Synthesis of Structured Triglycerides and Phospholipids from Palm Oil and Tuna Oil Using Immobilized Lipases and Phospholipases

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ชื่อวิทยานิพนธ์	การสังเคราะห์สตรัคเจอร์ไตรกลีเซอไรด์และฟอสโฟลิปิดจากน้ำมันปาล์มและน้ำมันปลาทูน่าโดยเอนไซม์ไลเปสตรึงรูปและฟอสโฟไลเปส
ผู้เขียน	นางสาวสิริรุ่ง วงศ์สกุล
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บทคัดย่อ

การผลิตสตรัคเจอร์ไตรกลีเซอไรด์ (ST) ที่มีกรดไขมันสายโซ่ปานกลาง (MCFA) คือ กรดคาปรีลิก (C8:0, Cy) และกรดลอริก (C12:0, La) ที่ตำแหน่ง 1,3 และกรดไขมันสายโซ่ยาว (LCFA) หรือกรดไขมันไม่อิ่มตัวสูง (PUFA) ที่ตำแหน่ง 2 (MLM) ทำการศึกษาโดยใช้กระบวนการแบบขั้นตอนเดียวและสองขั้นตอนที่ใช้ปฏิกิริยาแตกต่างกัน โดยใช้น้ำมันปาล์มและน้ำมันปลาทูน่าเป็นแหล่งของ LCFA และ PUFA โดยได้ใช้เอนไซม์ไลเปสตรึงรูปทางการค้า และได้ศึกษาปัจจัยต่าง ๆ

การสังเคราะห์ ST แบบขั้นตอนเดียวโดยการทำปฏิกิริยาแอซิโดไลซิสระหว่างน้ำมันปาล์มและน้ำมันปลาทูน่า กับ MCFAs สภาวะที่เหมาะสมต่อการเติม MCFAs ในน้ำมันปาล์ม คือ เร่งปฏิกิริยาด้วย Lipozyme TL IM ในเฮกเซน นาน 48 ชั่วโมง ที่อุณหภูมิ 40 และ 60 องศาเซลเซียส ให้การเติมกรดไขมันร้อยละ 34.1 และ 43.5 สำหรับกรดคาปรีลิกและกรดลอริก ตามลำดับ กรดไขมันสายโซ่ยาวที่คงอยู่ในตำแหน่งที่ 2 เป็นกรดโอเลอิกร้อยละ 32.2-36.4 สภาวะที่เหมาะสมสำหรับการทำปฏิกิริยาแอซิโดไลซิสของน้ำมันปลาทูน่า คือ ทำปฏิกิริยาในอะซิโตน ที่อุณหภูมิ 40 องศาเซลเซียส นาน 72 ชั่วโมง เร่งปฏิกิริยาด้วยเอนไซม์ตรึงรูปจาก *Candida antarctica* B (CAL-B) ให้การเติมกรดไขมันร้อยละ 31.6 และ 45.2 สำหรับกรดคาปรีลิกและกรดลอริก ตามลำดับ โดยมีปริมาณกรดดีเอชเอ (docosahexaenoic acid, DHA) เพิ่มขึ้นเป็นร้อยละ 30 จากการวิเคราะห์การกระจายตัวของกรดไขมัน พบว่ามีกรดคาปรีลิกร้อยละ 6-7 และกรดลอริกร้อยละ 6-8 ที่ตำแหน่งที่ 2 ของ ST

ผลผลิตที่สูงของการสังเคราะห์ ST ทำได้โดยกระบวนการ 2 ขั้นตอน ขั้นแรกเป็นการสังเคราะห์ 2-โมโนกลีเซอไรด์ (2-MAG) บริสุทธิ์ โดยการทำปฏิกิริยาเอทาโนไลซีสของน้ำมันปาล์มและน้ำมันปลาทูน่าในตัวทำละลายอินทรีย์ สภาวะที่เหมาะสมสำหรับการทำปฏิกิริยาแอลกอฮอล์ไลซิสของน้ำมันปลาทูน่า ทำโดยเร่งปฏิกิริยาด้วยเอนไซม์ไลเปสตรึงรูปจาก *Pseudomonas* (PS-

C) ในอะซิโตน ที่ค่ากิจกรรมของน้ำ 0.43 ที่ 60 องศาเซลเซียส นาน 12 ชั่วโมง ให้ผลผลิต 2-MAG ร้อยละ 81 ซึ่งมีปริมาณ PUFA ร้อยละ 80 สภาวะที่เหมาะสมสำหรับการทำปฏิกิริยาแอลกอฮอล์ไลซิสของน้ำมันปาล์ม คือ การเร่งปฏิกิริยาด้วยเอนไซม์ไลเปสจาก *Rhizopus delemar* ตรึงรูปบน EP100 (D-EP100) ใน เมททิลบิวทิลอีเทอร์ (MTBE) ที่ค่ากิจกรรมของน้ำ 0.43 ที่ 40 องศาเซลเซียส ใช้เวลาน้อยกว่า 12 ชั่วโมง ได้ผลผลิต 2-MAG ที่ตกผลึกได้ร้อยละ 60 และมีปริมาณกรดโอเลอิกร้อยละ 55.0-55.7 และกรดลิโนเลอิกร้อยละ 18.7-21.0 ขั้นตอนที่สองเป็นการทำปฏิกิริยาเอสเทอริฟิเคชั่นระหว่าง 2-MAG บริสุทธิ์ที่ได้จากน้ำมันปาล์ม กับกรดไขมันสายโซ่ปานกลาง สภาวะที่เหมาะสมใช้เอนไซม์ D-EP100 เป็นตัวเร่งปฏิกิริยาในเฮกเซน ที่ 40 องศาเซลเซียส และใช้อัตราส่วน 2-MAG ต่อกรดไขมัน 1:3 โมลต่อโมล ให้ผลผลิต ST ร้อยละ 88.0 สำหรับกรดคาปรีลิก และร้อยละ 82.9 สำหรับกรดลอริก สำหรับการทำปฏิกิริยาเอสเทอริฟิเคชั่น ของ 2-MAG ที่ได้จากน้ำมันปลาทูน่า ทำการเติมกรดไขมันสายโซ่ปานกลางลงไปในสารผสมที่ได้จากการทำปฏิกิริยาแอลกอฮอล์ไลซิสโดยตรง เนื่องจากไม่สามารถแยก 2-MAG จากน้ำมันปลาทูน่าด้วยการตกผลึกในตัวทำละลายที่เย็นได้ พบว่าเมื่อทำปฏิกิริยาโดยใช้เอนไซม์ CAL-B ในอะซิโตน ที่ 40 องศาเซลเซียส ให้ค่าการเติมกรดคาปรีลิกและกรดลอริกสูงสุดเท่ากับร้อยละ 47.2 และ 54.4 ตามลำดับ

วิธีการใหม่สำหรับการสังเคราะห์ ST ได้รับการพัฒนาและศึกษา โดยเริ่มจากการสังเคราะห์ 1,3-ไดลอริน หรือ –ไดคาปรีลิน ด้วยวิธีการที่แตกต่างกัน ซึ่งได้ตีพิมพ์แล้ว จากนั้น นำมาทำปฏิกิริยาเอสเทอริฟิเคชั่นตรงตำแหน่งที่ 2 กับกรดไขมันสายโซ่ยาวหรือไวนิลเอสเทอร์ของกรดไขมัน โดยใช้เอนไซม์ไลเปสต่างชนิดกัน จากวิธีการดังกล่าวนี้ ทำให้ได้ผลผลิต 1-capryloyl-2-oleyl-3-capryloyl-glycerol (CyOCy) และ 1-lauroyl-2-oleyl-3-lauroyl-glycerol (LaOLa) ร้อยละ 87 และ 78 ตามลำดับ เมื่อทำปฏิกิริยาเอสเทอริฟิเคชั่นกับไวนิลเอสเทอร์ของกรดโอเลอิก โดยใช้เอนไซม์ไลเปสตรึงรูปจาก *Pseudomonas cepacia* (PS-D) เป็นตัวเร่งปฏิกิริยาในเฮกเซน ที่ 60 องศาเซลเซียส อย่างไรก็ตาม ผลการวิเคราะห์การกระจายตัวของกรดไขมันบ่งชี้ว่ามีกรดคาปรีลิกร้อยละ 25.7 และกรดลอริกร้อยละ 11.1 ที่ตำแหน่งที่ 2 ของผลิตภัณฑ์ การทำปฏิกิริยาเอสเทอริฟิเคชั่น ระหว่าง 1,3-ไดกลีเซอไรด์กับกรดโอเลอิก ให้ผลผลิต ST ต่ำ (น้อยกว่าร้อยละ 20) เมื่อทำปฏิกิริยาในตัวทำละลายอินทรีย์ และให้ผลผลิต ST ปานกลาง (ร้อยละ 50) เมื่อทำปฏิกิริยาแบบปราศจากตัวทำละลายอินทรีย์ ภายใต้สภาวะความดันต่ำ

การสังเคราะห์ 1,2-ไดเอซิลฟอสโฟลิปิดจากกรดไขมันและกลีเซอโรฟอสโฟลิปิดหลายชนิดด้วยวิธีทางเคมี พบว่ามีเพียง 1,2-ไดลอริล-3-ฟอสฟาติดิลโคลีน (DLPC) เท่านั้นที่สามารถสังเคราะห์ได้ (ร้อยละ 82.3) จากการศึกษาปัจจัยที่มีผลกระทบต่อการดัดแปลง DLPC โดยการทำ

ปฏิกิริยาทรานฟอสฟาติดิลเลชั่นกับเอทาโนลามีน แสดงให้เห็นว่าเทคนิค salt-activation มีประสิทธิภาพในการเพิ่มกิจกรรมของเอนไซม์ฟอสโฟไลเปสดีถึง 8 ถึง 10 เท่า ในตัวทำละลายอินทรีย์ที่ปราศจากน้ำ และสามารถเปลี่ยน DLPC ไปเป็นฟอสฟาติดิลเอทาโนลามีนได้ร้อยละ 100 เกิดปฏิกิริยาอย่างสมบูรณ์ภายในเวลา 12 ชั่วโมง เมื่อทำปฏิกิริยาที่ 60 องศาเซลเซียส อุณหภูมิมีผลกระทบต่อการทำปฏิกิริยาอย่างมาก มากกว่าผลของปริมาณเอนไซม์ ตัวแลกเปลี่ยนประจุบวก เรซิน และความเข้มข้นของเอทาโนลามีนที่ใช้

Thesis Title	Synthesis of Structured Triglycerides and Phospholipids from Palm Oil and Tuna Oil Using Immobilized Lipases and Phospholipases.
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Abstract

The production of structured triglycerides (ST) containing medium-chain fatty acid (MCFA), i.e. caprylic acid (C8:0, Cy) and lauric acid (C12:0, La), at the *sn*1,3-positions and long-chain fatty acid (LCFA) or polyunsaturated fatty acid (PUFA) at the *sn*2-position (MLM) was investigated in an one-step reaction and two different two-step reactions. Palm and tuna oils served as a source of LCFA and PUFA. Commercial immobilized lipases were used and several parameters were studied.

One-step synthesis of ST was carried out by acidolysis of palm and tuna oils with MCFAs. The highest incorporations of MCFA into palm oil were obtained at optimized conditions catalyzed by Lipozyme TL IM in *n*-hexane for 48 h at 40°C for C8:0 (34.1%) and at 60°C for C12:0 (43.5%). Most LCFA remained at the *sn*2-position was oleic acid (32.2-36.4%). Acidolysis of tuna oil at optimum condition was performed in acetone at 40°C for 72 h catalyzed by immobilized lipase from *Candida antarctica* B (CAL-B), resulting in 31.6% and 45.2% incorporation of C8:0 and C12:0, respectively, with moderate increasing in docosahexaenoic acid (DHA) content (30%). The regiospecific analysis revealed that C8:0 (6-7%) and C12:0 (6-8%) were also present at the *sn*2-position of the ST products.

ST was synthesized in high yield by a two-step process. First, pure 2-monoglycerides (2-MAGs) were synthesized by ethanolysis of palm and tuna

oils in organic solvents. The optimum conditions for alcoholysis of tuna oil were catalyzed by immobilized lipase from *Pseudomonas* sp. (PS-C) in acetone at water activity 0.43, 60°C for 12 h, yielding up to 81% of 2-MAG containing 80% PUFA. The optimum conditions for alcoholysis of palm oil were catalyzed by lipase from *Rhizopus delemar* immobilized on EP100 (D-EP100) in methyl-*tert*-butyl ether (MTBE) at water activity 0.43, 40°C for < 12 h, yielding crystallized 2-MAG of ~60% with 55.0-55.7% oleic acid and 18.7-21.0% linoleic acid. The purified 2-MAGs from palm oil were re-esterified with MCFAs in the second step. The optimum conditions were catalyzed by D-EP100 in *n*-hexane at 40°C using the molar ratio of 2-MAG:MCFA of 1:3, yielding 88.0% and 82.9% STs using C8:0 and C12:0, respectively. For the esterification of 2-MAG from tuna oil, MCFA was added directly to the reaction mixture obtained from alcoholysis reaction because 2-MAGs from tuna oil could not purified by crystallization in a cold solvent. The highest incorporation of C8:0 (47.2%) and C12:0 (54.4%) was obtained from the reaction catalyzed by CAL-B in acetone at 40°C.

A novel alternative method for the synthesis of ST was developed and studied. First, 1,3-dilaurin or -dicaprylin were enzymatically synthesized using different published methods. Next, these were esterified at the *sn*2-position with oleic acid or its vinylester using different lipases. Using this strategy, 1-capryloyl-2-oleyl-3-capryloyl-glycerol (CyOCy) and 1-lauroyl-2-oleyl-3-lauroyl-glycerol (LaOLa) were obtained at 87% and 78% yield by esterification with oleic acid vinyl ester using immobilized lipases from *Pseudomonas cepacia* (PS-D) in *n*-hexane at 60°C. However, regiospecific analysis indicated that 25.7% caprylic acid and 11.1% lauric acid were located at the *sn*2-position of the products. Esterification of *sn*1,3-diglycerides and free oleic acid gave very low yield (< 20%) of ST in a solvent system and moderate yield (> 50%) in a solvent-free system under reduced pressure.

1,2-Diacylphospholipids were chemically synthesized from various fatty acids and glycerophospholipids. The reaction was accomplished only with the synthesis of 1,2-dilauroyl-*sn*3-phosphatidylcholine (DLPC) (82.3%). The studies on factors effecting modification of DLPC by transphosphatidylation with ethanolamine showed that a salt-activation technique was successfully applied to increase the phospholipase D activity for 8-10 folds in anhydrous solvent system and 100% conversion of DLPC to phosphatidylethanolamine was achieved. Complete reaction was achieved within 12 h when carried out at 60°C. The reaction was strongly effected by temperature rather than the amount of enzyme, cation exchange resin and concentration of ethanolamine.

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Sirirung Wongsakul

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Abbreviations

1,3-DCy = 1,3-dicaprylin

1,3-DLa = 1,3-dilaurin

CAL-B = Immobilized lipase preparation from *Candida antarctica* fraction B

CDI = 1,1'-carbonyl diimidazol

Cy = Caprylic acid (C8:0)

CyVE = Caprylic acid vinyl ester

DAG = Diacylglycerol (diglyceride)

DBU = 1,8-Diazabicyclo[5.4.0]undec-7-en

D-EP100 = Lipase D (from *Rhizopus delemar*) immobilized on EP100

DHA = Docosahexaenoic acid (C22:6)

DLPC = *sn*1,2-dilauroyl-*sn*3-phosphatidylcholine

ELSD = Evaporated light scattering detector

EMK = Ethyl methyl ketone

EPA = Eicosapentaenoic acid (C20:5)

FA = Fatty acid

FAEE = Fatty acid ethyl ester

FAME = Fatty acid methyl ester

FAVE = Fatty acid vinyl ester

FFA = Free fatty acid

FID = Flame ionization detector

GC = Gas chromatography

GPC = Glycerophosphatidylcholine

GPE = Glycerophosphatidylethanolamine

HPLC = High performance liquid chromatography

La = Lauric acid (C12:0)

LaVE = Lauric acid vinyl ester

LCFA = Long-chain fatty acid

Abbreviations (continued)

LCT = Long-chain triglyceride

MAG = Monoacylglycerol (monoglyceride)

MCFA = Medium-chain fatty acid

MCT = Medium-chain triglyceride

MLM = Structured triglyceride containing medium-chain fatty acids at the *sn*1,3-positions and long-chain fatty acid at the *sn*2-position.

MTBE = Methyl-*tert*-butyl ether

O = Oleic acid

OAVE = Oleic acid vinyl ester

PA = Phosphatidic acid

PC = Phosphatidylcholine

PE = Phosphatidylethanolamine

PL = Phospholipid

PLD = Phospholipase D

PUFA = Polyunsaturated fatty acid

ST = Structured triglyceride

TAG = Triacylglycerol (triglyceride)

TLC = Thin layer chromatography

CHAPTER 1

INTRODUCTION

Introduction

The oil palm (*Elaeis guineensis* Jacq.) is one of the important sources of edible oil. It is an economic plant grown widely in Southern Thailand. Palm oil contains high amounts of long-chain fatty acids (LCFAs), especially oleic acid (C18:1) and linoleic acid (C18:2). However, palm oil is used mainly by the food industry as an ingredient in formulated products, such as margarines or shortenings, or directly in food processing as a cooking or frying fat. The large production capacity of palm oil leads to low prices, also seasonal fluctuations contribute to unstable production and value.

Tuna canning industry is an important export industry in Thailand. There are 21 Tuna Canning factories in Thailand, producing total tuna oil as by-product of approximately 1,500-2,000 tons a year (Srikamlaithong *et al.*, 1999 Quoted by Sarabok, 2001). Tuna oil is rich in ω-3 polyunsaturated fatty acid (PUFA), especially eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6), which are of high nutritional value.

Structured triglycerides (STs) are new triglycerides (TAGs) containing medium-chain fatty acid (MCFA) in the *sn* 1- and *sn* 3-positions, and essential LCFA in the *sn* 2-position of glycerol backbone, they are also called MLM. This type of ST has been developed as new lipids that meet both nutritional and energy requirement (Soumanou *et al.*, 1997). The specificity of enzyme provides a greater control over the fatty acid distribution, leading to high yield and purity of the desired product in comparison to chemical reaction. Additionally, the enzymatic reaction can be carried out under mild reaction conditions so that the sensitive compound, such as PUFA, would remain

unchanged. Therefore, enzymatic synthesis of ST is preferred over a chemical synthesis. Several approaches for the synthesis of MLM by immobilized lipases have been reported. The one-step synthesis has been carried out by acidolysis of TAG containing LCFA with MCFA, or interesterification of long-chain triglyceride (LCT) with medium-chain triglyceride (MCT). A two-step reaction has been reported as effective process for MLM synthesis, which results in high yield of almost pure MLM (Soumanou, *et al.,* 1998).

Therefore, the synthesis of ST from palm oil and tuna oil using immobilized lipase is an interesting topic to be studied. Not only that the increase in the usage and value of palm and tuna oil by producing a valuable product, ST can also be used in medical applications as well as for nutritional purposes.

Literature Review

1. Lipase

Lipases (triacylglycerol ester hydrolase, EC 3.1.1.3) are ubiquitous enzymes whose biological function is to catalyze the reversible hydrolysis of acylglycerols. In addition, lipases are also able to perform the hydrolysis of a variety of compounds containing carboxylic ester moieties which are not acylglycerols, and are capable of catalyzing a variety of alternative enzymatic reactions, many of which have considerable commercial potential (Malcata *et al.*, 1992; Montero *et al.,* 1993). The combination of broad substrate range and high selectivity makes lipases an ideal catalyst for organic synthesis (Bornscheuer and Kazlauskas, 1999).

1.1 Lipase-catalyzed reactions

Lipases are generally soluble in water, but the practical utilization of lipase-catalyzed reactions is restricted almost exclusively to water-insoluble compounds (Malcata *et al.*, 1992). Lipases bind to the oil/water interface and

catalyze hydrolysis at this interface. The binding not only places the lipase close to the substrate, but also increases the catalytic power of the lipase, a phenomenon called interfacial activation (Bornscheuer and Kazlauskas, 1999).

- **Mechanism of the lipase-catalyzed reaction**

The reaction is catalyzed by a catalytic triad composed of Ser, His and Asp (sometimes Glu) similar to serine proteases and carboxyl esterases (EC 3.1.1.1). The mechanism for ester hydrolysis or formation is essentially the same for lipases and esterases and is composed of four steps: First, the substrate reacts with the active-site serine yielding a tetrahedral intermediate stabilized by the catalytic His- and Asp-residues. Next, the alcohol is released and a covalent acyl-enzyme complex is formed. Attack of a nucleophile (water in hydrolysis, alcohol in (trans-)esterification) forms again a tetrahedral intermediate, which collapses to yield the product (an acid or an ester) and free enzyme (Figure 1).

Lipases are distinguished from esterases by their substrate specificity: lipases accept LCFA (in TAGs) as substrates, esterases prefer short-chain FAs. More generally it can be stated, that lipases readily accept water-insoluble substrates, esterases prefer water-soluble compounds. A further difference was found in the 3D structures of these enzymes, lipases contain a hydrophobic oligopeptide (often called *lid* or *flap*) covering the entrance to the active site, which is not present in esterases. Lipases preferentially act at a water-organic solvent (or oil) interface, which presumably accounts for a movement of the *lid* making the active site accessible for the substrate. This phenomenon is referred to as 'interfacial activation'. Further characteristic structural features of lipases are the α,β-hydrolase fold (Ollis et al., 1992) and a consensus sequence around the active site serine (Gly-X-Ser-X-Gly, where X denotes any amino acid). It could be shown that after removal of the lid by genetic engineering, the activity of a lipase was improved in solution, mainly for applications in the laundry/detergent area.

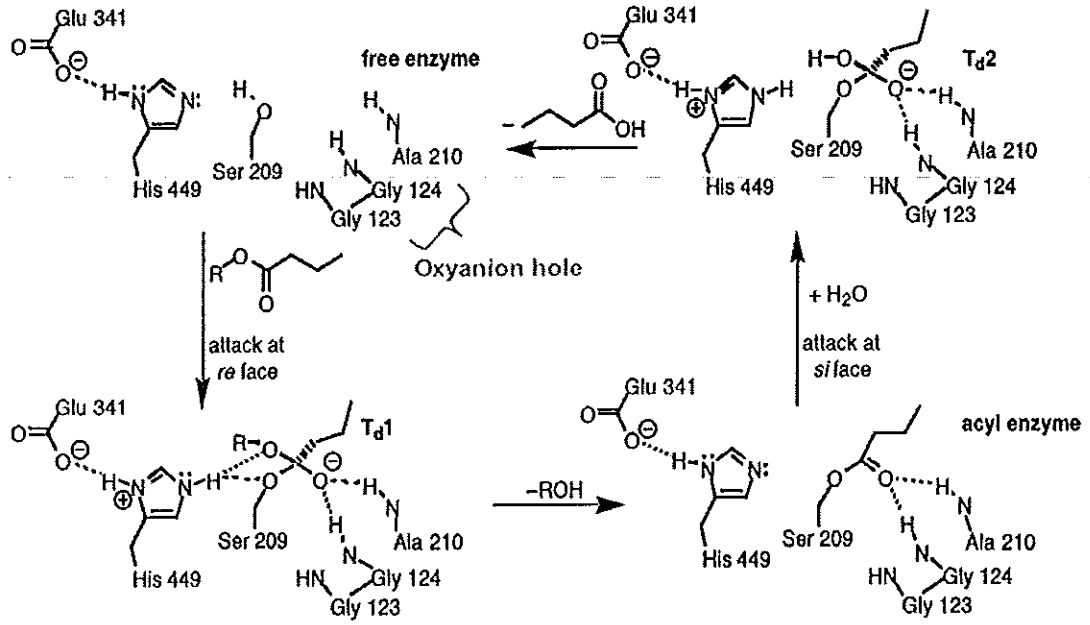


Figure 1 Mechanism of lipase-catalyzed ester hydrolysis of a butyrate ester. Numbering of amino acid residues is for lipase from *Candida rugosa* (CRL).

- **Types of the lipase-catalyzed reactions**

The lipase-catalyzed reactions can be categorized into three groups (Yamane, 1987).

(i) Hydrolysis reaction

Hydrolysis of ester refers to attack on the ester bond of glycerides in the presence of water molecules to produce both alcohol moiety and a carboxylic acid.

$$RCOOR' + H_2O \rightleftharpoons RCOOH + HOR'$$

(ii) Esterification reaction or synthesis of esters

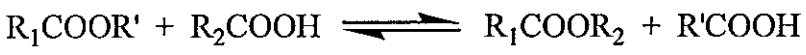
Esterification between polyhydric alcohols and free fatty acids (FFAs) are the reverse of the hydrolysis reaction of the corresponding glyceride. Generally, the relative rates of the forward and reverse reactions are controlled by the water content of the reaction mixture.

$$RCOOH + HOR' \rightleftharpoons RCOOR' + H_2O$$

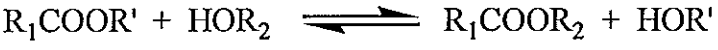
(iii) Interesterification reaction

Interesterification refers to the exchange of acyl residues between an ester and other molecules, such as acid (acidolysis), alcohol (alcoholysis), amine (aminolysis), and another ester (transesterification).

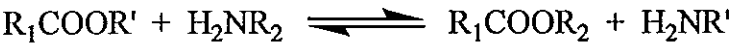
- Acidolysis



- Alcoholysis



- Aminolysis



- Transesterification

It is generally recognized that FFAs and alcohols tend to inhibit lipase-catalyzed hydrolysis reactions, while nonaqueous solvents can have a variety of effects on enzymes (Malcata *et al*, 1992).

1.2 Specificity of lipase

Because enzymes are specific to their substrates and operate at mild reaction conditions, they are preferred over chemical catalysts for the modification of lipids intended for modern consumers, who demand more natural and less synthetic food products and additives (Akoh, 1996).

Based on the specificity of lipases, three main groups of lipases can be defined;

(i) **random lipases** or **non-specific lipases**, which catalyze reactions at all three positions on the glycerol molecule, e.g. lipases from *Candida antarctica* and *C. rugosa*

(ii) ***sn*1,3-specific lipases** or **positional specific lipase**, which act preferentially at the *sn*1- and *sn*3-positions of the glycerol

molecule, e.g. lipases from *Rhizomucor miehei, Aspergillus niger* and *Rhizopus delemar*

(iii) **substrate specific lipases,** which show specificity toward specific types of FAs, e.g. lipase from *Geotrichum candidum* which acts specifically on oleic acid (C18:1, n-7).

The positional specificity of lipases is usually retained when they are placed in organic solvents (Fomuso and Akoh, 1998).

1.3 Microbial lipases

Lipases can be obtained from mammals, plants and microorganisms (Balcao *et al.*, 1996). Microbial lipases have been increasing interested due to the higher stability in comparison to lipases from mammals or plants. Microbial lipases can be produced in large scale according to a rapid growth of microorganisms and it is easy to control the productivity and quality of the produced lipases (Malcata *et al.*, 1992; Balcao *et al.*, 1996). Lipases obtained from yeast, fungi and bacteria show various properties according to the types of microorganism and the production conditions. There are a number of commercially available microbial lipases (Table 1) (Kazlauskas and Bornscheuer, 1997). The number of known microbial species that produce lipases is rather large and is, in fact, still increasing. For some important applications of lipase in food manufacturing and processing, immobilized rather than free lipases are required. Some lipases are manufactured and marketed in an immobilized state that is well suited to application in food-related processes such as modification of fats and oils (Godtfredsen, 1991). The areas of industrial application of microbial lipases are shown in Table 2.

Microbial lipases generally show a similar mode of action on TAGs as that exhibited by pancreatic lipase, which hydrolyzes only the primary ester at the *sn*1- and *sn*3-position of glycerides. As *sn*1,2- and *sn*2,3-diglycerides (DAGs) and 2-monoglycerides (MAGs) are unstable due to acyl migration to

Table 1 Examples of commercially available microbial lipases.

Origin of lipase	Other Name	Commercial source
Mammalian lipases		
Porcine pancreas		Amano, Sigma, Fluka, Boehringer Mannheim,
Pancreatic cholesterol esterase		Genzyme, Sigma
Fungal lipases		
Candida rugosa	*Candida cylindracea*	Altus Biologics, Amano, Sangyo, Boehringer Mannheim
Candida antarctica A		Boehringer Mannheim
Candida antarctica B		Novo Nordisk
Candida lipolytica		Boehringer Mannheim, Sigma, Novo Nordisk
Geotrichum candidum		Amano
Humicola lanuginosa	*Thermomyces lanuginosa*	Boehringer Mannheim, Novo Nordisk
Rhizomucor javanicus	*Mucor javanicus*	Amano
Rhizomucor miehei	*Mucor miehei*	Boehringer Mannheim, Amano, Fluka, Sigma, Novo Nordisk
Rhizopus oryzae	*R. javanicus, R. delemar, R. niveus*	Amano, Fluka, Sigma, Seikagaku Kogyo
Aspergillus niger		Amano
Penicillium roqueforti		Amano
Penicillium caamembertii	*P. cyclopium*	Amano

Table 1 Examples of commercially available microbial lipases (continued).

Origin of lipase	Other Name	Commercial source
Bacterial lipases		
Pseudomonas cepacia	*Burkholderia cepacia*	Altus Biologics, Fluka, Sigma, Amano, Boehringer Mannheim
Pseudomonas fluorescens		Amano, Biocatalysts
Pseudomonas fragi		Wako Pure Chemical
Chromobacterium viscosum	*Pseudomonas glumae*	Sigma, Genzyme, Asahi
Pseudomonas sp.		Chemical, Biocatalysts, Amano
Bacillus thermocantenulatus		Boehringer Mannheim,
Alcaligenes sp.		Meito Sangyo

From: Kazlauskas and Bornscheuer (1997).

yield the 1,3-DAG, prolonged reaction will cause complete hydrolysis of TAGs to glycerol (Wong, 1995). Most microbial lipases display maximum activity at pH values ranging from 5.6 to 8.5 and maximum stability in the neutral pH range. With respect to temperature, most lipases are optimally active between 30 and 40°C (Malcata *et al.*, 1992).

1.4 Immobilized lipases

Lipases are spontaneously soluble in aqueous solutions (as a result of their globular protein nature), but their natural substrates (i.e., lipids) are not. Although use of a proper organic solvent or an emulsifier helps overcoming the problem of intimate contact between substrate and enzyme, the practical use of lipases in such pseudohomogeneous reaction systems poses technological and economic difficulties. The former leads to constraints on the product level, because the final characteristics of the product depend on such postprocessing conditions as storage time and temperature. The latter leads to constraints on the process level, because the useful life of the enzyme is restricted to the space-time of the reactor. In both cases, part of the overall potential enzymatic activity is lost. If the lipase is immobilized, then it becomes an independent phase within the reaction system, which may be easily retained in the reactor via mechanical means with concomitant advantages in preventing contamination of the products and extending its useful active life (Balcao *et al.*, 1996).

Table 2 Industrial application areas for microbial lipases.

Industry	Effect	Product
Dairy	Hydrolysis of milk fat	Flavor agents
	Cheese ripening	Cheese
	Modification of butter fat	Butter
Bakery	Flavor improvement and shelf life prolongation	Bakery products
Beverage	Improved aroma	Beverages
Food dressing	Quality improvement	Mayonnaise, dressings and whipped topping
Health food	Transesterification	Health foods
Meat and fish	Flavor development and fat removal	Meat and fish products
Fat and oil	Transesterification	Cocoa butter, margarine
	Hydrolysis	Fatty acids, glycerol, mono- and diglycerides
Chemical	Enantioselectivity	Chiral building blocks and chemicals
	Synthesis	Chemicals
Pharmaceutical	Transesterification	Specialty lipids
	Hydrolysis	Digestive aids
Cosmetic	Synthesis	Emulsifiers, moisturing agents
Leather	Hydrolysis	Leather products
Paper	Hydrolysis	Paper products
Cleaning	Hydrolysis	Removal of cleaning agents, e.g. surfactants

From: Godtfredsen (1991).

Immobilization of lipases from several microbial and animal sources has been performed by different methods, including covalent attachment to activated supports, entrapment with photocrosslinkable resins and poly(vinyl) chloride or colloidion membranes, and adsorption on different materials such as ionic resins (Montero *et al.,* 1993). Immobilization by adsorption is usually carried out by incubation of the support and the enzyme in a buffer or by precipitating the lipase from a buffer with acetone onto the surface of the support (Mustranta *et al.,* 1993). The activity yields of the immobilized lipases so far reported are considerably lower than those reported for many other enzymes. The activity yield in immobilization of lipase is often only a few percent (Sarabok, 2000; Kaewthong *et al.*, 2004).

1.4.1 Effects of support

The catalytic activity of immobilized lipases is influenced by the properties of the support matrix and the method of immobilization (Mustranta *et al.,* 1993). The support can affect the partitioning of substrates, products, and water in the reaction mixture and, thereby, indirectly influence the catalytic properties of the enzyme (Castro *et al.,* 1999). When lipases are immobilized on porous solid supports, the highest retention of lipolytic activity is usually observed when the carrier possesses hydrophobic characteristics (Malcata *et al.*, 1992).

The use of a porous support material is recommended so that a suitable amount of lipase can be spread on a surface area without conformational changes. Comparative studies indicate that dramatic differences in activity are observed among lipases on supports of different materials. A good correlation was found between the water-attracting capacity (hydrophilicity) of the support and the reaction rate obtained with enzymes deposited on the support; low hydrophilicity resulted in high catalytic activity (Adlercreutz, 1991). It has been suggested that both ionic and hydrophobic interactions between the lipase and the surface are important for the non-covalent immobilization of lipase. A

number of bacterial lipases can be immobilized in a rapid and strong fashion on octyl-agarose gels. It has been shown that adsorption rates in the absence of ammonium sulfate are higher than in its presence, opposite to the observation for typical hydrophobic adsorption of proteins, and these immobilized lipase molecules show a dramatic hyperactivation. It is suggested, from these results, that lipases recognize these "well-defined" hydrophobic supports as solid interfaces and they become adsorbed through the external areas of the large hydrophobic active centers of their "open and hyperactivated structure" (Bastida *et al.,* 1998). It was found that interesterification activity was better using Resin or Celite as carrier compared to polypropylene (EP 100) and Hyflo Super Cel was the least suitable support for interesterification of triolein and caprylic acid (Soumanou *et al.*, 1999).

1.4.2 Effect of solvent

The solvent used in immobilization has been also shown to have a great effect on the applicability of a covalently immobilized *Rhizopus* species (Mustranta *et al.,* 1993). In the study of Castro *et al.* (1999), porcine pancreatic lipase was adsorbed on Celite, either by direct binding from aqueous solution or deposition from aqueous solution by the addition of organic solvent. Better activities of lipase were displayed when the immobilized lipase was prepared in an aprotic solvent such as n-hexane. It appeared that the use of a solvent, which had lower polarity (higher log *P* value) was capable of creating a specific microenvironment around the enzyme that may enhance its stability and activity.

Non-aqueous solvents can have a variety of effects on enzymes. A layer of bound water (or water hydration shell) plays a key role in maintaining the structural integrity and catalytically active conformation of lipases since it affects intramolecular salt bridges and hydrophobic interactions. Therefore, the existence of trace amounts of water in the immediate vicinity of the lipase is a prerequisite for successful functioning of a lipase in a microaqueous system, in

which lipase-catalyzed interesterification or ester synthesis reactions are occurring (Malcata, 1992).

Mustranta *et al.* (1993) studied the immobilization of lipases from *Candida cylindracea, Aspergillus niger,* and *Pseudomonas fluorescens* by adsorption on anion exchange resin and diatomaceous earth in buffer or n-hexane. It was found that immobilized *C. cylindracea* was more active in n-hexane. For *A. niger* lipase, n-hexane was also a better reaction medium and anion exchange resin was a better support than diatomaceous earth.

1.4.3 Effect of water on enzyme activity in organic media

In particular, the effects of water associated with the enzyme have been studied in some detail. It appears that different enzymes display vastly different requirements with regard to how much bound water they need to maintain an appreciable level of catalytic activity in non-aqueous media. It has also been shown that the stability of enzymes markedly increased under low water conditions, making it possible to perform biotransformations at temperatures higher than those used in conventional aqueous solutions (Turner and Vulfson, 2000). A layer of bound water plays a crucial role in maintaining the structural integrity and catalytically active conformation of lipases. Thus, the existence of trace amounts of water in the immediate vicinity of the lipase is a prerequisite for successful functioning of a lipase in a microaqueous system (Malcata *et al.*, 1992). The work of Turner *et al.* (2000) demonstrated that the temperature at which a protein undergoes thermal denaturation (T_d) was strongly dependent on the amount of water associated with the protein and could be correlated with the thermodynamic water activity (a_W) of enzymes. It was found that esterases pre-equilibrated over saturated salt hydrates to a fixed a_W had higher T_d at lower a_W. These observations suggested that at low water activity, enzymes should be catalytically active at temperatures of around 90-120°C.

1.5 Application of Immobilized Lipase

Lipases have high potential for hydrolysis, glycerolysis, and alcoholysis of bulk fats and oils because of their high specificity and the relative purity of the products derived therefrom (Balcao *et al.,* 1996). Immobilized lipase is used for the hydrolysis and partial hydrolysis of fats and oils for the industrial production of FA, DAGs, and MAGs, which are widely used as emulsifiers in food and pharmaceutical industrials. Examples of product obtained via lipase-catalyzed esterification include partial acylglycerol, TAGs, short chain esters, and terpene esters, which are used as flavor or fragrance. Sugar esters, which can be used as emulsifiers or fat substitutes, can be also synthesized by this reaction. Acidolysis and interesterification reactions have been extensively used for the production of partial acylglycerols, sugar esters, and designer fats such as cocoa butter and other STs. Lipases are also used for modifying the FA composition of vegetable oils according to several functional, health and nutritional reasons. Lipase is currently used for the production of optically active compounds for the fine chemicals and pharmaceutical industrial, such as hydroxymethyl glutaryl coenzyme A reductase inhibitor, which is a potential anticholesterol drug (Akoh, 1996).

The immobilized lipase from *Rhizopus delemar* on a ceramic carrier could be used for a continuous transesterification of borage oil with caprylic acid for more than 100 days without being destroyed in the packed-bed reactor. Thus the reaction system was effective for the industrial production of the STs (Shimada *et al.,* 1999).

Rhizopus arrhizus lipase immobilized on Celite was used to prepare isomerically pure 2-MAGs by alcoholysis of TAGs in organic media. The enzyme preparation showed high stability in repeated-batch reactions (Millqvist *et al.,* 1994).

2. Structured triglycerides

ST is TAG obtained by the hydrolysis and random transesterification of MCTs and LCTs. A physical mixture is merely a blending of two different fats with different absorption rates (Figure 2) (Schmidl, 1996). ST is a new TAG with a modified absorption rate. STs containing defined FAs at specified positions were synthesized to maximize their physical and nutritional properties.

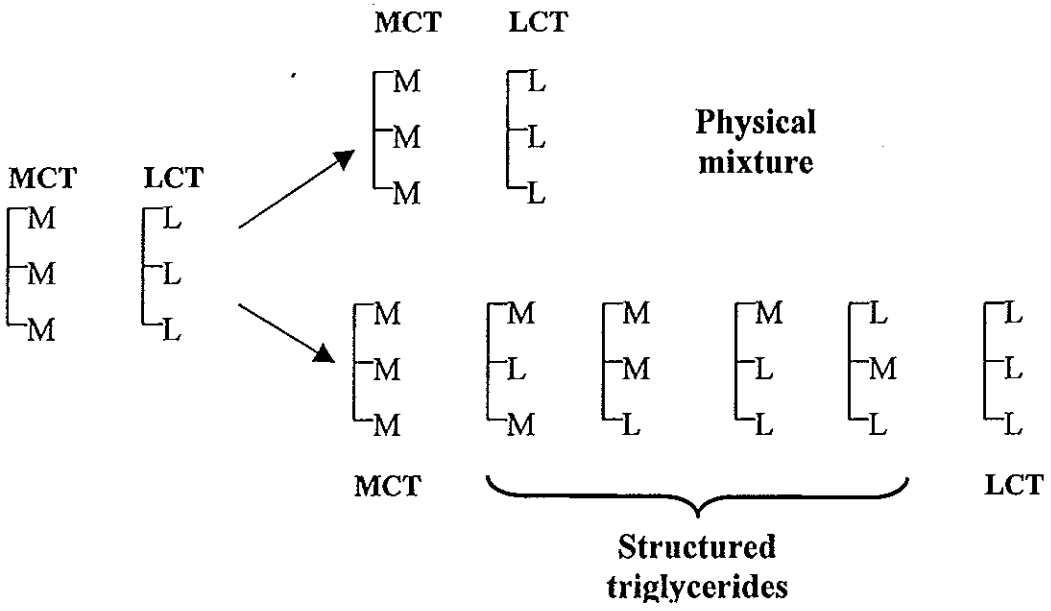


Figure 2 Structured triglyceride vs physical mixture (Schmidl, 1996).

MCT, composed of MCFAs, are readily hydrolyzed by lingual and gastric lipases and do not required pancreatic lipase and bile acids for digestion and absorption (Ikeda, 2000). Differ from regular fats and oils, MCT metabolized in the liver like carbohydrates and provide only 8.3 calories per gram, and they are not deposited as depot fat (Megremis, 1991). MCFA are absorbed via the portal system without resynthesis of TAG in intestinal cells. They are also subjected predominantly to β-oxidation in the liver and are not stored as fat (Ikeda, 2000). Therefore, MCT can be served as a good energy source in patients with pancreatic insufficiency and fat malabsorption, as well as

premature infants whose pancreatic juice and bile acids are insufficient. However, MCTs lack essential FAs, so they must be supplemented with essential LCFAs.

For years, the fats and oils industry has used rearrangements as a means of achieving physical and chemical modification in a fat or oil. By using the same techniques it is possible to obtain nutritional and health benefits from the same TAGs molecule. Thus, the health benefits of LCFA (the omega-3, 6 and 9 acids) are joined with the nutritional advantages of MCT (Matthews and Kennedy, 1990).

The metabolic advantages of STs over physical mixtures are likely due to the position, or stereospecificity, of certain FAs on the glycerol backbone. One theory is the ST with MCFA in the *sn*2-position of the glycerol molecule are more slowly removed from circulation than if they are given as standard MCT (Schmidl, 1996). Nutritional values and applications of STs with MCFA at the *sn*1- and *sn*3-positions and unsaturated LCFA at the *sn*2-position were investigated extensively. These STs are readily hydrolyzed by pancreatic lipases to give two molecules of free MCFA and one molecule of 2-MAG. These free MCFA are absorbed in intestines and are carried rapidly into the liver via the bloodstream to be consumed as a source of energy, while the remaining 2-MAGs becomes a source of essential FAs. Most TAGs found in vegetable oils are known to contain unsaturated FAs at the *sn*2-position and LCFA at the *sn*1- and *sn*3-positions. These TAG molecules are carried to adipose tissues via the bloodstream in form of chylomicrons. Therefore, unlike free LCFA, the STs are known as a concentrated and rapid source of calories and nutrients (Miura *et al.,* 1999). Figure 3 shows the digestion and absorption of fats and oils in human.

Four main properties of digestion and absorption of TAGs may contribute to the functions of STs (Ikeda, 2000). First, the rate and extent of hydrolysis by gastric and pancreatic lipases are different, depending on the variety of FAs.

Second, these lipases are 1,3-specific lipases. Therefore, FAs at the *sn*2-position are not hydrolyzed and retained during absorption. Third, free stearic and palmitic acids are less absorptive because they have relatively higher melting points and form hydrated acid soaps with divalent cations. Fourth, 2-MAG formed in the intestinal lumen by lipases is readily absorbed, even if the melting point is relatively high.

