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รายงานวิจัยฉบับสมบูรณ์

โครงการ "การสังเคราะห์ฟอสโฟลิปิดดัดแปลงจากน้ำมันปาล์ม และน้ำมันปลาโดยเอนไซม์ไลเปสตรึงรูปและฟอสโฟไลเปส"

Synthesis of Modified Phospholipids from Palm Oil and Tuna Oil by Immobilized Lipases and Phospholipases

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กรกฎาคม 2548

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บทคัดย่อ

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ชื่อโครงการ การสังเคราะห์ฟอสโฟลิปิดดัดแปลงจากน้ำมันปาล์มและน้ำมันปลาโดยเอนไซม์ไลเปสตรึงรูปและฟอสโฟไลเปส

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ระยะเวลาโครงการ 2 ปี

การสังเคราะห์ 1,2-ไดเอซิลฟอสโฟลิปิดจากกรดไขมันและกลีเซอโรฟอสโฟลิปิดหลายชนิด ด้วยวิธีทางเคมี พบว่ามีเพียง 1,2-ไดลอริล-3-ฟอสโฟโคลิน (DLPC) เท่านั้นที่สามารถสังเคราะห์ได้ (ร้อยละ 82.3) จากนั้นจึงทำการศึกษาการดัดแปลงฟอสโฟลิปิดโดยวิธีทางเอนไซม์ 2 วิธี คือ (1) การทำปฏิกิริยาทรานฟอสฟาดิดิลเลชั่น ระหว่าง DLPC กับเอทาโนลามีน ในระบบสองเฟสและในตัวทำละลายอินทรีย์ที่ปราศจากน้ำ โดยเอนไซม์ฟอสโฟไลเปสดี และ (2) การเติมกรดโอเลอิกที่ตำแหน่งที่ 1 ของ DLPC ในตัวทำละลายอินทรีย์ ด้วยเอนไซม์ไลเปสตรึงรูปชนิดต่าง ๆ ที่มีการควบคุมค่ากิจกรรมของน้ำ การศึกษาการเปลี่ยนหมู่แอลกอฮอล์ระหว่าง DLPC กับเอทาโนลามีนโดยใช้เอนไซม์ฟอสโฟไลเปสดีจาก *E. coli* ที่มีการแสดงออกของยีนจาก *Streptomyces antibioticus* โดยเปรียบเทียบระหว่างฟอสโฟไลเปสดีอิสระกับฟอสโฟไลเปสดีที่กระตุ้นด้วยเทคนิค salt-activation โดยเกลือโพแทสเซียมคลอไรด์ พบว่าเอนไซม์ที่ถูกกระตุ้นด้วยเทคนิค salt-activation มีค่ากิจกรรมเพิ่มขึ้น 10 ถึง 12 เท่า เมื่อเทียบเป็นปริมาณโปรตีนที่ใช้ จากการทำปฏิกิริยาในตัวทำละลายอินทรีย์ที่ปราศจากน้ำ พบว่า DLPC เปลี่ยนไปเป็น 1,2-ไดลอริล-3-ฟอสโฟเอทาโนลามีนได้ภายในเวลา 12 ชั่วโมง เมื่อทำปฏิกิริยาที่ 60 องศาเซลเซียส อุณหภูมิมีผลกระทบต่อการทำปฏิกิริยาอย่างมาก มากกว่าผลของปริมาณเอนไซม์ ตัวแลกเปลี่ยนประจุบวกเรซิน และความเข้มข้นของเอทาโนลามีนที่ใช้ ส่วนการทำปฏิกิริยาอะซิโดไลซิสระหว่าง DLPC กับกรดโอเลอิก พบว่าเอนไซม์ตรึงรูปจาก *Thermomyces lanuginosa* (Lipozyme TL IM) ทำปฏิกิริยาและเติมกรดโอเลอิกได้สูงที่สุด เมื่อทำปฏิกิริยาในเฮกเซน ที่ 40 องศาเซลเซียส ค่ากิจกรรมของน้ำ (a_W) 0.11 และใช้กรดโอเลอิกมากเกินพอ 8 เท่า

