solvent-free system during lipase-catalyzed reactions; however it had significant effect during reactions involving PLA₂. With both types of enzyme, the recovery of diacylphospholipids decreased with increase of water content due to parallel hydrolysis. Presence of solvent improves mixing in the system, and makes subsequent removal of enzyme more convenient; however increasing amounts of solvent was shown to reduce recovery of phospholipid more strongly than it increased fatty acid incorporation during batch operation.

During lipase-catalyzed acidolysis reaction between phosphatidylcholine (PC) and acyl donor, the formation of glycerophosphorylcholine (GPC), the presence of acyl donor in the intermediate lysophosphatidylcholine (LPC) and migration into the *sn*-2 position of PC were observed, which are consequences of acyl migration. GPC formation increased especially with increasing water content in the reaction system; whereas incorporation

into LPC and migration into *sn*-2 position increased with reaction time. Acyl migration should be minimized in the reaction system in order to achieve a high product yield and purity.

Production of structured phospholipids in packed bed reactors was affected by the same reaction parameters tested during batch operation. Continuous operation in packed bed reactor was very difficult with a solvent free system. A long reaction time combined with rapid deactivation of the enzyme makes the process unfavorable. Solvent system seems to provide good choice for acidolysis reaction, as high incorporation and yields are achieved. Recovery of diacylphospholipids is considerably higher when reactions are performed in packed bed reactors as compared to batch operation.

For the separation of structured phospholipids from free fatty acids, a downstream process involving ultrafiltration was developed during this work. In non-polar solvent phospholipids tend to form reverse micelles, which can be separated from free fatty acids and solvent by using appropriate membranes. Different commercial membranes with different cut-off values were screened in dead end operation. Polysulphone membrane with polyester support showed some good qualities in terms of flux and selectivity. Multiple steps with dilution of retentate to minimize the viscosity and fouling were done to improve the separation. Membrane performance was shown to be very dependent on the initial feed concentration, concentration factor in each step and applied pressure.

Two individual studies were made to examine the physical and chemical properties of specific structured phospholipids. In the first study, the ability of enzymatically synthesized structured phosphatidylcholine containing caprylic acid to form and stabilize oil-in-water emulsions, prepared with different triacylglycerols, was examined and compared with natural soybean PC and deoiled lecithin. In the other study, oxidative properties of structured phospholipid containing highly unsaturated docosahexaenoic acid were examined in liposome formulations. The two studies demonstrate the potential usages of structured phospholipids, which have properties differing from natural soybean phospholipids.

Overall, the study provides detailed information for practical application of enzyme catalyzed acidolysis of structured phospholipids including down stream processing, and property evaluation of specific structured phospholipids.

Sammenfatning

Dette projekt omhandler hovedsagligt enzymkatalyseret produktion af phospholipider med ændret fedtsyre profil (strukturerede phospholipider). Udover produktion af strukturerede phospholipider blev membranoprensning af strukturerede phospholipider, samt egenskaber af udvalgte strukturerede phospholipider i emulsioner og liposomformuleringer ligeledes undersøgt.

Udskiftning af eksisterende fedtsyrer i naturligt forekommende sojabønne phospholipid med andre ikke naturligt forekomne fedtsyrer blev udført ved acidolyse med anvendelse af forskellige kommercielle mikrobielle lipaser og phospholipase A₂ (PLA₂) fra svinebugspytkirtler. Lipaser blev anvendt til modificering af phospholipider i *sn*-1 positionen, mens PLA₂ blev anvendt til modificering af phospholipider i *sn*-2 positionen. Reaktionen blev udført i både ”packed bed” reaktorer og batchreaktorer med eller uden tilstedeværelse af solvent (organisk opløsningsmiddel). Indflydelse af forskellige reaktionsparametre på primære reaktioner samt sidereaktioner i diverse reaktionssystemer blev undersøgt. En TLC-FID metode blev udviklet i løbet af projektet for at assistere evalueringen af produkt- og biprodukt dannelsen.

I batchreaktorer var inkorporering af de ønskede fedtsyrer i phospholipid og genfindning påvirket af enzymmængde, reaktionstiden, reaktionstemperaturen, vandindholdet, det molære substratforhold og mængden af solvent. Indflydelsen af temperaturen og det molære substratforhold var tilsyneladende afhængig af det enkelte reaktionssystem. I solvent systemer med anvendelse af lipaser fra *Thermomyces lanuginosa* steg inkorporeringen af ønskede fedtsyre ved stigende temperatur (35-55°C) og molært substratforhold (3-15 mol/mol caprylsyre/phospholipid). I solvent frie systemer med anvendelse af lipaser fra *Rhizomucor miehei* faldt inkorporeringen derimod med lignende temperaturområde og substratforhold. Ved PLA₂ katalyseret acidolyse reaktioner viste det molære substratforhold ingen effekt på inkorporeringen og udbyttet, og maksimal inkorporering blev observeret ved 45°C. Vandindholdet viste ingen effekt på inkorporeringen i et solvent frit system under lipase-katalyseret reaktioner, men havde signifikant effekt under reaktioner med PLA₂. Med begge enzymtyper faldt genfindingen af diacylphospholipider ved stigende vandindhold på grund af parallelle hydrolyse reaktioner. Tilstedeværelse af solvent forbedrer blandbarheden i reaktionssystemerne, og letter den efterfølgende fjernelse af enzymet. Til gengæld viste øget solventmængde at reducere genfindingen af phospholipid mere end det øgede inkorporering af fedtsyrer i en batchproces.

Under lipase-katalyseret acidolyse reaktioner mellem phosphatidylcholin (PC) og acyldonor blev dannelsen af glycerophosphorylcholin (GPC), tilstedeværelsen af acyldonor i mellemproduktet lysophosphatidylcholin (LPC), og migrering ind i *sn*-2

positionen af PC observeret, hvilket er konsekvenser af acyl migrering. Dannelsen af GPC stiger særligt med stigende vandindhold i reaktionssystemet; mens inkorporeringen ind i LPC og migreringen til *sn*-2 positionen stiger med reaktionstiden. Acyl migrering skal minimeres i reaktionssystemet for at opnå højt produkt udbytte og renhed.

Produktion af strukturerede phospholipider i "packed bed" reaktorer var influeret af de samme parametre, der var undersøgt for batch processer. Kontinuerlig proces i "packed bed" reaktor var vanskelig med et solvent frit system. En lang reaktionstid kombineret med hurtig deaktivering af enzymet gør processen ufavorabel. Et solvent system virker tilsyndeladende til at være et godt valg for acidolyse reaktioner, da høj inkorporering og udbytte opnås. Udbyttet af diacylphospholipider er betydeligt højere når reaktioner udføres i "packed bed" reaktorer i sammenligning med batch proces.

Til adskillelse af strukturerede phospholipider fra fedtsyrer blev der under dette arbejde udviklet en "downstream" proces, som involverer ultrafiltrering. I apolære solventer har phospholipider tendens til at danne "reverse micelles", som kan adskilles fra fedtsyrer og solvent ved anvendelse af passende membraner. Forskellige kommercielle membraner med forskellige "cut-off" værdier blev screenet under "dead end" princippet. En polysulfon membran med polyester support udviste gode egenskaber med hensyn til flux og selektivitet. Gentagne trin med fortynding af retentatet blev gjort for at forbedre separationen og minimere viskositet og fouling. Membran kapaciteten blev vist at være afhængig af initiale føde koncentration, koncentreringsfaktoren i hvert trin samt det påførte tryk.

To individuelle studier blev udført for at undersøge fysiske og kemiske egenskaber af specifikke strukturerede phospholipider. I det første studie blev evnen af enzymatisk syntetiseret strukturerede phospholipid indeholdende caprylsyre til at danne og stabilisere olie-i-vand emulsioner, fremstillet med forskellige triacylglyceroler, undersøgt og sammenlignet med naturlig sojabønne PC og "deoiled" lecithin. I det andet studie blev de oxidative egenskaber af strukturerede phospholipider med et højt indhold af umættede docosahexaensyre undersøgt i liposom formuleringer. Disse to studier demonstrerer de potentielle anvendelser af strukturerede phospholipider, ved at udmærke sig med egenskaber forskellige fra naturligt forekommende sojabønne phospholipider.

Alt i alt, giver dette arbejde detaljeret information til praktisk anvendelse af enzym katalyseret acidolyse af strukturerede phospholipider inklusiv oprensning, og evaluering af fysiske egenskaber.

List of publications

This thesis is based on work reported in the following publications referred to in the text by their Roman numerals:

- I** Vikbjerg, A.F., Mu, H., Xu, X. Lipase-catalyzed acyl exchange of soybean phosphatidylcholine in n-hexane: a critical evaluation of both acyl incorporation and product recovery. *Biotechnol. Prog.* 21:397-404 (2005).
- II** Vikbjerg, A.F., Mu, H., Xu, X. Monitoring of monoctanoyl phosphatidylcholine synthesis by enzymatic acidolysis between soybean phosphatidylcholine and caprylic acid by thin-layer chromatography with flame ionization detector. *J. Agric. Food Chem.* 53:3937-3942 (2005).
- III** Vikbjerg, A.F., Mu, H., Xu, X. Parameters affecting incorporation and by-product formation during the production of structured phospholipids by lipase-catalyzed acidolysis in solvent-free system. *J. Mol. Catal. B* 36: 14-21 (2005).
- IV** Vikbjerg, A.F., Mu, H., Xu, X. Elucidation of acyl migration during lipase-catalyzed production of structured phospholipids, *J. Am. Oil Chem. Soc.* 83:609-614 (2006).
- V** Vikbjerg, A.F., Peng, L., Mu, H., Xu, X. Continuous production of structured phospholipids in a packed bed reactor with lipase from *Thermomyces lanuginosa*. *J. Am. Oil Chem. Soc.* 82: 237-242 (2005).
- VI** Vikbjerg, A.F., Mu, H., Xu, X. Synthesis of structured phospholipids by immobilized phospholipase A₂ catalyzed acidolysis. *J. Biotechnol.* (Accepted for publication, November 2006).
- VII** Vikbjerg, A.F., Jonsson, G., Mu, H., Xu, X. Application of ultrafiltration membranes for purification of structured phospholipids produced by lipase-catalyzed acidolysis. *Sep. Pur. Technol.* 50:184-191 (2006).
- VIII** Vikbjerg, A.F., Rusig, J.-Y., Jonsson, G., Mu, H., Xu, X. Strategies for production and purification of lipase-catalyzed structured phospholipids. *Eur. J. Lipid Sci. Technol.* 108: 802-811 (2006).
- IX** Vikbjerg, A.F., Rusig, J.-Y., Jonsson, G., Mu, H., Xu, X. Comparative evaluation of the emulsifying properties of phosphatidylcholine after enzymatic acyl modification. *J. Agric. Food Chem.* 54:3310-3316 (2006).
- X** Vikbjerg, A.F., Andresen, T.L., Jørgensen, K., Mu, H., Xu, X. Oxidative stability of liposomes composed of DHA-containing phospholipids. (In preparation).

Abbreviations

ANOVA	Analysis of variance
CLA	Conjugated linoleic acid
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
FA	Fatty acid
FFA	Free fatty acid
FID	Flame ionization detector
GC	Gas chromatography
GPC	Glycerophosphorylcholine
HLB	Hydrophilic/Lipophilic Balance
LPC	Lysophosphatidylcholine
LUV	Large unilamellar vesicles
MCT	Medium chain triacylglycerols
MLV	Multilamellar vesicles
MVV	Multivesicular vesicle
PA	Phosphatidic acid
PAF-AH	Platelet-activating factor acetylhydrolase
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PL	Phospholipid
PLA ₁	Phospholipase A ₁
PLA ₂	Phospholipase A ₂
PLB	Phospholipase B
PLC	Phospholipase C
PLD	Phospholipase D
PBR	Packed bed reactor
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acids
RSM	Response surface methodology
<i>sn</i>	Stereospecific numbering
SUV	Small unilamellar vesicles
TAG	Triacylglycerols
TLC	Thin layer chromatography
TBARS	Thiobarbituric acid reactive substances

1 Introduction

Phospholipids are used for different applications in food, pharmaceutical, and cosmetic products, where they function as emulsifiers, stabilizers and antioxidants. Most applications of phospholipids are based on natural products having complex composition, such as lecithin, which is a by-product from oil production. Chemical and enzymatic modifications of phospholipids to alter emulsifying and dispersing properties are common practice in industry, as the application range of phospholipids is widely extended (Schneider, 1997). The aim of these modifications is to adapt phospholipids for specific application requirements by giving it technical or physiological properties that the natural phospholipid substance does not possess. Chemical modification involves hydrolysis, hydroxylation, acylation, and hydrogenation (Bueschelberger, 2004). To large extent, commercial use of enzymes for phospholipid modification is known for only partially hydrolysis to produce lysophospholipids, which have improved emulsifying properties compared to natural phospholipids (Schneider, 1997). During the last two decades the interest for enzymatic acyl modification has increased continuously as enzymes are able to tailor phospholipids with defined fatty acid composition at the *sn*-1 and *sn*-2 positions. Replacement of existing fatty acids in the original phospholipid with desired fatty acids might improve physical and chemical properties or even nutritional, pharmaceutical and medical functions. Most of the work in this direction focus on incorporation of saturated fatty acids (including both medium and long chain) or polyunsaturated fatty acids (Hossen and Hernandez, 2005; Lyberg *et al.*, 2005; Reddy *et al.*, 2005; Peng *et al.*, 2002). The interest in the incorporation of saturated fatty acids is mainly to improve the heat stability, emulsifying properties and oxidation stability of the phospholipids (Chmiel *et al.*, 1999; Pedersen, 2001). The interest in incorporation of polyunsaturated fatty acids is due to claimed health promoting effects (Takahashi and Hosokawa, 2001). Lipases of microbial origin and phospholipase A₂ (PLA₂) from porcine pancreas have been the most commonly used enzymes for the exchange of fatty acids on phospholipids at *sn*-1 and *sn*-2 positions, respectively.

Enzymatic synthesis of phospholipids has some advantages compared to chemical synthesis such as selectivity and specificity. Further more, enzymatic reactions occur under mild temperature, pH and pressure conditions, as they generally require less energy and are conducted in equipment of lower capital cost than many other chemical processes. The limitation of industrial applications of enzymatic approaches is the high cost of enzymes. However with progress in enzyme technology it is believed that the future process costs of biocatalysts will decrease.

The increasing interest from industry to produce specific structured phospholipids by enzymatic modification inspired to this PhD project. With a practical process line it would dramatically increase application range of phospholipids. It was anticipated that through optimization of different reaction systems that it would be possible to set up practical processes, which potentially can be scaled up to pilot plant scale or production scale.

This thesis deals mainly with enzyme catalyzed production of phospholipids with modified fatty acid profile (structured phospholipids). Other parts of the thesis relate to purification of structured phospholipids, and physical/chemical properties of structured phospholipids in emulsions and liposome formulations. The participants in this project were: BioCentrum-DTU; Department of Chemical Engineering, and LiPlasome Pharma A/S. The project structure and involvement of participants are presented in Figure 1.1.

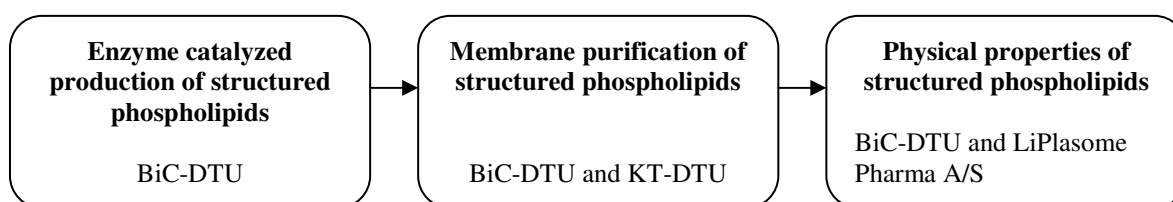


Figure 1.1: Project structure and participants. Abbreviations: BiC-DTU= BioCentrum-DTU; KT-DTU= Department of Chemical Engineering, DTU

1.1 Objectives and thesis outline

The objectives of this project were as following:

- 1) Development of potential processes based on enzymatic interesterification (acidolysis) to produce phospholipids with specified fatty acid profiles including process optimization.
- 2) Elucidation of side reactions and reduction of by-products.
- 3) Set-up membrane separation system for purification of structured phospholipids.
- 4) Examination of the emulsifying properties of enzymatically synthesized phospholipid.
- 5) Investigation of the possibilities of applying specific structured phospholipids in liposome formulations.

The present thesis is organized into three parts.

The first part covers the theoretical background for this study (Chapter 1-4). Chapter 1 is the introduction, which gives the background, and the objectives and main content of this thesis. Chapter 2 presents natural occurrence and structural characteristics of phospholipids, traditional physical and chemical approaches to modify phospholipids, and their applications in emulsions and liposome formulations. Chapter 3 presents the enzymes related to phospholipid modification, including enzyme sources, enzyme structures and functions, catalytic mechanism of the enzymes, and different techniques for their immobilization. Chapter 4 discusses the main reactions and side reactions for the production of structured phospholipids, effects of different parameters, tools for optimizing reaction processes, and downstream processing.

The second part of this thesis presents the applied experimental and analytical approaches (Chapter 5), a summary of obtained results and discussion of the findings (Chapter 6), and conclusion and future outlooks (Chapter 7).

Finally the third part consists of the ten research articles, where the results of the experimental work are presented. These articles are presented as appendices: Paper I-X.

2 Phospholipids: Properties and technological functions

2.1 Classification, structural characteristics and occurrence

Phospholipids are amphiphilic molecules that are ubiquitous in nature. Although a great variety of phospholipid structures have been found, they generally contain a glycerol or sphingosyl backbone (Gunstone *et al.*, 1994; Silvius, 1993; Vance and Vance, 2002). Phospholipids are classified into four main classes: glycerophospholipids, sphingolipids, ether phospholipids and phosphonolipids, depending on their backbones and bonding types (Hawthorne and Ansell, 1982). Of these only the glycerophospholipids and phosphosphingolipids occur naturally in amounts that have led to industrial applications.

The molecular structure and names of glycerophospholipids is presented in Figure 2.1. In naturally occurring glycerophospholipids fatty acids are esterified to the *sn*-1 and *sn*-2 positions, and a polar phosphate group is esterified to the *sn*-3 position of the glycerol backbone. The phosphoglycerols are all derivatives of phosphatidic acid (PA). Attached to the phosphate group is an amino alcohol, which may be serine, choline or ethanolamine. Alternatively, a polyhydroxy compound which is either glycerol or inositol is attached instead. The most abundant phospholipid class in nature is phosphatidylcholine (PC).

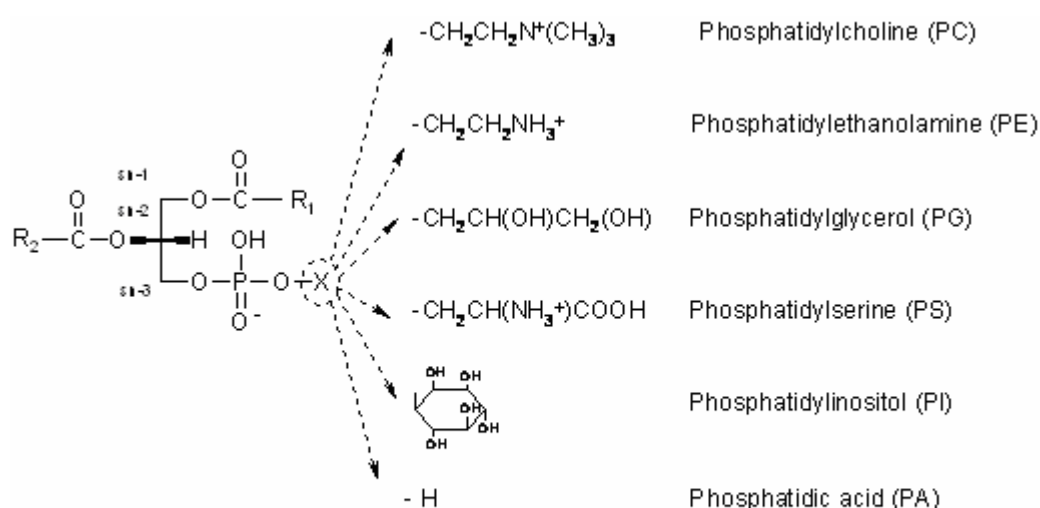


Figure 2.1: Schematic representation of chemical structures of natural glycerophospholipids. *sn* refer to international stereospecific numbering for natural stereoisomers. R_1 and R_2 refer to fatty acids.

The different phosphoglycerols are often named by placing the constituted attached to the phosphate group after 'phosphatidyl' e.g. phosphatidylcholine (3-*sn*-phosphatidylcholine or 1,2-diacyl-*sn*-glycero-3-phosphorylcholine). Glycerophospholipids only having one acyl group at *sn*-1 or *sn*-2 position are termed lysophospholipids.

As glycerophospholipids are essential components of biological membranes, they can be obtained from all types of biological tissue; however commercial sources are predominately from vegetable oil seeds. Egg phospholipids used in pharmaceutical and cosmetics, are very expensive in their production. Hence, in general these are not competitive with vegetable phospholipids in technology driven applications of the food industry. Fatty acids found in glycerophospholipids show variation in chain length (C₁₂-C₂₂) and degree of saturation (Bueschelberger, 2004). Saturated fatty acids are located dominantly at *sn*-1 position, whereas unsaturated fatty acids are located at *sn*-2 position. Glycerophospholipids from plant sources have a high level of polyunsaturation in fatty acid chains, and glycerophospholipids from mammalian sources contain higher portion of fully saturated chains. Due to the many possible variations in the fatty acid chains many different phosphoglycerides exist. When full chemical structure is known the apolar part of the phosphoglycerides is designated as well (e.g. 1-palmitoyl-2-oleoyl-phosphatidylcholine).

2.2 Structures and phases of phospholipids

Most amphiphilic molecules display a double affinity, on one hand for aqueous environment (polar) and on the other hand for organic media (non-polar) (Chopineau *et al.*, 1998). In water, phospholipid molecules self-associate, which eliminates the energetically unfavorable contact between the non-polar part of these molecules and water, while retaining their polar part in an aqueous compartment. Self-association of the phospholipid molecules results in molecular aggregates of very different size and geometries, depending on various intrinsic parameters (hydrophilic/lipophilic balance, degree of ionization, concentration, etc) and on external factors (temperature, pH, ion strength, etc.). Figure 2.2 represents some of the basic structures that are frequently found when phospholipids are mixed in aqueous solution. In the presence of organic solvent phospholipids tend to form reverse micelles (Walde *et al.*, 1990).

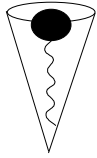
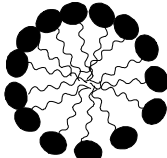
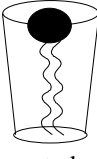
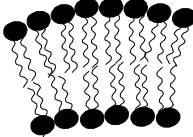
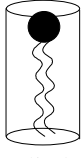
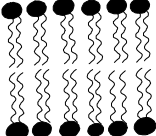
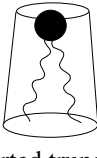
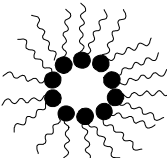
Shape	Organization	Phase	Amphiphiles
 Cone		Micelles Hexagonal I	Single-chained amphiphiles with large head group
 Truncated cone		Flexible lamellar (vesicle)	Single-chained amphiphiles with small head group or double-chained amphiphiles with large head group, fluid chains
 Cylinder		Lamellar cubic	Double-chained amphiphiles with small head group
 Inverted truncated cone		Reversed micelles Hexagonal II	Double-chained amphiphiles with small head group, nonionic amphiphiles, polyunsaturated chains

Figure 2.2: Structures and phases of amphiphiles (Modified from Chopineau *et al.*, 1998)

2.3 Production of Vegetable lecithins and their further processing

Vegetable phospholipids are manufactured exclusively from by-products of the vegetable oil refining process. Crude lecithin (a mixture of natural phospholipids) is obtained in the process of degumming vegetable oil (Bueschelberger, 2004; Schneider, 1997). Even though crude vegetable oil only contains 0.3-2.5 wt% phospholipids they must be removed in order to stabilize oil against sedimentation and off-taste development. Degumming of vegetable oil also reduces oil losses in subsequent phases of refining and helps avoid excessive darkening of the oil in the course of high-temperature deodorization.

In the first step of standard batch degumming warm water is added to the vegetable oil. The addition of water causes the polar lipids contained in the oil to hydrate, forming a heavy oil-insoluble sludge, which is separated from the oil by use of centrifugation. The

crude plant lecithin is obtained by careful drying. The lecithin has a typical composition of PC, 20%; PE, 15%; PI, 20%; neutral lipid, 35% and other components (van Nieuwenhuyzen, 1976).

There are various reasons for further purification of the phospholipids. Pure phospholipids are value-added nutritional and pharmaceutical compounds, and specific phospholipids have dedicated surface active properties, which support the processing of emulsion stability and shelf-life (van Nieuwenhuyzen, 1999). Further processing of lecithin involves solvent fractionation and chromatographic technology for preparation of high purity products (Doig and Diks, 2003b; Gunther, 1984a; Gober *et al.*, 1995; Napp, 1985). The neutral lipid in unrefined lecithin can be removed by extraction with acetone (polar lipids are insoluble in acetone) to give a dry granular product. Solubility difference of the phospholipids in different solvents makes it possible to obtain fractions enriched with selected phospholipid species. Ethanol fractionation of deoiled lecithin results in a soluble fraction consisting of PC and an insoluble fraction consisting of PE and PI (Pardun, 1984). Chromatographic purification is able to separate the phospholipids into different classes depending on polar head group of the molecule (Elsner and Lange, 1993; Lange *et al.*, 1994); however separation of molecules with defined fatty acid composition is by no means simple.

2.4 Phospholipid modification

Molecular structure of phospholipids can be altered by either chemical or enzymatic means. Aim of such processes is to obtain tailor-made technological and physiological properties that are somewhat different from those of natural products themselves. Chemical methods have long been used for modification of phospholipids mainly for the improvement of emulsification and oxidation properties. The chemical methods include acyl hydrolysis to produce lysophospholipids, hydrogenation and hydroxylation of double bonds in the fatty acids, and acetylation of the amino group of phosphatidylethanolamine (PE) (Doig and Diks, 2003b; Vandana *et al.*, 2003; Brois *et al.*, 1992; Betzing, 1980; Gunther, 1984b). For many years, the commercial use of enzymes for lecithin modification is only known with phospholipase A₂ from porcine pancreas for partial hydrolysis to produce lysophospholipids (Chung and Yang, 2004; Colarow *et al.*, 1994; Buikstra *et al.*, 1997; Yesair, 1998). However, since the late 1980s a large number of publications and patent applications related to other enzymes acting on phospholipids and the reactions they can catalyze have emerged. For overview of the enzymatic phospholipid modification the reader is recommended the following reviews:

Adlercreutz *et al.*, 2003; Ulbrich-Hofmann, 2003; Servi, 1999; D'Arrigo and Servi, 1997.

2.5 Synthesis of phospholipids with defined fatty acid chains

The preparation of phospholipids with specific polar head group and different and defined acyl chains at the *sn*-1 and *sn*-2 position can be effected either by total synthesis from appropriate homochiral precursors or by a combination of chemical and enzymatic transformation starting from materials derived from natural phospholipids (D'Arrigo and Servi, 1997).

A simple synthesis of diacylated phospholipids is by acylation of *sn*-glycerophospholipids or *sn*-glycerophosphate, which can be chemically esterified with desired head group alcohols. Glycerophosphatidylcholine can be obtained by deacylation of crude phospholipid preparation with alkali treatment (Chadha, 1970). Acyl donors are usually fatty acid anhydrides or fatty acid imidazolides. For efficient acylation, reactions are performed in the presence of catalyst. Pyrrolidinopyridine and N,N,- dimethyl-4-aminopyridine have been used for reactions involving fatty acid anhydrides, and methylsulfinylmethide have been used for reactions involving fatty acid imidazolides (Patel *et al.*, 1979; Gupta *et al.*, 1977; Hermetter and Paltauf, 1981).

For synthesis of phospholipids with different fatty acid in the *sn*-1 and *sn*-2 position an enzymatic step can be applied. First symmetric acid phospholipids is hydrolyzed by phospholipase A₂ followed by chemical reesterification of the resulting 1-acyl-lysophospholipid (Gupta *et al.* 1977; Mason *et al.*, 1981; Nicholas *et al.*, 1983).

In principle hydrolytic enzymes can also catalyze the reverse reaction i.e. the ester bond formation, and thus be an alternative to chemical esterification. Compared to chemical modification, enzymatic modification of phospholipids has a few advantages. Selectivity or specificity of enzyme is one of the most important properties of enzymes, which can not be easily achieved by chemical methods. Enzymatic reactions are often conducted under mild conditions, which help retain the original properties of those heat- or oxygen-sensitive phospholipids.

With possible and available enzymes, the manipulation of the phospholipid structure can be complicated but versatile. Various reaction routes can be implemented, and have been used for the production of different products. In the next two chapters the possible enzymes and reaction routes for phospholipid modification are described in more detail.

2.6 Technological applications of phospholipids

Most applications of phospholipids are related to their technological functions. As a consequence of the amphiphilic character, phospholipids are surface active molecules. The fatty acid portion of the molecule is attracted to fats and the polar head group is attracted to water. Because of this dual nature, lecithin molecules tend to position themselves at the boundary between immiscible materials, such as oil and water. There they serve many useful functions through a surface modifying effect. Phospholipid products in various forms are used for wetting, solubilization, lubrication, emulsification, or dispersion of products from technical industry, in feed and food products, and in cosmetic and pharmaceuticals (Schneider, 1997).

Application in industrial products involves coatings (paints, magnetic tape coatings, waxes, and polishes), plastic and rubber industry, glass and ceramic processing, paper and printing, masonry and asphalt products, petroleum industry, metal processing, pesticides, adhesives, textiles, and leathers (Schneider, 1997).

Lecithin is also used in a large range of foods, such as margarine (for anti-spatter and as an emulsifier), chocolates (to control viscosity and crystallization), chewing gum (for its softening, plasticizing, and release effects), and instant food products (for wetting, dispersing, and emulsification) (van Nieuwenhuyzen, 1976, 1981)

Phospholipids are highly specialized lipids that are key constituents of the cell membranes and are essential for the growth, maturation, maintenance, and functional capacity of all cells of animal and human body (Szuhaj and van Nieuwenhuyzen, 2001). Much research has been done on the therapeutic use of phospholipids, especially in the prevention or treatment of neurological and cardiovascular disorders (Lohmeyer and Bittmann, 1994; Crook *et al.*, 1992; Suzuki *et al.*, 2001). Also the possibilities as potential drug carriers in the form as liposomes have been extensively studied (Kisel *et al.*, 2001; Andresen *et al.*, 2005)

Some of the most exploited aspects of phospholipids from scientific point of view have been their emulsifying properties and ability to self-assemble forming liposomal structures for uses in food, pharmaceuticals and cosmetics. More information on these issues is given in the following sections.

2.6.1 Physical characteristics of emulsions

Phospholipids have been extensively used as emulsifiers to stabilize food and industrial emulsions. Emulsions are defined as mixtures of at least two immiscible liquids, which in foods usually mean oil and water. One of these constitutes the continuous (or external) phase, within which the dispersed (discontinuous, internal) phase is found as fine droplets. Emulsions in which the oil droplet are dispersed in the continuous water phase are designated oil-in-water (o/w) emulsions (e.g. butter and margarine), whereas water-in-oil emulsions (w/o) are emulsions in which water droplets are dispersed in the continuous oil phase (e.g. dressings and mayonnaise) (Schneider, 1986). Emulsions are produced through dispersing one immiscible phase in another by mixing, colloidal milling or homogenization. Emulsions are thermodynamic unstable, and will transform into total phase separated system over time. As the interfacial area increases, either through a decrease in particle size or the addition of more dispersed phase material, i.e. higher fat, more energy is needed to keep the emulsion from coalescing. However, upon addition of suitable emulsifiers, such as phospholipids, emulsions become kinetically stable for longer period of time (Friberg *et al*, 1990). Because phospholipids contain both hydrophilic and hydrophobic group on the same molecule they will orient themselves at the oil-water interface. At the oil-water interface they will lower the interfacial tension between the two phases and reduce the pressure gradients required to disrupt the droplets during emulsification process. Thereby, tendency of droplet coalescence is reduced. Stability of emulsions depends on several factors such as quality of emulsifying agent, viscosity of the phases and volume ratio of phases (Zhang and Proctor, 1997).

The hydrophilic /lipophilic characteristics of emulsifiers are normally standardized by their HLB value (**H**ydrophilic/**L**ipophilic **B**alance) to predict preference for oil or water. The HLB scale is 0 to 20, with the lower end identifying emulsifiers with a strong lipophilic character, while the higher end identifies emulsifiers with a stronger hydrophilic character (Schneider, 1997). This system is used as a guide to emulsifier selection for a given application. Even though the HLB system does not fit perfectly for zwitterionic molecules, application technologists still use it as guideline for selecting appropriate phospholipid products for emulsification purposes (Figure 2.3).

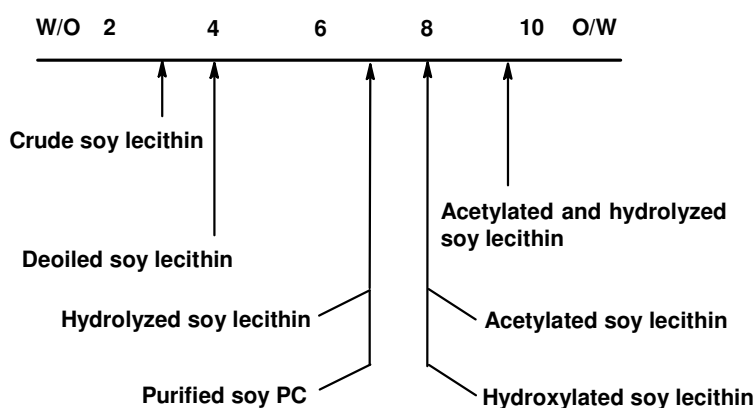


Figure 2.3: Location of different phospholipid products in the HLB system. Phospholipids having a low HLB are suitable for water-in-oil (w/o) emulsions and the ones having high HLB are for oil-in-water (o/w) emulsions.

Phospholipids have been applied in both water-in-oil (w/o) emulsions and oil-in-water (o/w) emulsions. For many industrial applications crude phospholipid products obtained from vegetable oil refining can be used directly, however usually it is desired to have some kind of purification. Deoiled lecithin is more commonly used as emulsifier as the phospholipid concentration is higher (neutral lipids have been removed), which means that significant lower dosage requirements are needed and higher functionality is achieved. PC-enriched lecithins have also been reported to deliver superior o/w emulsion capacity compared to the standard de-oiled product (Bueschelberger, 2004). The chemical and enzymatic methods commonly used for modification of phospholipids especially improve the emulsifying properties for o/w emulsions, as HLB value usually increases by these modifications.

2.6.2 Physical Characteristics of liposomes

When phospholipids are dispersed in aqueous media, they spontaneously form closed membrane capsules delimited by single or multiple lamellae commonly referred to as liposomes (New, 1990). The structure of liposomes offers separation of two aqueous compartments by a membrane. Liposomes can be used to entrap materials such as drugs both within the central aqueous compartment if they are water soluble and within the membrane if they are oil soluble. During the last thirty years the interest in liposomes for tissue specific transport of drugs has steadily grown and liposomal drug delivery systems are now being studied as promising drug carriers (Andresen *et al.*, 2005; Vemuri and Rhodes, 1995). Current applications of liposomes are vehicles to deliver drugs to specific parts of the body e.g. AmBisome®, an antifungal drug (liposomal

amphotericin B) (Boswell *et al.*,1998). Liposomes are also currently used extensively in cosmetics due to their claimed moisturizing effects.

Formation of liposomes results either from spontaneous curvature of a lamellar structure or require specific conditions (heating, mechanical constraints, solvent extraction, etc.) which bring energy to the system. Liposomes are usually classified according to their size range, number of lamellae and available internal volume. In Figure 2.4 a schematic illustration of liposomes of different size and number of lamellae is presented. One can distinguish between multilamellar vesicles (MLV), Multivesicular vesicles (MVV), large unilamellar vesicles (LUV), or small unilamellar vesicles (SUV). SUV show a diameter of 20 to approximately 100 nm. LUV, MLV, and MVV range in size from a few hundred nanometers to several microns.

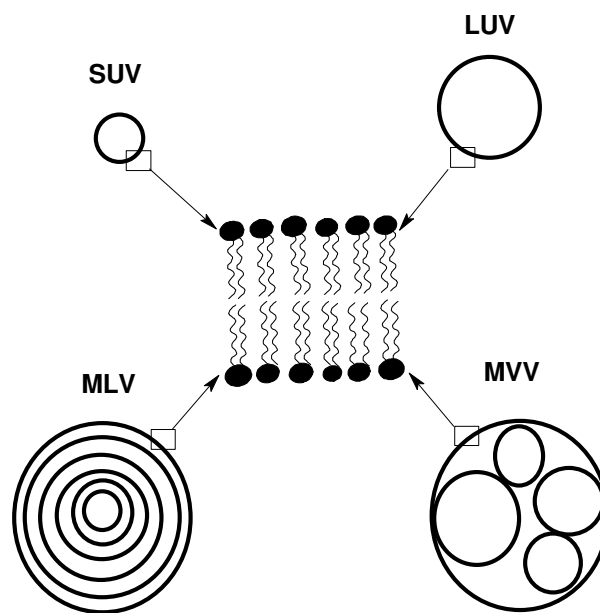


Figure 2.4: Schematic illustration of liposomes of different size and number of lamellae. SUV: Small unilamellar vesicles; LUV: Large unilamellar vesicles; MLV: Multilamellar vesicles; MVV: Multivesicular vesicles.

3 Enzymes related to phospholipid modification

Enzymes that utilize phospholipids as their natural substrate are called phospholipases and are named according to hydrolytic action on phospholipid molecules. A glycerophospholipid together with the names of the enzymes which hydrolyze the different ester bonds in the phospholipid molecules is shown in Figure 3.1.

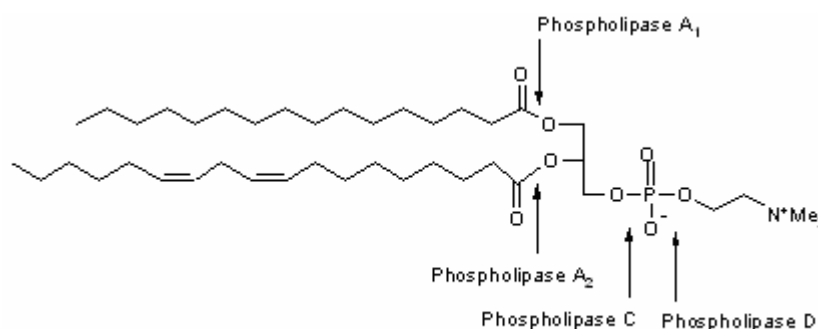


Figure 3.1: 1,2-diacyl-*sn*-glycerophosphorylcholine molecule and phospholipases which can be used to hydrolyse or form different ester bonds.

Phospholipase A₁ (PLA₁) and phospholipase A₂ (PLA₂) are acyl hydrolases, which hydrolyze acyl ester bonds at *sn*-1 and *sn*-2 positions of phospholipids, respectively. Phospholipase B (PLB) can hydrolyze both positional ester bonds (not shown in Figure 3.1). Phospholipase C (PLC) and phospholipase D (PLD) are enzymes that cleave the phosphorus-oxygen bond between glycerol and phosphate, or phosphate and head group, respectively. The diversity of phospholipases lies not only in their different enzyme species, but also in each family having many subgroups or even isozymes belonging to the same subgroups (Six and Dennis, 2000; Katan, 1998; Wang, 2000). Phospholipases are ubiquitous enzymes occurring from mammalian to bacteria. Up to date, hundreds of phospholipases have been purified, characterized and cloned (Frohman and Morris, 1999; Murakami and Kudo, 2002).

Besides phospholipases, there are other enzymes that can be used for phospholipid acyl modification such as *sn*-1,3 specific lipases, which act on the *sn*-1 position of phospholipids. Lipases have been more maturely developed than phospholipases in term of theoretical and practical understanding, and have in practice been the most common enzymes used for phospholipid acyl modification at the *sn*-1 position. PLB (EC 3.1.1.5) has not been used for phospholipid modification mainly because of its broad substrate selectivity, hydrolyzing and ester forming ability at the *sn*-1 and *sn*-2 positions. Head group exchange of phospholipids will also change the chemical and physical properties;

however it is beyond the scope of this thesis to describe all reactions and products possible for these enzymes. Focus will be on the enzymatic acyl modification.

There is no doubt that the understanding of the molecular characteristics and properties of phospholipases will be helpful for illustration of experimental phenomena and improvement of test design. However, it is impossible to present such big topics in a short length. In this writing, a basic description of the molecular characteristics and application properties of the most commonly used enzymes for phospholipid acyl modification are given.

3.1 Phospholipase A₁

PLA₁ (phosphatide 1-acyl hydrolase, EC 3.1.1.32) hydrolyzes the ester bonds at the *sn*-1 position of glycerophospholipids to produce lysophospholipids and fatty acids. Enzymes with PLA₁ activity have been isolated and characterized from various sources (Snijder and Dijkstra, 2000; Wen *et al.*, 2001; Ulbrich-Hofmann, 2000). PLA₁ can for example act as virulence factor contributing to the pathogenicity of certain bacteria and fungi (Brok *et al.* 1998, Merino *et al.*, 1999). PLA₁ has also been found in venom from arthropod and wasp (Ho *et al.*, 1999; King *et al.* 1984). PLA₁ is rarely used for phospholipid modification because *sn*-1,3 specific lipase performs the same task more efficiently and is more readily available (see table 4.1). Microbial PLA₁ is currently used for degumming during oil refining (Clausen, 2001), and perhaps in future more work will appear with the use of PLA₁ for phospholipid synthesis.

3.2 Lipases

Lipases (triacylglycerol hydrolases, EC 3.1.1.3) are enzymes that split fatty acid residue from the glycerol residue in neutral fat or phospholipid. Although widespread in nature, only few lipases are available in large quantities for industrial purposes. Microbial lipases have been the most attractive enzymes for the modification of phospholipids in the *sn*-1 position since they are more accessible commercially. With the use of genetic engineering it has become possible to develop recombinant microbial cultures having efficient expression of lipases (Schmid and Verger, 1998; Wong, 1995; Svendsen *et al.*, 1997).

Most lipases tend to have the same three dimensional structures. The amino acid sequence may differ, but they fold in similar fashion and have similar catalytic sites.

The active site of lipase consists of a catalytic triad formed by serine, aspartate/glutamate, and histidine (Norin *et al.*, 1994). All lipases are members of the “ α/β -hydrolase fold” family featuring a core of predominantly parallel β -sheets surrounded by α -helices. A unique feature of all the lipases is that the catalytic triad is covered by a lid structure composed of an amphiphilic α -helix peptide sequence that upon contact with substrate at interface undergoes conformational changes and opens up for the active site. The reaction mechanism involves two steps (acylation and deacylation step, respectively). In the first step the acyl donor binds to the active site and the carbonyl group is placed in close proximity to the hydroxyl group of the catalytic serine.

A tetrahedral acyl enzyme intermediate is formed by nucleophilic attack on the carbonyl carbon of the substrate by the serine hydroxyl group (see Figure 3.2). The oxyanion developed in the tetrahedral intermediate are stabilized by hydrogen bonds with amino acid residues in the so-called oxyanion hole. Subsequently, the substrate ester bond is cleaved and the tetrahedral intermediate broken down to the acyl-enzyme. In the second step the acyl-enzyme is subjected to a nucleophilic attack on the carbonyl carbon atom of the acyl group attached to the catalytic serine, and a tetrahedral intermediate is formed once again. Deacylation of the acyl-enzyme occurs with water being the nucleophile, resulting in the product and the enzyme.

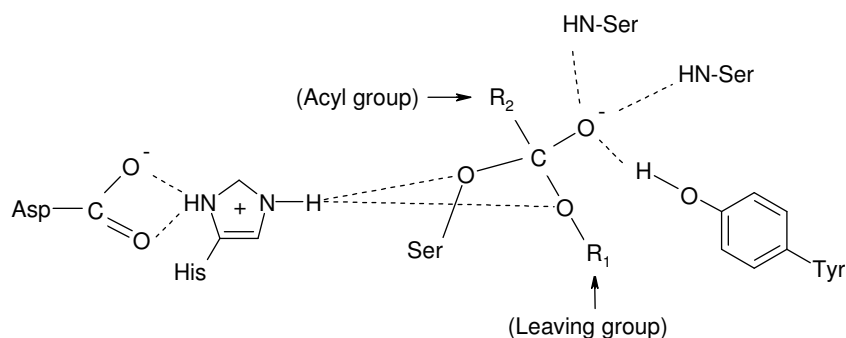


Figure 3.2: Schematic representation of the transition-state model in reaction center of lipases (Modified from Norin *et al.*, 1994)

3.3 Phospholipase A₂

PLA₂ (phosphatide 2-acyl hydrolase, EC 3.1.1.4) hydrolyzes the ester bond at the *sn*-2 position of glycerophospholipids to produce lysophospholipids and fatty acids. Mukakami and Kudo (2002) have categorized PLA₂ into four types: secretory PLA₂, (sPLA₂), cytosolic PLA₂ (cPLA₂), Ca²⁺-independent PLA₂ (iPLA₂), and platelet-activating factor acetylhydrolase (PAF-AH). Commercial sources of PLA₂ come from various sources such as mammalian pancreas (bovine and porcine), venom (snake and bee) and microorganisms (*Streptomyces violaceoruber*). Porcine pancreatic PLA₂ have been the most commonly used enzyme for phospholipid modification at the *sn*-2 position, since it has been the only commercial phospholipase available in larger quantities to the oil and fats industry. The supply of this enzyme is however limited by the availability of porcine glands. PLA₂ from mammalian pancreas is a digestive enzyme for hydrolysis of dietary phospholipids (Yuan and Tsai, 1999). PLA₂ derived from microbial source instead of an animal source would make it possible to produce unlimited quantities by fermentation.

Secreted PLA₂ from mammals, plants, and venoms are typically extracellular with molecular weight of 12-14 kDa, which are Ca²⁺-dependent and possess about six conserved disulfide bonds (Six and Dennis, 2000; Murakami and Kudo, 2002; Valentin *et al.*, 1999, Janssen *et al.*, 1999). The catalytic device of the secretory PLA₂ is characterized by Aspartate-Histidine dyad, a water molecule, a calcium-binding loop and possibly other determinants that stabilize the transition state (see Figure 3.3). Histidine and Aspartate have a function similar to that of the corresponding residues in the catalytic triad of well-known serine proteases. In these enzymes an additional serine is the attacking nucleophile, whereas in PLA₂ this function is performed by a water molecule. The oxyanion hole that stabilizes the transition state after nucleophilic attack is formed in PLA₂ by the backbone NH of glycine assisted by the charge of Ca²⁺ ion. Structures further show that essential co-factor is coordinated by two carboxylate oxygen atoms of Aspartate and three main-chain oxygen atoms from the so-called calcium binding loop. Moreover two tyrosines are involved in an extended hydrogen bonding network connecting the active site to the α -amino group (Janssen *et al.*, 1999). Unlike with lipases, an acyl-enzyme intermediate does not form.

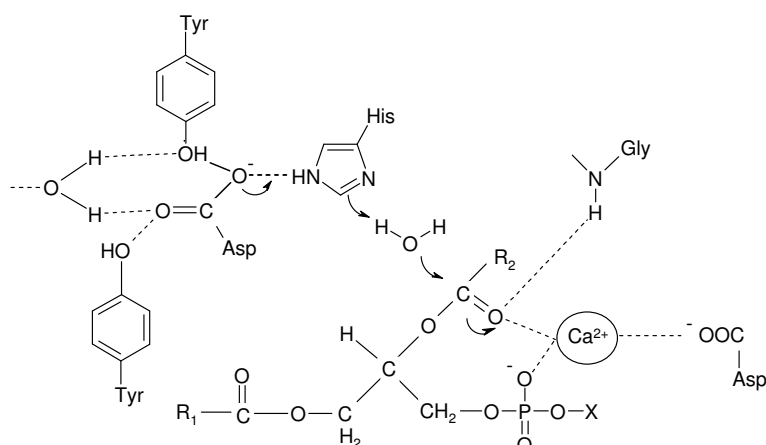


Figure 3.3: Schematic representation of the catalytic mechanism of secretory PLA₂ and part of the hydrogen bonding network present in the crystal structure of porcine pancreatic PLA₂ (Janssen *et al.*, 1999)

3.4 Immobilization of enzymes

For industrial applications, enzymes are preferred in an immobilized form because it is possible to isolate the biocatalyst from the product stream, and re-use it. Enzymes can bind to an insoluble matrix by variety of methods and still retain their catalytic activity. Use of immobilized enzymes often facilitates the development of a continuous reactor for enhancing productivity. Different immobilization technologies have been developed over the past decades as listed in the following (Christensen, *et al.*, 2003):

- Deposition onto hydrophilic inorganic material, such as celite or silica gel
- Encapsulation (for example, in alginate or carrageenan beads)
- Covalent-linkage to carrier, for example using epoxy functionalized polymer beads
- Adsorption onto polymer-based carriers
- Cross-linkage using for example glutaraldehyde

Of the various methods for immobilization physical adsorption of the enzyme onto solid support remains the simplest, least expensive, and least labour-intensive procedure. Adsorption involves relatively weak interactions, and therefore it can be anticipated that the immobilization will only have minor effect on activity.

The different carriers used for immobilization are not all suitable for large-scale productions. Preparations described on celite and certain other porous or powder inert materials have good initial activity but will often be difficult to handle or have

insufficient enzymatic and physical stability in industrial processes (Eigtved, 1992). Dust formation, displacement of the enzyme from the support and high pressure drops in packed bed columns are some of the problems that can occur using this type of carriers. Polymer or resin based carriers have been described, which offers strong adsorption, high activity, and stability of enzymes, which would accommodate enzymes and transport lipid substrate without major diffusion problems. Activity of the immobilized enzyme will depend on the selected parameters during immobilization such as fixation time, enzyme-to-carrier ratio, volume of the coupling medium, temperature during fixation, buffer concentration, and pH value.

So far only lipases can currently be obtained commercially in immobilized form for phospholipid modification; phospholipases in immobilized forms are unavailable. The use of enzyme for modification of phospholipids has not been sufficiently cost-effective to be introduced in large-scale production, especially due to the high cost of the immobilization procedure. A recently new process for immobilizing lipases based on granulation of silica has dramatically lowered process costs (Kirk *et al.*, 2002).

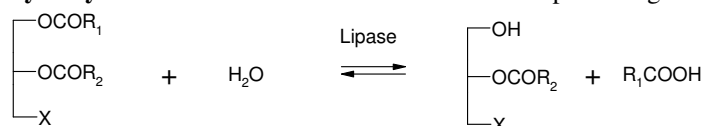
Commercial product of porcine pancreatic PLA₂ has only been provided in the free form (liquid solution), but some attempts have previously been made to immobilize the enzyme (Aura *et al.*, 1995; Doig and Diks, 2003a; Härrod and Elfman, 1995; Hossen and Hernandez, 2005; Lyberg *et al.* 2005). Secreted PLA₂ requires Ca²⁺ as co-factor; however the concentration of Ca²⁺ strongly influences the synthetic activity of these enzymes (Pernas *et al.*, 1990). In some cases the dependence of Ca²⁺ is simply overcome by doing the immobilization in buffer containing CaCl₂ (Egger *et al.*, 1997; Aura *et al.*, 1995; Lyberg *et al.*, 2005).

4 Enzymatic acyl modification of phospholipids

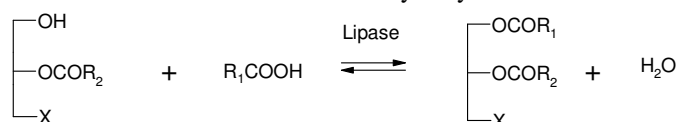
4.1 Possible reactions for acyl modification

Enzymatic acyl exchange reactions of glycerophospholipids can be carried out by using various reaction routes. Possible reactions, which can be catalyzed by lipases and phospholipases, are presented in Figure 4.1 (Reaction schemes are only illustrated for lipase-catalyzed reactions, however similar reactions can be performed with PLA₂ for exchange of fatty acid resided at *sn*-2 position).

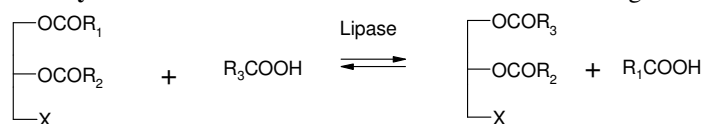
Hydrolysis: Reaction between an ester and water producing an acid and an alcohol



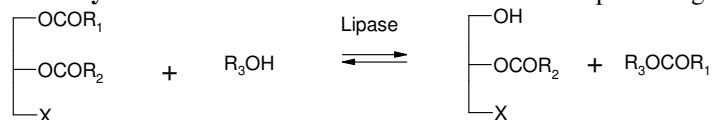
Esterification: Reverse reaction of hydrolysis. Production of an ester (and water) from an acid and an alcohol



Acidolysis: Reaction between an ester and an acid resulting in an exchange of acyl groups



Alcoholysis: Reaction between an ester and an alcohol producing an ester with different alkyl group.



Ester-ester exchange: Reaction of an ester with another ester leading to exchange of acyl groups.



Figure 4.1: Reactions catalyzed by lipase for phospholipid modification

In literature acidolysis reaction, alcoholysis reaction and the ester-ester exchange are also referred to as interesterification or transesterification. Commercial use of enzymes for phospholipid modification is significant only for partial hydrolysis to produce lysophospholipids. This reaction has found industrial application for the production of lysophospholipids and degumming of vegetable oil (see section 2.4 and 3.1).

Phospholipids that exist in nature contain a variety of fatty acids, the proportion depending on the source. In some cases it is desirable to have well-defined phospholipid products having specified fatty acid in one or both positions. By replacing the existing fatty acids in an original phospholipid molecule with desired ones, new physical properties and special functions can be achieved (Huang, 2001). It has for example been reported that the exchange of long-chain unsaturated fatty acids with short- or medium-chain fatty acids in phospholipids may improve their heat stability (Chmiel *et al.*, 1999). Medium-chain or long chain saturated fatty acids may be incorporated to modify emulsification properties, to modify the physiological value or to improve oxidation stability of the phospholipids (Pedersen, 2001). Long-chain polyunsaturated fatty acid (PUFA), such as linoleic, arachidonic, alpha-linolenic, eicosapentaenoic, docosahexaenoic or gamma-linolenic acids may be incorporated to improve the physiological or nutritional value of phospholipids. When polyunsaturated fatty acids (PUFA) are residues at the secondary position (*sn*-2 position) of a phospholipid molecule, PUFA are especially effective (Takahashi and Hosokawa, 2001). Docosahexaenoic acid (DHA)-containing phospholipids may have potential medical applications for instances in promoting cell differentiation in leukemia, enhancing survivals of tumor bearing mice, and preventing cerebral apoplexy (Eibl and Unger, 1988; Jensi *et al.*, 1995; Lason-Backstrom, 1995; Sakai *et al.*, 1992; Zerouga *et al.*, 2002).

Production of diacylphospholipids with modified fatty acid composition (structured phospholipids) can be done in one step by acidolysis or ester-ester exchange. Alternatively reactions are performed in two steps by first removing fatty acids by hydrolysis or alcoholysis, followed by re-esterification of the lysophospholipids. Purity of the final product will mainly determine the reaction route (Adlercreutz *et al.*, 2003). In the one step reactions, the fatty acid composition of the product will be a mixture of the original fatty acids and the ones to be incorporated. In the two step process, it is possible to isolate the lysophospholipids before the subsequent esterification reaction, which gives rise to phospholipid with high purity. Compared to the one step process the two step process takes considerable longer time as more steps for production and purification are needed.

The most commonly used approach for the exchange of fatty acids in the *sn*-1 position has been acidolysis, whereas hydrolysis/re-esterification approach has been the most common for the incorporation of fatty acids into the *sn*-2 position. Lipases are able to catalyze both esterification and interesterification reactions. Pancreatic PLA₂ functions reasonable well for esterification, but cannot catalyze interesterification/transesterification since no acyl-enzyme intermediate is formed (Adlercreutz *et al.*

2003). However it has been shown to be possible to use this enzyme to exchange the fatty acid in the *sn*-2 position in a one-step reaction anyway. Hydrolysis and re-esterification then occur in parallel.

To shift equilibrium to product side during acidolysis reaction, product should be removed from reaction system after its formation. However, usually the structured phospholipids are not easily separated from the system as their properties are very similar to the substrate material. Alternatively it may be possible to have high water content in beginning to facilitate formation of the intermediate LPC, and subsequently removal of water from the system will facilitate the formation of diacylphospholipids.

4.2 Applied biocatalysis

Several important parameters are considered to influence enzymatic esterification and transesterification for production of diacylphospholipids with modified fatty acid composition. These parameters include enzyme dosage, acyl donor, solvents, water content, reaction temperature and reaction time. Reactions have so far been performed mainly in small-scale laboratory experiments, and little effort has been made to up-scale the reactions to larger scale. In the following section, the different parameters important for these types of reactions are briefly discussed based on literature evaluation of enzyme-catalyzed production of structured phospholipids. Table 4.1 summarizes type of reaction, solvent and enzymes used in different studies for the production structured phospholipids by acidolysis or esterification of PC from LPC.

Several of the studies presented in table 4.1 have reported relative high yields under certain reaction conditions. Haraldsson and Thorarensen (1999) reported an incorporation of 53% and yield of 63% in a solvent free system by *Rhizomucor miehei* lipase-catalyzed acidolysis having a substrate molar ratio of 6.7 (mol/mol fatty acid/PC) and water content of 2% w/w. Similar incorporation and yield were reported by Svensson *et al.* (1992) using immobilized *Rhizopus oryzae* lipase in solvent system (toluene) with a substrate molar ratio of 40 mol/mol fatty acids/PC under controlled water activity ($a_w=0.06$). Even higher yields (~75%) have been reported with the same lipase by esterification of PC from LPC, when using higher substrate molar ratio (90 mol/mol) at higher water activity ($a_w=0.22$) in a solvent system (Adlercreutz *et al.*, 2002).

Enzymatic acyl modification of phospholipids

Table 4.1: Literature evaluation of the enzymatic production of structured and modified phospholipids with enzymes specific for *sn*-1 position

Enzyme	Enzyme (Source)	Reaction type	Acyl donor	Solvent	Reference
Lipase	<i>Candida cylindracea</i>	Acidolysis	EPA	Benzene	Yoshimoto <i>et al.</i> , 1986
	<i>Rhizomucor miehei</i> and others	Acidolysis	Margaric acid and others	Toluene	Svensson <i>et al.</i> , 1990
	<i>Candida cylindracea</i> and <i>Rhizopus delemar</i>	Ester-Ester exchange	Sardine oil	Hexane	Totani and Hara, 1991
	<i>Rhizopus delemar</i>	Acidolysis	Fish oil hydrolysate	Isooctane	Holmberg and Eriksson, 1992
	<i>Rhizopus oryzae</i> and <i>Rhizomucor miehei</i>	Acidolysis	Margaric acid	Toluene	Svensson <i>et al.</i> , 1992
	<i>Rhizomucor miehei</i>	Acidolysis and esterification	DHA and EPA	Hexane	Mutua and Akoh, 1993
	<i>Aspergillus niger</i> and <i>Rhizomucor. Miehei</i>	Acidolysis	Oleic acid	Toluene or solvent free	Mustranta <i>et al.</i> , 1994a,b
	<i>Rhizomucor miehei</i>	Acidolysis	EPA and DHA	Hexane + water mimics	Hosokawa <i>et al.</i> , 1995a, b
	<i>Rhizomucor miehei</i>	Acidolysis	Lauric and oleic acid	Solvent-free	Aura <i>et al.</i> , 1995
	<i>Rhizopus sp.</i>	Acidolysis	Margaric acid	Hexane	Hara and Nakashima, 1996
	<i>Rhizomucor miehei</i>	Acidolysis	DHA and EPA	Solvent-free	Haraldsson and Thorarensen, 1999
	<i>Rhizopus oryzae</i>	Acidolysis and esterification	Caproic acid	Toluene	Adlercreutz <i>et al.</i> , 2002
	<i>Candida rugosa</i> and <i>Rhizopus oryzae</i>	Ester-ester exchange	MAG and DAG oils	Hexane	Hara <i>et al.</i> , 2002
	<i>Thermomyces lanuginosa</i> , <i>Rhizomucor miehei</i> , <i>Candida antarctica</i>	Acidolysis	Caprylic acid, CLA, EPA and DHA	Solvent-free	Peng <i>et al.</i> , 2002
	<i>Rhizopus oryzae</i>	Ester-ester exchange	Methyl ester of lauric acid	Solvent-free	Doig and Diks, 2003a
	<i>Rhizopus oryzae</i>	Esterification	Capric acid	Toluene	Adlercreutz and Wehtje, 2004
	<i>Rhizomucor miehei</i> , <i>Thermomyces lanuginosa</i> , <i>Candida antarctica</i>	Acidolysis	Oleic acid	Hexane	Wongsakul <i>et al.</i> , 2004
	<i>Rhizomucor miehei</i> , <i>Thermomyces lanuginosa</i> , <i>Candida antarctica</i>	Acidolysis	CLA	Hexane	Hossen and Hernandez, 2005

Table 4.1(Continued)

	<i>Candida antarctica</i> , <i>Rhizopus arrhizus</i> , and <i>Rhizomucor miehei</i>	Esterification	DHA and EPA	Toluene	Lyberg <i>et al.</i> , 2005
	<i>Candida antarctica</i> <i>Thermomyces lanuginosa</i>	Acidolysis	Palmitic acid Stearic acid	Heptane	Reddy <i>et al.</i> , 2005
PLA1	<i>Fusarium oxysporum</i>	Ester-Ester exchange	Methyl esters of Ricinoleic acid	Hexane	Vijeeta <i>et al.</i> , 2004
PLA2	Snake venom and others	Esterification	Oleic acid	Toluene	Pernas <i>et al.</i> , 1990
	Porcine pancreas	Esterification	EPA and DHA	Isooctane	Na <i>et al.</i> , 1990
	Snake venom	Esterification	Palmitic acid	Chloroform	Lin <i>et al.</i> , 1993
	Porcine pancreas	Esterification	EPA	Glycerol+ water mimics	Hosokawa <i>et al.</i> , 1994
	Porcine pancreas	Esterification	Various	Toluene	Mingarro <i>et al.</i> , 1994
	Porcine pancreas	Acidolysis	Oleic acid	Toluene	Mustranta <i>et al.</i> , 1994 a,b
	Porcine pancreas	Acidolysis	Lauric acid	Solvent-free	Aura <i>et al.</i> , 1995
	Porcine pancreas	Esterification	PUFA	Isooctane, CO ₂ , propane	Härröd and Elfman, 1995
	Porcine pancreas	Esterification	EPA and DHA	Glycerol+ water mimics	Hosokawa <i>et al.</i> , 1995a
	Porcine pancreas	Esterification	Oleic acid	Toluene	Egger <i>et al.</i> , 1997
	Porcine pancreas	Acidolysis	EPA and DHA	Glycerol + water mimics	Hosokawa <i>et al.</i> , 1998
	Porcine pancreas	Ester-ester exchange	Ethyl ester of EPA	Toluene	Park <i>et al.</i> , 2000
	Porcine pancreas	Acidolysis	Caproic acid	Toluene	Adlercreutz <i>et al.</i> , 2001
	Porcine pancreas	Acidolysis	Myristic acid	Petroleum ether	Pedersen, 2001
	Porcine pancreas	Esterification	Caproic acid	Toluene	Adlercreutz <i>et al.</i> 2004
	Porcine pancreas	Acidolysis	CLA	Hexane	Hossen and Hernandez, 2005
	Porcine pancreas	Esterification	EPA and DHA	Toluene	Lyberg <i>et al.</i> , 2005

High yields (60%) have also been reported for esterification reaction using immobilized PLA₂ in toluene with substrate ratio of 180 mol/mol oleic acid/LPC (Egger *et al.*, 1997). For the same enzyme Hosokawa *et al.* (1995a) reported 40 % yield of PC during esterification with glycerol as solvent and low substrate molar ratio (~3 mol/mol EPA/PC).