STs, such as Stepan Co.'s Neobee® lipids, can be used in such foods to supply quick calories as well as the health advantages of the PUFA. This can also be used in sportsmen who need products that quickly give them high amount of energy. For the health-conscious consumers, products formulated with STs will be their best choice (Matthews and Kennedy, 1990).

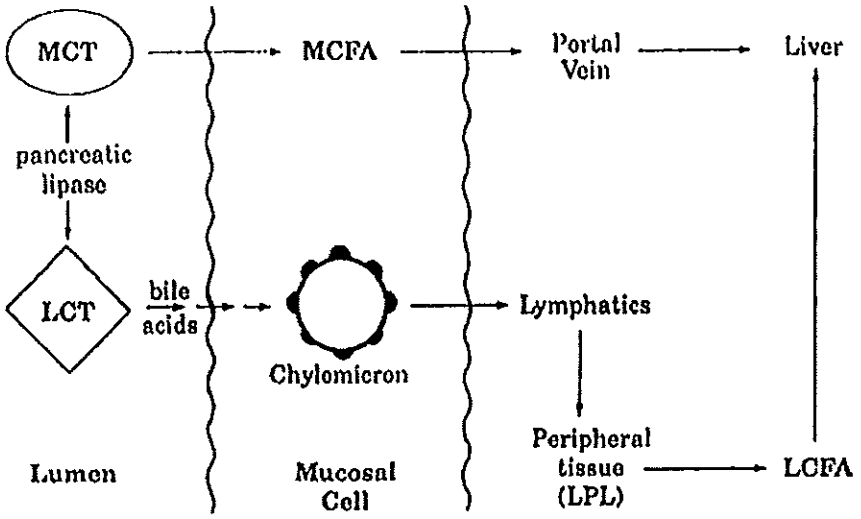


Figure 3 Simplified diagrammatic representation of digestion, absorption and transport of medium- and long-chain triglycerides (Schmidl, 1996).

2.1 Applications of ST

The reasons for utilizing STs are to increase or decrease the availability of FAs and to produce specific physiologic functions.

2.1.1 Prodrugs

In addition to nutritional and health benefits, STs can be used as carriers of therapeutically and nutritionally valuable acids, in form of "prodrugs". These forms are better adsorbed and have fewer side effects than the original drugs. For example, 1,3-DAGs are intermediates for the synthesis of various chemical compounds with pharmaceutical applications. DAG prodrugs can be obtained when the second position is esterified with a drug bearing a carboxylic group such as the anti-inflammatory niflumic acid (Rosu *et al.*, 1999).

2.1.2 Essential fatty acid-rich TAGs

Specialty vegetable oils containing the essential FA γ-linolenic acid (C18:3, n-6), which can not be synthesized *in vivo* in mammals and must be obtained from diet, have been developed and used in a number of therapeutic applications, including infant nutrition, dermatitis, high blood pressure and cancer (Clough, 2001).

According to the high nutritional benefits of PUFA, STs enriched with EPA and DHA have been widely synthesized from different sources of PUFA, especially fish oils, (Haraldsson and Hjaltason, 2001). The incorporation of n-3 PUFA into various TAGs using lipase was also reported (Lee and Akoh, 1998).

2.1.3 Low-calorie TAGs

Conventional TAG-based fats and oils represent the richest source of dietary calories (around 9 kcal/g), as compared to carbohydrates and proteins (4 kcal/g). Accordingly, the reduced-energy lipids are of interest, especially among health- and weight-conscious consumers (Auerbach *et al.*, 2001). By combination of FA structural features, such as the reduced energy content of short-chain FAs, the lowered energy content of MCFA, or the reduced gastric absorption of saturated LCFAs, several new reduced-calorie lipids have been enzymatically produced. The available commercial products are Salatrim, Caprenin, Captrin, and Bohenin.

2.1.4 Infant feeding

STs are also synthesized for utility in infant nutrition, e.g. 1,3-oleoyl-2-palmitoylglycerol (OPO). Human milk fats contain palmitic acid predominantly in the *sn*2-position of TAGs, but the fats in most infant formulas contain palmitic acid predominantly in *sn*1,3-positions, and their release during digestion may cause the formation of poorly absorbed calcium soaps, which results in indigestion and loss of calcium. In contrast, fats containing palmitic acid exclusively at the *sn*2-position are absorbed more efficiently. It was found that the absorption of FFAs varies greatly, depending on their chemical structure. Thus the nutritional value of TAGs and their physicochemical properties are determined not only by the FA compositions but also by the positional distribution of the acyl groups along the glycerol backbone (Schmid *et al.*, 1999).

2.1.5 Miscellaneous products

Cocoa butter equivalents have been designed to approximate the composition of cocoa butter so that they can replace expensive natural cocoa butter in any proportion. Although not generally thought of as a ST, it is the specific TAG structure that gives cocoa butter and cocoa butter equivalents their unique melting properties (Smith, 2001).

In order to achieve some of the textural and melting characteristics of margarines and spreads the modification techniques of original products are well defined (Podmore, 2001). The methods of modification are by relocating the FAs available (interesterification), by concentrating the higher and lower melting TAGs (fractionation), or by modifying the FAs present by way of unsaturation and generation of *trans* isomers. These techniques have been used singly and in combination in order to achieve the desired TAG structure.

2.2 ST production

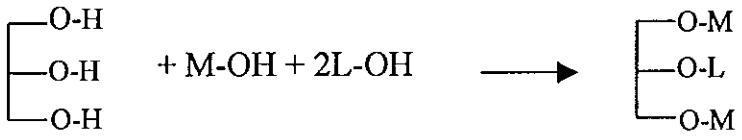
2.2.1 Chemical synthesis vs enzymatic synthesis

Nutritional properties, applications, benefits, and enzymatic approaches for the synthesis of STs have been recently reviewed, together with examples of already marketed, but chemically synthesized, products (Soumanou *et al.,* 1997). The first method reported for the production of MCT was chemical synthesis, which involved the fractionation of MCFA, obtained from the hydrolysis of high-grade vegetable oil, and their subsequent esterification with glycerol and catalysis. With such approach for the production of MLM it would be difficult to introduce the LCFA in the desired position (Soumanou *et al.,* 1998). In addition, these conventional chemical procedures are very expensive due to the extreme conditions, thus enzymatic synthesis was focused on the esterification of this STs from FAs and glycerol using regiospecific lipases. Lipase-catalyzed interesterification reactions, including transesterification and acidolysis, offer the advantage of greater control over the positional distribution of FAs in the final products (Willis and Marangoni, 1999). Very few by-products are formed in enzyme-catalyzed reaction since they are specific (Kwon *et al.,* 1996).

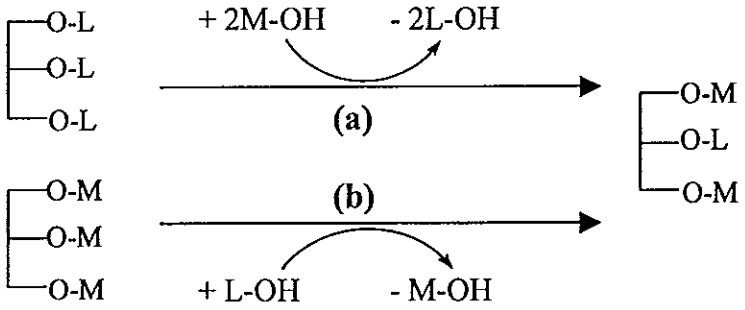
2.2.2 One-step process

Generally, there are three enzymatic routes to synthesize MLM (Soumanou *et al.,* 1997),

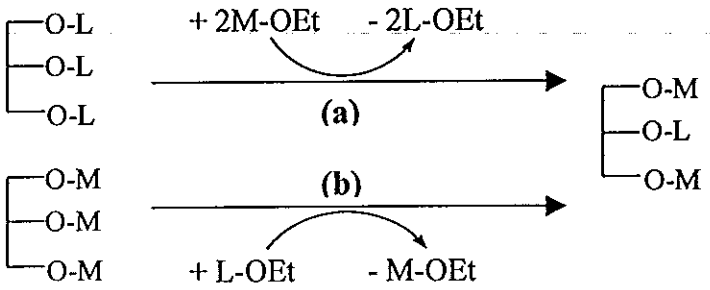
1) Direct esterification of glycerol with LCFA and MCFA.



2) Acidolysis between LCT with MCFA **(a)**, or MCT with LCFA **(b)**.



3) Interesterification, between LCT and MCT (see Figure 2), or LCT with MCFA esters **(a)**, or MCT with LCFA esters **(b)**.



There are many studies focused on ST synthesis with various processes and substrates used (Table 3).

Huang and Akoh (1996) found that STs were successfully synthesized by lipase-catalyzed transesterification of caprylic acid ethyl ester and triolein in organic solvents. It was shown that immobilized lipase IM60 from *R. miehei* converted most triolein into STs (41.7% dicaprylolein, 46.0% monocaprylolein, and 12.3% unreacted triolein). However, lipase SP435 from *C. antarctica* had a higher activity at higher temperature (62.0% dicaprylolein, 33.5% monocaprylolein, and 4.5% unreacted triolein at 55°C). They suggested that the use of MCFA ethyl ester as acyl donor had some advantage, such as high reaction rate, no inhibition at high substrate molar ratio and high substrate concentration, as opposed to the use of FFA.

Soumanou *et al.* (1997) studied the synthesis of STs containing MCFA and LCFA by lipase-catalyzed interesterification between tricaprylin and peanut oil. Among nine commercial lipases, lipases from *Rhizomucor miehei* (RML), *Candida* sp. (CSL), and *Chromobacterium viscosum* (CVL) gave good results for the hydrolysis of pure TAGs as well as natural oils. RML, which was adsorbed onto Hyflo Super Cel, gave the highest yields of ST (73% at 40°C), although its hydrolytic activity toward triolein was low. As the temperature was raised to 50°C, the yield of ST increased to 79%. After 120-hour reaction time, remaining activities were high for CSL (71%), moderate for CVL (48%), and low for RML (20%).

Table 3 Examples for the synthesis of structured triglycerides by one-step reaction.

Substrates	Lipases	Conditions	Yield	Reference
Arachidonic acid-rich TAG + Cy	*R. delemar* on ceramic carrier	n-hexane, 30°C, 1:2 (by mol)	60% Inc.	Shimada *et al.*, 1997
Safflower oil + Cy	*R. delemar* on ceramic carrier	solvent-free, 30°C, 1:2 (w/w), 2 days	45-50% Inc.	Shimada *et al.*, 1996
Perilla oil + Cy	Lipozyme RM IM	n-hexane, 55°C, 1:6 (by mol), 24 h	48-51% Inc.	Kim *et al.*, 2002
Triacetin + stearic acid	immobilized CAL-B (Chirazyme L-2)	solvent-free, water 0.65%, 80°C, 700 mmHg, 4h	88% ST	Yang *et al.*, 2001
Borage oil + Cy	Immobilized *Candida rugosa*	continuous, n-hexane, 30°C, 1:2 (w/w)	44.5% ST	Kawashima *et al.*, 2002
Borage oil + EPA + capric acid	immobilized *R. miehei* (IM60)	n-hexane, 55 °C, water 5%, 1:2:2 (by mol), 24 h with molecular sieves	36.3% Inc.	Akoh and Moussata, 1998
Triolein + La	Lipozyme IM	solvent-free, 70°C, 1:10 (by mol), 24h	70% ST	Miura *et al.*, 1999
Menhaden oil + C10:0	immobilized *R. miehei* (IM60)	n-hexane, 55°C, 1:8 (by mol), 72 h	50.7% Inc.	Jennings *et al.*, 2001
Single cell oil + Cy	*Pseudomonas* sp.	30°C, 1:19 (mol/mol), 6-7 days	> 60% Cy	Iwasaki *et al.*, 1999
Tripalmitin + ethyl ester of C2-C14	Papaya latex lipase	63-66°C, 1:2 (by mol), 24 h	32% Inc.	Gandhi and Mukherjee, 2001
Triolein + ethyl caprylate	immobilized *R. miehei* (IM60)	n-hexane, 45°C, 1:1 (by mol), 24 h	42% ST	Haung and Akoh, 1996
Tristearin + tricaprin	immobilized *R. miehei* (IM60)	n-hexane, 55°C, 1:1 (by mol), 24 h	44% ST	Akoh and Yee, 1997
Corn oil + tristearin	Lipozyme TL IM	solvent-free, 45°C, 4:1 (w/w), 24 h with molecular sieves	21% ST	Torres *et al.*, 2002

% Inc. = % incorporation, Cy = caprylic acid (C8:0), La = lauric acid (C12:0)

Akoh and Moussata (1998) also attempted to incorporate capric acid (C10:0) and EPA to borage oil, which is rich in γ-linolenic acid (GLA), using immobilized lipases in n-hexane in the presence of molecular sieve (4Å). Higher incorporation of EPA (10.2%) and capric acid (26.3%) was obtained with 1,3-specific lipase (IM60), compared to 8.8 and 15.5%, respectively, with nonspecific lipase (SP435). However, SP435 lipase was able to incorporate both capric acid and EPA at the *sn*2-position, but IM60 lipase did not.

Production of STs containing γ-linolenic acid achieved by the continuous acidolysis of borage oil with caprylic acid using 1,3-specific *Rhizopus delemar* lipase, which was immobilized on a ceramic carrier (Shimada *et al.*, 1999). When the substrate mixture was fed at 30°C and a flow rate of 4.5 mL/h into a column packed with 8 g of the carrier with immobilized lipase, the incorporation of caprylic acid was 50-55 mol%. Molecular distillation was employed to separate the transesterified TAGs from the reaction mixture.

Rhizopus delemar lipase immobilized on a ceramic carrier (SM-10) was also used for acidolysis between tuna oil and caprylic acid in a solvent-free system (Shimada *et al.*, 1996). Acidolysis proceeded effectively when a reaction mixture was incubated at 30°C for 2 days with shaking, but hydrolysis occurred simultaneously. This hydrolysis could be suppressed by removing water from the subsequent reaction mixture. The immobilized lipase could be reused 14 times without any significant loss of activity, and approximately 65% incorporation of caprylic acid into the *sn*1- and *sn*3-positions of tuna oil. The reaction did not proceed effectively with free lipase. Acidolysis between safflower oil or linseed oil and caprylic acid using the same condition as in a previous study resulted in 45-50 mol% of FAs in oils exchanged for caprylic acid, and the immobilized enzyme could be reused 45 and 55 times for safflower and linseed oils, respectively. All FAs at the *sn*1,3-position in the original oils were exchanged for caprylic acid by the repeated acidolysis for 3 times.

Acidolysis of menhaden oil with capric acid (C10:0) catalyzed by immobilized lipase from *Rhizomucor miehei* (Lipozyme IM60) was studied both in a solvent and a solvent-free systems (Jennings and Akoh, 2001). The incorporation of capric acid was increased as enzyme load, molar ratio of substrates and incubation time were increased, and the amount of water decreased. It was found that, the highest incorporation occurred at 72 h using oil/FFA molar ratio of 1:8 (50.7% and 36.7% for solvent and solvent-free system, respectively). However, a higher incorporation and less amount of enzyme used were found for acidolysis in hexane than in a solvent-free system.

Miura *et al.* (1999) suggested a rapid method for the preparation of 1,3-dilauroyl-2-oleoylglycerol (LaOLa) with a purity of over 95%. A fraction containing 70% LaOLa was obtained by enzymatic transesterification between triolein and lauric acid using Lipozyme IM. The fraction was passed through a Bond Elut SI Column to remove MAGs and DAGs produced during the transesterification. The ST fraction thus obtained was applied to a reversed-phase column, eluted with acetonitrile/tetrahydrofuran (8:2, vol/vol) to separate different ST species.

The modification of coconut oil by lipase-catalyzed acidolysis in n-hexane to incorporate n-3 or n-6 PUFA using immobilized lipase from *Rhizomucor miehei* (Lipozyme IM) was reported (Rao *et al.*, 2002). From the response surface methodology, it was found that the maximal incorporation of n-3 PUFA (14%) was found at a 1:4 molar ratio of TAG/FFA at 54°C for 34 h, while the maximal incorporation of n-6 PUFA (46%) was predicted at a 1:3 molar ratio of TAG/FFA when incubated at 39°C for 48 h.

Ghosh and Bhattacharyya (1997) studied the use of coconut oil as a starting raw material for the production of MCT. The process was based on the interchange reaction between coconut oil and methyl esters of MCFA by a chemical catalyst (sodium methoxide) or lipase (*Mucor miehei*) catalyst. Coconut oils with 25-28.3% (w/w) and 22.1-25% (w/w) MCFA were obtained

by chemical and lipase-catalyzed interchange reactions, respectively. It was found that coconut olein was a better raw material than coconut oil for production of MCFA-rich TAGs.

MAGs of erucic acid (C22:1, n-13) were reacted with caprylic acid using lipases as catalysts to synthesize caprucin, which was TAG containing two molecules of caprylic acid and one molecule of erucic acid (McNeill and Sonnet, 1995). Nonspecific lipase from *Pseudomonas cepacia* yielded approximately 17% caprucin together with a complex mixture of DAGs and TAGs, which resulted from random transesterification of erucic acid. Specific lipase from *Geotrichum candidum* resulted in a yield of 75% caprucin and promoted minimal transesterification of erucic acid. Caprucin was thereby converted into caprenin, a commercially available low-calorie TAG.

An immobilized *sn*1,3-specific lipase from *Rhizomucor miehei* (IM60) was used to catalyze the interesterification of tristearin with tricaprin (and tricaprylin) to produce low-calorie STs (Akoh and Yee, 1997). Acceptable product yields were obtained from a 1:1 molar ratio of both TAGs with 10% (w/w of reactants) of IM60 in n-hexane, as the molar ratio of tristearin to tricaprin increased yields for C_{41} and C_{49} dropped. The ST molecular species, based on total carbon number, were 44.2% C_{41} and 40.5% C_{49}, with 3.8 and 11.5% unreacted tristearin and tricaprin, respectively, remaining in the product mixture. The best yield of C_{41} was obtained when no water added.

Candida antarctica lipase showed high potential for the synthesis of TAG of PUFA by direct esterification of glycerol with PUFA or its vinyl ester derivatives, either in a solvent system (Kawashima *et al.*, 2001; Medina *et al.*, 1999) or a solvent-free system (Borg *et al.*, 2001; Kosugi and Azuma, 1994).

As mention above, the synthesis of STs by one-step process are usually based on a simple lipase-catalyzed transesterification between two different TAGs or between TAGs and FAs. Unfortunately, the yields of MLM-type STs were low and a formation of a variety of by-products led to difficulty in

separation and purification of the desired ST product (Soumanou, 1997; Schmid *et al.*, 1998; Irimescu *et al.*, 2001). Therefore, the two-step process for the synthesis ST has been recently developed to overcome the disadvantages of the one-step process.

2.2.3 Two-step process

The two-step enzymatic reactions for the synthesis of pure STs were suggested as an effective method for MLM synthesis (Soumanou *et al.*, 1998; Schmid *et al.*, 1999; Irimescu *et al.*, 2001). The principle of the two-step process is shown below (Figure 4).

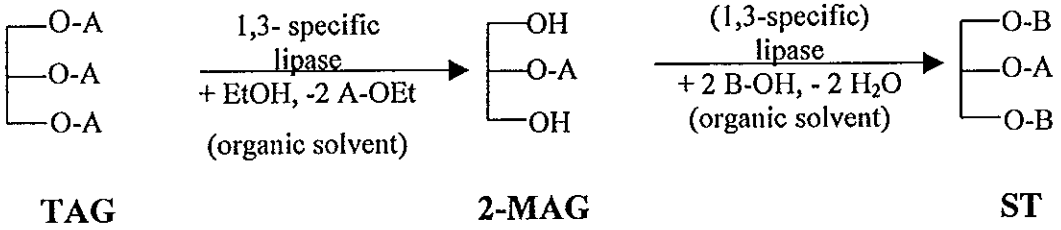


Figure 4 Principle of the two-step synthesis of structured triglyceride. (Soumanou *et al.*, 1997).
A = fatty acids, A-OEt = fatty acid ethyl ester, B-OH = any fatty acids other than A.

The two-step process allows a faster reaction and gives considerably higher final concentrations of the desired MLM in comparison to direct interesterification in one-step process. In addition, this two-step reaction was claimed to be superior to chemical methods and could also be applied to unsaturated FAs (Soumanou *et al.*, 1998). It would be difficult to introduce the LCFA in the desired position of MLM. The final products often contained a significant amount of LCFA in the *sn*1- and/or *sn*3-positions, due to differences in the regiospecificity of the lipases used and acyl migration during synthesis (Hayes and Gulari, 1992). To overcome these problems, esterification of 2-

MAGs with MCFA was reported for the esterification of 2-monopalmitin with caprylic acid using lipase from *Rhizopus delemar* in reverse micellar media.

1,3-oleoyl-2-palmitoylglycerol (OPO) was synthesized by a two-step process in high yields and purity using *sn*1,3-regiospecific lipases (Schmid *et al.*, 1999). The choice of solvent, carrier for lipase immobilization, and water activity, were very crucial factors and may need to be optimized for each new substrate to achieve the best results. In the first step, tripalmitin was subjected to an alcoholysis reaction in organic solvent catalyzed by 1,3-regiospecific lipases yielding the corresponding 2-monopalmitin (2-MP). It was found that, in contrast to the direct synthesis by an one-step interesterification, acyl migration could be suppressed efficiently in the alcoholysis reaction. The 2-MP was then easily isolated in up to 85% yield and >95% purity by crystallization in chilled acetone and esterified in the second step with oleic acid to form the OPO in up to 78% yield containing 96% palmitic acid in the *sn*2-position. The best results were achieved with lipases from *R. miehei* and *R. delemar* immobilized on EP 100 and equilibrated to a water activity of 0.43. At water activity of 0.11, conversion of tripalmitin was low and only a small amount of 2-MP was formed. After a 7-hour reaction time, 82% OPO was formed and further prolongation led to only a slight further increase in OPO concentration. Moreover, longer reaction times also caused the formation of undesired PPO, due to acyl migration and/or lack of 1,3-regioselectivity of the lipase.

Willis and Marangoni (1999) studied a two-step reaction to produce partial glycerides, which would subsequently be used as substrates in both lipase-catalyzed and chemically catalyzed esterification reactions with caprylic acid. The yields and kinetics of these two-step reactions were compared to lipase-catalyzed acidolysis and transesterification as well as to chemical transesterification reactions. Acyl migration did not occur during the hydrolysis or short-path distillation steps in the preparation of FFA-free partial glycerides for esterification reactions. No significant difference in final yields (59.9% to

82.8% w/w of total TAG) of new STs were detected among lipase-catalyzed (24 h) and chemically catalyzed (5 h) reactions. Thus, in contrast with other reports, they suggested that chemical esterification using hydrolyzed oil could represent the best synthetic option.

Soumanou *et al.* (1998) studied the enzymatic synthesis of MLM by two-step reaction. First, pure 2-MAGs were synthesized by alcoholysis of TAGs (triolein, trilinolein, or peanut oil) in organic solvent using 1,3-regiospecific lipases (from *Rhizomucor miehei, Rhizopus delemar*, and *Rhizopus javanicus*). The 2-MAGs were subsequently esterified with caprylic acid in n-hexane in a second reaction to form almost pure MLM. For 2-MAGs obtained from peanut oil, the final product contained more than 90% caprylic acid in the *sn*1,3-positions, whereas, the *sn*2-position was composed of 98.5% unsaturated LCFA. Highest initial rates and final concentrations of MLM were found with Lipozyme (89.1%) and *Rhizopus delemar* lipase immobilized on EP 100 (91.2%). It was demonstrated that, in the esterification of 2-MAGs with MCFA, the water content and/or activity must be low to avoid acyl migration and to convert all 2-MAGs to MLM. The optimal molar ratio of caprylic acid to 2-monoolein was 3:1.

Recently, an effective method for synthesis of DHA-rich STs via a two-step enzymatic reaction was reported (Irimescu *et al.*, 2001). First 2-MAGs rich in PUFA were produced by ethanolysis of bonito oil using *Candida antarctica* lipase B (Novozym 435). At optimum conditions, i.e. the bonito oil to ethanol weight ratio of 3:1, using 10% (based on total weight of substrates) Novozym 435 at 35°C, a reaction mixture composed of 69.5% ethylesters, 2.3% DAGs and 28.2% 2-MAGs (with 43.5% DHA content) was obtained at 2 h. The ethanolysis reaction mixture was used directly in the second step after the catalyst had been filtered out and excess ethanol had been removed. The 2-MAGs formed in the reaction mixture were reesterified with ethyl caprylate using Lipozyme IM, yielding 85.3% TAGs with two caprylic acid residues,

13% TAGs with one caprylic acid, and 1.7% tricaprylin in 1 h. It was found that higher ethyl caprylate to partial glyceride ratios improved the final reaction yield and the reaction equilibrium was pushed to higher yields by removing the resulting ethanol under reduced pressure.

Millqvist *et al.* (1994) found that MTBE was the best solvent for alcoholysis of tripalmitin at water activity 0.11. Ethanolysis gave the highest yield (97%) at an optimal ethanol concentration of 200-300 mM. At higher alcohol concentrations, the enzyme activity was substantially lowered.

Torres *et al.* (2003) studied the kinetics of the lipase-catalyzed ethanolysis of fish oil. The results indicated that the lipase from *Pseudomona cepacia* effectively discriminated against release of EPA and DHA residues regardless of the source of the fish acylglycerols.

Kawashima *et al.* (2001) tried to synthesized high-purity ST with caprylic acid at the *sn*1,3-positions and a PUFA at the *sn*2-position by a two-step enzymatic method. *Candida antarctica* lipase effectively catalyzed the synthesis TAG of PUFA in the first step. The reaction was carried out using a glycerol to PUFA molar ratio of 1:3 catalyzed by 5% immobilized lipase at 40°C and 15 mmHg for 24 h., yielding 88 and 83% TAG of EPA and DHA, respectively. The PUFA-TAG obtained from the first step was then subjected to acidolysis with caprylic acid using immobilized *Rhizopus delemar*lipase at 30°C for 48 h, resulting in 40% mol incorporation of caprylic acid. The caprylic acid content could be increased upto 66% mol with 98% isomeric purity after three repeated acidolysis reactions.

STs were also synthesized starting from 1,3-DAGs (Rosu *et al.,* 1999). 1,3-DAGs containing MCFA were synthesized in the first step by direct esterification of glycerol with MCFA in a solvent-free system using 1,3-specific lipase. The 1,3-DAG thus obtained was used for the synthesis of 1,3-dicapryloyl-2-eicosapentaenoylglycerol by chemical esterification with EPA in chloroform catalyzed by dicyclohexylcarbodiimide and 4-dimethylamino-

pyridine (4-DMAP). The yield after purification was 42%, and the purity of the TAG was 98% (as determined by GC analysis), of which 90% was 1,3-dicapryloyl-2-eicosapentaenoylglycerol (as confirmed by silver ion HPLC analysis).

A chemoenzymatic approach for synthesis of STs containing EPA or DHA at the *sn*2-position and stearic acid at the *sn*1,3-positions was presented in a recent paper (Haraldsson *et al.*, 2000). 1,3-Distearin was synthesized enzymatically in diethyl ether with 74% yield in 48 h and then esterified with DHA or EPA chemically in dichloromethane in 24 h with 91 and 94 % yield, respectively. The reaction times of this method were quite long. Another drawback was the use of toxic chemical catalysts for the esterification step, i.e. 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide and 4-DMAP.

3. Acyl migration

Acyl migration is the intramolecular transfer of an acyl group to an adjacent hydroxyl group (Bornscheuer and Kazlauskas, 1999). It is a well-known reaction in the glyceride and carbohydrate field and has to be taken into account as an undesired side-reaction degrading the regioselectivity of lipases (Sjursnes and Anthonsen, 1994). The equilibrium favors acylation of the less hindered primary position in both MAGs and DAGs (Bornscheuer and Kazlauskas, 1999).

Acid or base catalyze acyl migration, but acyl migration remains fast even at neutral pH in polar solvents (Bornscheuer and Kazlauskas, 1999). The rate of migration was increased with decreasing solvent polarity. To minimize or suppress acyl migration, it is necessary to work in aprotic solvents, such as ketones, ethers, toluene or alkanes (Sjursnes and Anthonsen, 1994; Millqvist-Fureby *et al.*, 1996), and to carefully control the water activity (Schmid *et al.*, 1999).

It was also found that both in the presence of enzymes or FA, the migration rate increased with increasing water activity. Additionally, FA exerted the strongest influence, but using a less basic salt can probably reduce this problem (Sjursnes and Anthonsen, 1994). Studies on the effects of solid salt hydrates showed that the rate of acyl migration was faster in the presence of hydrogen phosphate salts in comparison to sulfate salts (Sjursnes *et al.*, 1995). The reaction velocity of the rearrangement of 2-MAG to 1-MAG in hexane was found to be dependent on the FA chain length in the MAG (Boswinkel *et al.*, 1996). The acyl migration velocity increased with increasing the chainlength of the FA moiety in MAG.

4. Synthesis of DAGs

Synthesis of 1,3-DAGs from glycerol and various acyl donors in MTBE using 1,3-specific lipase from *Rhizomucor miehei* have previously been described (Berger *et al.*, 1992; Waldinger and Schneider, 1996). The products were obtained in high yields (>80%) and high purity and could also be synthesized in large amount (100 g). Because glycerol is immiscible with hydrophobic organic solvents, it was adsorbed onto a solid support, such as silica gel. The highest reaction rates and product yields were achieved when vinyl esters were used as acyl donors due to the irreversibility of these esterification reactions. The direct esterification of glycerol with FFAs with the addition of molecular sieves was quite successful, while FAME was a bad acyl donor.

Solvent-free systems can be also used for synthesis of 1,3-DAGs in high yields (84.6% for 1,3-DCy, 84.4% for 1,3-dicaprin and 67.4% for 1,3-DLa) by direct esterification of glycerol with FFAs (Rosu *et al.*, 1999). When the reaction was performed at normal pressure in a closed vessel, only 60% conversion of FA was achieved at equilibrium. With efficient removal of the produced water by a stream of nitrogen gas or applying vacuum, the conversion

of caprylic acid to 98% at 12 h was obtained with maximum 1,3-DCy yield of 84.6%.

Synthesis of DAG from TAGs and glycerol in a solid-state reaction in a solvent-free system was successfully done (Yamane *et al.*, 1994). Glycerolysis of hydrogenated beef tallow was catalyzed by *Pseudomonas* lipase. It was found that the yield of DAG depended strongly on the reaction temperature. The optimum molar ratio of glycerol to TAG was 1:2. At this ratio, the enzyme-catalyzed reaction was highly efficient and utilized essentially all of the glycerol. The maximum DAG yield of 90% was achieved after 3 days. About 95% of total DAG was 1,3-DAG.

Preparation of DAGs by lipase-catalyzed alcoholysis of TAGs was also studied (Fureby *et al.*, 1997). Different from previous methods, the major DAG obtained from this method was 1,2-DAG. Alcoholysis of trilaurin was carried out in organic media using lipase from *Penicillium roquefortii* immobilized on porous polyethylene particle and 75% yield of dilaurin was obtained. The highest lipase activity was observed at low water activity, but the yield increased with increasing water activity. It was also found that the best solvents were ethers, such as MTBE and diisopropyl ether, in which high enzyme activities were obtained, while the yield and regio-isomer purity of DAG product was low probably due to acyl migration. However, the reaction rate was highest in hexane and isooctane, in which a rapid acyl migration was observed.

5. Phospholipids

5.1 Properties of PL

Many complex lipids found in nature contain phosphorus called phospholipids, or phosphatides, are based either on glycerol (phosphoglycerides) or on sphingosine (sphingolipids) (Chapman, 1969). PL from natural sources contain several FA and their proportion depends on the

source (Svensson *et al.*, 1990). PL have the general structure shown in Figure 5.

Phosphoglyceride (phosphatide) Sphingolipids

Figure 5 Schematic of phospholipid structure (R, R_1, R_2 = fatty alkyl groups, X = base, sugar, etc.) (Ulbrich-Hofmann, 1999; Chapman, 1969).

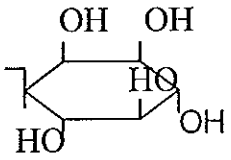
The glycero-phosphate residue is common to the structure of all natural phosphoglycerides. The 1,2-diacyl derivatives are the most abundant. The 1-mono-acyl derivatives are called the lysophosphatidyl derivatives. The plasmogens have been found in animal tissues but not in plant lipids. The substituents on *sn*1- and *sn*2-positions are derived from LCFA with variation on chain length and unsaturation. Most PL contain an appropriate amount of saturated FA having a chain length between 12-26 carbon atoms. In general, stearic and palmitic acids serve as major FA constituents of mammalian PL. It has been shown that unsaturated FA (i.e. oleic, linoleic, linolenic and palmitoleic acids) are usually located preferentially at the *sn*2-position in the lecithin (or PC) molecule (Chapman, 1969). Generally, different PL classes are distinguished based on their head group (Table 4).

5.2 Applications of PL

By exchanging FA asymmetrically in the PL molecule, new physical properties can be achieved. These modified lipids can be used in lipid/membrane research or for application as pharmaceuticals, food additives, cosmetics, medical substances, in liposome technology and in gene transfer

therapy (D'Arrigo and Servi, 1997). A special application of enzymatic transesterification is the position-specific labeling of PL with radioactive or photoactive acyl groups. Furthermore, biologically active PUFA that are chemically unstable can be incorporated under mild condition (Parnham, 1996; Svenssen *et al.*, 1992).

Table 4 Phospholipids commonly found in nature.

Head group (X)	**PL**
- H	Phosphatidic acid (PA)
- $CH_2CH_2N^+(CH_3)_3$	Phosphatidylcholine (PC)
- $CH_2CH_2N^+H_3$	Phosphatidylethanolamine (PE)
- $CH_2CH(OH)CH_2OH$	Phosphatidylglycerol (PG)
- $CH_2CH(N^+H_3)COO^-$	Phosphatidylserine (PS)
	Phophatidylinositol (PI)

From: Chapman (1969).

Various PL may act as antioxidants or prooxidants. Nwosu *et al.* (1997) investigated the effects of three PL classes, (i.e., sphingomyelin, PC, and PE) and their FA composition on antioxidant activity. Antioxidant properties were measured by oxidation induction time. It was shown that lipids with a choline head group had oxidation induction times greater than 60 hours in the salmon oil system. The choline-containing PL also offered better protection from oxidation to the n-3 and total PUFA in salmon oil. PL containing more saturated FA have shown better antioxidant properties and had longer oxidation induction times (>84 hours) and higher antioxidant index (>9). Chain length of FA may have contributed to the observed index, as PL with longer chains (i.e.,

C_{18} and above) had longer oxidation induction times. Radioactive-labeled PL are valuable for physical studies of model membrane system (Akoka *et al.*, 1985).

- **Nutritional use:** PL, especially PC, is used both as emulsifiers in processed foodstuffs and in high concentrations as a nutritional supplement. PC has been confirmed as important precursor of choline and beneficial effect for the neurological health of the elderly. It also has been suggested that infant formulas should contain adequate concentrations of various forms of choline, including PC, to meet the infants' dietary requirements (Parnham, 1996).
- **Cosmetic use:** The major use of PL in cosmetics is in the preparation of liposomes, which are use as skin moisturizing agents or as carriers to facilitate skin penetration of other cosmetic ingredients (Parnham, 1996).
- **Pharmaceutical use:** Purified PL from natural sources, together with semisynthetic and synthetic PL, are employed as pharmaceutical excipients and drug carriers, as well as forming the active ingredients of various pharmaceutical products administered orally, topically, or parentally (Parnham, 1996).

5.3 Modification of PLs

It is desirable to have PL containing special FA for scientific purposes and possibly for some practical applications. PL with special FA compositions can be obtained by solvent fractionation of natural PL, but the most common approach is to synthesize the desired compounds, by chemical or enzymatic conversion or a combination of both. One promising approach is the use of natural PL as starting material and replace the existing FA with the desired ones (D'Arrigo and Servi, 1997; Hara and Nakashima, 1996; Svensson *et al.*, 1990).

Synthesis of PL is difficult by chemical means since control of regio- and stereoselectivity must be ensured (Bornscheuer and Kazlauskas, 1999). Therefore, the normal hydrolytic action of lipases and phospholipases has been used for preparing PL with different FA in two carboxyl ester bond positions. After hydrolysis, the lyso-PL formed can be nonenzymatically esterified with the desired FA (Svensson *et al.,* 1992). Lyso-PL are industrially prepared on a large scale from complete or partial hydrolysis of lecithins catalyzed by lipases or PLA_2, yielding *sn*1-lyso-PL and *sn*2-lyso-PL, respectively (Sarney and Vulfson, 1995). *sn*2-lyso-PL were also obtained by acyl migration of *sn*1-lyso-PL by ammonia vapour.

There are several studies about the synthesis of PL with different FA compositions from original PL. The modification of PL can be effected in different phases of the synthesis and with different methodology (Servi, 1999). The possible combinations are outlined in Figure 6.

5.4 Modification of PL head groups

Although PC is usually used as a substrate, other PL are subject to transphosphatidylation reaction with external alcohol as nucleophiles. Therefore, the studies on head group exchange of PL have been interesting (Table 5).

Subramani *et al.* (1996) reported the characteristics of PLD in reverse micelles. PLD from cabbage showed activity in reverse micelles formed from its substrate PC and Triton X-100 in diethyl ether. The activity of PLD in this system was strongly dependent on the water content included into the reverse micelles. The optimum water content was 12.5%. Increasing in the molar ratio of Triton X-100 to substrate resulted in decreasing activity by 25%.

Juneja *et al.*(1987) studied the PG synthesis by transphosphatidylation of PC and glycerol using cabbage PLD. The comparative investigation was conducted between PC micelles in glycerol-buffer and emulsion of glycerol-buffer in ether containing dissolved PLD to minimize formation of the

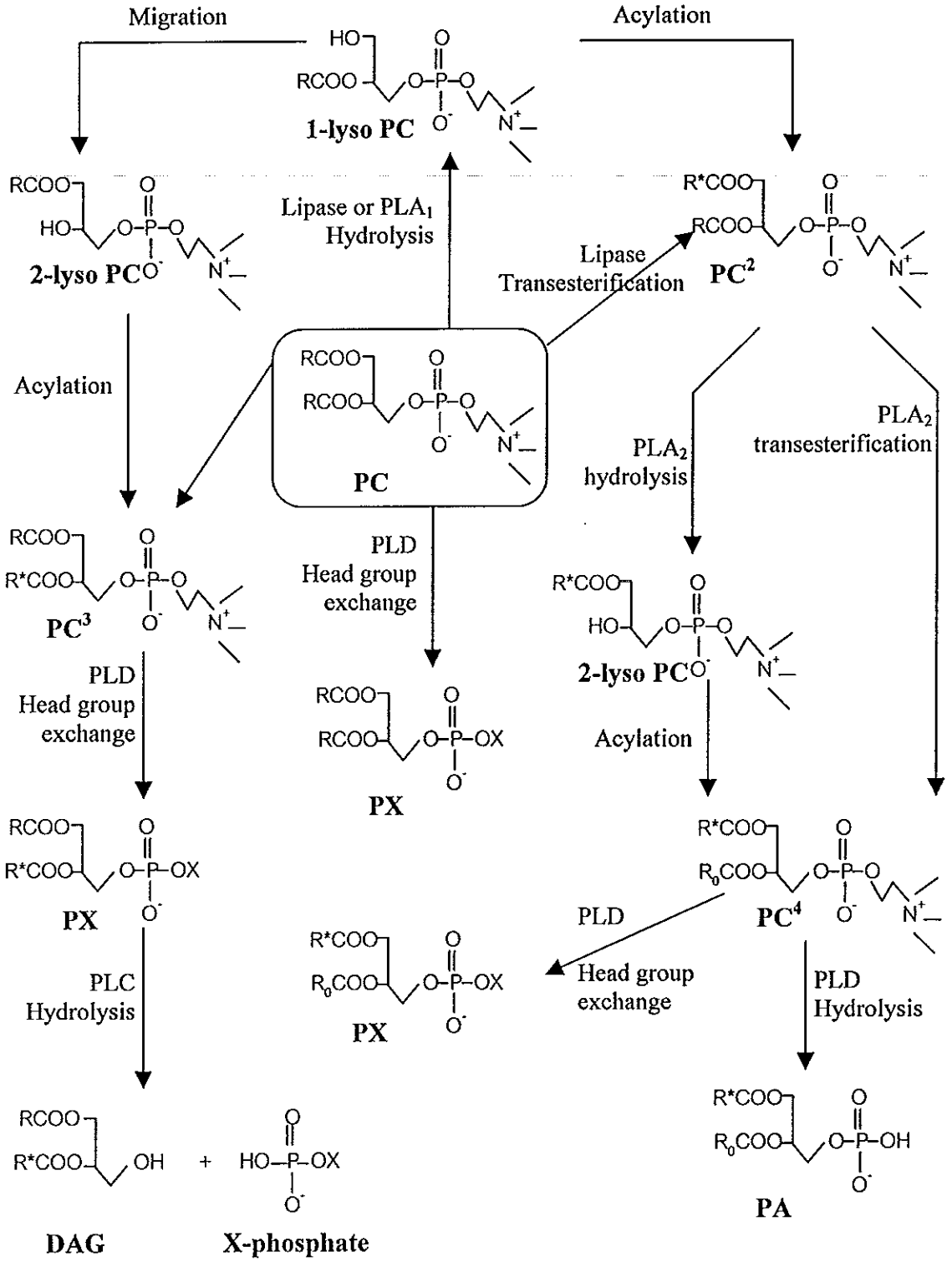


Figure 6 Phospholipase- and lipase- catalyzed enzymatic synthesis of modified phosphatidylcholine and lyso-phosphatidylcholine.
(Sarney and Vulfson, 1995).

Table 5 Transphosphatidylation of phospholipids by phospholipase D.

Substrate	Solvent	PLD type	Condition	Yield	References
PC/glycerol	ether/buffer	cabbage	0.04 M Ca^{2+}, 30% glycerol	100%[C]	Juneja *et al.* (1987)
Lecithin/ glycerol	ether/water	immobilized (Ca-alginate gel enveloped PEI-GA)	25°C, 1 h	80%	Wang *et al.* (1997)
PC/butanol	DE/buffer	cabbage	pH 5.6, 25°C, 120 mM Ca^{2+}	60% PA	Hirche *et al.* (1998)
PC/serine	DE/buffer	cabbage	pH 5.6, 45°C, 90 min	40-50%	Comfurius and Zwaal (1977)
PG	DE/water	*B. cereus*	pH 5.5, 25°C	100%[C]	Virto and Adlercreutz (2000)
PC	micelles	cabbage	12.5% water content	-	Subranani *et al.* (1996)
PL/Inhibitor Conjugates	chloroform	*Streptomyces* sp.	pH 6.5, 30°C, 4h	85%	Wang *et al.* (1993)
DPPC/ROH	chloroform		40°C, with cation resin	100%[C]	Rich and Khmelnitsky (2001)

[C] = % conversion

by-product PA. The initial rate of PG formation was higher in the PC micelles in glycerol-buffer, but so was the proportion of byproduct PA formation. However, the emulsion of glycerol-buffer in ether containing the dissolved PLD system seems to be more attractive because almost 100% conversion of PC and 100% selectivity of PG could be achieved at 30% (w/w) glycerol without any significant formation of PA. The optimum condition was at pH 5.6, 0.04 M Ca^{2+} concentration and 15 g/l PC. Diethyl ether was the most effective not only for reaction rate but also for enzyme stability.