คำหลัก ฟอสโฟลิปิด, เอนไซม์ไลเปสตรึงรูป, ฟอสโฟไลเปสดี, salt-activation, ทรานฟอสฟาดิดิลเลชั่น

Abstract

Project Code : BGJ4480016

Project Title : Synthesis of Modified Phospholipids from Palm Oil and Tuna Oil by Immobilized Lipases and Phospholipases

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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-phosphocholine (DLPC) (82.3%). Next, two approaches on enzymatic phospholipid modification were studied: (1) transphosphatidylation of the 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC) and ethanolamine in biphasic and anhydrous organic solvent systems by phospholipase D (PLD) and (2) incorporation of oleic acid into the *sn*1-position of DLPC in organic solvents with different immobilized lipases at controlled water activity. PLD-catalyzed head group exchange of DLPC with ethanolamine was studied using an enzyme from *Streptomyces antibioticus* expressed recombinantly in *E. coli*. A comparison of the free PLD with the biocatalyst activated by a salt-activation technique using KCl showed that the salt-activated enzyme (PLD-KCl) was 10-12 folds more active based on the amount of protein used. Thus, DLPC was quantitatively converted to 1,2-dilauroyl-*sn*-glycero-3-phosphoethanolamine in an anhydrous solvent system within 12 h at 60°C. The reaction was strongly effected by temperature rather than the amount of enzyme, cation exchange resin and concentration of ethanolamine. For the acidolysis of DLPC with oleic acid, Lipozyme TL IM showed the highest activity and incorporation of oleic acid. A quantitative incorporation was achieved at 40°C using a 8-fold molar excess of oleic acid in *n*-hexane at a water activity of 0.11.

Keywords : phosphocholine, immobilized lipases, phospholipase D, salt-activation, transphosphatidylation

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Sirirung Wongsakul

Aran H-Kittikun

Content

บทคัดย่อ (1)

Abstract (2)

Acknowledgment (3)

Introduction 1

1. Phospholipids 1
    - 1.1 Properties of phospholipids 1
    - 1.2 Applications of phospholipids 1
    - 1.3 Modification of phospholipids 3
    - 1.4 Modification of phospholipid head groups] 5
    - 1.5 Chemoenzymatic modification of phospholipids 7
2. Phospholipases 8
    - 2.1 Phospholipase A_1 (PLA_1) 8
    - 2.2 Phospholipase A_2 (PLA_2) 9
    - 2.3 Phospholipase C (PLC) 9
    - 2.4 Phospholipase D (PLD) 9

Objective 10

Methodology 11

Analytical methods 11

Experimental methods 12

Results and Discussion 14

1. Chemical synthesis of 1,2-diacyl-phosphocholine 14
2. Phospholipid modification by PLD-catalyzed transphosphatidylation 14
    - 2.1 Biphasic system vs anhydrous solvent system 15
    - 2.2 Effect of salt activation of the PLD 16
    - 2.3 Effect of cation exchange resin 16
    - 2.4 Effect of ethanolamine concentration 19
    - 2.5 Effect of temperature 19
    - 2.6 Effect of buffer concentration 21

Conclusion 22

Suggestion 22

Output 22

References 23

Table contents

Table		Page
1	Phospholipids commonly found in nature.	2
2	Transphosphatidylation of phospholipids by phospholipase D.	5
3	Conversion and selectivity of phospholipase D catalyzed transphosphatidylation of different phospholipids in an emulsion system.	10
4	Products from chemical synthesis of different phospholipids with different fatty acids.	14
5	Transphosphatidylation in biphasic and anhydrous solvent systems.	15
6	Actual amount of phospholipase D in salt-activated phospholipase D powder.	16