4.2.1 Possible enzymes and substrate selectivity

Lipase-catalyzed acidolysis reactions between phospholipids and free fatty acids have, in several publications, been reported to be an effective way to modify fatty acid profile of phospholipids. Several different commercial immobilized *sn*-1,3 specific lipases have been used for acyl modification of phospholipids. Most of the studies for lipase-catalyzed acyl modification have used the commercial lipase preparation Lipozyme RM IM (*Rhizomucor miehei* lipase immobilized on exchange resin by adsorption).

A screening of different immobilized lipases showed that Lipozyme TL IM (*Thermomyces lanuginosa* lipase) had higher activity compared to Lipozyme RM and Novozym 435 (*Candida antarctica* lipases) during lipase-catalyzed acidolysis between soybean lecithin and caprylic acid under solvent free conditions (Peng *et al.*, 2002). Novozym 435 has in certain cases been claimed to be non-specific for triacylglycerols, however with phospholipids, it works exclusively for *sn*-1 position (Lyberg *et al.*, 2005). In the case where acidolysis reaction was performed with PC and conjugated linoleic acid (CLA) in presence of hexane, Lipozyme RM IM resulted in higher incorporation as compared to Lipozyme TL IM and Novozym 435 (Hossen and Hernandez, 2005). During acidolysis reaction between PC and palmitic or stearic acids in heptane, the highest incorporation of acyl donor was obtained with Novozym 435 as compared to Lipozyme TL IM (Reddy *et al.*, 2005). For esterification reaction between 2-acyl LPC and fatty acids from fish oil in toluene, higher incorporation was achieved with Novozym 435 as compared to Lipozyme RM IM (Lyberg *et al.*, 2005). Comparison of the catalytic activity is rather complicated as the lipases specificity towards different fatty acids and temperature optimum may differ. Reactivity of the enzyme also depends on phospholipid head group composition and purity of substrates. No general conclusion can be made as to which immobilized enzyme would perform better for acidolysis reactions. However each of the lipases has shown to be superior over the others in certain reaction systems. Lipase from *Rhizopus oryzae* has also been reported to work well for phospholipid modification at the *sn*-1 position (Adlercreutz *et al.* 2002); however this lipase cannot be obtained commercially in the immobilized form.

In most studies PLA₂ from porcine pancreas have been used for the modification of the *sn*-2 position. Only few studies have compared PLA₂ from different sources. Pernas *et al.* (1990) reported that PLA₂ from *Naja Naja* snake venom performed better during synthesis of PC from LPC in toluene system as compared to PLA₂ isolated from *Streptomyces violaceoruber*, bee venom, porcine and bovine pancreas.

High enzyme dosages are normally required for effective incorporation of the novel fatty acids into phospholipids, however based on economic considerations and mass transfer limitations of the reaction system; the dosage of enzyme is restricted. Very high loads of enzyme will make the reaction mixture difficult to mix in batch reactor. High enzyme-to-substrate ratios can on the other hand be obtained by using continuous column reactor, containing immobilized enzyme. For reactions performed in organic solvent the enzyme dosage is lower compared to solvent-free system to obtain the same degree of incorporation.

Different fatty acids may be applied as acyl donor for acidolysis reaction. However the fatty acids usually results in different reactivity, due to fatty acid specificity or possible inhibition effects. Under same conditions, different fatty acids often results in different incorporation into phospholipids or different yields. High incorporation can usually be obtained with saturated fatty acids; however fatty acids with high degree of unsaturation are usually more difficult to incorporate during lipase-catalyzed acidolysis (Peng *et al.*, 2002; Hossen and Hernandez, 2005; Reddy *et al.*, 2005). Different lipases show discrimination of long-chain PUFA. For example Lipozyme RM IM, Lipozyme TL IM and Novozym 435 give higher incorporation of EPA compared to DHA during acidolysis and esterification (Peng *et al.*, 2002; Haraldsson and Thorarensen, 1999; Lyberg *et al.*, 2005).

The reaction rates of PLA₂-catalysed reactions have shown to be influenced by both chain length and degree of saturation in a toluene system (Egger *et al.*, 1997). Reaction rates were reported to be the same for saturated fatty acids of length between six and twelve carbon atoms, and decrease in the reaction rates for myristic and palmitic acids. The decrease might be caused by a diminished solubility of these fatty acids. Highest reaction rate was obtained with oleic acid, but higher degree of unsaturation resulted in lower reaction rates. Similarly, it was observed that with saturated fatty acids the esterification rate markedly decreased with increase of chain lengths, almost being negligible for stearic acid (Mingarro *et al.*, 1994). The number of unsaturation in the molecule did however not result in significant change in esterification reaction (Mingarro *et al.* 1994).

Reactivity of enzymes also depends on the phospholipid head group. During acidolysis reaction with eicosapentaenoic acid (EPA) in hexane using Lipozyme RM IM as catalyst, the following order of reactivity was observed PC>PI>PE>PS (PC is phosphatidylcholine, PI is phosphatidylinositol, PE is phosphatidylethanolamine, and PS is phosphatidylserine) (Mutua and Akoh, 1993). The nature of phospholipid also affected the incorporation rates of caprylic acid by Lipozyme TL IM in hexane system in the following order PC>PE>PA>PI (Peng *et al.*, 2002). PLA₂ selectivity toward the phospholipid polar head group was shown to be altered in non-aqueous system relative to all-water medium during hydrolysis reactions (Mingarro *et al.*, 1994). PC rapidly converted to its lyso-derivative in water saturated chloroform, whereas PI was hydrolyzed much slower. As for PE, phosphatidylglycerol (PG), and PS even after several days of incubation the hydrolysis was rather low. In parallel experiment in water the order of hydrolysis was PS>PG>PI>>PC>PE. Doig and Diks (2003a) reported that porcine pancreatic PLA₂ hydrolyzed PC almost twice the rate of PE, and was not able to hydrolyze PI in a hexane system. Phospholipids can be obtained commercially at different purities. Enzymatic fatty acid exchanges have been made with both pure phospholipid compounds and deoiled-lecithin. Purified compounds have considerably higher price compared to deoiled lecithin. Selection will depend on purity requirements.

4.2.2 Effects of substrate molar ratio

In theory product yield under reaction equilibrium during enzyme-catalyzed acidolysis reaction of phospholipids is determined by substrate ratio. The maximum incorporation of acyl donors can be calculated at certain substrate ratios assuming no by-product formation and acyl migration. The equation is given in Equation 4.1.

$$Inc_{\max} (\text{mol}\%) = \frac{50 \cdot S_r}{S_r + 1} \quad (\text{Eq. 4.1})$$

Theoretical maximum of new fatty acids to be incorporated into PC is expected to reach 50% for the *sn*-1,3 specific lipase or a PLA₂.

Increasing fatty acid concentration increased yield both for lipase-catalyzed esterification and transesterification reactions as hydrolysis reaction is inhibited (Adlercreutz *et al.*, 2002). Reaction time for esterification reactions (to reach 95% conversion) was independent of the fatty acid concentration. However, during transesterification, the reaction time increased with increasing fatty acid concentration. Decreased reaction rates were thought to be caused by increased polarity of the reaction

medium upon addition of fatty acids. Decreased reaction rates have also been reported during the PLA₂-catalyzed esterification reactions with increasing amounts of fatty acids, and were speculated to be caused by changes in polarity or viscosity (Egger *et al.*, 1997). In the solvent system, high substrate ratios did not seem to be a problem until very high substrate ratios, whereas inhibition is observed usually at lower substrate ratios for solvent free system (Peng *et al.*, 2002).

The choice of substrate molar ratio also relates to the downstream processing cost and difficulties in separating the fatty acids (acyl donor and exchanged fatty acids) from the products. Therefore, a compromise usually has to be made for substrate molar ratio.

4.2.3 Control of water content/ activity

Water seems to have a complex role in terms of compromising the enzyme activity, hydrolysis side reactions, reaction rate, and extent of incorporation (Haraldsson and Thorarensen, 1999). Lipases have been reported to operate at even very low water content, which inhibits hydrolysis. PLA₂ on the other hand require a higher water activity to function and therefore the yield is expected to be lower (Adlercreutz *et al.*, 2003). From the engineering point of view there will be optimal water content in the system for optimal incorporation of fatty acids and yield of structured phospholipids.

In some cases it has been shown that with increased water content in the system, also results in increased incorporation of novel fatty acids, but with major hydrolysis in parallel. Two ways commonly used for controlling water activity are pre-equilibrium of reactants and enzyme with saturated salt solution or direct addition of salt hydrates with a definite a_w value (Adlercreutz *et al.*, 2002; Egger *et al.*, 1997). For up-scaled production this would be impractical from techniques developed so far. Others have simply added water directly to substrate or wetted the enzyme prior to addition to the reaction mixture (Peng *et al.*, 2002). Hosokawa *et al.* (1995a) used water mimics, such as formamide, ethylene glycol or polypropylene glycol for in situ control of water activity during acidolysis reaction. These polar solvents showed to activate enzyme, but did not participate in hydrolysis like water, which resulted in enhanced incorporation and recovery of diacylphospholipids.

In reaction mixture with toluene as solvent it was observed that increased water activity increased hydrolysis reaction rate to a greater extent compared to the synthesis reaction rate. In order to have a high productivity it is recommended that the water content should be low.

4.2.4 Solvent system or solvent-free systems

The use of solvent should be avoided in case that solvent-free system could satisfy the requirement of reactions. The use of solvent may increase reactivity due to lowered viscosity and thus increased mass transfer. An organic solvent environment allows better accessibility of enzymes to substrates and better availability of water at the active site of the enzyme. Due to the high dosage requirements of immobilized enzyme during phospholipid acidolysis reactions, problems with agitation occur and, product is not easily removed from immobilized enzyme without extraction during solvent free batch operation. Presence of solvent would improve mixing in the system, and make the subsequent removal of enzyme more convenient.

Solvents with high polarity are however not suitable in enzymatic esterification reactions as they are strong water distorters and thereby inactivate the enzyme (Laane *et al.*, 1986). The best measure of polarity is the logarithm of the partition coefficient ($\log P$) of the organic liquid between n-octanol and water; the higher the $\log P$, the less polar the solvent is. Solvents with $\log P < 2$ are not considered suitable for enzyme reactions. Commonly used solvents for the acidolysis of phospholipids are toluene and hexane, both having $\log P > 2$. For more detailed information on use of solvent for enzymatic acyl modification of phospholipids please refer to Table 4.1. It has been reported that reactions conducted in hexane were faster than reactions conducted in toluene during lipase catalyzed acidolysis reactions (Haraldsson and Thorarensen, 1999). Mingarro *et al.* (1994) showed that the highest activity of PLA₂ was obtained with aromatic hydrocarbons from a list of solvents with varying polarities. In general activity decreased with increased polarity. Park *et al.* (2000) carried out similar PLA₂-catalyzed interesterification of PC with ethyl esters of EPA in various organic solvents (toluene, n-hexane, n-heptane, isooctane, butyl acetate, petroleum ether, diethyl ether, cyclohexane, chloroform and ethyl acetate) and found toluene to give higher incorporation.

From a safety and health point of view it would be preferred to have a solvent free system especially if the reaction is up-scaled for large-scale production. In the solvent free system no organic solvent is present beside the substrate. During the acidolysis reaction free fatty acids (FFA) are normally used in large excess (high substrate ratio; mol/mol), whereby the FFA will function as solvent for the phospholipid. The solvent free system may have a higher volumetric productivity compared to the solvent system if they are compared based on enzyme used and volume of reaction mixture. Compared to the solvent system the reaction time needed may be longer since substrate amount is higher. In the solvent free system the phospholipid concentration will be very high probably causing major stripping of water from the enzyme and reaction system may

therefore require additional water (Haraldsson and Thorarensen, 1999). Incorporation of novel fatty acids into phospholipids using a solvent free system with lipase as catalyst have reported to be high (Haraldsson and Thorarensen, 1999; Peng *et al.* 2002), however using PLA₂ the incorporation is very low (Aura *et al.*, 1995).

4.2.4 Temperature selection for reaction systems

Even though the temperature is an important parameter during the enzymatic fatty acid exchange in phospholipids, it has in general received very little attention. The viscosity of the substrate can be dramatically reduced with higher temperatures resulting in increased mass transfer and thereby increased reaction rate. Reaction temperature should be selected with consideration of the thermostability of the enzyme. Too high temperatures will irreversibly denature the enzyme resulting in reduced enzyme performance. It was however reported that low temperatures were favoured to minimize the rate of hydrolysis and loss of product during interesterification of phospholipids (Doig and Diks, 2003a). The temperature may also influence the substrate. During enzymatic synthesis of PC from lyso-PC and PUFA it was observed that PUFA oxidized and non-enzymatic reactions occurred at temperatures above 65°C (Lilja-Hallberg and Härröd, 1995). It has been reported that enzymes in general exhibit dramatically enhanced thermostability in nearly anhydrous media (Mingarro *et al.*, 1994). This is explained on basis of both increased conformational rigidity and minimization of deleterious covalent processes involved in irreversible inactivation.

4.2.5 Reactor configurations

Different reactor configurations have been used for modification of oil and fats by lipase catalyzed reactions (Xu, 2000). Enzyme reactors are usually classified according to operation (batch or continuous) and mixing performance (from well mixed to plug flow).

The exchange of fatty acids in phospholipids has mainly been performed batch-wise in small screw cap vessels or glass bottles with either orbital shaking or magnetic stirring. Operating batch-wise the enzyme has to be separated from the medium containing the reaction product normally either by centrifugation or filtration. Product loss may occur in this step due to absorption to the enzyme carrier. The batch reactor has many advantages: it is simple to use and have flexibility to change the operation from batch to batch. In this kind of reactor the immobilized enzyme is dispersed in the reaction

solution. The main disadvantage of batch reactors is that the reactor has to be emptied and refilled at the end of the batch run. This leads to down-time and a loss of productivity. In addition batch reactors can suffer from batch-to-batch variations.

Packed-bed reactors (PBR) are the most frequently used reactor in commercial scale operations for immobilized enzymes because of high efficiency, low capital investment, ease of construction, continuous operation, and easy maintenance. In PBR, the immobilized enzyme particles are held in a column and substrate is pumped through under plug flow conditions. Heating is controlled with a water jacket or inside heating elements in larger scale. In a PBR with up-flow, the enzyme at the bottom of the column is exposed to substrate whereas the enzyme at the top is exposed largely to product. The main disadvantage with PBR is the difficulty of maintaining even flow and constant temperature.

Only few reports are available on enzymatic modification of phospholipids using PBR. Härröd and Elfman (1995) reported the enzymatic synthesis of PC from LPC and fatty acids using PLA₂ as catalyst in PBR under supercritical conditions. The highest yield obtained in these reactions was 26% with low water activity in a mixture of the PUFA and propane as solvent.

4.3 Acyl migration and by-product formation

Acyl migration is a non-enzymatic reaction often encountered in selective synthesis of regiospecific glycerophospholipids, i.e. intramolecular transfer of one fatty acid moiety from one hydroxyl group to an adjacent one. In lysophospholipids there is one free hydroxyl group that makes acyl migration possible. Free hydroxyl oxygen carries out a nucleophilic attack on a neighbouring ester carbonyl carbon, which results in five membered ring intermediate (Figure 4.2).

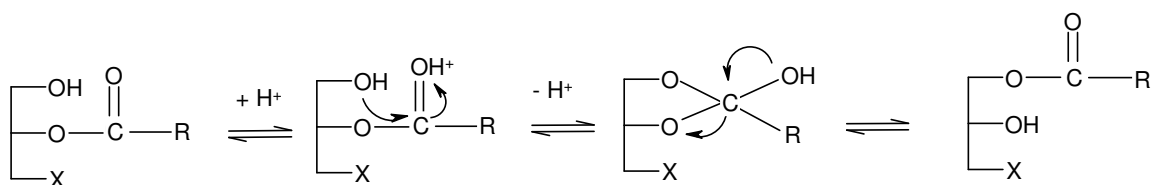


Figure 4.2: Possible mechanism of acyl migration between 2-acyl-lysophospholipid and 1-acyl-lysophospholipid.

Opening the ring forms a mixture of 1-acyl and 2-acylglycerol esters, with higher concentrations of the former. 2-acyl lysophospholipids is less stable than 1-acyl lysophospholipids, and is easily converted into the more stable 1-acyl lysophospholipids. The equilibrium between 1-acyl lysophospholipids and 2-acyl lysophospholipids is approximately 9:1 (Adlercreutz *et al.*, 2002).

Acyl migration results in lower yields during enzyme catalyzed reactions, especially involving lipases. The intermediate 2-acyl-LPC produced during lipase catalyzed reaction involving PC is converted to the more stable 1-acyl-LPC by acyl migration. LPC with fatty acids at the *sn*-1 position can be further hydrolyzed by the lipase to form glycerophosphorylcholine (GPC) which can be reacylated with novel fatty acids in the *sn*-1 position. If the acyl group migrates from the *sn*-1 position to the *sn*-2 position, the lipase has the possibility to incorporate yet another new fatty acid into the *sn*-1 position, which gives rise to PC with novel fatty acid at both positions. The detailed reaction scheme is presented in paper II (Figure 3). Acyl migration does occur during PLA₂ catalyzed reactions, however, only to a lower extent due to the more stable intermediate (Egger *et al.*, 1997).

Many factors possible influence acyl migration during enzyme catalyzed reactions including substrate concentrations, solvents in use, temperature, water content, and reaction time. Ion exchange resin used as carrier for enzyme immobilization can also catalyze acyl migration (Svensson *et al.*, 1992).

4.4 Tools for optimizing different reaction parameters

Several parameters important for the main reaction also affect by-product formation resulting in lower yield as described in the previous section. Usually compromises have to be made for specific production. Kinetic modelling is useful for the understanding of the reaction mechanism, however this does not rule out other ways of modelling. In a practical development, kinetic models never work properly for the precise prediction of the reaction system because of the multiple effects of many different factors. In such cases, statistical modelling makes it simple even though the models have no physical or chemical meaning in theory. Practical optimization of a real system can be made with mathematical tools such as response surface methodology (RSM) (Xu, 2002). RSM is an experimental strategy based on different mathematical and statistical methods. Commercial programs have been developed, and have been widely used by many researchers to assist optimization of different systems, especially in the field of enzymatic modification.

RSM can be used for modelling and analysis of response variable, which are influenced by multiple independent factors. Objective of RSM is to evaluate the effects of multiple independent factors and the interactions between individual factors, and to optimize them in terms of response variables targeted. Based on the continuity of the factor effect on response variables within the range designed, a surface that possibly contains the optimal points can be obtained for each response if the design is appropriate.

Often used model are in quadratic style that has been proved to be useful and sufficient for modelling of parameter effects:

$$Y = \beta_0 + \sum_{i=1}^n \beta_i X_i + \sum_{i=1}^n \beta_{ii} X_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^n \beta_{ij} X_i X_j + \varepsilon \quad (\text{Eq. 4.2})$$

Where Y is the response variables, X_i the i th independent variable, β_0 the intercept, β_i the first-order model coefficient, β_{ii} the quadric coefficient for variable i and β_{ij} is the model coefficient for the interaction between factor i and j , ε is the combination of the experimental error for the factors. The quadratic term make it possible to obtain information about curvatures in the response. Models with acceptable qualities should have $R_2 > 0.8$.

An advantage of using statistical experimental designs is the reduction of experimental numbers to obtain the same amount of information as with single experimental designs. However, it should be kept in mind that models are derived from defined ranges of each factor and extrapolation is impossible. Models can only be used in the defined ranges for optimization or prediction.

4.5 Down stream processing

The reaction mechanisms have so far been the main focus for the production of structured phospholipids. Little effort has been made to purify these products after reaction. For analytical purposes, solvent partitioning and solid phase extraction have been applied; however, for large scale application this would not be sufficient. It was reported that phospholipids can be extracted from reaction mixtures after interesterification reaction between lecithin and methyl and ethyl esters of fatty acids by dispersion in acetone (Doig and Diks, 2003a; Park *et al.*, 2000). Phospholipids, in contrast to neutral lipids, are usually considered to be acetone insoluble and thus

precipitate in acetone. However this generalization only holds for phospholipids containing long chain fatty acids. Lecithins with hydrocarbon chain length of less than 10 carbon atoms are soluble in acetone.

Some phospholipids recovered from reaction may contain lysophospholipids (intermediate during acidolysis reaction), which can be separated from diacylphospholipids by solvent extraction. Dissolving structured phospholipids in a mixture of hexane/ isopropanol/ water 2:1:1 v/v, the diacylphospholipids will be in top phase, whereas lysophospholipids will be in the lower phase (Doig and Diks, 2003a). Similarly, the solubility of lecithins in alcohol decrease considerably as the length of chain increases. The effect of chain length and unsaturation on lipid solubility has to be remembered when separation and analysis of different lipid classes are being carried out. Complete separation of individual phospholipids on the basis of their different solubility in certain solvents is often not possible, as each affects the solubility of the other.

Membrane technology is developing rapidly in the oil industry to supplement or replace conventional separation processes. The main advantages of the membrane technology are energy saving and better product quality. Micelle-enhanced ultrafiltration has been successfully applied by several researchers for the degumming of vegetable oils (Kim *et al.*, 2002; Koris and Vatai, 2002; Ochoa *et al.*, 2001; Pagliero *et al.*, 2001). Ultrafiltration membranes are considered as porous membranes where rejection is determined mainly by the size and shape of the solutes relative to the pore size in the membrane and where the transport of solvent is directly proportional to the applied pressure (Mulder, 1996). Theoretically, triacylglycerols and phospholipids have similar molecular weight, which would make it difficult to separate them by a membrane. However due to amphiphilic nature of the phospholipids they form reverse micelles with average molecular weight of 20,000 Da or more in non-polar media like hexane or natural oil (Gupta, 1986). This large molecular weight enables the separation of phospholipids from either crude oil/hexane or crude oil itself by ultrafiltration. As part of this study the possibilities of applying polymeric ultrafiltration membranes for separation of free fatty acids from phospholipids after acidolysis reaction were examined (Paper VII and VIII). It was expected that separation of FFA from phospholipids would be possible similar to degumming where phospholipids are separated from triacylglycerols.

An important point which must be considered is that the process performance is not equal to the intrinsic membrane properties in actual separations. The reason for this is the occurrence of concentration polarization and fouling. The macromolecular solute

retained by the membrane accumulates at the surface of the membrane resulting in a concentration build-up. Further pressure increase will not result in an increase in flux because resistance of the boundary layer has increased so that a limiting flux value (J_{∞}) is attained. The boundary layer phenomena mainly determine the process performance. Thus, intrinsic properties are not all that important in membrane development, but rather its chemical and thermal resistance and ability to reduce fouling tendency. Until now, major limitations for membrane degumming have been poor membrane stability in organic solvent and the low permeate flow compared to the scale of oil processing industry.

5 Experimental and analytical approaches

In the present PhD study three different types of experiments were conducted. The first and major part of the work was related to production, which included parameter evaluation and optimization of different enzyme-catalyzed acidolysis reactions. The second type of experiments was related to membrane purification for free fatty acids removal from the structured phospholipid. The last type of experiments was related to the physical properties of structured phospholipids in emulsions and liposome formulations. Figure 5.1 presents an overview of the experimental work through out this thesis and the related papers in the appendix section.

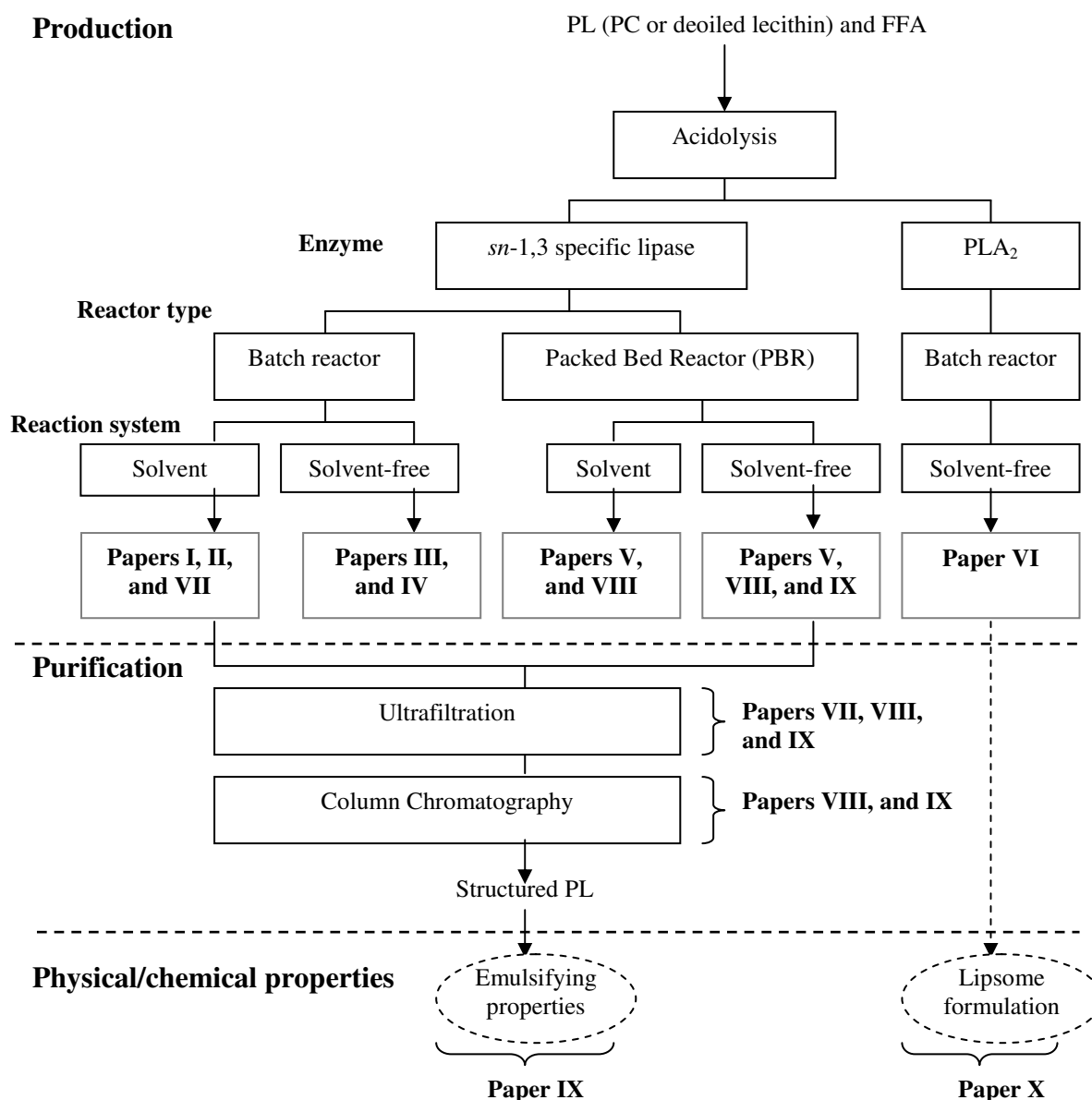


Figure 5.1: Flow sheet of experimental work in this thesis.

5.1 Production of structured phospholipids

Enzymes, used in this study for production of structured phospholipids, were provided by Novozymes A/S. Lipases were used for modification of fatty acid chain in the *sn*-1 position of glycerophospholipid molecules (Papers I, II, III, IV, V, VII, VIII and IX). Two different immobilized *sn*-1,3 specific lipases were used for acidolysis reaction involving phospholipids: Lipozyme TL IM, a granulated silica preparation of *Thermomyces lanuginosa* lipase, and Lipozyme RM IM, an immobilized granulate of *Rhizomucor miehei* lipase. Both of these lipases are produced by submerged fermentation of a genetically modified *Aspergillus oryzae* microorganism.

Lecitase 10L was used for the modification of fatty acid chain resided at *sn*-2 position of glycerophospholipids (Paper VI). Lecitase 10L is purified phospholipase A₂ manufactured from porcine pancreas. This enzyme could not be obtained in the immobilized form, and the study on modification of phospholipids at the *sn*-2 position therefore included a screening of carrier materials for immobilization of PLA₂.

With enzymatic modification, it would be able to produce phospholipids with defined fatty acid compositions, which can be targeted for specific applications in foods, pharmaceuticals, and cosmetics. Modified phospholipids with altered emulsifying properties and dispersing properties extend the application range in phospholipids in these areas. During this work two different types of phospholipids were used as substrate for the enzyme catalyzed acidolysis reaction, namely the purified PC and deoiled lecithin. In food industry, deoiled lecithin would be preferred over the purified phospholipids, since the price is considerably lower. In cosmetic and pharmaceutical industry purified phospholipids of high purity are used as well. Caprylic acid was used as acyl donor for the acidolysis reactions. It was anticipated that phospholipids containing high content of caprylic acid would have increased emulsifying properties in oil-in-water emulsions as compared to natural phospholipid.

Main focus of this thesis was on the lipase-catalyzed reactions, which were performed in both packed bed and batch reactors with or without presences of solvent. PLA₂ catalyzed reactions were performed by solvent-free batch operation. For the production of structured phospholipids, the use of solvent would increase the capital investment when the process is scaled up. The use of solvent should be avoided in the case that solvent-free system could satisfy the requirement of reactions such as employing excessive acyl donor as solvent. In some cases, solvents are absolutely necessary when system viscosity, mass transfer, and product separation considerably affect the process efficiency. From literature it was not possible to determine, whether solvent or solvent

free operation would be the better choice for production of structured phospholipids, as both type of operation have been demonstrated to work for phospholipid acyl modification in batch. For larger scale production, it would be convenient to operate in packed bed reactor as it allows continuous operation, however only few reports are available on such operation. Therefore, stability of immobilized lipases was examined during continuous operation for solvent and solvent free systems in this study to determine the preferred system for potential large-scale production. In the production of foodstuffs and food ingredients few extraction solvents are allowed; however, hexane is generally accepted in the fat and oil industry, and was therefore the choice of solvent for the lipase-catalyzed acidolysis reaction.

Selection of reaction parameters and their ranges during production of structured phospholipids was based on previous work within this field. Important parameters during acidolysis reactions were considered to be enzyme dosage, substrate molar ratio, reaction temperature, reaction time, water content, solvent amount, and reactor design. Factorial experimental designs were widely used during this study for evaluation of the effect of these factors and their interactions on incorporation, byproduct formation, and acyl migration. Otherwise, the conventional approach, where one variable is varied at a time while keeping all other variables constant, was used.

5.1.1 Synthesis of structured phospholipids by lipase-catalyzed acidolysis reaction in solvent batch operation (Papers I and II)

Reactions between soybean PC and caprylic acid were carried out in varying amounts of hexane with Lipozyme TL IM as catalyst. Reactions were performed batchwise in brown bottles with tight screw cap. A three-level five-factor fractional factorial design with two star points was employed. The independent variables and their levels were: enzyme dosage (10-30 wt% based on amount of substrate), Reaction temperature (35-55°C), Solvent amount (5-25 ml hexane), Reaction time (10-50h), and substrate molar ratio (mol/mol caprylic acid/PC). The number of experimental settings generated by the applied software Modde 6.0 (Umetri, Umeå, Sweden) was 29. Incorporation of caprylic acid into phospholipids (PC and LPC) and recovery of PC were determined for the lipase-catalyzed acidolysis reactions and were used as responses (Paper I). PC molecular distribution determined by TLC-FID was reported in paper II. Experimental data were analyzed by response surface regression procedure to fit second order equation as presented in section 4.4. Further information on the experiments and analysis please refer to paper I and II in the appendix section.

5.1.2 Synthesis of structured phospholipids by lipase-catalyzed acidolysis reaction in solvent free batch operation (Papers III and IV)

Reactions between soybean PC and caprylic acid were carried out with Lipozyme RM IM as catalyst. Reactions were performed batchwise in brown bottles with tight screw cap. A three-level five-factor fractional factorial design with two star points was employed. The independent variables and their levels were: enzyme dosage (10-50% based on substrate amount), reaction temperature (40-60°C), water addition (0-4 wt% based on total substrate), reaction time (10-90h), and substrate molar ratio (mol/mol caprylic acid/PC). The number of experimental settings generated by the applied software Modde 6.0 (Umetri, Umeå, Sweden) was 29. Incorporation of caprylic acid into PC and LPC together with phospholipid distribution (PC, LPC and GPC) were determined for the lipase-catalyzed acidolysis reactions and were used as responses (Paper III). Acyl migration into the *sn*-2 position of PC was also examined for these samples, and used as response for the quadratic response surface model (Paper IV).

5.1.3 Continuous production of structured phospholipids in packed bed reactor (Papers V and VIII)

PBR was applied for the production of structured phospholipids from acidolysis reaction between deoiled soybean lecithin and caprylic acid with Lipozyme TL IM as catalyst (paper V), and for production of structured phospholipids from acidolysis reactions between purified PC and caprylic acid using Lipozyme RM IM as catalyst (paper VIII). Bioreactors were jacketed columns (either glass or stainless steel) packed with the immobilized lipases. During operation, substrate mixture was fed upward into the column, and column temperature was held constant by circulating water bath. Figure 5.2 shows an image of a stainless column used as bioreactor for continuous production of structured phospholipids.



Figure 5.2: Image of packed bed reactor described in paper VIII

Different parameters were studied for the acidolysis reaction in PBR using either a solvent- or a solvent-free system. Effects of molar ratio of reactants, reaction temperature, residence time, and water content on caprylic acid incorporation into phospholipids were monitored as major variables. Operative stability of the enzyme during continuous operation over several days was also examined. Incorporation of caprylic into phospholipids were reported in both studies, and in the study using purified PC, information regarding the recovery (phospholipid distribution) was also included.

For detailed column dimensions and applied settings used in the different studies please refer to papers V and VIII in the appendix section.

5.1.4 Synthesis of structured phospholipids by immobilized PLA₂-catalyzed acidolysis in solvent free system (Paper VI)

Acyl modification of *sn*-2 position in phospholipids was conducted by acidolysis reaction using immobilized phospholipase A₂ (PLA₂) as catalyst (Paper VI). In the first stage different carrier materials were examined for their ability to immobilize PLA₂ by adsorption. Table 5.1 show the characteristics of the seven enzyme carriers screened.

The fixation levels of enzyme on carriers were estimated subtracting the protein remaining in the supernatant after binding compared to the initial protein concentration. Evaluation of the catalytic activity was determined by hydrolysis of PC as described by Kim *et al.* (2001).

Table 5.1: Carriers screened and their characteristics

Carrier	Supplier	General description
Amberlite XAD7	Sigma-Aldrich Chemie GmbH, Steinheim, Germany	Nonionic weakly polar macroreticular resin (matrix: acylic ester), Particle size: 0.25-0.85 mm (wet)
Superlite DAX8	Supelco, Bellefonte, USA	Resin with moderate polarity (matrix: acrylic ester), Particle size: 0.25-0.45mm
Celite 545	BHD, Poole, UK	Diatomaceous Earth, Particle size: 0.02-0.1 mm
Dowex 50W	Dow Chemical Company, Michigan, USA	Strongly acidic cation exchange (maxtrix:resinstyrene-divinylbenzene; functional group: sulfonic acid), Particle size: 0.15-0.30mm
Lewatit VPOC1600	Lanxess AG, Leverkusen, Germany	Divinyl benzene crosslinked polymer (Marix: methacrylate), Particle size: 0.3-1.2 mm
Duolite A568	Rohn and Haas, Chauny, France	Polymerized phenol-formaldehyde anionic exchange resin, Particle size: 0.15-0.85 mm
Accurel EP 100	Akzo, Obernburg, Germany	Macroporous polypropylene, Particle size: 0.6-0.8µm

A promising carrier (Amberlite XAD7) was selected and further experiments were performed to maximize the catalytic activity before subsequent usage for acidolysis reactions.

A three-level three-factor fractional experiment with 2 star points (17 experiments) was carried out for evaluation of PLA₂ catalyzed acidolysis reaction between PC and caprylic acid. The independent variables and their levels were reaction temperature (25-65°C), water addition (0-4wt% based on total substrate), and substrate molar ratio (3-15 mol/mol caprylic acid/PC). Enzyme dosage and reaction time were kept constant. Incorporation of caprylic acid into PC and LPC together with phospholipid distribution (PC, LPC and GPC) were determined for the for the PLA₂-catalyzed acidolysis reactions and were used as responses for the quadratic response surface model. Conjugated linoleic acid and docosahexaenoic acid (DHA) were also tested as acyl donors.

5.2 Purification of structured phospholipids by ultrafiltration

Membrane purification study was done in collaboration with Department of Chemical Engineering, DTU. Possibilities of applying polymeric ultrafiltration membranes to remove free fatty acids (FFA) and solvent from the reaction mixture after lipase-catalyzed production of structured phosphatidylcholine (PC) were examined. Since phospholipids form reverse micelles with large molecular weight in hexane it was anticipated that it would be possible separate FFA from PC by ultrafiltration. A stirred dead-end ultrafiltration cell with magnetic stirrer (Millipore, Glostrup, Denmark) was used for the separation of FFA and solvent from the reaction mixture (see figure 5.3). Nitrogen cylinder provided the driving force (pressure) for the permeation. Cell capacity was 300 ml with an effective membrane area of 40 cm². All membrane experiments were conducted at room temperature (20-25°C), and with a constant rotation speed of the spin bar (250 rpm). Permeates were collected through a port beneath the membrane support. Feed, retentate and permeate were analyzed for PC and FFA for calculation of their retention by the membranes.

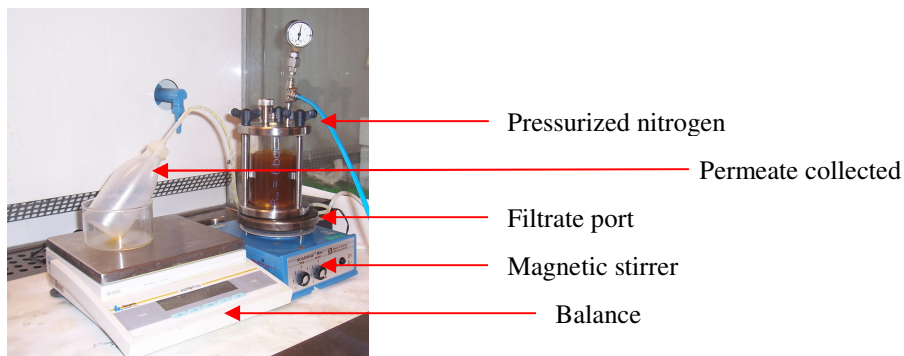


Figure 5.3: Membrane apparatus used for purification of structured phospholipids

5.2.1 Screening of different membranes for separation of FFA from structured phospholipids (Paper VII)

Commercial polymeric membranes with different cut-offs (1,000-20,000 Da) were screened for their abilities to separate free fatty acids (FFA) from structured phospholipids. Characteristics of the commercial membranes employed in this study are shown in Table 5.2.

Table 5.2: Membranes screened for purification of structured phospholipids and their characteristics

Membrane designation	Selective layer	Support layer	Cut-off ² (MW)	Membrane state
GR70PE	PSf	PET	20,000	Dry
GR90PP	PSf	PP	2,000	Wet
GR81PP	PSf	PP	10,000	Dry
GR61PP	PSf	PP	20,000	Dry
Hekla 10A	PSf ¹	PP	10,000	Dry
ETNA01PP	PVDF ¹	PP	1,000	Dry
ETNA10A	PVDF ¹	PP	10,000	Dry
ETNA20A	PVDF ¹	PP	20,000	Dry
FS 61PP	PVDF	PP	20,000	Wet

Abbreviations: PSf, polysulphone; PVDF, Polyvinylidene fluoride, PP, polypropylene; PET, Polyethyleneterephthalate¹Hydrophilic coated. ² MWCO values of membranes reported by the supplier.

Suitable membranes were selected in terms of high selectivity between FFA and phospholipids. Reaction mixtures obtained after Lipozyme TL IM catalyzed acidolysis reaction between PC and caprylic acid in hexane system were used directly without further concentration or dilution. For detail information on the production of the structured phospholipids used for membrane screening please refer to paper VII in appendix section. Concentrations of PC and FFA in the reaction mixture were 0.04 M and 1.9 M, respectively (molar ratio, 1:48).

5.2.2 Discontinuous diafiltration (Papers VII, VIII and IX)

In dead-end operation all the feed is forced through the membrane, which implies that the concentration of rejected components in the feed increase and consequently the permeate quality decreased with time. To overcome this problem the filtration process was performed as discontinuous diafiltration. Discontinuous diafiltration involve the addition of solvent to retentate and continuing ultrafiltration in order to overcome lower fluxes at higher concentrations and improve removal of permeate species. Discontinuous diafiltration was performed on reaction mixtures obtained from lipase-catalyzed acidolysis in solvent system, and also for reaction mixture from lipase-catalyzed acidolysis in solvent free system. Reaction mixture obtained from solvent free

operation was dissolved in hexane prior to ultrafiltration to overcome problems with low permeate flux and concentration polarization. Discontinuous diafiltration was conducted at different initial feed concentrations and concentrated to different volumes before new hexane was added in order to determine optimal conditions for filtration process. These latter experiments were conducted with reaction mixture obtained from solvent free production (substrate molar ratio, 21 mol/mol free fatty acids/PC).

5.2.3 Purification by Column Chromatography (Papers VIII and IX)

Separation of individual phospholipid species (PC, LPC and GPC) was not possible by ultrafiltration. Further purification of product for separation of individual phospholipids and FFA residues was therefore done by column chromatography. A column was packed with silica (see Figure 5.4), and the phospholipid species were eluted with two different solvent systems. Chloroform /methanol /water (65:35:5 v/v/v) was used to separate FFA, PC and LPC. GPC was eluted from the column with methanol/water (90:10 v/v). Further details about purification by column chromatography are described in papers VIII and IX. Purified structured PC containing caprylic acid was used for the study on emulsifying properties of such product (Paper IX).



Figure 5.4: Set up for the column chromatography of structured phospholipids

5.3 Physical and Chemical properties of structured phospholipids

Two individual studies were made on the physical/chemical properties of structured phospholipids. One focused on the ability of enzymatically synthesized structured PC containing caprylic acid to form and stabilize oil-in-water emulsions prepared with different triacylglycerols. The other focused the ability of using DHA-containing PC in liposome formulations in terms of oxidation stability.

5.3.1 Emulsifying properties of structured phospholipids (Paper IX)

The primary objective of the study was to investigate the emulsifying characteristics of the synthesized structured PC containing caprylic acid in oil-in-water emulsions (prepared with various triacylglycerols), and compare with deoiled lecithin and purified soybean PC. Chain length and degree of saturation of oil and phospholipids are known to have significant effect on the emulsion prepared (Nii *et al*, 2005). Triacylglycerols (TAG) with considerable different fatty acid profile were thus selected for this study. These TAG included Medium chain triacylglycerols (MCT), soybean oil and enzymatically synthesized structured lipid. Fatty acid composition of oils and phospholipids used in this study is presented in table 5.3. For o/w emulsions it would be expected that purified soybean PC would result in more stable emulsions compared to deoiled soybean lecithin (see Figure 2.3). With the enzymatic exchange of the long chain fatty acids with medium chain fatty acids in the pure soybean PC it would become more hydrophilic, and thus it was anticipated to have improved function as emulsifier in o/w emulsions.

Table 5.3: Fatty acid distribution in oils and phospholipids used in the emulsion study

Fatty acid composition (mol%)	Triacylglycerols			Phospholipids		
	MCT	Soybean oil	Structured lipid	Deoiled lecithin	PC	Structured PC
8:0	60.2	-	35.0	-	-	36.6
10:0	39.8	-	-	-	-	-
14:0	-	-	4.2	-	-	.
16:0	-	11.4	8.2	19.9	12.8	2.8
16:1	-	-	4.9	-	-	-
18:0	-	3.4	0.8	4.4	3.9	0.7
18:1	-	22.8	10.6	10.1	9.4	5.8
18:2	-	55.6	1.3	59.2	65.8	48.8
18:3	-	6.9	4.9	6.6	8.1	5.3
18:4	-	-	5.1	-	-	-
20:1	-	-	2.5	-	-	-
20:4	-	-	0.7	-	-	-
20:5	-	-	7.7	-	-	-
22:5	-	-	1.1	-	-	-
22:6	-	-	13.0	-	-	-

For detailed information regarding production and purification of structured PC used in this study please refer to Paper IX in appendix section. Components of the emulsion except water were weighed according to the ratio (10% or 30% of oil and 0.5% or 2% of phospholipids) and were heated to 60°C in a beaker with gentle stirring until the phospholipids were completely dissolved in the oil. Then, the water was weighed and dispersed into the oil phase. The mixture was homogenized for 10 s at 13,500 rpm with an ultra-turrax T25 (Janke & Kunkel GmbH & Co., Staufen, Germany).

For evaluation of the emulsifying properties, the oil droplet size, viscosity, and stability against phase separation was determined. For further details of methods, please refer to paper IX in the appendix section.

5.3.2 Oxidative stability of Liposomes prepared from DHA-containing PC (Paper X)

Liposome formulation study was done in collaboration with LiPlasome Pharma A/S. Phospholipids with defined molecular structure provide excellent opportunities within liposome technology for drug delivery and construction of anticancer prodrugs. Construction of liposome with phospholipids containing docosahexaenoic acid (DHA) is of high interest in cancer research, as this fatty acid has been shown to be a potent anticancer agent. Especially when resided at the secondary position (*sn*-2 position) of a phospholipid molecule, DHA is especially effective (Jenski *et al.*, 1995). DHA is an all *cis* polyunsaturated fatty acid with multiple double bonds, and is therefore highly prone to oxidation. Oxidation of phospholipids has remarkable influence on structure, and physical properties of liposomes, and should be limited during preparation steps. Even small quantities of lipid oxidation would give rise to physically unstable liposome formulations.

Incorporation of DHA into PC molecule by acidolysis reaction was demonstrated in paper VI. The structured PC produced contained 20% DHA, and a mixture of other fatty acids. Even though this product may have some potential application in liposome formulations, it was decided to use a commercial high purity product of a structured PC containing DHA (1-palmitoyl-2-DHA, 99% purity). This made it possible to follow oxidation of DHA exclusively without the presences of other fatty acids prone to oxidation.

The objective of the study was to examine the possibilities of using DHA-containing phospholipid in liposome formulations in terms of oxidation stability. The influence of preparation and cold storage on oxidative degradation were examined for DHA-

containing PC in the form of large unilamellar vesicles (LUV) and multilamellar vesicles (MLV). Phospholipid oxidation was followed by measuring primary (conjugated) and secondary (thiobarbituric acid reactive substances, TBARS) products of oxidation.

For detailed information regarding analytical methods and liposome preparation please refer to Paper X in the appendix section.

5.4 Analysis of structured phospholipids

Different analytical techniques were used during this study of which the most widely used are discussed below. So far, there have been scarce reports for the analysis of the fatty acid composition of phospholipids containing short- or medium-chain fatty acids. Therefore this issue is given some further attention in this chapter. For other analytical methods please refer to the papers in the appendix section.

5.4.1 Fatty acid composition analysis

Fatty acid composition analysis was determined by gas chromatography (GC) after conversion of fatty acids into methyl esters derivatives. Fatty acid methyl esters were prepared either by acid-catalyzed transesterification for the determination of fatty acid composition in individual phospholipid molecules (i.e. PC and LPC), whereas base-catalyzed transesterification was used for the preparation of fatty acid methyl esters for determination of fatty composition of all ester-bonded fatty acids. Free fatty acids are esterified and *O*-acyl lipids transesterified by heating them with large excess of anhydrous methanol in the presence of acidic catalyst (BF_3). In that particular case, it is necessary to have separation of FFA from phospholipids after reaction. *O*-acyl lipids are transesterified very rapidly in anhydrous methanol in the presence of a basic catalyst, however free fatty acids are not normally esterified. Structured phospholipid reaction mixture could thus be directly methylated by KOH-catalyzed transesterification without removal of FFA. However, this gives no information regarding the distribution in the different phospholipid molecules (i.e. PC and LPC). Due to acyl migration incorporation of fatty acid donor into LPC and other reactions may occur as described in section 4.3.

5.4.2 Analysis of phospholipid profile by TLC-FID

Quantification of individual phospholipid compounds were performed by Chromarod thin-layer chromatography coupled to flame ionization detector (TLC-FID). A method was also developed for examining the fatty acid distribution in PC and LPC during enzyme catalyzed acidolysis between PC and medium chain fatty acid. Method could thus be used to follow primary reactions and side reactions.

At low concentrations (<2 mg/ml) it was observed that PC and LPC split into several peaks during lipase-catalyzed acidolysis reaction between soybean PC and caprylic acid (see Figure 5.5). 2-3 peaks were observed for PC on chromatograms for samples taken during acidolysis reaction. The peaks represented PC of LL-, ML-, and MM-type, respectively. Similar LPC was observed to split into two peaks, representing LPC containing either long or medium chain fatty acids.

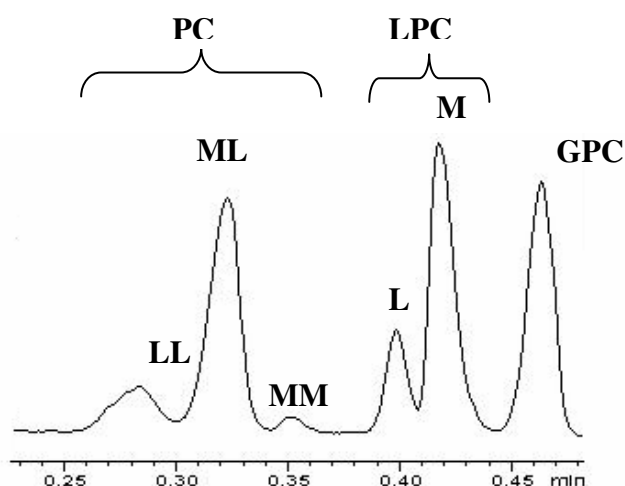


Figure 5.5: TLC-FID chromatogram of structured phospholipid produced from lipase-catalyzed acidolysis reaction between soybean PC and caprylic acid. Abbreviations: L, long-chain fatty acids; M, medium-chain fatty acids.

Calibration curves were prepared for soybean PC (LL-type PC) and 1,2-dioctanoyl-PC (MM-type PC). The response of the PC compounds showed to depend very much on the fatty acid composition and concentration. The signal from the FID usually corresponds to the mass of each component. However at concentration below 2 mg/ml the response of LL-type PC and MM-type PC were significantly different. When the concentrations were calculated into molar concentrations instead the response showed to be very similar for the two PC-types. Two-ways analysis of variance (ANOVA) showed that

there was no significant difference in response between the soybean PC and 1,2-octanoyl-PC. The results illustrates that at low concentrations the signal from the FID does not follow mass of the phospholipid components. Since the LL-type PC and MM-type PC had similar response factor, based on molar concentration, it is expected that ML-type PC will have it as well. Therefore the calibration curve would suit for LL-, MM- and ML-type PC.

From the analysis conducted by TLC-FID the distribution between the PC species was known making it possible to calculate the overall incorporation of caprylic acid in the product by the following equation:

$$Inc (mol\%) = 0.5 \cdot mol\% LM + 1 \cdot mol\% MM \quad (\text{Eq. 5.1})$$

Where LM = LM-type PC and MM = MM-type PC.

The method was successfully used for the evaluation of reaction conditions assisted by RSM experimental design (see paper II in appendix section). Correlation between TLC-FID data and GC-data was also compared (see paper IV in appendix section).

5.4.3 Regiospecific analysis (Papers I, III, and IV)

Determination of positional distribution of FA in phospholipids was done by enzymatic hydrolysis (Christie, 2003). Phospholipase A₂ from either snake venoms or porcine pancreases was used to hydrolyze the ester bond in *sn*-2 position of phospholipids releasing the fatty acids in this position. LPC containing the FA in *sn*-1 position was isolated for fatty acid composition analysis. Alternatively enzymes specific for the *sn*-1 position was be used to hydrolyze fatty acids in *sn*-1 position, and the hydrolysis product (LPC) was examined in a similar way. The accuracy of the hydrolysis procedures was checked by summing the results for the concentration of each fatty acid in *sn*-1 and *sn*-2 positions, dividing with two, and comparing this quantity with the analysis for each component in the original PC. These results agreed well, showing enzymes specific for *sn*-1 and *sn*-2 position are suitable for determining the positional distribution of natural phospholipids and structured phospholipids containing a mixture of long- and medium-chain fatty acids (see Papers I and III in the appendix section).

6 Summary and discussion of experimental work

This chapter is divided into three sections representing production, purification and physical properties of structured phospholipids. The results of the experimental work represented in papers I-X (appendix section) will be summarized and discussed in following sections of this chapter.

6.1 Production of structured phospholipids

The PhD project was initiated by previous work at BioCentrum-DTU using Lipozyme TL IM as catalyst for acidolysis reaction between caprylic acid and deoiled lecithin (Peng *et al.*, 2002). Many studies have applied Lipozyme RM IM; however the price of Lipozyme TL IM is much lower, which would make commercial modifications more economically feasible. Furthermore it has been reported that Lipozyme TL IM exhibits higher activity for the acidolysis of phospholipids compared to Lipozyme RM IM (Peng *et al.*, 2002). Therefore Lipozyme TL IM was the initial choice of catalyst for modification at the *sn*-1 position of phospholipids in this study.

Even though lipases show a good performance, the stability of the enzyme carrier is important. Lipozyme TL IM can easily be removed from the reaction medium in the presences of solvent during batch operation, however, in solvent free systems; the immobilized lipase is not easily removed. During reaction silica granulates are crushed, and do not easily precipitate in the solvent free system due to the highly viscous reaction medium. Lipozyme RM IM is more mechanically stable, and can more easily be removed from product mixture after solvent-free acidolysis reaction, and was therefore used for reactions conducted in solvent-free batch.

Lipozyme TL IM was reported to work well with deoiled lecithin; however reactions did not proceed very fast with purified PC as substrate at low levels of solvent and no water added (Paper I). Under such condition Lipozyme RM IM has better performance (unpublished data). During continuous operation in the PBR with deoiled lecithin as substrate it was decided to use Lipozyme TL IM as catalyst in both solvent and solvent free system (Paper V). Similar Lipozyme RM IM was used as catalyst for continuous operation in PBR with purified PC as substrate in solvent and solvent free system (Paper VIII).

In all studies where RSM was employed it was possible to generate satisfactory models for explaining the relationship between the parameters and responses. The best-fitting

models were determined by multiple regression and backward elimination, whereby insignificant factors and interactions were removed from the models. The coefficient of determination (R^2) of the models were >0.8 , and there was no lack of fit according to ANOVA. For detail information regarding models please refer to the individual papers in the appendix section.

6.1.1 Synthesis of structured phospholipids by lipase-catalyzed acidolysis reaction in solvent batch operation (Papers I and II)

With RSM it was shown that enzyme dosage, reaction temperature, solvent amount, reaction time and substrate ratio had a positive influence on incorporation during Lipozyme TL IM-catalyzed reaction in hexane system (see section 5.1.1 for the examined parameter levels). Enzyme dosage had the most significant effect followed by time, temperature, solvent amount, and substrate ratio. All parameters had a negative effect on PC recovery except for substrate ratio, which had no significant effect. However significant negative effect was observed for interaction between solvent amount and substrate ratio. Increasing solvent amount had the most negative influence on PC recovery followed by enzyme dosage, temperature, and reaction time. For optimal reaction conditions, a compromise, therefore, has to be made concerning enzyme dosage, reaction temperature, substrate ratio and solvent amount. Since increasing amount of solvent reduced the recovery of PC more strongly than it increased the incorporation, it is recommended that this should be kept as low as possible. The reaction temperature increased the incorporation of caprylic acid more strongly than it decreased the PC recovery, and therefore the temperature should be kept in a higher usable range. Individually substrate ratio had no effect on the yield; whereas it had significant effect on incorporation. Therefore higher substrate ratios are definitely better solutions in terms of only incorporation and yield. In reality, productivity and downstream processing should be considered as well. According to the models, it should be possible to have an incorporation of 46% with a recovery of 60%, when having a low solvent amount (5 ml) and the following reaction conditions: Enzyme dosage, 29%; Reaction temperature, 54°C; Reaction time, 50 h; and substrate molar ratio, 15 mol/mol. The prediction was confirmed from verification experiments.

The loss of PC in the system may be explained by hydrolysis, a side reaction, leading to the formation of LPC and GPC. Interestingly, it was observed that the PC/(PC+LPC) ratio (w/w%) was between 78-94% in all reaction mixtures after centrifugation, even for samples with very low recovery. This indicates that the hydrolysis products have very low solubility in hexane. Extraction of the immobilized enzyme after the reaction with

methanol-chloroform (50:50, v/v%) and further analysis revealed that large amounts of GPC was produced during reaction.

Samples from acidolysis reactions were also evaluated by TLC-FID with the method developed (For more detail see paper II). Measuring overall incorporation of fatty acid into phospholipids did not give information of incorporation into the different phospholipid species, and therefore further analyses were made. RSM design described above was used to examine the influence of the parameters on the formation of ML-type PC (desired product). According to the model generated, ML type PC formation was only affected by first order variables. All parameters selected for the study except for substrate ratio had positive effect on the formation of ML-type PC except for substrate ratio. Enzyme dosage had the most significant effect followed by solvent amount, reaction time, and reaction temperature. Substrate ratio showed no significant effect on the formation of ML-type PC within the tested range. Even higher settings for the other factors may increase ML-type PC formation since the studied effects increase over the entire range of values studied. According to the model the parameters should be on high level in order to obtain the highest degree of conversion. The generated model should be used with precaution since in certain cases MM-type PC was produced due to acyl migration.

6.1.2 Synthesis of structured phospholipids by lipase-catalyzed acidolysis reaction in solvent free batch operation (Papers III and IV)

All parameters showed to have an effect on either the incorporation of caprylic acid or the phospholipid distribution (see section 5.1.2 for the examined parameter levels). In order to have a practical operation system, some compromises have to be made for the different parameters since some of them not only have a beneficial effect on the incorporation into PC, but also lead to lower yields.

Enzyme dosage had the most significant effect on the incorporation into PC. Even though the increased enzyme load has beneficial effect on the incorporation into PC it also results in increased existence of caprylic acid in LPC. With increasing enzyme dosage the content of PC decreased whereas the content of LPC and GPC increased. Lipozyme RM IM uses anion exchange resin as lipase carrier. This type of carrier can catalyze acyl migration in the reaction system (Svensson *et al.*, 1992). It seems that acyl migration could be affected by enzyme carriers under the issue of enzyme dosage.

Higher temperature individually decreased the PC content and incorporation of caprylic acid into PC. Reaction temperature did not influence the formation of LPC; however it had significant effect on the formation of GPC. In addition with the increase in temperature the incorporation of caprylic acid into LPC also increased. It is therefore regarded most appropriate to apply temperatures at the low levels.

Water content had no significant influence on the incorporation into PC and LPC. Of all the parameters studied, water addition however had the most significant effect on formation of LPC and GPC. Increased water addition resulted in lower PC content and corresponding increase in LPC and GPC formation. It seems that excess water may act exclusively as a nucleophilic substrate for the hydrolysis rather than the esterification of desired fatty acids. Therefore water content is crucial for the optimization of the acidolysis reaction in terms of yield.

Reaction time was the parameter having most significant effect on the incorporation of caprylic acid into LPC. The formation of LPC was higher compared to that of GPC with increasing reaction time. A compromise is also needed for the reaction time since it has positive effect on the incorporation of caprylic acid into PC, however it also results in higher byproduct formation.

Incorporation into PC decreased with increasing substrate ratio. The incorporation of caprylic acid increased for LPC with increasing substrate ratio, which illustrates that acyl migration probably increases with increasing substrate ratio. The LPC content in the reaction system generally decreased with increasing substrate ratio, whereas GPC was not affected. A compromise therefore has to be made concerning the substrate ratio, even though incorporation of novel fatty acids decreases with increasing substrate ratio, the yield increases.

The optimal conditions were generated by the optimizer function of the software with interactive calculation within the low and high level of parameters studied (star points not included). The general conditions for optimal production were enzyme dosage, 40%; reaction temperature, 55°C; water addition, 1%; reaction time, 70 h; and substrate ratio 6 mol/mol. Under these conditions, an incorporation of caprylic acid into PC up to 49% with PC accounting for 51% of the phospholipid fraction can be obtained. Regiospecific analysis of the product revealed that caprylic acid was mainly incorporated into sn-1 position, accounting for 80% of the fatty acids incorporated.

Due to the complexity of the acidolysis reaction it is difficult to predict the influence of different parameters on the acyl migration into the *sn*-2 position of PC. The statistical experimental design was therefore also used to evaluate the individual parameters

mentioned above as well as their interactions on acyl migration into *sn*-2 position (Please refer to paper IV in the appendix section).

The reaction time was the most significant factor on the acyl migration into *sn*-2 of PC. Increased reaction time also resulted in an overall higher incorporation, and acyl migration therefore seems difficult to avoid in the present reaction system. Other parameters having an effect on the acyl migration were enzyme dosage and reaction temperature. Water addition and substrate ratio had no individual effect on the acyl migration. Some interaction was observed between the enzyme dosage and reaction temperature. High enzyme dosage together with high temperature resulted in increased acyl migration.

Correlation between the incorporation into the *sn*-2 position of PC and incorporation into LPC was set up with varying parameters (see Paper IV, Figure 3). As expected acyl migration into *sn*-2 position seems to increase with increased incorporation into LPC. When caprylic acid has been incorporated into LPC most FA will be in the *sn*-1 position, however some will migrate to the *sn*-2 position until some balance is reached. With migration to the *sn*-2 position this gives the lipase opportunity to incorporate a caprylic acid into the *sn*-1 position resulting in PC with caprylic acid on both positions.