Wang *et al.* (1993) successfully synthesized PL-inhibitor conjugates by transphosphatidylation with PLD. The reaction was performed in chloroform, with 50 mM $CaCl_2$ at pH 6.5, 30°C for 4 h. It was shown that PLD was chemoselective for primary hydroxy group (85% yield) but not reactive with amine and thiol groups. They also synthesized PG from soybean lecithin using PLD immobilized with calcium alginate gel-enveloped polyethyleneimine-glutaraldehyde (Wang *et al.*, 1997). The immobilized PLD could be used in 15 batches at similar conversions of PG. The conversion of 85% PG was obtained at optimum conditions carried out using the ratio of ether phase and water phase in the range of 1.5-2.5 at pH 8.2 at 25-30°C.

Hirche *et al.* (1998) studied the PLD-catalyzed exchange of choline in PC with various primary aliphatic alcohols. It was demonstrated that the reaction course depended on the organic solvent. The comparison of hexane and diethyl ether as organic solvent in two-phase systems showed great differences in the reactivity of PC as well as in the preference of the hydrolysis or transphosphatidylation reaction. In the system hexane/buffer an alcoholic component was necessary in order to make a catalytic reaction possible.

Recently, Rich and Khmelnitsky (2001) developed a new anhydrous reaction system which is highly efficient and synthetically useful for PLD-catalyzed transphosphatidylation of alcohols. The key innovation of the reaction system was a cation exchange resin serving as a scavenger for choline,

which was shown to have a strong inhibitory effect on PLD, that forms as a byproduct in the reaction. Due to the absence of water in this system, the reaction path dramatically shifts in favor of the target transphosphatidylated product. In contrast to commonly used biphasic water-organic systems, the undesirable hydrolysis side reaction of PC was completely suppressed. In addition, a salt activation technique was successfully applied to increase the catalytic activity of PLD in this anhydrous system. The new reaction system was successfully used (80-100% conversion was achieved after 2-day incubation) for transphosphatidylation of a wide range of primary, secondary, and aromatic alcohols catalyzed by PLD from *Streptomyces* sp. They also found that, though the initial rates observed were similar for the two enzyme preparations, the suspended enzyme was able to achieve higher alcohol conversion (100%) than the immobilized enzyme (80%).

Virto and Adlercreutz (2000) investigated a production of two lyso-PL using a combination of PLD and PLC. PLD from *Streptomyces* sp. allowed the formation of 1-lauroyl-phosphatidylglycerol and 1-lauroyl-phosphatidyl-dihydroxyacetone from PC and 1-monolauroyl-*rac*-glycerol and 1-lauroyl-dihydroxyacetone, respectively. Conversions of 100% could be achieved at low substrate alcohol concentrations. A two-phase system, diethyl ether/water, was chosen for the convenience in the recovery of the water insoluble products. A similar two-phase system was used for subsequent hydrolysis of the products by PLC from *Bacillus cereus* to obtain DAG and 1-lauroyl-*rac*-glycerophosphate and 1-lauroyl-dihydroxyacetonephosphate. Reactions were performed at 25°C, pH 5.5 for PLD and pH 7.5 for PLC.

5.5 Chemoenzymatic Modification of PL

Chemoenzymatic processes have been also suggested as an efficient tool for the synthesis of specific PL.

Baba *et al.* (1994) reported the synthesis of 1-stearoyl-2-[15'-(*S*)-hydroperoxyicosatetraenoyl]-phosphoserine by utilizing lipoxygenase catalyzed hydroperoxidation, protection of the hydroperoxy group, 1,3-dicyclohexylcarbodiimide (DCC)-mediated esterification and PLD-catalyzed transphosphatidylation. This process was found to proceed successfully and afforded a single desired product, PS-hydroperoxide, although the yield (48%) was not satisfactory.

Pisch *et al.* (1997) chemically synthesized 14-octadecynoic acid and 4-octadecynoic acid and incorporated them into GPC at the *sn*1- and *sn*2-positions using 1,1'-carbonyl-diimidazole (CDI) in dichloromethane and 1,8-diazabicylo[5.4.0]undec-7-ene (DBU) as chemical catalysts. The head group of the PC thus obtained (98% yield) was exchanged using PLD from various sources in a biphasic system consisting of chloroform/sodium acetate (0.1M)/$CaCl_2$(0.1M) buffer. PLD from cabbage was unsuitable for all nucleophiles and required much higher reaction time. Under optimized conditions (*Streptomyces* sp. PLD, 40°C, solvent:buffer ratio 1:1.5 (v/v), pH 5.6), the conversion of PC to the corresponding PL with the head groups glycerol, ethanolamine and *L*-serine reached 99% in 1 hour and yields of isolated products were between 85-87%. Changes in nucleophile concentrations gave no influence on the conversion, but the initial rates of product formation varied to some extent.

6. Phospholipases

6.1 Types of Phospholipase

Phospholipases are rapidly becoming one of the more useful tools in the analysis of PL, and they also have applications in synthetic work. These are enzymes which effect hydrolysis specifically at only one type of ester linkage in the phosphoglyceride structure (Chapman, 1969). It takes four different phospholipases to cleave a PL, such as lecithin. They are phospholipase A_1, A_2,

C and D. PLB exhibits the combination of PLA_1 and PLA_2 activity. They attack a PL as shown in Figure 7.

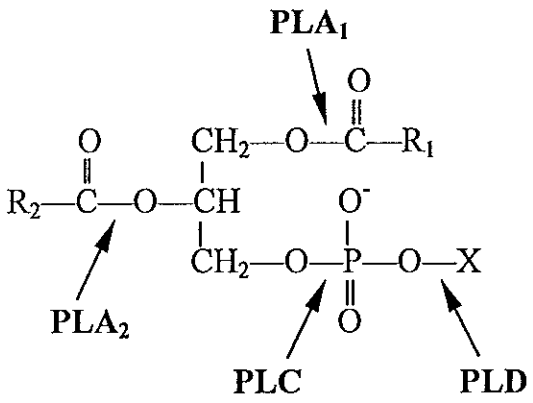


Figure 7 Possible sites of enzymatic hydrolysis of phospholipids.
(Chapman, 1969).

6.1.1 Phospholipase A_1 (PLA_1)

PLA_1 (phosphatidylcholine 1-acyl-hydrolase, EC 3.1.1.32), usually obtained from animal sources such as the pancreas, catalyses the hydrolysis of the 1-lysophosphatides. It was the only phospholipase, which was rarely used because the resulting PL was unstable due to facile acyl migration of residues at the *sn*2-position to the *sn*1-position. In addition similar reactions are catalyzed by a number of lipases, e.g., from *Rhizopus* sp, *Mucor* sp, which are better available and more thoroughly investigated (Ulbrich-Hofmann, 2000; Bornscheuer and Kazlauskas, 1999). Recently, PLA_1 from *Fusarium oxysporum* (Novozymes) has been used for degumming of oils.

6.1.2 Phospholipase A_2 (PLA_2)

PLA_2 (phosphatidylcholine 2-acyl-hydrolase, EC 3.1.1.4), which hydrolyzes FA bound at the *sn*-2 position of PL, has been detected universally in a variety of mammalian tissues and cells. It releases FA from the 2-acyloxy group in glycerophosphatides. The enzyme needs calcium ions for activity and

the hydrolytic degradation, which has an optimum pH of 7.2. (Kudo *et al.*, 1993; Chapman, 1969).

It is possible that the water activity influences not only the enzyme but also the molecular organization of the PL substrate. The packing density of PL molecules increases with decreasing water activity, then leads to decreasing in PLA_2 activity (Egger *et al.*, 1997). It is also detected, that a small amount of ether or alcohol increases the hydrolysis rate, but large amount of it inhibits the hydrolysis (Lin *et al.*, 1993).

6.1.3 Phospholipase C (PLC)

PLC (phosphatidylcholine phosphohydrolase, EC 3.1.4.3) hydrolyses PC to DAG and choline phosphate and appears to be mainly confined to the bacterial kingdom (Chapman, 1969). Cultures of *Bacillus cereus* and *B. thuringiensis* are suitable for the production of PLC for biocatalytic applications (D'Arrigo and Servi, 1997). Because of its high stereospecificity, PLC can be used to obtain enantiomerically pure *sn*-1,2-diacylglycerols by hydrolysis of corresponding glycerophospholipids. These compounds are important in biomedical research and can also serve as substrates for the production of synthetic PL with natural configuration (Ulbrich-Hofmann, 2000).

6.1.4 Phospholipase D (PLD)

PLD (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4) is readily obtained from a variety of plant tissues and has been the most commonly used phospholipase due to its relatively strong tendency to catalyse transesterification at the terminal phosphate ester bond of PL (Ulbrich-Hofmann, 2000; Chapman, 1969). PLD was first discovered as a lipolytic enzyme in the extracts of cabbage leaves. It is known to hydrolyze saturated and unsaturated phophatides to PA in good yields and also catalyze a transfer of the phosphatidyl moiety between two nucleophilic compounds containing a primary hydroxylic group (polar head group) (Juneja *et al.*, 1987; Chapman,

1969). PLD has been extensively studied as a catalyst for the synthesis of PL that are naturally occurring in minor quantities, i.e. PS and PG, which have been prepared in excellent yields from PC (Sarney and Vulson, 1995). Table 6 reports data concerning the conversion and selectivity for the transphosphatidylation reaction using PC, PE, or PG as substrates and choline (C), glycerol (G), or ethanolamine (E) as alcohol acceptors. The reaction was run at 36°C in a biphasic system formed by acetate buffer at pH 5.5, 1 M in alcohol and the PL solution in ethyl acetate containing 30 U/g of PL. The data indicated that the selectivity was higher with PE and PG, both poorer substrates of the enzyme (Juneja *et al.*, 1987).

Table 6 Conversion and selectivity of phospholipase D catalyzed transphosphatidylation of different phospholipids in an emulsion system.

Reactants	T %*	PX/PA
PC + glycerol	77	19
PC + ethanolamine	96	23
PE + glycerol	49	∝
PE + choline	26	∝
PG + choline	7	∝
PG + ethanolamine	18	∝

From : Servi, 1999

* T %ratio = PX/(PL+PA+PX), where PX indicates PL with X-head group.

The pH-adjustment technique exerts the improvement of the catalytic activity of PLD in non-aqueous solvents. The pH-adjusted enzyme was approximately fivefold more active than untreated enzyme (Rich and

Khmelnitsky, 2001). The presence of high concentration of KCl also improved PLD activity in the anhydrous reaction system. Therefore, the combination of pH-adjustment and salt concentration technique can be applied as a simple and efficient method to activate enzymes in anhydrous solvent.

The best solvents for the PLD-catalyzed transphosphatidylation of 1,2-dipalmitoyl-3-*sn*-phosphatidylglycerol (conversion >75% after overnight incubation) was chloroform > methylene chloride > MTBE > diethyl ether ≈ ethylacetate and the poorest solvents were: *tert*-butyl alcohol < benzene ≈ n-hexane ≈ acetonitrile < n-hexane/acetonitrile mixture < toluene (conversion <25% after overnight incubation) (Rich and Khmelnitsky, 2001).

7. Palm oil

Palm oil has a successful production as an export crop, almost exclusively in Southeast Asia (Berger, 2001).

7.1 Physical and Chemical Properties

Two types of oils are obtained from palm fruit; palm oil from fruit mesocarp and palm kernel oil.

Palm oil contains almost equal proportions of saturated and unsaturated fatty acids. Palmitic and oleic acids are major fatty acids with small quantities of linoleic and stearic acids. Its fatty acid composition is comparable to that of human milk, lard, tallow and hydrogenated whale oil (Salunkhe *et al.,* 1992). The fractionation of palm oil into palm olein and stearin fractions has significant influence on its fatty acid composition (Table 7). The olein fraction resembles other edible vegetable oils and contains a high proportion of unsaturated fatty acids, particularly oleic acid. The stearin fraction contains more saturated fatty acids, particularly palmitic acid, and resembles other saturated fats (Salunkhe *et al.,* 1992).

Neutralization process of crude palm oil, using just a sufficient quantity of caustic soda, was found to produce good refined oil yields and quality. After

mixing preheated-crude palm oil with caustic soda, then heating to 82-88°C, the soap phase was immediately separated from neutralized palm oil (Salunkhe, *et al.,* 1992).

Table 7 Fatty acid composition of processed palm oil and palm oil fractions.

Palm oil	Fatty acid (%)							
or fraction	14:0	16:0	18:0	**Total saturated**	18:1	18:2	18:3	**Total unsaturated**
Neutralized	0.8	41.0	5.1	**46.9**	39.0	13.8	0.2	**53.0**
Interesterified	0.7	41.2	5.0	**46.9**	38.6	13.8	0.6	**53.0**
Olein	0.5	9.9	1.4	**11.8**	63.1	23.9	0.2	**87.2**
Stearin	1.0	66.0	8.4	**75.9**	18.6	5.4	0.1	**24.1**

Source: Salunkhe, *et al.* (1992).

Palm oil is stable and resistant to rancidity. It is solid at ambient temperatures in temperate climates and fluid in tropical and subtropical climates with certain fractions held in crystalline form. Interesterification reaction, using a basic catalyst (e.g. NaOH), provides a method of transferring saturated fatty acid to predominately unsaturated glycerides and vice versa. Fractionation of palm oil produces two fractions, one with a very low melting point (olein) and the other with a high melting point (stearin). The iodine value of palm oil is lower (44-58) than other vegetable oils because of a high proportion of saturated fatty acids. The saponification value of palm oil is higher (195-205) than other edible vegetable oils.

Palm oil is a rich source of vitamins A and E. It contains 5-11% linoleic acid which is known to be essential for growth and health in animals and humans. Deficiency symptoms in humans include skin disease, loss of weight and increased metabolic rate. Palm oil is easily digested, absorbed and utilized for the support of healthy growth (Salunkhe *et al.,* 1992).

7.2 Application of palm oil

Palm oil is one of the world's most widely consumed edible oils, next only to soybean. It is mostly used as shortening, margarine, vanaspati (hydrogenated fat) and frying fat. It is also used in non-food products, for the manufacture of soaps and candles, or in tin-plate industry (Salunkhe *et al.*, 1992).

Certain TAG, with cocoa butter being the outstanding example, have high value because of their physical properties and comparative rarity. Acidolysis reactions, using lipases as biocatalyst, may be used for increasing the value of palm oil and sunflower oil by increasing their content of oleic acid residues. Cocoa butter is a relatively expensive fat used in confectionery, because of its sharp melting point between room temperature and body temperature, thus chocolate literally melts in the mouth. This is due to the fairly small variation in the structured of the constituent TAG; 80% have palmitic acid or stearic acid in the *sn*1- and 3-positions, with oleic in the *sn*2-position. For the production of cocoa butter substitute from palm oil, a process which increases the value of the product three-fold, the acidolysis utilizes stearic acid in hexane containing just sufficient water to activate the lipase (Chaplin and Bucke, 1990).

8. Tuna oil

Around 1.5 million metric tons of fish oils from a variety of species is produced annually worldwide (Bimbo, 1990). Marine oil is predominantly used for the production of salad oils, frying fats, table margarine, low-calorie spreads, and industrial margarine and shortenings for bakery products. Other uses include feed for livestock, pets, aquaculture species as well as numerous industrial products.

8.1 Physical and Chemical Properties

Fish oils and marine-animal oils are generally characterized by a rather large group of saturated and unsaturated FAs, which are commonly associated

with mixed TAGs. The FAs derived from fish oils are of three principal types: saturated, monounsaturated, and polyunsaturated. The formula,

$$CH_3(CH_2)_x(CH{=}CHCH_2)_n(CH_2)_yCOOH$$

where $n = 0$ to 6, illustrates the type of FA structures common to fish oils.

The saturated FAs have carbon chain lengths that generally range from C_{12} to C_{24}. The carbon chain lengths of the unsaturated FAs range generally from C_{14} to C_{22}. The PUFAs are preferentially located in the *sn*2-position of both TAGs and PLs from fish oils (Stansby *et al.*, 1990). Fatty acid compositions of some fish oils are shown in Table 8.

Table 8 Major fatty acid compositions of fish oils.

Fatty acid	Menhaden oil (Jennings *et al.*, 2001)	Cod liver oil (Medina *et al.*, 1999)	Tuna oil (Shimada *et al.*, 1997)	Tuna oil (Sarabok, 2000)
C14:0	1.9	3.5	2.6	4.0
C16:0	2.5	10.8	17.9	20.8
C16:1	2.7	7.4	4.9	5.8
C18:0	3.5	2.7	3.9	6.6
C18:1	9.9	18.2	23.0	10.7
C18:2	1.8	1.8	-	1.7
C18:3	1.5	0.8	-	-
C20:1	5.0	12.0	-	-
C20:5 (EPA)	34.7	10.1	5.8	6.4
C22:6 (DHA)	34.4	12.2	23.2	27.2

8.2 Applications and Health Benefits

In the past, fish oil was used in the diet to minimize the chance of acquiring cardiac heart failures. After intensive studies, research has shown that

fish oils were far superior to any vegetable oils in their ability to lower serum cholesterol levels (Stansby, 1990a). Nowadays, much research has shown that in the presence of omega-3 FAs in the diet, a significant reduction in platelet aggregation as well as lower blood pressure occurred, which in turn could reduce the possibility of heart attacks (Stansby, 1990b). The PUFAs of the omega-3 (EPA and DHA) and omega-6 (linoleic acid) series are of interest because they serve as precursors for the synthesis of eicosanoids, which are potent regulators of immune function and differentiation (Schmidl, 1996).

There are also a number of researches declaring the effects of omega-3 FAs on diseases other than coronary-type diseases. It was found that consumption of an appropriate amount of omega-3 FAs could result in diminishing the undesirable effects of inflammatory diseases, beneficial to rheumatoid arthritis and strokes, reduction in the severity of glomerular nephritis, prevention of multiple sclerosis, favorable effects on breast or colon cancers, and probably relieved certain skin diseases. Recently, the function of omega-3 FAs in the brain and retina was published (Stansby, 1990b).

Objectives

1. To study the synthesis of structured triglycerides based on palm oil and tuna oil by one-step or two-step reactions catalyzed by immobilized lipases
2. To study the modification of phospholipids by enzymatic method

Scope of Research Work

1. Optimization of structured triglycerides production by one-step reaction.
2. Optimization of structured triglycerides production by two-step reaction.
3. Synthesis of structured triglycerides by a novel two-step reaction.
4. Modification of phospholipids by transphosphatidylation catalyzed by phospholipase D.

CHAPTER 2

MATERIALS AND METHODS

Materials

1. Enzymes

Various commercial enzymes, available in powder or immobilized forms, used in this study, were from different suppliers, as shown in Table 9 and Table 10.

Table 9 Commercial enzymes powder used in this work.

Enzyme	Trade name	Supplier
Lipase from		
Candida antarctica A	Chirazyme L-1	Roche, Germany
Candida rugosa	Lipase OF	Amano, Japan
Penicillium roquefortii	Lipase R	Amano, Japan
Porcine pancreas		Amano, Japan
Pseudomonas sp.	Lipase PS	Amano, Japan
Rhizopus delemar	Lipase D	Amano, Japan
Rhizopus javanicus	Lipase F-AP15	Amano, Japan
Rhizopus niger	Lipase M	Amano, Japan
Phospholipase from		
Peanut (phospholipase D)		Sigma, Germany
Bee venum (phospholipase A_2)		Sigma, Germany
Recombinant *E. coli* containing PLD-gene		Nagoya University, Japan

Table 10 Commercial immobilized lipases used for the synthesis of structured triglycerides.

Lipase from	Matrix	Trade name	Supplier
Candida antarctica B	Carrier 2	Chirazyme L-2	Roche, Germany
Pseudomonas sp.	Celite	Lipase PS-C	Amano, Japan
Pseudomonas sp.	Diatomaceous earth	Lipase PS-D	Amano, Japan
Rhizomucor miehei	Anion exchange resin	Lipozyme RM IM	Novo, Denmark
Rhizopus delemar	Accurel (EP-100)	-	Unilever, The Netherlands
Thermomyces lanuginosa	Silica	Lipozyme TL IM	Novo, Denmark

2. Chemicals

All chemicals and solvents used were reagent grade and purchased from common commercial suppliers.

Chemical	Supplier
1,1'-carbonyl diimidazol (CDI)	Fluka, Switzerland
1,2-Dipalmitin	Fluka, Switzerland
1,8-Diazabicyclo[5.4.0]undec-7-en (DBU)	Fluka, Switzerland
Accurel EP-100	Akzo Nobel, Germany
Acetic acid (glacial)	Lab-scan, Ireland
Acetone	Lab-scan, Ireland
Acetonitrile	Lab-scan, Ireland
Allylmagnesium bromide	Fluka, Switzerland
Ammonium hydroxide	Carlo Erba, Italy
Benzene	Merck, Germany
Boric acid	Merck, Germany
Calcium chloride	Berker Analyzed, USA
Capric acid vinyl ester	TCI, Japan

Chemical	Supplier
Caprylic acid	Fluka, Switzerland
Caprylic acid vinyl ester	TCI, Japan
Celite	Wako Pure, Japan
Chloroform	Lab-scan, Ireland
Deoxycholic acid sodium salt	Fluka, Switzerland
Dichloromethane	Lab-scan, Ireland
Diethylether	Lab-scan, Ireland
Disodium hydrogenphosphate	Univar, UK
Dowex 1x8	Merck, Germany
Dowex 50	Merck, Germany
Ethanol absolute	Merck, Germany
Ethyl acetate	Lab-scan, Ireland
Ethyl methyl ketone	Fluka, Switzerland
Ethylenediamine tetraacetic acid (EDTA)	Univar, UK
Formic acid	Carlo Erba, Italy
Glycerol	Carlo Erba, Italy
Glycerophosphatidylcholine	Euticals, Italy
Glycerophosphatidylethanolamine	Euticals, Italy
Gum arabic	Fluka, Switzerland
Hydrochloric acid	J.T.Beker, USA
Iodine resublimed	Carlo Erba, Italy
Isooctane	Carlo Erba, Italy
Lauric acid	Fluka, Switzerland
Lauric acid vinyl ester	Fluka, Switzerland
Lecithin	Fluka, Switzerland
Linoleic acid	Fluka, Switzerland
Lithium chloride	Fluka, Switzerland
Magnesium chloride	Merck, Germany

Chemical	Supplier
Magnesium nitrate	Merck, Germany
Methanol	Lab-scan, Ireland
Methyl-*tert*-Butyl ether	Fluka, Switzerland
Molecular sieves 4A	Wako Pure, Japan
Myristic acid	Fluka, Switzerland
n-Hexane	Lab-scan, Ireland
Oleic acid	Fluka, Switzerland
Palladium (II) acetate	Fluka, Switzerland
Palm oil	Morrakot, Thailand
Palmitic acid	Fluka, Switzerland
Petroleum ether	Lab-scan, Ireland
Phosphatidylcholine	Euticals, Italy
Potassium acetate	Riedel-de Haën, Germany
Potassium carbonate	Merck, Germany
Potassium chloride	Merck, Germany
Sephadex SP250	Toyopearl, Japan
Siliga gel 60	Merck, Germany
*sn*1,2-dilauroyl-*sn*3-phosphatidylcholine	Euticals, Italy
Sodium acetate-3-hydrate	Riedel-de Haën, Germany
Sodium hydrogenphosphate	Univar, UK
Sodium hydroxide	Merck, Germany
Stearic acid	Fluka, Switzerland
Toluene	Carlo Erba, Italy
Trilaurin	Fluka, Switzerland

Crude tuna oil was obtained from a tuna canning factory in Songkla. Crude coconut oil was purchased from local supplier.

3. Equipments

Equipments	Supplier
Hydrolytic activity	
pH-Meter (702 SM Titrino)	Metrohm, Switzerland
Stirrer (703 Ti Stand)	Metrohm, Switzerland
Reaction	
Thermomixer (comfort)	Eppendorf, Germany
Magnetic stirrer	KIA, Malaysia
Thin-layer chromatography (TLC)	
Silica gel 60 F_{254}	Merck, Germany
Development chamber	Roth, Germany
TLC-FID (Iatroscan)	
Iatroscan MK-5^{new} TLC-FID analyzer	Iatron Laboratories, Japan
Chromarod SM-III	Iatron Laboratories, Japan
Chromarod holder SD-5	Iatron Laboratories, Japan
Developing tank DT-150	Iatron Laboratories, Japan
GC	
GC Hewlett-Packard 5890 (series II)	Hewlett-Packard, USA
Column 25 m x 0.25 mm (OPTIMA-5)	Macherey-Nagel, Germany
HPLC	
HPLC	Agilent, Germany
ELS detector	Polymer labs, Germany
Column (Nucleosil ODS 5 µm, 250 x 4.6 mm)	Agilent, Germany
Evaporator	
Rotary vacuum evaporator (N-N Series)	Eyela, Japan
Aspirator A-3S	Eyela, Japan
Digital water bath	Eyela, Japan

Analytical Methods

1. Hydrolytic activity of lipases

The hydrolytic activity of the free and immobilized lipases was determined by means of a pH-Stat assay using 5% (w/v) oil emulsions that contained 2% (w/v) gum arabic. Twenty mL of the emulsified solution, 470 µl of calcium chloride solution (22 % w/v), and a known amount of immobilized lipase were mixed, and liberated fatty acids were titrated automatically with 0.01 M sodium hydroxide to maintain the pH constant at pH 7.5 for 20 min. One unit (U) of lipase activity was defined as the amount of enzyme releasing 1 µmol FA per minute under assay conditions (Soumanou *et al.*, 1997).

2. Activity of PLD

Activity of PLD was determined by pH-Stat method. The assay solution consisted of 10 mg PC dissolved in 2 mL ether, 14 mL water and 1.2 mL calcium chloride (1 M, pH 5.6). Culture medium or concentrated culture medium (100-200 µl) or 5-10 mg lyophilized cultured powder dissolved in 1 mL sodium acetate buffer (0.1 M, pH 5.6) was added to the reaction mixture. The pH of the mixture was kept constant at 5.6 using 0.01 M sodium hydroxide solution. PLD activity was calculated from the initial rate of acid formation. One unit of hydrolytic activity was defined as the amount of enzyme that hydrolyzed 1 mmol of pure PC per minute at 40°C (Pisch *et al.*, 1997).

3. Determination of glyceride and PL compositions of the reaction mixture by TLC-FID analysis

Changes in glycerides and PL compositions during the reaction were quantitatively determined using a Thin Layer Chromatography equipped with Flame Ionization Detector (TLC-FID)

For determination of glyceride composition, Chromarods were immerged in boric acid solution (3% w/v) and dried at 110°C for 5 min prior to use in

order to minimize acyl migration during developing period (Rosu *et al.*, 1998). The boric acid-treated Chromarods were scanned before analysis. One µl of the reaction medium (diluted in chloroform at appropriate dilution) was spotted onto the Chromarod and the spotted sample was developed in a solvent mixture of benzene:chloroform:acetic acid (50:30:0.5 v/v) until the solvent front reached to 10 cm (approximately 35 min). Then the Chromarods were dried at 110°C for 5 min. Scanning was performed using a hydrogen flow rate of 160 mL/min and an air flow rate of 2.0 l/min to produce a chromatogram. The compositions were calculated as %mol based on the peak areas of each component (Appendix B, 3).

For the reaction mixture containing FAVE, Chromarods were developed in two solvent systems. First, development was carried out in a solvent system composed of n-hexane:diethyl ether:formic acid (50:20:0.5 v/v) for 15 min (solvent front = 8 cm). After air dried for 30 sec, the Chromarods were subsequently developed in a second solvent system composed of benzene:chloroform (50:20 v/v) for 35 min (or until the solvent front reached 10 cm), then dried at 110°C for 5 min and scanned with TLC-FID.

For determination of PL composition, no boric acid treatment of Chromarods was performed. The Chromarods were scanned twice prior to use. An aliquot amount of the reaction mixture was mixed with 200 µl Folsh's solution and 200 µl distilled water for 30 s. After centrifugation, two µl of the organic layer was applied to Chromarods. Chromarods were then developed in a solvent mixture of chloroform:methanol:water (40:20:1 v/v) until the solvent front reached 10 cm (approximately 40 min). The Chromarods were air dried for 2 min, followed by scanning with TLC-FID.

4. Determination of fatty acid composition by GC analysis

The FA compositions of TAG were determined by converting all FAs of TAG into the corresponding FAMEs, followed by GC analysis. After

evaporation of excess solvent *in vacuo*, 10 mg of acylglycerol was methanolyzed with 0.5 % NaOH in methanol (500 µl) and then incubated for 15 min at 60°C. The methylesters were extracted with n-hexane (500 µl) for 1 min. The n-hexane layer was washed with 200 µl distilled water and dried over anhydrous sodium sulfate. Analysis was carried out with a Hewlett-Packard 5890 (series II) gas chromatograph (Hewlett-Packard, USA) on a capillary column (OPTIMA-5, 25 m x 0.25 mm i.d., Macherey-Nagel GmbH, Düren, Germany). Helium was used as the carrier gas. The temperature program was 150°C (4°C/min, 0.50 min), 170°C (10°C/min), 195°C (10°C/min) and 215°C (15 min). Injector and detector temperatures were 250°C. Response factors were determined using a standard mixture of FAMEs (Appendix B, 4).

5. HPLC separation of TAGs

The composition of TAGs formed during the enzymatic esterification and interesterification was characterized by HPLC using a nucleosil ODS column, (5µm, 250 x 4.6 mm, Agilent, Waldbronn, Germany) and an evaporative light scattering detector (ELSD) (Polymer Labs, Darmstadt, Germany) at a room temperature and a flow rate of 1.5 mL/min. The purpose of ELSD is to complement ultraviolet (UV) detection of solutes, and to detect solutes, which do not absorb UV radiation such as medium-chain TAGs. The three main processes that occur successively within an ELSD:

1) nebulization of the chromatography eluent
2) evaporation of the chromatography eluent
3) light scattering by the residual particles, ideally constituting the analytes.

The principle of ELSD applies to all solutes having a lower volatility than the mobile phase. Elution was performed using a gradient elution system of acetonitrile and dichloromethane (70 to 55% acetonitrile over 10 min, followed by 55 to 70% acetonitrile over 8 min).

6. Regiospecific analysis of TAGs

The regiospecific analysis of oil was conducted by Grignard degradation with allylmagnesium bromide (Soumanou, 1997) or by enzymatic partial degradation using porcine pancreatic lipase (Schmid *et al.*, 1999) followed by GC analysis.

For Grignard degradation (Soumanou, 1997), 10 mg of TAG was dissolved in dry diethyl ether (1 mL), a freshly diluted solution of ethyl magnesium bromide (250 µl) was added, and the mixture was shaken for 1 min before adding glacial acetic acid (6 µl) in hexane (5 mL) and water (2 mL), and mixed. Hexane phase containing a mixture of deacylated products was withdrawn and dried over anhydrous sodium sulfate. After evaporation, the mixture of deacylated products was separated by TLC plates (Silica gel 60, F_{254}) impregnated with boric acid using chloroform:methanol:ammonia (70:0.4:0.1, v/v/v) as developing system. The *sn*-1,2 (2,3)-DAG bands were scraped off and methanolyzed to determine their FA composition using the same method described above. The molar percentage of FA composition at *sn*1 (3)- and *sn*2-positions of the produced TAG were calculated. Equations for % FA in *sn*1 *(sn3)*-position and in *sn*2-position are shown below:

$$[\%\ FA_{sn1\ (sn3)\text{-position}}] = 3\ [\%\ FA_{TAG}] - 2\ [\%\ FA_{1,2\text{-DAG}}]$$

$$[\%\ FA_{sn2\text{-position}}] = 4\ [\%\ FA_{1,2\text{-DAG}}] - 3\ [\%\ FA_{TAG}]$$

where $[\%\ FA_{1,2\text{-DAG}}]$ and $[\%\ FA_{TAG}]$ indicated for each FA, its percentage found in 1,2-DAGs and in TAGs.

For enzymatic partial degradation, TAG was digested with porcine pancreatic lipase (Schmid *et al.*, 1999). To a 5-mL screw capped tube, 20 mg of purified TAG, 10 mg pancreatic lipase and 3 mL Tris-HCl buffer (pH 8, 1 M) were added. After shaking, 0.5 mL sodium deoxycholate solution (1 g/L) and 0.2 mL calcium chloride solution (220 g/L) were added. After vigorous shaking, the tube was immediately placed in a water bath (40°C) for 1 min. Then the tube was shaken for 2 min and cooled in running water before adding

1 mL hydrochloric acid (6 M) and 1 mL diethyl ether, followed by vigorous shaking for 1 min. After centrifugation, the resulting mixture of partial acylglycerols in ether phase was separated by preparative TLC using a solvent mixture of chloroform:acetone (96:4 v/v). The 2-MAG band was scraped off, methanolyzed, and analyzed by GC.

7. TLC

7.1 TLC separation of acylglycerols

Impregnated silica gel plates with fluorescent indicator (Silica gel 60, F_{254}) were prepared by flushing slowly with 3% boric acid in ethanol. The plates were dried 15 min at 105°C and kept in a desiccator until use. The lipid sample (30 µl) was applied to the plate, followed by developing in a solvent mixture of chloroform: methanol:ammonium (70:0.4:0.1 v/v) for separation of TAG, or in a mixture of chloroform:acetone (96:4 v/v) for separation of 2-MAG (Figure 8). The plate was then dried and the lipid spots were visualized under a UV lamp (254 nm) or under iodine vapor.

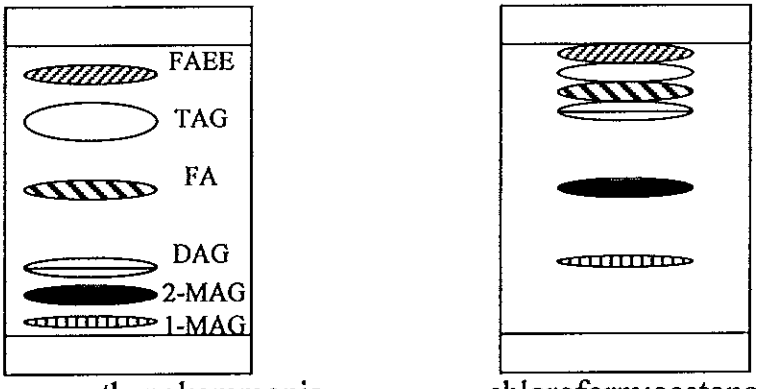


Figure 8 TLC separation of glyceride mixtures using different solvent system.

7.2 TLC separation of phospholipids

PL fractions were separated on a TLC plate developed in a solvent mixture of chloroform:methanol:water (65:24:5 v/v) (Figure 9). The localization of PL was done under iodine vapor (Park *et al.*, 2000).

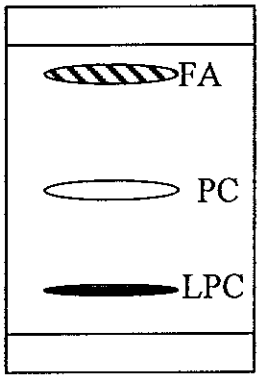


Figure 9 TLC separation of phopholipids mixture.

8. Separation of glyceride mixtures by column chromatography

A reaction sample containing a mixture of glycerides was fractionated in a glass column (7 x 120 mm) which was packed with 2 g silica gel and the loaded sample was eluted, as follows (Konishi *et al.*, 1993):

fraction 1, 25 mL diethyl ether:hexane = 2:98 (vol/vol), FAEE/FAVE;

fraction 2, 20 mL diethyl ether:hexane = 10:90 (vol/vol), TAG;

fraction 3, 15 mL methanol, DAG and MAG.

Isolation of each fraction was confirmed by TLC.

Experimental Methods

1. Purification of tuna oil and coconut oil

Refined tuna oil was prepared from crude tuna oil pressed from Skipjack tuna head by the method of Rungsilp (1998). The refining process was degumming, neutralization, fractionation, bleaching and deodorization (see appendix A).

Crude coconut oil was partially purified by degumming and neutralization using a method modified from Rungsilp (1998). Crude oil was melted at 40°C, then citric acid was added (0.15%, w/w) and the mixture was incubated for 5 min (40°C). Distilled water was added (2%, v/w based on weight of oil) and mixed at for 20 min 40°C. Wax and solid contaminants were removed from the oil layer by centrifugation (4,500 rpm, 15 min, 30°C). The degummed oil was saponified by adding 0.5% (v/w, based on weight of oil) NaOH solution (11.07% w/v) to oil layer. After mixing for 5 min, the mixture was immediately heat up to 70°C in a water bath, followed by cooling to room temperature and centrifuging (4,500 rpm, 15 min, 30°C). The obtained oil was re-saponified once again using 3% (v/w, based on weight of oil) NaOH solution (28.79% w/v). Before centrifugation, NaCl (2% w/w of oil) was added to the cooled mixture, followed by mixing for 5 min and centrifuging. The neutralized oil was subsequently bleached by heating to 80°C before 5% (w/w of neutralized oil) was added, followed by mixing at 90°C under a reduced pressure for 15 min. The mixture was then filtrated at 60°C. The partial purified coconut oil thus obtained was stored at 4°C until use.

2. One-step synthesis of ST by acidolysis of palm oil and tuna oil

Palm or tuna oil (0.35 mmol) and FA (0.9 mmol) were dissolved in different solvents (3 mL) and equilibrated to water activity 0.11 over saturated LiCl solution in a screw-capped vial. The reaction was started by addition of 10% (based on oil weight) of immobilized lipases (Lipozyme TL IM, Lipozyme RM IM, D-EP100, PS-C and PS-D) with the exception of CAL-B (15% based on oil weight) that was pre-equilibrated to water activity 0.11. The mixture was magnetically stirred at 40°C. After one hour of reaction, 3Å molecular sieve (activated by heating overnight at 250°C) was added. The reaction was stopped at 48 or 72 hours by centrifugation to remove immobilized enzyme and molecular sieves. The reaction mixture (40 µl) was

periodically withdrawn to determine the glyceride compositions by TLC-FID analysis. The final products at the end of each reaction were purified by column chromatography, followed by determination of FA compositions and regiospecific analysis.

Several factors were varied to find the optimum reaction condition.

- **Immobilized lipases:** Lipozyme TL IM, Lipozyme RM IM, D-EP100, PS-C, PS-D, and CAL-B
- **Solvent:** n-hexane, MTBE, and acetone
- **Temperature:** 40 and 60°C

3. Two-step synthesis of ST

A two-step synthesis of ST was studied. The reaction based on the alcoholysis of TAGs for the production of 2-MAGs and on the esterification of 2-MAGs with MCFAs (Figure 10).

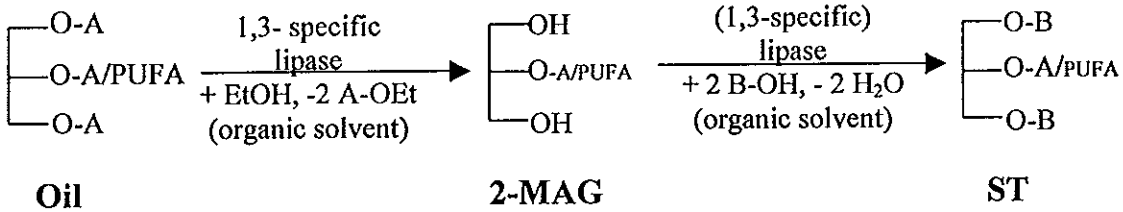


Figure 10 Principle of the two-step synthesis of structured triglycerides based on palm and tuna oils.

A = any fatty acid other than LCFA/PUFA, L = LCFA (mainly oleic/linoleic acids), PUFA mainly DHA/EPA, A-OEt = fatty acid ethyl ester, B-OH = fatty acid

3.1 Synthesis of 2-MAG by alcoholysis of palm oil and tuna oil

The 2-MAG was synthesized using a method modified from Soumanou *et al.* (1997).

Oil (0.35 mmol, 266 mg tuna oil or 285 mg palm oil) was dissolved in 2 mL of suitable solvents and equilibrated to a defined water activity (0.11- 0.43, Appendix A) over saturated salt solutions in a closed vessel for 48 h at room temperature. Dry ethanol (3.5 mmol, 161 mg), dried over activated molecular sieves (3 Å), was added and the mixture was stirred at 40°C for 15 min. The reaction was carried out in a 4-mL vial with a septum-lined screw cap using a magnetic stirrer operating at 400 rpm. The reaction was started by adding 10% (based on oil weight) of immobilized lipase PS-C, PS-D, D-EP100, Lipozyme TL IM and Lipozyme RM IM, or 15% (based on oil weight) of lipase CAL-B, pre-equilibrated to water activities of 0.11-0.43. An adequate amount of the reaction mixture was periodically withdrawn to determine the glyceride compositions by TLC-FID analysis. The contents of fatty acid ethyl ester, FA, TAGs, DAGs and MAGs were expressed as percentage of peak areas. The reaction was stopped by removing the immobilized lipases by centrifugation. After evaporation of excess solvent *in vacuo*, the oily residue was re-dissolved in 20 mL of n-hexane:MTBE (70:30 v/v) and stored overnight at –20°C. White crystal of 2-MAGs thus formed were collected by filtration at –20°C. To separate the non-crystallized form of 2-MAG for analysis, the supernatants were evaporated to remove excess solvent and the residue was applied to a TLC-plate, which was impregnated with boric acid to avoid acyl migration. After developing in chloroform:methanol:ammonia (70:0.4:0.1 v/v), the bands were visualized under iodine vapor and the 2-MAG band was scraped off and methanolyzed, followed by GC analysis to determine its FA composition.

Several factors were varied to find the optimum reaction condition.

- **Immobilized lipases:** Lipozyme TL IM, Lipozyme RM IM, D-EP100, PS-C, PS-D, and CAL-B
- **Initial water activity:** 0.11, 0.23, and 0.43
- **Solvent:** MTBE, acetone, and EMK
- **Temperature:** 30, 40, 50, and 60°C

3.2 Esterification of 2-MAG and MCFA

The purified 2-MAG obtained from alcoholysis reaction (60 mM) and caprylic acid (120 mM) were dissolved in 2 mL of n-hexane. The reaction was started by addition of 10% (based on the weight of 2-MAG) of immobilized lipase and 0.5 g molecular sieves. The mixture was incubated at 40°C and magnetically stirred at 400 rpm. An aliquot amount of the reaction medium was periodically withdrawn and the glyceride composition was determined by TLC-FID. The reaction was stopped at 24 h by centrifugation to remove immobilized enzyme and molecular sieve. The excess solvent was evaporated *in vacuo*. The oily residue was applied to a flash column chromatography. The TAG fractions were combined and methanolyzed, followed by GC analysis. Instead of caprylic acid, lauric acid was also tested.

Several factors were varied to find the optimum reaction condition.

- **Immobilized lipases:** Lipozyme TL IM, Lipozyme RM IM, and D-EP100
- **Solvent**: n-hexane, acetone, MTBE
- **Molar ratio of 2-MAG to MCFA:** 1:2, 1:3, 1:4, and 1:5

4. Two-step synthesis of structured triglyceride by novel method

Another alternative method for the synthesis of ST was studied. It is based on the synthesis of 1,3-DAGs and on the esterification of 1,3-DAGs with oleic acid. The concept of this two-step method is shown in Figure 11.