Figure Contents

Figure Page
1 Schematic of phospholipid structure 1
2 Phospholipase- and lipase- catalyzed enzymatic synthesis of modified phosphocholine and lyso-phosphocholine. 4
3 Possible sites of enzymatic hydrolysis of phospholipids. 8
4 TLC separation of phopholipids mixture. 11
5 Effect of amount of salt-activated phospholipase D (PLD-KCl) on transphosphatidylation of *sn*1,2-dilauroyl-*sn*3-phosphocholine and ethanolamine in anhydrous chloroform at 40°C. 17
6 Effect of types of cation exchange resin on transphosphatidylation of *sn*1,2-dilauroyl-*sn*3-phosphocholine and ethanolamine catalyzed by salt-activated phospholipase D in anhydrous chloroform at 40°C. 18
7 Effect of amount of cation exchange resin (Dowex 1x8) on transphosphatidylation of *sn*1,2-dilauroyl-*sn*3-phosphocholine and ethanolamine catalyzed by salt-activated phospholipase D in anhydrous chloroform at 40°C. 18
8 Effect of ethanolamine concentration on transphosphatidylation of *sn*1,2-dilauroyl-*sn*3-phosphocholine catalyzed by salt-activated phospholipase D in anhydrous chloroform at 40°C. 19
9 Effect of reaction temperature on transphosphatidylation of *sn*1,2-dilauroyl-*sn*3-phosphocholine and ethanolamine catalyzed by salt-activated phospholipase D in anhydrous chloroform. 20

Abbreviation

CDI – 1,1'-carbonyl diimdazole

DAG – diacylglycerol

DBU – 1,8-diazabicylo [5,4,0] undec -7-ene

DCC – 1,3-dicyclohexylcabodimide

DLPC – 1,2-dilauryl-*sn*-glycero-3-phosphocholine

FA – fatty acids

GPC – glycerol-3-phosphocoline

GPE – glycerol-3-phosphoethanolamine

LCFA – long chain fatty acids

PA - phosphatidic acid

PC - phosphocholine

PE - phosphoethanolamine

PG - phosphoglycerol

PLA$_1$ – phospholipas A$_1$

PLA$_2$ – Phospholisae A$_2$

PLB – phospholisae B

PLC – phospholipase C

PLD- phospholipase D

PI – phosphatidylinosital

PL – phospholipids

PS – phosphoserine

PUFA – polyunsaturated fatty acids

RPTG – isopropyl-β-D-thiogalactopyranoside

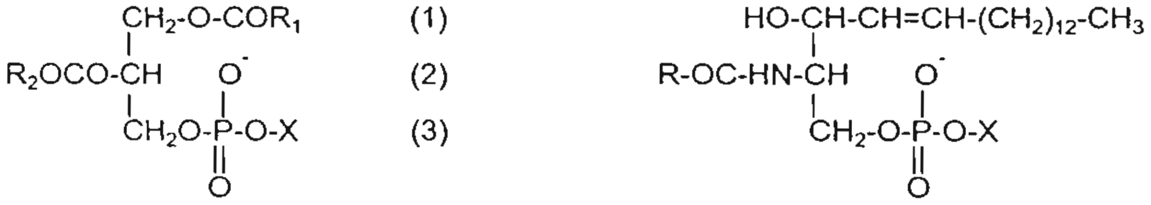
TLC-FID – thin layer chromatography with flame ionization detector

Introduction

1. Phospholipids

1.1 Properties of phospholipids

Many complex lipids found in nature contain phosphorus called phospholipids, or phosphatides, are based either on glycerol (phosphoglycerides) or on sphingosine (sphingolipids) (Chapman, 1969). Phospholipids (PL) from natural sources contain several fatty acids (FA) and their proportion depends on the source (Svensson *et al.*, 1990). PL have the general structure shown in Figure 1.



Phosphoglyceride (phosphatide)

Sphingolipids

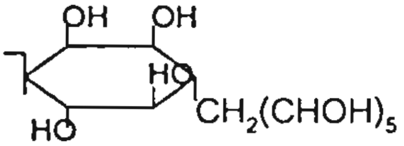
Figure 1 Schematic of phospholipid structure (R, R_1, R_2 = fatty alkyl groups, X = base, sugar, etc.).
(Ulbrich-Hofmann, 1999; Chapman, 1969).