6.1.3 Thermomyces lanuginosa lipase-catalyzed acidolysis reaction in packed bed reactor (Paper V)

The solvent free system was expected to have higher volumetric productivity than the solvent system if the comparison is based on enzyme load and volume of reaction mixture. However, the reaction time needed was expected to be longer in the solvent free system since substrate amount was higher. Therefore it was decided to use column with larger volume in the solvent free system compared to the solvent system.

Solvent ratio (ml/g hexane/total substrate) was varied to determine how it influences the incorporation of caprylic acid into phospholipids in the PBR. With the decrease of the solvent amount, the incorporation of caprylic acid decreased because the substrate/enzyme ratio is increased as well. Therefore a longer residence time will be needed to reach the same incorporation of the novel fatty acids. From the lowest to the highest solvent ratio there was a ten fold increase in the phospholipid concentration, however the incorporation was less than two fold. Highest rate of production would therefore be found for samples having the lowest substrate ratio. However if the conversion is low additional separation steps of the product and original phospholipid

substrate are needed if the goal is to obtain phospholipid with high incorporation of caprylic acid.

For the solvent system, the temperature was varied in the range of 30-50°C to minimize hexane evaporation. Higher temperature gave higher incorporation, and the highest incorporation of caprylic acid was seen at 50°C. Further increase of temperature could be interesting but is limited by the low boiling point of the solvent (boiling point for hexane: 66°C). In the absence of solvent, increasing column temperature can control viscosity of the substrates. At elevated temperatures, operation is easier, since higher temperatures increases solubility of reagents and decreases the viscosity of solutions. This is useful only within optimal temperature range of the enzyme involved, because higher temperature will deactivate the enzyme. For the solvent-free system the temperature was varied from 50-70°C with the following reaction conditions: substrate ratio, 6 mol/mol caprylic acid/phospholipid; water content, 0.5%; flow rate, 0.3 ml/min. The incorporation of caprylic acid into phospholipids was 14.6, 16.7 and 21.1% at 50, 60 and 70°C, respectively. The data shows that in the range 50-70°C the incorporation of caprylic acid increased with increasing temperature with the highest incorporation seen at 70°C.

The incorporation of caprylic acid increased with increasing substrate molar ratio. For the solvent-free system two substrate ratios were tested, 6 and 8 mol/mol caprylic acid/phospholipid with the following reaction conditions in the PBR: water content, 0.5%; flow rate, 0.3 ml/min; and reaction temperature, 60°C. The incorporation of caprylic acid into phospholipids was 13.1 and 24.4% with substrate ratio of 6 and 8, respectively. Varying the substrate ratio the phospholipid substrate applied to the enzyme per unit is also changed; therefore it should be noted that with high substrate ratios the overall productivity will decrease.

Residence time can be increased or reduced by varying the volume flow rates. For the solvent system, the flow rate was varied from 0.1-1.0 ml/min, giving residence time from 0.8 to 8 h. For the solvent-free system the residence time was varied from 1.5 to 13 h corresponding to flow rates between 0.1-1.1 ml/min. The results indicate that a low flow rate is required for a high incorporation.

The operative stability of the enzyme in a solvent system was followed over a week (168 h). The results show that the enzyme reached equilibrium within 48 h and thereafter was stable with only a slight decline in the incorporation of caprylic acid (enzyme activity). The influence of water in the solvent system was tested during the operative stability study by adding 0.25% water to the substrate after 168h of running. A

slight decrease could be observed for the incorporation of caprylic acid. Operative stability of the solvent-free system was tested for several days with two different water contents. With both substrates the incorporation was highest in the beginning and decreased until 30 h where it stabilized. The incorporation of caprylic acid was slightly higher when the water content was 0.25%. For the solvent free system, it seems that small amounts of water are beneficial for the incorporation. The results indicate that phospholipid amount in substrate mixture has a great influence on the catalytic activity of the enzyme. Increasing phospholipid concentration in the substrate will probably remove more water from the enzyme, thus reducing the catalytic activity. Therefore it is necessary to add water to the solvent-free system to increase the transacylation rate, and apparently the addition of water to the solvent system did not increase the incorporation at all.

6.1.4 Rhizomucor miehei lipase-catalyzed acidolysis reaction in packed bed reactor (Paper VIII)

Stability of Lipozyme RM IM during solvent free acidolysis reaction was followed over a few days. Flow rate through the PBR was adjusted to the lowest possible (0.1 ml/min) giving a residence time through the column of ~7h. Incorporation was seen to decrease continuously over time, and PC content increased over time. These results indicate that the catalytic activity of lipase decrease during running time. It is not uncommon during lipase-catalyzed reactions in packed bed to see a gradual decrease in the conversion degree over time (Rønne *et al.*, 2005). Problem like this is overcome by gradually increasing the residence time, which was not possible in this study. During lipase catalyzed production of structured triacylglycerols in PBR, an increase in water content in the substrate can in some cases help regain initial activity of the enzyme (Xu *et al.*, 1998). High phospholipid concentrations are known to slow down the acidolysis and interesterification reaction of triacylglycerols. The effect of phospholipids on the activity and stability of Lipozyme RM IM in organic media during batch operation was found to be very crucial (Wang and Gordon, 1991). Phospholipids have been reported to be totally absorbed by the enzyme bed during the first couple of days. Enzyme bed reactor retains the polar or complex compounds, depending on the hydrophobicity of the substrates. When shorter chain length fatty acids are used as acyl donors, the less retaining of the polar compounds would be expected. Compared to lipase catalyzed reactions with triacylglycerols as substrate, the reaction rate for reactions with phospholipids is considerably slower and deactivation is more rapid. The long reaction time required for the phospholipid acidolysis combined with the rapid loss of activity makes continuous operation for solvent free system very difficult. The yield was high showing that hydrolysis was minimal in the system.

In order to have high incorporation of acyl donor into PC under solvent free conditions batch operation still seems to be the best solution. Packed bed reactors have the advantage though, that the reaction mixture can simply be pumped out, whereas for the batch operation it needs sedimentation of the immobilized enzyme prior to collecting the reaction mixture for purification. A simple way to increase the conversion degree in the packed bed reactor under solvent free conditions is to recycle the reaction mixture through the packed bed as the contact time between substrate and enzyme column would increase. Different substrate mixtures were prepared with or without water addition, and reactions were performed at varying temperatures in PBR with recycle ($R=\infty$).

For reaction conducted in PBR with recirculation, incorporations of caprylic acid into PC were not significantly different when conducted at 40 and 55°C after 48h with no water addition to the substrate. The incorporation of caprylic acid into LPC was however higher when the reaction was conducted at 55°C. In both cases formation of LPC and GPC was observed, however recovery of PC was significantly higher when conducted at 40°C.

With addition of water, higher incorporation into PC can be obtained with shorter reaction time; however the recovery of PC was significantly lower. In paper III it was observed that the water addition did not affect the incorporation into PC and LPC; however from this study it seems that increased water addition increase the incorporation into PC when conducted at 55°C. Due to several reactions (hydrolysis, esterification, acyl migration) happening simultaneously and interactions between different reaction parameters make the prediction of incorporation and recovery during reaction complicated.

Reusability of the enzyme column was tested; however the incorporation was already very low after the second batch. Addition of water could probably improve conversion degree, however probably on the expense of the yield.

Selected samples with high incorporation were furthermore examined for fatty acid distribution in the *sn*-2 position. When the reaction at 40°C was prolonged to 120h, the overall incorporation of caprylic acid was not significantly increased as compared to 72h; however the occurrence of caprylic acid in the *sn*-2 position was dramatically increased. PC content was also observed to decrease.

Operative stability of the immobilized lipase was also examined in a solvent system. A decrease was also observed in the incorporation of novel fatty acids in the presence of

solvent during running time, however to a lower extent than under solvent free conditions. Solvent system seems to provide good choice for the acidolysis reaction, as high incorporation and yields are achieved. Activity of the lipase is maintained for longer time in the presence of solvent as compared to under solvent free conditions. Looking at the relative distribution of PC, LPC and GPC the recovery of PC is usually very high during bath operation, however looking at the actual concentration of phospholipids in the reaction mixture there is usually seen a low recovery, as mentioned in section 6.1.1. These low yields could be explained by the binding of substrate to enzyme carrier. It seems that running the reaction in PBR with solvent is quit beneficial as the recovery of PC is considerably higher as compared to batch operation. The reason for this dramatic increase in recovery needs to be exploited more in detail in order to give good explanation for this phenomenon. These data imply that there is potential in larger-scale continuous production of the structured phospholipids.

6.1.5 Synthesis of structured phospholipids by immobilized PLA₂-catalyzed acidolysis reaction in solvent free batch operation (Paper VI)

Seven different carriers for immobilization of PLA₂ were screened (see table 5.1 for the carriers screened and their characteristics). Based on protein fixation and hydrolytic activity, Amberlite XAD7 was found to be a suitable carrier and was selected for the further study. Protein binding to the carrier increased with increased ratio between enzyme and carrier. However activity only increased with increasing fixation level until a certain protein loading; and the specific activity decreased with increase in fixation level of PLA₂ (see paper VI, Figure 2A). Optimal fixation of PLA₂ in terms of activity as observed with an initial enzyme/carrier ratio of approximately 100 mg/g.

Reaction temperature, substrate ratio and water addition were selected as variables, whereas enzyme dosage and reaction time were held constant in the current study (see section 5.1.4 for examined parameter levels). Responses and variable settings were fitted to each other with multiple regressions.

Water addition was the most significant factor on the PLA₂ catalyzed acidolysis reactions in terms of incorporation and recovery. A continuous increase in the incorporation was observed until water level of 2%. Higher water addition had no significant effect on incorporation. The recovery of PC was seen to decrease with increased water addition. With increase of water in the reaction system both LPC and GPC increased.

Reaction temperature also had significant effect on the acidolysis reaction. Maximum incorporation was observed at 45°C. At higher and lower temperatures a decrease in the incorporation of caprylic acid into PC was observed. Lowest yields were obtained at 45°C. At higher and lower temperatures PC content increased. In general an increase in temperature increase the rate of all chemical reactions, including those catalyzed by enzymes, but at the same time it increase the rate of denaturation of enzyme protein. These processes probably explain the characteristic temperature profile of PLA₂ and high value for the second order value in the models. Temperature had an effect on formation of GPC. Highest content of GPC was at 45°C. With higher LPC content in the reaction system, formation of GPC seems to increase especially at elevated temperatures.

Substrate ratio had no significant effect on either incorporation of caprylic acid or the phospholipid distribution, and no interaction was seen for this factor. Even though no differences are seen in the phospholipid distribution, it should be remembered that the phospholipid concentration is higher at lower substrate ratios. In terms of production it would be recommended to have low substrate ratio.

Incorporation of caprylic acid into PC could reach 36% accounting for 29% of the phospholipid fraction by having reaction temperature, 45°C; water addition 2%; and substrate ratio, 9 mol/mol caprylic acid/PC. The incorporation of caprylic acid into LPC was also examined, however it was less than 4% for all samples (data not shown), and therefore no attempts were made to model these data.

Comparisons of the incorporation of DHA and CLA with that of caprylic acid were done under similar reaction conditions (reaction temperature, 45°C; water addition, 2%; substrate molar ratio, 3 mol/mol fatty acid/PC). CLA resulted in the highest degree of incorporation (30%), followed by caprylic acid (25%) and DHA (20%). With CLA as acyl donor, the phospholipid distribution after reaction was 21, 74, 5% for PC, LPC and GPC, respectively. With caprylic acid as acyl donor, the phospholipid distribution after reaction was 29, 62, 9% for PC, LPC and GPC, respectively. With DHA as acyl donor, the phospholipid distribution was 22, 77, and 1% for PC, LPC and GPC respectively. Yields were thus lower when using CLA and DHA as acyl donors, however the formation of GPC was also lower as compared to reactions performed with caprylic acid. The results indicate that caprylic acid may cause more acyl migration in the reaction system compared to DHA and CLA, however further experiments would be required to verify this observation.

6.2 Purification of structured phospholipids

6.2.1 Screening of membranes for separation of FFA from structured phospholipids (Paper VII)

Commercial polymeric membranes with different cut-offs (1,000-20,000 Da) were screened for their abilities to separate free fatty acids (FFA) from structured phospholipids (see table 5.2 for the membranes screened and their characteristics). Suitable membranes should be resistant to the solvents in use to avoid swelling or even solubilization. From previous publications on degumming, it seems that membranes made of PVDF and PSf have been among the most successful (Subramanian and Nakajima, 1997), and were therefore the primary choice of membranes selected for the screening.

Suitable membranes were selected in terms of high selectivity between FFA and phospholipids. Several membranes showed to be able to reject more than 90% of phosphatidylcholine (PC), however based on the solubility parameters of the polymers many of the membranes would not be suitable for long term use with the solvents. In order to determine polymer solubility in different solvents the three dimensional model proposed by Hansen was applied (Paper VII, equation 3). One membrane was more stable with the solvents compared to the other membranes screened; it was a polysulphone (PSf) membrane on polyester (PE) support (GR70PE). GR70PE showed similar retention of PC as that of few other membranes, but showed relatively higher retention of FFA, resulting in lower selectivity. Solute retention was measured with different MW PEG for selected membranes. For GR70PE it could be observed that the MWCO was between 3,000-8,000 Da, which show that cut-off value was lower than stated by the supplier.

Increased pressure increased the retention of both PC and FFA, however the selectivity was improved. In order to have good separation as well as good retention of PC, the strategy was to operate at higher pressures as larger selectivity is obtained. However multiple steps, especially with dilution of retentate to minimize the viscosity and fouling, will be required for a better separation.

6.2.2 Discontinuous diafiltration (Papers VII, VIII and IX)

In Paper VII the product mixture obtained after lipase-catalyzed reaction in solvent batch operation was used directly without further concentration or dilution. Reaction mixture was dissolved in hexane prior to ultrafiltration for product mixture obtained from solvent free production (Paper VIII).

Discontinuous diafiltration was conducted at different initial feed concentration and concentrated to different volumes before new hexane was added (Paper VIII, Figure 4). Diafiltrations were performed in nine steps. During concentration step the permeate flux continuously decreased, however with dilution of retentate the permeate flux was increased again. FFA in permeate decreased over the nine steps. Phospholipid concentration in the collected permeate fractions were very low, and from calculation retention was more than 99% (data not shown). With the discontinuous diafiltration it was possible to change the molar ratio between PC and FFA from 1:48 to 1:1.6 (paper VII).

With the same initial concentration, higher flux is obtained at lower volume reduction in each step. With higher initial concentration the flux is also lower at fixed volume reduction. When having larger volume reduction in each step this will reduce the number of filtrations. The results however shows that it is more important to collect smaller volumes of permeate in each step compared to the initial feed concentration of the solutes. Highest productivity (mol FFA removed/ mol PC h) was observed during diafiltration at low initial feed concentration and with smaller volume reduction during concentration step (see Paper VIII, Figure 5).

Upon a certain concentration, filtrate flux rates become prohibitively slow, and it may take longer to diafiltrate the concentrated sample than it would if the sample were first diluted. Even though diafiltration of the diluted sample requires a greater diafiltration volume the processing time would be less due to the faster filtrate flux rate (Process time= Filtrate flow rate x volume). The accumulation of retained molecules may form a concentrated gel layer. The impact of gel-layer formation is that it can significantly alter the performance characteristics of the membrane. It was observed that the gel layer concentration was almost constant when volume reduction was 30% during filtration step, regardless of the initial feed concentration (30-40 wt% of product mixture). However, when the volume reduction was increased to 40% in each step a continuous increase in gel layer concentration was observed, and therefore high volume reduction can not be recommended.

Examining the phospholipid distribution of the retentate it was observed that it was not significantly different from the initial feed. GPC, LPC and PC are all retained by the membrane. In order to have separation of these compounds additional purification techniques are required.

6.2.3 Purification by column chromatography (Papers VIII and IX)

Phospholipid mixture consisting of PC, LPC, GPC and small amount of FFA (retentate from ultrafiltration process) was subjected to column chromatography for further purification. FFA, PC and LPC were separated with the solvent system chloroform/methanol/water (42/22/3, v/v/v). After collecting LPC the solvent system was changed to methanol/water (90/10, v/v) to elute GPC. The column chromatography provided a good separation of all phospholipid species. Some FFA was still present after membrane filtration, however possessed no problem due to the low concentration. If only PC is the desired product and no collection of LPC and GPC are required, the solvent system may be changed after PC. LPC and GPC will elute faster, however it will not be separated (data not shown). Column chromatography could probably be used for the separation of FFA from phospholipids, however compared to the ultrafiltration method this requires longer time and larger amount of solvent to have this kind of separation. Structured PC containing caprylic was purified by ultrafiltration and column chromatography, and a final purity of 92% was achieved.

6.3 Physical and chemical properties of structured phospholipids

6.3.1 Emulsifying properties of structured phospholipids (Paper IV)

The structures of all the phospholipid-stabilized o/w emulsion were similar. All the emulsions prepared showed round droplets uniformly dispersed in the system. Particle size of o/w emulsions are known to depend on various factors such as dispersed oil and its ratio to the continuous water phase, the emulsifier and its concentration, and the method of emulsion preparation. With all the emulsions there could be observed particles with varying particle sizes. In general, the particle size increased with increase in oil concentration and with decrease in phospholipid concentration. Particle size analysis confirmed the microscopic examination. The particle size varied within each emulsion prepared. In general, particle size decreased with increase in phospholipid concentration and decrease in oil concentration. When the sauter diameter decreases, there was usually also a decrease in the span (For definition of sauter diameter and span please refer to Paper IX in the appendix section). Increasing the lecithin concentration

reduced the size of large vesicles, and had little effect on small emulsified droplets. Emulsion with an oil/water ratio of 10:90 generally showed smaller particle size and span with structured lipid compared with emulsions prepared with MCT and soybean oil. In most cases largest particle size and span was observed for emulsions prepared with soybean oil. Largest particles were observed for emulsions prepared with PC and soybean oil, and the smallest for emulsions with deoiled lecithin and structured lipid. Emulsions prepared with structured PC usually had a larger particle size and span compared to deoiled lecithin, except with structured lipid where there was no significant difference in particle size. Structured PC gave smaller particles and span than soybean PC in emulsions prepared with soybean oil at low phospholipid concentration and emulsions prepared with structured lipid at high phospholipid content.

For emulsion with oil/water ratio 30:70 the smallest particles could in general also be observed for emulsions containing structured lipid. Largest particle size was found for emulsion prepared with deoiled lecithin and structured lipid; however this was not the emulsion with the largest span, which was found for emulsions prepared with structured PC and soybean oil. At low concentrations of deoiled lecithin, the largest span was found for emulsions prepared with MCT followed by soybean oil and structured lipid, respectively. However when the phospholipid concentration was increased to 2% the reverse was observed. Determining which oil will result in the smallest particle size distribution in the emulsion is therefore highly dependent on the phospholipid concentration. With soybean PC used as emulsifier soybean oil gave the largest particle size and span. With the structured PC, the largest particle size was found in MCT followed by soybean oil and structured lipid. Structured PC produced smaller particles in emulsions prepared with soybean oil at both low and high phospholipid content compared to soybean PC, however the span was higher for the structured PC. At high concentration of phospholipid, the structured PC gave smaller particle size compared to the deoiled lecithin in emulsions prepared with MCT and structured lipid.

TAG having long acyl chains are highly lipophilic and, thus more difficult to emulsify. Soybean oil also has longer average chain length compared to the other oils used in this study (see table 5.3), and could explain why it has larger particle size in general. Soybean oil also has the highest degree of unsaturation.

Viscosity was generally higher for emulsion prepared with deoiled lecithin compared to other emulsifiers used. With an increase in phospholipid and oil concentration the viscosity increased. Viscosity was dramatically increased for emulsions containing soybean oil and structured lipid at oil/water ratio 30:70. Viscosity was also significantly higher for structured PC compared to soybean PC in emulsions containing structured

lipid with high phospholipid content at oil/water ratio 10:90. In other emulsions structured PC gave similar or lower viscosity.

Creaming occurs when dispersed particles either settle or float with respect to the continuous phase and when either the lower or upper portion, respectively, becomes more opaque or creamier. All the emulsions exhibit a tendency to creaming, except emulsion prepared with 10% MCT and 2% deoiled lecithin. During 32 days of storage creaming was not observed for this emulsion. Higher phospholipid concentration usually increased the cream volumes and slowed the creaming process. Initially all emulsions seemed stable by visual inspection as there was not observed any phase separation immediately after preparation. In most cases emulsions remained opaque at the base of the sample, while a concentrated cream layer developed at the top of the sample. No oil separation was observed during the 32 days the emulsions were followed. Destabilization kinetics of the different emulsions were very different. For some emulsions the phase separation was not evident until prolonged storage time. However, many of the emulsions separated in two phases within two days. In some cases the cream layer changed little over time and in other cases dramatic changes could be observed during storage. With low phospholipid concentration (0.5%) phase separation usually happened fast and the cream layer changed very little over time. In emulsions prepared with soybean oil the phase separation also happened within two days. During the whole storage time the cream layer volume was much higher for emulsion prepared with structured PC compared to the other emulsions prepared with 10% soybean oil. Similar phenomenon was observed for the MCT where structured PC resulted in a much higher cream layer compared to soybean PC. With structured lipid the structured PC was able to maintain a stable emulsion for at least 16 days. After day 32 the cream layer was similar to emulsions prepared with PC.

Emulsions prepared with 30% oil showed considerably higher cream layer compared to emulsions prepared with 10% oil. Some of these emulsions were able to maintain a stable emulsion for a few days, however after 16 days they all had separated into two phases. At 32 days of storage, the highest cream layer was observed for 2% deoiled lecithin with MCT. With soybean oil, the largest cream layer was observed for emulsion prepared with structured PC, and with structured lipid it was deoiled lecithin. For some emulsions phase separation happened fast and only change slightly over time. Other emulsions showed to decrease gradually over time, as seen for emulsion prepared with soybean oil and structured lipid with 2% deoiled lecithin. This was also observed for the structured PC in soybean oil. In most cases, emulsions prepared with PC had rapid phase separation, however with emulsion containing structured lipid it was possible to stabilize the emulsion for more than 8 days. Emulsions prepared with MCT and structured lipids were most stable when prepared with deoiled lecithin; however

soybean oil was more stable with structured PC. The cream layer volume at 32 days showed to vary greatly among the emulsions prepared. In general, the cream layer volume was high for emulsions prepared with deoiled lecithin. We observed that in most cases deoiled lecithin was better in stabilizing the emulsion compared to soybean PC. However at oil/water ratio 30:70 the PC was able to stabilize emulsion with structured lipid for longer time compared to the deoiled lecithin. Therefore it seems difficult to make clear conclusions about which phospholipid gives better stability as it depends on the particular formulations.

The findings of this study give some useful information on the phospholipid stabilized o/w emulsions. At oil/water ratio 10:90 the structured PC showed to be superior to the other emulsifiers tested when soybean oil and structured lipid were used. Furthermore, structured lipid had interesting emulsifying properties as it was able to produce smaller particle size compared to MCT and soybean oil, which are more commercially accessible. Deoiled lecithin and structured PC seemed to have the ability to stabilize all oils tested for several days. Soybean PC only produced stable emulsion with structured lipid. The optimal concentration of phospholipid and oil will depend on the actual application.

6.3.2 Oxidative stability of Liposomes prepared from DHA-containing PC (Paper X)

Liposomes were prepared from 1-palmityl-2-DHA-PC. Immediately after opening the vial containing the phospholipid, chloroform was removed under a stream of nitrogen until apparent dryness, and samples were taken, to determine the initial content of conjugated dienes and TBARS in the phospholipid. To eliminate traces of solvent from the lipid film, two different approaches were tested, continued flushing with nitrogen for addition 30 minutes, or under vacuum for 6h. From experience even with extensive nitrogen flushing there is often still traces of solvent in the lipid film as determined by NMR (data not shown). To ensure complete removal of trace solvent, vacuum evaporation for several hours is required. MLV and LUV were subsequently prepared from the dried lipid films.

The UV absorption spectra of the phospholipid immediately after opening the vial did not present any peak at 233 nm, and did therefore not contain appreciable quantities of conjugated dienes. After preparation of the liposomes, the concentration of conjugated dienes and TBARS were significantly higher compared to the initial values. Especially when vacuum evaporation was used for the removal of solvent traces in the lipid film, the concentration of conjugated dienes and TBARS significantly increased ($P < 0.001$), as compared to lipid film only dried under nitrogen ($P < 0.01$). Flushing with nitrogen for

solvent evaporation seems to minimize the oxidation, however further work needs to be done to determine if the solvent traces are below an acceptable threshold limit.

Significant differences in the formation of conjugated dienes in LUV and MLV were observed during storage in the dark at 4°C. Formation of conjugated dienes remained more or less constant during storage in LUV, whereas an increase in MLV was seen. This observation was made for liposomes prepared with different methods for solvent evaporation of lipid film. Even though the liposomes prepared from vacuum dried lipid film had a significant higher initial concentration of conjugated dienes, then this apparently did not induce further oxidation in LUV. However formation of conjugated dienes was seen to increase more rapidly in MLV prepared from vacuum dried lipid film as compared to MLV prepared from nitrogen flushed lipid film. MLV prepared from nitrogen flushed lipid film showed hardly any increase in conjugated dienes for the first 7 days. Higher initial concentration of conjugated dienes in freshly prepared MLV thus seems to induce further oxidation.

Similar observations made for the formation of conjugated dienes in MLV and LUV could be made for the formation of TBARS. Concentration of TBARS increased more in MLV than LUV during storage. Hardly any changes were observed in the TBARS concentration in LUV, whereas in MLV an increase in TBARS concentration was seen over the 21 days of storage.

Peroxidation may be prevented by adding antioxidants to the buffer solution used for the preparation of liposomes. Hepes buffer used in the current study, have been reported to scavenge hydroxyl radicals (Genot *et al.*, 1999). The buffer solution also contained EDTA, a metal chelator, which convert iron and copper ions into insoluble complexes or sterically hinder formation of the complexes between metals and lipid hydroperoxides (Jacobsen, 1999). Even with these antioxidants present in the buffer solution, oxidation was not prevented.

Koga *et al.* (1997) compared the rate of hydroperoxide formation in MLV and LUV from egg yolk PC by water-soluble and lipid-soluble radical generators. When peroxidation was induced by water-soluble radical generator, the rate of hydroperoxides formation in LUV was larger than in MLV. Aqueous radical seemed hardly to penetrate outer bilayers and reach inner bilayers in MLV. Due to the larger surface area of LUV it was considered that they were more likely attacked by aqueous radical generators, thus resulting in larger amount of hydroperoxides during storage.

Opposite trend was observed when a lipid-soluble radical generator was used (Koga *et al.*, 1997). LUV was more resistant to the formation of hydroperoxides than its MLV,

when initiating radicals were generated within the membranes. These radicals can react not only within one membrane layer but also with another neighbouring membrane layer in MLV.

As LUV, in contrast to MLV, was hardly oxidized in the liposome formulation prepared in the current study, it seems that oxidation of phospholipid is generated by radicals within the membranes, and not caused by radical present in the aqueous environment.

In conclusion, liposomes could be prepared from 1-palmityl-2-DHA PC with only minor consequences on oxidation level during storage in the form of LUV. Solvent evaporation of lipid film should preferably be done under nitrogen as vacuum evaporation causes oxidation of phospholipids. The UV absorbance method and the TBA method were shown to complement each other for determining oxidation degradation of DHA containing liposomes. With increase of primary oxidation products (conjugated dienes), there was also seen an increase in secondary oxidation product (TBARS). Information from this study can help in the further pursue of using polyunsaturated fatty acids in liposome formulations. For future work, the issue of solvent evaporation needs to be examined more closely. Solvent traces are difficult to remove with nitrogen flushing, however may be below acceptable values. Possibilities of using antioxidants in the prevention of oxidation during preparation and storage could be examined if liposomes are desired in the MLV form.

7 Conclusion and future outlooks

The studies in this thesis provide better understanding on some important aspects concerning enzyme catalyzed acidolysis of structured phospholipids, including downstream processing, and property evaluation of specific structured phospholipids. The main achievements are summarized in the following.

- TLC-FID method was developed for examining the fatty acid distribution in PC and LPC during enzyme catalyzed acidolysis between PC and medium chain fatty acid. It was found that structured PC (ML-type: L, long-chain fatty acids; M, medium-chain fatty acid) fractionated into three distinct bands on Chromarod TLC and plate TLC. These three bands represented PC of LL-type, ML-type and MM-type, respectively. Similar LPC was observed to split into two peaks, representing LPC containing long or medium chain fatty acids. Method could thus be used to follow primary reactions and side reactions.
- Immobilized lipases were used for modification of *sn*-1 position of phospholipids. Reactions were performed in both packed-bed and batch reactors with or without the presences of organic solvent. The incorporation of desired fatty acids into phospholipids and recovery in batch reactors were affected by enzyme load, reaction time, reaction temperature, water content, substrate molar ratio and solvent amount. Influence of temperature and substrate ratio seemed to depend on the particular reaction system. In solvent systems using immobilized *Thermomyces lanuginosa* lipase incorporation of desired fatty acid increased with increased temperature (35-55°) and substrate molar ratio (3-15 mol/mol), whereas in solvent free system using immobilized *Rhizomucor miehei* lipase, incorporation decreased with increase of these parameters in similar range. Individually, water content showed no effect on incorporation during solvent-free operation; however, it had positive effect in solvent system. The recovery of diacylphospholipids decreased with increase of water addition, due to parallel hydrolysis, and therefore can not be recommended. Presence of solvent improves mixing in the system, and makes subsequent removal of enzyme more convenient; however increasing amounts of solvent was shown to reduce recovery of phospholipid more strongly than it increased fatty acid incorporation during batch operation. Under certain conditions an incorporation of 46% with PC accounting for 53% of the phospholipid fraction. Regiospecific analysis of the product revealed that the caprylic acid was mainly incorporated into the *sn*-1 position accounting for 80% of the fatty acids incorporated.

- During acidolysis reaction between phosphatidylcholine (PC) and caprylic acid, the formation of glycerophosphorylcholine (GPC), the presence of acyl donor in the intermediate lysophosphatidylcholine (LPC) and migration into the *sn*-2 position of PC were observed, which are consequences of acyl migration. Formation of GPC especially increased with increasing water content in the reaction system; whereas incorporation into LPC and migration into *sn*-2 position increased with reaction time. Enzyme dosage, reaction temperature and reaction time showed increased effect on acyl migration into *sn*-2 position; whereas water addition and substrate ratio had no individual effect in the ranges tested (0-4% and 3-15 mol/mol, respectively). Correlation was observed between acyl donor in *sn*-2 position of PC and incorporation of acyl donor into LPC.
- Lipase catalyzed production of structured phospholipids in packed bed reactor was affected by the same reaction parameters tested during batch operation. Continuous operation in packed bed reactor was very difficult with a solvent free system. A long reaction time combined with rapid deactivation of the enzyme makes the process unfavorable. Solvent system seems to provide good choice for acidolysis reaction, as high incorporation and yields are achieved. Recovery of diacylphospholipids is considerably higher when reactions are performed in PBR as compared to batch operation.
- PLA₂ immobilized on carrier selected from screening was used for modification of *sn*-2 position by acidolysis. Reaction temperature and water addition had significant effect on acidolysis reaction, however no effect was observed for substrate ratio (mol caprylic acid/mol PC) in range tested. The highest incorporation obtained during acidolysis reactions was 36%. Such incorporation could be obtained under reaction temperature, 45°C; substrate ratio, 9 mol/mol caprylic acid/PC; and water addition of 2%; enzyme dosage 30%; and reaction time, 48h. The yield under these conditions was however only 29%. Lysophosphatidylcholine (LPC) was the major by-product formed during the reaction. Incorporation of acyl donor into LPC was very low (<4%), which indicates that acyl migration is only a minor problem for PLA₂ catalyzed synthesis reaction.
- For the purification of structured phospholipids from free fatty acids, a downstream process involving ultrafiltration was developed during this work. In non-polar solvent phospholipids tend to form reverse micelles, which can be separated from free fatty acids and solvent by using appropriate membranes. Different commercial membranes with different cut-offs were screened in dead end operation, and polysulphone membrane with polyester support showed some good qualities in

terms of flux and selectivity. Multiple steps with dilution of retentate to minimize the viscosity and fouling were done to improve the separation. Membrane performance was shown to be very dependent on the initial feed concentration, concentration factor in each step and applied pressure.

- The ability of enzymatically synthesized structured phosphatidylcholine (PC) containing caprylic acid to form and stabilize oil-in-water emulsions prepared with different triacylglycerols (Medium chain triacylglycerols, MCT; soybean oil; and enzymatically synthesized structured lipid) were examined and compared with natural soybean PC and deoiled lecithin. In certain cases structured PC showed to be superior to deoiled lecithin and soybean PC as emulsifier. This observation was made for emulsions prepared with soybean oil or structured lipid at oil/water ratio 10:90. At oil/water ratio 30:70 the deoiled lecithin performed better compared to the other phospholipids with all oil types. However structured PC produced more stable emulsions compared to natural soybean PC in MCT and soybean oil.
- Oxidative stability of liposomes made of docosahexaenoic acid (DHA)-containing phosphatidylcholine (PC) was examined during preparation and storage. After preparation of the liposomes, the concentration of primary (conjugated dienes) and secondary (thiobarbituric acid reactive substances, TBARS) oxidation products were significantly higher compared to the initial value. During cold storage formation of conjugated dienes and TBARS remained more or less constant in large unilamellar vesicles (LUV), whereas in multilamellar vesicles (MLV) they were seen to increase over a period of 21 days. Evaporation of solvent traces from lipid film should preferably be done under nitrogen as vacuum evaporation was shown to increase oxidation of the phospholipid.

Future outlooks

Many possible variations in the fatty acid chains can be imagined by enzyme catalyzed modification of natural phospholipids. If practical process line could be setup, this would dramatically increase application range of phospholipids. Enzymes as catalyst for the fatty acid exchange, although far from mature and long way from achieving widespread implementation in industry, have been a focus for extensive studies for decades. Fast increase in the applications of enzymatic acyl modification of phospholipids in industry is not expected, however the interest in using enzyme processes and realization of their benefits have increased in the industrial sector. Before these types of reactions can be implemented industrially, a lot more work is needed to

increase efficiency. For these reactions to be applicable their operative stability needs to be better understood, and a longer lifetime of the immobilized enzyme is desired. In terms of stability during continuous operation, it seems to be more beneficial to use non-polar solvent as higher incorporation can be obtained for prolonged time compared to solvent-free system. Although major by-product formation may occur during enzyme catalyzed reactions, it should be kept in mind that these by-products themselves are valuable products having wide applications in the same area as the original material. These compounds can be purchased in purified form from different companies, and are usually sold at considerable higher price compared to natural phospholipids. Until now, the reaction mechanisms have been the main focus. Little effort has been made to purify the structured phospholipids after reaction, and characterize their physical and chemical properties. In this work promising aspects of membrane implementation in the purification of structured phospholipids were demonstrated, and results hereof will probably spur the interest for further studies.

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Appendices

- Paper I:** Vikbjerg, A.F., Mu, H., Xu, X. Lipase-catalyzed acyl exchange of soybean phosphatidylcholine in n-hexane: a critical evaluation of both acyl incorporation and product recovery. *Biotechnol. Prog.* 21:397-404 (2005).
- Paper II:** Vikbjerg, A.F., Mu, H., Xu, X. Monitoring of monoctanoyl phosphatidylcholine synthesis by enzymatic acidolysis between soybean phosphatidylcholine and caprylic acid by thin-layer chromatography with flame ionization detector. *J. Agric. Food Chem.* 53:3937-3942 (2005).
- Paper III:** Vikbjerg, A.F., Mu, H., Xu, X. Parameters affecting incorporation and by-product formation during the production of structured phospholipids by lipase-catalyzed acidolysis in solvent-free system. *J. Mol. Catal. B* 36:14-21 (2005).
- Paper IV:** Vikbjerg, A.F., Mu, H., Xu, X. Elucidation of acyl migration during lipase-catalyzed production of structured phospholipids, *J. Am. Oil Chem. Soc.* 83:609-614 (2006).
- Paper V:** Vikbjerg, A.F., Peng, L., Mu, H., Xu, X. Continuous production of structured phospholipids in a packed bed reactor with lipase from *Thermomyces lanuginosa*. *J. Am. Oil Chem. Soc.* 82: 237-242 (2005).
- Paper VI:** Vikbjerg, A.F., Mu, H., Xu, X. Synthesis of structured phospholipids by immobilized phospholipase A₂ catalyzed acidolysis. *J. Biotechnol.* (Accepted for publication, November 2006).
- Paper VII:** Vikbjerg, A.F., Jonsson, G., Mu, H., Xu, X. Application of ultrafiltration membranes for purification of structured phospholipids produced by lipase-catalyzed acidolysis. *Sep. Pur. Technol.* 50:184-191 (2006).
- Paper VIII:** Vikbjerg, A.F., Rusig, J.-Y., Jonsson, G., Mu, H., Xu, X. Strategies for lipase-catalyzed production and the purification of structured phospholipids. *Eur. J. Lipid Sci. Technol.* 108:802-811 (2006).
- Paper IX:** Vikbjerg, A.F., Rusig, J.-Y., Jonsson, G., Mu, H., Xu, X. Comparative evaluation of the emulsifying properties of phosphatidylcholine after enzymatic acyl modification. *J. Agric. Food Chem.* 54:3310-3316 (2006).
- Paper X:** Vikbjerg, A.F., Andresen, T.L., Jørgensen, K., Mu, H., Xu, X. Oxidative stability of liposomes composed of DHA-containing phospholipids. (In preparation).

PAPER I

Title: Lipase-catalyzed acyl exchange of soybean phosphatidylcholine in n-Hexane: a critical evaluation of both acyl incorporation and product recovery

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Lipase-Catalyzed Acyl Exchange of Soybean Phosphatidylcholine in *n*-Hexane: A Critical Evaluation of Both Acyl Incorporation and Product Recovery

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Lipase-catalyzed acidolysis was examined for the production of structured phospholipids in a hexane system. In a practical operation of the reaction system, the formation of lyso-phospholipids from hydrolysis is often a serious problem, as demonstrated from previous studies. A clear elucidation of the issue and optimization of the system are essential for the practical applications in reality. The effects of enzyme dosage, reaction temperature, solvent amount, reaction time, and substrate ratio were optimized in terms of the acyl incorporation, which led to the products, and lyso-phospholipids formed by hydrolysis, which led to the low yields. The biocatalyst used was the commercial immobilized lipase Lipozyme TL IM and substrates used were phosphatidylcholine (PC) from soybean and caprylic acid. A response surface design was used to evaluate the influence of selected parameters and their relationships on the incorporation of caprylic acid and the corresponding recovery of PC. Incorporation of fatty acids increased with increasing enzyme dosage, reaction temperature, solvent amount, reaction time, and substrate ratio. Enzyme dosage had the most significant effect on the incorporation, followed by reaction time, reaction temperature, solvent amount, and substrate ratio. However the parameters had also a negative influence on the PC recovery. Solvent amount had the most negative effect on recovery, followed by enzyme dosage, temperature, and reaction time. Individually substrate ratio had no significant effect on the PC recovery. Interactions were observed between different parameters. On the basis of the models, the reaction was optimized for the maximum incorporation and maximum PC recovery. With all of the considerations, the optimal conditions are recommended as enzyme dosage 29%, reaction time 50 h, temperature 54 °C, substrate ratio 15 mol/mol caprylic acid/PC, and 5 mL of hexane per 3 g substrate. No additional water is necessary. Under these conditions, an incorporation of caprylic acid up to 46% and recovery of PC up to 60% can be obtained from the prediction. The prediction was confirmed from the verification experiments.

Introduction

Phospholipids (PLs) have wide applications in food, pharmaceutical, and cosmetic products where they function as emulsifiers, stabilizers, and antioxidants (1, 2). Phospholipids used for these applications are predominantly produced as byproducts of the production and refining of vegetable oils. The interest in production of structured PLs containing special fatty acids in one or both positions has increased continuously. Replacement of existing fatty acids in an original PL with desired fatty acids might improve physical and chemical properties or even nutritional, pharmaceutical, and medical functions. Especially, the incorporation of polyunsaturated fatty acids into PLs has gained much attention because of the possibilities for medical applications (3–5). Also the incorporation of other fatty acids into PLs has obtained increasing interest, especially within food applications where PLs with short- and medium-chain saturated fatty acids have better heat stability as well as emulsifying properties and improved oxidation stability (6, 7).

Chemical methods for synthesis of phospholipids with defined fatty acid composition exist based on previous studies (8); however, these methods require toxic chemicals and lack the selectivity and specificity of enzymes (9). Lipases of microbial origin and phospholipase A₂ (PLA₂) from porcine pancreas have been the most commonly used enzymes for the exchange of fatty acids on PLs at *sn*-1 and *sn*-2 positions, respectively (10–14). Usually, lipases work well for both esterification and transesterification, whereas pancreatic PLA₂ functions reasonably only for esterification (15).

Little effort has so far been made to upscale the enzymatic acyl exchange of phospholipids to pilot plant scale or production scale because of a number of reasons such as mass transfer limitations and low yields. Previous studies on enzymatic acyl modification of phospholipids have been focusing more on possibilities rather than applications, especially concerning choice of enzymes and solvents. Price of the applied enzymes is very high and would not be sufficiently cost-effective to be introduced into larger-scale applications. Many studies have applied lipase from *Rhizomucor miehei* (Lipozyme RM IM); however, the price of the lipase from *Thermomyces lanuginosa* (Lipozyme TL IM) is much lower, which

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Table 1. Fatty Acid Distributions in Soybean PC and Structured PC (mol %)

fatty acids	<i>sn</i> -1 position mol % ^a	<i>sn</i> -2 position mol % ^b	total calcd from <i>sn</i> -1 and <i>sn</i> -2 positions	direct analysis soybean PL	direct analysis structured PL ^c	theoretical composition structured PL ^d
C8:0	0.0	0.0	0.0	0.0	50.8	50.8
C16:0	27.0	2.9	15.0	13.7	4.0	6.7
C18:0	7.5	0.0	3.8	3.6	1.4	1.8
C18:1	9.9	10.2	10.0	9.5	5.5	4.7
C18:2	49.6	79.2	64.4	66.0	34.7	32.5
C18:3	6.0	7.7	6.8	7.2	3.6	3.5

^a The fatty acid composition (mol %) at *sn*-1 position after enzymatic hydrolysis with snake venom. ^b The fatty acid composition (mol %) at *sn*-2 position after enzymatic hydrolysis with Lipozyme RM IM. ^c Sample taken at 72 h during preliminary study (see Figure 1 for details). ^d Based on data from fatty acid composition analyzed from material PL (the fifth column).

would make commercial modifications more economically feasible. Furthermore it has been reported that Lipozyme TL IM exhibits higher activity for the acidolysis of PLs compared to Lipozyme RM IM (11).

Solvents usually selected for these types of reactions in previous studies were often not allowed for food applications. From the safety and health point of view it would be preferred to have a solvent-free system. However, because of the high viscosity in the solvent-free system, mixing and mass transfer become problematic, resulting in low reaction rates. In addition, high PL concentrations during enzymatic modifications result in major stripping of water from the enzyme, and additional water may be required (10). The additional water will, on the other hand, result in increased hydrolysis, which in turn leads to lower yields. The use of solvents can dramatically reduce viscosity of the substrates and as a consequence increase the reaction rate by increasing the mass transfer of substrates. The most commonly used solvents for modification of PLs by acidolysis have been toluene (17, 18) and hexane (19–21). In the production of foodstuffs and food ingredients few extraction solvents are allowed; however, hexane is generally accepted in the fat and oil industry, where it is used during extraction and fractionation.

Other important parameters affecting the main reactions and side reactions are enzyme dosage, water content, reaction temperature, reaction time, and substrate ratio (11). The individual and interactive effects between organic solvents and the parameters mentioned above are not well understood, and further work is required in order to make large-scale production feasible. In general there is a tendency of decrease in yields with increase in the fatty acid incorporation of these types of reactions (16). With high quantities of solvent over substrate it has been reported necessary to control water activity and to use a high substrate ratio in order to minimize hydrolysis reactions (18).

Response surface methodology (RSM) was used to evaluate the effects of enzyme dosage, reaction temperature, amount of solvent, and molar ratio of reactants on caprylic acid incorporations into PLs and the corresponding recovery of PC. The objective of this study is to optimize a practical reaction system that could be useful for food or pharmaceutical industries. A cheap lipase was selected together with a hexane system in order to have a reasonable high reaction rate. A solvent-free system was also studied for comparison. Both the incorporation of caprylic acid and the recovery of PC are evaluated during the optimization.

Materials and Methods

Materials. Granulated phosphatidylcholine (PC, purity 95%) was obtained from Avanti Polar-Lipids, Inc. (Alabaster, USA). The fatty acid composition of PC (mol %) can be seen in Table 1. Caprylic acid (C8:0, purity

97%) was purchased from Riedel-de-Haen (Seelze, Germany). Lysophosphatidylcholine (LPC) standard from soybean was purchased from Larodan Fine Chemicals (Malmö, Sweden). Lipozyme TL IM, a silica granulated *Thermomyces lanuginosa* lipase and Lipozyme RM IM, an immobilized lipase from *Rhizomucor miehei*, were donated by Novozymes A/S (Bagsvaerd, Denmark) with a water content of 5.6% and 2.8%, respectively. Both lipases are *sn*-1,3 specific. Snake venom from *Crotalus adamanteus* was purchased from Sigma (St. Louis, MO). All solvents and chemicals used were of analytical grade.

Acidolysis Reaction in Hexane. Reactions between soybean PC and caprylic acid were carried out using a 3-g reaction mixture in varying amounts of hexane in a brown flask with tight screw cap. Reactions were conducted in a water bath with magnetic stirring at 300 rpm, and reaction was started by the addition of lipase (wt % based on total substrates). After reaction the samples were centrifuged at 4000 rpm for 5 min. All samples were stored at -20°C before analysis.

Acidolysis Reaction in Solvent-Free Systems. For acidolysis reactions conducted in solvent-free systems, 3 g of PC was used together with varying amount of caprylic acid to obtain substrate ratios of 3, 6, 9, and 12 mol/mol caprylic acid/PC. Lipase was added according to total substrate amount. Reactions were conducted at 60°C for 72 h. Other reaction conditions were as described above except that no solvent was added for these reactions.

Experimental Design. A three-level five-factor fractional factorial design with two star points was used according to the principle of RSM with the assistance of the commercial software Modde 6.0 from Umetri (Umeå, Sweden). The factors chosen were enzyme dosage (E_d , wt %, based on substrates), temperature (T_e , $^{\circ}\text{C}$), solvent amount (S_a , mL hexane), reaction time (T_i , hours), and substrate ratio (S_r , mol/mol caprylic acid/PC). Incorporation of caprylic acid and PC recovery were used as responses. The variables and the applied ranges are presented in Table 2. All analyses were performed in duplicate, and mean values are reported.

Fatty Acid Composition Analysis. Samples were directly methylated by KOH-catalyzed interesterification, and the fatty acid methyl esters were analyzed with an HP6890 series gas-liquid chromatograph (Hewlett-Packard, Waldbronn, Germany) equipped with a fused-silica capillary column (Supelco Wax-10, 60 m \times 0.25 mm i.d., 0.20 μm film thickness; Supelco Inc., Bellefonte, PA) (11). Oven temperature was programmed from 70 to 225 $^{\circ}\text{C}$. Initial temperature was held for 0.5 min, increased to 160 $^{\circ}\text{C}$ at a rate of 15 $^{\circ}\text{C}/\text{min}$, then increased to 200 $^{\circ}\text{C}$ at a rate of 1.5 $^{\circ}\text{C}/\text{min}$, held for 15 min, and finally increased to 225 $^{\circ}\text{C}$ at a rate of 30 $^{\circ}\text{C}/\text{min}$ and held for 10 min. A flame-ionization detector was used at 300 $^{\circ}\text{C}$. The injector was used in split mode with a ratio of 1/20. Carrier gas was helium with a column flow of 1.2 mL

Table 2. Experimental Setup for Five-Factor, Three-Level Surface Response Design and the Responses

expt no.	factors ^a					incorporation of caprylic acid (mol %)	PC recovery (%)
	<i>E_d</i>	<i>T_e</i>	<i>S_a</i>	<i>T_i</i>	<i>S_r</i>		
1	15	40	10	20	12	10.1	87.5
2	25	40	10	20	6	15.3	63.8
3	15	50	10	20	6	11.5	73.0
4	25	50	10	20	12	25.3	54.1
5	15	40	20	20	12	10.6	66.6
6	25	40	20	20	6	23.5	41.6
7	15	50	20	20	6	22.2	45.3
8	25	50	20	20	12	25.9	37.9
9	15	40	10	40	12	13.4	81.3
10	25	40	10	40	6	23.5	69.7
11	15	50	10	40	6	17.6	100.9
12	25	50	10	40	12	33.7	47.3
13	15	40	20	40	12	20.0	61.5
14	25	40	20	40	6	27.9	44.3
15	15	50	20	40	6	20.6	49.9
16	25	50	20	40	12	53.4	10.6
17	10	45	15	30	9	10.0	74.9
18	30	45	15	30	9	41.1	27.4
19	20	35	15	30	9	17.1	62.2
20	20	55	15	30	9	33.3	41.0
21	20	45	5	30	9	14.7	92.2
22	20	45	25	30	9	30.7	28.3
23	20	45	15	10	9	12.4	62.7
24	20	45	15	50	9	37.0	41.4
25	20	45	15	30	3	16.4	54.9
26	20	45	15	30	15	36.2	41.2
27	20	45	15	30	9	25.7	47.6
28	20	45	15	30	9	24.7	46.6
29	20	45	15	30	9	23.3	52.6

^a Abbreviations: *E_d*, enzyme dosage (wt %, based on amount of substrates); *T_e*, reaction temperature (°C); *S_a*, solvent amount (mL of hexane); *T_i*, reaction time (h); *S_r*, substrate ratio (mol/mol caprylic acid/PC).

min. The fatty acid methyl esters were identified by comparing their retention times with that of authentic standards (Sigma; St. Louis, MO), and the molar composition was calculated together with response factors and molecular weight of the fatty acids. The relative measurement error associated with this method was ±3% based on five independent repeated injections (95% confidence interval).

Fatty Acid Position Analysis of PC. PC was hydrolyzed to LPC with Lipozyme RM IM (*Rhizomucor miehei*) to remove the fatty acids at the *sn*-1 position or with *Crotalus adamanteus* snake venom for the *sn*-2 position. A 5-mg portion of PC was dissolved in diethyl ether (2 mL) and incubated with 30 mg of Lipozyme RM IM dissolved in 0.1 mL of water or 2.5 mg of snake venom dissolved in 0.1 mL of 0.5 mM Tris buffer solution (pH 7.5) containing 4 mM CaCl₂. After shaking vigorously for 1 h, the mixtures were washed into conical flasks with methanol (10 mL) followed by chloroform (20 mL), and the solution was dried over anhydrous sodium sulfate. The mixture was filtrated, dried, and applied on TLC plates (Kieselgel 60, 0.2 mm, Merck, Darmstadt, FRG). The solvent system used to separate LPC from other constituents was a mixture of chloroform/methanol/water (65:35:5 v/v/v). Spots were visualized by spraying the plate with 0.2% of 2,7-dichlorofluorescein in ethanol (96%). Fatty acids on LPC were analyzed in the same way as described for fatty acid composition analysis.

Phospholipid Profile Analysis. Samples were applied to Chromarod SIII (Iatron Laboratories Inc; Tokyo, Japan) and developed in a mixture of chloroform/methanol/water (45:20:2 v/v/v). Amounts of the phospholipid species PC and LPC were analyzed by thin-layer chromatography coupled to a flame-ionization detector (TLC-FID)

(Iatroscan MK6s, Iatron Laboratories; Tokyo, Japan). Peaks were identified by external standards. Standard curves between weight and area (FID response) were constructed for PC and LPC. Recoveries of PC were calculated as follows:

$$\text{PC recovery (\%)} = \left[\frac{\text{recovered PC(mg)}}{\text{applied PC before acidolysis reaction (mg)}} \right] \times 100 \quad (1)$$

The relative measurement error associated with this method was ±8% based on five independent repeated analysis (95% confidence interval).

Statistical Analysis. The data were analyzed by means of response surface methodology using commercial software, Modde 6.0 from Umetri (Umeå, Sweden). Second-order coefficients were generated by regression with backward elimination. Responses were fitted to the factors by multiple regressions. The fit of the model was evaluated by coefficients of determination (*R*²) and the analysis of variance (ANOVA). The insignificant coefficients were eliminated after examining the coefficients, and the model was finally refined. The quadric response surface model was fitted to the following equation:

$$Y = \beta_0 + \sum_{i=1}^4 \beta_i X_i + \sum_{i=1}^3 \beta_{ii} X_i^2 + \sum_{i=1}^4 \sum_{j=i+1}^5 \beta_{ij} X_i X_j \quad (2)$$

where *Y* is the response variables (incorporation or PC recovery), *X_i* the *i*th independent variable, β_0 is the intercept, β_i is the first-order model coefficient, β_{ii} is the quadric coefficient for variable *i*, and β_{ij} is the model coefficient for the interaction between factor *i* and *j*. For process factors the main effect plot displays the predicted changes in the response when the factor varies from its low to its high level, all other factors in the design being set at their averages.

Results and Discussion

Preliminary Study. A preliminary study was conducted to evaluate incorporation and PC recovery during the time course of acidolysis reaction between PC and caprylic acid. Reaction conditions selected were a substrate ratio of 6 mol/mol caprylic acid/PC in 20 mL of hexane together with 30% lipase at 45 °C. No water was added since previous results have reported that the enzyme contains sufficient water to maintain the activity (11, 22). High enzyme dosages are usually required to have high incorporation of the novel fatty acids (10, 11). However, too high enzyme dosage will complicate the mixing, especially if little or no solvent is used.

The results show that, after 72 h, it is possible to have 50% total incorporation of caprylic acid into the phospholipid (Figure 1). However, with increasing incorporation, the recovery of PC decreased. It is known that the water content in these types of systems has a significant influence on the yield (18). In water-abundant systems, the hydrolysis will be the main reaction. Even though additional water was not added to the system, recovery of PC decreased with increased incorporation of caprylic acid. Lecithin had the effect of decreasing water activity by increasing the polarity of the solvent (hexane) so as to limit water availability by stripping it off from the immobilized catalyst during lecithin hydrolysis (10). This is probably also the case for transesterification reactions.

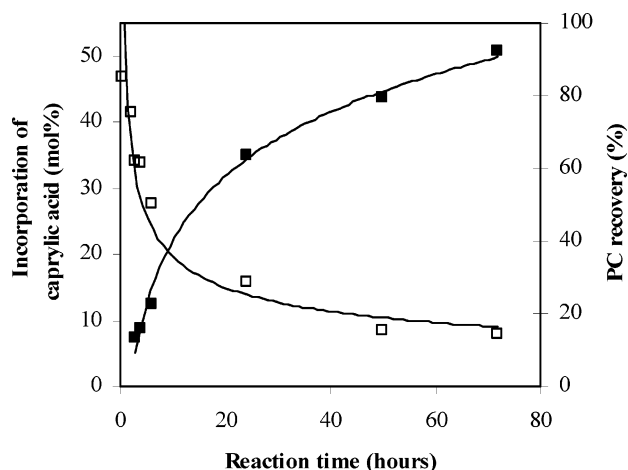


Figure 1. Time course for acidolysis reaction between PC and caprylic acid in hexane. Reaction conditions: enzyme dosage (E_d), 30%; substrate ratio (S_r), 6 mol/mol; solvent amount (S_a), 20 mL of hexane; temperature (T_e), 45 °C. (■) Incorporation of caprylic acid (mol %), (□) PC recovery (%).

Haraldsson and Thorarensen reported that water addition was necessary to obtain reasonable incorporation of novel fatty acids for transesterification reactions in the solvent-free system (12). Water addition may be required to increase enzyme activity; however, this will at the same time increase hydrolysis, which results in lower yields. The hydrophilic carrier used for Lipozyme TL IM may prove to effectively preserve water, which explains the higher activity observed from the enzyme used compared to the commonly used Lipozyme RM IM (11).

Enzymatic reactions conducted in solvent systems have been reported to be highly depended on the polarity of solvents (23). Enzymes in solvents with high polarity have in general lower catalytic activities since the solvent will remove bound water from the enzyme and, as a consequence, suppress PL synthesis. Different parameters are known to suppress the hydrolysis reaction; these include employment of high substrate ratios and having low water activity (16). In solvent-free systems, it has been reported that excessive amounts of fatty acids may decrease reaction rate and even result in substrate inhibition (11).

Table 1 contains the fatty acid composition of the final product from the preliminary study and the calculated theoretical composition through renormalization after subtracting the amount of caprylic acid incorporated. The results from analysis and calculation are relatively similar, indicating the lipase has no significant fatty acid selections during the reaction.

Model Fitting. An optimization of the process was made to evaluate effects of different parameters on both incorporation and yield. The objective was to obtain high incorporation of caprylic acid into PLs as well as to have high PC recovery. For the response surface optimization, a central composite rotatable design was selected with five factors: enzyme dosage, reaction temperature, solvent amount, reaction time, and substrate ratio. Table 2 lists the experimental parameter settings and the results based on the experimental design.

The best quadric models were determined by multiple regressions and backward elimination for both incorporation of caprylic acid and PC recovery. The statistics for the model coefficients and probability values for the two response variables were calculated. The two model equa-

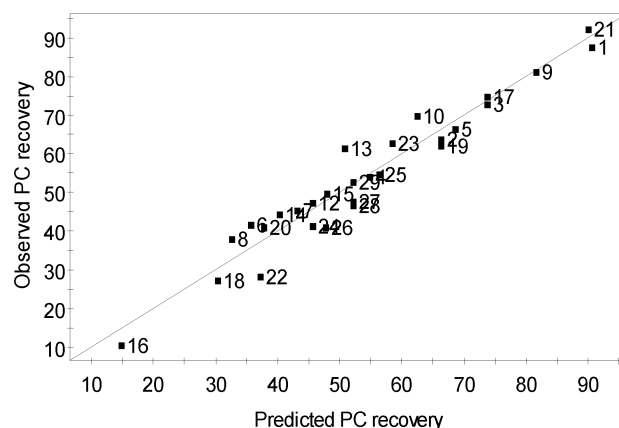
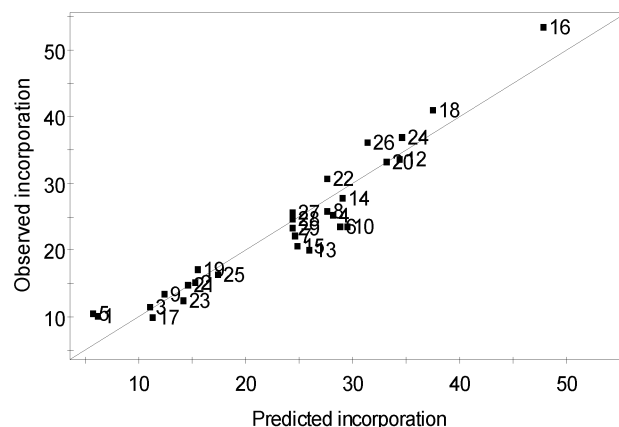


Figure 2. Relationship between observed responses and the results predicted by the developed models for incorporation of caprylic acid (upper) and PC recovery (lower). Numbers inside the figure are experimental setting numbers

tions for incorporation (mol %) and PC recovery (%) can, therefore, be written as follows:

$$\text{incorporation} = 24.277 + 6.538E_d + 4.420T_e + 3.253S_a + 5.098T_i + 3.502S_r - 0.808S_aS_a + 1.500S_aS_r \quad (3)$$

$$\text{PC recovery} = 52.011 - 10.867E_d - 7.118T_e - 13.203S_a - 3.241T_i - 2.138S_r + 2.880S_aS_a - 3.472S_aS_r \quad (4)$$

All P -values of the coefficients were below 0.05 for both models after model refining (data not shown). The coefficients of determination (R^2) of the models for incorporation and PC recovery were 0.91 and 0.94, respectively. According to ANOVA, there was no lack of fit. The observed and predicted values were sufficiently correlated except for no. 11, which was treated as an outlier (Figure 2).

Main Effects of Parameters. The major influence of parameters can be evaluated from the plots of main effects for incorporation and PC recovery (Figures 3 and 4). All five parameters studied affected the incorporation of caprylic acid. It could be seen that all parameters had a positive influence on incorporation. Enzyme dosage had the most significant effect, followed by time, temperature, solvent amount, and substrate ratio.

All parameters had a negative effect on PC recovery except for substrate ratio, which had no significant effect.

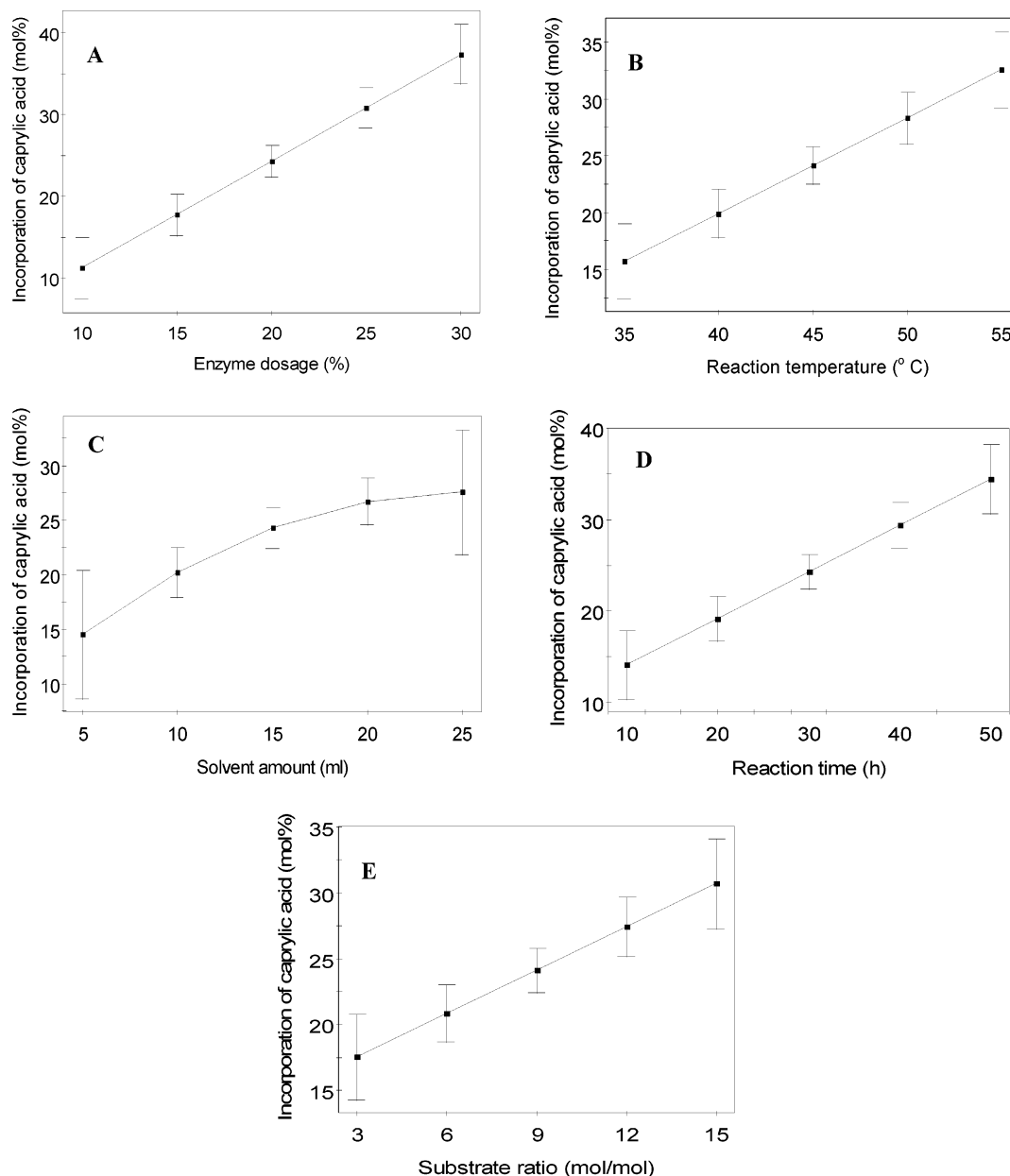


Figure 3. Main effects of parameters on the incorporation of caprylic acid into the phospholipid catalyzed by Lipozyme TL IM in solvent system: (A) enzyme dosage, (B) reaction temperature, (C) solvent amount, (D) reaction time, and (E) substrate ratio.