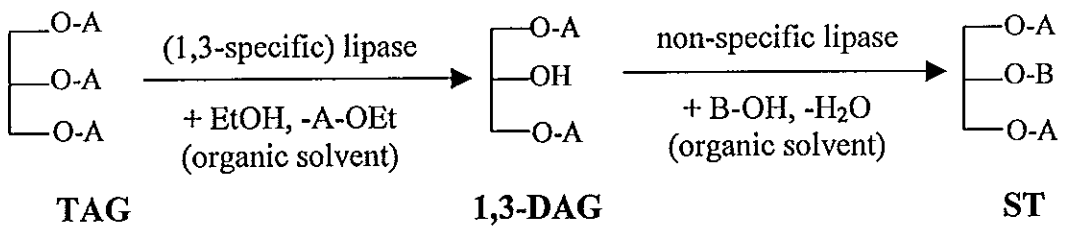


Figure 11 Principle of a novel two-step synthesis of structured triglyceride. A = medium-chain fatty acid, B = long-chain fatty acid, EtOH = ethanol, A-OEt = fatty acid ethyl ester, B-OH = fatty acid

4.1 Synthesis of 1,3-DAG by esterification of glycerol and FAVE/FFA

4.1.1 Esterification using immobilized glycerol

1,3-DAG was synthesized from esterification of glycerol (1 mmol, immobilized on 4 g silica gel) and FAVE (2 mmol) in 8 mL MTBE at room temperature using Lipozyme RM (10% based on glycerol weight) as catalyst (Berger *et al.*, 1992). The reaction was carried out in a 10-mL screw-capped vial at 40°C and mixed by magnetic stirrer (500 rpm). The reaction was stopped after 24 h by separation of enzyme and silica gel by filtration. The filtrate was evaporated under vacuum. The 1,3-DAG in oily residue was recovered and purified by crystallization in dry methanol at 4°C or –20°C for several hours and filtration at 4°C or –20°C for 1,3-DLa or 1,3-DCy, respectively.

4.1.2 Esterification using non-immobilized glycerol

1,3-DAG was synthesized by esterification of glycerol (1 mmol) and FFA or FAVE (2.5 mmol) in a solvent-free condition at 0°C using lipase CAL-B (10% based on glycerol weight) as biocatalyst, unless indicated otherwise. The reaction was carried out in a 4-mL vial with Teflon-lined screw cap. The reaction mixture was magnetically stirred at 400 rpm. Molecular sieves were added in a reaction with FFA to remove produced water from the reaction mixture. In some experiments, organic solvent (2 mL) was added to the reaction mixture to dissolve a solid FFA. The reaction was stopped by centrifugation to separate immobilized enzyme and molecular sieve from the reaction mixture. In case of solvent-free reactions, the reaction mixtures were dissolved in n-hexane prior to centrifugation. The 1,3-DAG product was recovered and purified by crystallization at –20°C. A further recrystallization in dry methanol at –20°C was necessary when high amount of MAG was present in the reaction mixture. A sample of the reaction mixture (10 μl) was periodically withdrawn to determine the glyceride composition. The sample was pretreated before analysis by adding 0.3 mL Folsh's solution (chloroform:methanol 2:1 v/v) and 0.3 mL distilled water, mixed for 30 sec,

followed by centrifugation (10,000 rpm, 2 min). The organic layer was applied to TLC-FID analysis.

4.2 Synthesis of 1,3-DAG by alcoholysis of pure TAG and coconut oil

DAGs were synthesized from alcoholysis of trilaurin or coconut oil, by modification of the method for 2-MAG synthesis from alcoholysis of palm oil and tuna oil described above (2.3.1),

TAG (3 mmol) was dissolved in organic solvent (2 mL) and pre-equilibrated over a saturated-salt solution at a_W 0.11 for 48 h (only for alcoholysis reaction). Dry ethanol or water (3 mmol) was added and the reaction mixture was incubated at 40°C for 15 min. The reaction was started by adding immobilized lipase (10% based on TAG weight) and carried out in a 4-mL screw-capped vial by stirring at 400 rpm. An aliquot amount of the reaction mixture was periodically withdrawn and diluted with chloroform to an appropriate dilution, followed by analysis with TLC-FID to determine its glyceride composition. The reaction was stopped by centrifugation to separate immobilized lipase from the reaction mixture.

4.3 Induction of acyl migration

Effect of temperature, FA (oleic acid), matrix (celite) and ion-exchanger resin on acyl migration of 1,2-dipalmitin (1,2-DP) were studied.

1,2-DP was dissolved in n-hexane (8 mg/mL). Oleic acid (2-4 mmol) or celite (8 mg) or ion-exchanger (10-100 mg) was added directly to the reaction mixture. All reactions were carried out in a 1.5-mL microcentrifuge tube with shaking (1,400 rpm) at room temperature (25°C), except for the study on effect of temperature, the reaction was carried out at 40 or 60°C.

4.4 Esterification of 1,3-DAG and oleic acid/oleic acid vinyl ester

ST was synthesized by esterification of 1,3-DAG and oleic acid (OA) or oleic acid vinyl ester (OAVE) in n-hexane at 60°C using immobilized lipase from *Pseudomonas* sp. (PS-D) as biocatalyst.

0.1 mmol of 1,3-DAG (45.7 mg of 1,3-dilaurin or 34.4 mg of 1,3-dicaprylin) and 0.2 mmol of OA (28.2 mg) or OAVE (60.2 mg) were dissolved in 1 mL n-hexane in a 2-mL screw-capped vial. Molecular sieves were added when OA was used as acyl donor. The reaction was started by addition of lipase PS-D (10% weight of 1,3-DAG). The vials were shaking on a thermomixer at 1,400 rpm at 60°C. An aliquot amount of the reaction mixtures was withdrawn for analysis with TLC-FID. ST thus obtained was purified on a TLC plate and the TAG band was scrapped off and methanolyzed, followed by GC analysis.

5. Chemical synthesis of PLs

PC was synthesized according to the method of Pisch *et al.* (1997). Briefly, 2.75 mmol FA and 3 mmol CDI were dissolved in 20 mL dichloromethane (dried over 3Å molecular sieve at least 48 h prior to use) in a 50-mL round bottom flask. The reaction mixture was magnetically stirred at room temperature for 30 min until no carbon dioxide was formed. Then 1 mmol GPC and 2.75 mmol DBU were added and the reaction continued at room temperature for 48 h. Solvent was evaporated under vacuum and the oily residue was precipitated in chloroform:acetone (1:9 v/v) at –20°C for several hours. The DLPC crystals were filtered and washed with cold acetone. DLPC thus obtained was freeze-dried and kept at 4°C until use.

6. PLD-catalyzed transphosphatidylation

6.1 Production of recombinant PLD from *Escherichia coli*

Synthetic medium containing (per liter) 5 g glucose, 2 g glycerol, 5 g K_2HPO_4, 5 g KH_2PO_4, 11 g $Na_2HPO_4 \cdot 12H_2O$, 3 g $(NH_4)_2SO_4$, 3 g $MgSO_4 \cdot 7H_2O$, 40 mg $FeSO_4 \cdot 7H_2O$, 4 mg $CaCl_2$, 1 mg $MnSO_4 \cdot 7H_2O$, 1 mg $ZnSO_4 \cdot 7H_2O$, 0.05 mg $Na_2MoO_4 \cdot 7H_2O$ was used for the cultivation of recombinant *E. coli* containing PLD-encoding gene of *Streptomyces antibioticcus* (Iwasaki *et*

al., 1995). One mL overnight culture was inoculated into 100 mL of the medium containing 50 mg/l kanamycin. After incubation at 30°C with shaking until OD_{660} reached 3.0 (approx. 15 h), isopropyl-β-D-thiogalactopyranoside (IPTG) was added to give a final concentration of 1 mM and incubation continued for 24 h. The culture supernatant was collected by centrifugation and concentrated by membrane filtration (Centricon) followed by lyophilization to obtain a powder of PLD.

6.2 Transphophatidylation in biphasic system

A conventional transphosphatidylation was modified from the method of Pisch *et al.* (1994).

50 mg DLPC and 1.8 g ethanolamine were dissolved in 6 mL chloroform and incubated at 40°C for 15 min in a screw-capped vial. Reactions were started by adding 9 mL PLD solution (0.05 mg/mL of 0.1 M sodium acetate buffer containing 0.1 M $CaCl_2$, pH 5.6). The reaction was carried out at 40°C and magnetically stirred at 500 rpm for 48 h. The organic layer was periodically withdrawn to determine the PL composition by TLC-FID. To terminate the reaction, organic and aqueous phase were separated and the water layer was extracted with Folsh's solution (chloroform:methanol, 2:1 v/v). The combined organic layers were washed with distilled water and 0.1 M EDTA solution (pH 7.4). The organic layer was then dried over anhydrous sodium sulfate and solvent was evaporated *in vacuo* to obtain the crude PE.

6.3 Preparation of salt-activated PLD (PLD-KCl)

The salt-activated enzyme was prepared by lyophilization from sodium acetate buffer (pH 5.6). According to the method of Ru *et al.* (1999), 50 mg PLD and 8.65 g KCl were dissolved in sodium acetate buffer (0.1 M or 0.2 M) containing calcium chloride (0.1 M or 0.08 M). The buffer pH was adjusted to 5.6 using 100 mM HCl solution. The solution mixture was lyophilized at 200 μm Hg, –50°C for 8 h to achieve a final dry preparation of 98% (w/w) KCl. The salt-activated enzyme powder was kept in a closed vessel at 4°C until used.

6.4 Transphosphatidylation in anhydrous organic solvent

Transphosphatidylation in anhydrous organic solvent was performed using a modified method of Rich and Khmelnitsky (2001).

To a 4-mL screw-capped vial, 25 mg DLPC and 0.6 g ethanolamine dissolved in 3 mL chloroform (dried over 3Å molecular sieve at least 48 h prior to use) were added and incubated at 40°C for 15 min. The reaction was started by adding 0.5 mg PLD or 50 mg salt-activated PLD (PLD-KCl) and 25 mg cation exchanger resin (Dowex 1x8, pH 5.6). The reaction mixture was magnetically stirred at 500 rpm, 40°C for 48 h, unless otherwise indicated. Samples were periodically withdrawn to determine the PL composition by TLC-FID. The reaction was stopped by centrifugation to separate the enzyme and resin from the reaction mixture. Crude PL was isolated by evaporation of solvent under vacuum.

CHAPTER 3

RESULTS AND DISCUSSIONS

The results from the studies on the synthesis of MLM-type STs and the modification of PL by enzymatic reactions are summarized in this chapter.

1. Characterization of Oils and Enzymes

1.1 Hydrolytic activity of enzymes

Hydrolytic activity of commercial immobilized lipases was determined by means of a pH-Stat assay (Table 11). Among the three 1,3-regiospecific immobilized lipases used Lipozyme TL IM showed higher hydrolytic activity toward palm, tuna, coconut and palm kernel oils than Lipozyme RM IM and D-EP100. For the non-regiospecific immobilized lipases, i.e. CAL-B, PS-C and PS-D, the hydrolytic activity toward palm oil and tuna oil was also determined. PS-D showed the highest hydrolytic activity. The hydrolytic activities were increased when increasing the temperature to 60°C, except for D-EP100 that was denatured at 60°C. However, it was found that the interesterification activity of lipase was not corresponding to its hydrolytic activity similar to the observation made in literature (Soumanou, 1997).

1.2 Glycerides and fatty acid compositions of oils

The major component in the raw material oils was TAG (> 96.6%), while minimal amounts of FFA and MAG were present (Table 12). Table 13 shows the FA compositions and distribution of palm oil and tuna oil before modification. Palm oil used in this studied is palm olein fraction. The major FAs in palm oil were oleic acid (38.42%) and palmitic acid (28.65%). The prominent FAs at the *sn*2-position were unsaturated FAs. Tuna oil used here contained 6.24% EPA and 23.63% DHA, which were almost exclusively located at the *sn*2-position. The most prominent FAs at the *sn*1,3-position are

myristic and palmitic acid. Coconut and palm kernel oils were rich in MCFA (63.5% and 58.3%, respectively). Chromatograms of oils are shown in Figure 12 - Figure 14.

Table 11 Hydrolytic activity of commercial immobilized lipases.

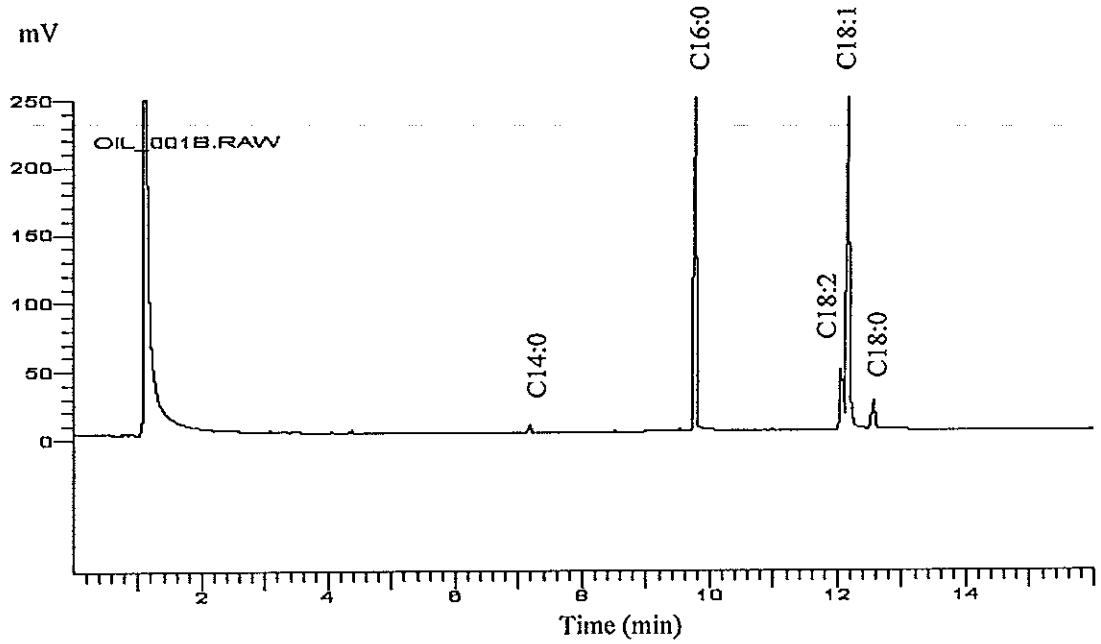
Enzyme	Activity (U/mg enzyme)							
	Palm		Palm kernel		Coconut		Tuna	
	40°C	60°C	40°C	60°C	40°C	60°C	40°C	60°C
RM	1.27	7.55	0.22	3.26	0.29	0.76	0.00	0.26
TL	4.44	10.69	0.26	0.71	0.71	10.17	3.61	4.87
D-EP100	0.72	0	0.13	0	0.29	0	0.14	0
CAL-B	0.00	0.07	n.d.	n.d.	n.d.	n.d.	0.00	0.05
PS-C	2.58	3.44	n.d.	n.d.	n.d.	n.d.	2.05	2.17
PS-D	58.07	59.14	n.d.	n.d.	n.d.	n.d.	40.36	49.25

n.d. = not done; RM = Lipozyme RM IM, TL = Lipozyme TL IM

Table 12 Glyceride compositions of oils.

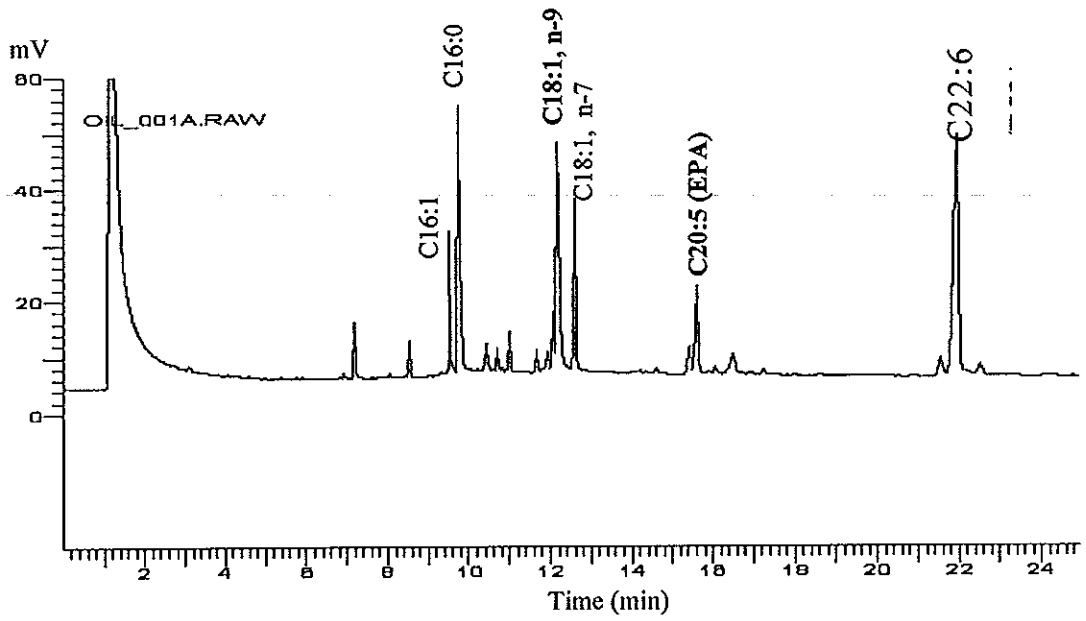
Oil	%TAG	%FFA	%DAG	%MAG
Palm	97.25	1.79	0.67	0.28
Tuna	98.62	n.d.	n.d.	1.37
Coconut	99.50	0.50	n.d.	n.d.
Palm kernel	96.60	2.83	n.d.	0.57

n.d. = not detectable



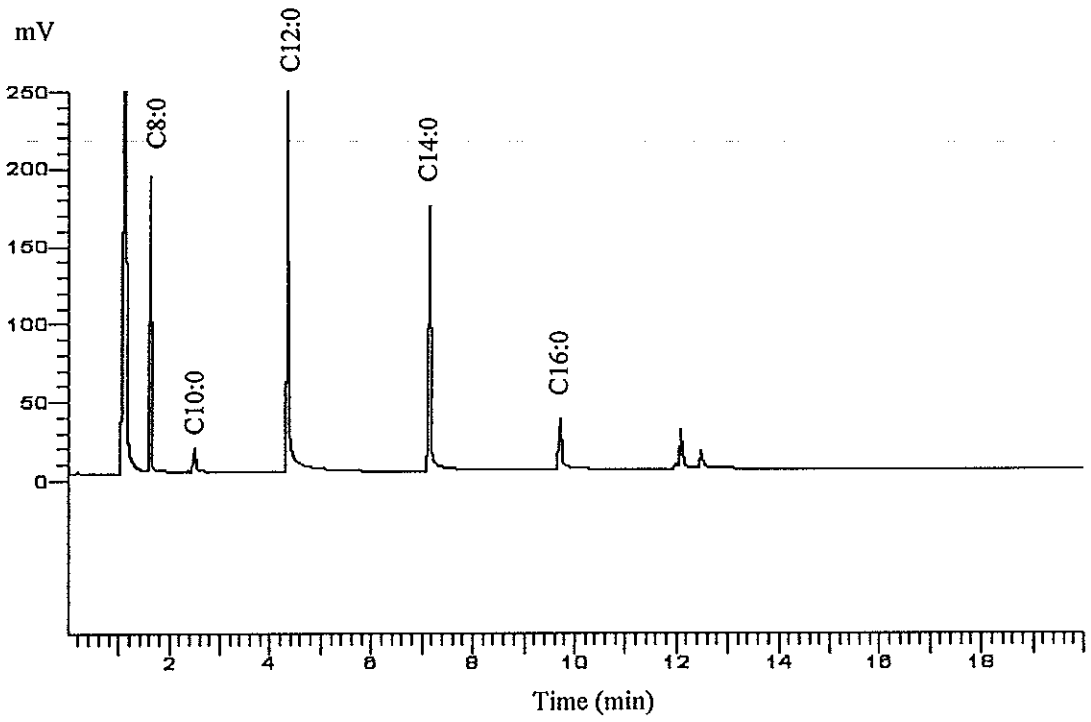
Peak #	Time (min)	Area (μV.s)	Height (μV)	Area (%)	Fatty acid
1	7.169	13444.80	6966.01	0.81	C14:0
2	9.766	642664.18	326040.84	38.68	C16:0
3	12.056	163319.61	52179.56	9.83	C18:2
4	12.182	778556.65	256683.35	46.86	C18:1
5	12.559	63370.63	21905.53	3.81	C18:0
		1661355.87	663775.29	100.00	

Figure 12 Chromatogram from GC analysis of palm oil.



Peak #	Time (min)	Area (μV.s)	Height (μV)	Area (%)	Fatty acid
1	7.174	38763.56	19332.28	3.29	C14:0
2	8.509	12125.91	6498.48	1.03	
3	9.509	57917.25	26899.80	4.91	C16:1
4	9.755	256756.15	127981.36	21.78	C16:0
5	10.416	16753.98	8167.70	1.42	
6	10.692	7242.25	3624.21	0.61	
7	10.997	14505.60	6926.68	1.23	
8	11.659	8639.77	3820.17	0.73	
9	11.908	5882.81	2727.68	0.50	C18:3
10	12.050	29430.72	10270.70	2.50	C18:2
11	12.156	205292.11	72248.57	17.41	C18:1, n-9
12	12.236	25351.48	10010.17	2.15	C18:1, n-7
13	12.572	84077.69	30051.98	7.13	C18:0
14	15.392	20535.37	5462.07	1.74	
15	15.568	72645.29	18637.88	6.16	C20:5, EPA
16	21.900	323110.04	41924.74	27.40	C22:6, DHA
		1179029.99	394584.45	100.00	

Figure 13 Chromatogram from GC analysis of tuna oil.



Peak #	Time (min)	Area (µV.s)	Height (µV)	Area (%)	Fatty acid
1	1.631	158958.7	190991.7	8.36	C8;0
2	2.502	110098.5	98312.75	5.79	C10:0
3	4.355	10665546	531975.3	56.07	C12:0
4	7.104	341440	175150.4	17.95	C14:0
5	9.707	139362.1	78876.43	7.33	C16:0
6	12.073	65930.94	25682.7	3.47	C18:0
7	12.487	19936.12	9421.19	1.05	C18:1
		1902272.73	1.11e+06	100.00	

Figure 14 Chromatogram from GC analysis of coconut oil.

Table 13 Compositions and distribution of fatty acids in oils.

Oil/ position	Fatty acid (%mol)										
	C8:0	C10:0	C12:0	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	EPA	DHA
Palm	0	0	0	**0.67**	**28.65**	0	**4.21**	**38.42**	**26.53**	n.d.	n.d.
*sn*1,3-				0	51.69		4.57	35.82	14.37		
*sn*2-				6.39	17.13		3.49	43.62	29.37		
Tuna	0	0	0	**5.40**	**25.18**	**5.01**	**6.90**	**13.48**	**3.23**	**6.24**	**28.27**
*sn*1,3-				13.90	46.71	15.87	7.73	11.30	0	0	4.21
*sn*2-				0.70	10.98	0	6.03	13.91	4.69	21.22	32.20
Coconut	**8.57**	**6.39**	**48.51**	**18.51**	**8.47**	0	**2.82**	**5.59**	**1.14**	n.d.	n.d.
*sn*1,3-	10.69	5.07	42.65	17.61	7.23		8.46	4.87	0		
*sn*2-	2.33	6.03	54.43	19.31	9.95		0	7.03	1.04		
Palm kernel	**3.94**	**3.81**	**50.54**	**15.43**	7.77	0	**2.00**	**14.17**	**2.28**	n.d.	n.d.
*sn*1,3-	5.82	6.83	74.40	19.09	1.83		0.42	0	0.50		
*sn*2-	0.18	0	2.82	8.11	18.65		5.16	59.69	5.84		

n.d. = not done

2. Synthesis of ST by One-Step Reaction

ST containing MCFA at the *sn*1- and *sn*3-positions and LCFA at the *sn*2-position (MLM) was synthesized by acidolysis of LCFA-containing TAGs with MCFA, such as caprylic acid (C8:0) and lauric acid (C12:0).

2.1 Screening of Immobilized Enzymes

The results from the acidolysis of palm oil with caprylic acid and lauric acid in n-hexane at initial water activity 0.11, 40°C for 48 hours are shown in Table 14 and Figure 15. Among three commercial immobilized lipases used, Lipozyme TL IM showed highest incorporation of caprylic acid (34.1%) and lauric acid (23.2%) into palm oil. D-EP100 gave slightly higher incorporation than Lipozyme RM IM. Incorporation of caprylic acid was approximately 2-fold higher than lauric acid in all cases.

Besides three immobilized regiospecific lipases used for acidolysis of palm oil, the immobilized non-specific lipases PS-C and CAL-B were also tested for acidolysis of tuna oil. In this preliminary screening, Lipozyme TL IM showed highest incorporation, while CAL-B and D-EP100 gave moderate incorporation of caprylic acid and lauric acid (Table 15 and Figure 16). Lipozyme RM IM was selected for screening due to its good potential in incorporation of MCFA into fish oil (Jennings and Akoh, 2001). However, in contrast with other studies (Jennings and Akoh, 2001; Iwasaki *et al.*, 1999; Akoh and Moussata, 1998), Lipozyme RM IM and PS-C showed very low activity in the acidolysis of tuna oil. Although CAL-B is not a 1,3-regiospecific lipase and gave only moderate activity in hydrolysis reactions using tuna oil, it turned out to be rather suitable to be investigated in subsequent optimization studies together with Lipozyme TL IM. Accordingly, by using CAL-B instead of using other 1,3-regiospecific lipases, which were originally and commonly used in the ST synthesis, the opportunity to use the non-specific lipases in ST synthesis should be wide-awake.

Table 14 Screening of immobilized lipases for acidolysis of palm oil with caprylic and lauric acid (n-hexane, 40°C, 48 h).

Acyl donor	Enzyme	Fatty acid composition after acidolysis (%mol)					
		C8:0	C12:0	C16:0	C18:0	C18:1	C18:2
Cy	TL	**33.93**	-	14.40	3.13	35.80	9.74
	RM	**19.30**	-	24.83	3.17	41.03	10.46
	D-EP100	**22.93**	-	18.72	1.93	44.98	11.44
La	TL	-	**23.20**	23.62	3.07	38.19	10.46
	RM	-	**6.89**	33.25	4.46	40.56	10.47
	D-EP100	-	**11.52**	32.15	4.72	38.97	10.18

* TL = Lipozyme TL IM, RM = Lipozyme RM IM

Table 15 Screening of immobilized lipases for acidolysis of tuna oil with caprylic and lauric acid (n-hexane, 40°C, 72 h).

Acyl donor	Enzyme	Fatty acid composition after acidolysis (%mol)						
		C8:0	C12:0	C16:0	C18:0	C18:1	EPA	DHA
Cy	TL	**9.54**	-	19.70	5.50	9.00	5.69	40.52
	RM	**8.66**	-	19.33	6.16	9.55	5.09	34.11
	D-EP100	**8.16**	-	20.49	6.10	7.49	5.66	36.76
	PS-C	**1.26**	-	26.30	12.08	17.62	4.47	21.71
	CAL-B	**5.13**	-	32.76	10.16	15.88	3.84	23.44
La	TL	-	**17.88**	15.74	5.48	14.02	5.66	24.04
	RM	-	**3.27**	19.37	6.84	12.29	6.92	27.13
	D-EP100	-	**4.83**	19.51	6.47	13.92	5.94	22.55
	PS-C	-	**1.05**	19.91	7.05	13.63	6.32	23.57
	CAL-B	-	**4.12**	18.50	6.47	11.47	5.83	23.26

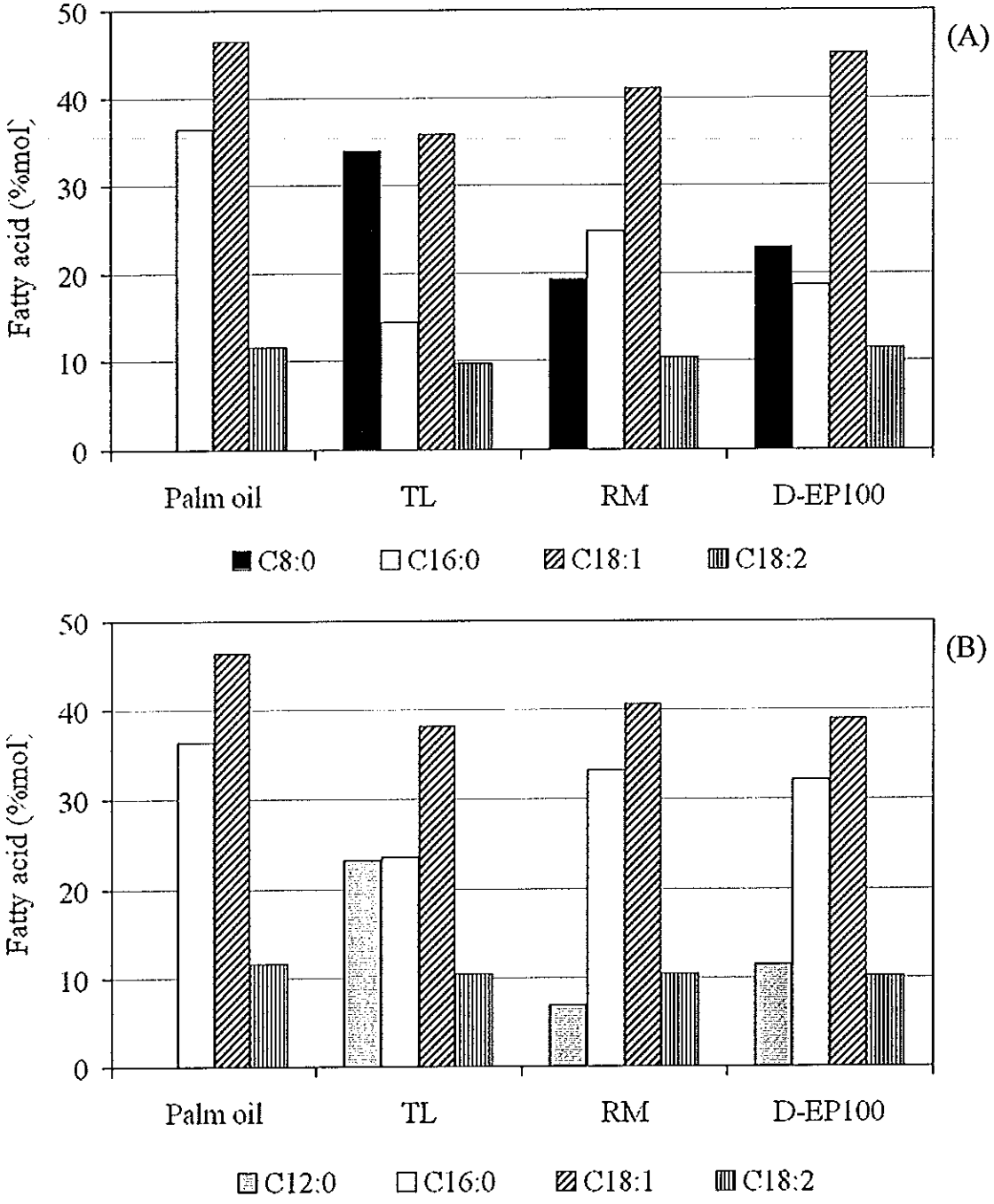


Figure 15 Acidolysis of palm oil with caprylic acid (A) and lauric acid (B) catalyzed by different immobilized lipases.
(n-hexane, 40°C, 48 h) (Lipozyme TL IM (TL), Lipozyme RM IM (RM) and D-EP100).

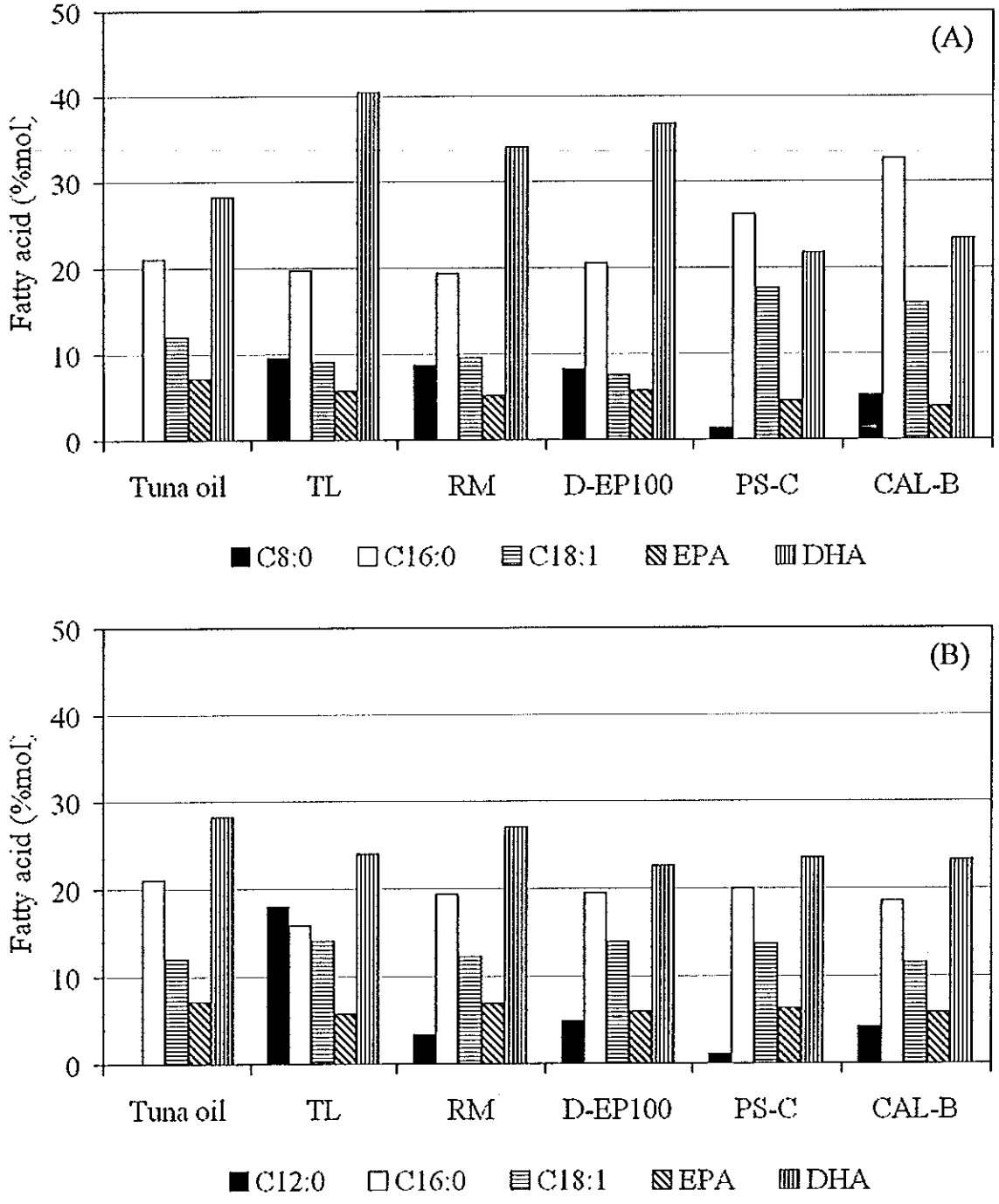


Figure 16 Acidolysis of tuna oil with caprylic acid (A) and lauric acid (B) catalyzed by different immobilized lipases.
(n-hexane, 40°C, 72 h) (Lipozyme TL IM (TL), Lipozyme RM IM (RM), D-EP100, PS-C and CAL-B).

2.2 Effect of Solvents

Organic solvents increase the solubility of non-polar substrates, shift the thermodynamic equilibrium toward synthesis rather than hydrolysis, but also can considerable influence the yield and FA composition in ST synthesis (Soumanou, 1997). The effect of various organic solvents on acidolysis of palm oil and MCFA was investigated using Lipozyme TL IM (Figure 17). The highest incorporation of caprylic acid (34.1%) and lauric acid (23.2%) was obtained in n-hexane, while acidolysis in MTBE was rather good. Among three organic solvents tested, acetone appeared to be a poor solvent for acidolysis of palm oil. This result was corresponding to the synthesis of 1,3-capryloyl-2-oleyl triglyceride from triolein and caprylic acid (Soumanou *et al.*, 1999).

In contrast to the acidolysis of palm oil and other natural plant oils (Rosu *et al.*, 1998; Schmid *et al.*, 1998; Jennings and Akoh, 2001), n-hexane was a rather poor solvent for the acidolysis of tuna oil. The relationship between transesterification activity of immobilized lipases and the type of organic solvent is shown in Figure 18A. Lipozyme TL IM gave higher incorporation of caprylic acid in MTBE (25.0%) compared to acetone (17.3%) and n-hexane (5.4%). In sharp contrast, CAL-B gave considerably higher incorporation of caprylic acid in acetone (31.6%) compared to MTBE (25.2%). However, higher DHA contents in ST were determined when using Lipozyme TL IM. Similar trends for solvents were found using lauric acid (Figure 18B). Incorporation of lauric acid was 45.2% using CAL-B in acetone, while 46.9% incorporation was obtained using Lipozyme TL IM in MTBE. However, no significant differences in the DHA content in ST were found using Lipozyme TL IM or CAL-B for acidolysis of tuna oil with lauric acid. Because CAL-B gave not only high incorporations of both caprylic and lauric acids into tuna oil but also high amount of DHA was obtained, it was chosen for further studies.

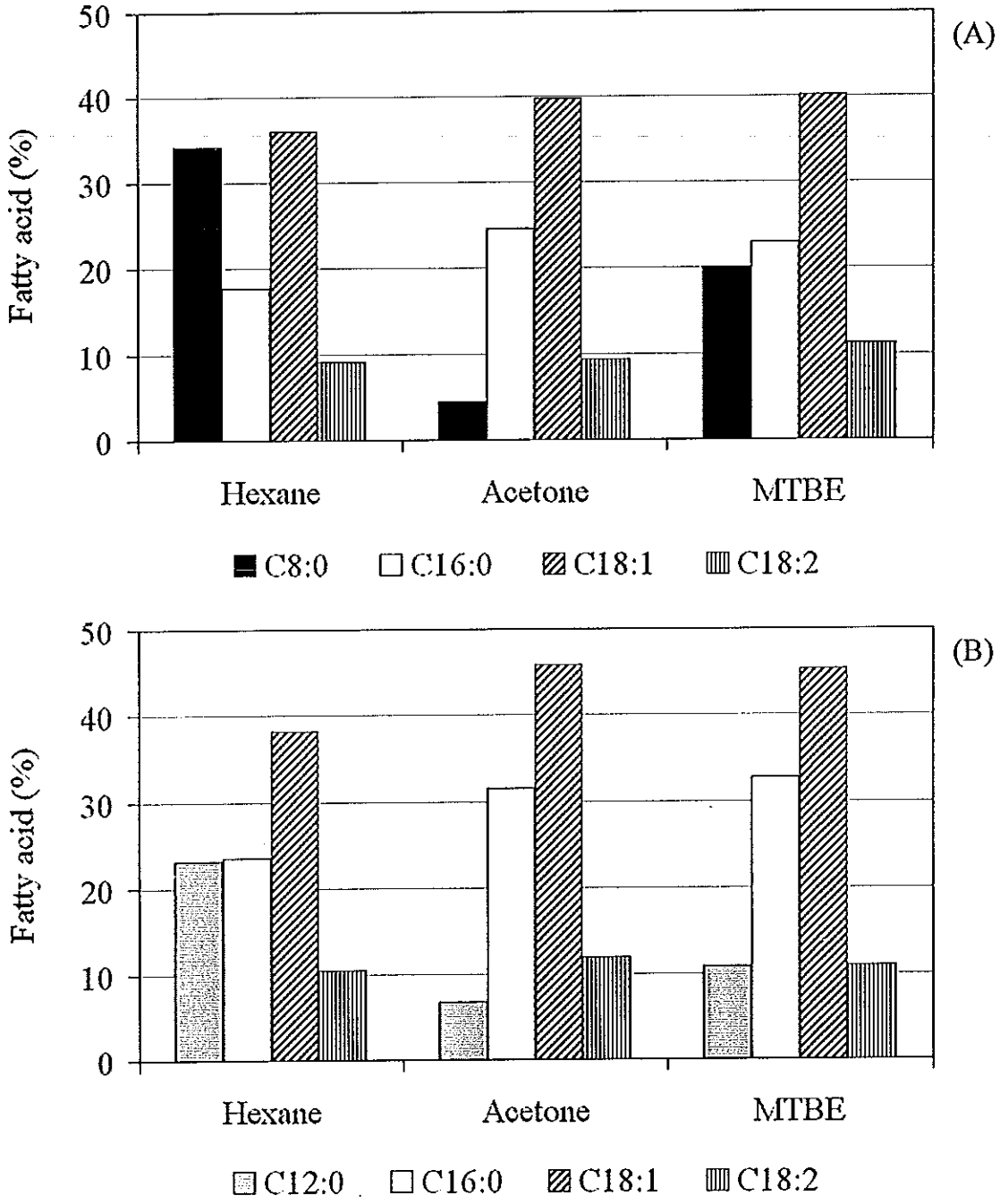


Figure 17 Effect of solvents on acidolysis of palm oil with caprylic acid (A) and lauric acid (B).
(Lipozyme TL IM, 40°C, 48 h).

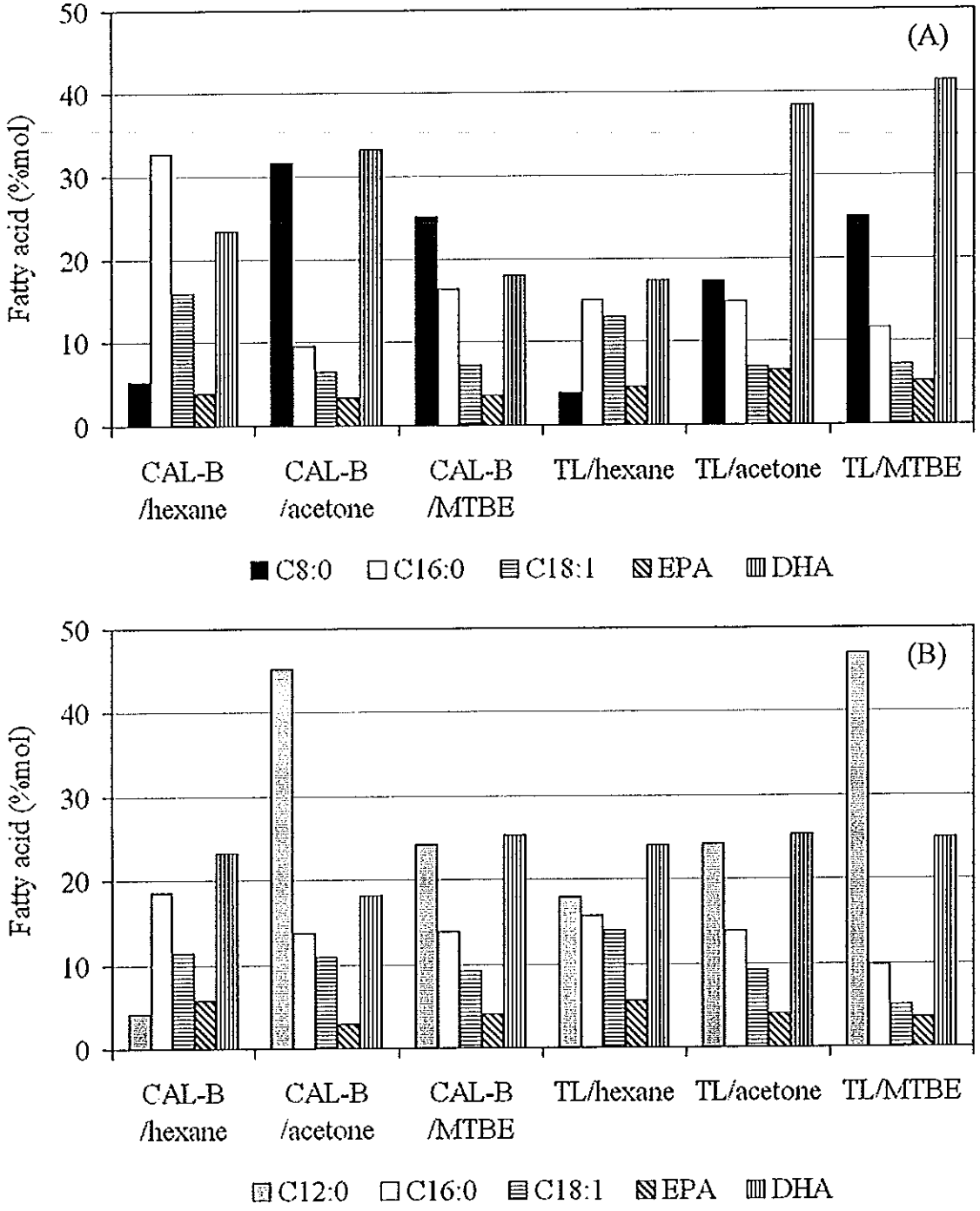


Figure 18 Effect of solvents on acidolysis of tuna oil with caprylic acid (A) and lauric acid (B).
(CAL-B and Lipozyme TL IM (TL), 40°C, 72 h).