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 long chain fatty acids (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 phosphocholine, PC) molecule (Chapman, 1969). Generally, different PL classes are distinguished based on their head group (X)(Table 1).

1.2 Applications of phospholipids

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 polyunsaturated fatty acids (PUFA) that are chemically unstable can be incorporated under mild condition (Parnham, 1996; Svenssen *et al.*, 1992).

Table 1 Phospholipids commonly found in nature.

Head group (X)	**PL**
- H	Phosphatidic acid (PA)
- $CH_2CH_2N^+(CH_3)_3$	Phosphocholine (PC)
- $CH_2CH_2N^+H_3$	Phosphoethanolamine (PE)
- $CH_2CH(OH)CH_2OH$	Phosphoglycerol (PG)
- $CH_2CH(N^+H_3)COO^-$	Phosphoserine (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 PUFA 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).

1.3 Modification of phospholipids

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 2.

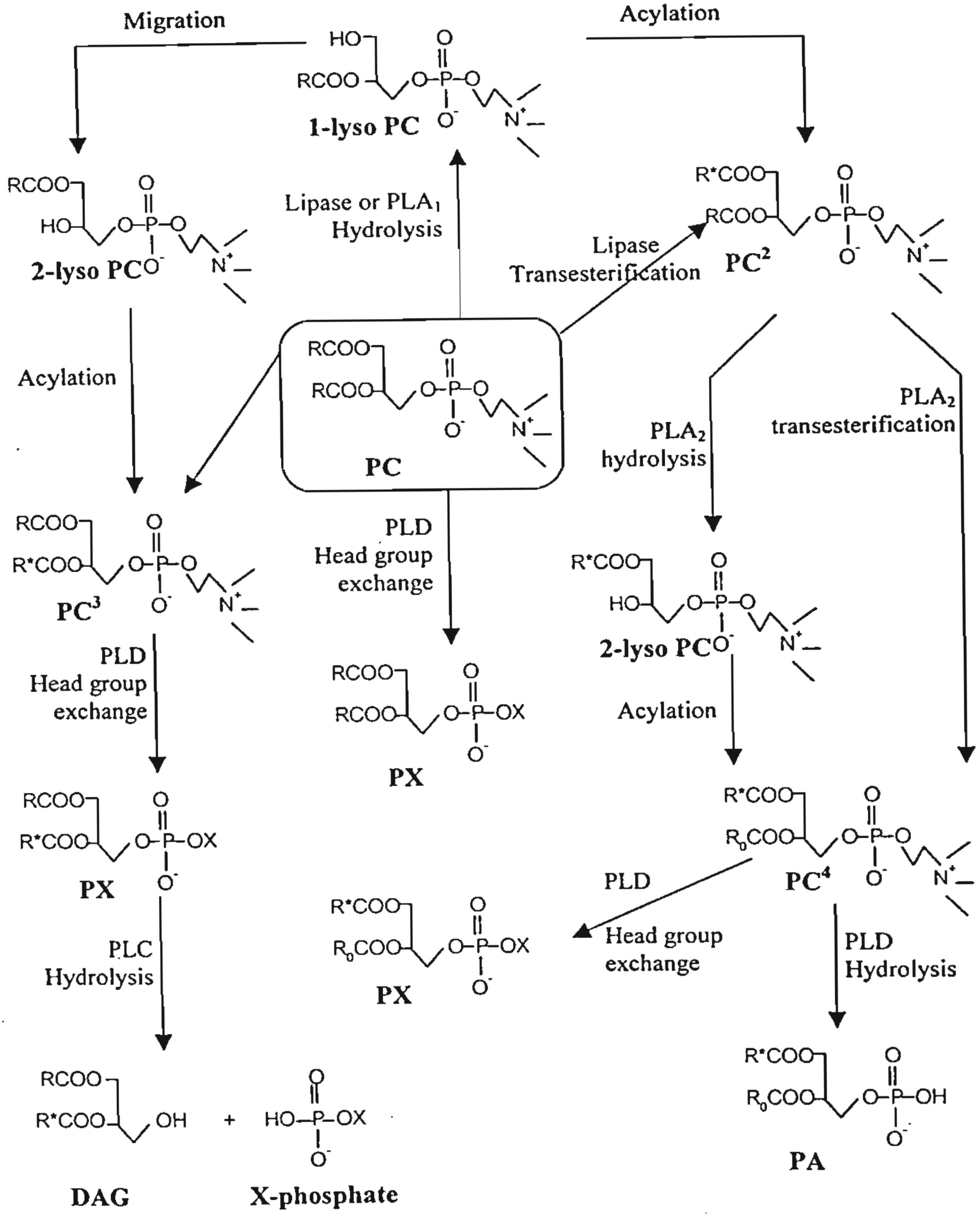


Figure 2 Phospholipase- and lipase- catalyzed enzymatic synthesis of modified phosphocholine and lyso-phosphocholine.
(Sarney and Vulfson, 1995).

1.4 Modification of phospholipid 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 2).

Table 2 Transphosphatidylation of phospholipids by phospholipase D (PLD).

Substrate	Solvent	PLD source	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

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 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 diacylglycerol (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.

1.5 Chemoenzymatic modification of phospholipids

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-diazabicyclo[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.

2. Phospholipases

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. Phospholipase B (PLB) exhibits the combination of PLA_1 and PLA_2 activity. They attack a PL as shown in Figure 3.

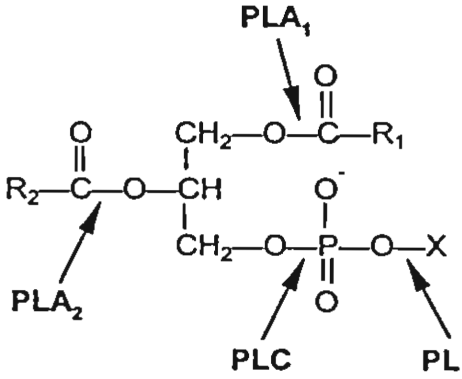


Figure 3 Possible sites of enzymatic hydrolysis of phospholipids. (Chapman, 1969).

2.1 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.

2.2 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).

2.3 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).