However, a significant negative effect was observed for interaction between solvent amount and substrate ratio. Solvent amount had the most negative influence on PC recovery, followed by enzyme dosage, temperature, and reaction time. Reaction time had only slight influence on the recovery of PC. This may be understood by examining Figure 1. The recovery only changed slightly after the first couple of hours and then equilibrium was reached for the PC content. The equilibrating rate is dependent on the selected parameters during the reaction.

The loss of PC in the system may be explained by hydrolysis, a side reaction, leading to the formation of LPC and totally deacylated PC (glycerophosphorylcholine (GPC)). LPC and GPC have low solubility in hexane and will probably precipitate during reaction. Interestingly, it was observed that the PC/(PC + LPC) ratio (w/w %) was between 78% and 94% in all reaction mixtures after centrifugation, even for samples with very low recovery (data not shown). This indicates that the hydrolysis products had very low solubility in hexane. Solubility of

LPC probably increased with the increase of PC in the reaction mixture.

According to the model predictions, all parameters had a positive effect on incorporation; however, at the same time they resulted in lower recovery of PC. Hydrolysis increased with increasing incorporation as a result of higher lipase dosage and temperature in accordance with previous publications (10, 12). From the present study as well as previous studies, it is clear that the solvent amount has significant influence on both incorporation and recovery (yield). In the esterification reaction of LPC under supercritical conditions, an additional 10% v/v propane as solvent gave maximum yields of PC (24). Both higher and lower amounts of solvent resulted in lower yields. For optimal reaction conditions, a compromise, therefore, has to be made concerning enzyme dosage, reaction temperature, substrate ratio, and solvent amount.

It has been reported that the yield of the structured PL increased with decreasing water activity and increasing substrate ratios for acidolysis reactions conducted in

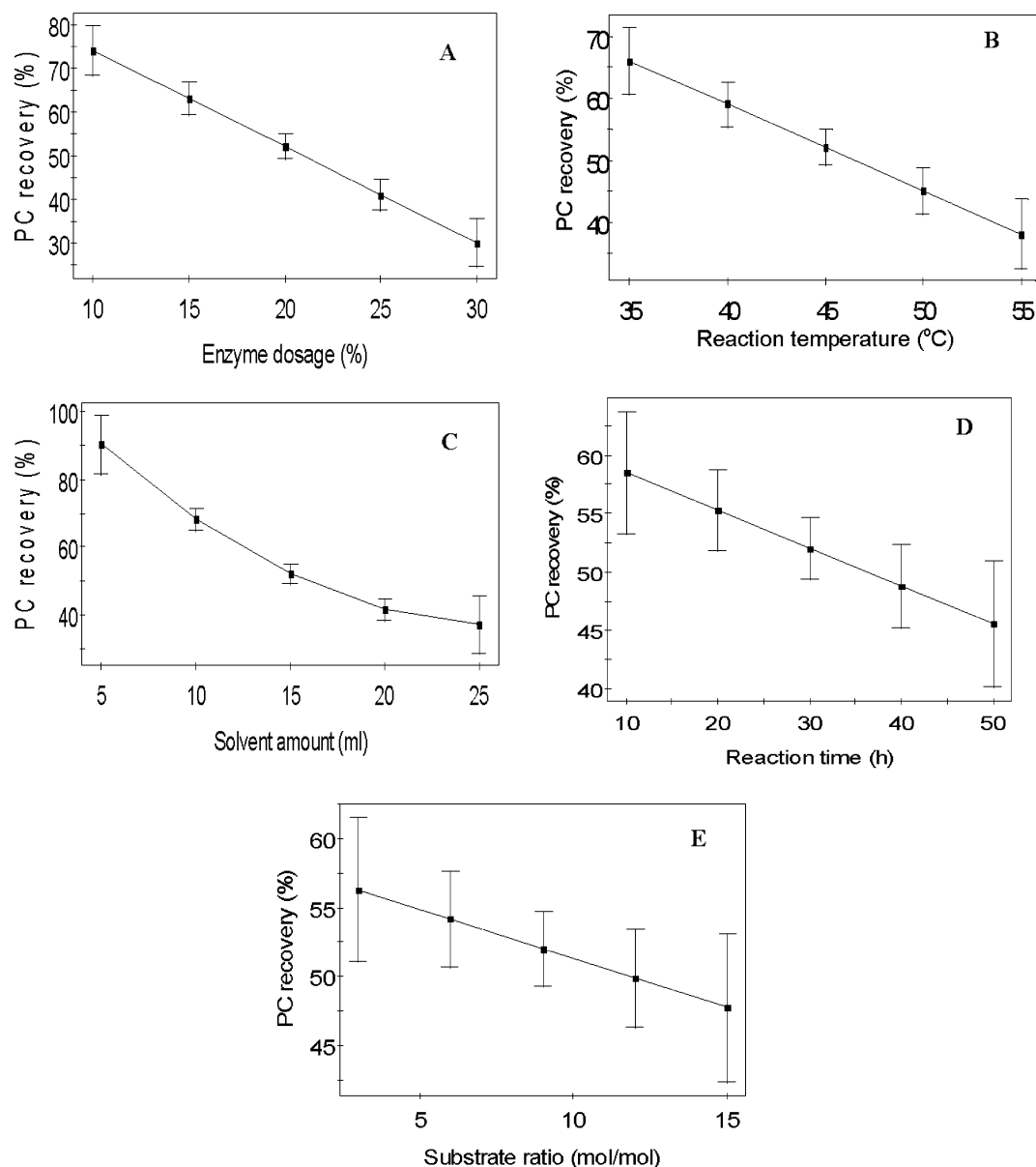


Figure 4. Main effects of parameters on PC recovery during Lipozyme TL IM catalyzed acidolysis reaction between phosphatidylcholine and caprylic acid: (A) enzyme dosage, (B) reaction temperature, (C) solvent amount, (D) reaction time, and (E) substrate ratio.

toluene (18). In this study the substrate ratio had a significant effect on incorporation; however, it had little effect on PC recovery.

Optimization of the Reaction. According to the models generated, incorporation and PC recovery were affected not only by first-order variables but also by the second-order and parameter interactions. Incorporation and PC recovery have a complex relationship with parameters that encompasses both first- and second-order polynomials and may have more than one maximum. Thus, the maximum incorporation and recovery cannot be directly obtained by solving the two equations because more than one solution might exist.

To evaluate the relationship and interactions of parameters, contour plots give good predictions. Typical contour plots between each parameter were generated as Figures 5 and 6, for incorporation of caprylic acid and PC recovery, respectively. All four plots in Figure 5 gave similar relationships with respect to effects of parameters. The higher the enzyme dosage, reaction tempera-

ture, solvent amount, reaction time, and substrate ratio, the higher the incorporation could be obtained. From contour plots between parameters for PC recovery (Figure 6), higher enzyme dosage, solvent amount, temperature, and reaction time resulted in lower recoveries. These results are in agreement with conclusions for the evaluation of the main effects. The results clearly show that some kind of compromise has to be made for these reactions, with several parameters having positive influences on incorporation of caprylic acid but at the same time having negative influences on the recovery of PC.

For the production of structured PLs, the use of solvents would increase the capital investment when the process is scaled up. Preferably the reaction should be conducted in a solvent-free system. Therefore, acidolysis reactions were conducted in a solvent-free system as well for comparison. The results showed that even after 72 h of reaction with 30% lipase at 60 °C, the incorporation of caprylic acid was only 11.5, 11.6, 13.8, and 14.8 mol

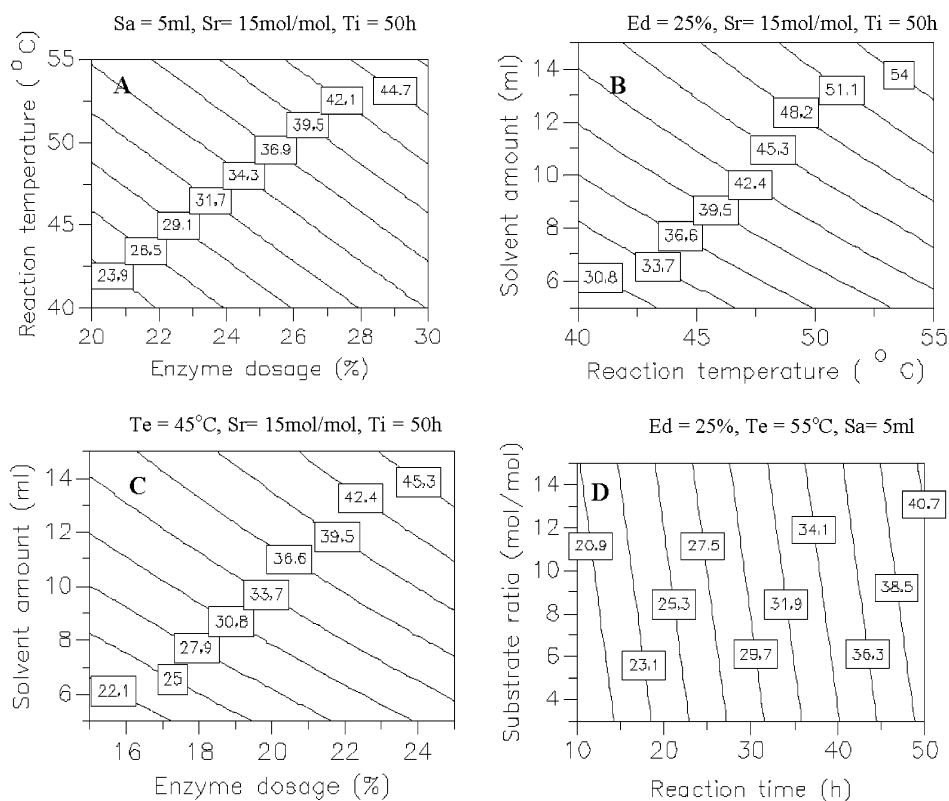


Figure 5. Selected contour plots between parameters for incorporation of caprylic acid into phospholipids catalyzed by Lipozyme TL IM. Numbers inside the contour plots indicate the incorporation of caprylic acid into phospholipids: (A) enzyme dosage (E_d) vs reaction temperature (T_e), (B) reaction temperature (T_e) vs solvent amount (S_a), (C) enzyme dosage (E_d) vs solvent amount, and (D) reaction time (T_i) vs substrate ratio (S_r). For abbreviations, see Table 2.

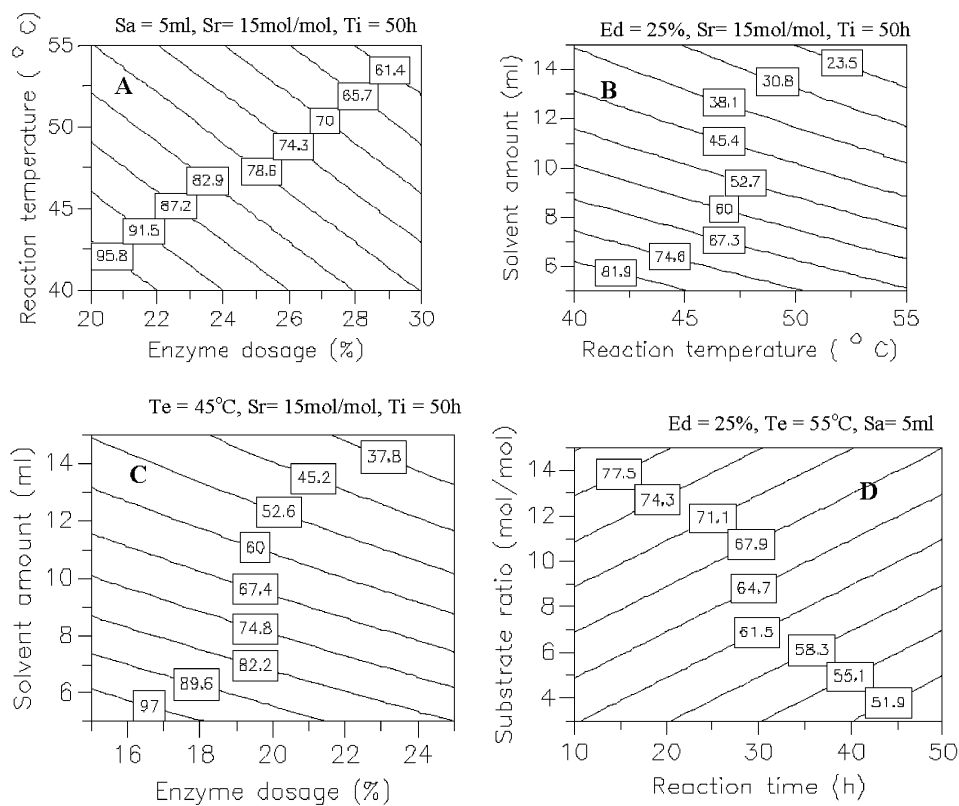


Figure 6. Contour plots between parameters for PC recovery during Lipozyme TL IM catalyzed acidolysis reaction between phosphatidylcholine and caprylic acid. Numbers inside the contour plots indicate PC recovery: (A) enzyme dosage (E_d) vs reaction temperature (T_e), (B) reaction temperature (T_e) vs solvent amount (S_a), (C) enzyme dosage (E_d) vs solvent amount, and (D) reaction time (T_i) vs substrate ratio (S_r). For abbreviations, see Table 2.

%, respectively, when substrate ratios of 3, 6, 9, and 12 mol/mol were used. These results clearly show that

reaction rates were very slow without solvent, and a higher substrate ratio increased incorporation only slightly.

Small amounts of solvent are, therefore, considered to be beneficial for the reaction.

According to the models, it should be possible to have an incorporation of 46% with a recovery of 60%, when having a low solvent amount (5 mL) and the following reaction conditions: E_d , 29%; T_e , 54 °C; T_i , 50 h; and S_r , 15 mol/mol. Verification experiments under these reaction conditions were conducted. The incorporation and PC recovery agreed well with the range of prediction.

Conclusion

Satisfactory quadric models were set up for both the incorporation of caprylic acid into phosphatidylcholine and its recovery in the Lipozyme TL IM-catalyzed acidolysis, including enzyme dosage, reaction temperature, solvent amount, reaction time, and substrate ratio in a batch reaction system. The results clearly show that the amount of hexane had a very significant effect on the yield obtained after acidolysis reaction. With an increasing amount of hexane the recovery decreased, probably as a result of increased hydrolysis. Since the amount of solvent reduced the recovery of PC more strongly than it increased the incorporation, it is recommended that this should be kept as low as possible. However it cannot be totally omitted as demonstrated in this study. The reaction temperature increased the incorporation of caprylic acid more strongly than it decreased the PC recovery, and therefore the temperature should be kept in a higher usable range. Individually the substrate ratio had no effect on the yield, whereas it had significant effect on incorporation. Therefore higher substrate ratios are definitely better solutions in terms of only incorporation and yield. In reality, productivity and downstream processing should be considered as well. With increased enzyme dosage and reaction temperature, higher incorporation can be obtained with higher losses of PC at the same time. According to the optimization, it is possible to obtain 46% incorporation of caprylic acid into PLs and still have recovery of 60% by using 29% enzyme dosage, 50 h reaction time, temperature 54 °C, for a reaction mixture containing 3 g of substrate with a substrate ratio of 15 mol/mol caprylic acid/PC in 5 mL of hexane.

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PAPER II

Title: Monitoring of monoctanoylphosphatidylcholine synthesis by enzymatic acidolysis between soybean phosphatidylcholine and caprylic acid by thin-layer chromatography with a flame ionization detector

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Monitoring of Monoctanoylphosphatidylcholine Synthesis by Enzymatic Acidolysis between Soybean Phosphatidylcholine and Caprylic Acid by Thin-Layer Chromatography with a Flame Ionization Detector

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Thin-layer chromatography with a flame ionization detector (TLC-FID) was used for monitoring the production of structured phospholipids (ML type: L, long-chain fatty acids; M, medium-chain fatty acids) by enzyme-catalyzed acidolysis between soybean phosphatidylcholine (PC) and caprylic acid. It was found that the structured PC fractionated into two to three distinct bands on both plate thin-layer chromatography (TLC) and Chromarod TLC. These three bands represented PC of the LL type, ML type, and MM type, respectively. The TLC-FID method was applied in the present study to examine the influence of enzyme dosage, reaction temperature, solvent amount, reaction time, and substrate ratio (caprylic acid/PC, mol/mol) on formation of ML-type PC in a batch reactor with *Thermomyces lanuginosa* lipase as the catalyst. The formation of ML-type PC was dependent on all parameters examined except for the substrate ratio. The ML-type PC content increased with increasing enzyme dosage, reaction temperature, solvent amount, and reaction time. The substrate ratio had no significant effect on the formation of ML-type PC within the tested range (3–15 mol/mol). The formation of MM-type PC was observed in some experiments, indicating that acyl migration is taking place during reaction since the lipase is claimed to be 1,3-specific. The TLC-FID method offers a simple and cheap technique for elucidation of product and byproduct formation during enzyme-catalyzed reactions for production of phospholipids containing mixtures of long- and medium-chain fatty acids.

KEYWORDS: *Thermomyces lanuginosa* lipase; acidolysis; response surface methodology; structured phospholipids; phosphatidylcholine; TLC-FID

INTRODUCTION

Phospholipids containing medium-chain fatty acids have received increased attention (1, 2). Phospholipids with medium-chain fatty acids are more water soluble than natural phospholipid and have better heat stability. In the native form soybean phospholipids contain more than 70% mono- or polyunsaturated fatty acids. For some applications, particular those involving very long shelf lives, more saturated grades of phospholipids may be desired.

In the area of liposome formulation it has been reported that the release of drug is very fast when small amounts of phospholipids containing medium-chain fatty acids are incorporated into the carrier liposome due to instantaneous activation of phospholipase A₂ (PLA₂) (3). The more rapidly PLA₂ is activated, the faster the drug release and the larger the drug absorption during the time which the carrier spends near the

target. Elevated PLA₂ activity is often seen in inflamed and cancerous tissue. Furthermore, it has been observed in disorders such as epilepsy, bipolar disorders, and some types of pain and migraine associated with inflammatory processes (4, 5).

In recent years there has also been an increasing interest in the synthesis of phospholipids containing drug molecules. Compounds comprising the anticonvulsant valproic acid bonded to the phospholipid moiety at the sn-2 position by chemical synthesis have been produced (6). These compounds were found to be effective at much lower equivalent molar doses compared to the doses currently used for valproic acid. The reduced therapeutic doses in turn reduce the toxicological risk, accompanying side effects, and the risk of undesirable interactions with other drugs. Depending on the fatty acid located at the sn-1 position of these phospholipid derivatives, different pharmacokinetic profiles were observed (6). The length of the alkyl moiety esterified at the sn-1 position of the phospholipid may determine the lipophilicity of the phospholipid derivatives, and thus also transport across the cellular membrane. Other drug molecules may be inserted into the phospholipids, and therefore, there will be a demand to have phospholipids with varying fatty

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acids, giving the opportunity to change pharmacokinetic properties for individual pseudo-phospholipids.

Structured phospholipids with a defined fatty acid profile can be manufactured by enzyme-catalyzed synthesis reactions. The most commonly used enzymes for these purposes have been sn-1,3-specific lipases and PLA₂ for exchange of fatty acids at the sn-1 position and the sn-2 position, respectively (7–10). In many studies the overall incorporation of novel fatty acids into phospholipids has been determined during reactions, which unfortunately does not give any information concerning the distribution of the novel fatty acids. The sn-2 position on the phospholipid may be involved in the lipase-catalyzed acidolysis reaction, which could lead to the false assumption that higher incorporation at sn-1 has occurred since these enzymes are stated to be specific for the sn-1 position. This is based on observations of higher incorporation of novel fatty acids than theoretically possible (11). Incorporation of novel fatty acids has also been determined for the whole reaction mixture including both phospholipids and lysophospholipids, without any fractionation into individual compounds (1). During acidolysis it has been reported that the intermediate lysophosphatidylcholine (LPC) may have high incorporation of novel fatty acids as a result of acyl migration.

Chromarod thin-layer chromatography with an Iatroscan flame ionization detector (TLC-FID) has become more accepted as standard and is being used routinely for lipid analysis in several fields, including food, medical, environmental, toxicological, and ecological studies. TLC is a fast, easy, and cost-saving method for the qualitative determination of most compounds. It can also be used quantitatively to determine the purity of a sample, after reaction to determine recovery and purity, and has in several cases been used to evaluate the lipase-catalyzed hydrolysis and esterification reactions of phospholipids (12–14).

We observed that phosphatidylcholine (PC) splits into two to three bands by TLC corresponding to the differences in the fatty acid composition for samples taken during lipase-catalyzed acidolysis reaction between soybean PC and caprylic acid. These three bands represented PC of the LL type, ML type, and MM type (L, long-chain fatty acids; M, medium-chain fatty acids). This observation was made on both Chromarod TLC and Kieselgel silica plate TLC. LPC was also observed to separate into two bands depending on the fatty acid composition. Fractionation of triacylglycerols into several bands on TLC has also been reported. This separation is based on the difference in fatty acid chain length of triglycerides as well as a result of stereospecific distribution of fatty acids within the triglycerides (15).

In this paper we describe a TLC-FID method for examining the fatty acid distribution in PC during the lipase-catalyzed acidolysis between soybean PC and medium-chain fatty acid. Mixtures of the products of the reaction could be spotted directly on the Chromarods without any preparation. With the aid of response surface methodology (RSM) the developed analysis method was applied for the evaluation of parameter effects during acidolysis reactions.

MATERIALS AND METHODS

Materials. Granulated soybean PC (purity 95%) was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). The fatty acid composition (mol %) of soybean PC was C16:0, 13.7; C18:0, 3.6; C18:1, 9.5; C18:2, 66.0; C18:3, 7.2. 1,2-Octanoyl-sn-glycero-3-phosphatidylcholine (purity, 99%), soybean LPC (purity 98%), and glycerophosphorylcholine (GPC; purity 99%) were purchased from Larodan Fine Chemicals (Malmö, Sweden). Caprylic acid (C8:0; purity 97%) was purchased

Table 1. Experimental Setup for Five-Factor, Three-Level Surface Response Design and the Responses^a

experiment no.	factors					concn of ML-type PC (mol %)
	E_d	T_e	S_a	T_i	S_r	
1	15	40	10	20	12	4.6
2	25	40	10	20	6	23.1
3	15	50	10	20	6	16.3
4	25	50	10	20	12	32.9
5	15	40	20	20	12	15.7
6	25	40	20	20	6	33.3
7	15	50	20	20	6	32.7
8	25	50	20	20	12	42.0
9	15	40	10	40	12	18.9
10	25	40	10	40	6	31.4
11	15	50	10	40	6	17.7
12	25	50	10	40	12	48.2
13	15	40	20	40	12	25.9
14	25	40	20	40	6	41.9
15	15	50	20	40	6	26.6
16	25	50	20	40	12	63.2
17	10	45	15	30	9	14.5
18	30	45	15	30	9	61.6
19	20	35	15	30	9	21.1
20	20	55	15	30	9	45.3
21	20	45	5	30	9	6.5
22	20	45	25	30	9	48.2
23	20	45	15	10	9	11.9
24	20	45	15	50	9	54.3
25	20	45	15	30	3	27.2
26	20	45	15	30	15	55.0
27	20	45	15	30	9	38.1
28	20	45	15	30	9	41.5
29	20	45	15	30	9	35.0

^a Abbreviations: E_d , enzyme dosage (wt %, based on the amount of substrates); T_e , reaction temperature (°C); S_a , solvent amount (mL of hexane); T_i , reaction time (h); S_r , substrate ratio (caprylic acid/PC, mol/mol).

from Riedel-de-Haen (Seelze, Germany). Lipozyme TL IM, silica granulated *Thermomyces lanuginosa* lipase, was donated by Novozymes A/S (Bagsvaerd, Denmark). All solvents and chemicals used were of analytical grade.

Acidolysis. Reactions between soybean PC and caprylic acid were carried out using a 3 g reaction mixture in varying amounts of hexane in a brown flask with a tight screw cap. Reactions were conducted in a water bath with magnetic stirring at 300 rpm, and reaction was started by the addition of lipase (wt % based on total substrates). After reaction the samples were centrifuged at 4000 rpm for 5 min. All samples were stored at –20 °C prior to analysis.

Experimental Design. A fractional factorial design based on the principle of RSM was used in this work with the assistance of the commercial software Modde 6.0 (Umetri, Umeå, Sweden). The five factors chosen were enzyme dosage (E_d , wt % based on substrates), reaction temperature (T_e , °C), solvent amount (S_a , mL of hexane), reaction time, (T_i , h), and substrate ratio (S_r , caprylic acid/PC, mol/mol). The variables and their levels are presented in Table 1. The mole percent of ML-type PC of the total PC was used as the response.

Analysis of the Phospholipid Profile by TLC-FID. A 1 μ L aliquot of diluted sample was spotted onto Chromarod SIII (Iatron Laboratories Inc., Tokyo, Japan), which was developed in a mixture of chloroform/methanol/water (42:22:3, v/v/v). After the development, the Chromarods were dried at 120 °C for 5 min, and phospholipid species PC, LPC, and GPC were analyzed by TLC-FID (Iatroscan MK6s, Iatron Laboratories, Tokyo, Japan). Flow rates of 2 L/min and 160 mL/min were used during the analysis for air and hydrogen, respectively. Peaks were identified by external standards.

Plate Thin-Layer Chromatography. Analytical separations were also performed on silica gel 60 thin-layer plates (20 cm \times 20 cm; Merck, Darmstadt, Germany). Double determinations were performed for each sample. After development in chloroform–methanol–water (65:35:5, v/v), the plate was sprayed with 0.2% 2,7-dichlorofluorescein in ethanol (96%), making the lipid bands visible under UV light. The

following bands were observed: LPC of the M type ($R_f = 0.07$), LPC of the L type ($R_f = 0.15$), PC of the MM type ($R_f = 0.24$), PC of the ML type ($R_f = 0.30$), and PC of the LL type ($R_f = 0.35$), where L refers to long-chain fatty acids and M refers to medium-chain fatty acids (caprylic acid), and fatty acids ($R_f = 0.78$). The lipid bands were scraped off, methylated, and analyzed by GC.

Methylation of Phospholipid Species. The scrapings from TLC were transferred to test tubes with tight screw caps. A 1 mL sample of 0.5 M NaOH in methanol was added to each tube, and the tubes were kept at 80 °C for 5 min. Then 1 mL of 20% BF_3 in methanol and 0.5 mL of 0.5% hydroquinone in methanol were added, and the tubes were kept at 80 °C for 2 min. A 2 mL sample of 0.73% NaCl solution was added and subsequently 1 mL of heptane. The upper phase was transferred to a new tube. A 1 mL sample of a saturated salt solution was added to the new tube, and the upper phase was taken for GC analysis.

GC Analysis of the Fatty Acid Composition. The methyl esters were analyzed on an HP6890 series gas-liquid chromatograph (Hewlett-Packard, Waldbronn, Germany) equipped with an FID, as described elsewhere (1).

Statistical Analysis. Data were analyzed by means of response surface methodology using the commercial software Modde 6.0 from Umetri (Umeå, Sweden). Responses were fitted to the factors by multiple regression, and the fit of the model was evaluated by the coefficient of determination (R^2) and analysis of variance (ANOVA). R^2 above 0.8 indicates that the model has acceptable qualities. The significance of the results was established at $P \leq 0.05$. The response surface model was fitted to the following equation:

$$Y = \beta_0 + \sum_{i=1}^5 \beta_i X_i + \sum_{i=1}^5 \beta_{ii} X_i^2 + \sum_{i=1}^4 \sum_{j=i+1}^5 \beta_{ij} X_i X_j \quad (1)$$

where Y is the response variable of the sample (ML-type PC content), X_i the i th independent variable, β_0 the intercept, β_i the first-order model coefficient, β_{ii} the quadric coefficient for variable i , and β_{ij} the model coefficient for the interaction between factors i and j . The insignificant coefficients were eliminated after the coefficients were examined, and the model was finally refined. For process factors the main effect plot displays the predicted changes in the response when the factor varies from its low to its high level, all other factors in the design being set at their averages.

RESULTS AND DISCUSSION

Acidolysis Reaction Between Soybean PC and Caprylic Acid. The 1,3-specific lipase was used for synthesis of PC with medium-chain fatty acids at the sn-1 position by acidolysis between soybean PC and caprylic acid. TLC-FID analysis of the acidolysis product (Figure 1) illustrates how the PC composition changed on Chromarods for a sample taken at different reaction times. Two to three peaks were observed on the chromatograms for samples taken during acidolysis reaction. The peaks might represent PC of the LL, ML, and MM types. A mixture of 1,2-octanoyl-PC (MM-type PC) and soybean PC (LL-type PC) was spotted on Chromarods, and it was observed that they were separated into two separate peaks, suggesting that the retention value of PC on TLC depends on the fatty acid composition (Figure 2).

To verify that the peaks observed represented PC of the LL, ML, and MM types, the sample taken at 72 h (reaction conditions described in Figure 1) were separated by plate TLC. Similarly, PC was observed to split into three bands. The fatty acid composition of each PC band was measured, after conversion to methyl esters (Table 2). These data confirm that the three bands represent PC of the LL type, ML type, and MM type, since the first band contains practically no caprylic acid and the second and third bands contain approximately 50% and 100% caprylic acid, respectively.

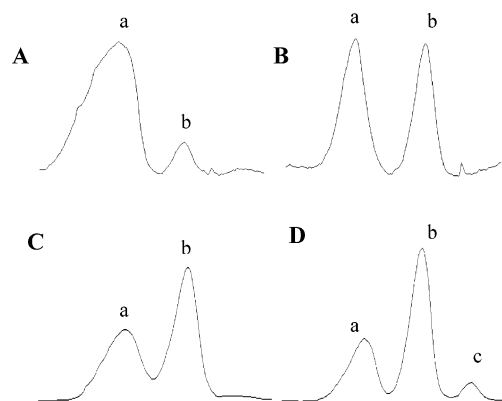


Figure 1. Separation of acidolysis products using TLC-FID. Observed changes in PC during lipase-catalyzed acidolysis reaction between soybean PC and caprylic acid at different reaction times. Reaction conditions: enzyme dosage (E_d), 30%; substrate ratio (S_r), 6 mol/mol; solvent amount (S_a), 20 mL of hexane; reaction temperature (T_e), 45 °C. Key: (A) 6 h, (B) 24 h, (C) 50 h, (D) 72 h. Peaks a, b, and c represent LL-type PC, ML-type PC, and MM-type PC.

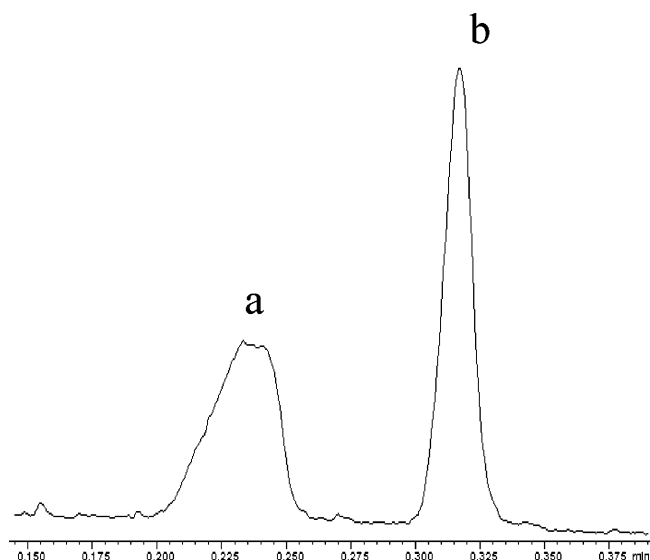


Figure 2. Separation of soybean PC and 1,2-dioctanoyl-PC. Peaks a and b represent soybean PC and 1,2-dioctanoyl-PC.

Table 2. Fatty Acid Distribution (mol %) in Structured Phosphatidylcholine for a Sample Taken at 72 h^a Measured by GC

fatty acid	band 1	band 2	band 3	[PC] _{total} ^b
C8:0	1.3	49.0	92.1	38.7
C16:0	9.7	2.7	1.7	4.8
C18:0	4.8	0.6	0.8	1.8
C18:1	11.1	5.8	1.3	6.9
C18:2	66.5	38.3	3.6	43.5
C18:3	6.6	3.7	0.5	4.3

^a For reaction conditions see Figure 1. ^b Mole percent of caprylic acid incorporated into PC when all PC bands were methylated together (bands 1–3).

The content of ML-type PC increased with reaction time, whereas that of LL-type PC decreased as expected. However, after 72 h MM-type PC was produced (Figure 1D). This is an undesirable byproduct formed during acidolysis reaction. Acyl migration is a serious problem with these types of reactions, leading to lower yields and formation of byproducts as illustrated in Figure 3 (2, 11). Acyl migration is a problem often

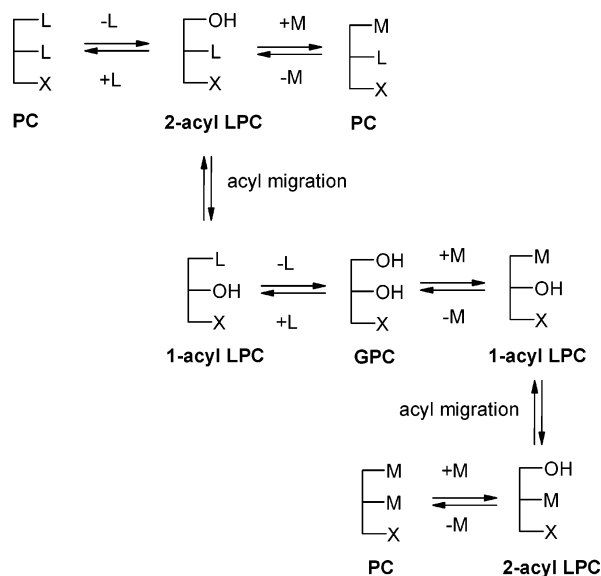


Figure 3. Diagram of the reaction and principle of the lipase-catalyzed acidolysis and side reactions for the production of specific structured PC (L, long-chain fatty acids; M, medium-chain fatty acids).

encountered in selective synthesis of regiospecific glycerophospholipids, i.e., intramolecular transfer of one fatty acid moiety from one hydroxyl group to an adjacent one. In the intermediate LPC, there is a free hydroxyl group, making the reaction possible. 2-Acyl-LPC is less stable than 1-acyl-LPC and converts into the more stable 1-acyl-LPC by acyl migration. Even though an *sn*-1,3-specific lipase is used for the production of the structured PC, caprylic acid on both positions may therefore occur. In addition, it was observed from GC analysis that acidolysis products contain LPC with caprylic acid incorporated, which further illustrates that acyl migration is taking place (data not shown). Acyl migration cannot be simply avoided in applied systems. Many factors possibly influence acyl migration. Often balancing acyl incorporation and migration is necessary to have optimal conditions since an important parameter for acyl incorporation may result in an increase in acyl migration as well.

Calibration. Calibration curves were prepared for soybean PC (LL-type PC) and 1,2-dioctanoyl-PC (MM-type PC). The response of the PC compounds was shown to depend very much on the fatty acid composition and concentration. The signal from the FID usually corresponds to the mass of each component. However, at a concentration below 2 mg/mL the responses of LL-type PC and MM-type PC were significantly different. When the concentrations were calculated into molar concentrations instead, the response was shown to be very similar for the two PC types. Two-way ANOVA showed that there was no significant difference in response between the soybean PC and 1,2-octanoyl-PC. The results illustrate that at low concentrations the signal from the FID does not follow the mass of the phospholipid components. The relationship between the peak area and the concentration of PC is shown in **Figure 4**. Calibration curves for these types of analysis are known to be nonlinear, and are usually represented by a power law equation, $y = ax^b$ (16).

Since the LL-type PC and MM-type PC had similar response factors based on molar concentration, it is expected that ML-type PC will as well. Therefore, the calibration curve would be suited for LL-, MM-, and ML-type PC.

From the analysis conducted by TLC-FID the distribution between the PC species was known, making it possible to

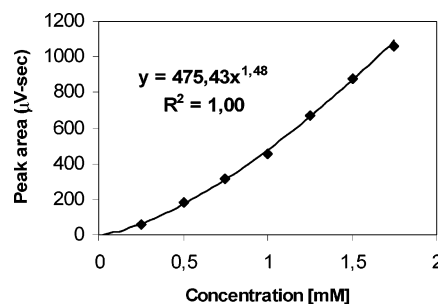


Figure 4. Standard calibration curve for PC. Lines were fitted to a power law equation. Calibration is based on soybean PC ($n = 3$) and 1,2-dioctanoyl-PC ($n = 3$).

Table 3. Multiple Linear Regression Coefficients Describing the Influence of Different Parameters on the Formation of ML-Type PC^a

	ML-type PC formation			ML-type PC formation	
	regression coefficient	P ^b		regression coefficient	P ^b
constant	31.40	3.64×10^{-18}	S_d	7.15	2.43×10^{-5}
E_d	10.49	7.81×10^{-8}	T_i	6.59	6.78×10^{-5}
T_e	5.54	4.61×10^{-4}			

^a For abbreviations see **Table 1** and the text. ^b The 95% confidence limit on each regression coefficient was ± 2.81 (± 2.60 for the constant).

calculate the overall incorporation of caprylic acid into the product by the following equation:

$$\text{Inc (mol \%)} = 0.5[[\text{LM}] (\text{mol \%})] + [\text{MM}] (\text{mol \%}) \quad (2)$$

where LM = LM-type PC and MM = MM-type PC. By applying eq 2, the incorporation of caprylic acid into PC for the 72 h sample (see **Figure 1** for details) was calculated as 38%. From GC analysis the same result was obtained when all PC bands from the TLC plate were methylated together (**Table 2**).

The TLC-FID method was applied in the present study to examine the influence of enzyme dosage, reaction temperature, solvent amount, reaction time, and substrate ratio on the formation of ML-type PC (mol %) during acidolysis reaction between soybean PC and caprylic acid with *T. lanuginosa* lipase as catalyst. RSM was used for evaluating the relationships of the parameters and predicting the results and behavior under the given reaction conditions.

Model Fitting. A central composite rotatable design was selected with five factors: enzyme dosage, reaction temperature, solvent amount, reaction time, and substrate ratio. **Table 1** lists experimental parameter settings and the results based on the experimental design, which were obtained by the method developed above. The best model was determined by multiple regression and backward elimination. According to the model generated, ML-type PC formation was only affected by first-order variables. The model coefficients and *P* values for the regression variables are given in **Table 3**. All *P* values of the coefficient were below 0.05 after the model was refined. The coefficient of determination (R^2) of the model was 0.85 ($Q^2 = 0.77$). The observed and predicted values were sufficiently correlated as can be seen in **Figure 5**, except for no. 26, which was treated as an outlier. According to ANOVA, there was no lack of fit. This indicates that the model represents the actual relationship of the reaction parameters well within the ranges selected.

Main Effects of the Parameters. The effects of the parameters can be evaluated by the plots of the main effects

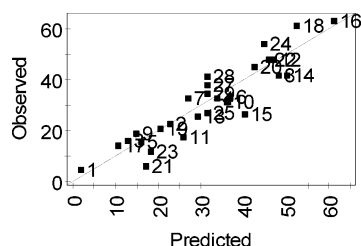


Figure 5. Relationship between the observed results and the data predicted by the models. The numbers inside the graph represent the experimental numbers. The solid line was obtained by regression.

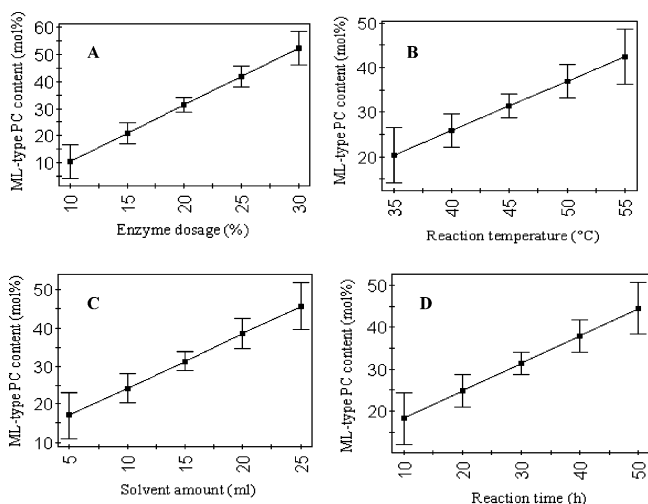


Figure 6. Main effects of the parameters on the ML-type PC content during Lipzyme TL IM-catalyzed acidolysis reaction between PC and caprylic acid: (A) enzyme dosage, (B) reaction temperature, (C) solvent amount, (D) reaction time.

(Figure 6). All parameters selected for the study except for the substrate ratio had a positive effect on the formation of ML-type PC. The enzyme dosage had the most significant effect followed by the solvent amount, reaction time, and reaction temperature. The substrate ratio showed no significant effect on the formation of ML-type PC within the tested range. Even higher settings for the other factors may increase ML-type PC formation since the studied effects increase over the entire range of values studied. According to the model the parameters should be on a high level to obtain the highest degree of conversion.

Typical contour plots between different parameters were generated as Figure 7 for the ML-type PC content (mol %). All the plots in Figure 7 gave similar relationships with respect to the effects of the parameters. The higher the enzyme dosage, reaction temperature, solvent amount, and reaction time, the higher the incorporation obtained. These results are in agreement with the conclusions for the evaluation of the main effects. The generated model should be used with precaution since in certain cases MM-type PC is produced due to acyl migration. In the experimental design only in the sample from experiment 16 MM-type PC was detected. This sample also had the highest formation of ML-type PC.

It should be kept in mind that the yield (recovery) of the total PC is also important for the reaction performance. The incorporation and the recovery of PC were examined for the reaction mixture with all parameters on a high level. The results show that with an increase in ML-type PC formation a decrease in the recovery of PC was observed (Figure 8). An explanation for the loss of product is the formation of byproducts with low

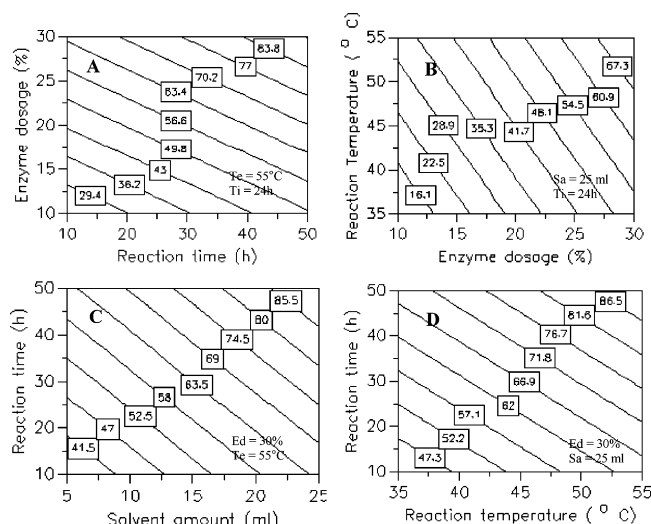


Figure 7. Contour plots of the ML-type PC content during Lipzyme TL IM-catalyzed acidolysis reaction between soybean PC and caprylic acid. The numbers inside the contour plots indicate the ML-type PC content (mol %). Key: (A) enzyme dosage vs solvent amount, (B) enzyme dosage vs reaction temperature, (C) solvent amount vs reaction time, (D) reaction temperature vs reaction time. For abbreviations see Table 1.

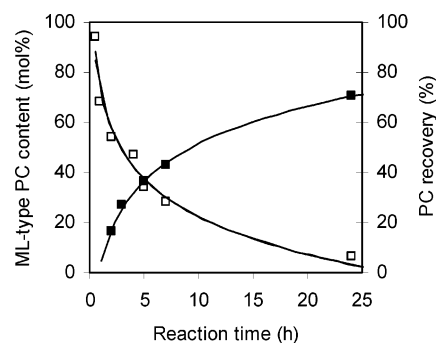


Figure 8. Time course for acidolysis reaction between PC and caprylic acid in hexane. Reaction conditions: enzyme dosage (E_d), 30%; substrate ratio (S_r), 15 mol/mol; solvent amount (S_a), 25 mL; reaction temperature, 55 °C. Key: (■) ML-type PC content (mol %), (□) PC recovery (%).

solubility in hexane, which are lost during removal of the enzyme. This was confirmed by extraction of the immobilized enzyme after the reaction with methanol–chloroform (50:50, v/v) and further analysis, which revealed that large amounts of GPC (totally deacylated PC) were produced. With all parameters on high levels during the acidolysis reaction, MM-type PC was not observed probably due to the rapid hydrolysis to GPC. According to the model having a reaction time of 48 h with other parameters on a high level, 90% of the PC would be of the ML type. However, with these conditions no PC could be observed in the reaction mixture. With a reaction time of 24 h the ML-type content should be 74% according to the generated model, which agrees well with the experimental value. The overall yield was however very low.

Optimal conditions for incorporation of novel fatty acids should be compromised with the consideration of recovery. Readers should thus make their own decisions concerning whether to have a high purity of ML-type PC or a compromise between the ML-type PC purity and recovery of PC.

In conclusion, the TLC-FID method developed has been shown to be suitable for analysis of enzymatic reactions for synthesis of structured phospholipids with mixtures of long- and

medium-chain fatty acids, since it is possible to follow the formation of both products and byproducts. The method was successfully used for the evaluation of reaction conditions assisted by RSM experimental design. The response model developed in this study satisfactorily expressed the formation of ML-type PC with regard to the selected parameters in the batch system. MM-type PC, an undesirable byproduct, is also formed during the lipase-catalyzed acidolysis reaction, due to acyl migration as seen from the developed method.

ABBREVIATIONS USED

FID, flame ionization detector; GC, gas chromatography; GPC, glycerophosphorylcholine; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; RSM, response surface methodology; TLC, thin-layer chromatography.

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PAPER III

Title: Parameters affecting incorporation and by-product formation during the production of structured phospholipids by lipase-catalyzed acidolysis in solvent-free system

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Parameters affecting incorporation and by-product formation during the production of structured phospholipids by lipase-catalyzed acidolysis in solvent-free system

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Abstract

By-product formation is a serious problem in the lipase-catalyzed acyl exchange of phospholipids (PL). By-products are formed due to parallel hydrolysis reactions and acyl migration in the reaction system. A clear elucidation of these side reactions is important for practical operation in order to minimize by-products during reaction. In the present study we examined the lipozyme RM IM-catalyzed acidolysis for the production of structured phospholipids between phosphatidylcholine (PC) and caprylic acid in the solvent-free system. A five-factor response surface design was used to evaluate the influence of major factors and their relationships on a number of responses reflecting the turnover of main reactions as well as side reactions. The five factors, including enzyme dosage, reaction time, reaction temperature, substrate ratio (mol/mol caprylic acid/PC) and water addition, were varied at three levels with two star points. All parameters besides water addition had an effect on the incorporation of caprylic acid into PC and lysophosphatidylcholine (LPC). Reaction time and enzyme dosage showed increased effect on incorporation into PC, while substrate ratio and reaction temperature showed opposite effect. The PC content decreased with increase of all parameters except for substrate ratio. Optimal conditions are recommended as enzyme dosage 40%, reaction temperature 55 °C, water addition 1%, reaction time 70 h, and substrate ratio 6 mol/mol caprylic acid/PC. Under these conditions an incorporation of 46% with PC accounting for 53% of the PL fraction can be obtained. Regiospecific analysis of the product revealed that the caprylic acid was mainly incorporated into the sn-1 position accounting for 80% of the fatty acids incorporated.

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Keywords: Acidolysis; Solvent-free system; Lipase; Response surface methodology; Structured phospholipids

1. Introduction

Applications of structured phospholipids (PLs) in food, pharmaceuticals and cosmetics have increased interest in lipase-catalyzed interesterification for production of such compounds. Several attempts have been made over the last two decades for the enzymatic acyl exchange of phospholipids, however in general the yield for these reactions has been low [1–3]. Lipase-catalyzed interesterification (acidolysis) is a two-step reaction involving hydrolysis and esterification. Lysophosphatidylcholine (LPC) produced in the first step is reactant in the second step. The amount of LPC

in the reaction mixture therefore affects the overall reaction rate. However, LPC also causes acyl migration or the formation of by-products, and as a consequence the formation of LPC decreases the yield and purity of the structured PLs. Acyl migration, a non-enzymatic reaction, is a problem often encountered in selective synthesis of region-specific glycerol-PLs, i.e. intramolecular transfer of one fatty acid moiety from one hydroxyl group to the adjacent one [4]. The intermediate 2-acyl-LPC during the lipase-catalyzed interesterification is less stable than 1-acyl-LPC, and is easily converted into the more stable 1-acyl-LPC by acyl migration, which can be further hydrolyzed by the lipase producing glycerophosphorylcholine (GPC) [5,6]. GPC can then be reacylated with the novel fatty acid in the sn-1 position. If the acyl group migrates from the sn-1 to the sn-2 position the

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lipase has the possibility to incorporate yet another new fatty acid into the sn-1 position, which would give rise to PC with novel fatty acids on both positions. In several studies the recovery of the phospholipid after the reaction have been measured without consideration of the distribution of by-products formed [7,8]. The formation of GPC and the LPC containing the novel fatty acids is a direct consequence of acyl migration and should be minimized in the reaction system in order to have high product yield and high product purity.

For the lipase-catalyzed production of structured PLs, the use of solvents would increase the capital investment when the process is scaled up. Preferably the reaction should be conducted in solvent-free systems. So far only few studies have looked into using solvent-free systems for the production of structured PLs [1,5,9]. Usually the reactions have been conducted with the assistance of organic solvents such as hexane or toluene [10–12]. However it has been reported that lipase-catalyzed acidolysis is faster in solvent-free systems compared to solvent systems [5].

Reaction conditions should be selected with care during lipase-catalyzed acyl exchange of PLs as demonstrated from previous studies [13]. Several parameters important for the main reaction also affect by-product formation resulting in lower recoveries. Usually there is a tendency of decrease in yields with increase in acyl incorporation for these types of reactions. With increasing lipase amount and reaction time the incorporation as well as the hydrolysis increases during lipozyme RM IM-catalyzed acidolysis reaction in solvent-free systems [1,5]. Water has been reported to have a complex role during acidolysis reaction. Varying the water content showed that 5% water addition to the enzyme resulted in the highest incorporation of novel fatty acids; however this water addition also resulted in the highest degree of hydrolysis [5]. Others have reported that water addition had no influence on the incorporation of novel fatty acids during lipase-catalyzed acidolysis reactions [9]. Substrate ratio and reaction temperature are other important parameters during the reactions [9,14]. It was claimed that too high temperature resulted in deactivation of the enzyme, and too high substrate ratios caused substrate inhibition in solvent-free systems [9].

In this study, the incorporation of caprylic acid into PC and LPC together with the PL distribution was determined for the lipase-catalyzed acidolysis reaction between soybean PC and caprylic acid using a solvent-free system with lipozyme RM IM as catalyst. The parameters examined for their effects on incorporation and PL distribution were enzyme dosage, reaction time, reaction temperature, water addition, and substrate ratio (mol/mol caprylic acid/PC). Response surface methodology (RSM) was used to minimize the numbers of experiments. The objective of the study is to optimize a practical reaction system for lipase catalyzed acyl exchange of PC using the solvent-free system, and to have a clear elucidation of the by-product formation.

2. Materials and methods

2.1. Materials

Soybean PC (Epikuron 200, purity 93%) was obtained from Degussa Texturant Systems Deutschland GmbH & Co. KG (Hamburg, Germany). The fatty acid composition (mol%) of PC can be seen in Table 1. Caprylic acid (C8:0, purity 97%) was purchased from Riedel-de-Haen (Seelze, Germany). Lipozyme RM IM, an immobilized sn-1,3 specific lipase from *Rhizomucor miehei* and *Lecitase Novo* (phospholipase A₁), was donated by Novozymes A/S (Bagsvaerd, Denmark). *Crotalus adamanteus* snake venom (phospholipase A₂) was purchased from Sigma (St. Louis, MO). All solvents and chemicals used were of analytical grade.

2.2. Acidolysis reaction

Reactions between soybean PC and caprylic acid were carried out using a 10 g reaction mixture in a brown flask with tight screw cap. Reactions were conducted in a water bath with magnetic stirring at 300 rpm and reaction was started by the addition of lipase (wt.% based on total substrates). After reaction the samples were centrifuged at 4000 rpm for 5 min, and the supernatants were collected. All samples were stored at –20 °C prior to analysis.

Table 1
Fatty acid distribution in PC and structured PC (mol%)

Fatty acids	Soybean PC				Structured PC ^c			
	Direct analysis	sn-1 position (mol%) ^a	sn-2 position (mol%) ^b	Total calcd from sn-1 and sn-2	Direct analysis	sn-1 position (mol%) ^a	sn-2 position (mol%) ^b	Total calcd from sn-1 and sn-2
8:0	0.0	0.0	0.0	0	46.3	71.9	18.0	44.9
16:0	12.8	24.4	1.5	12.9	3.4	5.6	2.5	4.1
18:0	3.9	6.7	0.5	3.6	0.8	1.6	0.7	1.2
18:1	9.4	8.6	13.1	10.8	6.3	3.1	10.3	6.7
18:2	65.8	53.0	77.8	65.4	39.0	15.7	62.1	38.9
18:3	8.1	7.3	7.1	7.2	4.2	2.0	6.4	4.2

^a The fatty acid composition (mol%) at the sn-1 position after enzymatic hydrolysis with snake venom.

^b The fatty acid composition (mol%) at the sn-2 position after enzymatic hydrolysis with *Lecitase Novo*.

^c Structured PC produced under optimal conditions (for details see exp 12 in Table 2).

2.3. Plate thin-layer chromatography (TLC)

Analytical separations were performed on Silica Gel 60 thin-layer plates (20 cm × 20 cm, Merck, Darmstadt, Germany). After development in chloroform–methanol–water (65:35:5, v/v), the plate was sprayed with 0.2% of 2,7-dichlorofluorescein in ethanol (96%), making the lipid bands visible under UV-light. The lipid bands were scraped off, and methylated for analysis by GC.

2.4. Methylation of phospholipid species

The scrapings from TLC were transferred to test tubes with tight screw caps. One milliliter 0.5 M NaOH in methanol were added to each tube and placed at 80 °C for 5 min. Then 1 ml 20% BF₃ in methanol and 0.5 ml 0.5% hydroquinone in methanol were added and placed at 80 °C for 2 min. Two milliliters of 0.73% NaCl solution was added and subsequently 1 ml heptane. The upper phase was transferred to a new tube. One milliliter of saturated salt solutions was added to the new tube. After mixing and phase separation the upper phase was taken for GC analysis.

2.5. GC analysis

The methyl esters were analyzed on a HP6890 series gas–liquid chromatograph (Hewlett-Packard, Waldbronn, Germany) equipped with a flame-ionization detector (FID), as described elsewhere [9].

2.6. Phospholipase hydrolysis of phospholipids

Caprylic acid-enriched PC was isolated on the TLC plates as described above, extracted with Chloroform-Methanol-water (20:10:0.5, v/v), and dried in a rotary evaporator. Water was removed by adding acetone during evaporation. Isolated PC was hydrolyzed to LPC with Lecitase Novo to remove the fatty acids at the sn-1 or with *Crotalus adamanteus* snake venom for the sn-2 position. A 2.5 mg portion of PC was dissolved in diethyl ether (2 ml) and incubated with 10 μl Lecitase Novo dissolved in 0.1 ml of water or 2.5 mg snake venom dissolved in 0.1 ml 10 mM Tris buffer (pH 8.0) containing 10 mM CaCl₂. After shaking vigorously for 5 min, the mixtures were washed into conical flasks with methanol (10 ml) and chloroform (20 ml), and the solution was dried over anhydrous sodium sulfate. The mixture was filtrated, dried and applied to TLC plates. The solvent system used to separate LPC from the other constituents was the same as described above.

2.7. Analysis of phospholipid profile by TLC–FID

One microliter of diluted sample were spotted to Chromarod SIII (Iatron Laboratories Inc.; Tokyo, Japan) and developed in a mixture of chloroform–methanol–water (45:20:2, v/v/v). After the development, chromarods were

dried at 120 °C for 5 min, and PL species (PC, LPC and GPC) were analyzed by TLC coupled to a flame-ionization detector (TLC–FID) (Iatroskan MK6s, Iatron Laboratories; Tokyo, Japan). Flow rates of 200 ml/min for air and 160 ml/min for hydrogen were used during analysis. Peaks were identified by external standards. From GC-analysis the average molecular weight of PC and LPC were calculated in order to recalculate the TLC–FID data into molar distribution.

2.8. Viscosity measurements

Viscosity was carried out using a concentric cylinder bob cup CC25 measuring system by Stresstech rheometer (Version 3.8, Reologica Instruments AB, Sweden). A constant temperature of 50 °C was maintained during the measurements with a circulatory water bath. Shear stress was increased progressively from 0.5 up to 300 Pa in 20 logarithmic steps with continuous upward sweep direction. The viscosity was determined as the slope of shear stress versus shear rate curve.

2.9. Experimental design and statistical analysis

Experiments were conducted using a central composite design to investigate the linear, quadratic, and cross-product effects of five factors, each varied at five levels and also includes three center points for replication. The five factors chosen were enzyme dosage (E_d , wt.% based on substrate), reaction temperature (T_e , °C), water addition (W_a , wt.% based on total substrate), reaction time (T_i , h) and substrate ratio (S_r , mol/mol caprylic acid/PC). The design of the experiments employed is presented in Table 2. A software package (Modde 6.0, Umetri, Umeå, Sweden) was used to fit the second-order model to the independent variables. Where it was possible, the model was simplified by dropping terms which were not statistically significant ($P > 0.05$) by analysis of variance. The coefficient of determination (R^2) and the lack-of-fit test were used to determine whether the constructed model was adequate to describe the observed data. For process factors the main effect plot displays the predicted changes in the responses when factor varies from low to its high level, all other factors in the design being on their average.

3. Results and discussions

3.1. Model fitting

It has previously been demonstrated that LPC containing the fatty acids to be incorporated into PC was observed in the products during lipase catalyzed acidolysis reactions [5,16]. This is related to the acyl migration in the system. Therefore the amount of such LPC will indirectly indicate the extent of acyl migration. In the present study RSM was used to evaluate the effects of enzyme dosage, reaction temperature, water

Table 2
Actual experimental settings of the factors and the responses

Exp number	Factors					Incorporation of caprylic acid (mol%)			PL distribution (mol%)		
	E_d	T_e	W_a	T_i	S_r	PC	LPC	PC	LPC	GPC	
1	20	45	1	30	12	12.6	28.7	76.7	11.9	11.4	
2	40	45	1	30	6	31.2	46.5	69.5	18.5	12.0	
3	20	55	1	30	6	28.2	50.4	67.4	21.5	11.1	
4	40	55	1	30	12	27.1	61.9	66.8	18.9	14.3	
5	20	45	3	30	6	36.6	34.5	57.0	26.6	16.4	
6	40	45	3	30	12	23.9	48.4	59.6	22.8	17.5	
7	20	55	3	30	12	8.5	47.3	65.9	17.9	16.3	
8	40	55	3	30	6	33.0	65.9	43.0	32.3	24.7	
9	20	45	1	70	6	30.6	54.8	65.2	24.8	10.0	
10	40	45	1	70	12	38.2	66.1	58.2	21.8	20.1	
11	20	55	1	70	12	15.8	60.3	71.4	16.4	12.2	
12	40	55	1	70	6	46.3	66.2	53.2	29.9	16.9	
13	20	45	3	70	12	22.3	67.7	49.8	27.0	23.2	
14	40	45	3	70	6	19.5	47.0	47.0	24.8	28.2	
15	20	55	3	70	6	25.4	64.3	42.9	28.1	29.0	
16	40	55	3	70	12	27.6	75.9	41.6	25.6	32.8	
17	10	50	2	50	9	16.7	39.9	69.1	16.9	14.1	
18	50	50	2	50	9	32.6	60.6	54.5	20.7	24.8	
19	30	40	2	50	9	35.3	53.1	57.6	23.5	18.9	
20	30	60	2	50	9	19.7	78.2	40.3	22.6	37.2	
21	30	50	0	50	9	28.9	59.1	80.6	10.9	8.5	
22	30	50	4	50	9	33.2	58.8	37.0	24.3	38.7	
23	30	50	2	10	9	10.7	24.4	74.2	15.1	10.7	
24	30	50	2	90	9	34.3	74.2	41.5	31.8	26.7	
25	30	50	2	50	3	31.1	46.0	49.3	31.4	19.4	
26	30	50	2	50	15	22.7	68.0	54.4	18.9	26.7	
27	30	50	2	50	9	30.5	58.0	52.6	27.2	20.1	
28	30	50	2	50	9	28.5	63.2	53.4	24.9	21.7	
29	30	50	2	50	9	31.7	64.4	57.3	25.5	17.2	

Abbreviations: E_d , enzyme dosage (wt.% based on substrate); T_e , reaction temperature ($^{\circ}\text{C}$); W_a , water addition (wt.% based on total substrate); T_i , reaction time (h); S_r , substrate ratio (mol/mol caprylic acid/PC).

content, molar ratio of reactants, and reaction time on incorporation of caprylic acid into PC as well as the existence of caprylic acid in LPC. Additionally the PL species distribution was examined in order to understand how the parameters

influence on the product recovery or by-product formation. The best-fitting quadric models by multiple regression and backward elimination were determined. The observed and predicted values were sufficiently correlated except for no.