2.3 Effect of reaction temperature

The reaction temperature was tested at 40°C and 60°C. Although according to the manufacturers of both Lipozyme TL IM and CAL-B show the highest catalytic activity around 60°C, reactions were also performed at 40°C to diminish possible acyl migration.

Figure 19 shows the effect of temperature on acidolysis of palm oil with MCFA. Whereas variation of temperature did not effect acidolysis reactions using caprylic acid, higher incorporations were found for lauric acid at 60°C, possibly due to its higher melting point. Under optimized conditions, i.e. acidolysis of palm oil in n-hexane at an initial water activity of 0.11 using Lipozyme TL IM, the highest incorporation were obtained at 40°C for caprylic acid (34.1%) and 60°C for lauric acid (43.5%).

For acidolysis of tuna oil in n-hexane, the incorporations of MCFA were dramatically increased when the reaction temperature increased from 40°C to 60°C (Figure 20). On the other hand, when the reactions were carried out in acetone, the incorporations at 60°C were slightly less than at 40°C. Under optimized conditions, i.e. acidolysis of tuna oil in acetone at an initial water activity of 0.11 using CAL-B, the best result was obtained at 40°C for caprylic acid (32% incorporation, Figure 20A) and lauric acid (45% incorporation, Figure 20B).

STs were synthesized in larger scale (starting from 2.85 g palm oil and 2.66 g tuna oil) using the optimized conditions (acidolysis of palm oil in n-hexane at 40°C using Lipozyme TL IM; acidolysis of tuna oil in acetone at 40°C using CAL-B). The ST products were purified by column chromatography and subjected to regiospecific analysis by enzymatic method. Regiospecific analysis by enzymatic method revealed that minor amounts of caprylic or lauric acid were present at the *sn*2-position of the ST produced (Table 16).

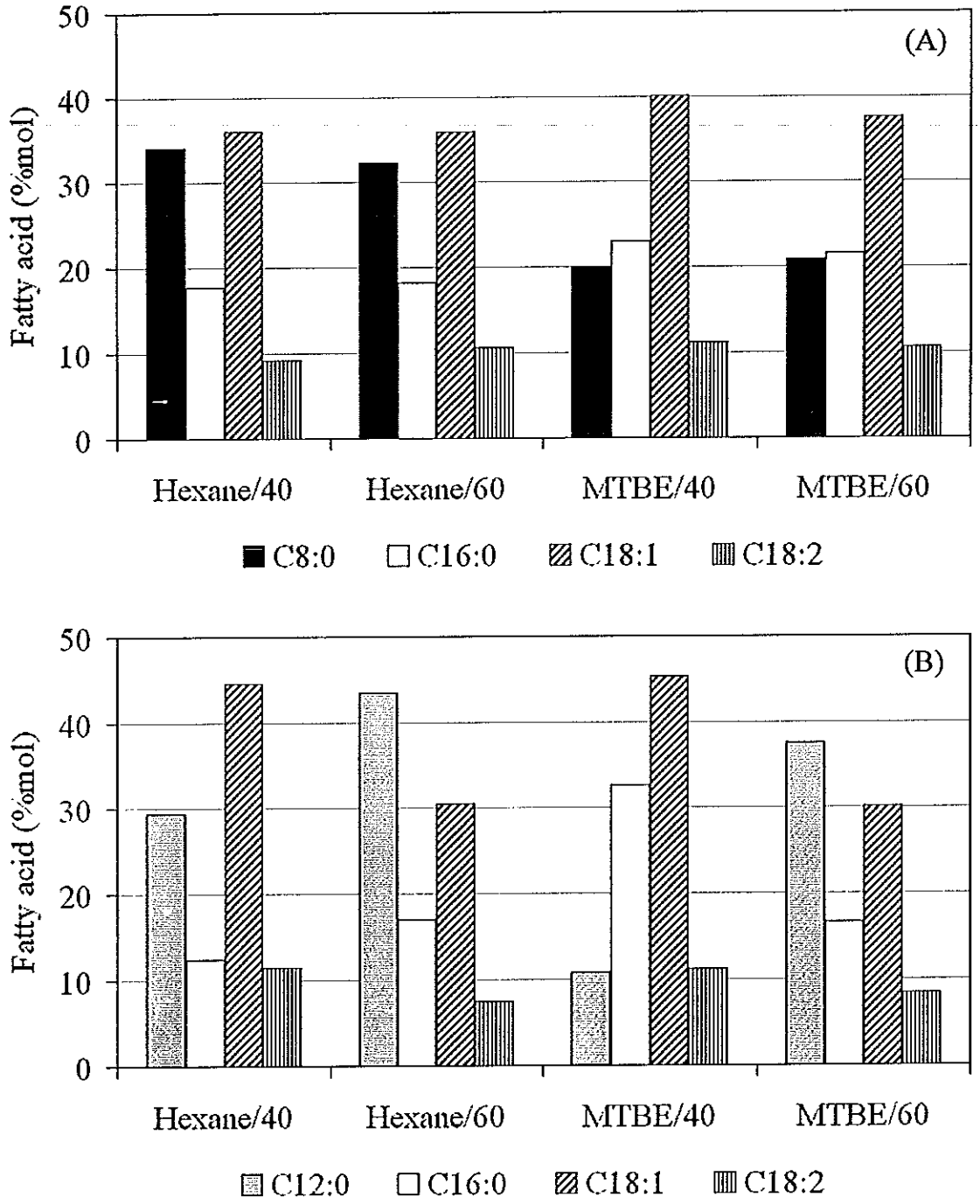


Figure 19 Effect of temperature on acidolysis of palm oil with caprylic acid (A) and lauric acid (B).
(Lipozyme TL IM, 40°C, 48 h).

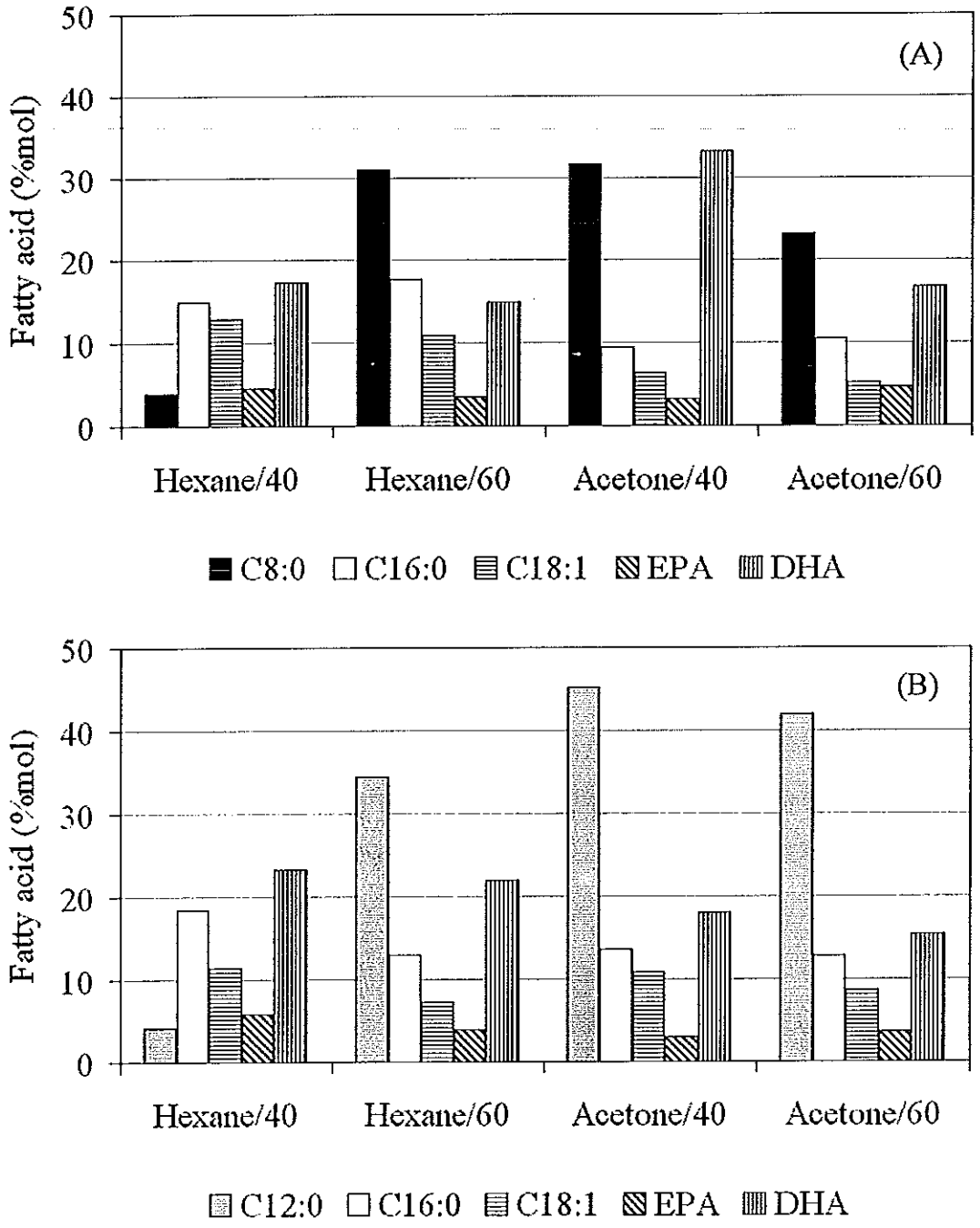


Figure 20 Effect of temperature on acidolysis of tuna oil with caprylic acid (A) and lauric acid (B).
(CAL-B, 72 h).

Table 16 Major fatty acid compositions at the *sn*2-position of structured triglyceride products from acidolysis of palm and tuna oils with medium-chain fatty acids under optimized conditions.

Oil	FA	FA composition (%mol)						
		C8:0	C12:0	C16:0	C18:1	C18:2	EPA	DHA
Palm	Cy	7.12	-	8.48	77.19	7.21	-	-
	La	-	8.42	9.07	76.12	6.39.	-	-
Tuna	Cy	5.89	-	14.77	9.30	6.45	5.28	57.31
	La	-	6.48	22.61	10.52	5.46	3.19	50.74

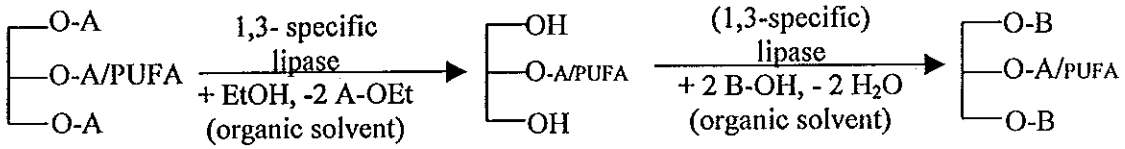
*Optimized conditions for acidolysis

1. Palm oil: Lipozyme TL IM, n-hexane, 40°C, 48 h.
2. Tuna oil: CAL-B, acetone, 40°C, 72 h.

As outlined above, CAL-B is a non-regioselective lipase and therefore should not be suitable for the synthesis of ST. However, this study shows that by using CAL-B, under optimized conditions, similar and even higher incorporation of MCFAs in tuna oil are possible compared to 1,3-regioselective lipases. This can be attributed to the rather esterase-like properties of CAL-B, as this enzyme preferentially accepts on short and medium chain FAs as substrates and also lacks a lid covering the active site, which is a typical structural feature of 'true' lipases (Bornscheuer, 2000). In addition, CAL-B apparently does not act on PUFAs like DHA and EPA. Thus, its suitability for the acidolysis of tuna oil rather stems from its fatty chain length specificity than its non-regioselectivity.

3. Synthesis of ST by Two-Step Reaction

The two-step reaction was reported as an effective and rapid method for the production of ST in high yield and purity (Soumanou *et al.*, 1997). The reaction was based on the synthesis of 2-MAG containing LCFA by alcoholysis of TAG in the first step. The 2-MAG thus obtained was then esterified with MCFA in the second step, as shown below.



TAG **2-MAG** **ST**

In this study, palm and tuna oils were used as a source of LCFA and PUFA for the production of 2-MAGs.

3.1 Alcoholysis of oils

3.1.1 Screening of immobilized enzymes

Three commercial immobilized 1,3-specific lipases, i.e. Lipozyme TL IM, Lipozyme RM IM and D-EP100, were tested for alcoholysis of palm oil with ethanol in MTBE. Besides the 1,3-specific lipases, which are normally used in transesterification reaction, non-specific lipases were also found to be effective in the enrichment of PUFA in fish oils (Rakshit *et al.*, 2000; Medina *et al.*, 1999). Therefore, three non-specific lipase preparations, i.e. PS-C, PS-D and CAL-B, were also employed in the alcoholysis of tuna oil.

Lipozyme TL IM and D-EP100 showed higher activity in the alcoholysis of palm oil, while Lipozyme RM IM showed almost no activity (Figure 21A). However, these 1,3-regiospecific lipases, with exception of D-EP100, showed very low activity towards tuna oil (Figure 21B). In contrast, the non-regiospecific lipase preparations from CAL-B, PS-C and PS-D were found to catalyze the alcoholysis of tuna oil with moderate activity. Notably, it was suggested that the regiospecificity of *Candida antarctica* lipase B (Novozyme 435) depended on the type of reaction and the initial composition of the reaction medium and showed strict 1,3-regiospecificity in an excess amount of ethanol (Irimescu *et al.*, 2001). Thus, Lipozyme TL IM and D-EP100 were selected for further optimization of palm oil alcoholysis, while CAL-B and PS-C were selected for tuna oil alcoholysis. Only trace amounts of 1-MAG were detected in all cases as confirmed by Grignard degradation.

3.1.2 Effect of initial water activity

Water activity is recognized as an important factor in transesterification reactions. A certain amount of water is necessary to preserve the catalytically active conformation of the enzyme and to allow the formation of an acyl-enzyme complex. In contrast, excess water causes acyl migration and leads to a decrease in 2-MAG yield (Sjursnes and Anthonsen, 1994). In addition, the

absolute amount of water required for catalysis of each enzyme significantly varied from one solvent to another (Zaks and Klibanov, 1987). Ferreira-Dias and da Fonseca (1995) found that the hydrolytic side-reaction was completely suppressed at an initial water activity level lower than 0.43, while glycerolysis still occurred. Schmid *et al.* (1999) found that alcoholysis of tripalmitin was low at an initial water activity of 0.11, while highest 2-monopalmitin yield without forming undesired 1-monopalmitin was achieved at an initial water activity of 0.43.

Figure 22 shows the effect of initial water activity on the alcoholysis of palm and tuna oils in MTBE using the selected immobilized lipases. The reaction rate and 2-MAG yield tended to increase with increasing initial water activity from 0.11 to 0.43. In the alcoholysis of palm oil, both Lipozyme TL IM and D-EP100 produced the highest concentration of 2-MAG (28.1% and 40.5%, respectively) at an initial water activity of 0.43 (Figure 22A). For alcoholysis of tuna oil, the effect of initial water activity was not clearly seen. However, the highest yield was obtained at initial water activity of 0.43 for both PS-C (14.4%) and CAL-B (8.8%) (Figure 22B). The influence of initial water activity was also underlined by the determination of the initial rate in the production of 2-MAG particularly in the alcoholysis of palm oil.

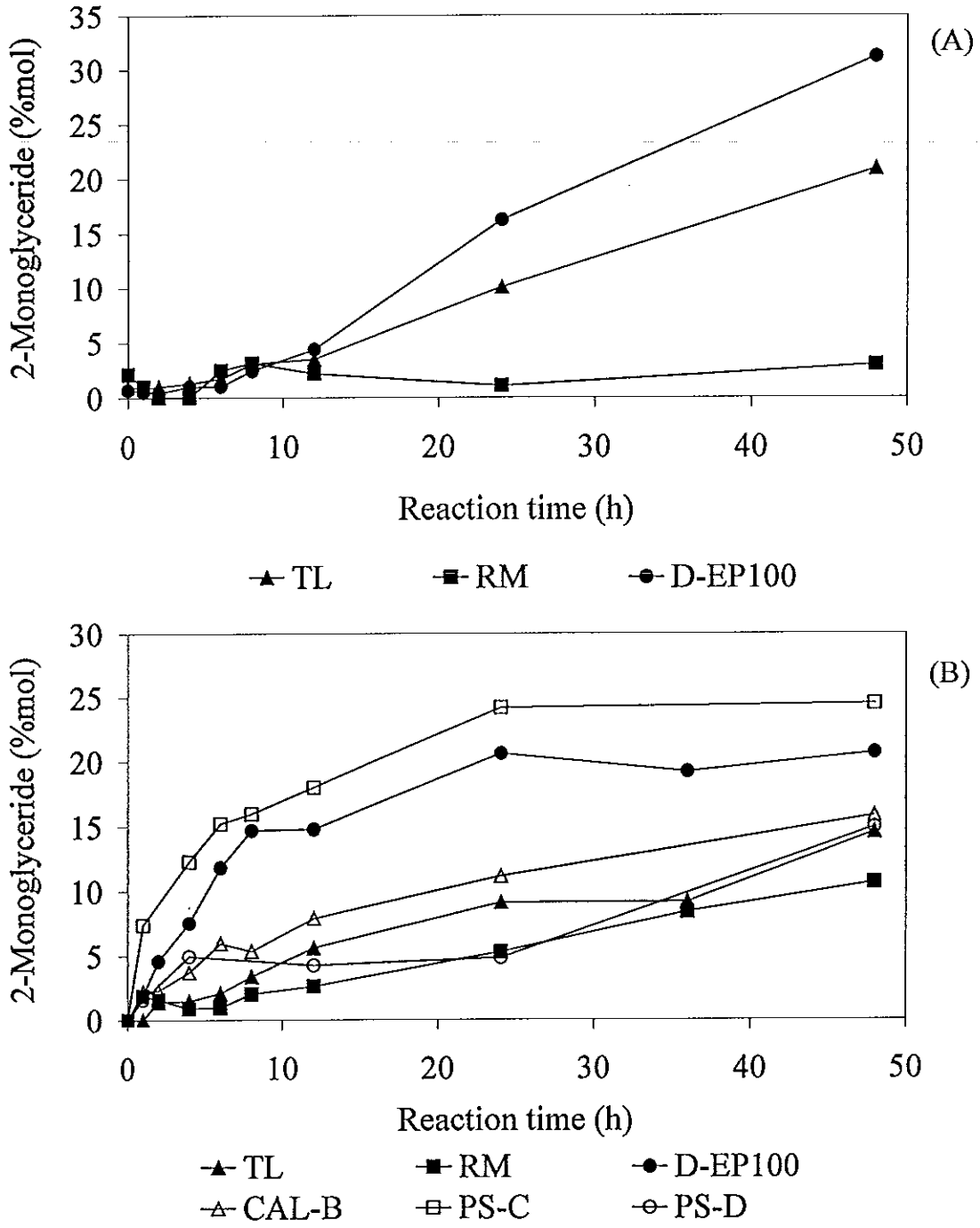


Figure 21 Screening of immobilized lipases for 2-monoglyceride production from palm oil (A) and tuna oil (B) by alcoholysis.
(a_W 0.43, MTBE, 40°C) ((Lipozyme TL IM (TL), Lipozyme RM IM (RM)).

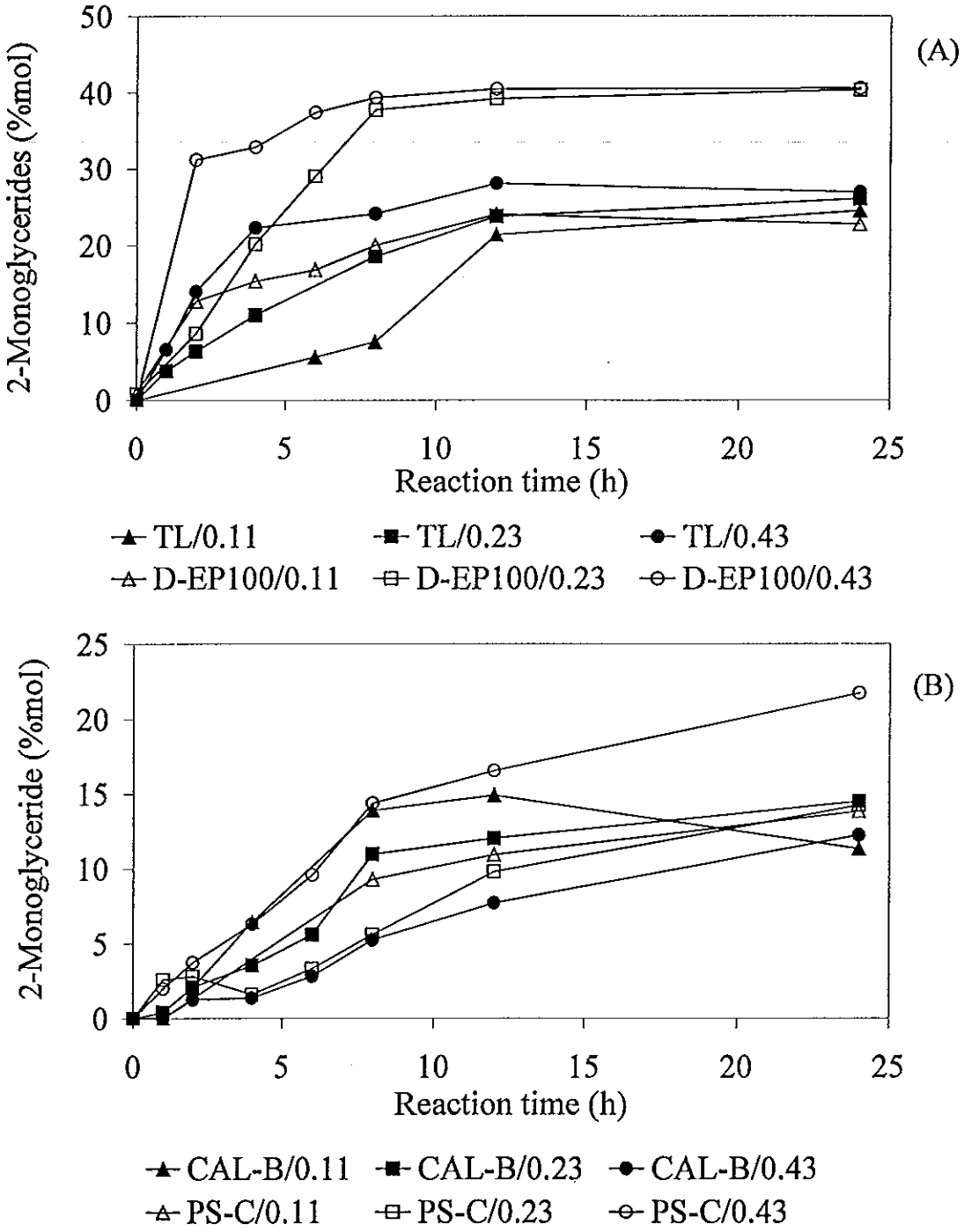


Figure 22 Effect of initial water activity on the alcoholysis of palm oil (A) and tuna oil (B) catalyzed by selected immobilized lipases. (MTBE, 40°C).

3.1.3 Effect of solvents

The effect of organic solvents on alcoholysis of palm and tuna oils was studied in acetone, MTBE and ethyl methyl ketone (EMK). For alcoholysis of palm oil (Figure 23A), a small influence of solvents on the production of 2-MAG was observed using D-EP100. However, the highest initial reaction rate was found in MTBE, which is accordance with the observation reported by Millqvist *et al.* (1994). In contrast, acetone was found to be the most suitable solvent for the alcoholysis catalyzed by Lipozyme TL IM.

In the alcoholysis of tuna oil (Figure 23B), the highest initial reaction rate and concentration of 2-MAG were obtained using PS-C in acetone. CAL-B exhibited moderate activity in MTBE and acetone but almost no activity in EMK.

3.1.4 Effect of temperature

For the production of MAG by the glycerolysis of fats and oils, the yield was greatly affected by the reaction temperature (McNeill and Yamane, 1991). Here the alcoholysis of palm oil in MTBE gave the highest initial reaction rate at 30 and 40°C. Although the initial reaction rate of alcoholysis by D-EP100 in acetone was lower (Figure 24), the higher concentration of 2-MAG was found after 24 h at both temperatures (data not shown). At 50°C, the ethanolysis of palm oil by D-EP100-catalyzed reactions was slowed down, presumably due to the low thermostability of this lipase (Amano product information). The alcoholysis of tuna oil by PS-C in acetone gave the highest reaction rate and concentration of 2-MAG at 40°C (29.3%) (Figure 25). With PS-C, both in acetone and MTBE, it was found that the yield of 2-MAG was decreased with increasing temperature. It could be assumed that PS-C was not stable at high temperature. As CAL-B is known to be very thermostable (Novozymes product information), it is not surprising that the alcoholysis of tuna oil using CAL-B in acetone gave the highest yields at 60°C (21.3%) (Figure 26). No significant effect of temperature on alcoholysis of tuna oil using CAL-B in MTBE was observed.

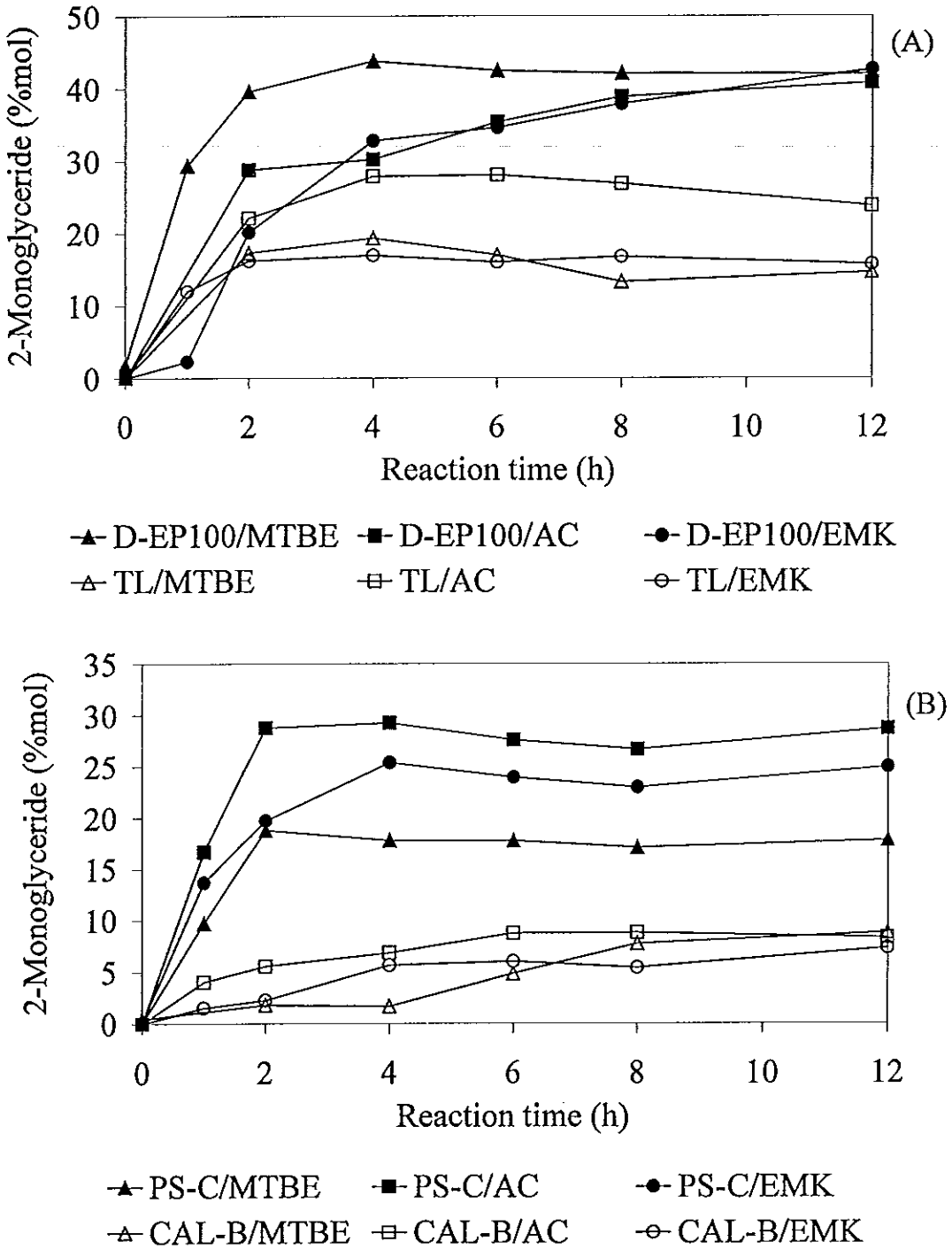


Figure 23 Effect of organic solvents on the alcoholysis of palm (A) and tuna oils (B) catalyzed by selected immobilized lipases.
(a_W 0.43, 40°C) (MTBE = methyl-*tert*-butyl ether, AC = acetone, EMK = methyl ethyl ketone).

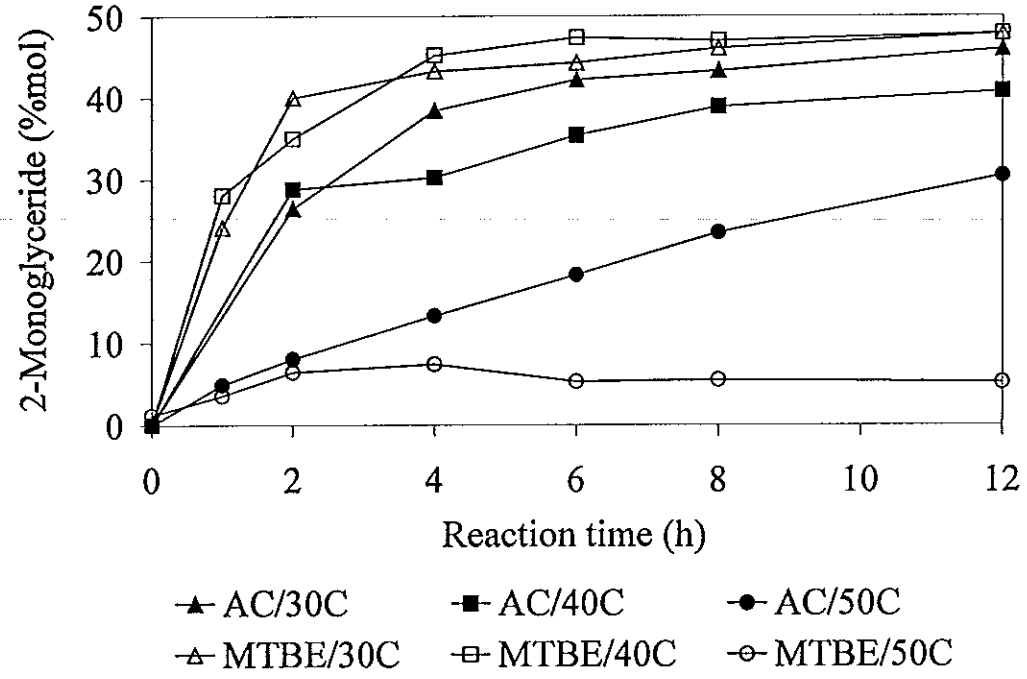


Figure 24 Effect of temperature on the alcoholysis of palm oil catalyzed by D-EP100.

(a_W 0.43, MTBE and acetone (AC)).

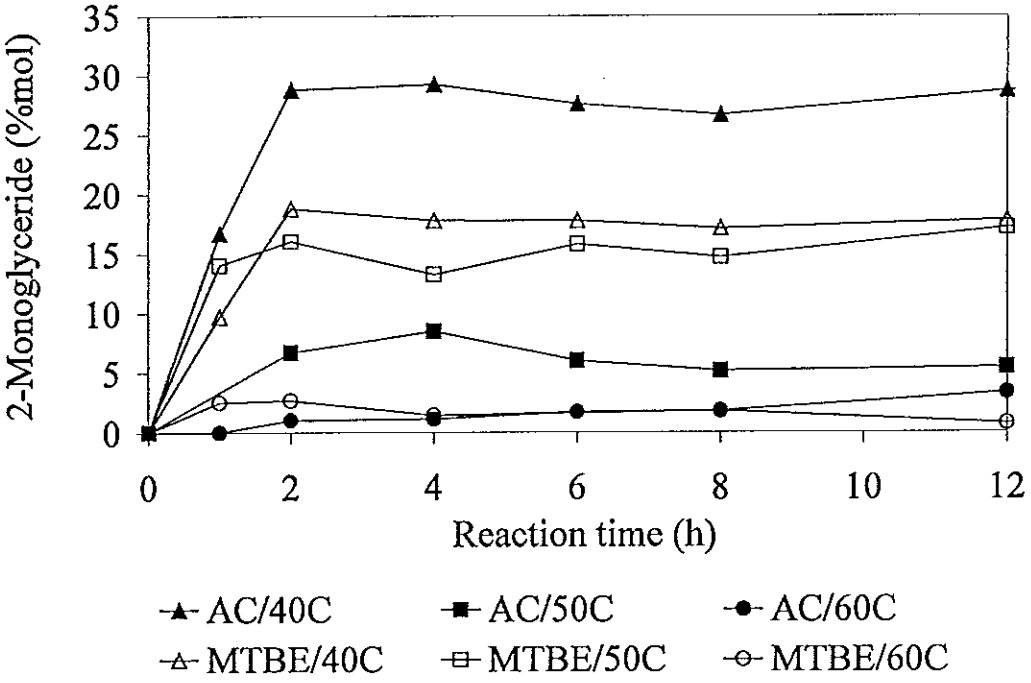


Figure 25 Effect of temperature on the alcoholysis of tuna oil catalyzed by PS-C.

(a_W 0.43, MTBE and acetone (AC)).

3.1.5 Characteristics of 2-MAG

After the successful identification of the optimum reaction conditions, 2-MAGs were produced on a preparative scale by lipase-catalyzed alcoholysis. The production of 2-MAG by the alcoholysis of palm oil was conducted using D-EP100 at an initial water activity of 0.43 in MTBE at 40°C for 12 h. The alcoholysis of tuna oil was carried out in acetone at an initial water activity of 0.43 using CAL-B at 60°C or using PS-C at 40°C for 24 h. After the reaction, the 2-MAG fractions were isolated by crystallization in n-hexane:MTBE (70:3 v/v) at –20°C and analyzed to determine their FA compositions (Table 17). In the alcoholysis of palm oil catalyzed by D-EP100, only minor differences in the FA compositions of crystallized 2-MAGs were found independent of whether acetone or MTBE was used as the solvent. However, the filtrate contained 2-MAG with an approximately 1.8-fold enriched concentration of linoleic acid (C18:2) compared to that of palm oil substrate at the *sn*2-position (Table 13). Unfortunately, it was not possible to crystallize these 2-MAG fractions due to their low melting point. It was also found that the reactions carried out in MTBE gave higher initial reaction rate, however, slightly higher 2-MAG yield was obtained from alcoholysis of palm and tuna oil in acetone (Figure 27).

The difficulty in crystallization was even more pronounced in the isolation of 2-MAG fractions obtained by the alcoholysis of tuna oil, because the MAG of EPA and DHA have even lower melting points. However, the non-desired 2-MAGs of palmitic (C16:0), stearic (C18:0) and oleic acids (C18:1) preferentially crystallized at –20°C and thus the filtrate contained high levels of 2-MAG fractions composed of PUFA (Table 17). After separation of the filtrate by TLC, the content of DHA in the 2-MAG fraction was found to be substantially increased (48.5-67.6%) compared to the starting material (28.2%). With CAL-B, the amount of EPA was reduced to only 2.5%. Using PS-C in acetone, the 2-MAG fractions in the filtrate contained approximately 80% EPA and DHA.

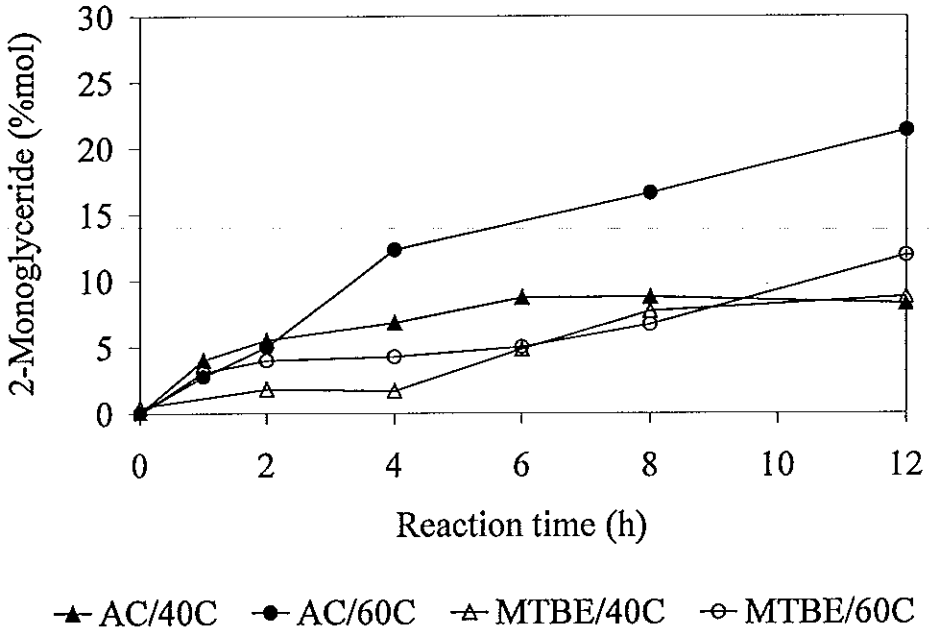


Figure 26 Effect of temperature on the alcoholysis of tuna oil catalyzed by CAL-B.
(a_W 0.43 MTBE and acetone (AC)).

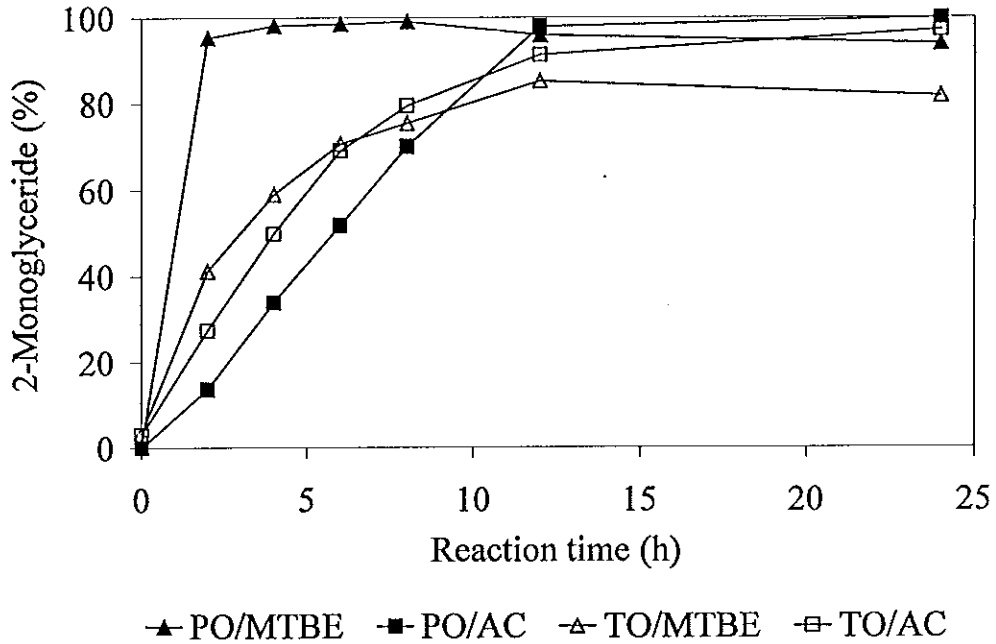


Figure 27 Time course of the production of 2-MAG by alcoholysis of palm oil (PO) and tuna oil (TO) in MTBE or acetone (AC) under optimized condition.
(% 2-monoglycerides were calculated based on the maximum theoretical yield of 33.33%).

Table 17 Fatty acid compositions and yield of 2-monoglyceride obtained after alcoholysis of palm and tuna oils under optimized conditions.

Oil	Enzyme	Solvent	Fraction	FA content of 2-MAG (%)					Yield
				C16:0	C18:1	C18:2	EPA	DHA	(%)*
Palm	D-EP100	MTBE	crystal	22.2	55.0	18.7	n.d.	n.d.	56.7[a]
			filtrate	1.8	48.1	50.1	n.d.	n.d.	52.8[b]
	D-EP100	acetone	crystal	19.2	55.7	21.0	n.d.	n.d.	64.5[a]
			filtrate	n.d.	42.7	57.3	n.d.	n.d.	57.4[b]
Tuna	PS-C	MTBE	crystal	58.7	2.4	n.d.	n.d.	n.d.	6.0[a]
			filtrate	6.0	8.4	2.3	16.0	48.5	63.6[b]
	PS-C	acetone	crystal	61.7	2.2	7.2	n.d.	n.d.	5.1[a]
			filtrate	n.d.	9.3	n.d.	21.3	59.2	81.3[b]
	CAL-B	acetone	crystal	51.7	4.8	1.4	1.6	8.4	4.8[a]
			filtrate	8.1	8.5	n.d.	2.5	67.6	66.9[b]

n.d. = not detectable

* % yield as molar concentration based on maximum theoretical yield of 33.33%

a = yield of 2-MAG recovered from crystallization

b = yield as determined by TLC-FID analytical method

3.2 Esterification of 2-MAGs obtained from palm oil with MCFA

The 2-MAGs thus obtained from alcoholysis of palm oil was purified by crystallization in n-hexane:MTBE (70:3 v/v) at –20°C (65.4% yield). The white crystal of 2-MAGs (> 98% pure) were subsequently esterified with caprylic and lauric acid in organic solvent. Several factors were studied.

3.2.1 Screening of immobilized lipases

Three commercial immobilized lipases (Lipozyme TL IM, Lipozyme RM IM and D-EP100) were used for esterification of 2-MAGs and caprylic or lauric acid. In contrast to other studies (Schmid *et al.*, 1999; Soumanou *et al.*, 1998), Lipozyme RM IM showed very low activity in this study (Figure 28).

D-EP100 gave the highest initial esterification rate with high incorporation of caprylic acid (65.4 %) and the highest incorporation of lauric acid (46.5 %). However, D-EP100 allowed slightly higher initial reaction velocity than Lipozyme TL IM. Therefore, D-EP100 were used for further experiments on the effect of solvents on esterification of 2-MAG with MCFA.

3.2.2 Effect of solvents

Esterification of 2-MAG from palm oil with MCFAs was achieved only when the reaction was carried out in n-hexane. No esterification was occurred in acetone or MTBE (data not shown).

3.2.3 Effect of substrate ratios

The theoretical ratio for esterification of 2-MAG and MCFA is 1:2 mol/mol. However, from the literature review, it was found that the optimum ratio was higher than the theoretical ratio (Soumanou *et al.*, 1998). Less amount of MCFA lead to slow esterification. In contrast an increase in concentration of MCFA above the critical value of MCFA in substrate mixture resulted in a decreased initial rate and incorporation due to acidic substrate inhibition. It was reported that high amounts of caprylic acid used as substrate in acidolysis of peanut oil might inhibit the activity of Lipozyme IM60 during the transesterification reaction (Lee and Akoh, 1998).