2.4 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 3 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 PLD solution in ethyl acetate containing 30 U/g. The data indicated that the selectivity was higher with PE and PG, both poorer substrates of the enzyme (Juneja *et al.*, 1987).

Table 3 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 five folds 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*-phosphoglycerol (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).

Objective

To synthesize new phospholipids base on palm oil and tuna oil using immobilized enzymes

Methodology

Analytical Methods

1. 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 of recombinant *Escherichia coli* containing PLD-encoding gene of *Streptomyces antibioticus* was dissolved in 1 mL sodium acetate buffer (0.1 M, pH 5.6) and 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).

2. Determination of phospholipids compositions of the reaction mixture by TLC-FID analysis

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

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, 2 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.

3. 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 4). The localization of PL was done under iodine vapor (Park *et al.*, 2000).

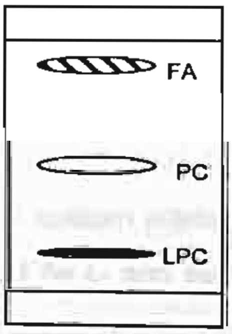


Figure 4 TLC separation of phopholipids mixture.

Experimental Methods

1. Chemical synthesis of phospholipids

PC was synthesized according to the method of Pisch *et al.* (1997). Briefly, 2.75 mmol FA and 3 mmol carbonyldiimidazol (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 glycerol-3-phophocholine (GPC) and 2.75 mmol 1,8-diazabicylo[s,4,0]undec-7-ene (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 1,2-dilauryl-*sn*-glycero-3-phosphocholine (DLPC) crystals were filtered and washed with cold acetone. DLPC thus obtained was freeze-dried and kept at 4°C until use.

2. PLD-catalyzed transphosphatidylation

2.1 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.

2.2 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 phases 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.

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

The salt-activated enzyme was prepared by lyophilization of enzyme and salt solution in 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 –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.

2.4 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.