Table 3
Regression coefficients and significance (P) values of the second-order polynomials after backward elimination

Term	Incorporation of caprylic acid (mol%)				PL distribution (mol%)						
	PC		LPC		PC		LPC		GPC		
	Regression coefficient	P	Regression coefficient	P	Regression coefficient	P	Regression coefficient	P	Regression coefficient	P	
Constant	28.06	2.31×10^{-11}	61.2	2.45×10^{-14}	52.51	8.85×10^{-14}	25.07	6.72×10^{-14}	22.42	9.19×10^{-9}	
E_d	5.51	9.47×10^{-6}	5.88	5.74×10^{-5}	-4.20	7.06×10^{-4}	1.72	1.94×10^{-3}	2.48	0.03	
T_e	-2.84	3.2×10^{-3}	4.96	2.75×10^{-4}	-2.12	0.04	-0.11	0.80	2.24	0.05	
W_a	0.37	0.64	1.89	0.08	-9.32	2.35×10^{-7}	3.40	3.67×10^{-6}	5.91	6.20×10^{-5}	
T_i	4.39	8.99×10^{-5}	10.33	1.31×10^{-7}	-6.53	1.18×10^{-5}	3.11	9.45×10^{-6}	3.41	5.27×10^{-3}	
S_r	-2.41	9.2×10^{-3}	4.19	1.11×10^{-3}	1.69	0.10	-2.32	1.66×10^{-4}	0.64	0.54	
$E_d \times E_d$	-1.25	0.10	-3.03	5.19×10^{-3}	2.64	8.71×10^{-3}	-1.37	4.45×10^{-3}	-1.27	0.19	
$W_a \times W_a$	0.35	0.63	-0.85	0.37	1.90	0.05	-1.67	1.12×10^{-3}	-0.23	0.81	
$T_i \times T_i$	-1.80	0.03	-3.26	3.18×10^{-3}	1.65	0.08	-0.21	0.62	-1.45	0.14	
$E_d \times T_e$	4.95	2.4×10^{-4}	3.45	0.02	-2.69	0.04	2.41	8.34×10^{-4}	0.28	0.83	
$E_d \times W_a$	-4.87	2.8×10^{-4}	-3.32	0.02	1.44	0.25	-1.36	0.03	-0.08	0.95	
$E_d \times T_i$	-1.59	0.13	-5.23	1.16×10^{-3}	0.82	0.50	-1.39	0.03	0.57	0.66	
$T_e \times W_a$	1.32	0.21	2.68	0.05	-1.49	0.24	0.39	0.50	1.10	0.41	
$T_e \times T_i$	2.86	0.01	-0.38	0.77	-0.37	0.76	0.27	0.64	0.10	0.94	
$W_a \times T_i$	-4.55	4.9×10^{-4}	-1.94	0.15	0.16	0.90	-1.84	5.93×10^{-3}	1.67	0.21	

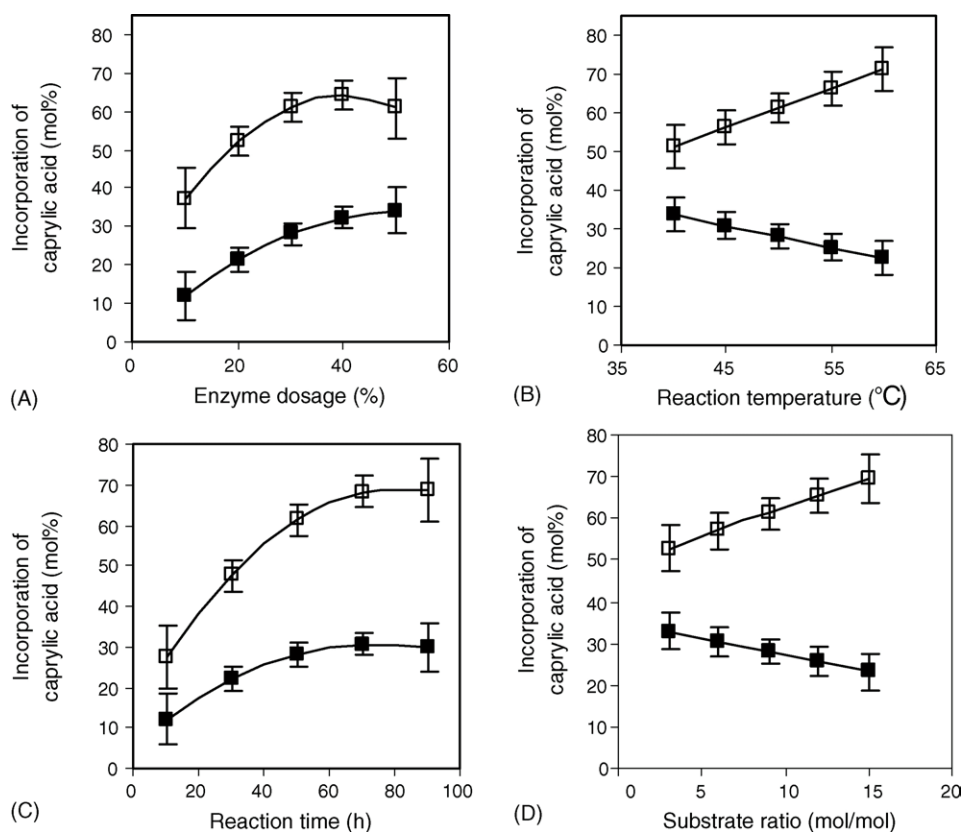


Fig. 1. Main effects of parameters on the incorporation of caprylic acid catalyzed by lipozyme RM IM in solvent-free system (■) PC and (□) LPC. (A) Enzyme dosage, (B) reaction temperature, (C) reaction time, and (D) substrate ratio.

3, which was treated as an outlier. The statistics for the model coefficients and probability (P) values for the response variables were calculated (Table 3). The coefficients of determination (R^2) of the models were 0.92, 0.94, 0.93, 0.94, and 0.83 for the five responses, i.e. caprylic acid incorporation into PC, caprylic acid existence in LPC, PC content, LPC content and GPC content, respectively. According to the analysis of variance there was no lack of fit for all the models.

3.2. Main effects of parameters on incorporation

Plots of main effects can be used to evaluate the major influence of parameters (Figs. 1 and 2). All parameters showed to have an effect on either the incorporation of caprylic acid or the PL distribution. In order to have a practical operation system, some compromises have to be made for the different parameters since some of them not only have a beneficial effect on the incorporation into PC, but also lead to lower yields.

3.2.1. Enzyme dosage

Enzyme dosage had the most significant effect on the incorporation into PC. Incorporation into PC increased for increasing enzyme dosage (Fig. 1A). It has been reported that high enzyme dosages are needed for effective incorporation of novel fatty acids into PLs by acidolysis in solvent-free

system [1,5]. The use of high enzyme loads however gives problems with agitation and decrease the mass transfer. Even though the increased enzyme load has beneficial effect on the incorporation into PC it also results in increased existence of caprylic acid in LPC. A compromise has to be made since increased enzyme concentrations not only favour incorporation into PC, but LPC as well. With increasing enzyme dosage the content of PC decreased whereas the content of LPC and GPC increased (Fig. 2A). Only few lipases are commercially available in the immobilized form. Lipozyme RM IM is the most commonly used enzyme for the lipase-catalyzed production of structured PLs [1,5,7,10]. Lipozyme RM IM uses anion exchange resin as lipase carrier. This type of carrier can catalyze acyl migration in the reaction system [16]. With the lipase from *Rhizopus oryzae* immobilized on polypropylene support no incorporation of acyl donor into LPC was observed [16]. It seems that acyl migration could be affected by enzyme carriers under the issue of enzyme dosage.

3.2.2. Reaction temperature

The effect of the temperature in solvent-free systems has received very little attention. Commonly the temperature has been kept at 60 °C in order to decrease viscosity of the reaction mixture [1,5]. Previous study performed at our lab has shown that the incorporation of caprylic acid into soybean lecithin using lipozyme TL IM, a silica granulated

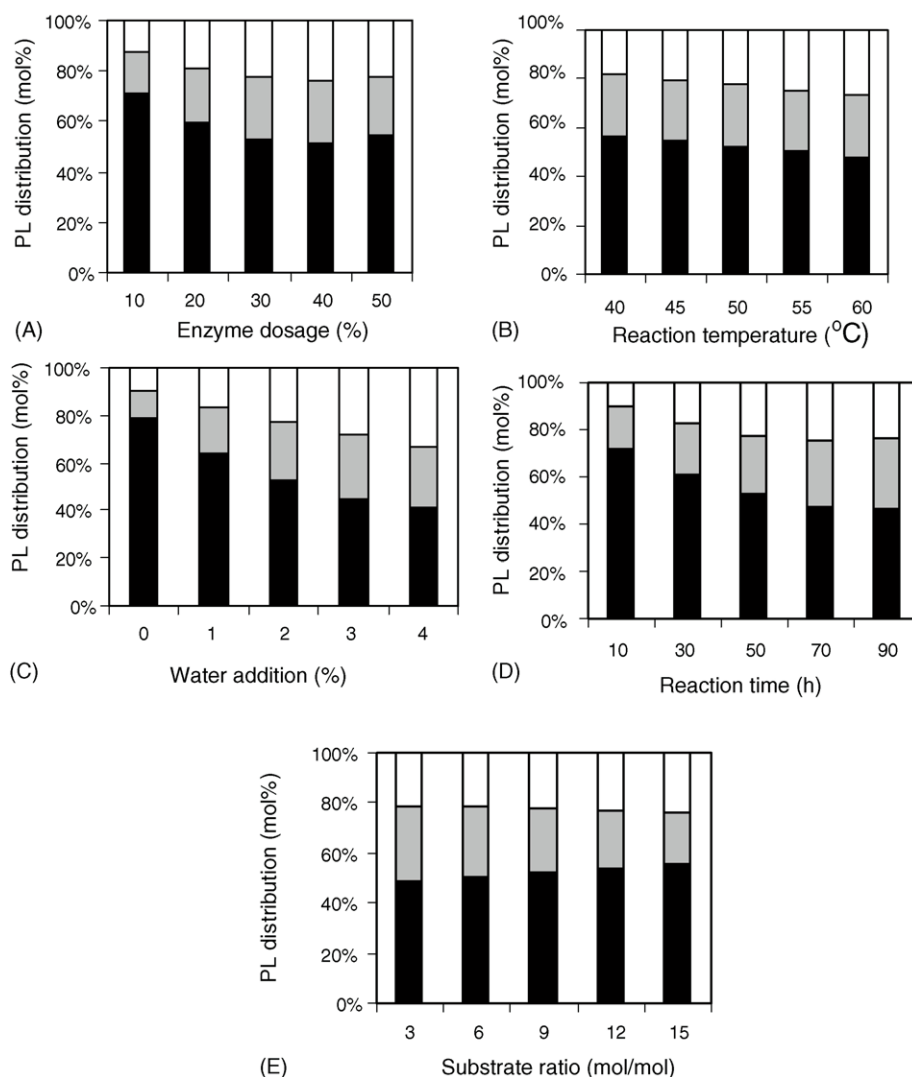


Fig. 2. Main effects of parameters on PL distribution during lipzyme RM IM catalyzed acidolysis reaction between PC and caprylic acid (■) PC, (▒) LPC, and (□) GPC. (A) Enzyme dosage, (B) reaction temperature, (C) water addition, (D) reaction time, and (E) substrate ratio.

Thermomyces lanuginosa lipase, as catalyst had maximum performance at 57 °C [9]. From synthetic reaction using phospholipase A₂ (PLA₂) as catalyst it is known that elevated temperatures resulted in increased acyl migration and by-product formation [15]. It was reported that acyl migration was not observed at 25 °C. In this study it was observed that higher temperature individually decreased the PC content and incorporation of caprylic acid into PC (Figs. 1B and 2B). Reaction temperature did not influence the formation of LPC; however it had significant effect on the formation of GPC. In addition with the increase in temperature the incorporation of caprylic acid into LPC also increased. It is therefore best to apply temperatures at the low levels.

3.2.3. Water content

In this study the water content had no significant influence on the incorporation into PC and LPC. Of the parameters studied, water addition however had the most significant effect on

formation of LPC and GPC (Fig. 2C). Increased water addition resulted in lower PC content and corresponding increase in LPC and GPC formation. It seems that excess water may act exclusively as a nucleophilic substrate for the hydrolysis rather than the esterification of desired fatty acids. Therefore water content is crucial for the optimization of the acidolysis reaction in terms of yield. Others have reported that the water content had significant influence on both incorporation and the yield. Haraldsson and Thorarensen reported that 5% water addition resulted in the highest incorporation into both PC and LPC; both also gave the highest degree of hydrolysis [5]. Aura et al. reported that the minimal water content of the reaction mixture for incorporation of novel fatty acids into soybean PL by lipzyme RM IM was below 0.5% (w/w) based on substrate [1]. The incorporation and degree of hydrolysis was not greatly influenced by the amount of water in the range 0.5–2.5%. Similar for the lipzyme TL IM-catalyzed acidolysis, the incorporation was not influenced by addition of

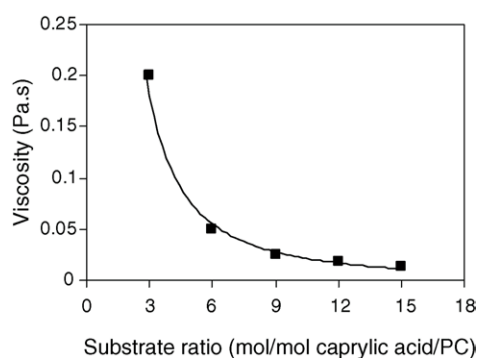


Fig. 3. Viscosity of the initial reaction mixture at different substrate ratios (mol/mol caprylic acid/PC). Two percent of water was added to substrate material. Measurements were conducted at 50 °C without enzyme addition.

1–5% water based on enzyme (0.2–1% based on total substrate) [9]. In reaction mixture with toluene as solvent it was observed that increased water activity increased hydrolysis reaction rate to a greater extent compared to the synthesis reaction rate. Water seems to have a complex role in terms of compromising the lipase activity, hydrolysis side reactions, reaction rate, and extent of incorporation. In order to have a high productivity it is however recommended that the water content should be low.

3.2.4. Reaction time

Usually there is an increase in incorporation of new fatty acids into both PC and LPC during progress in reaction time [5]. Increasing reaction time also results in higher degree of hydrolysis. Similar results were obtained in this study. Reaction time was the parameter having most significant effect on the incorporation of caprylic acid into LPC (Fig. 1C). The formation of LPC was higher compared to that of GPC with increasing reaction time (Fig. 2D). A compromise is also needed for the reaction time since it has positive effect on the incorporation of caprylic acid into PC, however it also results in higher by-product formation.

3.2.5. Substrate ratio

Increasing fatty acid concentration increased yield both for esterification and transesterification reactions [6]. Reaction rates for esterification reactions were independent of the fatty acid concentration. However, during transesterification, the reaction rates increased with increasing fatty acid concentration. Decreased reaction rates were thought to be caused by increased polarity of the reaction medium upon addition of fatty acids. Decreased reaction rates have also been reported during the PLA₂-catalyzed esterification reactions with increasing amounts of fatty acids, and were speculated to be caused by changes in polarity or viscosity [15].

In order to determine if the viscosity had any relationship with reaction rate the viscosity of the initial substrate materials at different substrate ratios were measured (Fig. 3). It was observed that the viscosity decreased with increasing substrate ratio. With higher substrate ratios the mass

transfer would expect to increase due to the decrease in viscosity and thus resulting in higher reaction rates. However it was observed that the incorporation into PC decreased with increasing substrate ratio (Fig. 1D), and therefore mass transfer limitations do not seem to be the explanation for the decrease in reaction rate. The incorporation of caprylic acid increased for LPC with increasing substrate ratio, which illustrates that acyl migration probably increases with increasing substrate ratio.

In theory the product yield under reaction equilibrium during acidolysis is determined by the substrate ratio. The maximum incorporation of acyl donors can be calculated at certain substrate ratios assuming no by-product formation and acyl migration. The equation is given below:

$$\ln c_{\max} (\text{mol}\%) = 50 \frac{S_r}{S_r + 1} \quad (1)$$

Theoretical maximum of new fatty acids to be incorporated into PC is expected to reach 50% for the sn-1,3 specific lipase. Theoretically having substrate 3–15 mol/mol will result in conversion of 75–94% (incorporation of 38–47 mol% based on total PL). Higher substrate ratios will in theory result in higher incorporation of acyl donors. The LPC content in the reaction system generally decreased with increasing substrate ratio, whereas GPC was not affected (Fig. 2E). A compromise therefore has to be made concerning the substrate ratio, even though incorporation of novel fatty acids decreases with increasing substrate ratio, the yield increases.

3.3. Optimization of reaction system

The most important responses for the optimization of the process are the incorporation into PC and PC content. Optimization with these two related responses and five variables cannot be calculated mathematically as more than one optimum condition may exist. Possible optimum conditions, however, can be iteratively calculated in the set ranges and target responses of incorporation into PC and PC content (mol%). The best way to evaluate the relationships between responses and parameters and interactions that exist herein is to analyze the contour plots (Fig. 4). The tendency being the same for parameters as those discussed above. The optimal conditions were generated by the optimizer function of the software with interactive calculation within the low and high level of parameters studied (star points not included). The general conditions for optimal production were enzyme dosage 40%, reaction temperature 55 °C, water addition 1%, reaction time 70 h, and substrate ratio 6 mol/mol. Under these conditions, an incorporation of caprylic acid into PC up to 49% with PC accounting for 51% of the PL fraction can be obtained from the prediction. From Table 2 it can be observed that experiment no. 12 has the settings that are predicted to be the optimal conditions. Regiospecific analysis was performed on this sample (see Table 1). As could be observed most of the caprylic acid was found on the sn-1 position, accounting for 80% of the fatty acid incorporated. Due to acyl migration

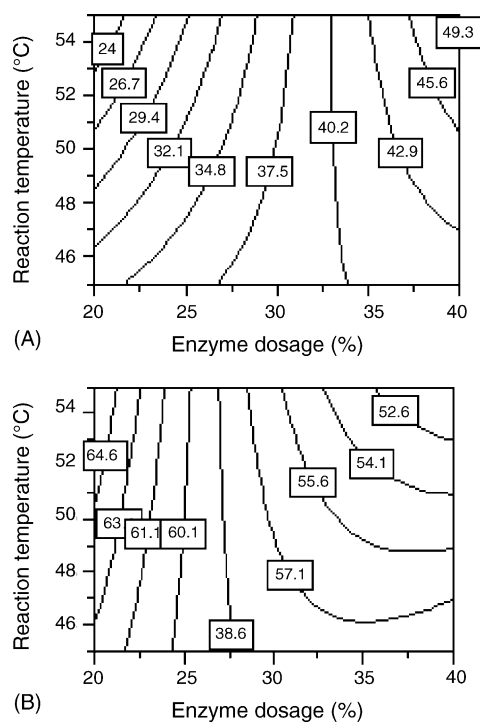


Fig. 4. Contour plots between enzyme dosage and reaction temperature for (A) incorporation of caprylic acid into PC and (B) PC content. Other conditions were as follows: water addition 1%, substrate ratio 6 mol/mol and reaction time 70 h.

caprylic acid could also be observed in the sn-2 position as well.

4. Conclusion

The quadric response models satisfactorily expressed the incorporation of caprylic acid and PL distribution in lipase-catalyzed acidolysis between PC and caprylic acid regarding enzyme dosage, reaction time, reaction temperature, substrate ratio and water content in the batch reactor. Several compromises have to be made in order to have high product yield since many of the parameters favouring acyl incorporation also results in increased hydrolysis and acyl migration in the reaction system. Increased temperature and substrate ratio decreased incorporation into PC, but increased the incorporation into LPC. Increasing all other parameters except

for water, however, increased incorporation into both PC and LPC. With an increase of parameters there was seen a decrease in PC content, except for substrate ratio with no individual effect. According to the optimization, it is possible to obtain 49% incorporation of caprylic acid into PC with PC accounting for 51 mol% of the PL fraction by using 40% enzyme, 70 h reaction time, 55 °C temperature for reaction mixture with substrate ratio of 6 mol/mol caprylic acid/PC in the solvent-free system. Regiospecific analysis of structured PC produced under optimal conditions revealed that caprylic acid was not exclusively incorporated into the sn-1 position. Twenty percent of the caprylic acid incorporated could be found in the sn-2 position.

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PAPER IV

Title: Elucidation of acyl migration during lipase-catalyzed production of structured phospholipids
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Elucidation of Acyl Migration During Lipase-Catalyzed Production of Structured Phospholipids

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ABSTRACT: Elucidation of acyl migration was carried out in the Lipozyme RM IM (*Rhizomucor miehei*)-catalyzed transesterification between soybean phosphatidylcholine (PC) and caprylic acid in solvent-free media. A five-factor response surface design was used to evaluate the influence of five major factors and their relationships. The five factors—enzyme dosage, reaction temperature, water addition, reaction time, and substrate ratio—were varied on three levels together with two star points. Enzyme dosage, reaction temperature, and reaction time showed increased effect on the acyl migration into the *sn*-2 position of PC, whereas increased water addition and substrate ratio had no significant effect in the ranges tested. The best-fitting quadratic response surface model was determined by regression and backward elimination. The coefficient of determination (R^2) was 0.84, which indicates that the fitted quadratic model has acceptable qualities in expressing acyl migration for the enzymatic transesterification. Correlation was observed between acyl donor in the *sn*-2 position of PC and incorporation of acyl donor into the intermediate lysophosphatidylcholine. Furthermore, acyl migration into the *sn*-2 position of PC was confirmed by TLC-FID, as PC with caprylic acid was observed on both positions. Under certain conditions, up to 18% incorporation could be observed in the *sn*-2 position during the lipase-catalyzed transesterification.

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KEY WORDS: Acyl migration, lipase-catalyzed acidolysis reaction, response surface methodology, *Rhizomucor miehei*, solvent-free system, structured phospholipids.

Production of structured phospholipids (PL) by lipase-catalyzed transesterification has attracted increased attention during the last couple of decades, and several publications have recently appeared on the subject (1–4). Unfortunately, many of these publications do not consider the problem of acyl migration, which in a practical reaction system cannot be simply avoided. No comprehensive reports have to our knowledge been published on acyl migration into the *sn*-2 position of PL during the lipase-catalyzed production of structured PL. Most commonly, the overall incorporation of novel FA has been measured, which gives no information about the location in the PL molecule. It is generally agreed that *sn*-1,3-specific lipases are specific for the *sn*-1 position of PL. However, during the transesterification reaction between PC and acyl donor, the formation of glycerophosphorylcholine (GPC) and the presence

of the acyl donor in lysophosphatidylcholine (LPC) are usually observed, which are consequences of migration of the acyl group from the *sn*-2 position to the *sn*-1 position (5). The main reason for acyl migration is the existence of LPC, which is the intermediate produced during the lipase-catalyzed transesterification. The mechanism proposed for the acyl migration suggests that LPC goes through a cyclic ortho ester intermediate (6). Acyl migration from the *sn*-2 position to the *sn*-1 position or vice versa continues until a dynamic balance is reached.

Plückthun and Dennis (6) investigated acyl migration in LPC and reported that about 90% of thermodynamically stable 1-acyl LPC and 10% 2-acyl LPC were present in mixture at equilibrium under the conditions of their investigation. During the lipase-catalyzed acidolysis reaction between PC and EPA under solvent-free conditions, 85% of the FA in LPC were on the *sn*-1 position at equilibrium (5). Similar findings were reported for lipase-catalyzed acidolysis between PC and oleic acid where 90% of the FA were found on the *sn*-1 position in LPC at equilibrium (7).

Several factors possibly influence acyl migration. Increasing solvent polarity or addition of water to nonpolar solvents has been reported to cause lower rates of acyl migration (8). To prevent losses due to acyl migration, the acidolysis reaction should be carried out at high water activity (8). Increased water content in the reaction system, however, influences LPC formation and thus results in lower yields. In many cases excessive amounts of acyl donor have been applied to push the main reaction toward product formation. However, with increased substrate ratios, there would be a higher content of FFA, which have been reported to cause acyl migration on partial acylglycerols (8). Reaction temperature also influences the equilibrium of acyl migration. Higher temperatures decrease the overall incorporation of acyl donor into PC in a solvent-free system, but not into LPC (9). This indicates that the reaction rate for the acidolysis reaction becomes slower than acyl migration rates at elevated reaction temperatures. Certain supports for the immobilization of enzymes may cause increased acyl migration in the reaction system as well (10).

Response surface methodology (RSM) enables the evaluation of effects of multiple parameters, alone or in combination, on response variables. The objective of this study was to examine the relationship between five factors (enzyme dosage, reaction temperature, water addition, reaction time, and substrate ratio) and their effects on acyl migration into the *sn*-2 position of PC during lipase-catalyzed acidolysis. These five factors have been shown previously to have an effect on either the

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overall incorporation of acyl donor or the recovery of PC during acidolysis reaction (9).

MATERIALS AND METHODS

Materials. Soybean PC (Epikuron 200, purity 93%) was obtained from Degussa Texturant Systems Deutschland GmbH & Co. KG (Hamburg, Germany). The FA composition of the PC (mol%) was 16:0 (12.8%), 18:0 (3.9%), 18:1 (9.4%), 18:2 (65.8%), and 18:3 (8.1%). Caprylic acid (purity 97%) was from Riedel-de-Haen (Seelze, Germany). *sn*-1,3-Specific lipase from *Rhizomucor miehei* immobilized on macroporous ion resin (Lipozyme RM IM) and phospholipase A₁ (PLA₁) from *Fusarium oxysporum* (Lecitase Novo) were donated by Novozymes A/S (Bagsværd, Denmark). All solvents and reagents for analyses were of analytical grade.

Experimental design. A three-level with two star points and partial five-factor fractional factorial design according to the principle of RSM was used in this study. The five factors and their levels were enzyme dosage (e_d , 10–50 wt% based on substrate), reaction temperature (t_r , 40–60°C), water addition (w_a , 0–4 wt% based on substrate), reaction time (t_i , 10–90 h), and substrate ratio (s_r , 3–15 mol/mol caprylic acid/PC). The design generated 29 experimental settings as determined by the use of the software Modde 6.0 (Umetri, Umeå, Sweden) (Table 1).

Transesterification. The transesterification (acidolysis) between soybean PC and caprylic acid was carried out in a system previously described (9). PC and caprylic acid (10 g reaction mixture) were mixed in a brown flask with tight screw cap. Reactions were started by the addition of lipase (wt% based on total substrate). Reactions were conducted in a water bath with magnetic stirring at 300 rpm. After reaction, the samples were centrifuged at $2879 \times g$ for 5 min, and the supernatants were collected. All samples were stored at –20°C prior to analysis.

TLC. Analytical separations of PC, LPC, and FFA were performed on Silica Gel 60 thin-layer plates (20 × 20 cm; Merck, Darmstadt, Germany). The solvent system used for the separations consisted of: chloroform/methanol/water (65:35:5, by vol). Lipid bands on TLC plates were visualized by spraying with 0.2% 2,7-dichlorofluorescein in ethanol. Lipid bands were scraped off and methylated for FA analysis.

FA position analysis of PC. Caprylic acid-enriched PC was separated from LPC and FFA on Silica Gel 60 thin-layer plates as described above. PC was extracted from the silica gel with 4 × 10 mL chloroform/methanol/water (65:35:5, by vol). After drying in a rotary evaporator, the PC was hydrolyzed to LPC with Lecitase Novo to remove the FA in the *sn*-1 position. A 2.5 mg portion of PC was dissolved in diethyl ether (2 mL) and incubated with 10 μL Lecitase Novo dissolved in 100 μL water. After shaking vigorously for 5 min, solvent was evaporated under nitrogen. Hydrolyzed PC was redissolved in chloroform and applied to the TLC plate for separation. The LPC band was scraped off and methylated for GC analysis.

Methylation and GC analysis. Methylation and GC analysis were performed on PC, LPC, and the PLA₁-catalyzed hydrolysis product of PC. Methyl esters were prepared by treating

scrapings from TLC with 0.5 M NaOH in methanol, followed by 20% boron trifluoride treatment, and analyzed by HP6890 series GC with FID (Hewlett-Packard) using a fused-silica capillary column (SUPELCO WAX, 60 m × 0.25 mm i.d., 0.20 mm film thickness; Supelco Inc., Bellefonte, PA) as described before (9).

Analysis of phospholipid profile by TLC–FID. Diluted sample (1 μL) was spotted to Chromarod SIII (Iatron Laboratories Inc., Tokyo, Japan) and developed in a mixture of chloroform/methanol/water (42:22:3 by vol). After the development, Chromarods were dried at 120°C for 5 min, and PL species were analyzed by TLC–FID (Iatronscan MK6s; Iatron Laboratories). Flow rates of 2 L/min and 160 mL/min were used during analysis for air and hydrogen, respectively. Peaks were identified by external standards. With TLC–FID, it is possible to monitor the replacement of long (L)-chain FA with medium (M)-chain FA during the lipase-catalyzed acidolysis reaction between soybean PC and caprylic acid as previously described (11). PC can split into three peaks: the LL-type, the ML-type (LM-type), and the MM-type. Overall incorporation of caprylic acid into PC can thus be calculated by Equation 1:

$$\text{Inc (mol\%)} = 0.5 \{[\text{ML}](\text{mol\%})\} + \{[\text{MM}](\text{mol\%})\} \quad [1]$$

Statistical analysis. Data were analyzed by means of RSM with Modde 6.0. Second-order coefficients were generated by regression analysis with backward elimination. Responses were fitted for the factors by multiple regression. The fit of the model was evaluated by the coefficients of determination (R^2) and ANOVA. Insignificant coefficients ($P > 0.05$) were eliminated after examining the coefficients, and the model was finally refined. Linear regression analysis was performed with assistance of Microsoft Office Excel 2003 (Microsoft Corporation, Redmond, WA). All samples were analyzed in duplicate, and mean values are reported.

RESULTS AND DISCUSSION

Phospholipase hydrolysis of PL. Determination of positional distribution of FA in PL is usually done by enzymatic hydrolysis (12). Different enzymes have been suggested in the literature for specific acyl hydrolysis of PC. Phospholipase A₂ from snake venoms and porcine pancreases has been used to hydrolyze the ester bond in the *sn*-2 position of PL, releasing the FA in this position (10,12). The FFA products from the *sn*-2 position and the LPC-containing FA in the *sn*-1 position can be isolated for analysis so the distribution of FA in both positions of the glycerol moiety is determined. Alternatively, enzymes specific for the *sn*-1 position can be used to hydrolyze FA in the *sn*-1 position, and hydrolysis products can be examined in a similar way. Recently Vijeeta *et al.* (13) proposed a method for determining positional distribution of PC by using phospholipase A₁ (Lecitase Novo). Hydrolysis reactions are performed over 6 h in tertiary alcohol. Acyl migration rates are usually lower in alcoholic solutions compared with nonpolar organic solvents (8); however, the reaction time is considered

TABLE 1
Set Factor Levels and Observed Responses in Response Surface Methodology Experiments for Acyl Migration into *sn*-2 Position of PC During Lipase Catalyzed Acidolysis Reactions Between Soybean PC and Caprylic Acid

Exp. no.	Factors ^a					Migration to <i>sn</i> -2 position (%)
	e_d	t_e	w_a	t_r	s_r	
1	20	45	1	30	12	3.8
2	40	45	1	30	6	6.0
3	20	55	1	30	6	3.8
4	40	55	1	30	12	8.2
5	20	45	3	30	6	4.6
6	40	45	3	30	12	4.3
7	20	55	3	30	12	2.0
8	40	55	3	30	6	11.9
9	20	45	1	70	6	14.0
10	40	45	1	70	12	12.5
11	20	55	1	70	12	8.8
12	40	55	1	70	6	18.0
13	20	45	3	70	12	12.2
14	40	45	3	70	6	5.9
15	20	55	3	70	6	9.9
16	40	55	3	70	12	12.9
17	10	50	2	50	9	3.7
18	50	50	2	50	9	9.4
19	30	40	2	50	9	5.1
20	30	60	2	50	9	12.2
21	30	50	0	50	9	12.3
22	30	50	4	50	9	5.9
23	30	50	2	10	9	0.9
24	30	50	2	90	9	14.5
25	30	50	2	50	3	6.0
26	30	50	2	50	15	8.8
27	30	50	2	50	9	13.3
28	30	50	2	50	9	16.1
29	30	50	2	50	9	16.9

^aAbbreviations: e_d , enzyme dosage (wt% based on substrate); t_e , reaction temperature (°C); w_a , water addition (wt% based on total substrate); t_r , reaction time (h); s_r , substrate ratio (mol/mol caprylic acid/PC).

very long from a practical point of view. The 2-acyl LPC formed is a thermodynamically unstable molecule that, over time, will convert to the more stable 1-acyl LPC, which can be further hydrolyzed to GPC by the lipase. We previously examined the regioselectivity of the Lipozyme RM IM-catalyzed incorporation of caprylic acid into PC (9). Structured PC was hydrolyzed with PLA₁ and snake venom to verify the FA composition in the *sn*-1 and *sn*-2 positions, respectively. The accuracy of the hydrolysis procedures were checked by summing the results for the concentration of each FA in *sn*-1 and *sn*-2 positions, dividing by two, and comparing this quantity with the analysis for each component in the original PC. These two results agreed well, showing that snake venom and PLA₁ are suitable for determining the positional distribution of structured PC containing a mixture of long- and medium-chain FA.

Acyl migration into the sn-2 position of PC. The effect of different parameters on the overall incorporation and distribution of PL has been examined during the lipase-catalyzed acidolysis reaction (9). Several compromises have to be made in order to have high product yield since parameters favoring acyl incorporation also result in increased by-product formation in

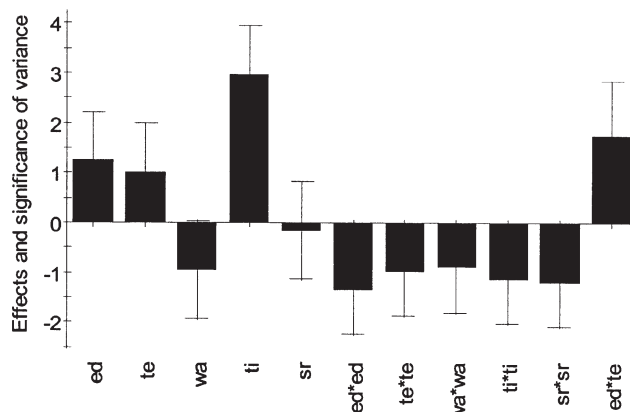


FIG. 1. Effect and significance plot of parameters and interactions on acyl migration into *sn*-2 position of PC during lipase-catalyzed acidolysis reaction between PC and caprylic acid. Abbreviations: e_d , enzyme dosage (wt% based on substrate); t_e , reaction temperature (°C); w_a , water addition (wt% based on total substrate); t_r , reaction time (h); s_r , substrate ratio (mol/mol caprylic acid/PC).

the reaction system. Under optimal conditions (based on overall incorporation), 20% of the FA were found in the *sn*-2 position. Owing to the complexity of the acidolysis reaction it is difficult to predict the influence of different parameters on the acyl migration into the *sn*-2 position of PC. A statistical experimental design was therefore set up with the assistance of RSM to evaluate the influence of the individual parameters mentioned above, as well as their interactions, on acyl migration into the *sn*-2 position. The practical experimental settings are given in Table 1 including responses from the experiments. The structured PC produced were hydrolyzed with PLA₁ so as to have a direct measure of the migration into the *sn*-2 position.

Partial least-squares regression was used to fit the responses. Insignificant variables were refined in steps of backward elimination. The coefficient of determination was 0.84 for acyl migration into the *sn*-2 position of PC, and according to the ANOVA there was no lack of fit. The effect of each parameter can be seen from the plot of the main effects (Fig. 1). The

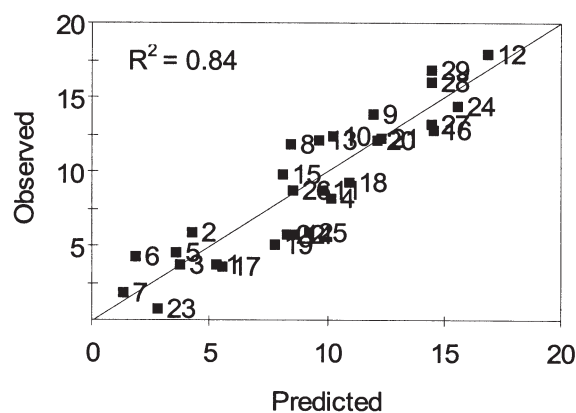


FIG. 2. Linear relationship between observed responses and those predicted for acyl migration into *sn*-2 position of PC. Numbers in figure are experimental setting number.

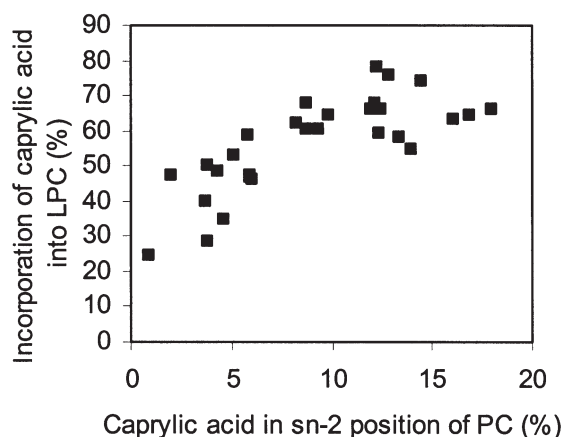


FIG. 3. Correlation between the caprylic acid in the *sn*-2 position of PC and the incorporation of caprylic acid into lysophosphatidylcholine (LPC).

model for the migration to the *sn*-2 position was generally satisfactory for the evaluation of such a system, as the observed and predicted results were well correlated (Fig. 2). According to regression analysis the relationship between observed and predicted results was significant ($P < 0.01$).

The reaction time was the most significant factor in the acyl migration into the *sn*-2 position of PC. Increased reaction time also resulted in an overall higher incorporation, and acyl migration therefore seems difficult to avoid in the present reaction system. Other parameters having an effect on the acyl migration were enzyme dosage and reaction temperature. Water addition and substrate ratio had no individual effect on the acyl migration. The acyl migration rate of LPC has previously been shown to decrease in toluene solution in the presence of increased amounts of water (8). Svensson *et al.* (10) observed the incorporation of novel FA into LPC at low water activity. At higher water activity, incorporation into the LPC was very low. The highest incorporation into LPC was reported at 5% water addition (5). Higher water content resulted in lower incorporation into LPC, indicating lower acyl migration. We previously observed that the overall incorporation into PC and LPC was not influenced by the water content (1,9). With increased water in the system, the hydrolysis rate increases and GPC becomes a major reaction product. In considering the yield, a high water content in the system cannot be recommended.

Acids can catalyze acyl migration in LPC. Adlercreutz (8) examined the dependence of acyl migration from the *sn*-2 to the *sn*-1 position in LPC on the FA concentration in toluene. The acyl migration rate was observed to increase with increasing FA concentration. The highest rate was observed when LPC was dissolved directly in the FA in the absence of solvent. In the current study no significant difference in acyl migration into the *sn*-2 position was seen within the range of substrate ratios tested. An increased substrate ratio decreased the amount of LPC in the reaction mixture during acidolysis in a solvent-free system, but incorporation into PC also was decreased (9).

A higher conversion degree can usually be obtained by increasing the temperature in the solvent system (14). However,

in a solvent-free system it was more beneficial to operate at low temperatures (9). Not only the yield decreased with increased temperature, but also the overall incorporation into PC. Temperature also was reported to have an effect on the acyl migration (8), as confirmed in the current study. With increase in temperature, the acyl migration in the reaction system increases. Enzyme dosage also had a significant effect on acyl migration into the *sn*-2 position. The anion exchange resin used for immobilization of the *R. miehei* lipase has been reported to cause the acyl migration (10). Acyl migration was also observed with the commercially available *Thermomyces lanuginosa* lipase immobilized on hydrophilic silica granules (11). For *Rhizopus oryzae* lipase immobilized on polypropylene, no acyl migration was observed into the *sn*-2 position of PC during PL transesterification (10); however this lipase cannot be obtained commercially in the immobilized form.

Some interaction was observed between the enzyme dosage and reaction temperature. High enzyme dosage together with high temperature resulted in increased acyl migration.

A correlation between incorporation into the *sn*-2 position of PC and incorporation into LPC was set up with varying parameters (Fig. 3). As expected, acyl migration into the *sn*-2 position seemed to increase with increased incorporation into LPC. When caprylic acid is incorporated into LPC, most FA will be in the *sn*-1 position; however, some will migrate to the *sn*-2 position until some balance is reached. With migration to the *sn*-2 position, the lipase has opportunity to incorporate caprylic acid into the *sn*-1 position, resulting in PC with caprylic acid on both positions. Haraldsson and Thorarensen (5) reported that the maximal incorporation into LPC was 70%. Even higher incorporation into LPC can be seen, depending on the reaction conditions (9). As for acyl migration to the *sn*-2 position of PC, the reaction time was the factor having the most significant effect on the incorporation of acyl donor into LPC (9).

PC molecular distribution. With TLC-FID, the distribution of different FA species in individual PC molecules can be followed. A typical chromatogram for PC molecular distribution is depicted in Figure 4.

The incorporation of caprylic acid into PC was calculated based on the distribution of individual PC species and compared with results obtained by GC (Fig. 5). The two different ways of analysis correlated fairly well ($R^2 = 0.82$). According to regression analysis, the intercept does not equal zero ($P < 0.01$). This indicates that incorporation of caprylic acid into PC should be above a certain level in order to be detected by TLC-FID. The P -value for slope was less than 0.01 showing that there is a significant relationship between the two data sets. Incorporation of caprylic acid into PC (determined by GC) was also correlated with the PC molecular distribution (Fig. 6). LL-type PC was observed to decrease with increase of incorporation of caprylic acid, primarily with formation of ML-type PC. However MM-type PC was also observed under certain reaction conditions, confirming acyl migration to the *sn*-2 position. In Table 1, experiment no. 12 resulted in the largest migration to the *sn*-2 position. TLC-FID analysis showed that the PC dis-

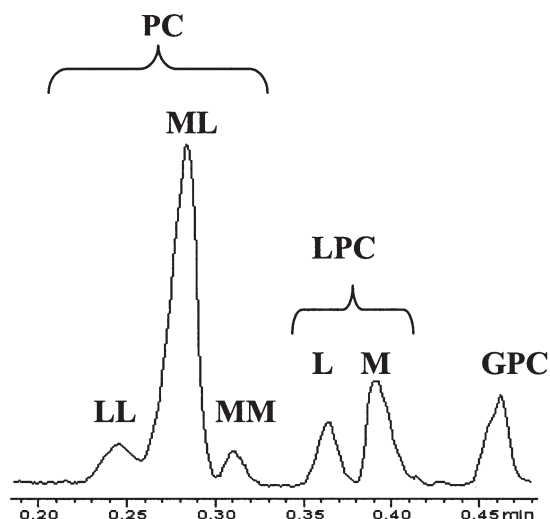


FIG. 4. TLC-FID chromatogram of structured PC. Structured PC produced with conditions given for Experiment 12 (enzyme dosage, 40%; reaction temperature, 55°C; water addition, 1%; reaction time, 70 h; substrate ratio, 6 mol/mol caprylic acid/PC). Abbreviations: L, long-chain FA; M, medium-chain FA. For other abbreviation see figure 3.

tribution of this product was 75% ML-type PC, 16% LL-type PC, and 9% MM-type PC. Combined, the GC and TLC results show that the majority of the caprylic acid is incorporated into the *sn*-1 position of PC during the lipase-catalyzed acidolysis reaction with the ML-type as the major product.

Acyl migration has been demonstrated to be a serious problem during lipase-catalyzed acyl exchange of PL. Lipase-catalyzed acyl exchanges of PL are normally conducted over several days; however, the present study has shown that reaction time is the factor having the most significant effect on acyl migration into the *sn*-2 position of PC. To increase the conversion

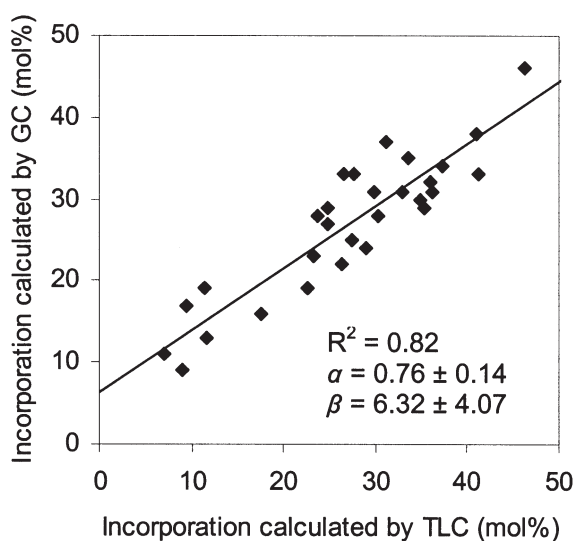


FIG. 5. Correlation between incorporation of caprylic determined by GC and TLC-FID. α and β represent the slope and intercept, respectively calculated from linear regression analysis.

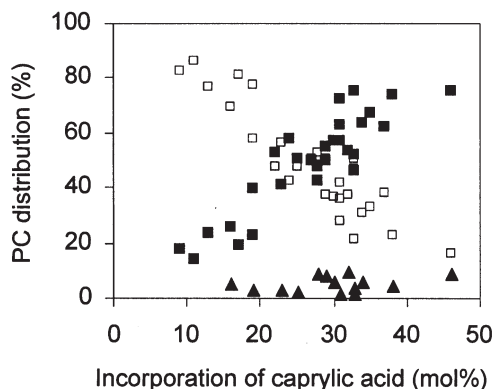


FIG. 6. Correlation between incorporation of caprylic acid into PC and PC molecular distribution. (□) LL-type PC, (■) ML-type PC, and (▲) MM-type PC. For abbreviations see Figure 4.

rate, a higher enzyme load may be used; however, for the current reaction system this is not advisable as the enzyme concentration already is very large. With higher enzyme loads, mixing will be extremely difficult. Alternatively the reactions could be conducted in packed bed reactors. Packed bed reactors were demonstrated to be advantageous over stirred tank reactors during lipase-catalyzed production of structured lipid, since the former had a much lower level of acyl migration (15). For optimal conditions it is recommended that temperature, substrate ratio, and water addition should be low.

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PAPER V

Title: Continuous production of structured phospholipids in a packed bed reactor with lipase from *Thermomyces lanuginosa*

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Continuous Production of Structured Phospholipids in a Packed Bed Reactor with Lipase from *Thermomyces lanuginosa*

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ABSTRACT: The possibilities of producing structured phospholipids between soybean phospholipids and caprylic acid by lipase-catalyzed acidolysis were examined in continuous packed-bed enzyme reactors. Acidolysis reactions were performed in both a solvent system and a solvent-free system with the commercially immobilized lipase from *Thermomyces lanuginosa* (Lipozyme TL IM) as catalyst. In the packed bed reactors, different parameters for the lipase-catalyzed acidolysis were elucidated, such as solvent ratio (solvent system), temperature, substrate ratio, residence time, water content, and operation stability. The water content was observed to be very crucial for the acidolysis reaction in packed bed reactors. If no water was added to the substrate during reactions under the solvent-free system, very low incorporation of caprylic acid was observed. In both solvent and solvent-free systems, acyl incorporation was favored by a high substrate ratio between acyl donor and phospholipids, a longer residence time, and a higher reaction temperature. Under certain conditions, the incorporation of around 30% caprylic acid can be obtained in continuous operation with hexane as the solvent.

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KEY WORDS: Enzyme bed reactor, lipase-catalyzed acidolysis, Lipozyme TL IM, structured phospholipids, *Thermomyces lanuginosa*.

Phospholipids (PL) are widely used in the food, pharmaceutical, and cosmetic industries. The uses of PL are mostly based on natural products having a very complex composition, such as lecithins, which usually are by-products from soybean oil production. These products consist of many individual molecules with FA having chain lengths between 16 and 22 carbon atoms. Desired PL with new physical and chemical properties can be produced by exchanging FA in the PL. Modified PL with different FA compositions and distributions may be used in various applications. Incorporation of medium-chain FA into natural PL by interesterification will result in better emulsifying and better heat-stability properties for some emulsion systems such as salad dressings and mayonnaise (1). Besides being food additives, the structured or modified PL, into which different functional FA have been specifically incorporated, also

can be used in lipid membrane research or for application as pharmaceuticals and cosmetics (2).

Several studies have been published on the modification of PL with phospholipases or lipases (3–6). Compared with chemical methods, enzymatic modifications of PL have the advantage that enzymes are selective and specific. Phospholipase A₁ (PLA₁) and lipases are specific for the ester bond in the *sn*-1 position of the lipids, whereas phospholipase A₂ (PLA₂) is specific for the ester bond in the *sn*-2 position. So far, little effort has been made to scale up the enzymatic modifications of PL to pilot plant or production scale because of problems such as mass transfer limitations and side reactions, which result in low yields. For industrial applications, enzymes are preferred in an immobilized form because it is possible to reuse the enzymes. However, only lipases can currently be obtained commercially in immobilized form for the PL modification; phospholipases are unavailable.

Most of the investigations reported to date have studied the enzyme-catalyzed reactions for production of structured PL in laboratory vessels. Almost all previous studies on lipase-catalyzed acidolysis of PL used pure PC as the substrate (3–6). For commercial considerations, deoiled soybean lecithin might be favored since the price is considerably lower than purified PC. Natural soybean PL are usually a mixture of several PL species including PC, PE, and PI.

Packed (or fixed) bed bioreactors (PBR) have been investigated and applied in the lipase-catalyzed production of structured lipids (7,8). These studies have demonstrated that PBR have promise in developing lipase-catalyzed lipid modifications. The application of PBR for production of structured PL may also be promising for further scale-up. PBR are used for most large-scale enzymatic processes because of high efficiency, low capital investment, ease of construction, possibility of continuous operation, and easy maintenance. Only a few reports are available on the enzymatic modification of PL using PBR. Härröd and Elfman (9) reported the synthesis of PC from lyso-PC and FA using PLA₂ as catalyst under supercritical conditions.

The use of an enzyme in an organic media has attracted increased interest in recent years. One reason is that in organic media, hydrophobic compounds such as lipids can be dissolved and thereby made accessible for enzymatic conversion. In this study we looked into the possibilities of producing structured PL in PBR with or without solvent using Lipozyme TL IM as

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TABLE 1
FA Distribution in Soybean Lecithin and Structured Lecithin

FA	Soybean lecithin	Structured lecithin ^a
8:0	0.0	35.4
16:0	19.8	9.7
18:0	4.4	2.4
18:1	10.1	6.3
18:2	59.2	41.9
18:3	6.6	4.6

^aReaction conditions: solvent ratio, 7.5 mL/g (hexane/substrate); substrate ratio, 36 mol/mol caprylic acid/phospholipids; reaction temperature, 40°C; flow rate, 0.2 mL/min; water content, 0%.

the catalyst. Effects of the molar ratio of reactants, reaction temperature, residence time, and water content on caprylic acid incorporation into PL were monitored as major variables.

MATERIALS AND METHODS

Materials. Natural PL in the form of soybean PL (Sterninstant PC-30) were donated by Stern Lecithin & Soja GmbH (Hamburg, Germany). The PL profile (area%) was PC 54.8, PE 15.6, PI 15.3, phosphatidic acid (PA) 4.3, lyso-PC 0.4, and others 9.6 (unidentified). The FA composition (mol%) of the soybean lecithin can be seen in Table 1. Caprylic acid (8:0, purity 97%) was purchased from Riedel-de-Haen (Seelze, Germany). Lipozyme TL IM, a silica-granulated *Thermomyces lanuginosa* lipase, was donated by Novozymes A/S (Bagsvaerd, Denmark). All solvents and chemicals used were of analytical grade.

PBR. The lipase-catalyzed acidolysis was performed using caprylic acid and soybean PL as substrates. Acidolysis reaction was conducted in both a solvent system and a solvent-free system. With each new experiment with specific parameters, preheated and conditioned substrates were pumped into the enzyme bed for the acidolysis reaction. Approximately 4–5 enzyme bed void volumes (V_s) were discarded before sampling. When a new enzyme bed was used, short-time equilibration with new substrate was performed to stabilize the bed. The substrate mixture was fed upward into the column, and the column

temperature was held constant by a circulating water bath (Fig. 1). The reaction substrates, kept in a circulating water bath, were pumped through the enzyme reactor by a pump from Fluid Metering Inc. (New York, NY). For the acidolysis in the solvent system, the bioreactor was a jacketed stainless steel column ($l = 200$ mm, i.d. = 21 mm) packed with 36 g of Lipozyme TL IM ($l = 196$ mm). For the experiments using a solvent system, the enzyme was replaced once with new material during the study (as described in the Results and Discussion section). For acidolysis in the solvent-free system, the bioreactor was a jacketed glass column ($l = 230$ mm, i.d. = 26 mm) packed with 48 g of Lipozyme TL IM ($l = 180$ mm). The enzyme was reused throughout the study. For stability studies, substrates were passed through the columns for several days, and samples were collected continually.

Determination of bed void fraction (ϵ). The bed void fraction was determined in the stainless steel column used for the solvent system experiments. Hexane was fed into the newly packed column at room temperature. The column was weighed before and after filling the bed with hexane. The volume of the enzyme bed (V) was calculated from the diameter of the column and length of the bed and the volume of substrate (V_s) was calculated using the density and weight of hexane. The void fraction was then calculated as $\epsilon = V_s/V$. The same void fraction was used for the calculations in both the solvent-free and solvent-containing systems. The residence time of the substrate in the bed was calculated as $V \cdot \epsilon / V_f$, where V is the enzyme bed volume, ϵ the void fraction, and V_f the substrate flow rate. The void fraction was calculated to be 0.67. Measurement was conducted at room temperature.

Analysis of FA composition. Samples were directly methylated by KOH-catalyzed esterification as described elsewhere (10). The FAME were analyzed with an HP6890 series gas-liquid chromatograph (Hewlett-Packard, Waldbronn, Germany) equipped with a fused-silica capillary column (SUPELLOWAX-10, 60 m \times 0.25 mm i.d., 0.20 μ m film thickness; Supelco Inc., Bellefonte, PA). The oven temperature was programmed from 70 to 225°C. The initial temperature was held for 0.5 min, increased to 160°C at 15°C/min, then increased to 200°C at a rate of 1.5°C/min, held for 15 min, and finally the temperature was increased to 225°C at a rate of 30°C/min and held for 10 min. An FID was heated at 300°C. The injector was used in split mode with a ratio of 1:20. The carrier gas was helium with a column flow of 1.2 mL/min. The FAME were identified by comparing their retention times with authentic standards (Sigma Chemical Co., St. Louis, MO), and the molar composition was calculated together with response factors and M.W. of the FA.

RESULTS AND DISCUSSION

Different parameters were studied for the acidolysis reaction in PBR using either a solvent-containing or a solvent-free system. The solvent-free system was expected to have higher volumetric productivity than the solvent system if the comparison is based on enzyme load and volume of reaction mixture. However, the reaction time needed was expected to be longer in the

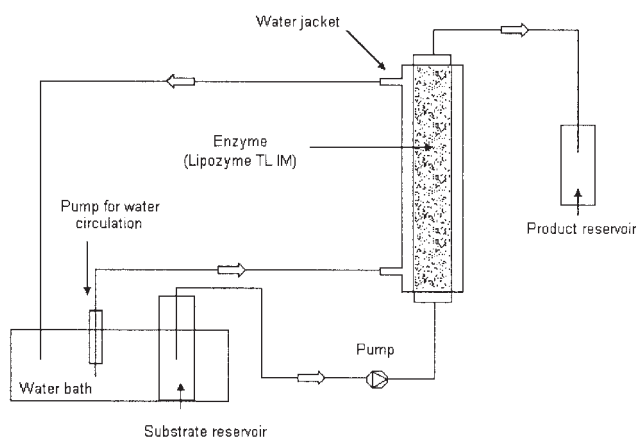


FIG. 1. Process diagram of lipase-catalyzed acidolysis for production of structured phospholipids (PL) in a packed bed reactor.

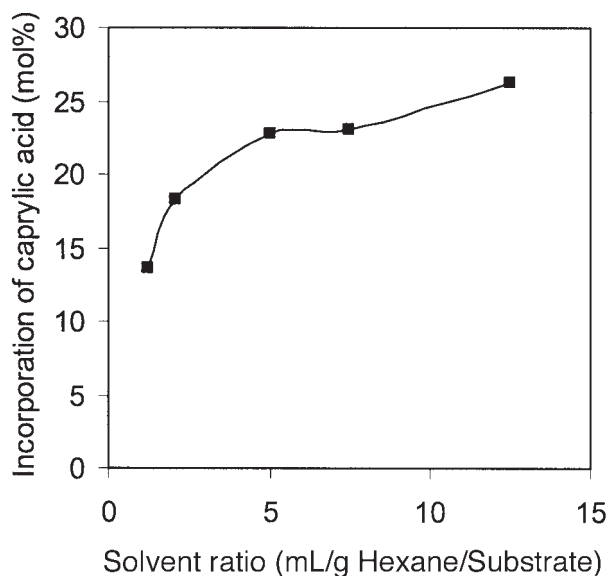


FIG. 2. Effect of solvent ratio (mL/g, hexane/substrate) on the incorporation of caprylic acid (mol%). Reaction conditions: substrate ratio, 3.5 mol/mol caprylic acid/PL; flow rate, 0.2 mL/min; water content, 0%; reaction temperature, 40°C. For abbreviation see Figure 1.

solvent-free system since the substrate amount was higher. Therefore, it was decided to use a column with a larger volume in the solvent-free system compared with the solvent system.

Effect of solvent ratio. The use of solvents in the reaction system can dramatically reduce the viscosity of the substrates and thus increase the reaction rate by increasing the mass transfer of the substrates. Solvents with too high a polarity are, however, not suitable in enzymatic esterification reactions because they are strong water distorters and thereby inactivate the enzyme (11). The best measure of polarity is the logarithm of the partition coefficient ($\log P$) of the organic liquid between *n*-octanol and water; the higher the $\log P$, the less polar the solvent is. Solvents with $\log P < 2$ are not considered suitable for enzyme reactions. Commonly used solvents for the acidolysis of PL are toluene and hexane, both having $\log P > 2$ (12–15). It was reported that reactions conducted in hexane were faster than reactions conducted in toluene (6). Hexane was selected for further studies since it is generally accepted in the fat and oil industry. The immobilized enzyme is compatible with this solvent. The solvent ratio (mL/g, hexane/total substrate) was varied to determine how it influences the incorporation of caprylic acid into PL in the PBR. Five different solvent ratios were tested with the same substrate ratio. The substrates were run through the column with the highest solvent ratio first and then in the order of decreasing solvent ratios. With the decrease of the solvent ratio, the incorporation of caprylic acid decreased (Fig. 2) because the substrate/enzyme ratio was increased as well. Therefore, a longer residence time will be needed to reach the same incorporation of the novel FA. From the lowest to the highest solvent ratio, there was a 10-fold increase in the PL concentration; however, the incorporation was less than twofold. The highest rate of production would therefore be found for samples having the

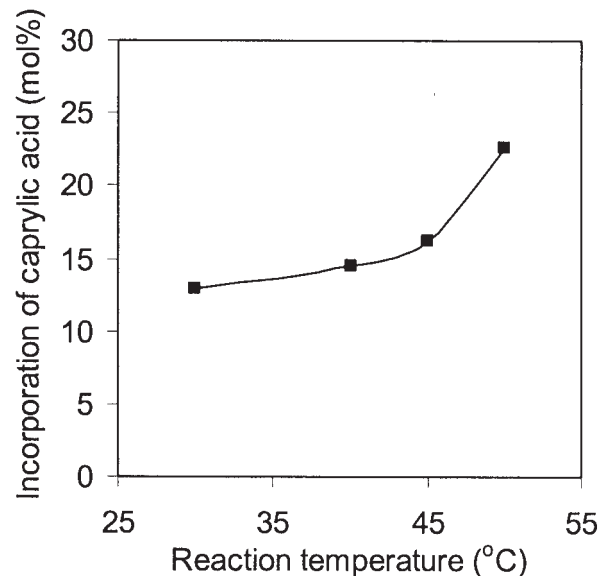


FIG. 3. Effect of temperature on the incorporation of caprylic acid (mol%) in a solvent system. Reaction conditions: solvent ratio, 12.5 mL/g, hexane/substrate; substrate ratio, 3.5 mol/mol caprylic acid/PL; flow rate, 0.2 mL/min; water content, 0%. Immobilized enzyme was reused from previous experiments. For abbreviation see Figure 1.

lowest solvent ratio. However, if the conversion is low, additional separation steps of the product and original PL substrate are needed if the goal is to obtain PL with a high incorporation of caprylic acid. For practical applications, the hexane amount should be kept as low as possible, because of downstream processing and environmental considerations. Therefore, a compromise has to be made concerning the amount of solvent.

Effect of temperature. According to the supplier of Lipozyme TL IM, the enzyme is most active in the temperature range of 55–70°C. Usually, an increase of reaction temperature results in an increased reaction rate, according to the Arrhenius law, during enzyme-catalyzed reactions. A high temperature favors higher yields for endothermic reactions owing to the shift of thermodynamic equilibrium. When lipase activity decreases, it is possible to compensate by increasing the operating temperature at a rate that permits the system to maintain a constant conversion rate. For the solvent system, the temperature was varied in the range of 30–50°C to minimize hexane evaporation. A higher temperature gave higher incorporation, and the highest incorporation of caprylic acid was seen at 50°C (Fig. 3). A further increase in temperature could be interesting but is limited by the b.p. of the solvent (b.p. for hexane: 66°C). In the absence of solvent, increasing the column temperature can control the viscosity of the substrates. At elevated temperatures, operation is easier, since higher temperatures increase the solubility of reagents and decrease the viscosity of solutions. This is useful only within the optimal temperature range of the enzyme involved, because higher temperatures will deactivate the enzyme. For the solvent-free system, the temperature was varied from 50 to 70°C with the following reaction conditions: substrate ratio, 6 mol/mol caprylic acid/PL; water content, 0.5%; flow rate, 0.3 mL/min. The incorporation of caprylic acid into

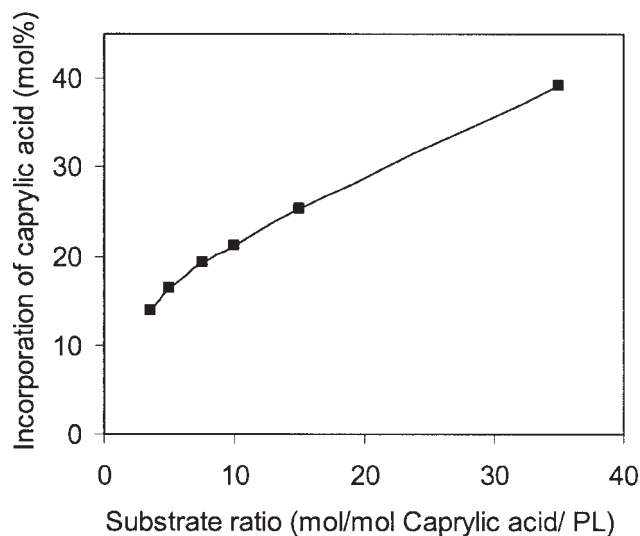


FIG. 4. Effect of substrate ratio on the incorporation of caprylic acid (mol%). Reaction conditions: solvent ratio, 7.5 mL/g, hexane/substrate; reaction temperature, 40°C; flow rate, 0.2 mL/min; water content, 0%. Immobilized enzyme was reused from previous experiments. For abbreviation see Figure 1.