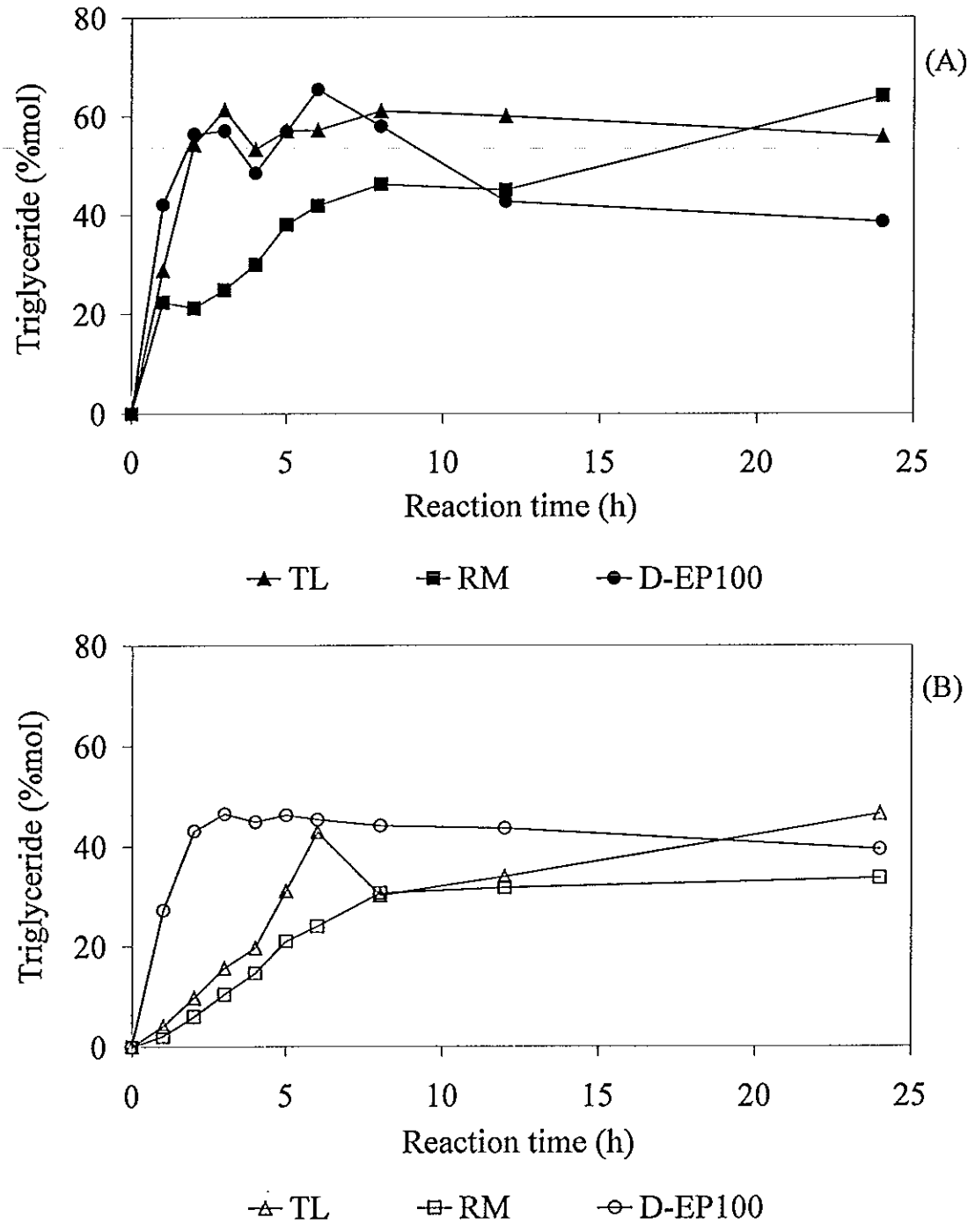


Figure 28 Synthesis of structured triglycerides by esterification of 2-monoglyceride obtained from palm oil with caprylic acid (A) and lauric acid (B) catalyzed by different immobilized lipases. (n-hexane, molar ratio of 2-monoglyceride:fatty acid = 1:3, 40°C) (Lipozyme TL IM (TL), Lipozyme RM IM (RM) and D-EP100).

Figure 29 shows the production of ST by esterification of 2-MAG from palm oil and MCFA catalyzed by D-EP100 in n-hexane at 40°C using different ratio of 2-MAG to MCFA. The 2-MAG to MCFA ratio of 1:3 (mol/mol) gave the highest initial reaction rate. However, no significant difference of the ST yield was found at different molar ratio used. The GC analysis results (Table 18) revealed that ST products obtained from esterification of 2-MAG from palm oil with MCFA contained slightly higher content of MCFA (71.6-79.4%) than the theoretical content (66.7%).

3.3 Esterification of 2-MAGs obtained from tuna oil with MCFA

Differently from the synthesis of 2-MAGs from palm oil, 2-MAGs obtained from alcoholysis of tuna oil were difficult to separate and purify from the reaction mixture due to their very low melting point. Therefore, the partially purified filtrate containing a mixture of FAEEs, TAGs, FAs, DAGs and 2-MAGs was directly used in an esterification reaction with caprylic and lauric acids. The reaction mixture obtained from alcoholysis of tuna oil using PS-C in acetone under optimum conditions (initial water activity 0.43, 40°C) composed of 46% FAEE, 7% FA, 9% DAG and 38% 2-MAG.

The result showed that CAL-B gave higher incorporation of MCFA in acetone rather than in n-hexane (Table 19). In contrast, Lipozyme TL IM showed better incorporation of MCFA in n-hexane than in acetone. Incorporation of lauric acid was slightly higher than for caprylic acid in every case using the same reaction conditions, in exception for the esterification reaction catalyzed by Lipozyme TL IM in acetone.

The highest incorporations of caprylic acid (47.2%) and lauric acid (54.4%) into 2-MAG from tuna oil were obtained using CAL-B in acetone. The ST products contained 17.3% and 16.1% PUFA, respectively. When the reaction was carried out in n-hexane the ST product contained 19.2% and 21.0% DHA, respectively.

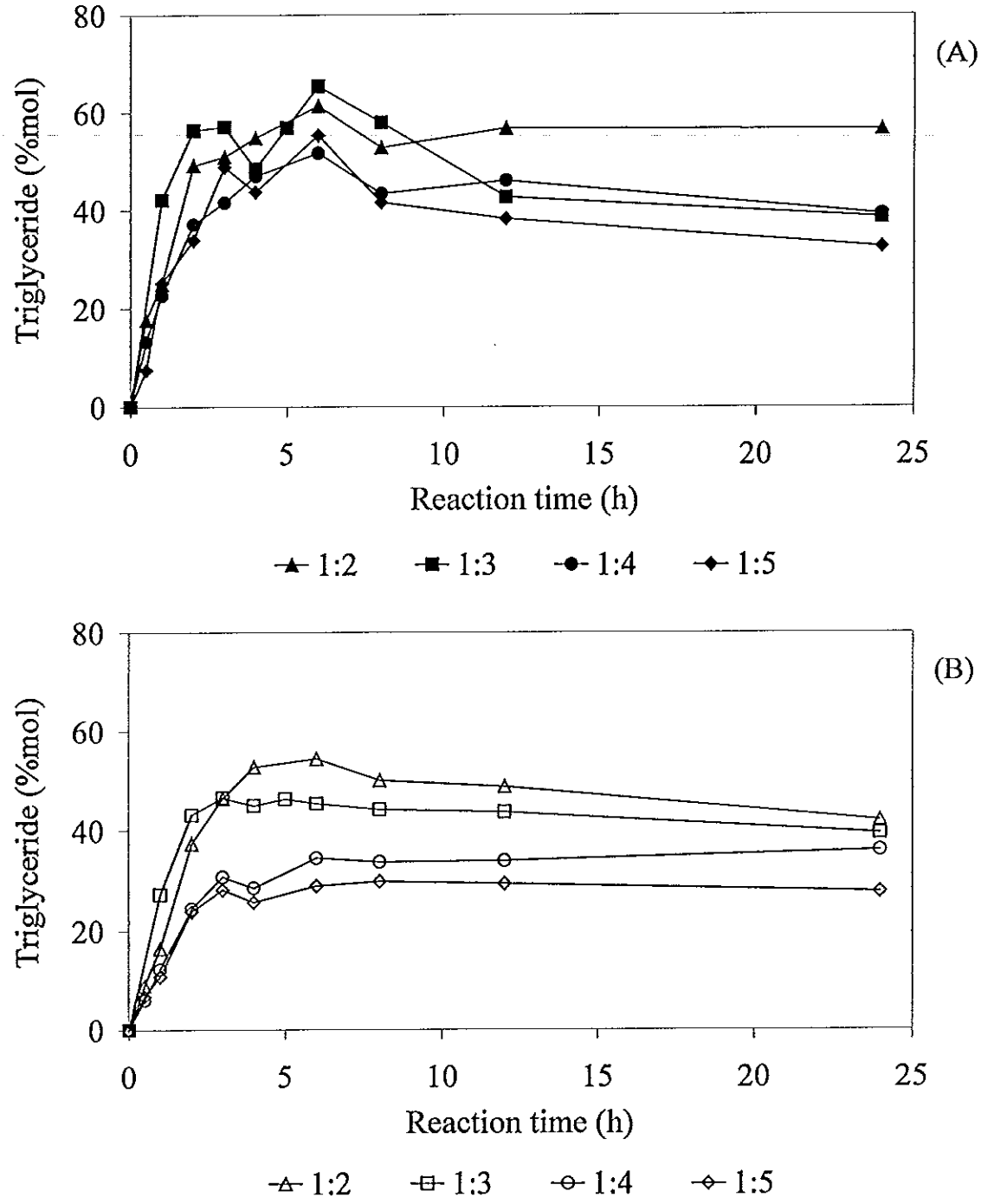


Figure 29 Effect of the molar ratio of 2-monoglyceride to medium-chain fatty acid on esterification of 2-monoglyceride from palm oil and caprylic acid (A) and lauric acid (B).
(D-EP100, n-hexane, 40°C).

Table 18 Fatty acid compositions of structured triglycerides obtained from esterification of 2-monoglycerides from palm oil with medium-chain fatty acids.

MCFA	Fatty acid composition (%)					
	C8:0	C12:0	C16:0	C18:0	C18:1	C18:2
C8:0	71.6	0	8.3	1.2	18.1	0.1
C12:0	0	79.4	4.4	0.9	14.8	0.5

Note: D-EP100, n-hexane, molar ratio of 2-monoglyceride:medium-chain fatty acid = 1:3, 40°C, 24 h.

Table 19 Major fatty acid compositions of structured triglyderides obtained from esterification of 2-monoglycerides from tuna oil with caprylic and lauric acids.

Enzyme	Solvent	MCFA	Major fatty acids of structured triglycerides (% mol)				
			C8:0	C12:0	C16:0	EPA	DHA
CAL-B	hexane	C8:0	44.5	0	13.9	4.71	19.2
	acetone	C8:0	47.2	0	13.4	4.25	13.1
	hexane	C12:0	0	43.7	7.8	3.66	21.0
	acetone	C12:0	0	54.4	7.7	3.53	12.6
TL	hexane	C8:0	35.6	0	12.2	5.28	23.8
	acetone	C8:0	33.3	0	19.7	6.43	20.7
	hexane	C12:0	0	45.5	7.3	4.07	24.1
	acetone	C12:0	0	19.1	4.2	5.56	52.9

Note: Molar ratio of 2-monoglyceride to medium-chain fatty acid = 1:3, 40°C, 24 h.

4. Synthesis of ST by Novel Two-Step Reaction

To overcome the problem of the difficulty in the separation of 2-MAGs containing very long-chain FAs, such as PUFAs, from the resulting reaction mixture from alcoholysis of palm and tuna oil, an alternative method for the synthesis of ST by two-step reaction was created. The concept of this two-step reaction is, instead of a non-stable 2-MAG, the 1,3-DAG containing MCFA, which is quite stable and easy to purify, was produced in the first step, followed by re-esterification in the second step to incorporate the LCFA to the mid-position of 1,3-DAG. The overall scheme of this two-step reaction is shown in Figure 30.

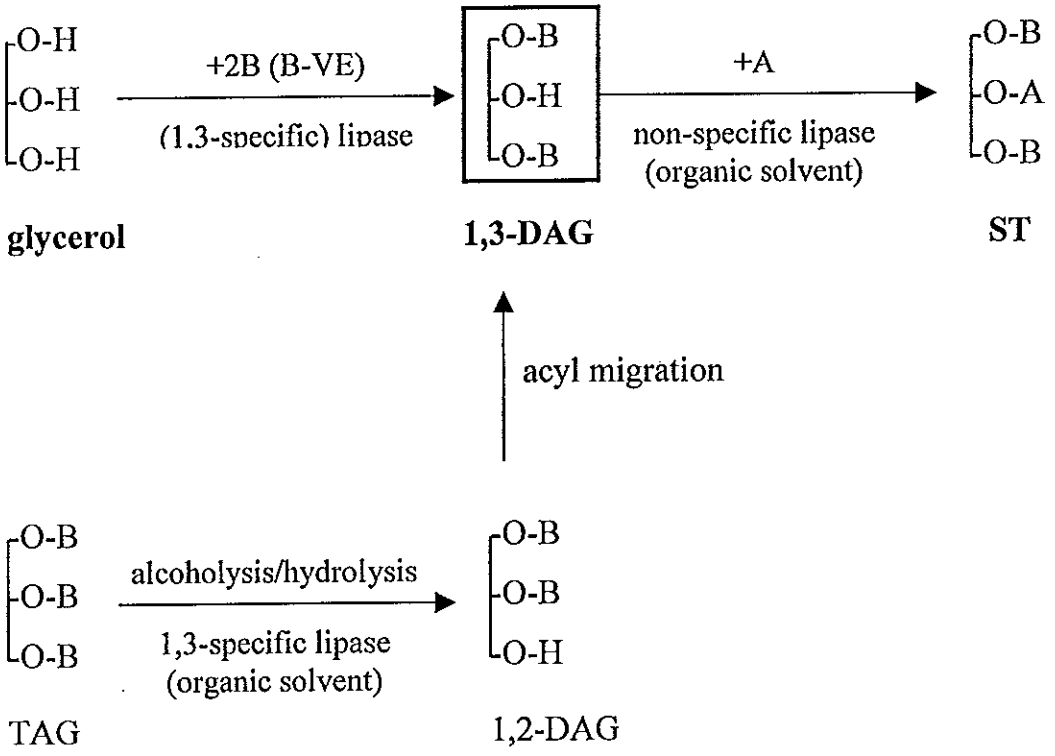


Figure 30 Principle of the synthesis of ST by a novel two-step reaction.
B = medium-chain fatty acids, B-VE = vinyl ester of medium-chain fatty acids, A = long-chain fatty acids.

4.1 Synthesis of 1,3-DAGs by esterification

Several published methods were modified and used for the synthesis of 1,3-DAGs containing the same MCFA at the *sn*1- and *sn*3-positions, i.e. 1,3-dicaprylin (1,3-DCy) and 1,3-dilaurin (1,3-DLa). FFA and its vinyl ester were used as acyl donor both in organic solvent and a solvent-free system.

Synthesis of 1,3-DAG from alcoholysis and hydrolysis of medium-chain TAGs, such as trilaurin, and natural oil rich in MCFA were also studied.

4.1.1 Synthesis of 1,3-DCy

The highest 1,3-DCy yield (92.9%), which was even higher than the 80% yield of the published method (Berger *et al.*, 1992), were obtained from esterification of glycerol and caprylic acid vinyl ester (CyVE) in a solvent-free reaction catalyzed by CAL-B at 0°C for 24 h (Figure 31).

Prevention of the accumulation of the by-product vinyl alcohol in the reaction favored the esterification-side reaction. Vinyl alcohol is not stable and is automatically degraded into a volatile substance, i.e. aldehyde. Therefore, with modification by increasing reaction temperature as well as using an open system for esterification between CyVE and glycerol in a solvent-free system, higher 1,3-DCy yield and faster reaction rate was achieved (Table 20). The highest 1,3-DCy yield (96.2%) was obtained from esterification in an open vial (open system) at room temperature using CAL-B, while 89.2% 1,3-DCy was obtained from the reaction carried out in a screw-capped vial (close system).

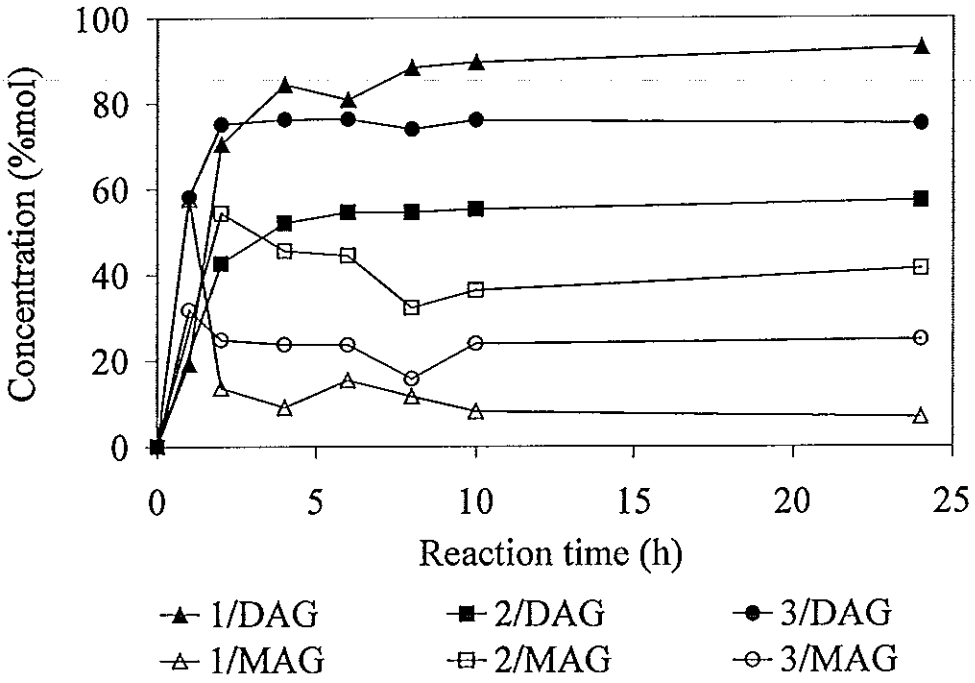


Figure 31 Synthesis of 1,3-dicaprylin by different methods.

1 = esterification of glycerol and caprylic acid vinyl ester in a solvent-free system using CAL-B at 0°C.

2 = esterification of glycerol and caprylic acid in n-hexane using CAL-B at 0°C.

3 = esterification of glycerol (immobilized on silica gel) and caprylic acid vinyl ester in MTBE using Lipozyme RM IM at room temperature (Berger *et al.*, 1992).

Esterification between caprylic acid and glycerol in n-hexane gave moderate yield of 1,3-DCy at a slower reaction and a high amount of MAG was produced (Figure 31). The yield of 1,3-DCy was increased with increasing the reaction temperature from 0°C (45%) to room temperature (55%). The yield could be increased up to 63% within 4 h by carrying out the reaction in MTBE (Table 21). It was also found that the esterification in a solvent-free condition resulted in higher yield of 1,3-DCy in comparison to the reaction carried out in organic solvent (Table 20 and Table 21).

Studies on the effect of the molar ratio of glycerol to caprylic acid on esterification reaction in a solvent-free condition catalyzed by CAL-B showed that there was no difference in the yield of 1,3-DCy when increased the ratio increasing ratio from 1:2.5 to 1:4 when the reaction was performed at room temperature (Table 22). However, when the reaction temperature increased to 40°C highest yield (68.2%) was obtained using glycerol to caprylic acid ratio of 1:3 (mol/mol). It was possible that CAL-B was inactivated by a high concentration of caprylic acid.

4.1.2 Synthesis of 1,3-dilaurin

Berger *et al.* (1992) reported the highest 1,3-DLa yield of 85% by esterification of LaVE and glycerol adsorbed onto silica gel in MTBE using Lipozyme RM IM. Similarly to the synthesis of 1,3-DCy, however, the highest 1,3-DLa yield (90%) in this study was obtained from esterification of glycerol and LaVE in a solvent-free condition catalyzed by CAL-B at room temperature (Figure 32). The reaction was complete in 4 h. When the reaction was carried out at 0°C, the yield was low because LaVE was solid at that temperature (Table 23). After purification by crystallization in n-hexane at – 20°C, 70% recovery yield of 1,3-DLa was obtained in high purity (>96%).

Table 20 Effect of temperature and reaction condition on the production of 1,3-dicaprylin by esterification of glycerol with caprylic acid vinyl ester (1:2.5 mol/mol) in a solvent-free system using CAL-B.

T (°C)	Time (h)	maximum 1,3-DCy yield (%mol)	special condition
0	12	89.6	-
0	12	90.9	open*
25	4	94.3	-
25	4	**96.2**	open

* open = open system

Table 21 Production of 1,3-dicaprylin by esterification of glycerol and caprylic acid (1:2.5 mol/mol) in organic solvent in the presence of molecular sieves.

Enzyme	solvent	T (°C)	Time (h)	maximum 1,3-DCy yield (%mol)
CAL-B	hexane	0	48	45.3
CAL-B	hexane	25	48	55.0
CAL-B	hexane	25	48	43.5*
CAL-B	MTBE	25	4	**63.1**
Lipozyme RM IM	MTBE	25	4	60.9

* without molecular sieves

Table 22 Effect of temperature and substrate ratio on the production of 1,3-dicaprylin by esterification of glycerol and caprylic acid in a solvent-free system under reduced pressure using CAL-B for 24 h.

T (°C)	glycerol to caprylic acid ratio (mol/mol)	maximum 1,3-DCy yield (%mol)
25	1:2.5	60.3
40	1:2.5	64.9
25	1:3	60.1
40	1:3	68.2
25	1:4	60.3
40	1:4	64.8

Table 23 Production of 1,3-dilaurin by esterification of glycerol with lauric acid vinyl ester (LaVE) in a solvent-free system (glycerol:LaVE = 1:2.5 mol/mol).

Enzyme	T (°C)	Time (h)	maximum 1,3-DLa yield (%mol)	special condition
CAL-B	0	24	79.1	-
Lipozyme RM IM	0	24	40.9	-
Lipozyme TL IM	0	24	5.8	-
CAL-B	25	24	76.9	-
CAL-B	25	8	**90.5**	open*
Lipozyme RM IM	25	24	81.1	-
Lipozyme RM IM	25	24	83.2	open

* open = open system

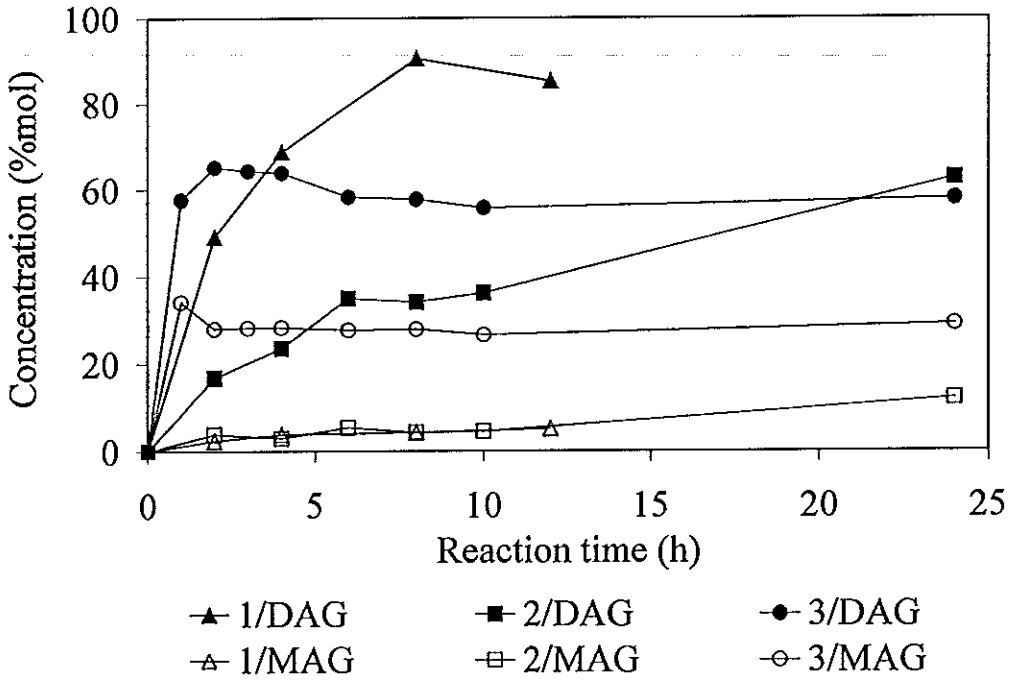


Figure 32 Production of 1,3-dilaurin by different published methods.

1 = esterification of glycerol and lauric acid vinyl ester in a solvent-free system using CAL-B at room temperature.

2 = esterification of glycerol and lauric acid in n-hexane using CAL-B at room temperature.

3 = esterification of glycerol (immobilized on silica gel) and lauric acid vinyl ester in MTBE using Lipozyme RM IM at room temperature (Berger *et al.*, 1992).

Esterification of glycerol and LaVE in a solvent-free system was also studied. Surprisingly a higher 1,3-DLa yield was obtained using non-specific lipase CAL-B (79.1%) than using a 1,3-specific lipase, i.e. Lipozyme RM IM (40.9%) or Lipozyme TL IM (5.8%) (Table 23).

The results from esterification of glycerol and lauric acid in a solvent-free condition under reduced pressure (500 mm Hg) using CAL-B showed that the 1,3-DLa yields were decreased with increasing the molar ratio of glycerol to lauric acid from 1:2.5 to 1:6 (Table 24 and Figure 33). Also, a decreasing tendency of the 1,3-DLa yield was observed after 6 h due to the synthesis of the by-product TAG (Figure 33). The highest 1,3-DLa yield (68.9%) was obtained using a molar ratio of 1:2.5, while high amounts of trilaurin were produced at molar ratio of 1:4. In addition, at optimum substrate ratio (glycerol:lauric acid = 1:2.5 mol/mol), the reaction carried out at 40°C gave higher 1,3-DLa yield than at 60°C and the maximum 1,3-DLa yield (74.9%) was obtained at 9 h.

The reaction temperature for the esterification of glycerol and lauric acid carried out in a solvent-free system was higher than in organic solvent and the esterification of caprylic acid due to the melting point of lauric acid (44-45°C). Similar to the reaction with caprylic acid, esterification of glycerol with lauric acid in a solvent-free system under reduced pressure gave higher 1,3-DLa yield than the reaction carried out in organic solvent (Table 24 and Table 25).

4.2 Synthesis of 1,3-DAGs by alcoholysis and hydrolysis of TAGs

Although 1,3-DAG was efficiently synthesized in an esterification reaction, the cost of starting material, especially FAVE, is still too high for an industrial-scale production. Therefore, the synthesis of 1,3-DAG from a lower cost raw material, such as TAG, was investigated.

Table 24 Effect of substrate ratio and temperature on the production of 1,3-dilaurin by esterification of glycerol with lauric acid in a solvent-free system under reduced pressure using CAL-B for 6 h.

T (°C)	glycerol to lauric acid ratio (mol/mol)	maximum 1,3-DLa yield (%mol)
40	1:2.5	56.4
60	1:2.5	68.9
60	1:4	52.3
60	1:6	43.0

Table 25 Effects of immobilized lipases and molar ratio of glycerol to lauric acid on the production of 1,3-dilaurin by esterification in n-hexane at 30°C in the presence of molecular sieves.

Enzyme	Time (h)	glycerol to lauric acid ratio (mol/mol)	maximum 1,3-DLa yield (%mol)
CAL-B	24	1:2.5	**62.6**
CAL-B	48	1:4	57.5
Lipozyme RM IM	8	1:4	43.0
D-EP100	24	1:4	40.4

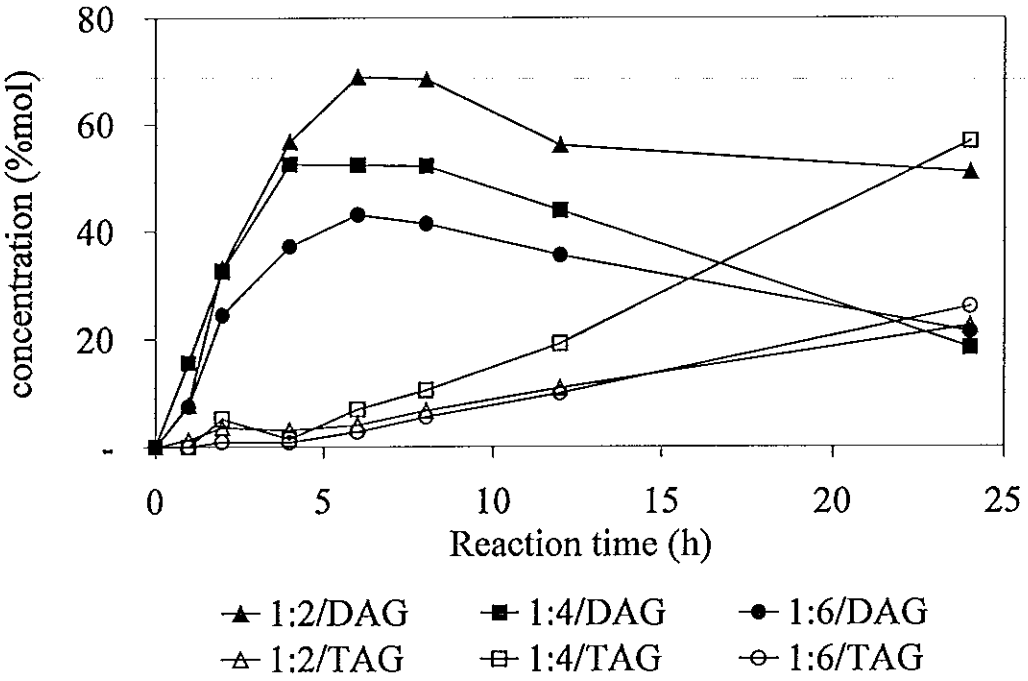


Figure 33 Effect of the molar ratio of glycerol to lauric acid on production of 1,3-dilaurin in a solvent-free system.
(CAL-B, 60°C).

4.2.1 Alcoholysis of Pure TAG

Trilaurin was subjected to alcoholysis reaction to produce DLa. Several parameters were studied.

4.2.1.1 Effect of initial water activity

An adequate water content in the reaction medium is critical and to optimize enzymatic activity, one should examine it at different water content (Zaks and Klibanov, 1988).

A higher yield of DAG and less MAG was obtained from alcoholysis with ethanol of trilaurin using Lipozyme RM IM in MTBE at a_W 0.11 (50.4% DAG, 17.0% MAG) than at a_W 0.43 (46.3% DAG, 26.3% MAG) (Figure 34). It was also found that the MAG content was increased with increasing a_W.

4.2.1.2 Effect of solvents

Fureby *et al.* (1997) reported that the best solvent for alcoholysis of trilaurin using immobilized lipase from *Penicillium roquefortii* was ether. Correspondingly, in this study the highest DAG yield (50.4%) was obtained from alcoholysis in MTBE. Alcoholysis in n-hexane and isooctane gave moderate yield (32.3% and 30.2%, respectively), while acetone (4.5%) was a poor solvent for Lipozyme RM IM (Figure 35).

The reaction performed in n-hexane showed slightly faster acyl migration rate than in MTBE (Figure 36).

4.2.1.3 Effect of substrate ratio

Highest DLa yield (49.4%) was obtained from alcoholysis using trilaurin to ethanol ratio of 1:1 (mol/mol), which was equal to the theoretical molar ratio for the production of DAG (Figure 37). DLa yields and initial reaction rates were decreased, while MAG was sharply increased with increasing in ethanol concentration.

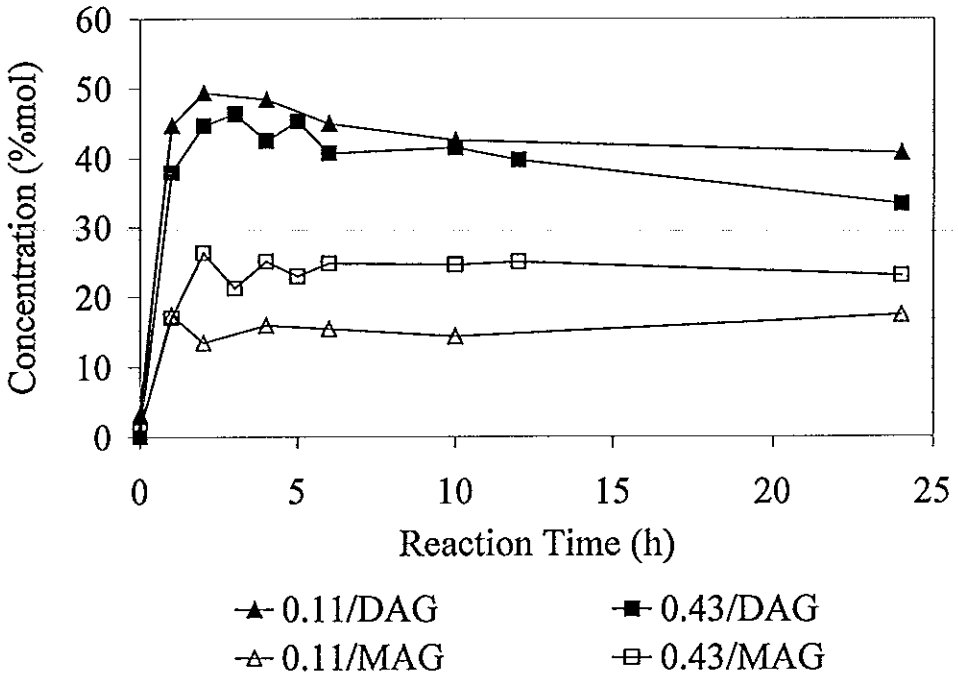


Figure 34 Effect of initial water activity on alcoholysis of trilaurin.
(Lipozyme RM IM, MTBE, molar ratio of trilaurin:ethanol = 1:1, 60°C).

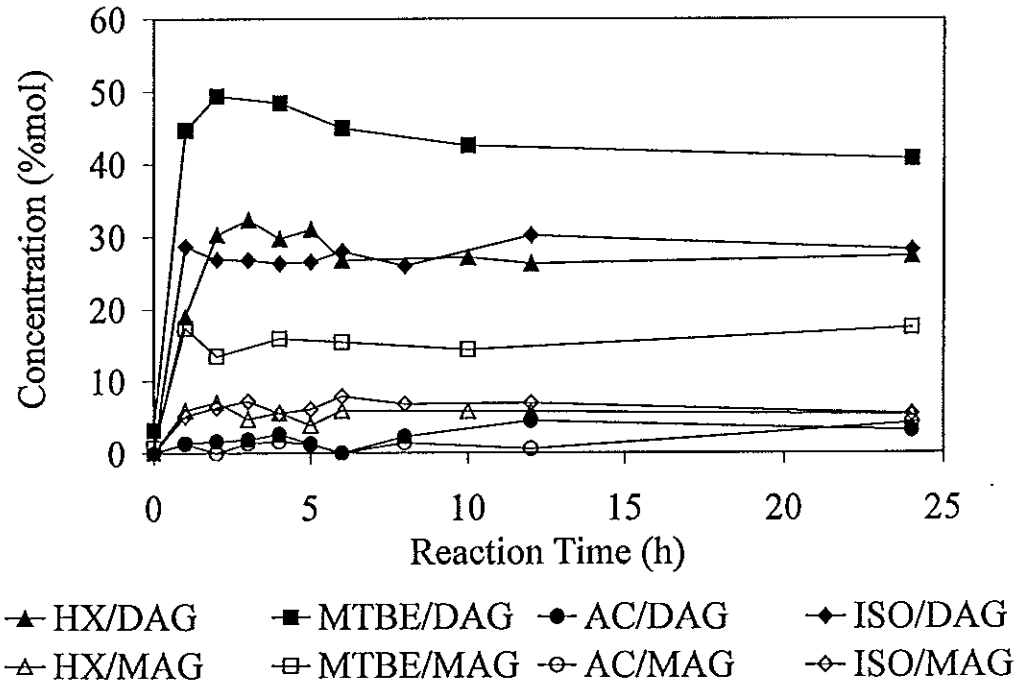


Figure 35 Effect of solvents on alcoholysis of trilaurin.
(Lipozyme RM IM, a_W 0.11, molar ratio of trilaurin:ethanol = 1:1, 60°C) (n-hexane (HX), MTBE, acetone (AC) and iso-octane (ISO)).

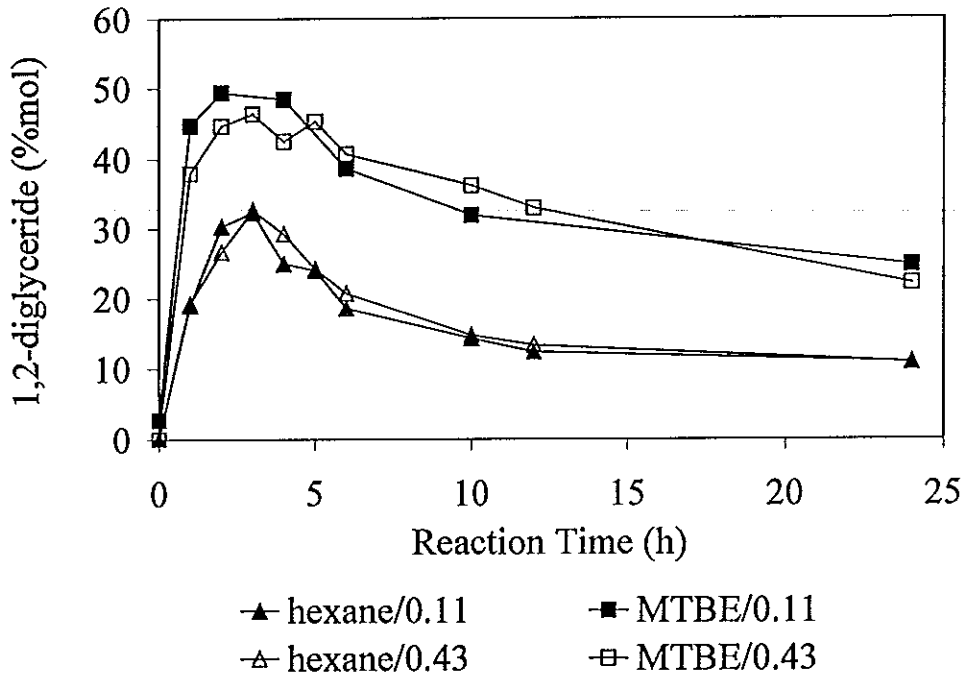


Figure 36 Effect of solvents on acyl migration of 1,2-diglycerides during alcoholysis of trilaurin.
(Lipozyme RM IM, a_W 0.11 or 0.43, 60°C).

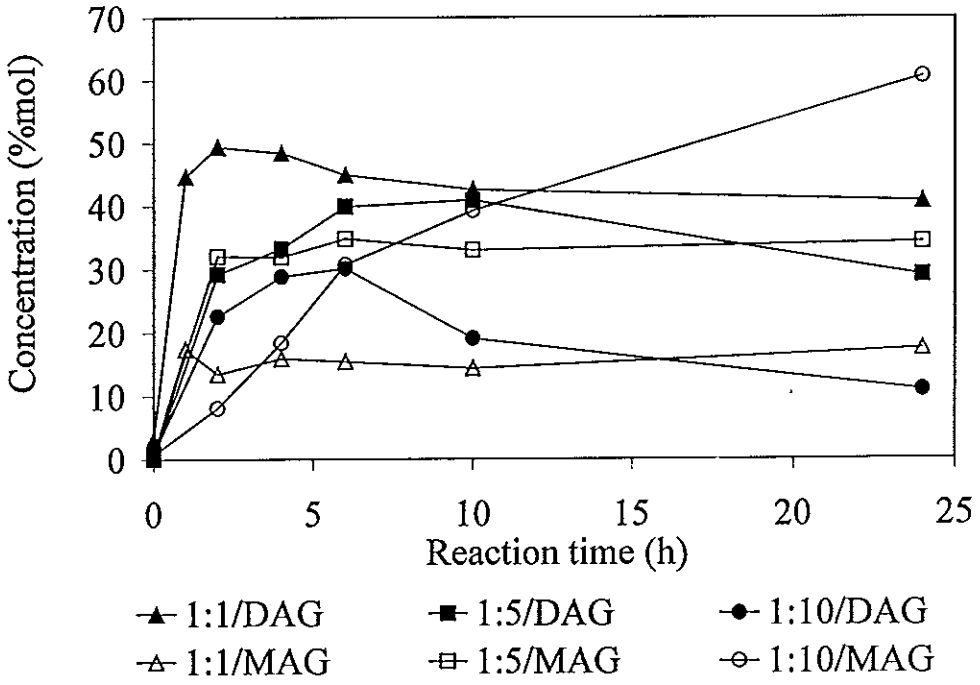


Figure 37 Effect of molar ratio of trilaurin to ethanol on alcoholysis in MTBE.
(Lipozyme RM IM, a_W0.11, 60°C).

4.2.2 Hydrolysis of Pure TAG

It was found that, instead of MAG from alcoholysis, a high amount of by-product FAs was produced during the hydrolysis reaction. Similar to alcoholysis reaction, a high amount of unreacted TAG (> 25%) remained in the reaction mixture and most DAG obtained was 1,2-DAG. However, a higher potential of acyl migration was found in hydrolysis in comparison to the alcoholysis reaction (Figure 36 and Figure 40).

4.2.2.1 Effect of amount of water

Highest yield of DAG (43.9%) was obtained from hydrolysis using a trilaurin to water ratio of 1:1 (mol/mol). It was also shown that the DLa yield decreased and the amount of MAG increased with increasing TAG to water ratio, especially in MTBE (Figure 38).

4.2.2.2 Effect of solvents

The result was corresponding to the result obtained from the alcoholysis reaction. Hydrolysis of trilaurin by Lipozyme RM IM at 60°C in MTBE (46.0%) allowed higher DAG yield than in n-hexane (23.2%), isooctane (27.0%) and acetone (8.5%), respectively (Figure 39). Although the reaction in MTBE gave higher DAG yield, a high amount of MAG was produced and lower acyl migration of 1,2-DAG was found in comparison to the reaction carried out in n-hexane (Figure 40).

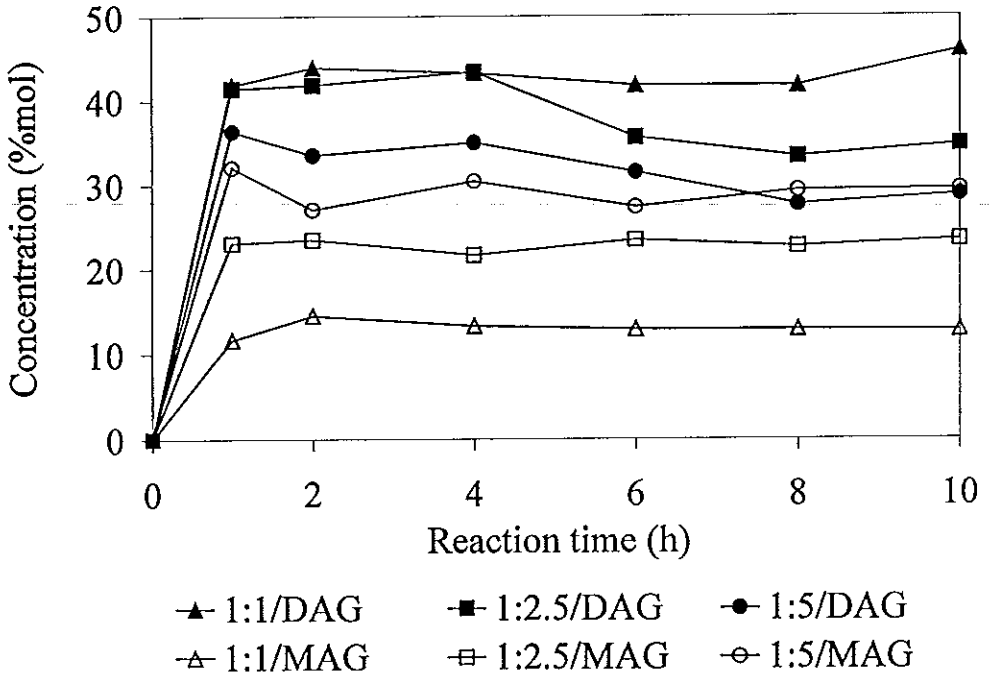


Figure 38 Effect of molar ratio of oil to water on hydrolysis of trilaurin.
(Lipozyme RM IM, MTBE, 60°C).