PL was 14.6, 16.7, and 21.1% at 50, 60, and 70°C, respectively. The data show that in the 50–70°C range, the incorporation of caprylic acid increased with increasing temperature, with the highest incorporation seen at 70°C. Lipase stability is influenced by temperature; a high temperature will greatly reduce the enzyme stability and its half-life. A higher temperature will also increase the lipid oxidation rate, especially if PUFA are used as acyl donors. In batch reactions for the same enzyme and substrate, the maximum incorporation was observed at 57.5°C (10). This was an optimized temperature, which is believed to be associated with other parameters. However, results presented in this study show that it is possible to increase the temperature to 70°C and still get an increase in the incorporation of caprylic acid in the solvent-free system.

Effect of substrate ratio. The PL composition of the product in the enzymatic acidolysis reaction depends on the substrate ratios (mol acyl donor/mol PL). The effect of the PL to caprylic acid mole ratio in the reaction mixtures on lipase-catalyzed acidolysis when using a solvent system is shown in Figure 4. The incorporation of caprylic acid increased with increasing substrate mole ratio. For the solvent-free system, two substrate ratios were tested, 6 and 8 mol/mol caprylic acid/PL with the following reaction conditions in the PBR: water content, 0.5%; flow rate, 0.3 mL/min; and temperature, 60°C. The incorporation of caprylic acid into PL was 13.1 and 24.4% with substrate ratios of 6 and 8, respectively. In batch reactions, substrate ratios above 5.5 gave rise to substrate inhibition when the same enzyme and substrate were used (10). These results indicate that an even higher molar ratio can increase the incorporation of caprylic acid into PL in PBR using a solvent-free system. The results from the solvent-containing and the solvent-free systems show that the substrate ratio certainly moves the reaction equilibrium to the product side and improves acyl incor-

poration. The choice of substrate mole ratio also relates to the downstream processing cost and difficulties in separating the FA (acyl donor and exchanged FA) from the products. Therefore, a compromise has to be made. By varying the substrate ratio, the PL substrate applied to the enzyme per unit is also changed; therefore, it should be noted that with high substrate ratios the overall productivity will decrease.

Effect of residence time. Residence time can be increased or reduced by varying the volume flow rates. The flow rate and residence time have a reciprocal relationship described by the following equation:

$$\text{residence time} = \frac{\pi \cdot r^2 \cdot l \cdot \epsilon}{V_f} \quad [1]$$

where r = inner radius of the column, l = column length, ϵ = the bed void fraction, and V_f = flow rate of substrates. For the solvent system, the flow rate was varied from 0.1 to 1.0 mL/min, giving a residence time from 0.8 to 8 h. For this study, a new enzyme column was prepared. For the solvent-free system, the residence time was varied from 1.5 to 13 h, corresponding to flow rates between 0.1 and 1.1 mL/min. In Figures 5A and 5B, the incorporation of caprylic acid as a function of the residence time is depicted.

The results indicate that a low flow rate is required for a high incorporation. Having low flow rates gives rise to the problem of external mass transfer limitations. This indicates that for an efficient operation, several steps should be used since a long reaction time is needed. Further study on this issue is necessary to optimize the system.

Water content and operative stability. The operative stability of the enzyme in a solvent system over a week (168 h) is shown in Figure 6A. The enzyme reached equilibrium within 48 h and thereafter was stable with only a slight decline in the incorporation of caprylic acid (enzyme activity). The influence of water in the solvent system was tested during the operative stability study by adding 0.25% water to the substrate after 168 h of running. A slight decrease could be observed for the incorporation of caprylic acid. Addition of excessive amounts of water should also be avoided since it would result in emulsion formation and complicate the product recovery (16). Operative stability of the solvent-free system was tested for several days with two different water contents (Fig. 6B). With both substrates, the incorporation was highest in the beginning and decreased until 30 h, where it stabilized. The incorporation of caprylic acid was slightly higher when the water content was 0.25%. For the solvent-free system, it seems that small amounts of water are beneficial for the incorporation. The results indicate that the PL amount in a substrate mixture has a great influence on the catalytic activity of the enzyme. Increasing PL in the substrate will probably remove more water from the enzyme, thus reducing the catalytic activity. Therefore, it is necessary to add water to the solvent-free system to increase the transacylation rate, and apparently the addition of water to the solvent system did not increase the incorporation at all.

Table 1 contains the FA composition for structured PL having a high incorporation of caprylic acid. From the present and

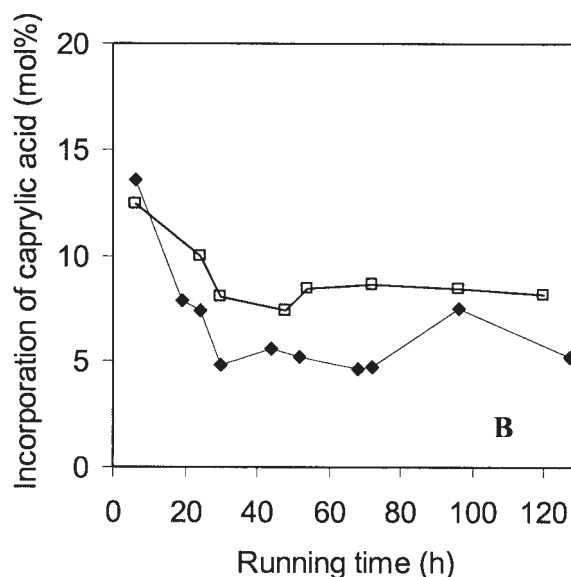
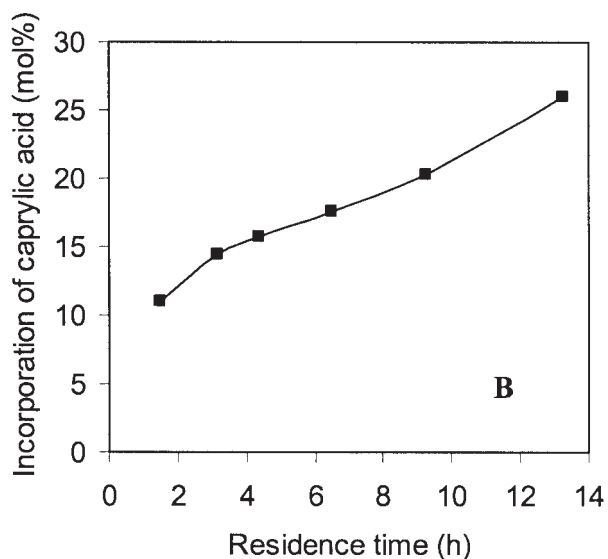
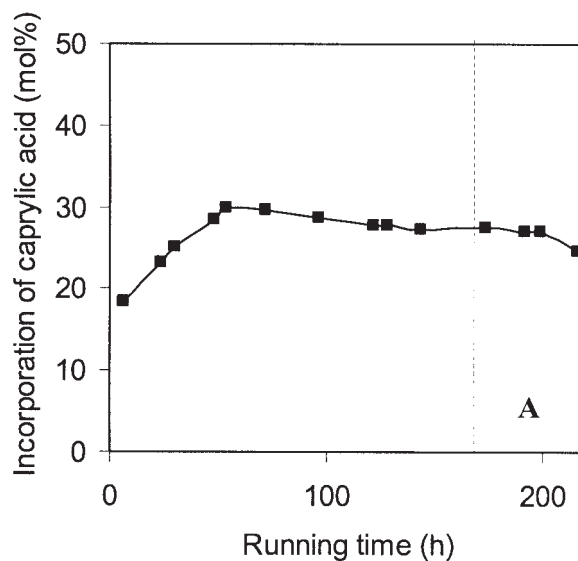
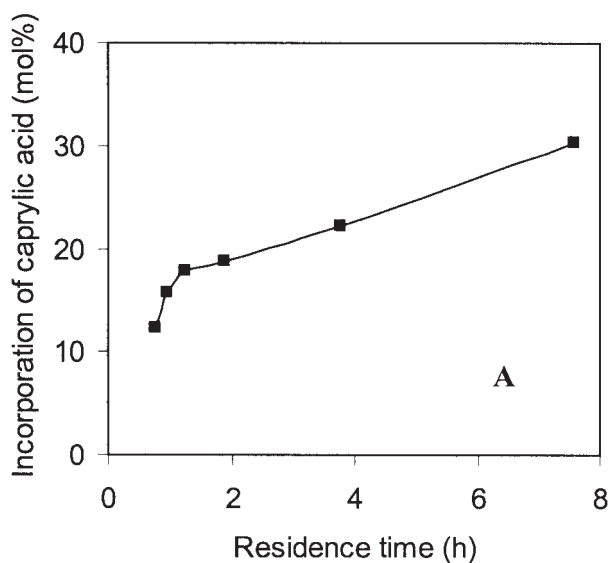


FIG. 5. Effect of residence time on the incorporation of caprylic acid (mol%). (A) Solvent system. Reaction conditions: solvent ratio, 7.5 mL/g, hexane/substrate; substrate ratio, 3.5 mol/mol caprylic acid/PL; reaction temperature, 40°C; flow rate, 0.2 mL/min; water content, 0%. (B) Solvent-free system. Reaction conditions: substrate ratio, 6 mol/mol caprylic acid/PL; reaction temperature, 60°C; water content, 0.50%. For abbreviation see Figure 1.

also previous publications, it has been reported that the maximum incorporation of caprylic acid into the deoiled soybean lecithin is 38–40% (10). The nature of the PL affects the incorporation rates of caprylic acid catalyzed by Lipozyme TL IM in hexane. The following order of reactivity was observed: PC > PE > PI > PS (10). Incorporation of caprylic acid into PC was high within 48 h, whereas the incorporation into other PL species was low. Since soybean lecithin actually is a mixture of PL species, this is probably the reason for the lower incorporation into this substrate compared with purified PC. Previously, we showed that the water content had no effect on the incorpo-

FIG. 6. Operative stability of Lipozyme TL IM. (A) Solvent system. Reaction conditions: solvent ratio, 7.5 mL/g, hexane/substrate; substrate ratio, 9.5 mol/mol caprylic acid/PL; reaction temperature, 40°C; flow rate, 0.2 mL/min; water content, 0 (0–168 h) and 0.25% (168–216 h). (B) Solvent-free system. Reaction conditions: substrate ratio, 6 mol/mol caprylic acid/PL; reaction temperature, 70°C; flow rate, 0.3 mL/min; (◆) water content: 0%, (□) water content: 0.25%. For abbreviation see Figure 1.

ration of caprylic acid under a solvent-free batch system (10). However, in this study we found that water has some influence on the incorporation using a PBR. Before these types of reactions can be implemented industrially, further work will need to be done to increase efficiency. However, this study provides a few clues for the importance of the system, e.g., that water addition is necessary for the solvent-free system, that the reaction needs a long residence time, and that an increase of temperature

within the thermostability of the enzyme gives higher incorporation. Furthermore, a high amount of solvent and substrate ratio will increase incorporation of novel FA as well.

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PAPER VI

Title: Synthesis of structured phospholipids by immobilized phospholipase A₂ catalyzed acidolysis

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Synthesis of structured phospholipids by immobilized phospholipase A₂ catalyzed acidolysis

Running title: PLA₂ catalyzed synthesis of structured phospholipids

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Abstract

Acyl modification of the *sn*-2 position in phospholipids (PLs) was conducted by acidolysis reaction using immobilized phospholipase A₂ (PLA₂) as the catalyst. In the first stage we screened different carriers for their ability to immobilize PLA₂. Several carriers were able to fix the enzyme and maintain catalytic activity; however the final choice of carrier for the continued work was a non-ionic weakly polar macroreticular resin. Response surface methodology was applied to evaluate the influence of substrate ratio, reaction temperature and water addition during acidolysis reaction between caprylic acid and soybean phosphatidylcholine (PC). Reaction temperature and water addition had significant effect on acidolysis reaction, however no effect was observed for substrate ratio (mol caprylic acid/mol PC) in range tested. In general an inverse relationship between incorporation of caprylic acid and PC recovery was observed. Highest incorporation obtained during acidolysis reactions was 36%. Such incorporation could be obtained under reaction temperature, 45°C; substrate ratio, 9 mol/mol caprylic acid/PC; and water addition of 2%; 30 wt % immobilized enzyme; and reaction time, 48h. The yield under these conditions was however only 29%. Lysophosphatidylcholine (LPC) was the major by-product formed during the reaction. Incorporation of acyl donor into LPC was very low (<4%), which indicates that acyl migration is only a minor problem for PLA₂ catalyzed synthesis reaction. Conjugated linoleic acid and docosahexaenoic acid were also tested as acyl donors, and were able to be incorporated into PC with 30 and 20%, respectively.

Key words: Immobilization; PLA₂ catalyzed synthesis; response surface methodology; solvent-free system; structured phospholipids

1. Introduction

Different enzymes can be used to tailor phospholipids (PLs) with defined fatty acid composition at the *sn*-1 and *sn*-2 positions. Using enzymatic acyl exchange it would be possible to acquire PLs for specific application requirements in food, pharmaceuticals and cosmetics by altering the technical or physiological properties of

the natural compounds. Most of the work in this direction focuses on incorporation of saturated fatty acids (including both medium chain and long chain) or polyunsaturated fatty acids into PLs (Hossen and Hernandez, 2005; Lyberg et al., 2005; Reddy et al., 2005; Vikbjerg et al., 2005). The interest in the incorporation of saturated fatty acids is mainly to improve the heat stability, emulsifying properties and oxidation stability of the PLs (Chmiel et al., 1999; Pedersen, 2001), while the incorporation of polyunsaturated fatty acids is due to the claimed health promoting effects (Takahashi and Hosokawa, 2001).

Compared to enzymatic acyl exchange at the *sn*-1 position of PLs, the enzymatic acyl exchange in the *sn*-2 position has received less attention. Porcine pancreatic phospholipase A₂ (PLA₂), which is the most commonly used enzyme for modification of PLs at the *sn*-2 position, is considerably more difficult for synthesis in comparison with lipases from microbial sources commonly used for modification of the *sn*-1 position of PLs. Pancreatic PLA₂ has requirement of calcium ions and a water activity above 0.2 to be catalytically active, which means that low yields can be expected compared to lipase-catalyzed reactions that can function in nearly anhydrous reaction systems without the presence of calcium ions (Pernas et al., 1990, Adlercreutz et al, 2003).

Despite these problems there remains a great interest in using PLA₂ for PL synthesis as fatty acids resided in the secondary position of PLs may have particular important influence on nutritional and medical functions (Takahashi and Hosokawa, 2001).

Commercial product of PLA₂ has so far only been provided in the free form (liquid solution), but some attempts have previously been made to immobilize the enzyme (Aura et al., 1995; Doig and Diks, 2003; Härrod and Elfman, 1995; Hossen and Hernandez, 2005; Lyberg et al. 2005). Main reason to use immobilized enzymes is the ability to isolate the biocatalyst from reaction mixture as well as to improve the stability. Some of the carriers selected in these previous studies would however not be suitable if having larger-scale production in mind. Enzymes immobilized on celite and certain other porous or powder inert materials have good initial activity, but are often difficult to handle or have insufficient enzymatic and physical stability in industrial processes (Eigtved, 1992). Dust formation, displacement of the enzyme from the carrier, and high pressure drops in packed bed columns are some of the problems that can occur using these types of carriers. Polymer or resin based carriers have been described, which offers strong adsorption, high activity, and stability of enzymes, which would accommodate enzymes and transport lipid substrate without major diffusion problems (Eigtved, 1992).

Most work described for the PLA₂ catalyzed synthesis of structured PLs are based on esterification of lyso-PLs in organic solvent (Adlercreutz et al., 2003; Guo et

al., 2005). In order to obtain lyso-PLs for this type of reaction it would require a hydrolysis step of the PL and subsequent purification step to remove free fatty acids. Direct transesterification (acidolysis) of PL with acyl donor would avoid these additional steps as reaction can be performed in a single step. Some attempts have previously been made for transesterification; however in general the incorporation of fatty acids into the *sn*-2 position is rather low (<15%) (Aura et al., 1995; Hossen and Hernandez, 2005; Park et al., 2000).

In this study we screened different carriers for immobilization of PLA₂. A promising carrier was selected and further experiments were performed to maximize catalytic activity of the immobilized enzyme. The immobilized PLA₂ was subsequently used for synthesis of structured PLs under solvent-free conditions. The reaction scheme for PLA₂-catalyzed acidolysis is depicted in Fig. 1. Different parameters were examined for their influence on incorporation and PL distribution during PLA₂ catalyzed synthesis of structured PLs. Response surface methodology was used to assist the evaluation.

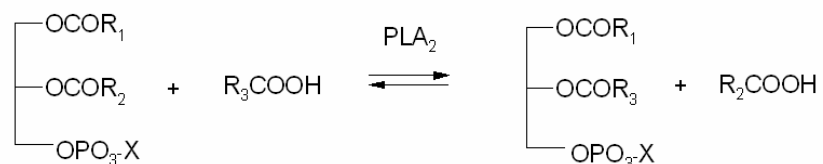


Fig.1. Schematic presentation of PLA₂-catalyzed acidolysis of phospholipid with free fatty acid. R₁, R₂ and R₃ refer to fatty acids and x refers to phospholipid head group (e.g. choline).

2. Materials and Methods

2.1. Materials

Epikuron 200 (PC, 93%) was purchased from Degussa Texturant Systems Deutschland GmbH & Co. KG (Hamburg, Germany). The fatty acid composition (mol%) of PC can be seen in Table 1. Caprylic acid (C8:0, purity 97%) was purchased from Riedel-de-Haen (Seelze, Germany). Conjugated linoleic acid (CLA, purity 80%) consisting of 38.8% 9c,11t isomer and 38.8% 10t,12c isomer was provided by Natural ASA (Hovdebygd, Norway). 4,7,10,13,16,19 all cis-Docosahexaenoic acid (DHA, purity 99+ %) was purchased from Loradan Fine Chemicals (Malmö, Sweden). Porcine pancreatic PLA₂ (Lecitase 10L, 10.000 U/ml) was supplied by Novozymes A/S (Bagsvaerd, Denmark). Carrier materials and their suppliers are listed in table 2. All solvent and chemicals were of analytical grade.

2.2. Immobilization of PLA₂

Varying amounts of PLA₂ solution was added to 5 ml buffer (10 mM Tris-HCl, 10 mM CaCl₂, pH 8) followed by the addition of 250 mg carrier. The enzyme solutions containing the carrier were incubated overnight by end-over-end mixing at room temperature followed by centrifugation at 4000 rpm for 5 minutes. The fixation level was estimated subtracting the protein remaining in the supernatant after binding compared to the initial protein concentration. Protein was determined according to the method of Lowry et al. (1951) using Bovine Serum albumin (BSA) as the standard. Enzyme preparation was removed by filtration and subsequently dried overnight in fume hood. Immobilized PLA₂ was stored at 5°C prior to use.

2.3. Hydrolytic activity of PLA₂

Evaluation of the catalytic activity was determined by hydrolysis of PC as described by Kim et al. (2001). Reactions were carried out in an ethanol-buffer (10 mM Tris-HCl, 10 mM CaCl₂, pH 8.0) (ratio, 70:30) with 0.4 g PC/ ml. Capped flasks containing the PC solution were incubated in water bath with magnetic stirring (300 rpm) at 40°C. Hydrolysis reactions were initiated by the addition of PLA₂. Samples were withdrawn during progress in reaction, and analyzed by TLC-FID. The activity was defined as the amount of LPC produced per min, and specific activity was defined as the amount of LPC produced per min and mg protein.

2.4. Acidolysis reaction

Reactions between fatty acid and PC were carried out using a 1 g reaction mixture in 5 ml glass vials. Vials were incubated in a water bath with magnetic stirring (300 rpm) and reactions were initiated by the addition of 300 mg immobilized PLA₂ (carrier: Amberlite XAD7; 72 mg PLA₂/g carrier). After reactions, samples were withdrawn from the reaction mixture for analysis. A three-level three-factor fractional experiment with 2 star points (17 experiments) was carried out. The three factors chosen were: reaction temperature (°C), water addition (wt% based on total substrate), and substrate ratio (mol/mol caprylic acid/PC). The incorporation of caprylic acid into PC, and the PL distribution (PC, LPC and glycerophosphorylcholine (GPC)) were used as responses. In table 4 are listed the factors used, the parameter ranges applied, and the responses.

2.5. Analysis methods

Analytical separations of PL species and fatty acids were performed on Silica Gel 60 thin-layer plates (20cm x 20cm, Merck, Darmstadt, Germany). After development in chloroform-methanol-water (65:35:5, v/v), the plates were sprayed with 0.2% of 2,7-dichlorofluorescein in ethanol (96%), making the lipid bands visible under UV-light. Bands representing PC and LPC were scraped off and methylated by BF_3 for analysis on a HP6890 series gas-liquid chromatograph (Hewlett-Packard, Waldbronn, Germany) equipped with a flame-ionization detector (FID) (Vikbjerg et al., 2005).

Phospholipid profile analysis was performed on product mixtures from acidolysis reactions using thin layer chromatography coupled with flame ionization detection (TLC-FID). Samples were spotted onto silica gel chromarods (Chromarod SIII, Iatron Laboratories Inc., Tokyo, Japan) and developed in a mixture of chloroform/methanol/water (42:22:3, v/v/v). After developing, chromarods were dried at 120°C for 5 min. Chromarods were then placed into the TLC-FID analyzer (Iatroscan MK6s, Iatron Laboratories Inc., Tokyo, Japan) and scanned at a rate of 30s/rod. Flow rates of 160 ml/min for hydrogen and 2 l/min for air were used during analysis. Peaks were identified by external standards.

2.6. Statistical analysis

Significance of the results was established at $P \leq 0.05$. Differences in the responses were determined by one-way analysis of variance, where 95% confidence intervals were calculated from pooled standard deviations (SD) using software Microsoft Office Excel 2003 (Microsoft Corporation, Redmond, WA). The computer program Modde 6.0 (Umetri AB, Umeå, Sweden) was used to aid the statistical design of the factorial experiments and to fit and analyze the data by multiple regressions. The fit of the models were evaluated by the coefficient of determination (R^2) and analysis of variance (ANOVA).

3. Results and discussion

3.1. Screening for carrier materials

In order to have a practical approach for PLA_2 catalyzed production of structured PLs the enzyme is preferred in the immobilized form. This would make it possible in sight to develop a continuous process as the enzyme can easily be recovered and reused, and would make the process more economically feasible. Of the various methods for immobilization physical absorption of the enzyme onto solid support

remains the simplest, least expensive, and least labour-intensive procedure. Secreted PLA₂ requires Ca²⁺ as co-factor; however the concentration of Ca²⁺ strongly influences the synthetic activity of these enzymes (Pernas et al., 1990). High concentrations of Ca²⁺ give rise to severe inhibition of synthesis reactions. In some cases the dependence of Ca²⁺ is simply overcome by doing the immobilization in buffer containing CaCl₂ (Egger et al., 1997; Aura et al, 1995; Lyberg et al. 2005). Pernas et al. (1990) reported that initial rate of PL synthesis conducted in organic solvent was dependent on the pH of the last aqueous solution in which the enzymes were exposed; however the maximum conversion was not dependent on the pH in the range 4-11. In most cases buffer has been adjusted to pH 8, when porcine pancreatic PLA₂ have been used as catalyst. Conditions for the buffer used in the current study were selected based on recommendations from the previous studies mentioned above.

Table 1 Fatty acid distribution in PC and structured PCs (mol%)

Fatty acids	Soybean PC	Structured PC ^a		
		Caprylic acid enriched PC	CLA enriched PC	DHA enriched PC
8:0	-	25.3	-	-
16:0	12.8	13.0	13.0	12.2
18:0	3.9	3.2	3.0	3.2
18:1	9.4	7.5	9.1	8.3
18:2	65.5	45.3	38.6	48.9
18:3	8.1	5.7	6.4	7.3
CLA (all isomers)	-	-	30.0	-
22:6	-	-	-	20.2

^a Reaction conditions: Reaction temperature, 45°C, Water addition, 2%; Substrate ratio, 3 mol/mol, enzyme dosage, 30%; Reaction time, 48h.

Seven different carriers were examined for their ability to immobilize PLA₂. Characteristics of enzyme carriers screened are presented in table 2. In all cases, the immobilization procedure was the same. Table 3 shows the protein absorption to different carriers. High fixation of PLA₂ to the carriers was observed except for Accural EP100 and Lewatit VP1600. These two carriers were also very hydrophobic, and did not suspend in the enzyme solution as the other carriers, but floated to the top. By pre-wetting these carriers with ethanol prior to immobilization it was possible to suspend these carriers in the enzyme solution, which also resulted in an increase of the fixation level of PLA₂ (table 3). The three carriers immobilized with PLA₂ having the highest protein fixation (Amberlite XAD7, Duolite A568, and Superlite DAX8) were tested for their hydrolytic activity (table 3). As there was seen some differences in the enzyme fixation, the immobilized enzymes were added to the reaction mixture with similar protein loading. One-way analysis of variance showed that there was significant difference in catalytic activity of PLA₂ when immobilized on these different carriers

($p < 0.01$). Having Amberlite XAD7 and Superlite DAX8 as carriers resulted in significant higher specific activity as compared to having Duolite A568 as the carrier; however there was no significant difference in the specific activity between Amberlite XAD7 and Superlite DAX8. Amberlite XAD7 had the highest protein fixation though, which means that lower dosage requirements were needed to obtain the same conversion degree. From considerations above Amberlite XAD7 was found to be a suitable carrier and was selected for the further study.

Table 2. Carriers screened and their characteristics

Carrier	Supplier	General description
Amberlite XAD7	Sigma-Aldrich Chemie GmbH, Steinheim, Germany	Nonionic weakly polar macroreticular resin (matrix: acylic ester), Particle size: 0.25-0.85 mm (wet)
Superlite DAX8	Supelco, Bellefonte, USA	Resin with moderate polarity (matrix: acrylic ester), Particle size: 0.25-0.45mm
Celite 545	BHD, Poole, UK	Diatomaceous Earth, Particle size: 0.02-0.1 mm
Dowex 50W	Dow Chemical Company, Michigan, USA	Strongly acidic cation exchange (maxtrix:resinstyrene-divinylbenzene; functional group: sulfonic acid), Particle size: 0.15-0.30mm
Lewatit VPOC1600	Lanxess AG, Leverkusen, Germany	Divinyl benzene crosslinked polymer (Marix: methacrylate), Particle size: 0.3-1.2 mm
Duolite A568	Rohn and Haas, Chauny, France	Polymerized phenol-formaldehyde anionic exchange resin, Particle size: 0.15-0.85 mm
Accurel EP 100	Akzo, Obernburg, Germany	Macroporous polypropylene, Particle size: 0.6-0.8 mm

Table 3 Fixation level of PLA2 on different carriers, and corresponding enzyme loading and specific activity.

Carrier ^a	Enzyme loading ^b (mg protein/g support)	Specific activity ^c ($\mu\text{mol mg}^{-1} \text{min}^{-1}$)
Amberlite XAD7	49.0	0.30
Superlite DAX8	44.4	0.28
Duolite A568	43.3	0.24
Dowex 50W	7.8	-
Celite 545	3.6	-
Accural EP 100	1.9	-
+ Prewetting	42.5	-
Lewatit VPOC 1600	1.3	-
+ Prewetting	40.3	-

^a Pre-wetting of Accural EP 100 and Lewatit VPOC 1600 were done by addition of 0.5 ml 96% ethanol/g support immediately before immobilization; ^b Pooled SD = 1.0 mg protein/g support; ^c Pooled SD = 0.015 $\mu\text{mol mg}^{-1} \text{min}^{-1}$; - n.d., not determined.

3.2. Conversion efficiency of the immobilized enzyme

Binding of enzyme to the carriers and the total amount bound will depend on the initial concentrations of the catalyst and the carrier, and ratio of the two components. In Fig.2 the influence of initial enzyme /carrier ratio on fixation level to Amberlite XAD7 is depicted. Protein binding to the carrier increased with increased ratio between enzyme and carrier.

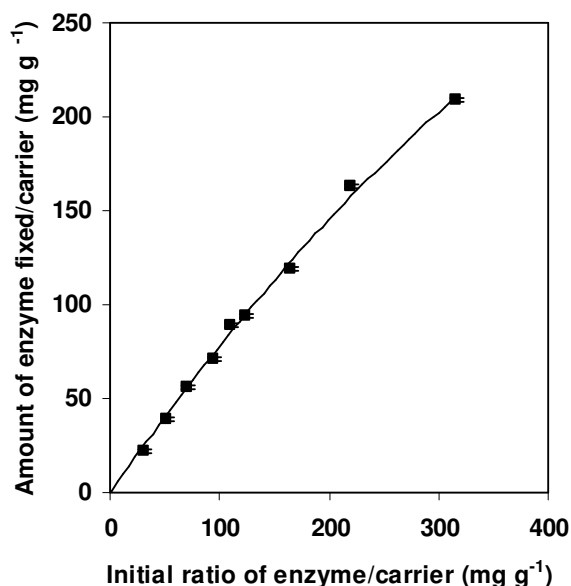


Fig. 2. Influence of initial enzyme/carrier ratio on fixation level to Amberlite XAD7. Varying amounts PLA₂ were incubated in the presence of 250 mg carrier. Bars represents mean \pm pooled SD.

However activity only increased with increasing fixation level until a certain protein loading; and the specific activity decreased with increase in fixation level of PLA₂ (Fig. 3A). Highest specific activity was observed at low fixation level of PLA₂. At high enzyme load only a fraction of the enzyme seems to be involved in the catalytic reaction. Higher enzyme load would contribute to increased limitation of substrate diffusion and therefore decreasing efficiency. From Fig. 3A it seems that an initial enzyme/carrier ratio of approximately 100 mg/g would give the optimal fixation of PLA₂ in terms of activity. Influence of enzyme loading on activity and specific activity with this fixation level was examined (Fig. 3B). This was mainly to confirm that the results obtained above were valid, and that the decline in activity was not related to for example substrate limitations. As expected the activity increased with increased enzyme dosage, and the specific activity was constant. For the subsequent acidolysis reactions PLA₂ was immobilized to Amberlite XAD7 with an initial enzyme/carrier ratio 100mg/g (72 mg/g enzyme fixed/carrier).

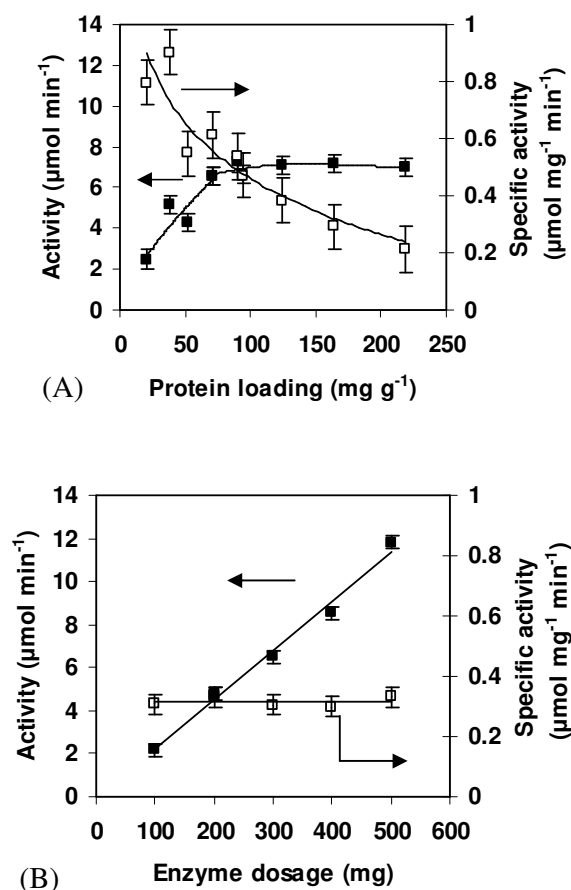
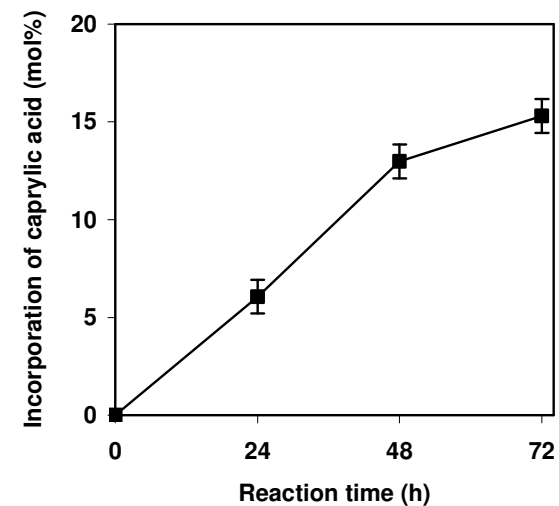


Fig.3. Bioconversion efficiency of PLA₂ immobilized Amberlite XAD7. A) Influence on enzymatic loading on activity and specific activity of immobilized system with different fixation level (mg enzyme per g support). B) Influence on enzymatic loading on activity and specific activity of immobilized system with same fixation level. Enzymatic assay and PLA₂ activity measurement were performed according to procedure described in material and methods. Bars represent mean ± pooled SD (n=2).

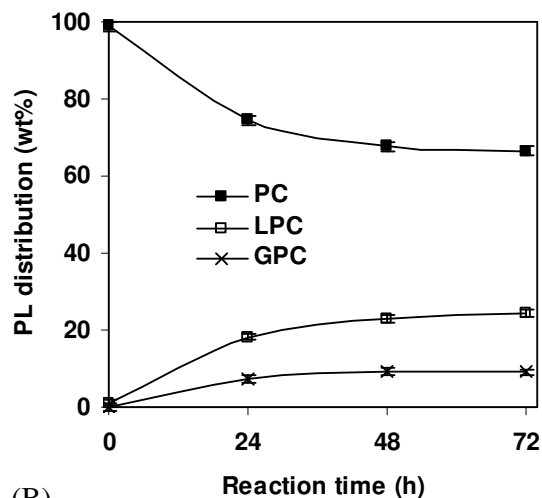
3.3. PLA₂ catalyzed acidolysis reaction

Reactions were performed in a single step, having both hydrolysis and esterification reactions that occur simultaneously. The fatty acids resided in the *sn*-2 position of PLs will therefore be a mixture of original fatty acids and the ones to be incorporated. Theoretically the presence of original fatty acids can be minimized by having high substrate ratio (mol acyl donor/mol PL). A preliminary study was conducted to evaluate incorporation and PL distribution during the time course of acidolysis reaction between PC and caprylic acid. Reaction conditions selected were a

substrate ratio of 6 mol/mol caprylic acid/PC, together with 30% enzyme dosage at 40°C. Some water was added to the reaction mixture (0.75%), as this enzyme requires some water to main activity (Adlercreutz et al., 2003).



(A)



(B)

Fig. 4. Time course for acidolysis reaction between PC and caprylic acid in solvent free system. Reaction conditions: substrate ratio, 6 mol/mol caprylic acid/PC, water addition, 0.75%; dosage of immobilized enzyme, 30 wt%; and reaction temperature, 40°C. A) Incorporation of caprylic acid into PC and B) PL distribution. Bars represent mean \pm pooled SD (n=2).

The results showed that, after 72h, it was possible to have 15% incorporation of caprylic acid into PC (Fig. 4A). However with increasing incorporation, the recovery of PC decreased. Complexity of the acidolysis reaction makes it difficult to predict the influence of different parameters on incorporation and PL distribution. A statistical experimental design was therefore set up with the assistance of response surface methodology (RSM) to evaluate the influence of individual parameters, as well as their interactions, on incorporation and PL distribution. Reaction temperature, substrate ratio and water addition were selected as variables, whereas enzyme dosage and reaction time were held constant in the current study. From Fig. 4B it can be observed that with a reaction time more than 48h there was only seen a small progress in the reaction.

Table 4 Settings of the RSM generated experimental design for the PLA₂ catalyzed acidolysis and measured responses.

Experiment no.	Factors			Responses ^a			
	T _e	W _a	S _r	Inc.	PC	LPC	GPC
1	35	1	6	8.0	70.0	22.1	7.9
2	55	1	6	5.9	72.1	19.8	8.1
3	35	3	6	28.3	20.6	62.4	17.0
4	55	3	6	32.9	24.1	64.9	11.0
5	35	1	12	11.3	62.4	28.1	9.5
6	55	1	12	7.2	76.0	16.8	7.2
7	35	3	12	32.1	22.4	63.8	13.8
8	55	3	12	28.6	25.1	58.3	16.6
9	25	2	9	9.8	54.8	40.2	5.0
10	65	2	9	3.1	74.0	19.4	6.6
11	45	0	9	0.6	90.0	3.0	7.0
12	45	4	9	30.5	17.7	65.0	17.3
13	45	2	3	25.3	29.2	62.3	8.5
14	45	2	15	35.0	25.2	56.2	18.6
15	45	2	9	33.5	30.7	56.4	12.9
16	45	2	9	35.9	28.7	58.3	13.0
17	45	2	9	33.5	30.3	60.4	9.3

Abbreviations: T_e, Reaction temperature (°C); W_a, water addition (wt% based on total substrate); S_r, substrate ratio (mol Caprylic acid/mol PC), Inc., Incorporation of caprylic acid (mol%), PC, phosphatidylcholine content; LPC, lysophosphatidylcholine content; GPC, glycerophosphorylcholine content. ^aValues reported for the PL distribution are based on weight percentages of PC + LPC+ GPC

From a process point of view it would be desirable to have as low a reaction time as possible. Responses and variable settings in Table 4 were fitted to each other with multiple regressions. The best-fitting models were determined by multiple regression and backward elimination, whereby insignificant factors and interactions were removed

from the models. The statistics for the model coefficients and probability values for response variables are presented in table 5.

Table 5 Regression coefficients and P-values describing the influence of different parameters on incorporation of caprylic acid into PC and PL distribution^a.

Term	Incorporation of caprylic acid into PC (mol%)		PL distribution (wt%)					
			PC		LPC		GPC	
	Regression coefficient	P-value	Regression coefficient	P-value	Regression coefficient	P-value	Regression coefficient	P-value
Constant	32.38	9.97×10^{-10}	28.37	1.45×10^{-9}	58.72	3.58×10^{-12}	12.91	9.68×10^{-7}
Te	-2.07	0.06	5.22	1.52×10^{-4}	-4.81	6.95×10^{-4}	-0.40	0.62
Wa	8.50	5.32×10^{-6}	-19.38	8.81×10^{-10}	16.55	1.32×10^{-8}	2.83	5.13×10^{-3}
Te x Te	-6.51	7.83×10^{-6}	8.83	2.01×10^{-7}	-7.18	4.10×10^{-6}	-1.65	0.03
Wa x Wa	-4.33	2.38×10^{-4}	6.19	5.34×10^{-6}	-6.16	1.59×10^{-5}	-0.03	0.97
Te x Wa	1.87	0.22	-3.72	0.02	3.60	0.03	0.12	0.92

^aValues reported for the PL distribution are based on weight percentages of PC + LPC+ GPC. The effect of each factor (linear and quadratic) and interaction effects are statistically significant when $P\text{-value} < 0.05$.

The coefficient of determination (R^2) of the models were 0.95, 0.99, 0.98, 0.67 for the four responses, i.e. incorporation into PC, PC content, LPC content and GPC content, respectively. Models with acceptable qualities should have $R_2 > 0.8$. Most of models therefore represent real relationship between responses and the reaction parameters. According to the analysis of variance there was no lack of fit for the generated models. Observed and predicted values were sufficiently correlated except for experiment no.1, which was treated as an outlier.

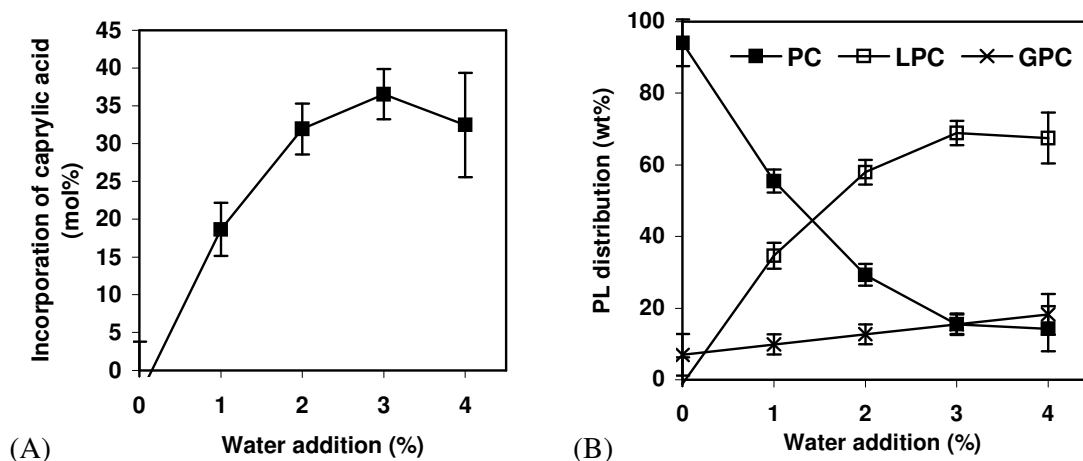


Fig.5. Effect of water addition on PLA₂ catalyzed acidolysis reaction when varied from low to a high level with all other factors being on their average. A) Incorporation of caprylic acid into PC and B) PL distribution. Error bars indicate 95% confidence interval.

Water addition was the most significant factor on the PLA₂ catalyzed acidolysis reactions in terms of incorporation and recovery (table 5). A continuous increase in the incorporation was observed until water level of 2% (Fig. 5A). Higher water addition had no significant effect on incorporation. The recovery of PC decreased with increased water addition (Fig.5B). With increase of water in the reaction system both LPC and GPC increased. GPC forms if acyl chain of LPC molecule migrates from the *sn*-1 position to the *sn*-2 position, and the formed 2-acyl LPC is hydrolyzed by PLA₂. It was previously demonstrated that water content had no effect on the incorporation in solvent-free system during lipase-catalyzed acidolysis reaction (Vikbjerg et al., 2005), which is in contrast to PLA₂ catalyzed acidolysis reaction. With both types of enzyme, the recovery of PC decreases with increasing water content due to parallel hydrolysis reaction. Water seems to have a complex role in terms of compromising enzyme activity, hydrolysis side reactions, reaction rate, and extent of incorporation. As PLA₂ require a higher water activity to function as compared to lipases, the yield is expected to be lower (Adlercreutz et al., 2003).

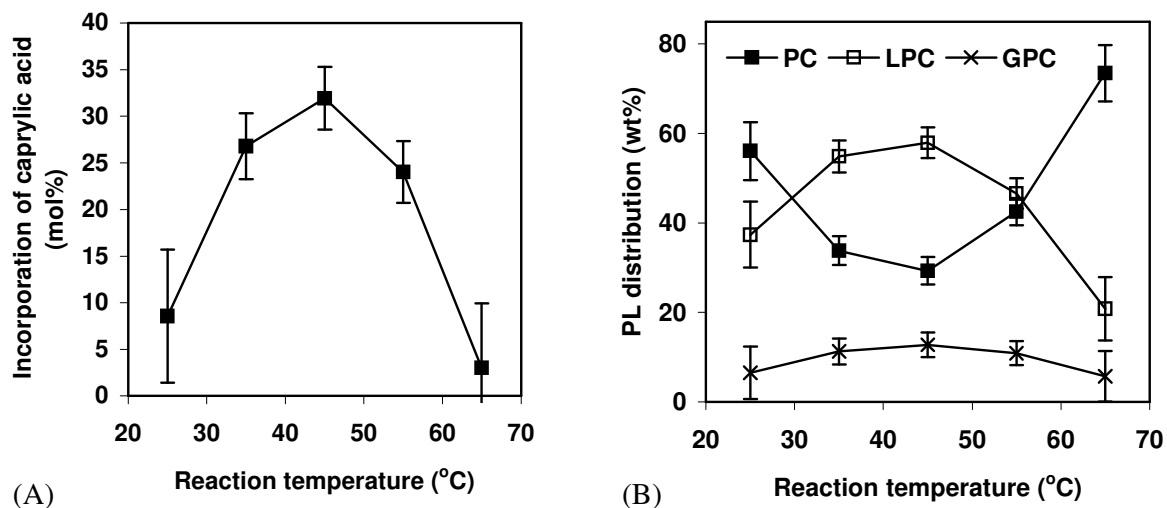


Fig.6. Effect of reaction temperature on PLA₂ catalyzed acidolysis reaction when varied from low to a high level with all other factors being on their average. A) Incorporation of caprylic acid into PC and B) PL distribution. Bars indicate 95% confidence interval.

Reaction temperature also had significant effect on the acidolysis reaction. Maximum incorporation was observed at 45°C (Fig.6A). At higher and lower temperatures there was a decrease in the incorporation of caprylic acid into PC. The lowest yield was obtained at 45°C (Fig.6B). At higher and lower temperatures PC content increased. In general an increase in temperature increases the rate of all

chemical reactions, including those catalyzed by enzymes, but at the same time it increases the rate of denaturation of enzyme protein. These processes probably explain the characteristic temperature profile of PLA₂ and high value for the second order value in the models. Park et al. (2000) examined the effect of reaction temperature on transesterification of PC and ethyl esters of EPA in toluene, and found that maximum reaction rate and yield were at 50°C. Enzyme activity was observed to drop sharply above 50°C. Egger et al. (1997) reported that during synthesis of PC from LPC highest reaction rate was observed at 40°C. At this temperature there was however observed a decrease in the amount of PC and LPC during the enzymatic reaction. This decrease was found to be due to formation of GPC. It was claimed that at this high temperature GPC formation occurred due to acyl migration. In this study the temperature had an effect on formation of GPC. Highest content of GPC was at 45°C. With higher LPC content in reaction system formation of GPC seems to increase especially at elevated temperatures.

Substrate ratio had no significant effect on either incorporation of caprylic acid or the PL distribution, and no interaction was seen for this factor. Even though no differences are seen in the relative PL distribution, it should be remembered that the PL concentration is higher at lower substrate ratios. In terms of production it would be recommended to have low substrate ratio.

Highest incorporation was obtained by having reaction temperature, 45°C; water addition 2%; and substrate ratio, 9 mol/mol caprylic acid/PC. Under these conditions the PC accounted for 29% of the PL fraction. The incorporation of caprylic acid into LPC was also examined, however was less than 4% for all samples (data not shown), and therefore no attempts were made to model these data.

3.4. Reactivity of different fatty acids

Different fatty acids may be applied as acyl donor for acidolysis reaction. However the fatty acids usually result in different reactivity, due to fatty acid specificity or possible inhibition effects. Under the same conditions, different fatty acids often result in different incorporation into PLs or different yields. Reaction rates have been reported to be the same for saturated fatty acids of length between 6 and 12 carbon atoms, but they were lower for myristic and palmitic acids (Egger et al. 1997). Highest reaction rate was obtained with oleic acid, but higher degree of unsaturation resulted in lower reaction rates. In this study we compared the incorporation of DHA and CLA with that of caprylic acid under similar reaction conditions (Te, 45°C; Wa, 2%; Sr, 3 mol/mol fatty acid/PL). The incorporations of the different fatty acids into PC are presented in table 1. CLA resulted in the highest degree of incorporation, followed by caprylic acid and DHA. PLA₂ showed little discrimination toward the two main isomers

of CLA (data not shown). With CLA as acyl donor the PL distribution after reaction was 21, 74, and 5% for PC, LPC and GPC, respectively. With DHA as acyl donor the PL distribution was 22, 77, and 1% for PC, LPC and GPC respectively. Yields were thus lower when using CLA and DHA as acyl donors, however the formation of GPC was also lower as compared to reactions performed with caprylic acid (see table 4, experiment no.13). The results indicate that caprylic acid may cause more acyl migration in the reaction system compared to DHA and CLA, however further experiments would be required to verify this observation.

In conclusion PC with modified fatty acid profile can be produced by PLA₂ catalyzed acidolysis. Water addition and reaction temperature were shown to have significant effect on both incorporation and yield. Both reaction temperature and water addition had an inverse relationship between incorporation and recovery of PC. Substrate ratio showed no effect on the PL distribution. Incorporation of caprylic acid into PC could reach 36% accounting for 29% of the PL fraction. Incorporation of new fatty acids was shown to depend on acyl donor. Polyunsaturated fatty acids DHA and CLA were incorporated into PC with 30 and 20%, respectively.

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PAPER VII

Title: Application of ultrafiltration membranes for purification of structured phospholipids produced by lipase-catalyzed acidolysis

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Application of ultrafiltration membranes for purification of structured phospholipids produced by lipase-catalyzed acidolysis

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Abstract

The possibilities of applying ultrafiltration for the purification of structured phospholipids (PLs) produced by lipase-catalyzed acidolysis in a hexane system were examined. Commercial polymeric membranes with different cut-offs (1000–20,000 Da) were screened for their abilities to separate free fatty acids (FFA) from structured PLs. Suitable membranes were selected in terms of high selectivity between FFA and PLs. Several membranes showed to be able to reject more than 90% of phosphatidylcholine (PC), however, based on the solubility parameters of the polymers many of the membranes would not be suitable for long term with the solvents in use. One membrane was more stable with the solvents compared with the other membranes screened; it was a polysulphone (PSf) membrane on polyester (PE) support (GR70PE). GR70PE showed similar retention of PC as that of few other membranes, but showed relatively higher retention of FFA, resulting in lower selectivity. Increased pressure increased the retention of both PC and FFA, however, the selectivity was improved. With a discontinuous diafiltration process (11 batches) using GR70PE, it was possible to change the molar ratio between PC and FFA from 1:48 to 1:1.6. The results of this study show that membrane separation may be a promising route for downstream processing of structured PLs.

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Keywords: Structured phospholipids; Polymeric membrane; Free fatty acids; Diafiltration

1. Introduction

Ultrafiltration membranes can be considered as porous membranes where rejection is determined mainly by the size and shape of the solutes relative to the pore size in the membrane and where the transport of solvent is directly proportional to applied pressure [1]. Most of the industrial applications of pressure-driven membrane processes are related to aqueous solutions. However, there is an enormous potential field for the application of ultrafiltration in non-aqueous systems, provided that there are commercially available membranes and modules that are suitable for applications involving organic solvents. Especially within lipid technology, the interest for membrane applications has increased in recent years. The major applications of membrane technology in oil and fat technology include recovery of solvent from micella, waste water treatment, separation in

degumming, refining and bleaching, condensate return, catalyst recovery [2–4] and hydrolysis of oils and fats [5,6] or syntheses of glycerides with two-phase membrane reactors [7].

The removal of phospholipids (PLs) is the first step of crude vegetable oil refining process (known as degumming). PLs are surfactants in nature and form reverse micelles with an average molecular weight (MW) of 20,000 Da or more in non-polar media like hexane or natural oil [8]. Membrane degumming processing has been applied to remove PLs from crude oil/hexane mixtures as well from crude oil itself without the addition of organic solvent [9–16]. Different membrane materials have been used for the degumming such as polyamide (PA), polysulphone (PSf), polyvinylidene fluoride (PVDF), and polyimide (PI). Generally, all of these polymers are well known for their good thermal and chemical resistances [1].

PLs are valuable by-products from the production of edible oils. Due to the amphiphilic nature of PLs they have wide applications in foods, pharmaceuticals, and cosmetics where they function as emulsifiers and liposome formers. Naturally occurring PLs consist of mixtures of long chain fatty acids. However, for certain applications there may be an interest in totally or

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partially replacing original fatty acids with others not naturally occurring. Replacement of existing fatty acids in original PLs results in changed physical and chemical properties. Lipases and phospholipases provide powerful tools for the synthesis of structured PLs with specific fatty acid composition [17]. Until now, the reaction mechanisms have been the main focus. Little effort has been made to purify these products after reaction. For analytical purposes, the preparative HPLC and solid phase extraction have been applied; however, for large scale application this would not be sufficient.

In this study, we looked into the possibilities of applying polymeric ultrafiltration membranes to remove free fatty acids (FFA) and solvent from the reaction mixture after lipase-catalyzed production of structured phosphatidylcholine (PC). Since PLs form reverse micelles with large molecular weight in hexane, it was expected that the separation of FFA from PLs would be possible similar to membrane degumming where PLs are separated from triglycerides.

2. Experimental

2.1. Materials

Soybean PC (Epikuron 200, purity 93%) was obtained from Degussa Texturant Systems Deutschland GmbH & Co. KG (Hamburg, Germany). The fatty acid composition (mol%) of PC can be seen in Table 1. Caprylic acid (C8:0, purity 97%) was purchased from Riedel-de-Haen (Seelze, Germany). Lipozyme

Table 1

Fatty acid distribution in soybean phosphatidylcholine and structured phosphatidylcholine (mol%)

Fatty acids	PC	Structured PC ^a
C8:0	0.0	37.8
C16:0	12.8	4.12
C18:0	3.9	1.2
C18:1	9.4	7.0
C18:2	65.8	44.9
C18:3	8.1	5.0

^a Reaction conditions during production: 75 g substrate in 125 ml hexane; substrate ratio, 15 mol/mol caprylic acid/PL; water content, 0.01 g/g substrate; enzyme dosage, 0.3 g Lipozyme TL IM/g substrate; reaction temperature, 55 °C; reaction time, 24 h.

TL IM, a silica granulated *Thermomyces lanuginosa* lipase was donated by Novozymes A/S (Bagsvaerd, Denmark). All membranes were donated by Alfa Laval A/S (Nakskov, Denmark). The details of the membranes are listed in Table 2. All solvents and chemicals used were of analytical grade.

2.2. Production of structured phospholipids

Structured phospholipids were produced by lipase-catalyzed acidolysis reaction between soybean PC and caprylic acid in the presence of hexane as described previously [18]. Seventy-five grams of substrate was mixed with 125 ml hexane in 250 ml blue cap bottles. Reaction conditions were as follows: substrate ratio (mol/mol caprylic acid/PC), 15 mol/mol; enzyme dosage, 30%

Table 2
Membrane screened and their characteristics^a

Membrane specifications					Conditioning, treatment ^b	Solvent flux (kg/m ² h)			Selectivity ^c		
Membrane designation	Selective layer	Support layer	Cut-off ^d (MW)	Membrane state		Water	Ethanol	Hexane	%R _{FFA}	%R _{PC}	α _{FFA/PC}
GR70PE	PSf	PET	20,000	Dry	A	36	27	No flux	–	–	–
					B			436	12	92	11.2
GR90PP	PSf	PP	2,000	Wet	A	66	52	No flux	–	–	–
					B			No flux	–	–	–
GR81PP	PSf	PP	10,000	Dry	A	8	22	No flux	–	–	–
					B			28	5	87	7.4
GR61PP	PSf	PP	20,000	Dry	A	No flux	219	207	n.d.	n.d.	n.d.
					B			273	5	19	1.2
Hekla 10A	PSf ^e	PP	10,000	Dry	A	23	22	No flux	–	–	–
					B			No flux	–	–	–
ETNA01PP	PVDF ^e	PP	1,000	Dry	A	43	26	51	2	92	12.4
					B			No flux	–	–	–
ETNA10A	PVDF ^e	PP	10,000	Dry	A	76	45	32	2	92	12.3
					B			4	n.d.	n.d.	n.d.
ETNA20A	PVDF ^e	PP	20,000	Dry	A	130	97	327	10	83	5.5
					B			717	n.d.	n.d.	n.d.
FS 61PP	PVDF	PP	20,000	Wet	A	767	445	585	14	19	1.1
					B			158	n.d.	n.d.	n.d.

Abbreviations: PSf, polysulphone; PVDF, polyvinylidene fluoride; PP, polypropylene; PET, polyethyleneterephthalate.

^a Operating conditions: temperature (20–25 °C); pressure, 0.3 MPa; stirring rate, 250 rpm.

^b Treatment: A, solvent flux measured in the following order: water, ethanol, and hexane with no pre-treatment; B, ethanol soaking 30 min prior to measurement of hexane flux.

^c n.d., not determined, (–) no permeation through the membrane.

^d MWCO values of membranes reported by supplier.

^e Hydrophilic coated.

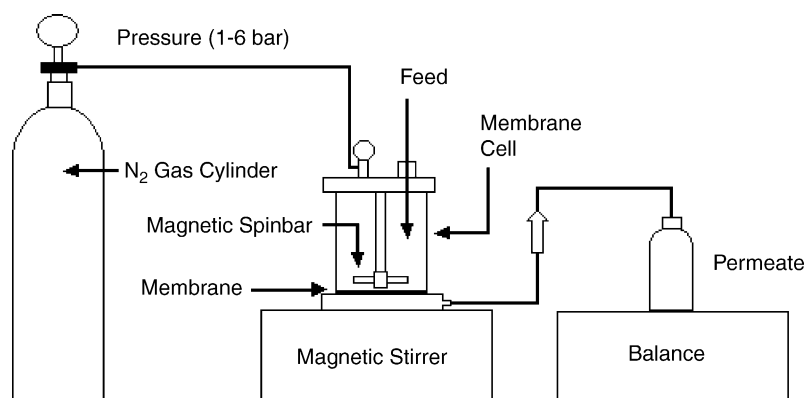


Fig. 1. Membrane apparatus used for the purification of structured phospholipids produced by lipase-catalyzed acidolysis in a hexane system.

(based on substrate); temperature, 55 °C; water addition, 1% (based on substrate); and reaction time, 24 h. Reactions were conducted in a water bath with magnetic stirring at 300 rpm. After the reaction, the samples were centrifuged at 4000 rpm for 5 min in order to remove the immobilized enzyme. Supernatants were collected and stored at –20 °C prior to ultrafiltration and analysis. All analyses were performed in duplicate, and mean values are reported.

2.3. Membrane apparatus

A stirred dead-end ultrafiltration cell with magnetic stirrer (Millipore, Glostrup, Denmark) was used for the separation of FFA and solvent from the reaction mixture. Pressurized nitrogen gas provided the driving force for the permeation. Experimental setup can be seen in Fig. 1. Cell's capacity was 300 ml with an effective membrane area of 40 cm². Membrane screening experiments were conducted at room temperature (20–25 °C) and pressure was kept at 0.3 MPa unless otherwise stated. All experiments were done at a constant rotation speed of the spin bar (250 rpm). Permeate was collected through a port beneath the membrane support. Membrane screening was conducted by charging the cell with 50 g feed (lipid/solvent mixture); each trial was continued until 20 g permeate was collected. Samples of feed, retentate, and permeate were analyzed for PC and FFA. Membrane retention (%R) was calculated as:

$$\%R = \frac{C_f - C_p}{C_f} \times 100 \quad (1)$$

where C_f and C_p are concentrations in feed and permeate, respectively. Selectivity factor (α) between FFA and PC was calculated as:

$$\alpha_{\text{FFA/PC}} = \frac{C_{p,\text{FFA}}}{C_{p,\text{PC}}} \quad (2)$$

2.4. Fatty acid composition analysis

Analytical separations were performed on Silica Gel 60 thin-layer plates (20 cm × 20 cm, Merck, Darmstadt, Germany). After the development in chloroform/methanol/water (65:35:5, v/v/v), the plate was sprayed with 0.2% of 2,7-dichlorofluorescein in ethanol (96%), making the lipid bands

visible under UV-light. The lipid bands were scraped off and transferred to test tubes with tight screw caps. One milliliter of 0.5 M NaOH in methanol was added to each tube and placed at 80 °C for 5 min. Then, 1 ml 20% BF₃ in methanol and 0.5 ml 0.5% hydroquinone in methanol were added and placed at 80 °C for 2 min. Two milliliters of 0.73% NaCl solution was added followed by 1 ml heptane. The upper phase was transferred to a new tube. One milliliter of saturated salt solutions was added to the new tube. After mixing and phase separation, the upper phase was taken for GC analysis. The methyl esters were analyzed on a HP6890 series gas-liquid chromatograph (Hewlett-Packard, Waldbronn, Germany) equipped with a flame-ionization detector (FID), as described elsewhere [18].

2.5. Phospholipid analysis

Samples were applied to Chromarod SIII (Iatron Laboratories Inc., Tokyo, Japan) and developed in a mixture of chloroform/methanol/water (42:22:3 v/v/v). PC, lysophosphatidylcholine (LPC), and glycerophosphorylcholine (GPC) concentrations were analyzed by thin-layer chromatography coupled to a flame-ionization detector (TLC-FID) (Iatrosan MK6s, Iatron Laboratories, Tokyo, Japan). Peaks were identified by external standards. Standard curves between weight and area (FID response) were constructed for PC.

2.6. Free fatty acids content

FFA content in feed, permeate, and retentate were determined with standard alkali titration using phenolphthalein indicator [19].

2.7. Analysis of membrane molecular weight cut-off

Solute rejection was measured at 0.3 MPa with solution containing polyethylene glycol (PEG) with varying size (MW 400–35,000 Da). Feed concentration of each PEG was 0.2 wt%. Permeate and feed concentrations were measured by HPLC equipped with a refractometer. Molecular weight cut-off (MWCO) curves for ultrafiltration membranes were constructed as described by Jonsson [20].

3. Results and discussion

3.1. Production of structured phospholipids for membrane screening

Reaction conditions for the lipase-catalyzed production of structured PC in this study were selected based on the recommended optimal conditions as described previously [18]. One percent water was added to the reaction mixture as we have observed that the reaction rate increases and thus higher incorporation can be obtained with shorter reaction time (data not shown).

For membrane screening, the structured PC was prepared with a reaction time of 24 h. Several batches were performed and the reaction mixtures were pooled and used for the subsequent membrane screening. Prolonged reaction time may result in higher incorporation, however, it was considered that the yield was too low (Fig. 2). The fatty acid distribution of the final product can be seen in Table 1. Seventy-seven percent of the PLs consisted of PC, where the remaining LPC and GPC. It was observed that the formation of LPC and GPC could not explain the loss of PC, which is caused by their precipitation in the reaction mixture as described previously [21]. Concentrations of PC and FFA in the final reaction mixture were 0.04 and 1.9 M, respectively (molar ratio, 1:48).

3.2. Membrane screening

Characteristics of the commercial membranes employed in this study are shown in Table 2. Practical and theoretical considerations for membrane selection are considered below. Suitable membranes should be resistant to the solvents in use to avoid swelling or even solubilization. From previous publications on degumming, it seems that membranes made of PVDF and PSf have been among the most successful [16] and were, therefore, the primary choice of membranes selected for the screening.