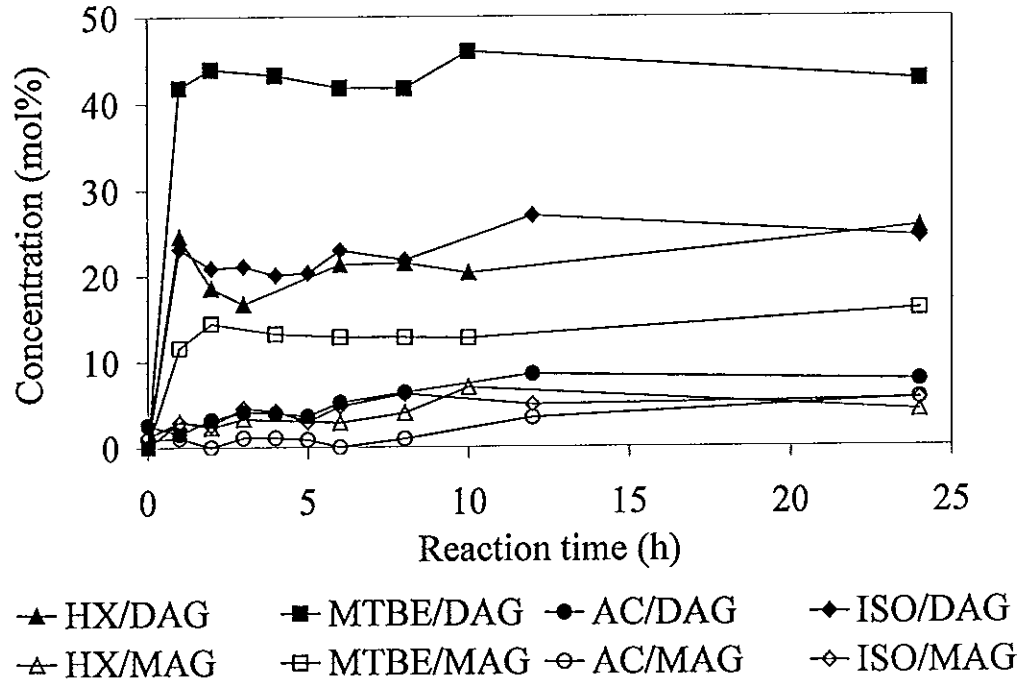


Figure 39 Effect of solvents on hydrolysis of trilaurin.
(Lipozyme RM IM, molar ratio of trilaurin:water = 1:1, 60°C)
(n-hexane (HX), MTBE, acetone (AC) and isooctane (ISO)).

4.2.3 Alcoholysis and hydrolysis of natural oils

Coconut oil contains approximately 63.5% MCFA (Table 13). Therefore, it is a good and cheap raw material for the synthesis of 1,3-DAGs containing MCFA. Several parameters were studied to optimize the reaction condition for the synthesis of DAG from coconut oil.

Alcoholysis and hydrolysis of coconut oil was carried out in n-hexane at 40°C using commercial 1,3-specific immobilized lipases (Lipozyme TL IM and Lipozyme RM IM). The ratio of oil to ethanol in alcoholysis and the ratio of oil to water in hydrolysis were 1:1 (mol/mol). The alcoholysis reaction gave slightly higher DAG yield with lower MAG than hydrolysis reaction (Figure 41). Moreover, the presence of water in the hydrolysis reaction resulted in high amount of undesired FAs and difficulties in the purification of the DAG product. Accordingly, further studies were emphasized on DAG synthesis by alcoholysis of coconut oil.

4.2.3.1 Screening of immobilized enzymes for DAG production

Lipozyme RM IM gave higher DAG yield (42.4%) than D-EP100 (29.0%), while Lipozyme TL IM showed low activity in the alcoholysis of coconut oil in n-hexane at a_W 0.11, 40°C (Figure 42). However, the results from alcoholysis of palm oil revealed that D-EP100 gave higher potential of 2-MAG synthesis. Therefore, D-EP100 was selected for further studies on the effect of other parameters.

4.2.3.2 Effect of solvent

Similar to alcoholysis of trilaurin, alcoholysis of coconut oil performed in MTBE gave higher yield (50.3%) and faster reaction rate than in acetone (44.1%) and n-hexane (29.0%), respectively (Figure 43).

4.2.3.3 Effect of Water Activity

It was reported that in the alcoholysis of trilaurin using lipase from *Penicillium roquefortii* the highest lipase activity was observed at low initial water activity, but the 1,2-DLa yield increased with increasing initial water

activity (Fureby *et al.*, 1997). In contrast, it was found in this study that high DAGs yield were obtained from alcoholysis of coconut oil by D-EP100 in MTBE at initial water activity 0.23 (41.7%) and 0.33 (40.4%) at 40°C. Moreover, using initial water activity above 0.33 resulted in lower reaction rate and DAG yield with an increase in the MAG yield (Figure 44).

4.2.3.4 Effect of Molar Ratio of Substrates

Although alcoholysis of coconut oil catalyzed by D-EP100 in MTBE using a molar ratio of oil to ethanol 1:1 showed slower initial reaction rate, the highest DAG yield (35.3%) was obtained. An increase in the ethanol concentration resulted in a decrease in DAG yield and an increase in MAG production (Figure 45).

Under optimized condition (a_W 0.23, 40°C), DAG obtained from alcoholysis of coconut oil using D-EP100 in acetone and MTBE contained approximately 81% MCFA with minimal amount of LCFA (< 7%) (Table 26). However, the yield of 1,2-DAG obtained from alcoholysis or hydrolysis of coconut oil was slightly less than the yield obtained from pure TAGs.

Table 26 Fatty acid compositions of diglycerides obtained from alcoholysis of coconut oil in organic solvent catalyzed by D-EP100.

Solvent	Fatty acid composition (%mol)							
	C8:0	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2
MTBE	1.83	2.33	76.43	12.80	1.58	n.d.	3.82	1.20
acetone	5.25	4.12	72.03	11.72	1.94	n.d.	3.78	1.15

n.d. = not detected

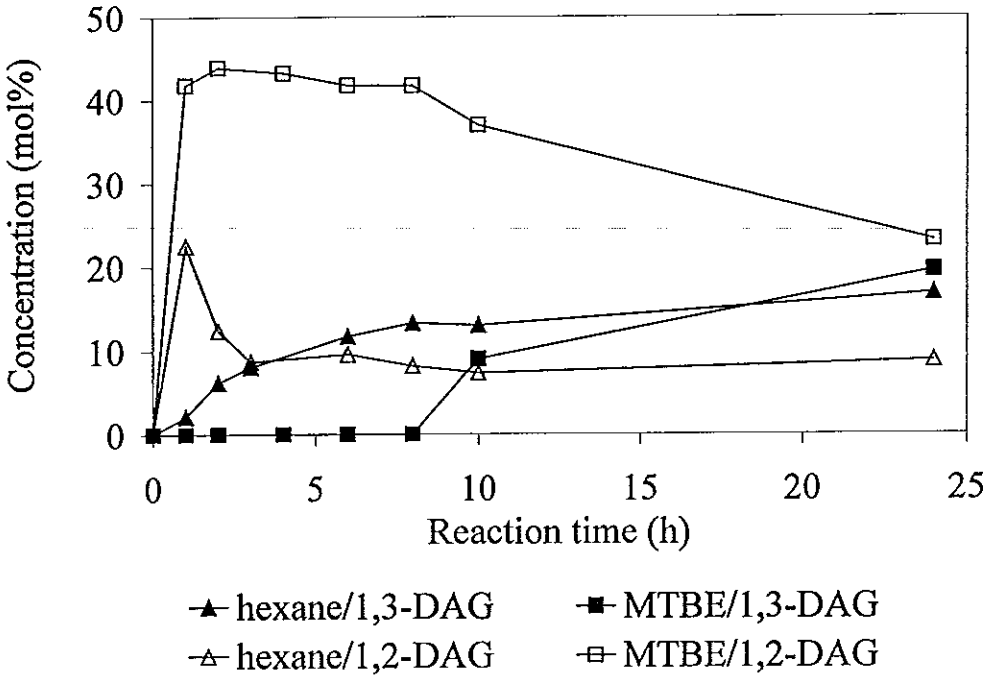


Figure 40 Acyl migration of 1,2-diglyceride during hydrolysis of trilaurin. (Lipozyme RM IM, n-hexane or MTBE, 60°C).

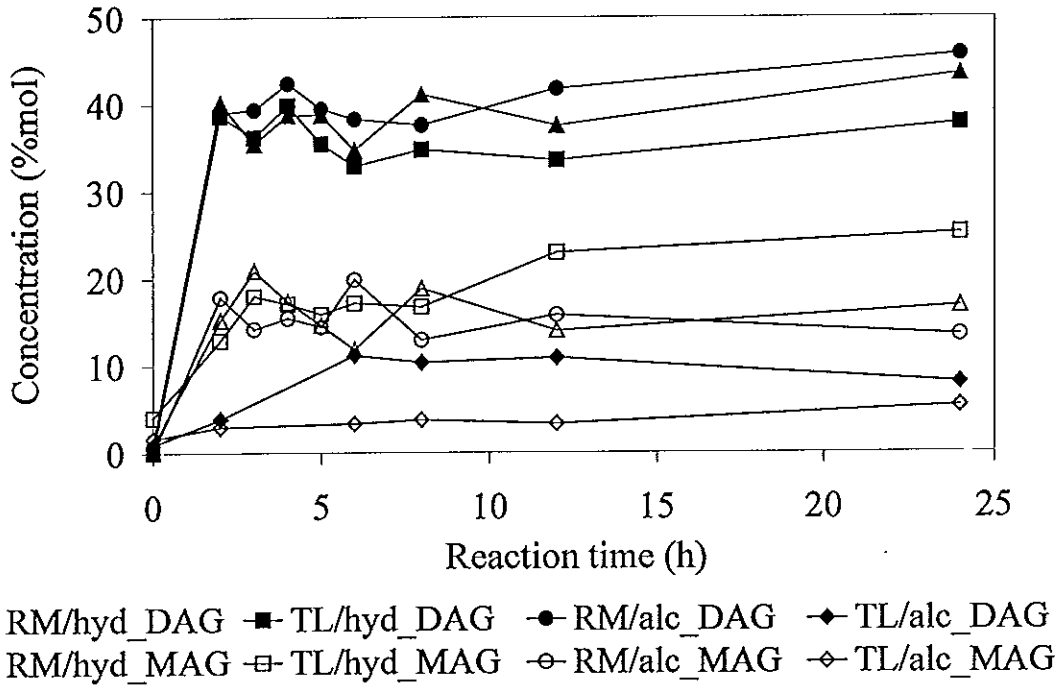


Figure 41 Synthesis of diglycerides by alcoholysis (alc) and hydrolysis (hyd) of coconut oil by Lipozyme TL IM (TL) and Lipozyme RM IM (RM).
(n-hexane, a_W 0.11, 40°C).

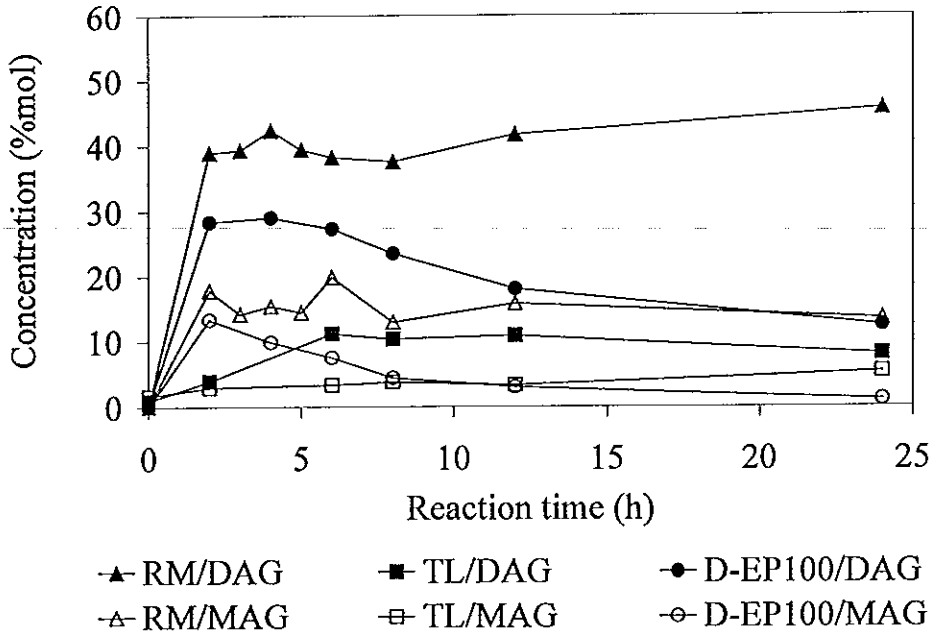


Figure 42 Screening of enzymes for the synthesis of diglyceride by alcoholysis of coconut oil.
(n-hexane, a_W 0.11, oil:ethanol = 1:1 mol/mol, 40°C) (Lipozyme RM IM (RM), Lipozyme TL IM (TL) and D-EP100).

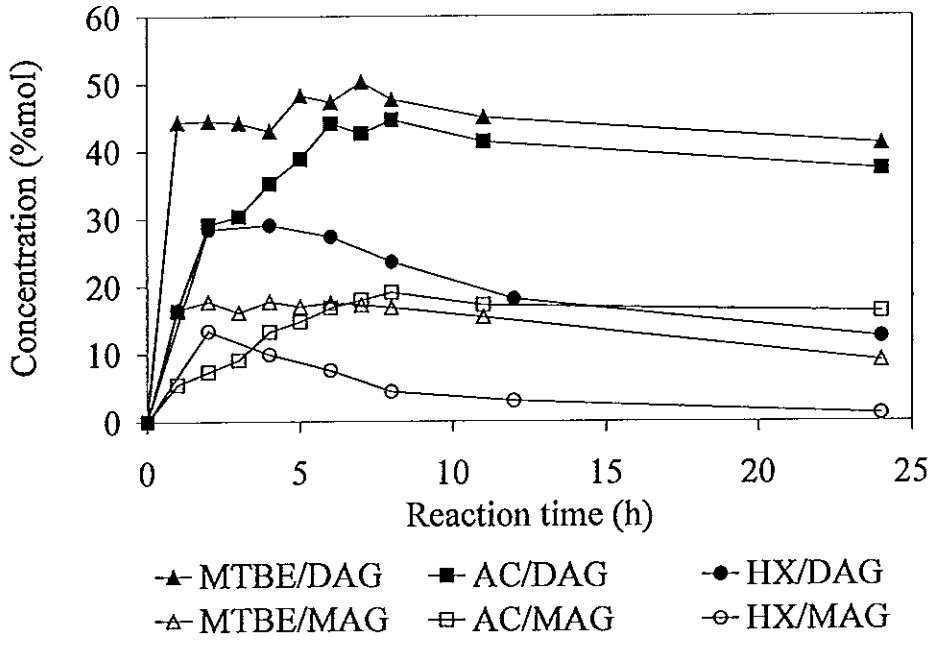


Figure 43 Effect of solvents on synthesis of diglyceride by alcoholysis of coconut oil.
(D-EP100, a_W 0.11, oil:ethanol = 1:1 mol/mol, 40°C).
(MTBE, acetone (AC) and n-hexane (HX)).

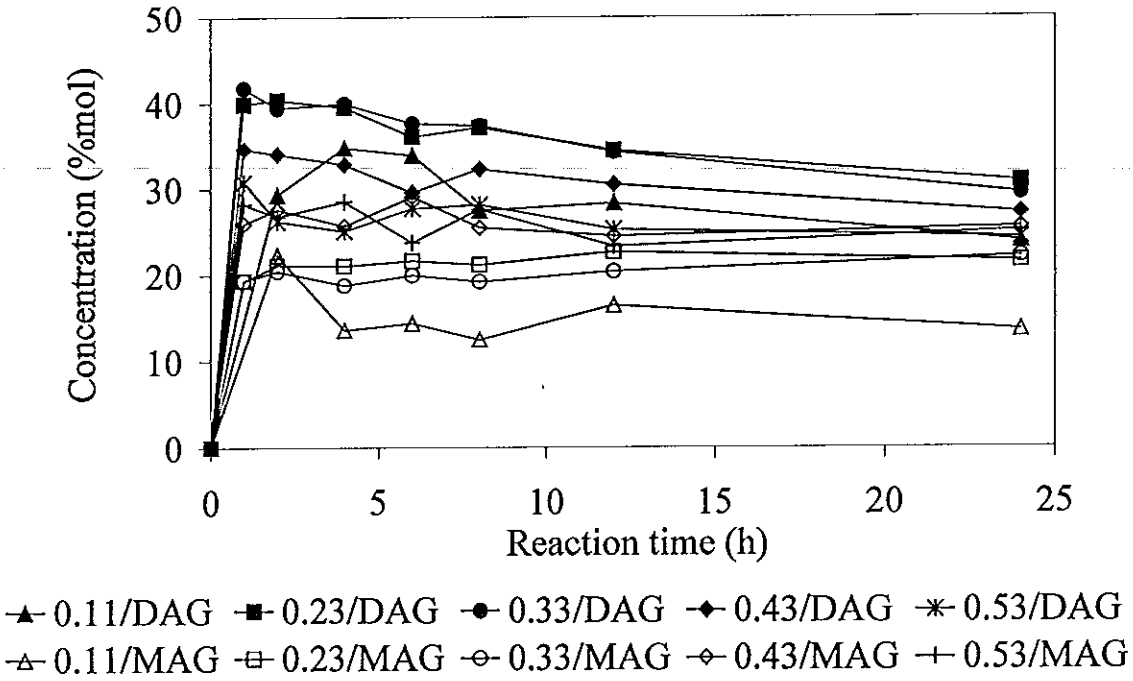


Figure 44 Effect of initial water activity on alcoholysis of coconut oil.
(D-EP100, MTBE, oil:ethanol = 1:1 mol/mol, 40°C).

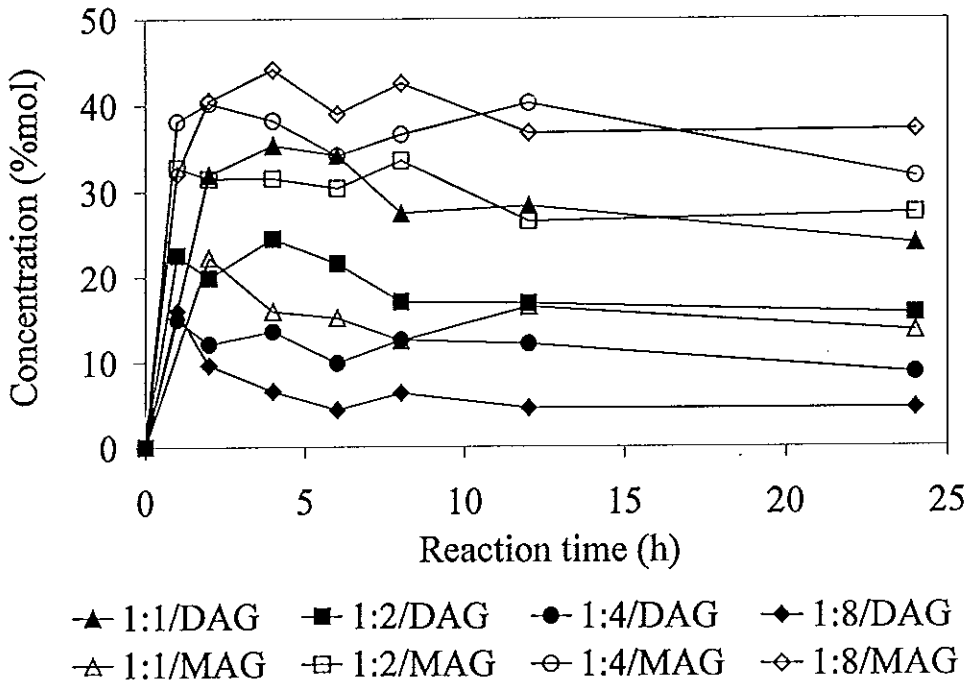


Figure 45 Effect of the molar ratio of coconut oil to ethanol on alcoholysis.
(D-EP100, MTBE, a_W 0.11, 40°C).

4.2.4 Induction of acyl migration of 1,2-DAGs

Acyl migration is a well-known reaction occurring during lipase-catalyzed esterification or transesterification of glycerides, in which DAGs and MAGs are reaction intermediates. Solvent, initial water activity, temperature, pH or the presence of acid or base, and ion-exchange resin were found to effect the degree of acyl migration (Sjursnes and Anthonsen, 1994; Bornscheuer and Kazlauskas, 1999). Generally, acyl migration has to be taken into account as undesired side-reaction leading to a decrease of selectivity. Instead of suppressing it, an induction of acyl migration of 1,2-DAG (a major product from the alcoholysis of coconut oil) to 1,3-DAG (a substrate for the synthesis of ST by this two-step method) was investigated in this study.

1,2-Dipalmitin (1,2-DP) was used as a model substrate. Addition of oleic acid, which also served as a substrate in the subsequent esterification reaction with 1,3-DAGs, to the reaction mixture slightly induced acyl migration at room temperature (Figure 46). The acyl migration rate was slightly increased with increasing oleic acid:1,2-DP ratio.

It was found that an anion exchange resin showed high induction of acyl migration, while a cation exchanger resin showed no effect. The acyl migration rate was increased with increasing amount of anion exchanger (Figure 47). However, a large amount of anion exchanger was required to induce a fast acyl migration.

Acyl migration of 1,2-DP gradually occurred when incubated at 60°C and 75.6% 1,3-DP was obtained after 96 h (Figure 48). Almost no acyl migration was observed in 1,2-DP solution in the presence of Celite even after incubation for 72 h (data not shown).

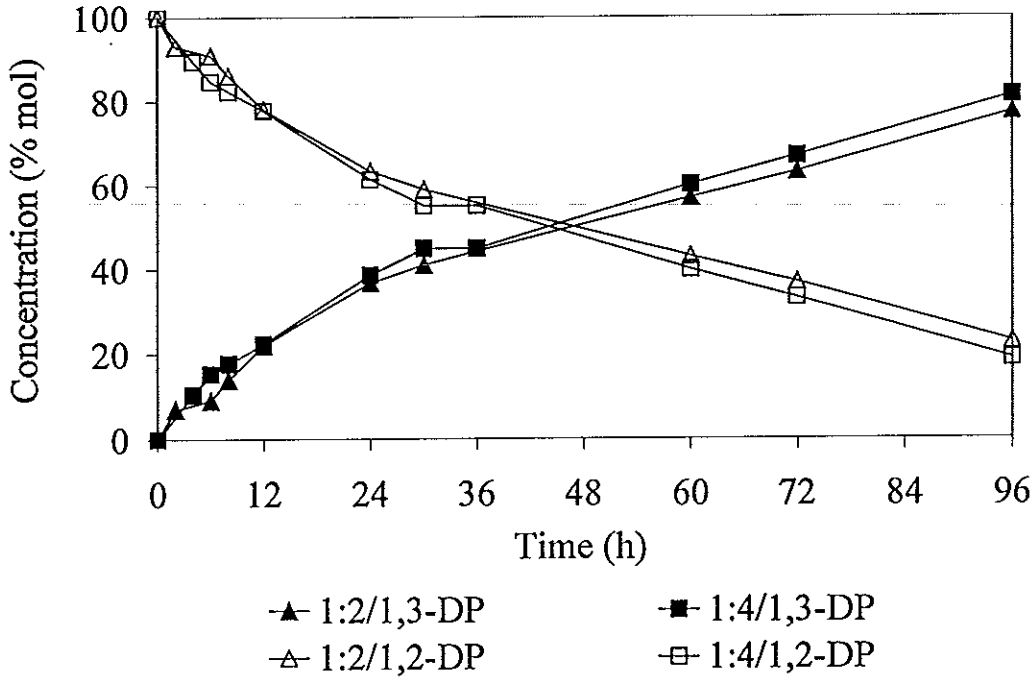


Figure 46 Acyl migration of 1,2-dipalmitin (DP) to 1,3-dipalmitin at different molar ratios of 1,2-dipalmitin to oleic acid.
(n-hexane, room temperature).

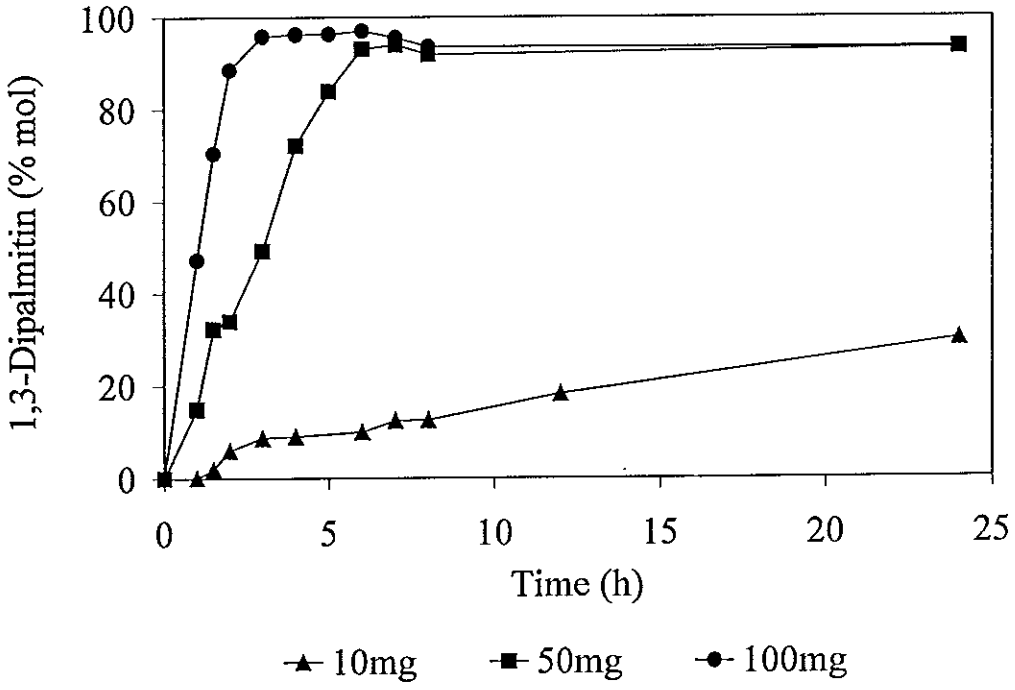


Figure 47 Effect of amount of anion exchange resin on acyl migration of 1,2-dipalmitin (8 mg).
(n-hexane, room temperature).

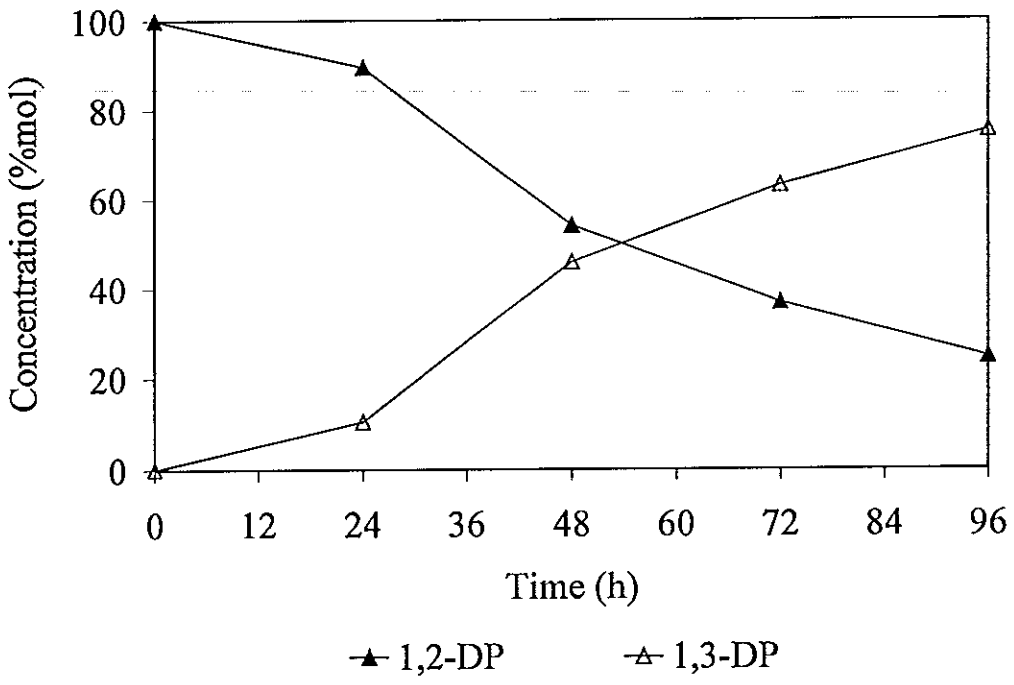


Figure 48 Acyl migration of 1,2-dipalmitin (8 mg/mL) in n-hexane at 60°C.

4.3 Esterification of 1,3-DAGs and OA/OAVE

The hypothesis of this alternative route for synthesis of ST was that the enzymatic synthesis of ST should also be feasible from 1,3-DAG using a lipase with fatty acid chain-length or fatty acid saturation specificity. Accordingly, this enzyme must not act on the fatty acids already present in the 1,3-DAG, but at the same time exhibit high selectivity and activity towards the fatty acid to be introduced at the *sn*2-position.

To demonstrate the practicability of this new strategy, the non-regiospecific PS-D was chosen as it preferably acts on LCFA rather than MCFA. Thus, by esterification of a 1,3-DAG composed of MCFA and a LCFA as acyl donor, ST synthesis should be feasible. Indeed, esterification of 1,3-DCy or -DLa with OAVE in n-hexane using PS-D at 60°C resulted in high yields of ST (87 and 78%, respectively) at very short reaction times (3 and 8 h) (Figure 49). GC analysis of these STs showed that the molar ratio of MCFA to LCFA was about 2:1 (Table 27). Esterification of 1,3-DCy with OAVE performed at gram-scale was finished after only 2 h (Figure 50). HPLC analysis of the product mixture revealed that the desired ST, CyOCy (45%) was most prominent followed by CyOO (25%). Triolein was formed in only very small amounts (0.5%).

Although ST was efficiently synthesized from transesterification of 1,3-DAGs and OAVE, the regiospecific analysis of the ST products revealed the presence of caprylic acid and lauric acid at the *sn*2-position of the ST products (Table 28). The presence of MCFA at the *sn*2-position could be on account of the non-specificity of PS-D or acyl migration during reaction.

Transesterification of 1,3-DAGs and OAVE were also carried out in a solvent-free system under reduced pressure. However, the yields and reaction rate were decreased to 32% (from 1,3-DCy) and 73% (from 1,3-DLa) (Figure 51).

When free oleic acid was used as acyl donor, water produced during the esterification was removed by applying vacuum (solvent-free system) or by adding molecular sieves (solvent system), to avoid acyl migration and an unfavorable equilibrium. Esterification between 1,3-DAG and oleic acid in n-hexane was very slow and only 20 % ST were obtained after 24 h in the esterification starting from 1,3-DLa, while almost no reaction occurred using 1,3-DCy (Figure 49).

A solvent-free system was a better alternative for esterification in industrial application, whereas vacuum was applied to remove the produced water in the reaction mixture without using molecular sieves. For esterification of 1,3-DAGs with oleic acid, yields and reaction rates were considerably increased to 56% (from 1,3-DCy) and 53% (from 1,3-DLa) in the solvent-free system under reduced pressure (Figure 52). In contrast, esterification between 1,3-DLa and linolenic acid in n-hexane was faster than in the solvent-free system under reduced pressure (Figure 53). Only 14.1% TAG was obtained from esterification of 1,3-DLa and linolenic acid catalyzed by PS-D at 60°C in a solvent-free system under reduced pressure, while 42.2% TAG was obtained from the reaction carried out in n-hexane. Higher yield of ST was achieved when increased the molar ratio of 1,3-DLa to linolenic acid, especially in a solvent system. TAG yields were increased to 73.7% and 29.1% for esterification in n-hexane and in solvent-free system, respectively, using molar ratio of 1,3-DLa to linolenic acid of 1:2.

Table 27 Fatty acid composition of structured triglyceride products from esterification of 1,3-diglycerides and oleic acid vinyl ester.

Structured triglycerides	Fatty acid composition (%)[a]		
	C8:0	C12:0	C18:1
CyOCy[b]	61.0	-	39.0
LaOLa[b]	-	63.3	36.7

[a]as determined by GC analysis

[b]CyOCy, 1-capryloyl-2-oleyl-3-capreloyl-glycerol; LaOLa, 1-lauroyl-2-oleyl-3-lauroyl-glycerol

Table 28 Regiospecific analysis of structured triglyceride products from esterification of 1,3-diglycerides and oleic acid vinyl ester.

ST	position	Fatty acid (% mol)		
		C8:0	C12:0	C18:1
CyOCy	*sn*2-	25.7	-	74.3
LaOLa	*sn*2-	-	11.1	88.9

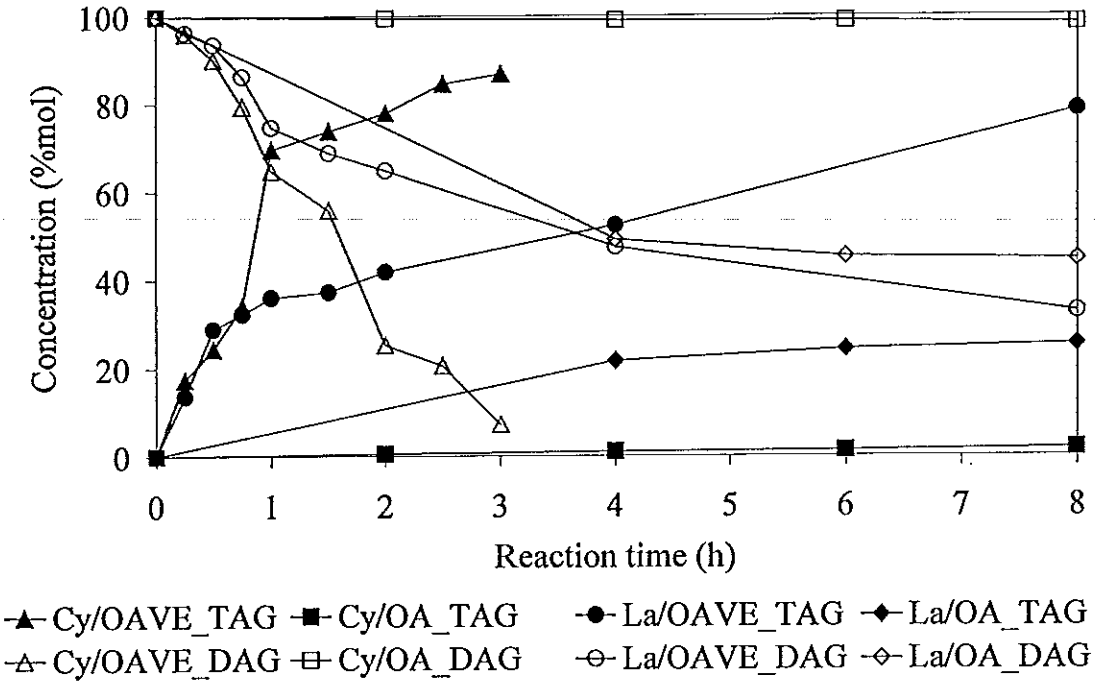


Figure 49 Time course of the esterification between 1,3-diglycerides with oleic acid vinyl ester or oleic acid.
(PS-D, n-hexane, 60°C).

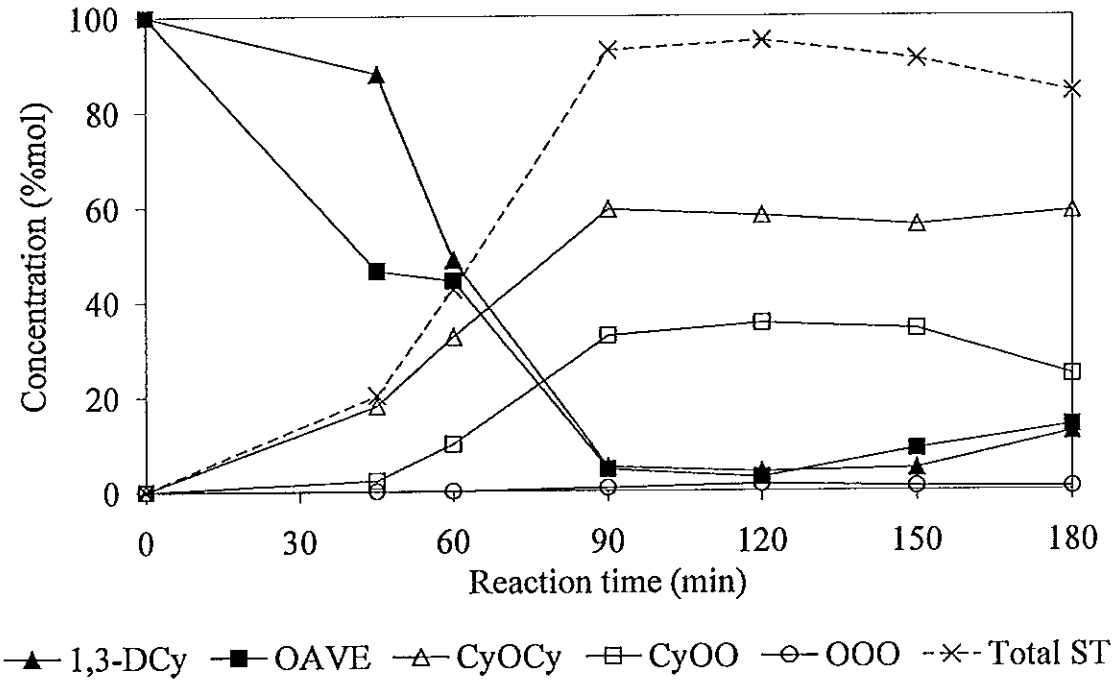


Figure 50 Time course of the esterification between 1,3-dicaprylin and oleic acid vinyl ester.
(PS-D, n-hexane, 60°C).

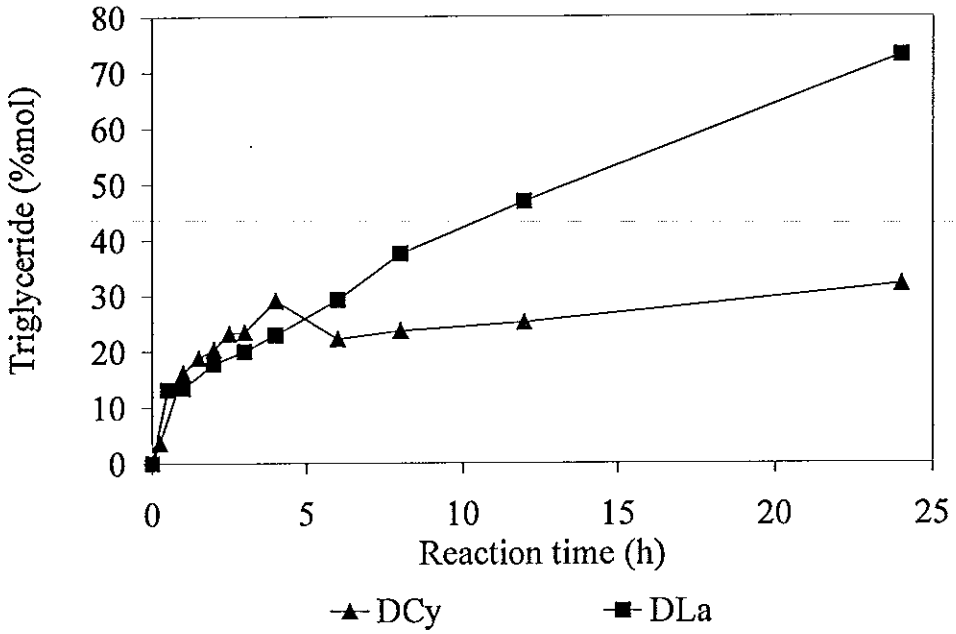


Figure 51 Structured triglyceride production by esterification of 1,3-dicaprylin (DCy) and 1,3-dilaurin (DLa) with oleic acid vinyl ester in a solvent-free system under reduced pressure.
(PS-D, 60°C)

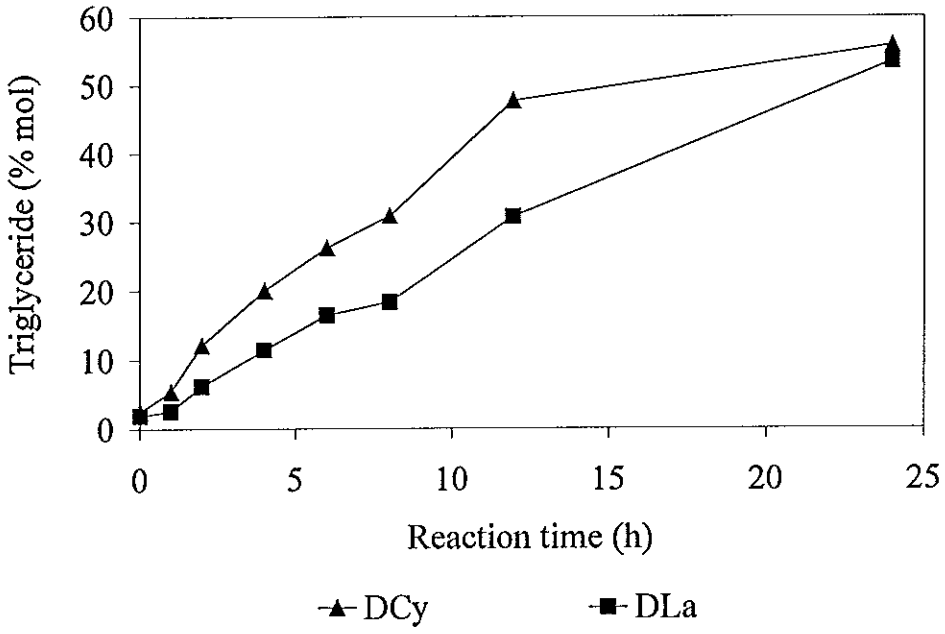


Figure 52 Structured triglyceride production by esterification of 1,3-dicaprylin (DCy) and 1,3-dilaurin (DLa) with oleic acid in a solvent-free system catalyzed under reduced pressure.
(PS-D, 60°C)

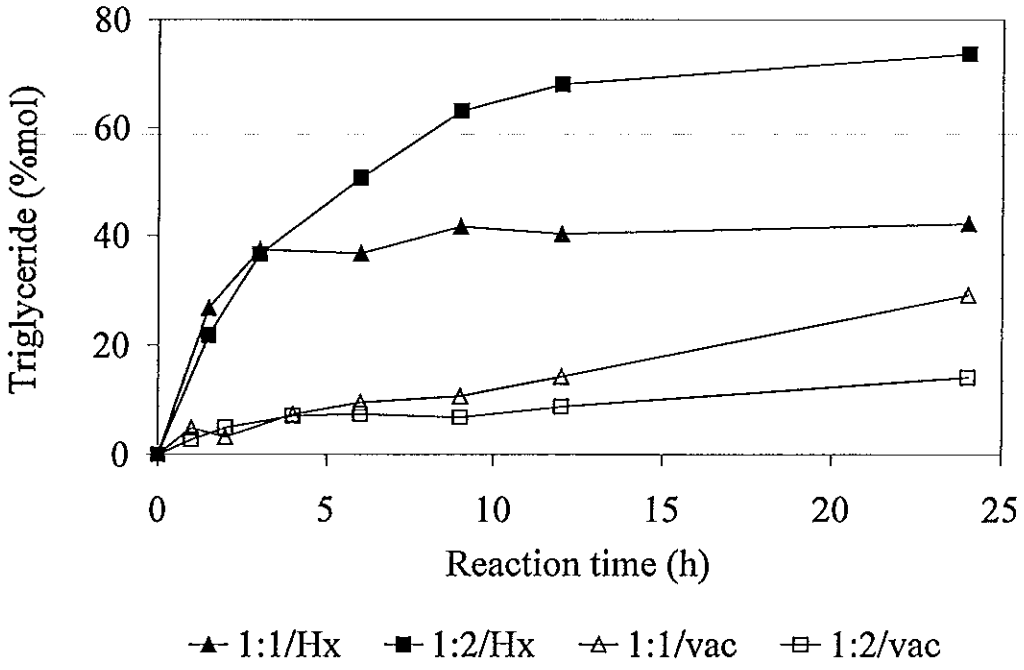


Figure 53 Structured triglyceride production by esterification of 1,3-dilaurin with linolenic acid in n-hexane (Hx) and in a solvent-free system under reduced pressure (vac).
(PS-D, 60°C).

5. Chemical Synthesis of 1,2-Diacyl-PC

Pisch *et al.* (1997) successfully synthesized 1,2-di-(octadecynoyl)-*sn*-glycero-3-phosphocholine in high yield (98%) by chemical reaction. First, octadecynoic acid was activated using 1,1'-carbonyl-diimidazole and then coupled with GPC in the presence of DBU.

In this study, the incorporation of one type of FA to the *sn*1- and *sn*2-positions of GPC was performed by chemical synthesis starting from GPC (Table 29). It was found that a high yield of the desired 1,2-dialkoyl-PC was only achieved using lauric acid yielding 82 % DLPC. Purification by crystallization from acetone:chloroform (9:1 v/v) at –20°C gave 95 % pure product. In contrast, with caprylic or linoleic acid only low yields of the corresponding PCs were found, which also did not crystallize at –20°C. In addition, GPE was used as alternative starting material, but only 10 % yield was observed in the incorporation of lauric acid. One alternative for the synthesis of PLs bearing an ethanolamine group is PLD-catalyzed head group exchange, which was subsequently studied.

6. PL Modification by PLD-Catalyzed Transphosphatidylation

PLD-catalyzed transphosphatidylation or a head-group exchange of natural PL with various nucleophiles or alcohols has been widely studied. Since most PLD utilizes alcohols rather than water, it is possible to accomplish transphosphatidylation reaction even in the presence of high amount of water (Pisch *et al.*, 1997). In two-phase or biphasic system, consisting of buffer and apolar solvent such as chloroform and ethyl ether, the aqueous phase is employed as the accepting-reservoir for choline, which exerts strong inhibitory action on PLD (Juneja *et al.,* 1992). However, the considerable amounts of water result in the undesirable hydrolytic side reaction. Thus, an anhydrous organic solvent system has been recently studied using a non-aqueous scavenger, specifically a cation exchange resin, which would be able to efficiently remove positively charged choline (Rich and Khmelnisky, 2001). In

this study, transphosphatidylation (or a head group exchange reaction) of DLPC and ethanolamine was carried out both in a conventional biphasic system and an anhydrous solvent system.

6.1 Biphasic System vs Anhydrous Solvent System

It was found that the initial reaction rate of transphosphatidylation of DLPC and ethanolamine using PLD carried out in biphasic system was higher than in non-aqueous solvent when the same amount of enzyme and substrate concentration was used (Table 30). Nevertheless, the reaction in biphasic system was constant after 24 h without any increase in PE yield. Although transphosphatidylation in anhydrous system required longer reaction time (48 h) to be completed, 100% conversion was obtained in comparison to a maximum conversion of 98% from the reaction in a biphasic system at 24 h. Therefore, subsequent studies concentrated on transphosphatidylation in the anhydrous system.

6.2 Effect of Salt Activation of the PLD

It has been reported that a salt activation technique can dramatically increase the catalytic activity of the dried powder of several enzymes in organic media relative to enzyme with no added salt. Moreover, the salt-activated enzyme exhibited greatest activity when lyophilized from a solution of a pH equal to the pH for optimal activity in water (Ru *et al.*, 1999). Therefore PLD-KCl preparation used in this work was lyophilized in a buffer of pH 5.6, which is an optimum pH for PLD (Juneja *et al.*, 1987). The mechanism of salt-induced activation of enzyme activity in organic solvents may be due to the fact that a highly polar salt matrix may help to maintain the native structure of the enzyme in organic media (Khmelnitsky *et al.*, 1994).

Table 29 Products from chemical synthesis of different phospholipids with different fatty acids.

GPX	Fatty acid	Composition (%)			
		PX	LPX	GPX	FA
GPC	Caprylic acid (C8:0)	46.82	trace	27.67	17.66
GPC	Lauric acid (C12:0)	82.33	7.49	3.7	6.65
GPC	Linoleic acid (C18:2)	22.94	19.68	12.56	46.28
GPE	Lauric acid (C12:0)	10.12	18.13	27.42	59.10

*PX = phospholipid, LPX = lysophospholipid, GPX = glycerophospholipid

Table 30 Transphosphatidylation in biphasic and anhydrous solvent systems. (phospholipase D, 40°C).

System	Phospholipase D sources	Reaction time (h)	PE (%)	Initial reaction rate (% PE/h)
biphasic	peanut	24	98.6	16.6
biphasic	*E. coli* strain	24	96.2	15.8
anhydrous	peanut	48	100	4.4
anhydrous	*E. coli* strain	48	100	3.9

It was found that the transphosphatidylation reaction increased with increasing amount of PLD-KCl (Figure 54). The reaction rate catalyzed by 5 mg of free PLD was comparable to the reaction catalyzed by 100 mg of salt-activated PLD. However, in consideration of the actual content of PLD in lyophilized powder, 100 mg salt-activated powder contained only 0.4-0.5 mg PLD (Table 31). That means salt-activated PLD was about 10-fold more active than free PLD.

6.3 Effect of Cation Exchange Resin

Different cation exchange resins were added to the reaction medium to collect choline produced during transphosphatidylation of DLPC and ethanolamine in anhydrous organic solvent. No obvious difference between all treatments using different types (Figure 55) as well as amounts (Figure 56) of cation exchange resin on transphosphatidylation reaction in anhydrous chloroform was found.

6.4 Effect of Ethanolamine Concentration

Transphosphatidylation of DLPC and ethanolamine in anhydrous solvent at 40°C was slightly increased with increasing ethanolamine concentration from 0.6 g to 0.9 g (Figure 57).

6.5 Effect of Temperatures

Transphosphatidylation of DLPC and ethanolamine in anhydrous organic solvent was greatly affected by the reaction temperature. It was found that the yield of PE was dramatically increased with increasing reaction temperature from 30°C to 60°C (Figure 58). The transphosphatidylation reaction carried out in anhydrous system at 60°C was completed in 12 h.

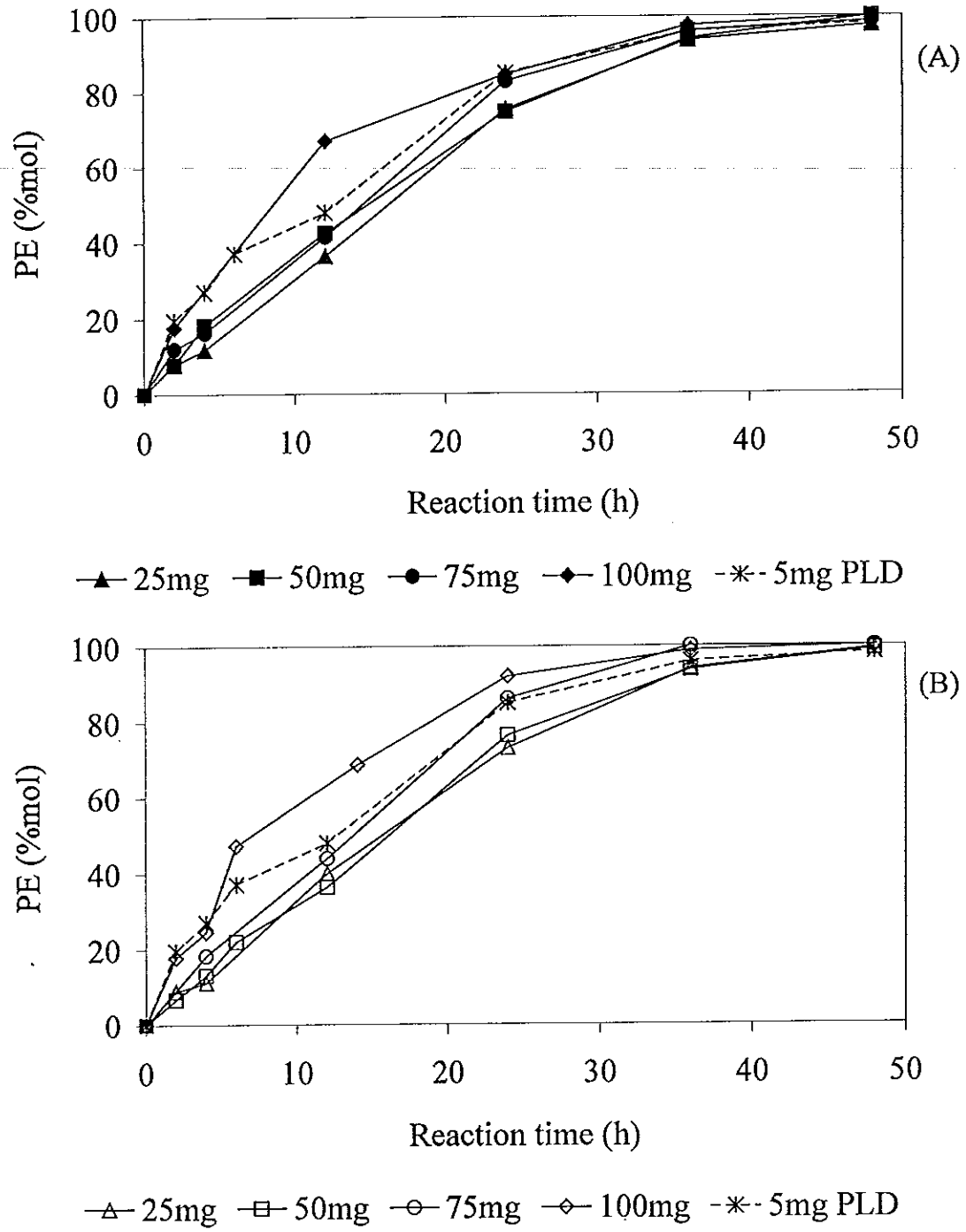


Figure 54 Effect of amount of salt-activated phospholipase D (PLD-KCl) on transphosphatidylation of *sn*1,2-dilauroyl-*sn*3-phosphatidylcholine and ethanolamine in anhydrous chloroform at 40°C.
(Phospholipase D was lyophilized in 0.1 M sodium acetate buffer containing 0.1 M $CaCl_2$ and KCl (A) or 0.2 M sodium acetate buffer containing 0.08 M $CaCl_2$ and KCl (B)).

Table 31 Actual amount of phospholipase D in salt-activated phospholipase D powder.

PLD-KCl weight (mg)	Actual PLD weight (mg)	
	0.1 M buffer	0.2 M buffer
25	0.17	0.14
50	0.34	0.28
75	0.51	0.42
100	0.68	0.56

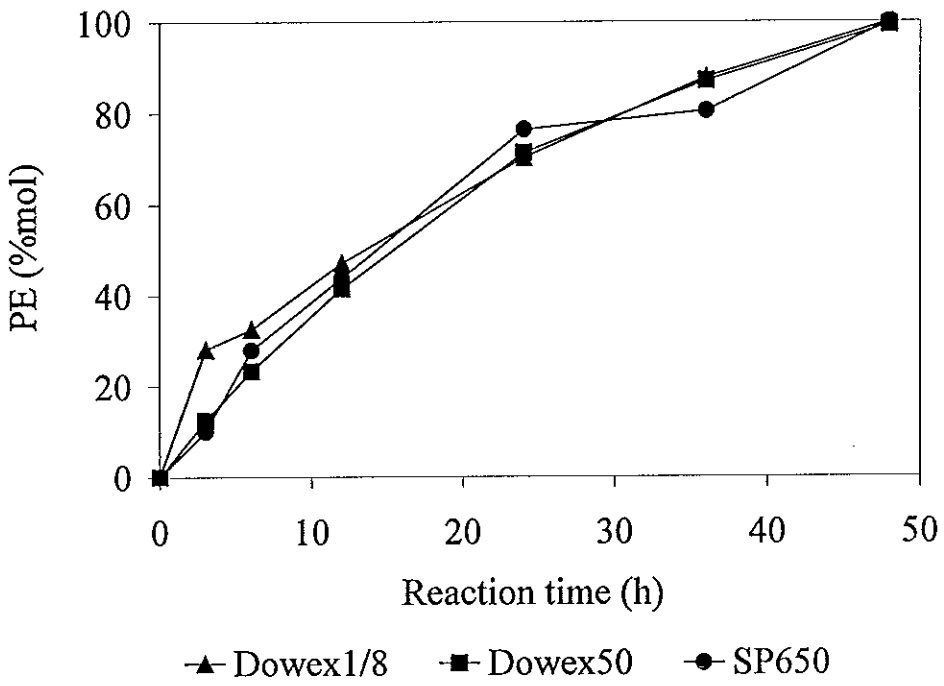


Figure 55 Effect of types of cation exchange resin on transphosphatidylation of *sn*1,2-dilauroyl-*sn*3-phosphatidylcholine and ethanolamine catalyzed by salt-activated phospholipase D in anhydrous chloroform at 40°C.

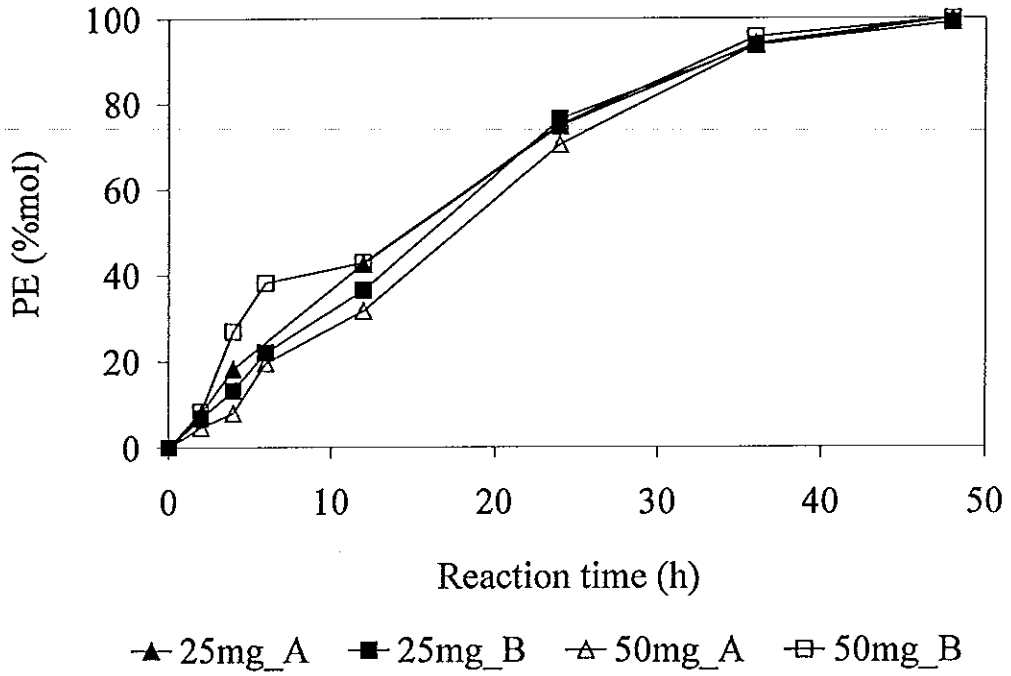


Figure 56 Effect of amount of cation exchange resin (Dowex 1x8) on transphosphatidylation of *sn*1,2-dilauroyl-*sn*3-phosphatidylcholine and ethanolamine catalyzed by salt-activated phospholipase D in anhydrous chloroform at 40°C.

(Phospholipase D was lyophilized in 0.1 M sodium acetate buffer (A) or 0.2 M sodium acetate buffer (B)).

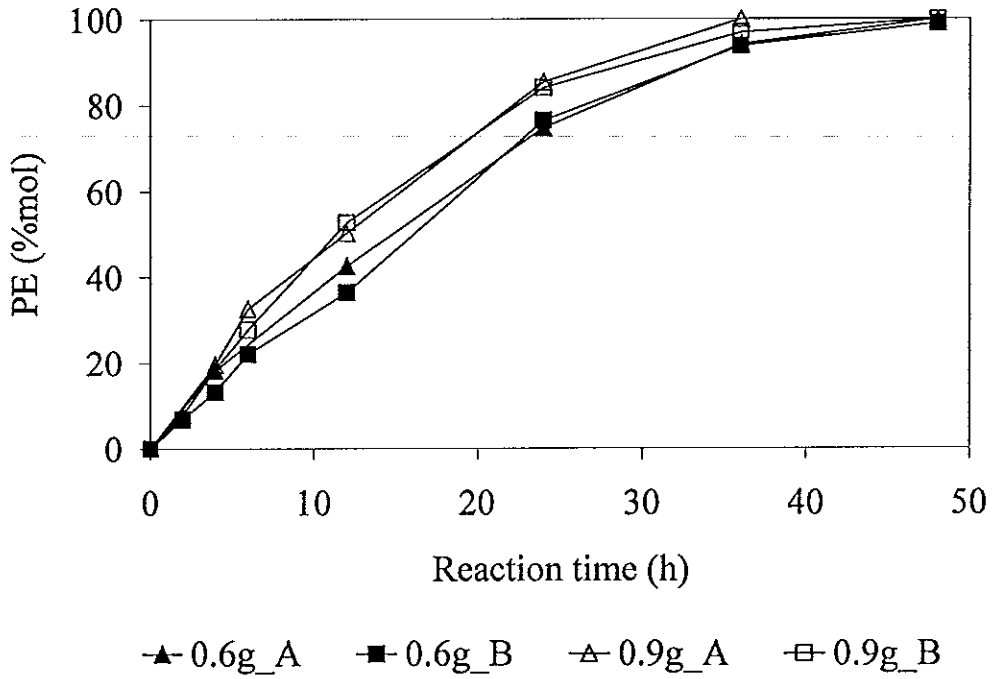


Figure 57 Effect of ethanolamine concentration on transphosphatidylation of *sn*1,2-dilauroyl-*sn*3-phosphatidylcholine catalyzed by salt-activated phospholipase D in anhydrous chloroform at 40°C.
(Phospholipase D was lyophilized in 0.1 M sodium acetate buffer (A) or 0.2 M sodium acetate buffer (B)).

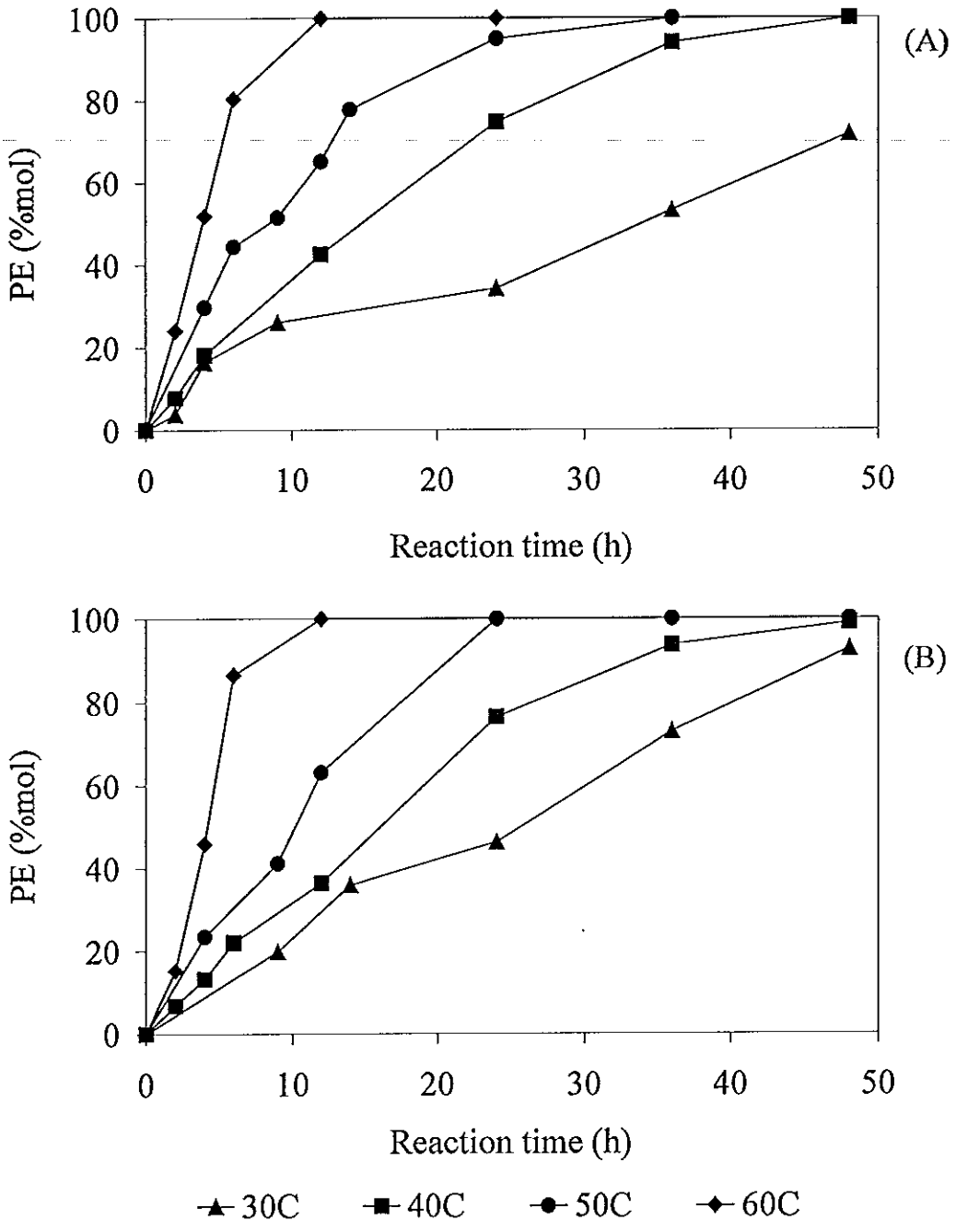


Figure 58 Effect of reaction temperature on transphosphatidylation of *sn*1,2-dilauroyl-*sn*3-phosphatidylcholine and ethanolamine catalyzed by salt-activated phospholipase D in anhydrous chloroform.
(Phospholipase D was lyophilized in 0.1 M sodium acetate buffer (A) or 0.2 M sodium acetate buffer (B)).

6.6 Effect of Buffer Concentration

Transphosphatidylation of DLPC and ethanolamine in anhydrous chloroform using PLD-KCl preparations from 0.2 M buffer showed slightly higher activity at low temperature than PLD-KCl preparation from 0.1 M buffer (Figure 58). No difference between the PLD-KCl preparations was obviously observed in other treatments or factors studied. However, by re-calculation the actual amount of PLD/mg dry powder of PLD-KCl from the total weight of dry powder (Table 31), it was found that the actual amount of PLD/mg dry powder lyophilized from 0.1 M sodium acetate buffer was more than the powder lyophilized from 0.2 M sodium acetate buffer. Therefore, it is inferred that PLD-KCl lyophilized from 0.2 M buffer gave higher activity than PLD-KCl lyophilized from 0.1 M buffer.

CHAPTER 4

CONCLUSION

In the present work, the synthesis of MLM-type STs based on palm and tuna oils using different enzymatic approaches have been investigated. Water activity, choice of solvents, substrate ratio, and reaction temperature were strongly effected the synthetic reactions. These factors were optimized to achieve the highest productivity of desired ST products and at the same time suppress the undesired side-reaction.

A screening for hydrolysis activity of the commercially available immobilized lipases were studied before their use in organic media. However, there was no relationship between the hydrolytic activity and the synthetic activity of enzymes.

An one-step process was performed by direct acidolysis of palm and tuna oils with caprylic and lauric acid in organic solvent. The method was simple but resulted in low yield of ST. Moreover, a mixture of TAGs in the final reaction mixture led to difficulties in purification of the desired product.

In a two-step process, the reaction was started from alcoholysis of palm or tuna oils, yielding 2-MAGs, which were subsequently esterified with MCFA. Although this process seemed to be more complicated in comparison to acidolysis reaction, a higher yield and purity of the desired ST was rewarded. The optimum conditions for alcoholysis of palm oil, were using 1,3-regiospecific lipase from *Rhizopus delemar* in MTBE for <12 h, at a_W 0.43, 40°C. In contrast, non-regiospecific lipases from *Pseudomonas* sp. (PS-C) and *Candida antarctica* B (CAL-B) were better biocatalysts for alcoholysis of tuna oil, for which the optimum reactions were carried out in acetone at a_W 0.43, 40°C for 12 h, yielding 81% 2-MAG. Despite these 2-MAGs were enriched in

PUFA, they were not crystallized in cold organic solvent and difficult to separate from the reaction mixture due to the low stability.

A novel two-step process was developed in this work to overcome the drawbacks of the 2-MAGs production. The process started from the production of 1,3-DAGs containing MCFA, which were esterified at the *sn*2-position with LCFA in the second step. The advantage of this novel two-step process is the high stability and ease of purification of 1,3-DAG. Furthermore, there was flexibility for the choices of starting raw material, i.e. FAVEs, FFAs or MCTs, since 1,3-DAG could be synthesized by several different methods, which have been well defined. Among these, the esterification of glycerol with fatty acid vinylesters in a solvent-free system gave the highest yield of 1,3-DAG and the fastest reaction. Non-regiospecific lipase, CAL-B, efficiently catalyzed the synthesis of 1,3-DAG. Key to a successful esterification step was the choice of a lipase with appropriate FA specificity, i.e. which does not act on the FAs already present in the 1,3-DAGs, but at the same time exhibits high selectivity and activity towards the fatty acid to be introduced. Immobilized lipase from *Pseudomonas cepacia* (PS-D) exhibited high potential on this task. However, some amount of MCFAs was still present at the *sn*2-position.

The overall conclusion and comparison of the three different enzymatic processes for the synthesis of ST is shown in Table 32.

The chemical synthesis of 1,2-diacyl-PLs gave very low yield of PL and it was complicated to recover the product. Transphosphatidylation of the natural PL should be a good alternative method for the production of PL with different head group. The reaction could also performed in anhydrous organic solvent. With the help of a salt-activation technique and addition of cation exchange resin, a 100% conversion of phosphatidylcholine to phosphatidylethanolamine was achieved within 24 h.

Table 32 Synthesis of structured triglycerides by different enzymatic processes.

Process	Optimized conditions	Reaction time (h)	Yield (%)	Content of MCFA in ST (%Cy / %La)
1. One-step				
(Acidolysis)				
palm	Lipozyme TL IM, a_w0.11, n-hexane, oil:MCFA = 1:3, 40/60°C	48	n.d.	34.1/43.5
tuna	CAL-B, a_w 0.11, acetone, oil:MCFA = 1:3, 40°C	72	n.d.	31.6/45.2
2. Two-step				
2.1 Alcoholysis				
palm	D-EP100, a_w 0.43, MTBE, 40°C	< 12	~ 60.0	
tuna	PS-C, a_w 0.43, acetone, 40°C	12	81.3	
2.2 Esterification				
palm	D-EP100, n-hexane, 40°C 2-MAG:MCFA = 1:3	6	88.0/82.9	71.6/79.4
tuna	CAL-B, acetone, 40°C 2-MAG:MCFA = 1:3	24	92.4/87.2	47.2/54.4
3. New two- step				
3.1 Synthesis of 1,3-DAG	CAL-B, room temperature, solvent-free, glycerol:FAVE = 1:2,	4/6	96.2/79.1	
3.2 Esterification	PS-D, n-hexane, 60°C 1,3-DAG:OAVE = 1:2.5	3/8	87.0/78.0	61.0/63.3

n.d. = not done; all ratios are molar ratio

Suggestions

From this work on the synthesis of structured triglycerides from natural oils such as palm and tuna oils using immobilized lipases, the suggestions for further studies are:

1. How to promote a high yield of structured triglycerides from the reaction carried out in a solvent-free system, which is safer for the food-related products.
2. Optimization of the novel two-step process, emphasizing on the choices of enzyme and types of fatty acid to be incorporated.
3. Transphosphatidylation of phosphatidylcholine with other head groups in anhydrous solvent system.

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APPENDIX

Appendix A

1. Determination of water activity

Water activity will be, by definition, equal in all phases at equilibrium. It is defined in any mixture as the ratio of the saturated vapor pressure of water present (p_w) to that of pure water (p^o_w) at the same temperature (see equation below).

$$a_w = p_w / p^o_w$$

In this way, water activity is replaced by the equilibrium relative humidity. The fixed water activity of the reaction medium was determined by pre-equilibation of the reaction mixtures using saturated salt solution.

To adjust the water activity, the immobilized enzymes or substrate solutions were equilibrated over saturated salt solutions in close vessels at room temperature for 48 hours. Salt solution with different water activity was used for this purpose (Table 31).

Table 33 Water activity at equilibrium of some saturated salt solutions (20°C).

Salt solution	Water activity (a_W)
LiCl	0.11
$KC_2H_3O_2$	0.23
$MgCl_2$	0.33
K_2CO_3	0.43
$Mg(NO)_3$	0.53
NaCl	0.75
K_2SO_4	0.97

From: Ono, *et al.* (1997); Svensson, *et al.* (1992).

2. Purification of tuna oil

Tuna oil was partially purified using the method of Rungsilp (1998) as follow:

2.1 Degumming

Crude tuna oil heated to 40°C

↓

Add citric acid 0.15% (w/w of crude oil)

↓

Mix (200 rpm, 40°C, 5 min)

↓

Add distilled water 2% (v/w of crude oil)

↓

Mix (200 rpm, 40°C, 20 min)

↓

Centrifuge (4,500 rpm, 30°C, 15 min)

↓

Collect oil layer (degummed oil)

2.2 Neutralization

Degummed oil

↓

Add 11.06% (w/v) NaOH solution (0.5%, v/w of degummed oil)

↓

Mix (200 rpm, room temperature, 5 min)

↓

Stop mixing and heat up to 70°C immediately

↓

Cooling to room temperature

↓

Centrifuge (4,500 rpm, 30°C, 15 min)

↓

Collect oil layer

↓

Add 28.72% NaOH solution (3%, v/w of degummed oil)

↓

Mix (200 rpm, room temperature, 5 min)

↓

Stop mixing and heat up to 70°C immediately

↓

Cooling to room temperature

↓

Add NaCl (2%, w/w of degummed oil)

↓

Mix (200 rpm, room temperature, 5 min)

↓

Centrifuge (4,500 rpm, 30°C, 15 min)

↓

Collect oil layer (Neutralized oil)

2.3 Bleaching

Neutralized oil in bleaching apparatus

↓

Heat up to 80°C

↓

Add activated earth (5%, w/w of neutralized oil)

↓

Bleaching (200 rpm, 90-95°C, 27 in Hg, 15 min)

↓

Cooling to 60°C at normal pressure

↓

Filtration (Whatman no.5)

↓

Bleached oil

2.4 Fractionation

Bleach oil + n-hexane (1:1, w/v)

↓

Fractionation (0°C, 24 h)

↓

Filtration (Whatman no.5)

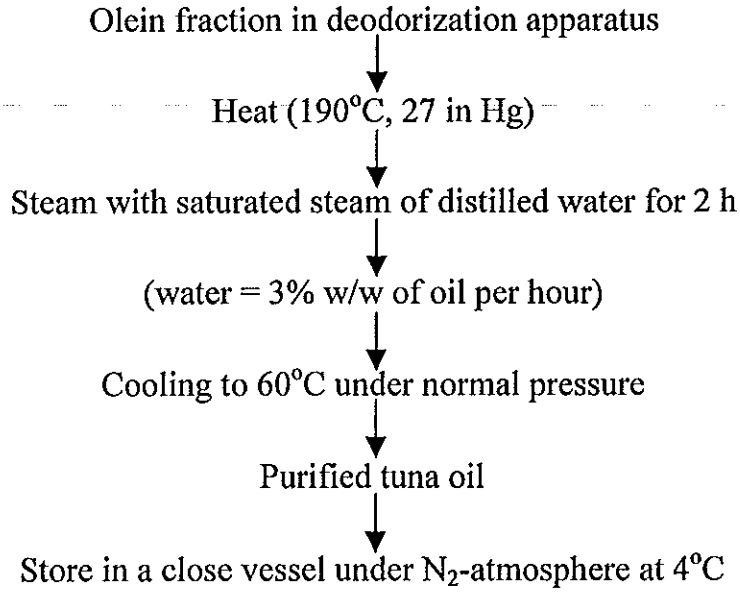
↓

Collect liquid oil (olein fraction)

↓

Evaporation of n-hexane

2.5 Deodorization



Appendix B

Analytical Method

1. The Folin-Lowry Method (Lowry *et al.*, 1951)

Protein content of enzymes were determined by Folin-Lowry method.

The copper reagent is prepared by adding 1.0 mL of 0.5 % copper sulfate pentahydrate and 1% sodium-potassium tartrate to 50 mL of 0.1 M sodium hydroxide, containing 2 % sodium carbonate. The protein solution (0.5 mL) is added to 5.0 mL of the alkaline copper reagent and the solution allowed to stand for 10 minutes. Then 0.50 mL of the Folin-Ciocalteu phenol reagent (which had been diluted 1:1 with water) is rapidly added and mixed. This solution is allowed to stand for 30 minutes, and the absorbance is measured at 750 nm. A standard curve is prepared using the protein under study or some other protein, such as serum albumin at concentrations of 20-400 μg/mL.

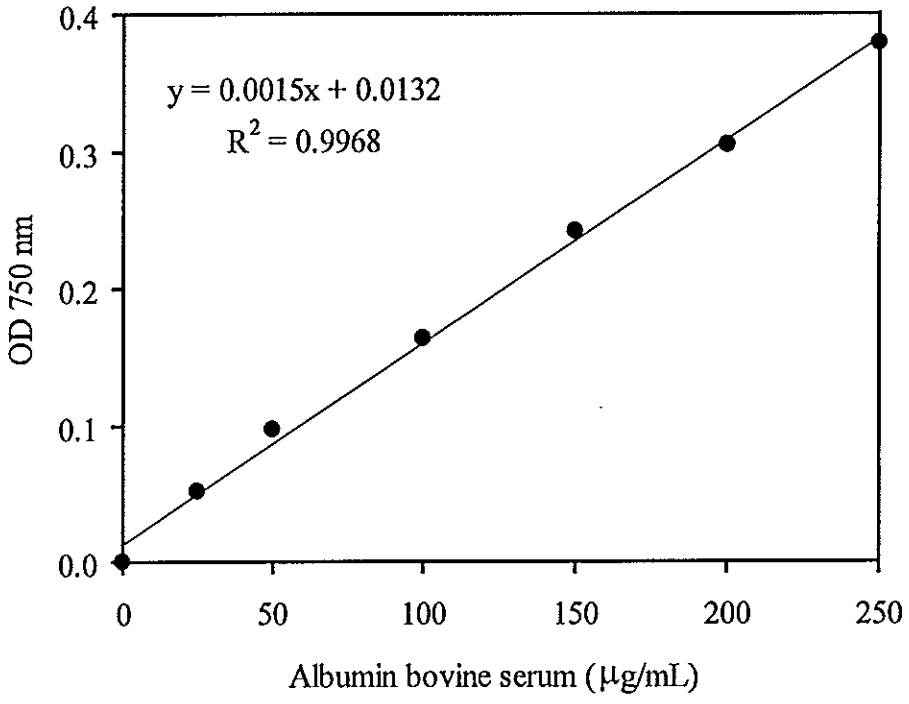


Figure 59 Standard curve of albumin bovine serum.

2. Hydrolytic activity of lipase (Lee and Rhee, 1993)

Hydrolytic activity of lipase was also assayed by the modified cupric acetate method. At first, 5 % (w/v) aqueous solution of cupric acetate was prepared and filtered, the pH being adjusted to 6.1 using pyridine. For the lipase reaction in two-phase system, 0.2 ml of enzyme solution (2.0 mg for immobilized enzyme), 1.0 ml of 0.1 M phosphate buffer (pH 7.0), and 1.5 mL of 10 % palm olein in isooctane was incubated at 500 rpm and 30°C for 30 min. The enzyme reaction was stopped by adding 0.3 ml of 6 M HCl.

The upper isooctane layer (1 ml) was taken out and mixed with cupric acetate solution (0.4 ml). Free fatty acids dissolved in isooctane were determined by measuring the absorbance of isooctane solution at 715 nm against the control, which contained no free fatty acid. Lipase activity was determined by measuring the amount of fatty acids from the standard curves of palmitic acid. One unit of enzyme activity was defined as the enzyme necessary to release 1 μmol of palmitic acid per minute at the specified condition.

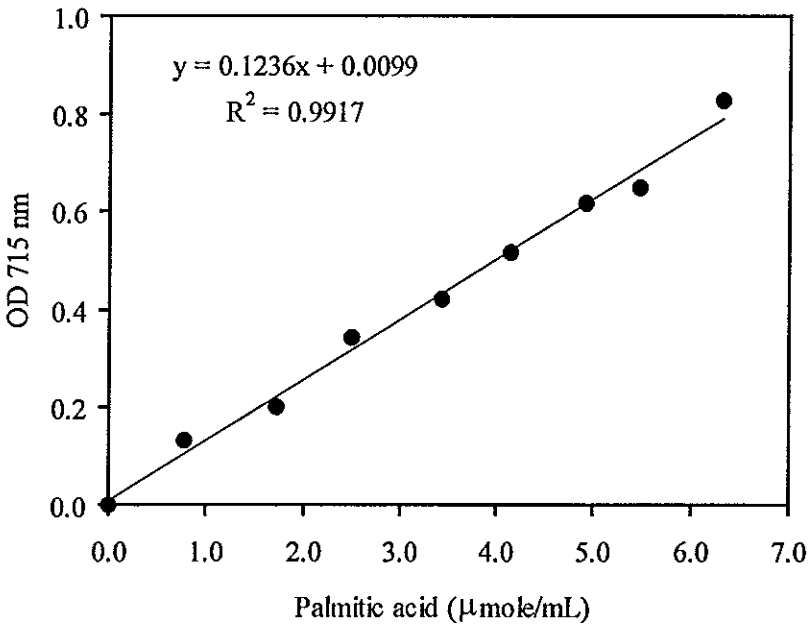
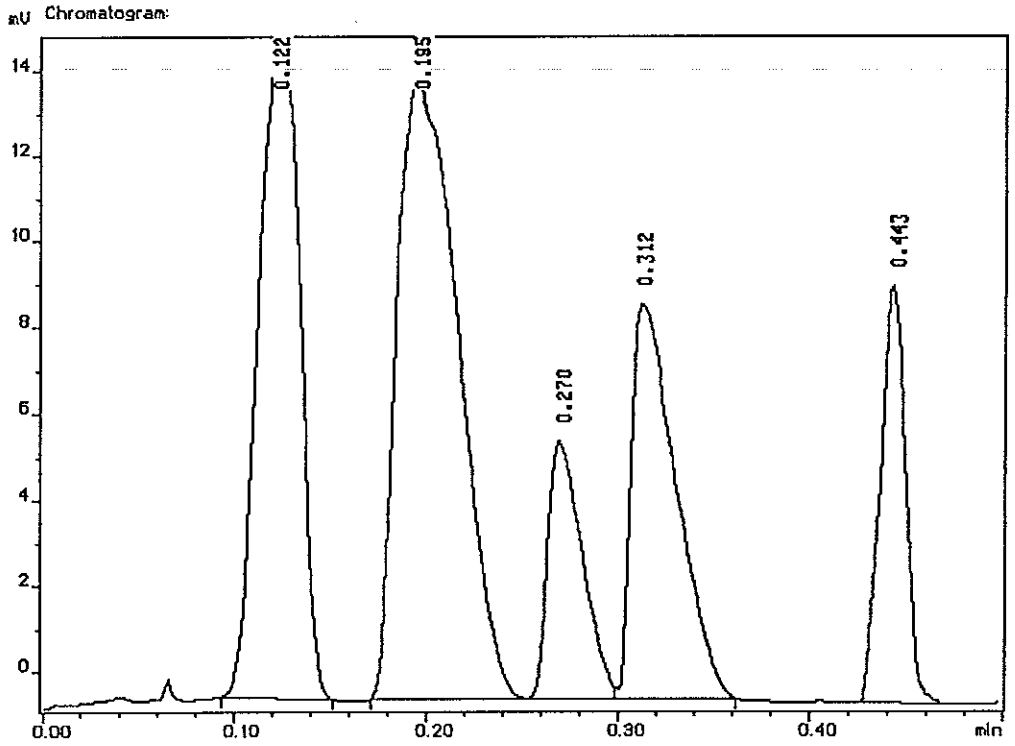


Figure 60 Standard curve of palmitic acid.

3. Determination of oil composition by TLC-FID analyzer



Peak No	Name	Ret. Time (min)	Pk. Start (min)	Pk. End (min)	Area	Height (mV)	Area %
1	Triolein	0.123	0.093	0.152	12059	15.26	26.731
2	Oleic acid	0.195	0.172	0.248	16658	14.41	36.923
3	1,3-Diolein	0.270	0.248	0.298	3913	5.99	8.674
4	1,2-Dioleoyl-rac-glycerol	0.313	0.298	0.362	7831	9.07	17.358
5	1-Monopalmitin	0.443	0.427	0.472	4654	9.67	10.315
					45115	54.39	100.000

Condition:

Mobile phase : benzene : chloroform : acetic acid (50:20:0.7)

Figure 61 TLC-FID chromatogram of standard oil compositions.

4. Determination of fatty acid composition by GC analysis

Table 34 Retention time of standard fatty acid methyl esters.

Fatty acid	Retention time (min)
Caprylic acid (C8:0)	1.63-1.68
Caproic acid (C10:0)	2.50-2.53
Lauric acid (C12:0)	4.33-4.36
Myristic acid (C14:0)	7.10-7.17
Palmitoleic acid (C16:1)	9.50-9.51
Palmitic acid (C16:0)	9.75-9.76
Linolenic acid (C18:3)	11.97-11.98
Linoleic acid (C18:2)	12.05-12.06
Oleic acid (C18:1)	12.15-12.18
Stearic acid (C18:0)	12.56-12.57
EPA (C20:5)	14.99-15.00
DHA (C22:6)	21.90-21.91
Behenic acid (C20:0)	22.30

Column	OPTIMA-5 (25 m x 0.25 mm i.d.)
Condition	T_1 = 150°C (4°C/min, 0.50 min)
	T_2 = 170°C (10°C/min)
	T_3 = 195°C (10°C/min)
	T_4 = 215°C (15 min)
	Injection temperature 250°C
Detection	FID (250°C)
Carrier gas	Helium (1.24 ml/min)

Vitae

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The Thailand Research Fund (BGJ/16/2544)

Presentations

Wongsakul, S., Bornscheuer, U.T. and Hanpongkittikun, A. 2001. Production of 2-Monoglycerides by Alcoholysis of Palm Oil and Tuna Oil Using Immobilized Lipases. RGJ-Congress II. 20-22 April 2001. Pattaya, Thailand.

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