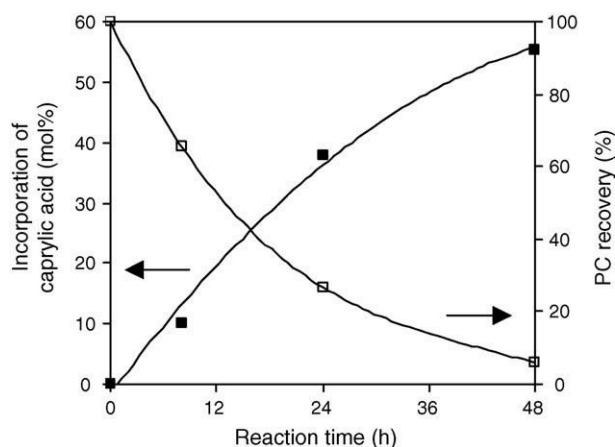


Fig. 2. Time course for the acidolysis reaction between PC and caprylic acid in hexane. For reaction conditions see Table 1.

Table 3

Solubility parameter data^a for selected solvents and polymers at 25 °C [1,22,23]

	δ_d	δ_p	δ_h	P_r
Solvent				
<i>n</i> -Hexane	14.9	0	0	–
Caprylic acid	15.1	3.3	8.2	–
Ethanol	15.8	8.8	19.4	–
Water	15.5	16.0	42.3	–
Polymer				
PP	16.6	–2.3	1.0	11.7
PET	19.1	6.3	9.1	4.8
PSf	18.4	4.7	5.5	7.0
PVDF	13.7	10.6	8.2	14.7

^a δ parameters and P_r are expressed in MPa^{1/2}. For abbreviations see Table 2.

3.2.1. Solvent–membrane interaction

In order to determine the polymer solubility in different solvents, the three-dimensional model proposed by Hansen [23] can usually be applied:

$$\delta^2 = \delta_d^2 + \delta_p^2 + \delta_h^2 \quad (3)$$

where δ_d is the solubility parameter due to dispersion forces; δ_p , the solubility parameter due to polar forces; and δ_h is the solubility parameter due to hydrogen bonding. The solubility parameter may be considered as a vector in a three-dimensional space. A solvent with given values of δ_{ds} , δ_{ps} , and δ_{hs} is represented as a point in space, with δ being the vector from the origin to this point. Polymers are characterized by a set of Hansen parameters (δ_{dp} , δ_{pp} , and δ_{hp}), and an interaction radius P_r . The distance $P^s r$ of the point representing the solvent from the center of the polymer solubility sphere is calculated as:

$$P^s r^2 = 4(\delta_{dp} - \delta_{ds})^2 + (\delta_{pp} - \delta_{ps})^2 + (\delta_{hp} - \delta_{hs})^2 \quad (4)$$

For solubility to be predicted, the value of P_r has to be less than $P^s r$. Even solvents located close to the Hansen's solubility sphere will have some swelling effect, thereby changing the pore sizes and solvent permeability. Hansen solubility parameters for selected polymers and solvents are presented in Table 3 and corresponding $P^s r$ values are presented in Table 4. From calculation, PVDF would not be stable in neither hexane nor caprylic acid as $P^s r < P_r$. The support layers of majority of the tested membranes were prepared from polypropylene (PP), which will not be stable with hexane or caprylic acid as well. GR70PE membrane consists of a PSf membrane on a polyethyleneterephthalate (PET) support. From the above discussion, the selective layer as well as the support layer of this membrane is stable in the presence

Table 4

$P^s r$ value calculated for different polymer materials and solvents

Polymer	Solvent			
	Hexane	Caprylic acid	Ethanol	Water
PP	4.2	9.6	21.6	45.2
PET	13.9	8.6	12.5	37.9
PSf	10.1	7.3	15.4	38.9
PVDF	13.6	7.8	12.1	34.7

For abbreviations see Table 2.

of hexane and caprylic acid as their P^S_r values are higher than the P_r values. These calculations demonstrate that the GR70PE membrane is less soluble in the solvents compared with the other membranes screened, and are therefore considered more suitable for long-time usage.

3.2.2. Membrane flux and retention

Results from the membrane screening can be found in Table 2. Even though it could be calculated that some of the membranes would solubilize in hexane and caprylic acid, then for most of the membranes, it was possible to collect a permeate with proper conditioning. The commercial membranes provided for this study were either in dry or wet state. In general, most ultra-filtration membranes are stored under wet conditions because structure of membrane changes when subjected to a drying procedure. For membranes in dry state, glycerol is usually filled into the pores of the membrane prior to drying to prevent changes of the porous structure. At the time of use, the dry membrane usually needs to be contacted with water or alcohol to flush out the glycerol, and rewet the membrane. Membranes were tested without preconditioning, but no permeation of the hexane–lipid mixture occurred (data not shown). Two different approaches were therefore employed for conditioning the membranes (see Table 2). In most cases, one of these treatments allowed the subsequent membrane separation of the hexane–lipid mixture. Preferred conditioning of PSf membranes was by soaking in ethanol, and for PVDF membranes it was by flushing with water and ethanol.

The permeate flux of the hexane–lipid mixture decreases in general with reducing MWCO (Fig. 3). For all membranes, the flux was stable with up to 40% (20 g) removal of the feed. ETNA01 and ETNA10A have high retention of PLs and low retention of FFA, and were also the membranes with the highest selectivity. With ETNA20A and ETNA01PP membranes, higher hexane flux was observed compared to water and ethanol flux; however, this was not observed with ETNA10A. However, the flux of water and ethanol increases with the increase in MWCO for these membranes. ETNA20A gave high permeate flux, however, the selectivity was lower compared to the other ETNA membranes.

With the PSf membranes (GR membranes) on a PP support, it was observed that the hexane flux increased with the increase in MWCO; however, the PL retention also decreased. PSf membrane on PET support (GR70PE) had higher hexane flux and a considerable higher selectivity compared to PSf membrane on PP support with similar MWCO.

Other membranes were tested as well, however, they either showed no hexane flux or had very low PL retention (Table 2 and Fig. 3). For the purpose of membrane degumming, comparison of the permeability of water and the permeability of solvent has been conducted to examine the presence of solvent–membrane interactions in various membranes [13]. If the ratio between hexane and water permeabilities is low, it has been claimed that high degree of solvent–membrane interaction exists since pore space shrinkage phenomenon may infer with such observations. For several membranes tested, there was no hexane flux through the membrane under the tested conditions. This could probably

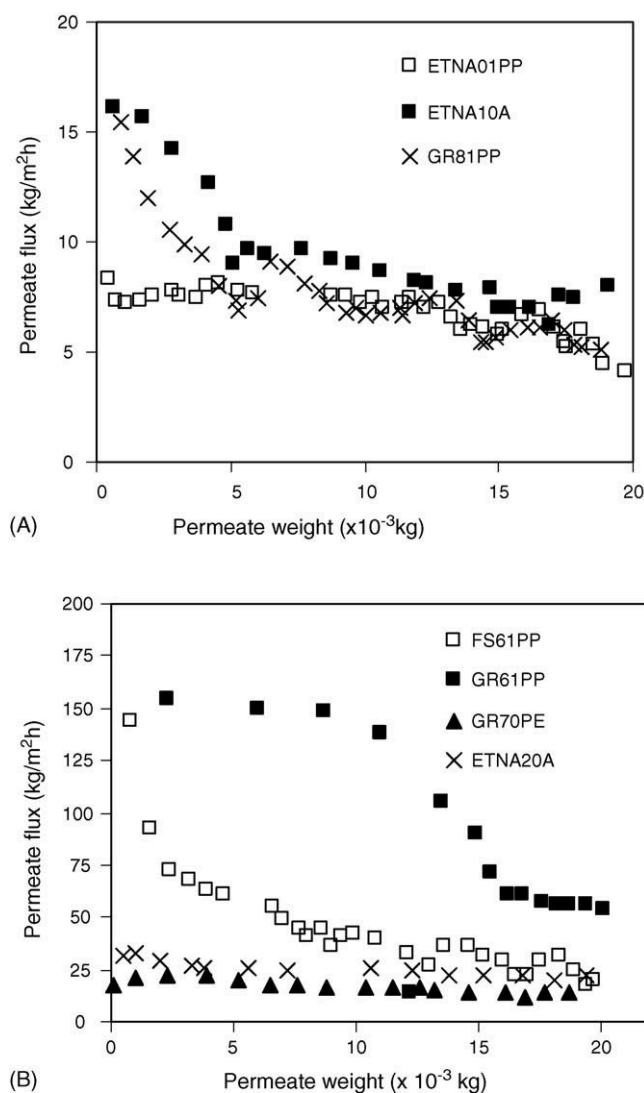


Fig. 3. Permeate flux for the different membranes screened vs. collected permeate weight during membrane filtration. (A) MWCO \leq 10,000 Da; (B) MWCO = 20,000 Da. For operating conditions see Table 2.

be explained by solvent–membrane interactions. However, with visual inspection of the membranes after use there could not be observed any change in the membrane surface structure.

The retention of PC was similar for ETNA01PP and GR70PE even though GR70PE had MWCO that was 20 times higher according to the supplier. From the other membranes, the retention significantly decreased with the increase in the MWCO. Cut-off values are often defined in different ways under different test conditions which makes it difficult to compare results. When different types of solutes with the same molecular weight are considered (e.g., branched versus linear flexibly molecules), different rejection characteristics can be observed as a function of the molecular weight. Solute retention was measured with different MW PEG for selected membranes with $\%R_{PC} > 90\%$ (Fig. 4). For GR70PE it could be observed that the MWCO was between 3000 and 8000 Da, for ETNA01PP it was between 1000 and 3000 Da, and for ETNA10A it was between 2000 and 8000 Da. Under these conditions, the cut-offs of GR70PE

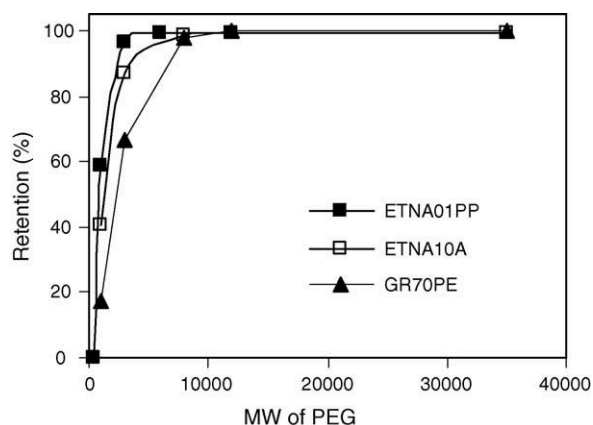


Fig. 4. Solute retention of different membranes at different MW of PEG.

and ETNA10A were lower than stated by the supplier. From these results, it seems that the membrane cut-off in the range of 1000–8000 Da would be suitable for the separation of FFA from structured PL containing caprylic acid.

From all above considerations of interactions, flux, retention, and selectivity, GR70PE was found to be a suitable membrane and was selected for further study for the separation of FFA from structured PLs.

3.3. Influence of the applied pressure

Flux of pure hexane through GR70PE was measured at different pressures (Fig. 5). Above 0.3 MPa compaction of the membrane was observed, and therefore the applied pressure should be limited to this level to avoid irreversible deformation of the membrane. In Fig. 6 the relationship between permeate flux of the hexane–lipid mixture and applied pressure is depicted. At higher pressure the permeate flux was higher as expected. During filtration the permeate flux gradually decreased being more significant at high pressure. Flux through the membrane is highly dependent on the concentration of solutes, and decrease

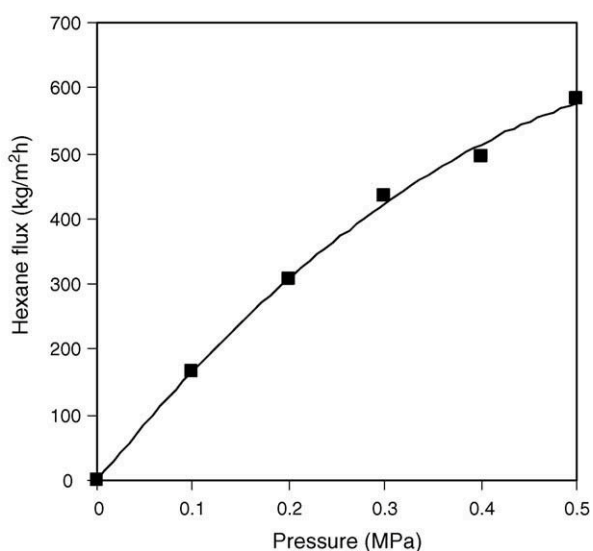


Fig. 5. Hexane flux through GR70PE membrane at different applied pressure.

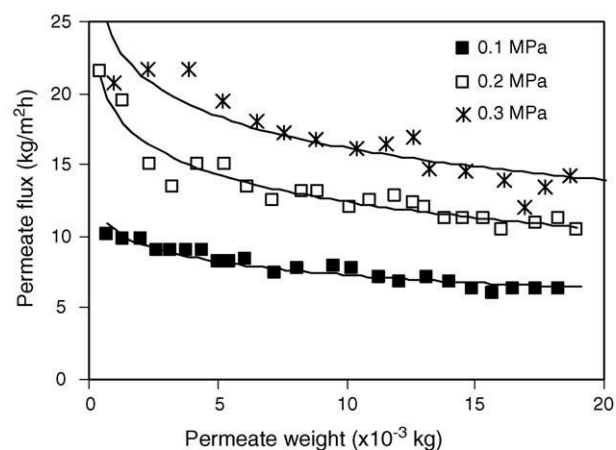


Fig. 6. Permeate flux through GR70PE membrane at different applied pressure.

with volumetric concentration factor. The accumulation of PLs and increase of retentate viscosity at high volumetric concentration factors lead to decrease in permeate flux. The retention of both PC and FFA was observed to increase with increase in pressure (Table 5). In order to have good separation as well as good retention of PC, the strategy was to operate at higher pressures as a larger selectivity was obtained. However, multiple steps, especially with dilution of retentate to minimize the viscosity and fouling as above discussed will be required for a better separation.

3.4. Discontinuous diafiltration

A complete separation between FFA and PLs cannot be achieved with the design given in Fig. 1. With the above discussed strategy, discontinuous diafiltration was applied with GR70PE membrane. Retentate was diluted continually with solvent (hexane) during permeation so the FFAs were washed out. Membrane cell was charged with 50 g of hexane–lipid mixture and after 20 g of feed had permeated through the membrane, the cell was depressurized and an equal weight of hexane was added to the retentate before the unit was restarted. Discontinuous diafiltration was conducted in 11 steps (Fig. 7). During each diafiltration, it could be observed that the flux gradually decreased. With addition of more solvent, the flux increased again. Diafiltrations with ETNA01PP and ETNA10A were also performed, however, the permeate fluxes did not increase with the addition of more hexane after each concentration step as seen for GR70PE (data not shown). ETNA membranes are therefore not recommended for purification of structured PC as the sepa-

Table 5
Change in FFA and PC retention and selectivity with applied pressure for GR70PE membrane

Pressure (bar)	% R_{FFA}	% R_{PC}	$\alpha_{\text{FFA/PC}}$
1	3.2	70.6	3.3
2	8.4	86.6	6.8
3	11.5	92.1	11.2

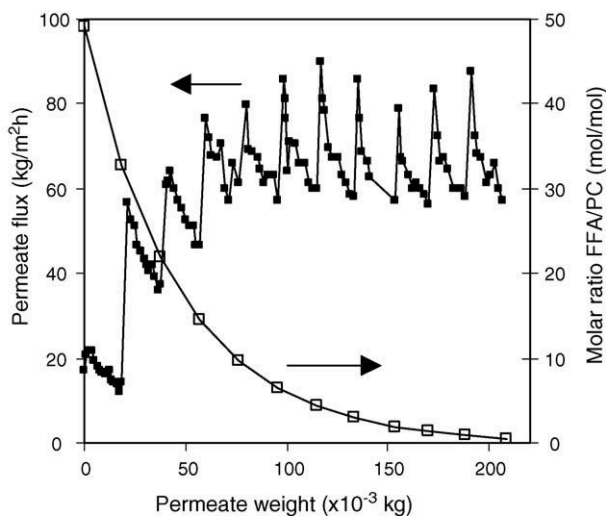


Fig. 7. Changes in permeate flux and molar ratio between FFA and PC during discontinuous diafiltration using GR70PE membrane.

ration would take very long time due to continuous irreversible decrease in the permeate fluxes.

Usually the relationship between the volume and concentration can be explained by the following equation during ultrafiltration:

$$\frac{C_r}{C_f} = \left(\frac{V_f}{V_r} \right)^R \quad (5)$$

where C_r is the retentate concentration; C_p , the permeate concentration; V_r , the retentate volume; V_f , the feed concentration; and R is the retention. Assuming that R is constant for GR70PE (0.92 and 0.12 for PC and FFA, respectively) during diafiltration, a predicted permeate concentration can be calculated for each batch collected (Fig. 8). The measured permeate concentration follow the trend of the predicted values; however, a perfect fit was not obtained which illustrates that the retention is probably not constant throughout the diafiltration.

The concentrations of PC and FFA in the final retentate were measured to be 0.03 and 0.05 M, respectively. With the discontinuous diafiltration, it was possible to change the molar ratio

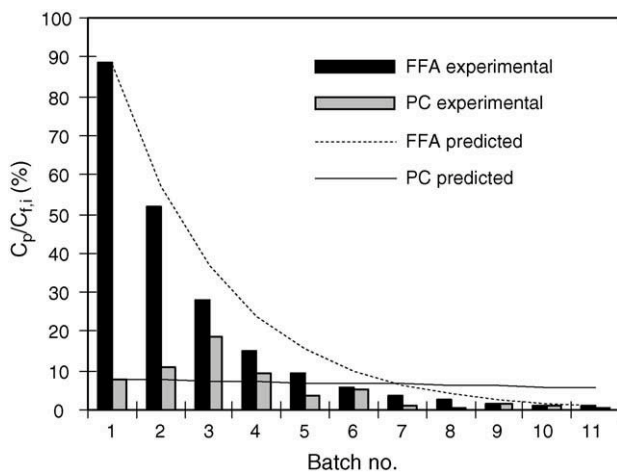


Fig. 8. Changes in permeates concentration during diafiltration using GR70PE.

between PC and FFA from 1:48 to 1:1.6. The results clearly demonstrate that diafiltration can be used for purification of structured PLs. However, further improvements of the method are required in order to have a feasible process. The PL distribution between PC, LPC, and GPC was not significantly changed by this procedure (data not shown), and alternative separation techniques are required in order to separate PC from LPC and GPC in the mixture.

4. Conclusion

The results from this study indicate that membrane filtration could be an effective method for FFA separation from structured PLs after lipase-catalyzed acidolysis reaction between PLs and FFA. The knowledge of PLs forming reverse micelles in non-polar solvents can be applied to other areas besides degumming of vegetable oils. Several membranes showed the ability to separate PLs and FFA, and the diafiltration operation could provide a good alternative compared to other ways of purification. Membranes selected should preferably be stable in hexane and free fatty acids. In most cases, we were able to collect a permeate; however, the long-term stability for many of these membranes screened are not known even though solvent–membrane interaction show certain aspects from the calculation. From this study, a PSf membrane with PET support showed some good qualities in terms of flux and selectivity. To improve PL retention and permeate flux, further work will be conducted in our laboratory. Information from this study can help the further progress in developing membrane reactors, where reaction and separation could be conducted simultaneously.

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PAPER VIII

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Strategies for lipase-catalyzed production and the purification of structured phospholipids

This work provides different strategies for the enzymatic modification of the fatty acid composition in soybean phosphatidylcholine (PC) and the subsequent purification. Enzymatic transesterification reactions with caprylic acid as acyl donor were carried out in continuous enzyme bed reactors with a commercial immobilized lipase (Lipozyme RM IM) as catalyst. Operative stability of the immobilized lipase was examined under solvent and solvent-free conditions. The long reaction time required to have a high incorporation, combined with rapid deactivation of the enzyme, makes the solvent-free transesterification reaction unfavorable. Performing the reaction in the presence of solvent (hexane) makes it possible to have high incorporation into PC and deactivation of the lipase is less pronounced as compared to solvent-free operations. For solvent-free operation, it is suggested to recycle the reaction mixture through the packed bed reactor, as this would increase incorporation of the desired fatty acids, due to increased contact time between substrate and enzyme in the column. Removal of free fatty acids from the reaction mixture can be done by ultrafiltration; however, parameters need to be selected with care in order to have a feasible process. No changes are observed in the phospholipid (PL) distribution during ultrafiltration, and other techniques as column chromatography may be required if high purity of individual PL species is desired. LC/MS analysis of transesterified PC revealed the presence of 8:0/8:0-PC, showing that acyl migration takes place during the acidolysis reaction.

Keywords: Enzyme bed reactor, transesterification, Lipozyme RM IM, structured phospholipids, ultrafiltration.

1 Introduction

Structured phospholipids (PL) with a defined fatty acid profile can be manufactured by enzyme-catalyzed synthesis based on the selective/positional recognition of different phospholipases and lipases. The aim to alter the existing fatty acids in original PL are to improve chemical and physical properties to meet particular functional requirements. In order to make the production of structured PL commercially feasible, it is essential to develop effective bioreactors and processes. Many kinds of enzyme bioreactors have been used for the modification of oil and fat, such as batch reactors, packed-bed reactors (PBR), and membrane reactors [1]. The advantages of the various types of available enzyme reactors can be exploited more readily by using immobilized enzymes.

Several different commercial immobilized lipases have been used for acyl modification of PL; however, comparison of the catalytic activity is rather complicated as the lipase specific-

ity towards different fatty acids and the temperature optimum may differ. A screening of different immobilized lipases showed that *Thermomyces lanuginosa* lipase (TLL) had a higher activity compared to *Rhizomucor miehei* lipase (RML) and *Candida antarctica* lipases (CAL) during lipase-catalyzed acidolysis between soybean lecithin and caprylic acid under solvent-free conditions [2]. However, in the case where the acidolysis reaction was performed with PC and conjugated linoleic acid (CLA) in the presence of hexane, RML resulted in higher incorporation as compared to TLL and CAL [3]. During the acidolysis reaction between PC and palmitic or stearic acids in heptane, the highest incorporation of the acyl donor was obtained with CAL as compared to TLL [4]. For the esterification reaction between 2-acyl lysophosphatidylcholine (LPC) and fatty acids from fish oil in toluene, higher incorporation was achieved with CAL as compared to RML [5]. No general conclusion can be made as to which immobilized enzyme would perform better for acidolysis reactions. However, each of the lipases has been shown to be superior over the others in certain reaction systems.

In a practical operation of a reaction system, the formation of by-products from hydrolysis and acyl migration can be a serious problem. Incorporation of the desired fatty acids

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into PL and the recovery are known to be affected by enzyme load, reaction time, reaction temperature, water content, substrate ratio, and solvent amount. The influence of temperature and substrate ratio seems to depend on the particular reaction system. In a solvent system using TLL, the incorporation of the desired acid increased with increasing temperature (35–55 °C) and substrate ratio (3–15 mol/mol) [6], whereas in a solvent-free system using RML, incorporation decreased with increase of these parameters in a similar range [7]. Clearly, optimization must be individually performed in each case.

Due to the high dosage requirements of immobilized enzyme during PL transesterification reactions, problems with agitation occur and separation from the product after reaction is not easily done during solvent-free batch operation. The presence of solvent would improve mixing in the system and would make the subsequent removal of enzyme more convenient. Increasing the amounts of solvent was previously reported to reduce the recovery of PL more strongly than it increased fatty acid incorporation during batch operation [6]. If possible, it is recommended that the reaction should be conducted solvent free. For larger-scale production, it is desirable to conduct the reaction in a PBR as this allows the continuous operation. The PBR has been applied to the production of structured PL from the reaction between deoiled soybean lecithin and caprylic acid with TLL as catalyst [8]. Due to the zwitterionic nature of PL, it was speculated that water might be stripped from the enzyme in the PBR, resulting in reduced catalytic activity. Addition of water to a solvent-free system also showed to increase the transacylation rate. Purified soybean PC is more hydrophilic as compared to deoiled lecithin, and to our knowledge there exists no information whether this can be used as substrate during continuous operation.

Equally important as the reaction is the subsequent purification. Membrane technology is developing rapidly in the oil industry to supplement or replace conventional separation processes. The main advantages of the membrane technology are energy saving and better product quality. Micelle-enhanced ultrafiltration has been successfully applied by several researchers for the degumming of vegetable oils [9, 10]. In nonpolar solvents, PL tend to form reverse micelles with a molecular weight of 20,000 or more, which can be separated from oil and solvent by using appropriate membranes. Recently, we reported the possibilities of applying ultrafiltration for the removal of free fatty acids (FFA) from PL after production of structured PL [11]; however, no attempt was made to optimize the filtration process.

In the present work, we provide different processing strategies for the production and purification of structured PL, which include several steps as illustrated in Fig. 1.

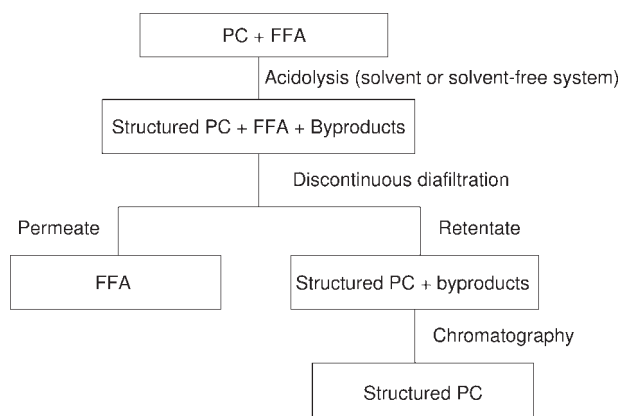


Fig. 1. Preparation of structured PL containing caprylic acid at the *sn*-1 position. FFA, free fatty acids; PC, phosphatidylcholine.

Important issues for the different processes are addressed, and future aspects for enzyme-catalyzed production of structured PL are considered.

2 Materials and methods

2.1 Materials

PC (Epikuron 200, purity 93%) was obtained from Degussa Texturant Systems Deutschland GmbH & Co. KG (Hamburg, Germany). The fatty acid composition of PC (mol-%) was 16:0 (12.8%), 18:0 (3.9%), 18:1 (9.4%), 18:2 (65.8%), 18:3 (8.1%). Caprylic acid (8:0, purity 97%) was purchased from Riedel-de-Haen (Seelze, Germany). Lipozyme RM IM, an immobilized *sn*-1,3-specific lipase from *Rhizomucor miehei*, and Lecitase Novo, a *Fusarium oxysporum* phospholipase A₁, were donated by Novozymes A/S (Bagsvaerd, Denmark). Membrane GR70PE (GR: polysulfone, PE: polyester) was donated by Alfa Laval A/S (Nakskov, Denmark). Silica gel 60 (particle size 0.035–0.070 mm) was purchased from Fluka Chemie GmbH (Buchs, Switzerland). All solvents and chemicals used were of analytical grade.

2.2 Enzyme stability during continuous operation

Enzyme stability was followed during the acidolysis reaction between PC and caprylic acid under solvent and solvent-free conditions. The bioreactor was a jacketed stainless-steel column (l = 200 mm, i.d. = 21 mm) packed with 25 g Lipozyme RM IM (l = 180 mm). Substrate molar ratio was 6 and 10 mol/mol caprylic acid/PC during solvent-free and solvent system operation, respectively. For the solvent system the proportion of hexane to substrate

Tab. 1. Incorporation of caprylic acid and PL distribution during Lipozyme RM IM-catalyzed acidolysis reaction between PC and caprylic acid in PBR with total recycle ($R = \infty$).^{§,§}

Water addition [%]	Reaction temperature [°C]	Reaction time [h]	Incorporation [mol-%]			PL distribution [mol-%]		
			PC	<i>sn</i> -2 pos [‡]	LPC	PC	LPC	GPC
0	55	24	27.3 ± 0.6		44.6 ± 1.6	69.2 ± 0.6	19.5 ± 0.8	11.3 ± 1.4
		48	31.8 ± 0.0		51.1 ± 0.7	54.8 ± 2.9	19.6 ± 0.8	25.6 ± 3.7
0	55 (2. batch)	24	9.1 ± 0.1		40.7 ± 0.1	67.8 ± 4.9	11.9 ± 0.3	20.2 ± 5.2
		48	12.6 ± 1.3		46.5 ± 0.0	68.5 ± 0.9	15.4 ± 1.8	16.1 ± 0.9
0	40	24	23.3 ± 1.0		28.4 ± 1.8	77.2 ± 0.2	13.3 ± 0.3	9.5 ± 0.1
		48	32.6 ± 0.7		40.3 ± 2.3	71.4 ± 0.4	17.6 ± 0.4	11.0 ± 0.1
		72	36.0 ± 0.1	1.4 ± 0.1	46.2 ± 0.5	67.1 ± 0.7	21.2 ± 0.7	11.6 ± 0.0
		120	36.6 ± 1.7	6.4 ± 1.0	52.7 ± 0.8	58.4 ± 0.1	24.8 ± 0.0	16.8 ± 0.0
3	55	24	30.5 ± 0.9		42.0 ± 2.7	37.4 ± 3.0	25.1 ± 1.3	37.5 ± 4.3
		48 [#]	37.4 ± 0.3	5.3 ± 0.2	55.1 ± 1.3	34.7 ± 0.7	29.1 ± 0.3	36.2 ± 1.0

[§] Reaction conditions: substrate molar ratio, 6 mol/mol caprylic acid/PC; flow rate, 3.5 mL/min.

[§] Data presented are mean values ± standard deviations of double determinations.

[‡] Fatty acid composition (mol-%) at the *sn*-2 position after enzymatic hydrolysis with Lecitase Novo.

[#] PL species in reaction mixture were purified by diafiltration and column chromatography.

was 7.5 mL/g. The substrate mixture was fed upward into the column, and the column temperature was held constant at 40 °C by a circulating water bath. The reaction substrates were pumped through the enzyme reactor by a pump from Fluid Metering Inc. (New York, NY). Flow rates were adjusted to 0.1 and 0.4 mL/min during solvent-free and solvent system operation, respectively. Substrates were passed through the column for several days, and samples were collected continually. The amount of PC treated was ~3 g/h and ~1 g/h for the solvent-free and solvent system, respectively.

2.3 Recycle operation for PBR

Reactions were carried out as acidolysis reactions between PC and caprylic acid under solvent-free conditions. The bioreactor was a jacketed stainless-steel column ($l = 300$ mm, i.d. = 21 mm) packed with 37 g Lipozyme RM IM ($l = 280$ mm). The reaction mixture coming out of the PBR was returned to the substrate reservoir, thus having a recycle ratio $R = \infty$ (R is defined as volume of fluid returned to substrate reservoir per volume leaving the system). The flow rate through the column was 3.5 mL/min. Other conditions were as described above. The initial substrate volume was 100 mL, and the enzyme void was estimated to be 68 mL. Samples were collected continually.

2.4 Removal of FFA by diafiltration

Structured PC produced under solvent-free conditions (see Tab. 1) were separated from FFA in a stirred dead-end ultrafiltration cell with magnetic stirrer (Millipore, Glostrup,

Denmark). Pressurized nitrogen provided the driving force for the permeation. The cell capacity was 300 mL with an effective membrane area of 40 cm². GR70PE (polysulfone membrane on polyester support) was used for the ultrafiltration process. The membrane was soaked in ethanol prior to filtration. Membrane separations were conducted at ambient temperature and pressure was kept at 3 bar. Permeate was collected through a port beneath the membrane support. Initially, the cell was charged with 100 g feed (30 or 40 wt-% reaction mixture in hexane). Of permeate, 30 or 40 g was collected, and new hexane was added into the ultrafiltration cell to reach the starting volume. Addition of more solvent was done in order to improve the permeation rate, since the flux is seen to continuously decrease with concentration factor. Nine batches of permeate were collected with subsequent addition of hexane to the retentate. The experiment was thus performed as a discontinuous diafiltration. Total volumes of hexane added during the nine steps were 410 and 550 mL when 30 and 40 g permeate was collected in each step, respectively. Samples of feed, retentate and permeate were analyzed for PC and FFA content.

2.5 Separation of PL species by column chromatography

In order to separate PC from LPC, glycerophosphorylcholine (GPC) and small amounts of FFA, the product was purified by column chromatography. A column was packed with 30 g silica, and the lipid species were eluted with two different solvent systems. Chloroform/methanol/water (65 : 35 : 5, vol/vol/vol) was used to

separate FFA, PC and LPC. GPC was eluted from the column with methanol/water (90 : 10, vol/vol). Fractions of 10 mL were collected. The samples were analyzed, PC-containing fractions were pooled, and the solvent was evaporated followed by lyophilization.

2.6 Fatty acid composition analysis

Fatty acid methyl esters were prepared by BF_3 methylation and analyzed on a HP6890 series gas-liquid chromatograph (Hewlett-Packard, Waldbronn, Germany) equipped with a flame ionization detector (FID) [7]. The fatty acid distribution at the *sn*-2 position of PC was determined by Lecitase Novo (phospholipase A_1) hydrolysis followed by isolation and methylation of the resulting LPC as described elsewhere [7].

2.7 FFA content

FFA content in feed, permeate and retentate from the ultrafiltration process was determined by Official and Recommended Practice of American Oil Chemists' Society (AOCS) method Ca 5a-40 (1998).

2.8 Analysis of the PL profile by TLC-FID

PL profile analysis was performed on product mixtures from the acidolysis reactions using thin-layer chromatography coupled with flame ionization detection (TLC-FID). Samples were spotted onto silica gel chromarods (Chromarod SIII; Iatron Laboratories Inc., Tokyo, Japan) and developed in a mixture of chloroform/methanol/water (42 : 22 : 3, vol/vol/vol). After developing, the chromarods were dried at 120 °C for 5 min. The chromarods were then placed into the TLC-FID analyzer (Iatroskan MK6s; Iatron Laboratories Inc.) and scanned at a rate of 30 s/rod. Flow rates of 160 mL/min for hydrogen and 2 L/min for air were used during analysis. Peaks were identified by external standards. TLC-FID data were calculated into molar concentrations based on average molecular weights of the PL species determined by GC.

2.9 Molecular species analysis of structured PC by API-ES LC/MS

The products and the PL standard mixture were separated on a silica column ($l = 15$ cm, i.d. = 4.6 mm, particle size = 5 μm ; Phenomenex). The column was fitted into an HP 1100 Series LC/MSD system, consisting of a quaternary pump, a vacuum degasser, an autosampler, and an MS detector (Hewlett-Packard). A binary solvent system of chloroform/methanol/ammonium acetate (90 : 10 : 0.5,

vol/vol/vol) and chloroform/methanol/water/ammonium acetate (60 : 35 : 5 : 0.5, vol/vol/vol/wt) was used. API-ES was used in the negative mode. The capillary and fragmentor voltages were 4000 and 250 V, respectively. The nebulizer gas pressure was 25 psi. The heated nitrogen drying gas temperature and flow rate were 350 °C and 10.0 L/min, respectively. Full mass spectra were taken in the mass range of 50–1000, and the step size was 0.1 m/z . System control and data evaluation were conducted by using HP ChemStation.

3 Results and discussion

Even though lipases may show a good performance in terms of reactivity, the stability of the enzyme carrier also needs to be considered. Commercially silica-granulated TLL can easily be removed from the reaction medium in the presences of solvent during batch operation; however, in solvent-free systems, the immobilized lipase is not easily removed. During reaction conducted with magnetic stirring, the silica granulates are crushed and do not easily precipitate in the solvent-free system due to the highly viscous reaction medium. In a previous study, it was therefore decided to use immobilized RML for optimization of the lipase-catalyzed acidolysis reaction during solvent-free batch operation, as the mechanical stability of the carrier for RML is considerably higher and thus more easily recovered from the reaction mixture [7]. Under optimal conditions, up to 46% incorporation of caprylic acid into PC was made during that study.

In this study, we used a PBR for the production of structured PL from high-purity PC with Lipozyme RM IM (RML) as catalyst, to examine the possibilities of continuous operation under solvent and solvent-free conditions similar to work done with silica-granulated TLL using deoiled lecithin as substrate [8]. During continuous operation in the PBR, the stability of the silica-granulated carrier material for TLL could be maintained. The conversion degree was very low when having solvent-free conditions for the Lipozyme TL IM-catalyzed acidolysis without the addition of water. In the presence of solvent, a considerably higher conversion degree could be obtained and maintained for several days. RML and TLL have both been demonstrated to be able to incorporate high levels of caprylic acid into PL, but to our experience, RML performs slightly better when purified PC is used as substrate (data not shown).

3.1 Enzyme stability during continuous operation

Data obtained from batch operation can be used to determine the enzyme dosage and flow rate required for the PBR. Assuming the same reaction rate and conver-

sion degree in batch reactor and PBR, the following equation can be used to describe the relationship between the two types of reactors [12]:

$$\frac{w_b}{V_b} t = \frac{w_p}{F_p} \quad (1)$$

where w_b is the enzyme dosage during batch operation, V_b is the amount of substrate during batch operation, t is the reaction time during batch operation, w_p is the amount of enzyme in the PBR, and F_p is the flow rate through the PBR. Batch operation is usually performed over several days together with high enzyme dosages [7], implying that a long residence time is required in the PBR.

3.1.1 Stability in solvent-free system

Several compromises concerning the reaction parameters are required in order to have good performance [7]. The most critical parameter to control during reaction is the water content. With a higher water content in the reaction system, especially the formation of GPC increases. Incorporation of novel fatty acids was previously shown not to be influenced by water addition [7]. With increased substrate ratio, the incorporation into PC decreased in the range 3–15 mol/mol FFA/PC. In this work, we used a 6-molar excess which would give a theoretical conversion of 86% (or 43% incorporation) at equilibrium. With a lower substrate ratio, the theoretical conversion would decrease. At a higher substrate ratio, the reaction rate is decreased and purification of the product becomes increasingly more difficult. Flow rate through the PBR was adjusted to the lowest possible (0.1 mL/min), and stability was followed over a few days. The residence time in the column was ~7 h.

Fig. 2 shows the incorporation of caprylic acid and the PL distribution during solvent-free operation in the PBR. Incorporation was seen continuously to decrease over time, and PC content increased over time. These results indicate that the catalytic activity of the lipase decreased during running time. It is not uncommon during lipase-catalyzed reactions in a packed bed to see a gradual decrease in the conversion degree over time [13]. A problem like this is overcome by gradually increasing the residence time, which was not possible in this study. During lipase-catalyzed production of structured triacylglycerols in a PBR, an increase in water content in the substrate can in some cases help regain the initial activity of the enzyme [14]. High PL concentrations are known to slow down the acidolysis and interesterification reaction of triacylglycerols. The effect of PL on the activity and stability of Lipozyme RM IM in organic media during batch operation was found to be very crucial [15]. PL have been reported to be

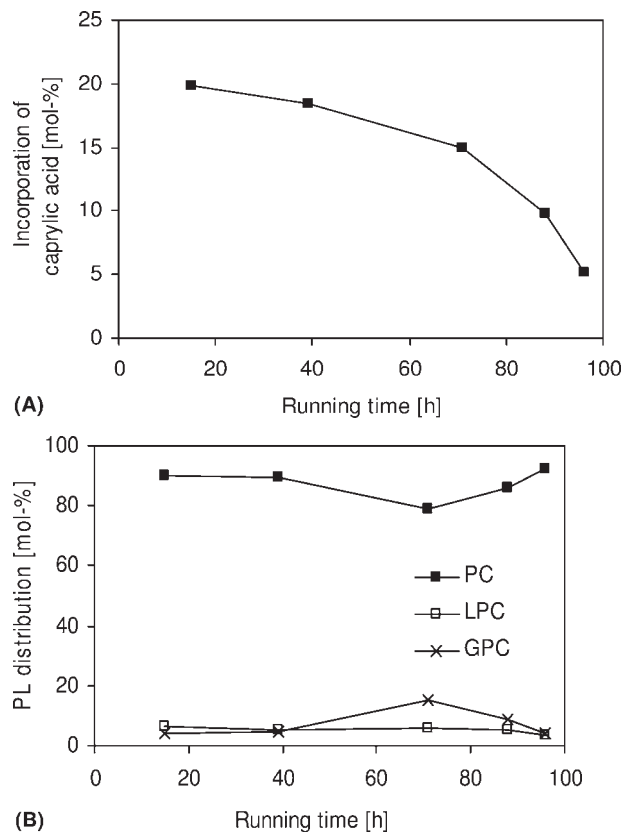


Fig. 2. Operative stability of Lipozyme RM IM during solvent-free conditions. (A) Incorporation of caprylic acid (mol-%) and (B) PL distribution (mol-%). Reaction conditions: substrate ratio, 6 mol/mol caprylic acid/PC; reaction temperature, 40 °C; flow rate, 0.1 mL/min. GPC, glycerophosphorylcholine; LPC, lysophosphatidylcholine; PC, phosphatidylcholine.

totally absorbed by the enzyme bed during the first couple of days. The enzyme bed reactor retains the polar or complex compounds, depending on the hydrophobicity of the substrates. When shorter-chain length fatty acids are used as acyl donors less retaining of the polar compounds would be expected. Compared to lipase-catalyzed reactions with triacylglycerols as substrate, the reaction rate is considerably slower and deactivation is more rapid with PL as substrate. The long reaction time required for the PL acidolysis, combined with the rapid loss of activity, makes continuous operation for solvent-free systems very difficult. The yield was high, showing that hydrolysis was minimal in the system.

3.1.2 Stability in solvent system

Operative stability of the immobilized lipase was also examined in a solvent system (Fig. 3). A decrease was also observed in the incorporation of novel fatty acids in

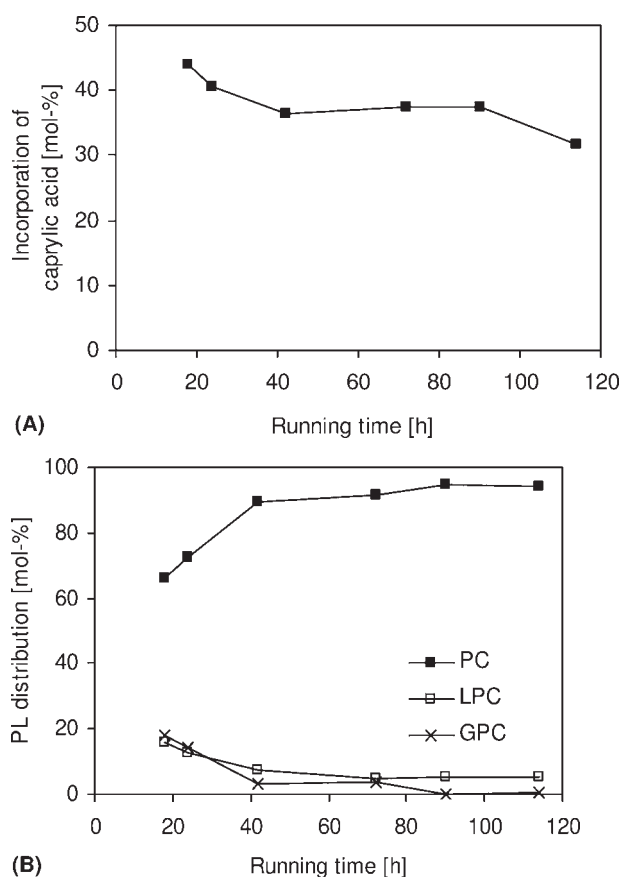


Fig. 3. Operative stability of Lipozyme RM IM in solvent system. (A) Incorporation of caprylic acid (mol-%) and (B) PL distribution (mol-%). Reaction conditions: substrate ratio, 10 mol/mol caprylic acid/PC; reaction temperature, 40 °C; solvent ratio, 7.5 mL/g hexane/substrate; flow rate, 0.4 mL/min. For abbreviations see Fig. 2.

the presence of solvent during running time, but to a lower extent than under solvent-free conditions. The solvent system seems to provide a good choice for the acidolysis reaction, as high incorporation and yields are achieved. Activity of the lipase is maintained for a longer time in the presence of solvent as compared to the solvent-free conditions. Looking at the relative distribution of PC, LPC and GPC, the recovery of PC is usually very high during batch operation; however, looking at the actual concentration of PL in the reaction mixture, a low recovery is usually seen [7]. These low yields could be explained by the binding of substrate to the enzyme carrier. It seems that running the reaction in a PBR with solvent is quite beneficial, as the recovery of PC is considerably higher as compared to batch operation (data not shown). The reason for this dramatic increase in recovery needs to be explored in more detail in order to give a good explanation for this phenomenon. These data imply that there is potential in larger-scale continuous production of the structured PL.

3.2 Recycle operation for solvent-free system

Surely, lipases can be used to exchange the fatty acids in PL; however, continuous operation of enzymatic acidolysis in a solvent-free system does not represent a practical manufacturing route. In order to have high incorporation of the acyl donor into PC under solvent-free conditions, batch operation still seems to be the best solution. PBR have the advantage, though, that the reaction mixture can simply be pumped out, whereas for the batch operation it needs sedimentation of the immobilized enzyme prior to collecting the reaction mixture for purification. A simple way to increase the conversion degree in the PBR under solvent-free conditions is to recycle the reaction mixture through the packed bed, as the contact time between substrate and enzyme column would increase. When the cycle ratio is raised, the operation shifts from continuous ($R = 0$) to resemble that of batch ($R = \infty$) [16]. PBR with total recycle ($R = \infty$) was examined for the production of structured PL under solvent-free conditions. Tab. 1 lists the different substrate mixtures prepared, together with the temperature settings during the reactions. Other parameters used during reactions are based on previous recommendations from batch operation [7].

As expected, the incorporation into PC and by-product formation in the PBR with total recycle were similar to results previously obtained for batch operation [7]; however, some differences were observed. Individually, water seemed not to have any effect on the incorporation during batch operation, but interactions were observed with other parameters [7]. As a general rule, it was better to perform the reaction at lower temperatures; however, with high enzyme dosage, incorporation of the desired fatty acids increased at elevated temperatures.

From Tab. 1 it can be observed that for reactions conducted in a PBR with total recirculation, incorporation of caprylic acid into PC was not significantly different when conducted at 40 or 55 °C after 48 h with no water addition to the substrate. The incorporation of caprylic acid into LPC was, however, higher when the reaction was conducted at 55 °C. In both cases, formation of LPC and GPC was observed, but recovery of PC was significantly higher when conducted at 40 °C. Incorporation of caprylic acid into LPC and GPC formation are direct consequences of acyl migration in the system.

With the addition of water, higher incorporation into PC can be obtained with shorter reaction times, but the recovery of PC was significantly lower. We previously reported that water addition did not affect the incorporation into PC and LPC [7], but from this study it seems that increased water addition increases the incorporation into PC when conducted at 55 °C. Due to several reactions (hydrolysis, esterification, and acyl migration) happening

simultaneously and interactions between different reaction parameters, the prediction of incorporation and recovery during the reaction is complicated.

Reusability of the enzyme column was tested; however, the incorporation was very low already after the second batch. Addition of water could probably improve the conversion degree, but probably on the expense of the yield.

Selected samples with high incorporation were furthermore examined for fatty acid distribution in the *sn*-2 position. When the reaction at 40 °C was prolonged to 120 h, the overall incorporation of caprylic acid was not significantly increased as compared to 72 h; however, the occurrence of caprylic acid in the *sn*-2 position was dramatically increased. The PC content was also observed to decrease.

From batch reactions, it was possible to have 46% incorporation of the desired fatty acid; however, regiospecific analysis revealed that 20% of the fatty acids were found in the *sn*-2 position [7]. When calculating the net incorporation (difference between the overall incorporation and acyl migrated to the *sn*-2 position) it was 37 mol-%. In this study, net incorporation was higher at 72 h when conducted at 40 °C with no water addition compared to the reaction performed at 55 °C for 48 h with 3% water added to the substrate. However, with increasing reaction time the net incorporation decreased.

3.3 Separation of FFA from structured PL by membrane filtration

Development of convenient methods for the separation of structured PC from FFA is required since traditional PL refining is not possible. Separation of PL from neutral lipids is usually done by acetone extraction. PL have low solubility in acetone and will therefore precipitate in the presence of acetone. Attempts were made to extract the transesterified PC in the solvent-free reaction mixtures from FFA with acetone according to the procedure described by Doig and Diks [17], but no precipitation was observed. It seems that this procedure may not apply for extraction of PL from all neutral lipids. Solubility of PL in acetone may increase with the presence of certain neutral lipids, *e.g.* medium-chain fatty acids.

As an alternative, membrane separation can be applied. We previously screened different ultrafiltration membranes for their ability to separate PL and FFA, and found that a polysulfone membrane on a polyester support (GR70PE) showed some good qualities in terms of flux and selectivity [11]. During that study, the reaction mixture was used directly without further concentration or dilution after the lipase-catalyzed reaction. The acidolysis reaction was directly performed in the presence of hexane.

In this study, we purified the structured PC having the highest net incorporation in the final product from solvent-free recycle operation in PBR (reaction conditions: substrate ratio, 6 mol/mol; water addition, 3%; temperature, 40 °C; enzyme load, 37 g; flow rate, 3.5 mL/min; $R = \infty$). Even though the reaction mixture contained different by-products, it should be considered that these compounds are high-value products themselves in the purified form. The acidolysis reaction is therefore not limited to the production of structured PC, but can also be applied to production of other interesting compounds.

The reaction mixture was dissolved in hexane prior to ultrafiltration. Even though degumming of vegetable oil has been conducted without the presence of solvent, we believe that it is helpful to use solvent to overcome issues with low permeate flux and concentration polarization. The membrane design selected was dead-end operation. Here, all the feed is forced through the membrane, which implies that the concentration of rejected components in the feed increased and, consequently, the permeate quality decreased with time. To overcome this problem, the filtration process was performed as diafiltration. Discontinuous diafiltration was conducted at different initial feed concentrations and concentration to different volumes before new hexane was added (Fig. 4). During the concentration step, the permeate flux continuously decreased, but with dilution of the retentate, the permeate flux was increased again. FFA in the permeate decreased over the nine steps in which the diafiltrations were performed. PL concentrations in the collected permeate fractions were very low, and from calculation retention was more than 99% (data not shown).

From Fig. 4 it is observed that it is better to have a lower feed concentration and to collect smaller fractions of permeate in terms of permeate flux. With the same initial concentration, higher flux is obtained at lower volume reduction in each step. With higher initial concentration, the flux is also lower at a fixed volume reduction. When having a larger volume reduction in each step, this will reduce the number of filtrations. However, the results show that it is more important to collect smaller volumes of permeate in each step compared to the initial feed concentration of the solutes. Highest productivity (mol FFA removed/mol PC h) was observed during diafiltration at low initial feed concentration and with smaller volume reduction during the concentration step (Fig. 5).

Upon a certain concentration, filtrate flux rates become prohibitively slow, and it may take longer to diafiltrate the concentrated sample than it would if the sample were first diluted to reduce the concentration. Even though diafiltration of the diluted sample requires a greater diafiltration volume, the processing time would be less due to the

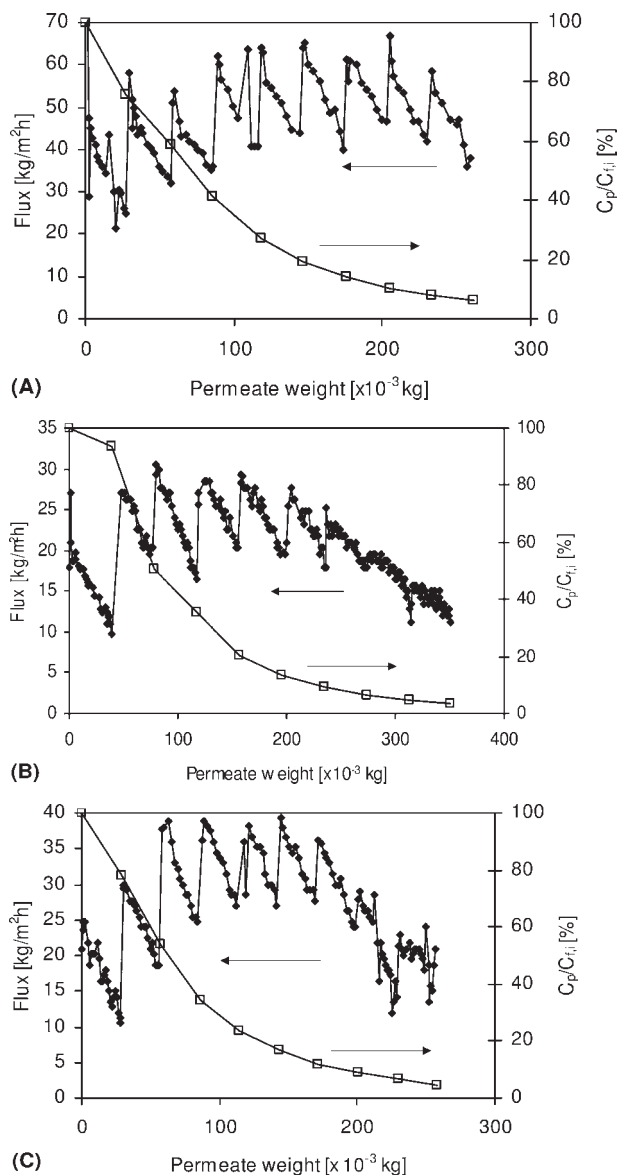


Fig. 4. Changes in permeate flux and FFA concentration in permeate during discontinuous diafiltration using GR70PE membrane at ambient temperature and pressure of 3 bar. (A) Initial feed concentration: 30 wt-% reaction mixture in hexane; permeate collected in each step: 30 g. (B) Initial feed concentration: 30 wt-% reaction mixture in hexane; permeate collected in each step: 40 g. (C) Initial feed concentration: 40 wt-% reaction mixture in hexane; permeate collected in each step: 30 g. $C_{f,i}$, initial feed concentration; C_p , permeate concentration.

faster filtrate flux rate (process time = filtrate flow rate \times volume). The accumulation of retained molecules may form a concentrated gel layer. The impact of gel layer formation is that it can significantly alter the performance characteristics of the membrane. This is commonly called concentration polarization. Fundamentally, the gel layer

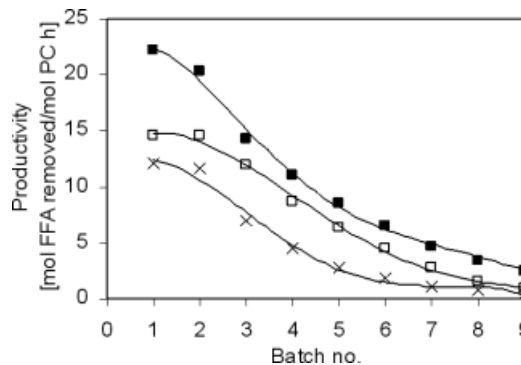


Fig. 5. Productivity of discontinuous diafiltration using GR70PE membrane at ambient temperature and pressure of 3 bar. (■) Initial feed concentration: 30 wt-% reaction mixture in hexane; permeate collected in each step: 30 g. (x) Initial feed concentration: 30 wt-% reaction mixture in hexane; permeate collected in each step: 40 g. (□) Initial feed concentration: 40 wt-% reaction mixture in hexane; permeate collected in each step: 30 g. For abbreviations see Fig. 1.

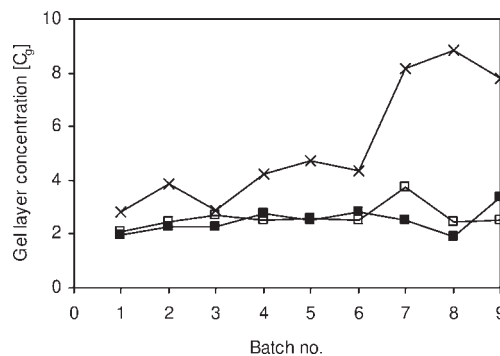


Fig. 6. Changes in gel layer concentration during discontinuous diafiltration using GR70PE membrane at ambient temperature and pressure of 3 bar. (■) Initial feed concentration: 30 wt-% reaction mixture in hexane; permeate collected in each step: 30 g. (x) Initial feed concentration: 30 wt-% reaction mixture in hexane; permeate collected in each step: 40 g. (□) Initial feed concentration: 40 wt-% reaction mixture in hexane; permeate collected in each step: 30 g.

will limit the filtrate flow rate, and any increase in pressure will have no beneficial effect. Gel concentration depends on size, shape, chemical structure and degree of solvation, and may also depend on bulk concentration and cross-flow velocity [18]. The gel layer concentration can be determined by concentrating a sample on a membrane and plotting data for filtrate flux rate vs. log concentration (or concentration factor). The curve can then be extrapolated to filtrate flux rate = 0. The gel layer concentration was observed to be almost constant when the volume reduction was 30% during the filtration step, regardless of the initial feed concentration (Fig. 6). How-

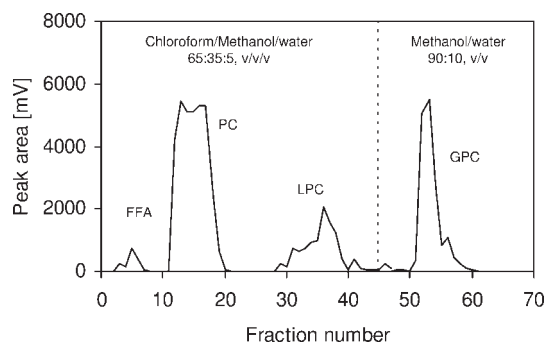


Fig. 7. Separation of lipid species by column chromatography using two different solvent mixtures. Concentrations of lipid species were measured by TLC-FID. For abbreviations see Fig. 2.

ever, when the volume reduction was increased to 40% in each step, a continuous increase in gel layer concentration was seen, and therefore high volume reduction cannot be recommended.

Examining the PL distribution in the retentate, it was observed that it was not significantly different from the initial feed. GPC, LPC and PC are all retained by the membrane (data not shown). In order to have separation of these compounds, additional purification techniques are required.

3.4 Column chromatography

Column chromatography provided a good separation of all PL species, as can be observed from Fig. 7. Some FFA were still present after membrane filtration, but presented no problem due to their low concentration. If only PC is the desired product and no collection of LPC and GPC is required, the solvent systems may be changed after collecting PC. LPC and GPC will elute faster, but will not be separated (data not shown). Column chromatography could probably be used for the separation of FFA from PL, but compared to the ultrafiltration method this requires longer time and larger amounts of solvent to have this kind of separation. The final purity of structured PC was determined by TLC-FID to be 92%. The fatty acid composition of the structured PC (mol-%) determined by GC was 8:0 (36.6%), 16:0 (2.8%), 18:0 (0.7%), 18:1 (5.8%), 18:2 (48.8%), and 18:3 (5.3%).

3.5 Characterization of PC molecular species

The structured PL containing caprylic acid was also separated into molecular species by high-performance liquid chromatography conjugated with mass spectrometry (MS), to provide a complete structural analysis. In

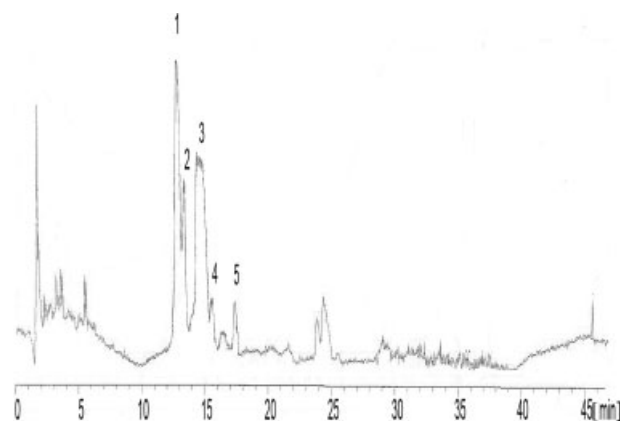


Fig. 8. LC/MS total ion chromatogram of structured PC (according to GC analysis, 37% caprylic acid was incorporated into the soybean PC). Peaks numbered 1–5 represent different molecular species of PC (for identification of individual species see Tab. 2).

natural soybean PC, 16 molecular species have been identified by LC-MS with five species accounting for more than 90 mol-% [18:2/18:3 (9.0%), 18:2/18:2 (42.4%), 18:1/18:2 (11.1%), 16:0/18:2 (21.2%), and 18:0/18:2 (7.0%)] [19]. Mass spectra of structured PC showed the pseudo-molecular ions $[M+Ac]^-$ and $[M+Cl]^-$, and fragment ions $[R_1-1]^-$ and $[R_2-1]^-$ (M, molecular species; Ac, acetate; Cl, chloride; R, acyl groups). For all PC species, fragment ions could be detected at m/z 168 and 224. The caprylic acid-enriched PC was identified to contain different molecular species (Fig. 8, Tab. 2). The main molecular species of the structured PC was 8:0/18:2. Other caprylic acid-containing PC molecules were identified as well (8:0/16:0 and 8:0/8:0). Identification of molecular species 8:0/8:0-PC confirms that acyl migration occurs during production of structured PL as was also demonstrated in Tab. 1.

4 Conclusion

The present paper provides some useful information for the modification of PL. Compared to previous methods described, the yield is considerably higher with a lower excess of acyl donor. Due to the long residence time required in order to have high incorporation, we recommend to conduct the reactions in a PBR with addition of hexane to the reaction mixture. For solvent-free operation, recirculation may be applied in order to have high incorporation. Continuous operation is difficult for the solvent-free system. The long reaction time, combined with rapid deactivation of the enzyme, makes the process not very favorable. Ultrafiltration can be applied for separation of FFA; however, parameters need to be

Tab. 2. LC/MS analysis of structured PC.

Retention time	Species number	Molecular species	[M+Ac] ⁻	[M+Cl] ⁻	[R ₁ -1] ⁻	[R ₂ -1] ⁻
12.8	1	18:2/18:2	840.5	817.5	279.1	–
13.4	2	18:2/16:0	816.5	792.5	279.3	255.2
14.7	3	18:2/8:0	704.5	680.3	279.3	143.1
15.5	4	16:0/8:0	680.3	656.3	255.2	143.1
17.3	5	8:0/8:0	568.2	544.2	143.1	–

Ac, acetate; Cl, chloride; M, molecular species; R₁ and R₂, acyl groups.

selected with care to have a feasible process. Other techniques as column chromatography may be required if high purity of individual PL species is desired, as there are seen no changes in the PL distribution during ultrafiltration. Lipid modifications still have important and promising applications for lipases, regardless of the slow progress made compared to other applications such as the use of detergents.

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PAPER IX

Title: Comparative evaluation of the emulsifying properties of phosphatidylcholine after enzymatic acyl modification
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Comparative Evaluation of the Emulsifying Properties of Phosphatidylcholine after Enzymatic Acyl Modification

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The ability of enzymatically synthesized structured phosphatidylcholine (PC) containing caprylic acid to form and stabilize oil-in-water emulsions prepared with different triglycerides [medium chain triglycerides (MCT), soybean oil, and enzymatically synthesized structured lipids] was examined and compared with natural soybean PC and deoiled lecithin. Emulsions were prepared with varying oil and emulsifier concentrations. The particle size distribution, creaming stability, and viscosity were measured for the evaluation of the emulsifying properties. With an increase in the oil concentration, there was an increase in particle size, viscosity, and creaming layer. With an increase in the phospholipid (PL) concentration, there was usually a decrease in particle size and an increase in viscosity, where the emulsion stability was increased. General emulsions prepared with structured lipids resulted in smaller particle sizes as compared to MCT and soybean oil. Deoiled lecithin was able to increase the viscosity more significantly and give smaller particle sizes as compared to the other emulsifiers, thus producing more stable emulsions. However, in certain cases, structured PC was superior to deoiled lecithin and soybean PC. This observation was made for emulsions prepared with soybean oil or structured lipid at an oil/water ratio of 10:90. At an oil/water ratio of 30:70, the deoiled lecithin performed better as compared to the other PLs with all oil types. However, structured PC produced more stable emulsions as compared to natural soybean PC in MCT and soybean oil.

KEYWORDS: Emulsions; structured phospholipids; triglycerides; particle size; viscosity; stability

INTRODUCTION

Phospholipids (PLs) have been applied in both water-in-oil (w/o) emulsions and oil-in-water (o/w) emulsions for the production of foods, pharmaceuticals, and cosmetics. The ability of PLs to simultaneously interact with water and oil makes it an effective emulsifier. PLs help maintain stable emulsions between miscible liquids. The surface tension between the two liquids is decreased, which allows them to mix and form a stable heterogeneous dispersion. PLs in o/w emulsions are absorbed at the oil droplet surface forming a multilayer lamellar structure, whereas in w/o emulsions, PLs stabilize the emulsion by forming reverse micellar structures (1). For many industrial applications, crude PL products obtained from vegetable oil refining can be used directly; however, usually, it is desired to have some kind of purification. Crude vegetable lecithins contain 30–40% neutral lipids, predominantly triglycerides; the remainder consists of polar lipids, mainly a mixture of different PLs. To improve the handling of the highly viscous crude lecithin and to improve dispersability, industry commonly makes an acetone deoiling (2). Triglycerides (TAGs) dissolve in acetone, in

contrast to the other more polar components of lecithin. With acetone extraction, PLs become more concentrated, which results in significantly lower dosage requirements and higher functionality.

There are various reasons for further purification of the PLs. Pure PLs have specific nutritional and pharmaceutical values, and specific PLs have dedicated surface active properties, which support the processing of emulsion stability and shelf life (3). Phosphatidylcholine (PC)-enriched lecithins have also been reported to deliver superior o/w emulsions capacity as compared to the standard deoiled product (4).

The molecular structure of PLs can be changed by either enzymatic or chemical means. The aim of these processes is to obtain tailor-made technological and/or physiological properties that differ from the natural substrate. Especially, enzymatic modification has gained increasing interest as enzymes can be used to modify PLs in a wide variety of ways. Commercial use to a large extent is known only with phospholipase A₂ (PLA₂) for partial hydrolysis to produce lyso-PLs. Partially hydrolyzed lecithin products possess improved emulsifying properties (2). The higher the degree of hydrolysis, the smaller the droplets are that are generated in a comparative emulsion process (4). Desired PLs with new physical and chemical properties can also be obtained by exchanging fatty acids in PLs. It has been

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Table 1. Fatty Acid Distribution in Oils and PLs

fatty acid composition (mol %)	triglycerides			PLs		
	MCT	soybean oil	structured lipid	deoiled lecithin	PC	structured PC
8:0	60.2		35.0			36.6
10:0	39.8					
14:0			4.2			
16:0		11.4	8.2	19.9	12.8	2.8
16:1			4.9			
18:0		3.4	0.8	4.4	3.9	0.7
18:1		22.8	10.6	10.1	9.4	5.8
18:2		55.6	1.3	59.2	65.8	48.8
18:3		6.9	4.9	6.6	8.1	5.3
18:4			5.1			
20:1			2.5			
20:4			0.7			
20:5			7.7			
22:5			1.1			
22:6			13.0			
degree of unsaturation (mol %)	0	85.2	51.8	75.7	83.3	59.9
average chain length	8.8	17.8	14.9	17.6	17.7	14.3

claimed that C₂–C₁₈ saturated fatty acids may be incorporated to modify emulsification properties, to modify the physiological value, or to improve oxidation stability (5). For emulsions prepared with pure soybean PC, phase separation happens very rapidly; however, it is less pronounced for emulsions containing PC enriched with caproic acid (6). Several attempts have been made over the last two decades for the enzymatic acyl exchange; however, in general, the yield for these reactions has been low. During lipase-catalyzed acidolysis reactions, major byproduct formation usually occurs (7, 8). It should be kept in mind that these byproducts are valuable products themselves as they also have wide applications in the same areas as the original material. These compounds can be purchased in purified form at different companies and are usually sold at considerably higher prices as compared to the natural PLs.

So far, there has been little effort to examine the emulsifying properties of structured PLs. The primary objective of the current study was to investigate the emulsifying characteristics of the synthesized structured PL containing caprylic acid using various triglycerides and to compare it with deoiled lecithin and purified soybean PC. Chain length and degree of saturation of oil and PLs are known to have a significant effect on the emulsion prepared (9). TAGs with considerable different fatty acid profile were thus selected for this study. These TAGs included MCT, soybean oil, and enzymatically synthesized structured lipids. Concerning emulsion properties, we determined the oil droplet size, the viscosity, and the stability.

MATERIALS AND METHODS

Materials. Deoiled lecithin (Sterninstant, PC-30) was donated by Stern Lecithin & Soja GmbH (Hamburg, Germany). PC (Epikuron 200, purity 93%) was obtained from Degussa Texturant Systems Deutschland GmbH & Co. KG (Hamburg, Germany). Caprylic acid (C8:0, purity 97%) was purchased from Riedel-de-Haen (Seelze, Germany). MCT (medium chain triglycerides) oil was purchased from Cognis Deutschland GmbH & Co. KG (Illertissen, Germany). Soybean oil was obtained from Aarhus United A/S (Aarhus, Denmark). Structured lipids were synthesized by acidolysis between fish oil and caprylic acid as previously described (10) and purified by thin film distillation (TFD). The fatty acid compositions of lipids and PLs used in the present study are presented in **Table 1**. Lipozyme RM IM, an immobilized sn-1,3 specific lipase from *Rhizomucor miehei*, was donated by Novozymes

A/S (Bagsvaerd, Denmark). Membrane GR70PE (polysulfone membrane on a polyester support) was donated by Alfa Laval A/S (Nakskov, Denmark). Silica gel 60 (particles size 0.035–0.070 mm) was purchased from Fluka Chemie GmbH (Buchs, Switzerland). All solvents and chemicals used were of analytical grade.

Structured PC Synthesis. The reaction was carried out by an acidolysis reaction between PC and caprylic acid. The bioreactor was a jacketed stainless steel column ($l = 300$ mm, i.d. = 21 mm) packed with 37 g of Lipozyme RM IM ($l = 280$ mm). The substrate mixture was fed upward into the column, and the column temperature was held constant by a circulating water bath. The reaction substrates were pumped through the enzyme reactor by a pump from Fluid Metering Inc. (New York, NY). Reaction conditions were as follows: substrate volume, 100 mL; substrate ratio, 6 mol/mol caprylic acid/PC; reaction temperature, 55 °C; and water addition, 3% based on total substrate. The substrate was passed through the column for 48 h by recirculation. After reaction, the incorporation of caprylic acid was 37.4 mol %, and the PL distributions were 52, 28, and 20 wt % for PC, lysophosphatidylcholine (LPC), and glycerophosphorylcholine (GPC), respectively.

Removal of FFA from Reaction Mixture. A stirred dead-end ultrafiltration cell with magnetic stirrer (Millipore, Glostrup, Denmark) was used for the separation of free fatty acids (FFAs) from the reaction mixture. Pressurized nitrogen gas provided the driving force for the permeation. The cell's capacity was 300 mL with an effective membrane area of 40 cm². A GR70 PE flat sheet membrane was used for the separation of PLs and FFA. The membrane was soaked in ethanol prior to filtration. Membrane filtration was conducted at room temperature (20–25 °C), and the pressure was kept at 3 bar. The permeate was collected through a port beneath the membrane support. Initially, the cell was charged with 100 g of feed (30 w/w% reaction mixture in hexane), 30 g of permeate was collected, and a new 30 g of hexane was added. The experiment was thus performed as a discontinuous diafiltration. The addition of more solvent was done in order to improve the permeation rate, since the flux was seen to continuously decrease with concentration factor. Twelve batches of 30 g of permeate were collected with subsequent addition of hexane. Ninety-two percent of the FFAs were removed by this procedure. The diafiltration was conducted three times. The PL distribution was not changed by the diafiltration, and further purification was required in order to have separation of PC from other PL constituents.

Separation of PC from Other PL Species. To separate PC from LPC, GPC, and small amounts of FFAs after ultrafiltration, column chromatography was applied. The column was packed with 30 g of silica. The PL species were eluted with two different solvent systems. Chloroform/methanol/water (65:35:5 v/v/v) was used to elute FFA and PC, and methanol/water (90:10 v/v) was used to elute LPC and GPC. Fractions of 10 mL were collected. Fractions were analyzed, PC-containing fractions were pooled, and the solvent was evaporated followed by lyophilization. The purity of the structured PC was 92% after purification. The fatty acid composition of the structured PC can be seen in **Table 1**.

Emulsion Preparation. Components of the emulsion except water were weighed according to the ratio (10 or 30% of oil and 0.5 or 2% of PLs) and were heated to 60 °C in a beaker with gentle stirring until the PLs were completely dissolved in the oil. Then, the water was weighed and dispersed into the oil phase. The mixture was homogenized for 10 s at 13500 rpm with an ultraturax T25 (Janke & Kunkel GmbH & Co., Staufen, Germany).

Fatty Acid Composition Analysis. Fatty acid methyl esters (FAMES) were prepared by methylation and analyzed on a HP6890 series gas–liquid chromatograph (Hewlett-Packard, Waldbronn, Germany) equipped with a flame ionization detector (FID), as described elsewhere (7).

FFA Content. This was determined by AOCS official methods (11). FFAs were 0.11, 0.05, and 0.74% for MCT, soybean oil, and structured lipids, respectively.

Microscopic Examination. An Optiphot light microscope (Olympus Co., Tokyo, Japan) was used to observe the structure of the emulsions. The emulsion samples were smeared on microscope slides and observed at 20× magnification.

Particle Size Distribution. Particle size analysis was performed with a laser diffractometer Mastersizer 2000 (Mastersizer S, Malvern Instruments, Malvern, United Kingdom) using standard optical parameters. Each sample was measured in triplicate. The surface mean diameter (Sauter diameter), $D[3,2] = \frac{\sum d^3}{\sum d^2}$, and the span, particle diameter at 90% cumulative size – particle diameter at 10% cumulative size, were calculated. The Sauter diameter is the equivalent spherical diameter by surface area per unit volume to the full distribution, i.e., the particle diameter that has the same specific surface as that of the full distribution. The span provides a measure between the points of distribution and therefore signals the quality of the distribution. A small span indicates a narrow size distribution.

Rheological Properties. Viscosity was measured using a concentric cylinder bob cup CC25 measuring system by Stresstech rheometer (Version 3.8, Reologica Instruments AB, Sweden). A constant temperature of 25 °C was maintained during the measurements with a circulatory water bath. Shear stress was increased progressively from 0.5 up to 2 Pa in 20 logarithmic steps with a continuous upward sweep direction. The viscosity was determined as the slope of shear stress vs shear rate curve.

Determination of Oil Density. Masses of the oils were determined by weighing into a 100 mL volume. The density was then calculated as the mass/volume. The densities of the oils were 0.94, 0.92, and 0.93 g/mL for the MCT, the soybean oil, and the structured lipids, respectively.

Emulsion Stability. For each emulsion, two test tubes were filled with 10 mL of the emulsion and closed with a cap. Samples were stored at 2 °C. The height of the total system and the height of cream separated out at the top were measured at 2, 4, 8, 16, and 32 days. A larger value of the cream layer was an indication of a more stable emulsion. If no macroscopic changes were observed, the creaming volume percentage was set at 100.

Statistics. Differences in particle size distribution, viscosity, and emulsion stability were determined by one-way analysis of variances, where 95% confidence intervals, that is, $P \leq 0.05$ significance level, were calculated from pooled standard deviations using software Microsoft Office Excel 2003 (Microsoft Corp., Redmond, WA). Data are expressed as the average of at least double determinations.

RESULTS AND DISCUSSION

The stability of an emulsion is controlled by interfacial surface forces, the size of the disperse phase droplets, viscous properties of the continuous phase, and the density difference between the two phases. To have a good o/w emulsion, the surfactant should orient most of the molecule in the water dispersion medium to maximize the reduction in the interfacial tension. An HLB system (hydrophilic/lipophilic balance) is often used for the selection of emulsifiers and is a measure of the surfactant's preference for oil or water, with the higher the number corresponding to a greater hydrophilicity-to-lipophilicity ratio. A high HLB number is preferred for o/w emulsions. The HLB value for purified soybean PC is approximately 7, and for deoiled soybean lecithin, it is 4 (2). For o/w emulsions, it would thus be expected that purified soybean PC would result in more stable emulsions as compared to deoiled soybean lecithin. With the enzymatic exchange of the long chain fatty acids with medium chain fatty acids in the pure soybean PC, it would become more hydrophilic and thus have an improved function as an emulsifier in o/w emulsions.

Emulsion instability is a complex process, which involves different mechanisms contributing to the transformation of a uniformly dispersed emulsion into a totally phase-separated system. Stokes' law equation gives a quantitative indication of the physical factors that influence the stability of an emulsion:

$$v = \frac{2r^2 \Delta \rho g}{9\eta} \quad (1)$$

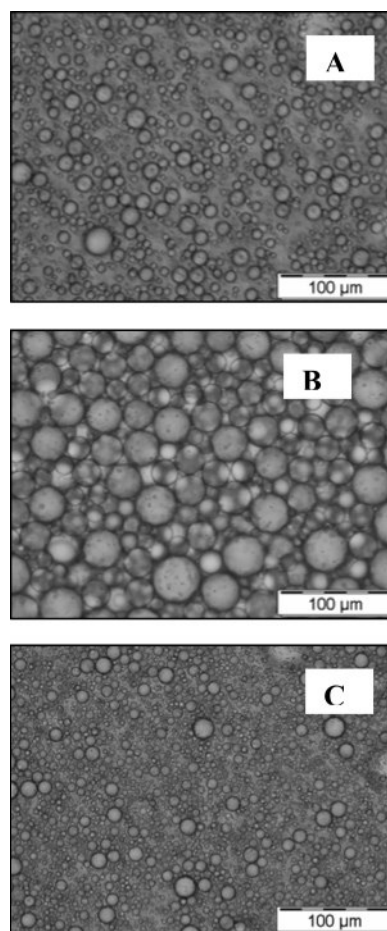


Figure 1. Microscopic view of emulsion with 10% oil and 2% structured PC: (A) MCT, (B) soybean oil, and (C) structured lipid.

where v is the rate of phase separation, r is the radius of the particles, $\Delta \rho$ is the difference in density between the two liquids, g is the gravity, and η is the viscosity of the medium. The stability of the emulsion is enhanced by small settling velocities of the dispersed oil particles. From the equation, it can be seen that especially the particle size is of critical importance as it occurs as a squared term. Emulsions are also more stable when density differences are small and when the viscosity of the medium is high. For the evaluation of the PL-stabilized emulsions, the particle size of the dispersed droplets, the viscosity, and the oil density were determined in order to calculate a theoretical separation phase rate based on these physical factors. Furthermore, the creaming stability of the emulsions was followed during cold storage (2 °C).

Microscopic Examination. The structures of all of the PL-stabilized o/w emulsions were similar. All of the emulsions prepared showed round droplets uniformly dispersed in the system. Structures of selected emulsions observed with a microscope are presented in **Figures 1** and **2**. Particle sizes of o/w emulsions are known to depend on various factors such as dispersed oil and its ratio to the continuous water phase, the emulsifier and its concentration, and the method of emulsion preparation. With all of the emulsions, there could be observed particles with varying particle sizes. In general, the particle size increased with an increase in oil concentration and with a decrease in PL concentration. It has previously been reported that $\geq 0.5\%$ (wt/vol of the continuous phase) lecithin is required in order to have spherical structures in the emulsion (12). At lower lecithin concentrations, oil droplets can be observed in the aqueous continuous phase.

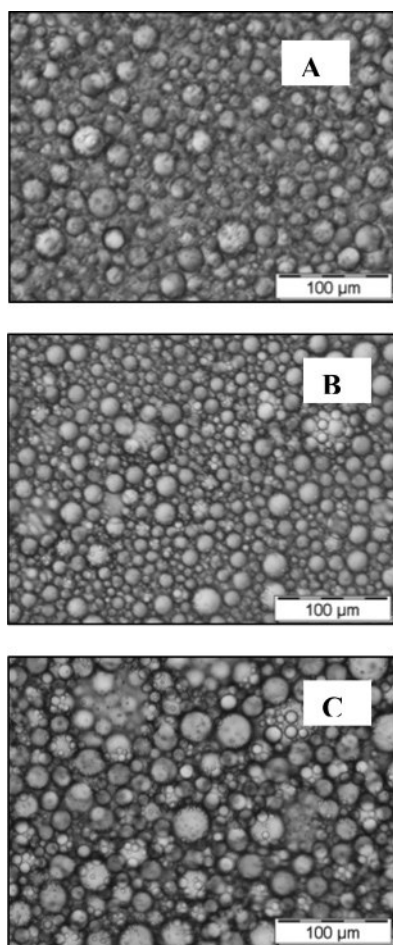


Figure 2. Microscopic view of emulsion with 30% soybean oil and 2% emulsifier: (A) deoiled lecithin, (B) PC, and (C) structured PC.

Particle Size Distribution. The particle size analysis confirmed the microscopic examination. The particle size varied within each emulsion prepared. In general, the particle size decreased with an increase in PL concentration and a decrease in oil concentration (Figures 3 and 4). When the Sauter diameter decreased, there was usually also a decrease in the span. Increasing the PL concentration reduced the size of large vesicles and had little effect on small emulsified droplets. Emulsion with an o/w ratio of 10:90 generally showed a smaller particle size and span with structured lipids as compared with emulsions prepared with MCT and soybean oil. In most cases, the largest particle size and span was observed for emulsions prepared with soybean oil. The largest particles were observed for emulsions prepared with PC and soybean oil, and the smallest particles were observed for emulsions with deoiled lecithin and structured lipids. Emulsions prepared with structured PCs usually had a larger particle size and span as compared to deoiled lecithin, except with structured lipids where there was no significant difference in particle size. Structured PCs gave smaller particles and spans than soybean PC in emulsions prepared with soybean oil at low PL concentrations and emulsions prepared with structured lipids at high PL contents.

For emulsions with an o/w ratio of 30:70, the smallest particles could in general also be observed for emulsions containing structured lipids. The largest particle size was found for emulsions prepared with deoiled lecithin and structured lipids; however, this was not the emulsion with the largest span, which was found for emulsions prepared with structured PCs and soybean oil. At low concentrations of deoiled lecithin, the

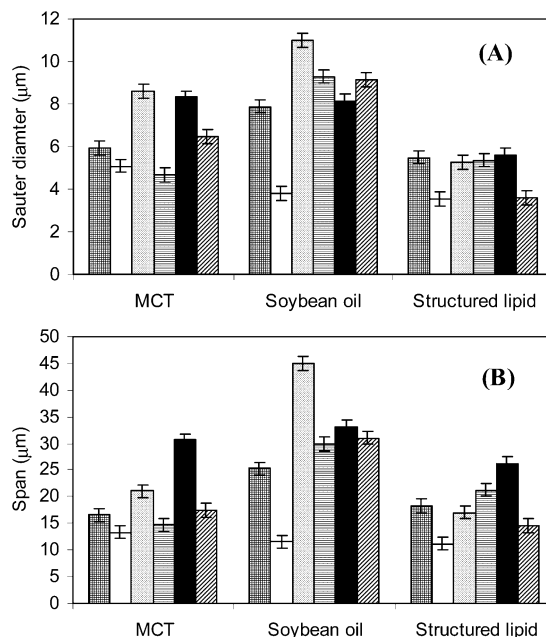


Figure 3. Particle size distribution of PL-stabilized emulsions with an o/w ratio of 10:90: (A) Sauter diameter and (B) span. Key: 0.5% deoiled lecithin, checked bar; 2% deoiled lecithin, white bar; 0.5% soybean PC, dotted bar; 2% soybean PC, horizontally striped bar; 0.5% structured PC, black bar; and 2% structured PC, diagonally striped bar. Bars indicate a 95% confidence interval based on pooled standard deviation.

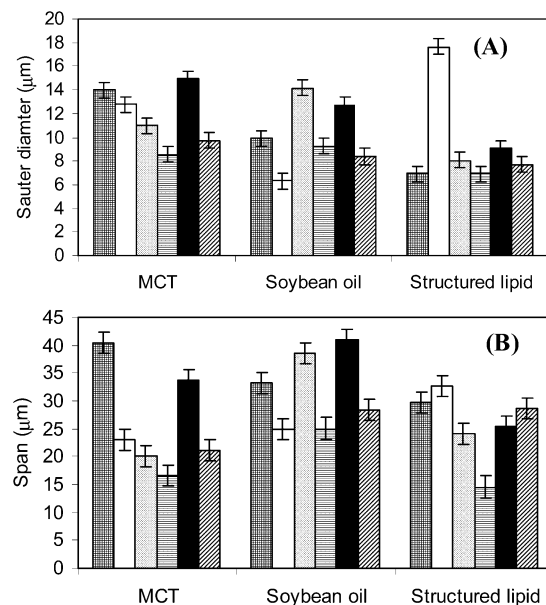


Figure 4. Particle size distribution of PL-stabilized emulsions with an o/w ratio of 30:70: (A) Sauter diameter and (B) span. Key: 0.5% deoiled lecithin, checked bar; 2% deoiled lecithin, white bar; 0.5% soybean PC, dotted bar; 2% soybean PC, horizontally striped bar; 0.5% structured PC, black bar; and 2% structured PC, diagonally striped bar. Bars indicate a 95% confidence interval based on pooled standard deviation.

largest span was found for emulsions prepared with MCT followed by soybean oil and structured lipids, respectively. However, when the PL concentration was increased to 2%, the reverse was observed. Determining which oil will result in the smallest particle size distribution in the emulsion is therefore highly dependent on the PL concentration. With soybean PCs used as an emulsifier, soybean oil gave the largest particle size and span. With the structured PC, the largest particle size was

found in MCT followed by soybean oil and structured lipids. Structured PCs produced smaller particles in emulsions prepared with soybean oil at both low and high PL contents as compared to soybean PC; however, the span was higher for the structured PCs. At a high concentration of PL, the structured PCs gave smaller particle sizes as compared to the deoiled lecithin in emulsions prepared with MCT and structured lipids.

Characteristics of various saturated and unsaturated PC used for emulsifying MCT have previously been reported (9). Particle sizes were shown to be influenced by the length and degrees of unsaturation of the acyl chain of the PC. The mean diameter of emulsion droplets increases as the number of carbons in the acyl chain of PC and TAG increases. The particle size also tends to increase with increased saturation degree (9). PCs with 6–10 carbons in their acyl group were better to form stable o/w emulsions, because these PCs are still able to form bilayer structures and also have stronger hydrophilic properties (9). TAGs having long acyl chains are highly lipophilic and, thus, more difficult to emulsify. Soybean oil also has a longer average chain length as compared to the other oils used in this study (Table 1), which could explain why it has a larger particle size in general. Soybean oil also has the highest degree of unsaturation. However, the results of this study indicate that in order to have small particles the oil should rather have a shorter chain length as compared to a high degree of unsaturation. On the basis of Stokes law equation (eq 1), it would be expected that emulsions prepared with structured lipids would have increased stability as compared to emulsions prepared with other oils since the particle sizes generally are smaller. Only in soybean oil, the particle size was smaller for structured PCs as compared to PCs. Exchange of long chain fatty acids with medium chain fatty acids in PC does not necessarily result in smaller particle size in the emulsions as it also seems to depend on the oil in use.

Rheological Characteristics. The rheological parameters are reflections of interactions and of the particle structure. The presence of lecithin changes the attractive forces between the particles; therefore, the rheological characteristics of the emulsion are affected by the type and amount of emulsifier. Shear stress and viscosity values of an emulsion change as the shear rate is increased (13). Shear stress is inversely proportional to viscosity. Viscosity was generally higher for the emulsion prepared with deoiled lecithin as compared to other emulsifiers used (Figure 5). With an increase in PL and oil concentration, the viscosity increased. Viscosity was dramatically increased for emulsions containing soybean oil and structured lipids at an o/w ratio of 30:70. Viscosity was also significantly higher for structured PCs as compared to soybean PCs in emulsions containing structured lipids with high PL content at an o/w ratio of 10:90. In other emulsions, structured PCs gave similar or lower viscosities.

Emulsion Stability. Creaming occurs when dispersed particles either settle or float with respect to the continuous phase and when either the lower or the upper portion, respectively, becomes more opaque or creamier. Creaming volume in the emulsions during cold storage is shown in Tables 2 and 3. All of the emulsions exhibit a tendency to creaming, except emulsions prepared with 10% MCT and 2% deoiled lecithin. During 32 days of storage, creaming was not observed for this emulsion. Higher PL concentrations usually increased the cream volumes and slowed the creaming process. Initially, all emulsions seemed stable by visual inspection as there was not observed any phase separation immediately after preparation. In most cases, emulsions remained opaque at the base of the

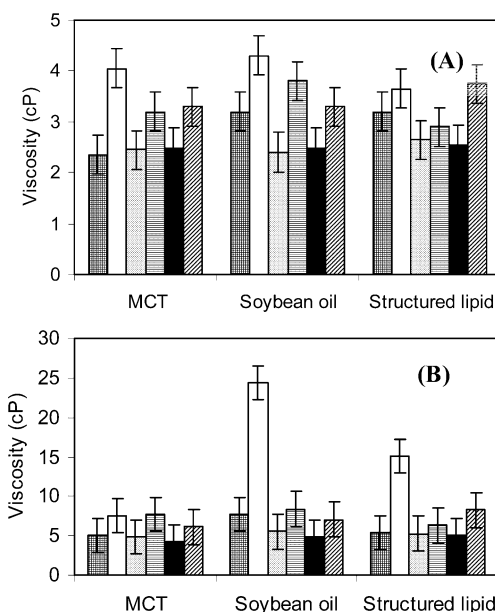


Figure 5. Viscosity of PL-stabilized emulsions with an (A) o/w ratio of 10:90 and an (B) o/w ratio of 30:70. Key: 0.5% deoiled lecithin, checked bar; 2% deoiled lecithin, white bar; 0.5% soybean PC, dotted bar; 2% soybean PC, horizontally striped bar; 0.5% structured PC, black bar; and 2% structured PC, diagonally striped bar. Bars indicate a 95% confidence interval based on pooled standard deviation.

Table 2. Stability under Cold Storage (2 °C) for PL-Stabilized Emulsions with an o/w Ratio of 10:90 and Calculated Phase Separation Rate

oil	emulsifier	emulsifier content (%)	creaming volume (%) ^a					phase separation rate ($\mu\text{m s}^{-1}$) ^b
			day 2	day 4	day 8	day 16	day 32	
MCT	deoiled lecithin	0.5	20	21	23	24	26	0.46
		2	100	100	100	100	100	0.20
	PC	0.5	15	15	15	15	15	0.92
		2	5	7	9	8	10	0.21
	structured PC	0.5	21	19	18	18	15	0.84
		2	43	42	42	41	35	0.38
soybean oil	deoiled lecithin	0.5	17	17	17	17	16	0.59
		2	12	15	15	15	18	0.10
	PC	0.5	12	13	13	13	14	1.54
		2	13	14	14	14	14	0.69
	structured PC	0.5	21	19	19	18	18	0.81
		2	39	38	38	35	32	0.77
structured lipid	deoiled lecithin	0.5	24	24	24	21	20	0.29
		2	100	100	22	23	23	0.10
	PC	0.5	16	16	17	17	18	0.32
		2	13	14	14	14	15	0.30
	structured PC	0.5	23	21	21	19	15	0.38
		2	100	100	100	100	16	0.11

^a The 95% confidence limit was ± 2.0 (based on pooled standard deviation).

^b Calculated according to Stokes' law equation (eq 1).

sample, while a concentrated cream layer developed at the top of the sample. No oil separation was observed during the 32 days that the emulsions were followed. Destabilization kinetics of the different emulsions were very different. For some emulsions, the phase separation was not evident until prolonged storage time. Many of the emulsions, however, separated in two phases within 2 days. In some cases, the cream layer changed little over time, and in other cases, dramatic changes could be observed during storage. With low PL concentration (0.5%), phase separation usually happened fast and the cream layer changed very little over time. In emulsions prepared with soybean oil, the phase separation also happened within 2 days.

Table 3. Stability under Cold Storage (2 °C) for PL-Stabilized Emulsions with an o/w Ratio of 30:70 and Calculated Phase Separation Rate

oil	emulsifier	emulsifier content (%)	creaming volume (%) ^a					phase separation rate ($\mu\text{m s}^{-1}$) ^b
			day 2	day 4	day 8	day 16	day 32	
MCT	deoiled lecithin	0.5	47	48	51	51	49	1.18
		2	94	94	90	90	90	0.66
	PC	0.5	45	45	43	43	42	0.77
		2	51	49	46	44	44	0.29
soybean oil	structured PC	0.5	41	39	39	39	39	1.60
		2	100	100	53	52	45	0.47
	deoiled lecithin	0.5	53	49	48	46	46	0.38
		2	100	68	63	59	56	0.05
structured lipid	PC	0.5	45	45	43	42	42	1.10
		2	48	46	45	42	45	0.31
	structured PC	0.5	43	43	41	41	41	1.03
		2	75	68	63	60	57	0.3
structured lipid	deoiled lecithin	0.5	53	53	50	48	47	0.27
		2	92	84	74	62	56	0.63
	PC	0.5	56	54	51	50	50	0.38
		2	100	100	100	55	54	0.23
	structured PC	0.5	44	42	41	41	40	0.50
		2	100	45	43	43	41	0.22

^a The 95% confidence limit was ± 3.2 (based on pooled standard deviation).

^b Calculated according to Stokes' law equation (eq 1).

During the whole storage time, the cream layer volume was much higher for emulsions prepared with structured PCs as compared to the other emulsions prepared with 10% soybean oil. Similar phenomena were observed for the MCT where structured PCs resulted in a much higher cream layer as compared to soybean PC. With structured lipids, the structured PC was able to maintain a stable emulsion for at least 16 days. After day 32, the cream layer was similar to emulsions prepared with PC.

Emulsions prepared with 30% oil showed considerably higher cream layers as compared to emulsions prepared with 10% oil (Table 3). Some of these emulsions were able to maintain a stable emulsion for a few days; however, after 16 days, they had all separated into two phases. At 32 days of storage, the highest cream layer was observed for 2% deoiled lecithin with MCT. With soybean oil, the largest cream layer was observed for emulsions prepared with structured PCs, and with structured lipids, it was deoiled lecithin. For some emulsions, phase separation happened fast and only changed slightly over time. Other emulsions were shown to decrease gradually over time, as seen for emulsions prepared with soybean oil and structured lipids with 2% deoiled lecithin. This was also observed for the structured PC in soybean oil. In most cases, emulsions prepared with PC had rapid phase separation; however, with emulsions containing structured lipids, it was possible to stabilize the emulsion for more than 8 days. Emulsions prepared with MCT and structured lipids were most stable when prepared with deoiled lecithin; however, soybean oil was more stable with structured PCs. The cream layer volume at 32 days was shown to vary greatly among the emulsions prepared. In general, the cream layer volume was high for emulsions prepared with deoiled lecithin. Previously, it has been reported that 10% structured lipid o/w emulsions were not stable to creaming when 0.25–1% deoiled soybean lecithin was used (14). With low concentrations of pure PC in o/w emulsions, oil separation also occurred immediately with soybean oil (1). Pan et al. reported that emulsions containing 0.5% sunflower lecithin presented a faster creaming process than systems containing 1.0% for emulsions prepared with 30% sunflower seed oil (12). At levels of 2.5 and 5%, clarification was hardly detectable, which was

explained by vesicles that occluded a greater part of the sample. The ability of PLs to stabilize emulsions has been known for decades; however, the stabilization mechanisms remain controversial. Rydhag and Wilton stated that the effectiveness of lecithin was mainly determined by the proportion of negatively charged PLs (1). However, Van der Meeren et al. reported that the stabilizing effect of PC-enriched lecithin was better as compared to deoiled lecithin (15). Deoiled lecithin is highly negatively charged, whereas the PC-enriched lecithin is rather zwitterionic. We observed that in most cases deoiled lecithin was better in stabilizing the emulsion as compared to soybean PC. However, at an o/w ratio of 30:70, the PC was able to stabilize emulsions with structured lipids for longer times as compared to the deoiled lecithin. Therefore, it seems difficult to make clear conclusions about which PL gives better stability as it depends on the particular formulations.

Applying Stokes' law equation (eq 1) with the measured data, the theoretical phase separation rate was calculated (Tables 2 and 3). The calculated phase separation rate was higher for emulsions prepared with 0.5% PL as compared to emulsions prepared with 2%. The phase separation rate was generally lower in emulsions prepared with structured lipid, which indicates that the presence of this oil may help increase its stability by physical means. Because the radius has a squared term in Stokes law equation, the calculated phase separation rate will be highly correlated to the particle size. Emulsions prepared with 10% soybean oil and 2% deoiled lecithin had a low calculated phase separation rate, however, already after 2 days the emulsion had separated. Emulsions prepared with 10% structured lipid and 2% structured PC also had a low phase separation rate; however, this emulsion was able to remain stable up to 16 days. For emulsions with an o/w ratio of 30:70, the phase separation rate were slowest in emulsions prepared with soybean oil and deoiled lecithin. This emulsion was stable for 2 days, and the cream volume was seen to gradually decrease during cold storage. In some cases, the emulsion had a higher phase separation rate as compared to other emulsions prepared; however, it was still able to maintain stability, e.g., emulsion prepared with 30% MCT and 2% deoiled lecithin. With an increase of PL concentration, the particle size usually become smaller, the viscosity increases, and also, the stability increases. However, it seems difficult to compare between different oils and PL compounds, as different kinds of interactions may occur. The tendency that can be observed for 10% oil emulsion may be completely different when the oil concentration is increased. With higher o/w ratios (0.4–0.6) and more than 0.5% PC, it was reported that emulsions became very viscous and did not separate spontaneously within 24 h (1). The emulsion was mechanically stabilized with respect to creaming while coalescence was not prevented. Coalescence is observed as the increase of emulsion droplets over time. Oil-in-water emulsions having the same lecithin concentration but varying levels of oil and water are not as good as w/o emulsions (16). A better emulsion stability is associated with emulsions having a high ratio of o/w (>60:40). With low oil concentrations, the destabilization occurs rapidly, whereas with higher oil concentrations, the oil separation is slowed. The stability of the emulsion is not only determined by the physical factors according to Stokes' law but is also related to interfacial forces within the emulsion. PL packing type (spontaneous curvature) is known to affect both the phase behavior of microemulsions and the coalescence energy barrier for the macroemulsions and has been correlated with the stability of emulsions formed (17). As a general trend, o/w emulsions become more stable as the spontaneous curvature increases. The

spontaneous curvature is known to increase with decreasing temperature, higher PC content, higher degree of saturation of the PL chains, long chain oils, and low penetration of oil into the surfactant "brush" (17).

The findings of this study give some useful information on the PL-stabilized o/w emulsions. At an o/w ratio of 10:90, the structured PC was shown to be superior to the other emulsifiers tested when soybean oil and structured lipids were used. Furthermore, structured lipids had interesting emulsifying properties as they were able to produce smaller particle sizes as compared to MCT and soybean oil, which were more commercially excisable. Deoiled lecithin and structured PC seemed to have the ability to stabilize all oils tested for several days. Soybean PC only produced stable emulsions with structured lipids. The optimal concentration of PL and oil will depend on the actual application. In the current study, we used high-shear homogenization; however, in order to increase the stability, high-pressure homogenization may be applied instead as smaller particles can be obtained (6). It is apparent that PLs are surface-active components, which through processes such as deoiling, fractionation, and modification, can be tailored to special applications. In the food industry, the deoiled lecithin is preferred over the purified PLs, since the price is considerably lower. In the pharmaceutical and cosmetic industry, further chromatographic purification exists and often different chemical modifications of PLs are made, e.g., hydroxylation, acetylation, and hydrogenation. Modified PLs with altered emulsifying and dispersing properties extend the application range of PLs in these areas. With enzymatic modification, it would be able to produce PLs with defined fatty acid compositions, which can be targeted for specific applications in foods, pharmaceuticals, and cosmetics. In this study, the substrate was produced from purified PC; however, acyl exchange can also be done for deoiled lecithin as previously demonstrated (18).

ABBREVIATIONS USED

FAMEs, fatty acids methyl esters; FFA, free fatty acids; FID, flame ionization detector; GC, gas chromatography; GPC, glycerophosphorylcholine; HLB, hydrophilic/lipophilic balance; LPC, lysophosphatidylcholine; MCT, medium chain triglycerides; o/w, oil-in-water; PC, phosphatidylcholine; PL, phospholipid; TAG, triacylglyceride; TFD, thin film distillation; TLC, thin-layer chromatography; w/o, water-in-oil.

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PAPER X

Title: Oxidative stability of liposomes composed of DHA-containing phospholipids

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(Manuscript in preparation)

Oxidative stability of liposomes composed of Docosahexaenoic acid-containing phospholipids

Running title: Oxidation of liposomes with DHA-containing phospholipids

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ABSTRACT

Oxidative stability of liposomes made of DHA-containing PC was examined during preparation and storage. After preparation of the liposomes, the concentration of primary (conjugated dienes) and secondary (TBARS) oxidation products were significantly higher compared to the initial value. During cold storage formation of conjugated dienes and TBARS remained more or less constant in large unilamellar vesicles (LUV), whereas in multilamellar vesicles (MLV) they were seen to increase over a period of 21 days. Evaporation of solvent traces from lipid film should preferably be done under nitrogen as vacuum evaporation was found to increase oxidation of the phospholipid.

KEYWORDS: Liposomes, Phosphatidylcholine, DHA, Oxidation stability.

INTRODUCTION

Phospholipids with defined molecular structure provide excellent opportunities within liposome technology for drug delivery and construction of anticancer prodrugs (1,2). Fatty acid profile and head group distribution of the phospholipid can be varied in numerous ways making it possible to optimize the biophysical membrane properties and thereby obtain liposomes that are stable with respect to drug encapsulation and circulation.

Liposomal drug delivery systems have been developed in an attempt to improve the pharmacokinetics and biodistribution of chemotherapeutic agents after systematic administration, as many of these compounds often give rise to inadequate delivery of therapeutic concentrations to the cancer tissue at doses causing severe toxic effects on normal organs (2). To fulfil their potential as targeted microcarriers the liposomes should be tailored to accumulate and release the encapsulated drug in the tumour tissue. Secretory phospholipase A₂ (sPLA₂) is highly over-expressed in

inflammatory and cancer tissue (3), and is considered to be an ideal tumor specific trigger enzyme that can be used to disrupt liposomes once they accumulate in cancer tissue due to the enhanced permeability and retention effect (1,4). sPLA₂ catalyzes the hydrolysis of ester-linkage in the *sn*-2 position of glycerophospholipids, producing free fatty acids and lysophospholipids. The hydrolysis products of diacylglycerophospholipids can synergistically act as locally generated permeability enhancers that increase diffusion of released drugs across the cellular membrane of the target cancer cell (5). sPLA₂ trigger mechanism may furthermore prove useful in development of lipid based prodrugs, which can be used to make liposomal drug carriers, that selectively target the tumor and undergo a site-specific triggered activation and release. Fatty acid released from the *sn*-2 position of the diacylglycerophospholipid can for example be substituted with an anticancer agent, such as docosahexaenoic acid (DHA) (6,7).

The long term stability or shelf-life of a drug product containing lipids can be dramatically affected by the lipid species used in the formulation. While saturated lipids offer the greatest stability in terms of oxidation, they also have much higher transition temperatures and thus present other difficulties in formulation. However, the more unsaturated a compound, the easier the product is oxidized, and thus the shorter the shelf life of the product. DHA is an *all-cis* polyunsaturated fatty acid with multiple double bonds, and is therefore highly prone to oxidation.

Determination of lipid oxidation poses an analytical problem as there is no single product to measure (8). Many intermediates and products are produced during lipid oxidation reactions (9). In the initial step an abstraction of hydrogen atom from the fatty acid chain occurs. The lipid radical form (R[•]) rapidly reacts with oxygen to form a peroxy radical (ROO[•]) via a free radical chain reaction. The peroxy radical (ROO[•]) can gain a hydrogen atom to form a lipid hydroperoxide (ROOH) (primary oxidation product). Hydroperoxides formed may be decomposed by homolytic β-scission to alkoxy radicals. Subsequently, the alkoxy radical may undergo further homolytic cleavage whereby secondary volatile products such as hydrocarbon, furans, alcohols, aldehydes and ketones are formed. Additional processes can occur, such as formation of endoperoxides, and rearrangement of double bonds in the fatty acid molecule. Different analytical procedures have been developed to measure classes of compounds formed during phospholipid oxidation (8). Some products (e.g. volatile aldehydes) are formed relatively late in the reaction, and is only detected after high degree of oxidation while others (e.g. conjugated dienes) may form in large numbers early in the reaction but breakdown to immeasurable levels in later stage.

The objective of this work was to study the influence of liposome preparation and storage on the oxidative degradation of phospholipids with DHA attached to the *sn*-2 position. Oxidation of phospholipids has remarkable influence on structure, and

physical properties of liposomes, and should be limited during preparation steps. Even small quantities of lipid oxidation would give rise to physically unstable liposome formulations. Phospholipid oxidation was followed by measuring primary (conjugated dienes) and secondary (thiobarbituric acid reactive substances, TBARS) products of oxidation (8).

MATERIALS AND METHODS

Preparation of liposome. 1-palmitoyl-2-DHA-PC (PL, 16:0, 22:6-PC) (25 mg/ml chloroform) was obtained from Avanti Polar Lipids Inc. (Alabaster, USA). 50 mg of PL in chloroform was transferred into two tubes with tight screw cap. Solvent was evaporated under a stream of nitrogen. One of tubes was maintained under nitrogen 30 min after apparent dryness, whereas the other tube was placed under vacuum for 6h. Nitrogen flushed Hepes buffer solution (10 mM Hepes, 150 mM KCl, 30 μ M CaCl₂, 10 μ M EDTA, pH7.5) was then added to the lipid films to give a concentration of 5 mM. The lipid suspensions were kept at 30°C for 1h in order to ensure complete hydration. During this period the lipid suspensions were vortex every 15 min for the preparation of multilamellar vesicles (MLV). Large unilamellar vesicles (LUV) were subsequently prepared by extrusion of MLV through two stacked 100 nm pore size polycarbonate filters 10 times (10). MLV and LUV were transferred to 10 ml plastic vials with screw caps, and kept in darkness at 4°C. Aliquots of liposomes were taken on the day of preparation and after 1, 3, 7, 14, and 21 d of storage for subsequent analysis. Vials were flushed with nitrogen for 30 s immediately after sampling.

Oxidation stability. Oxidation of phospholipids was monitored by analysis of conjugated dienes and cyclic peroxides according to methods described by New (8). Conjugated dienes were measured directly by diluting liposomes in absolute ethanol. The absorption spectrum (190 nm < λ < 350 nm) of the diluted liposome suspension was recorded with a Lambda2 UV/VIS Spectrophotometer (Perkin Elmer & Co, Überlingen, Germany) using 1-cm quartz cuvettes. Conjugated dienes were expressed as absorbance at 233 nm per mg mL⁻¹ of phospholipid (A₂₃₃/mg mL⁻¹ PL). Ethanol was used as the blank. Cyclic peroxides (or endoperoxides) were detected by reaction of their breakdown product at elevated temperatures (malondialdehyde) with thiobarbituric acid (TBA) giving a pink chromophore which absorbs at 532 nm. TBA reactivity was determined from standard curve of 1,1,3,3-tetraethoxypropane (TEP). Aliquots of diluted liposome suspension or TEP solution were transferred into test tubes with screw caps and mixed with 0.1 ml ferric chloride solution (2.7 mg/ml), 0.1 ml butylated hydroxytoluene solution (2.2 mg/ml), 1.5 ml 0.2M glycine buffer (pH 3.6), and 1.5 ml TBA reagent (5 mg/ml TBA and 3 mg/ml sodium dodecyl sulphate). Tubes were capped and placed in boiling water for 15 min. Tubes were then cooled to room temperature, and to each tube 1 ml acetic acid and 2 ml chloroform was added. Samples

were mixed well and subsequently centrifuge at 2879 x g for 2 min to separate the two phases. The upper phase was taken off and absorbance at 532 nm was measured with a Lambda2 UV/VIS Spectrophotometer (Perkin Elmer & Co, Überlingen, Germany). TBARS were expressed as μmol of TEP equivalent per μmol PL. All measurements were performed in duplicate, and mean values are presented.

Statistical analysis. The observed effects were tested for significance by analysis of variance (ANOVA), and considered significant when $P < 0.05$. 95% confidence intervals were calculated from pooled standard deviation (SD) using software Microsoft Office Excel 2003 (Microsoft Corporation, Redmond, WA).

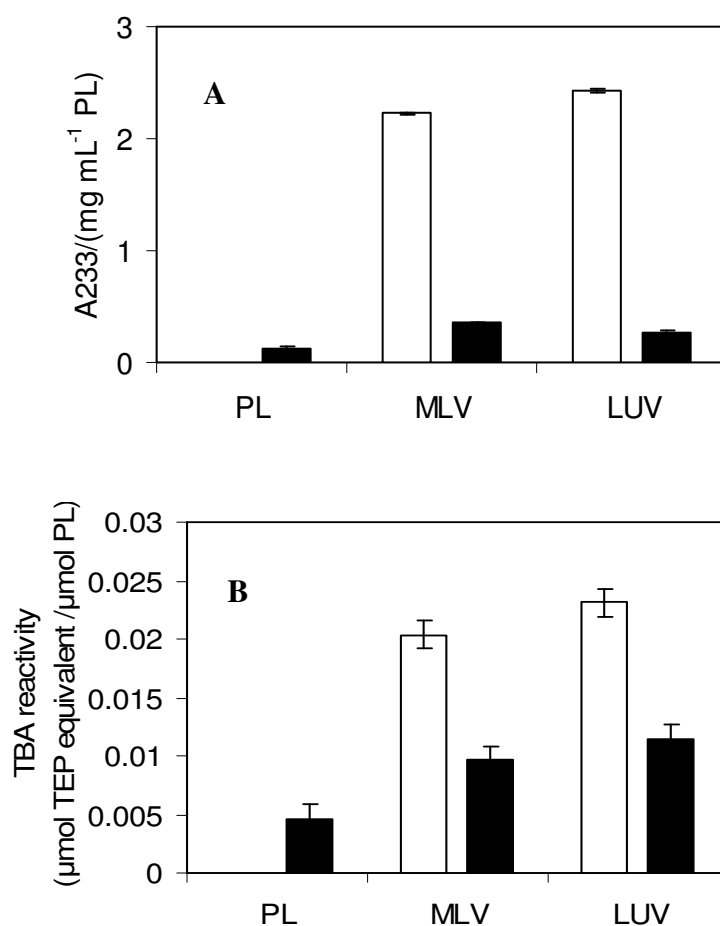


Fig. 1. Conjugated dienes (a) and TBARS (b) in PL directly after opening vial and in freshly prepared MLV and LUV. Results correspond to different procedures for removing solvent traces from lipid film (□: vacuum drying; ■: nitrogen flushing). Bars represent 95% confidence interval (n=2). Abbreviations: LUV, large unilamellar vesicles; MLV, multilamellar vesicles; PL, phospholipids; TBARS, thiobarbituric acid reactive substances.

RESULTS AND DISCUSSION

Lipid oxidation in freshly prepared liposomes. Immediately after opening the vial containing the PL, chloroform was removed under a stream of nitrogen until apparent dryness, and samples were taken, to determine the initial content of conjugated dienes and TBARS in the PL. To eliminate traces of solvent from the lipid film, two different approaches were tested, continued flushing with nitrogen for addition 30 minutes, or under vacuum for 6h. From experience even with extensive nitrogen flushing there is often still traces of solvent in the lipid film as determined by NMR (data not shown). To ensure complete removal of trace solvent, vacuum evaporation for several hours is required. MLV and LUV were subsequently prepared from the dried lipid films. Fig. 1 shows the initial concentration of conjugated dienes and TBARS in the PL, and in the freshly prepared liposomes (MLV and LUV).

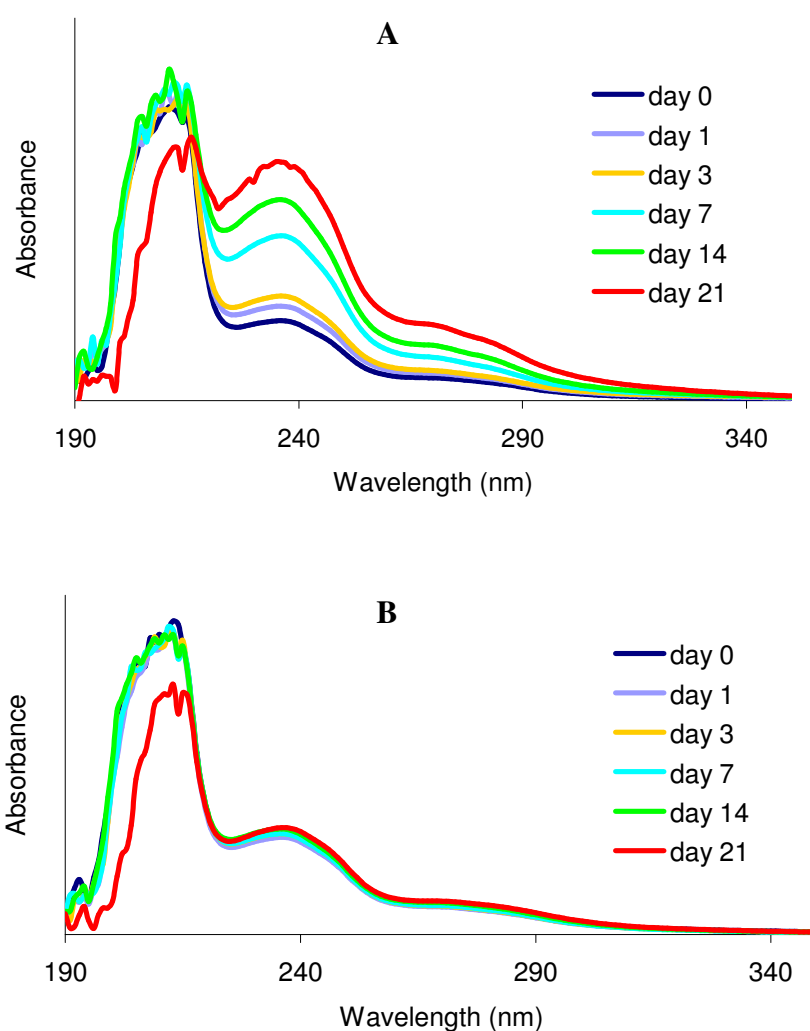


Fig. 2. UV spectra of 1-palmitoyl-2-DHA-PC liposomes (prepared from vacuum dried lipid film) during cold storage. Conjugated dienes is indicated by appearance of peak at 233 nm. A) MLV B) LUV. For abbreviations see Figure 1.

The UV absorption spectra of PL immediately after opening the vial did not present any peak at 233 nm, and did therefore not contain appreciable quantities of conjugated dienes.

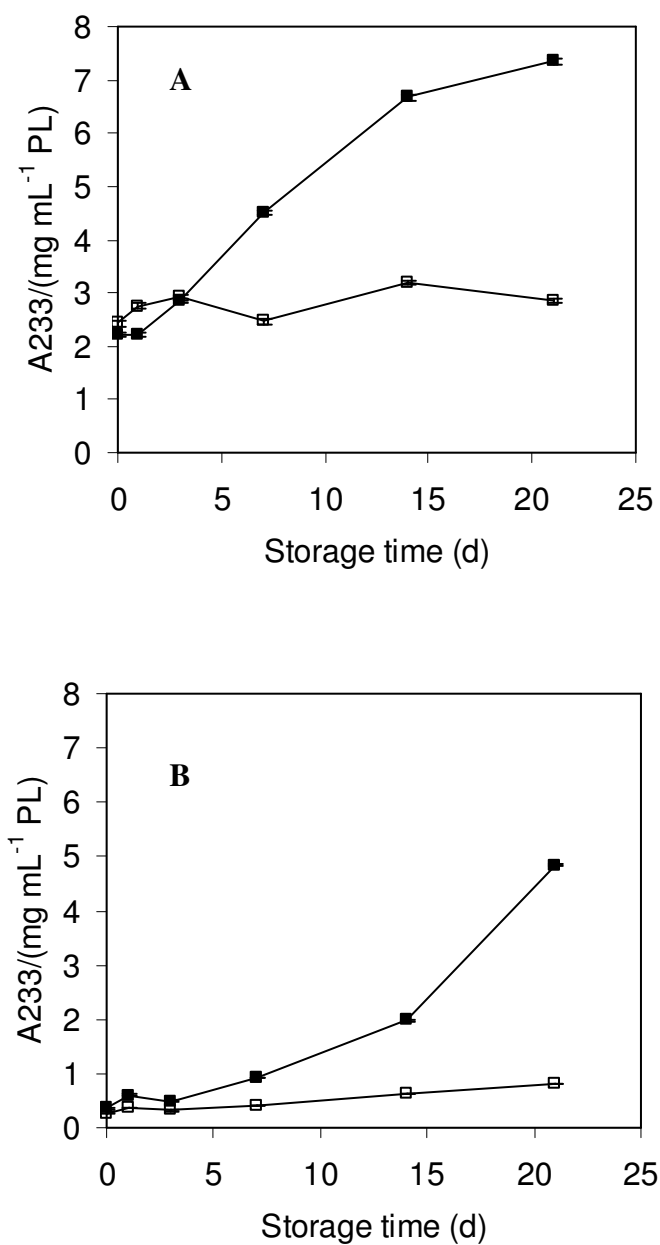


Fig. 3. Conjugated dienes in MLV (■) and LUV (□) made of 1-palmitoyl-2-DHA-PC during storage in the dark at 4°C A) Liposomes prepared from vacuum evaporated lipid film, B) Liposomes prepared from lipid film dried by nitrogen flushing. Bars represent 95% confidence interval (n=2). For abbreviations see Figure 1.

After preparation of the liposomes, the concentration of conjugated dienes and TBARS were significantly higher compared to the initial value. Especially when vacuum evaporation was used for the removal of solvent traces in the lipid film, the concentration of conjugated dienes and TBARS significantly increased ($P < 0.001$), as compared to lipid film only dried under nitrogen ($P < 0.01$). Flushing with nitrogen for solvent evaporation seems to minimize the oxidation, however further work needs to be done to determine if the solvent traces are below an acceptable threshold limit.

Lipid oxidation in liposomes during cold storage. Significant differences in the formation of conjugated dienes in LUV and MLV were observed during storage in dark at 4°C (Fig. 2 and Fig. 3.). Formation of conjugated dienes remained more or less constant during storage in LUV, whereas in MLV there was seen an increase. This observation was made for liposomes prepared with different methods for solvent evaporation of lipid film. Even though the liposomes prepared from vacuum dried lipid film had a significant higher initial concentration of conjugated dienes, then this apparently did not induce further oxidation in LUV. However formation of conjugated dienes was seen to increase more rapidly in MLV prepared from vacuum dried lipid film as compared to MLV prepared from nitrogen flushed lipid film. MLV prepared from nitrogen flushed lipid film showed hardly any increase in conjugated dienes for the first 7 days. Higher initial concentration of conjugated dienes in freshly prepared MLV thus seems to induce further oxidation.

Similar observations made for the formation of conjugated dienes in MLV and LUV could be made for the formation of TBARS. Concentration of TBARS increased more in MLV than LUV during storage (Fig. 4). Hardly any changes were observed in the TBARS concentration in LUV, whereas in MLV there was seen an increase in TBARS concentration over the 21 days of storage.

Peroxidation may be prevented by adding antioxidants to the buffer solution used for the preparation of liposomes. Hepes buffer used in the current study, have been reported to scavenge hydroxyl radicals (11). The buffer solution also contained EDTA, a metal chelator, which convert iron and copper ions into insoluble complexes or sterically hinder formation of the complexes between metals and lipid hydroperoxides (9). Further more phospholipids themselves have been reported to be efficient antioxidants (12). Even with these different antioxidants present in the buffer solution, oxidative decomposition of the DHA alkyl chain was not prevented.

Lipids in bulk are more likely prone to oxidation than lipids incubated in solvent or aqueous solutions, because it exposes a larger surface to the air (13). Difference in the oxidative stability of PL in bulk and organic solvent system would be due to the different rate of hydrogen abstraction by free radicals from intermolecular or intramolecular alkyl groups and not due to the PL confirmation, because PL takes no

packed confirmation in such systems (14). The removal of solvent prior to hydration during liposome preparation is thus probably one of the most critical steps in terms of peroxidation.

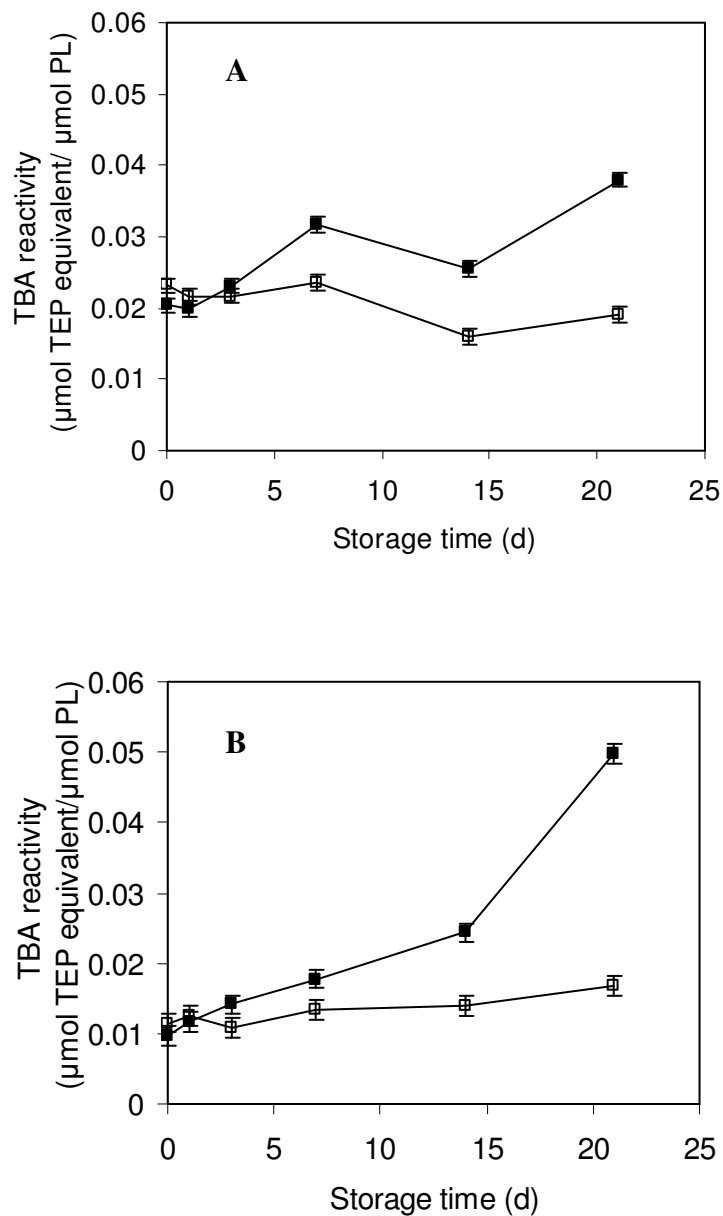


Fig.4. TBARS in MLV (■) and LUV (□) made of 1-palmitoyl-2-DHA-PC during storage in the dark at 4°C A) Liposomes prepared from vacuum evaporated lipid film, B) Liposomes prepared from lipid film dried by nitrogen flushing. Bars represent 95% confidence interval (n=2). For abbreviations see Figure 1.

The oxidative stability of PL in bulk and organic solvent was reported to decrease with increasing degrees of unsaturation; however had little effect on the stability of PC in aqueous solution as MLV (14). Oxidative stability of PL in liposomes seems to be affected not only by degree of unsaturation, but also the conformation of fatty acid components in PL bilayer. No correlation could be made for mean vesicle size diameter of MLV and degree of oxidation of polyunsaturated fatty acids in PL (14), however it was speculated to be caused by packing degree of PL bilayer. In aqueous solution DHA form a tighter intermolecular packing conformation of each bilayer, which makes it more difficult for free radicals and/or oxygen to attack (14). Lyberg et al. (13) reported that formation of hydroperoxides in DHA was almost completely prevented by incorporating DHA into one position of PC in bulk and in chloroform solution. In contrast, PL containing DHA on both positions should be avoided, since it is highly oxidized. Similar observations have also been made by Araseki et al. (14), and were explained by the idea that an intramolecular free radical chain reaction between PUFA of esters occurred more rapidly than intermolecular chain reaction.

Koga et al. (15) compared the rate of hydroperoxide formation in MLV and LUV from egg yolk PC by water-soluble and lipid-soluble radical generators. When peroxidation was induced by a water-soluble radical generator, the rate of hydroperoxides formation in LUV was larger than in MLV. Aqueous radical seemed hardly to penetrate outer bilayers and reach inner bilayers in MLV. Due to the larger surface area of LUV it was considered that they were more likely to be attacked by aqueous radical generators, thus resulting in larger amount of hydroperoxides during storage. Opposite trend was observed when a lipid-soluble radical generator was used (15). LUV was more resistant to the formation of hydroperoxides than its MLV, when initiating radicals were generated within the membranes. These radicals can react not only within one membrane layer but also with another neighbouring membrane layer in MLV.

The results obtained in this study clearly demonstrate that packing conformation of DHA-containing phospholipids in aqueous solution has significant effect on the oxidative stability. As LUV, in contrast to MLV, was hardly oxidized in the liposome formulation prepared in the current study, it seems that oxidation of PL is generated by radicals within membranes, and not caused by radicals present in aqueous environment. Solvent evaporation of lipid film should preferably be done under nitrogen as vacuum evaporation causes oxidation of PL. The UV absorbance method and the TBA method were shown to complement each other for determining oxidation degradation of DHA containing liposomes. With increase of primary oxidation products (conjugated dienes), there was also seen an increase in secondary oxidation product (TBARS). Information from this study can help in the further pursue of using polyunsaturated fatty acids in liposome formulations. For future work, the issue of

solvent evaporation needs to be examined more closely. Possibilities of using antioxidants in the prevention of oxidation during preparation and storage could be examined if liposomes are desired in the MLV form.

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