

Results and Discussion

1. Chemical synthesis of 1,2-diacyl-phosphocholine

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 4). 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.

Table 4 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

2. Phospholipid 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.

2.1 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 5). 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.

Table 5 Transphosphatidylation in biphasic and anhydrous solvent systems.
(phospholipase D, 40°C).

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

* Phosphoethanolamine

2.2 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).

It was found that the transphosphatidylation reaction increased with increasing amount of PLD-KCl (Figure 5). 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 0.5-0.7 mg PLD (Table 6). That means salt-activated PLD was about 10-fold more active than free PLD.

2.3 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 6) as well as amounts (Figure 7) of cation exchange resin on transphosphatidylation reaction in anhydrous chloroform was found.

Table 6 Actual amount of phospholipase D in salt-activated phospholipase D (PLD) 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

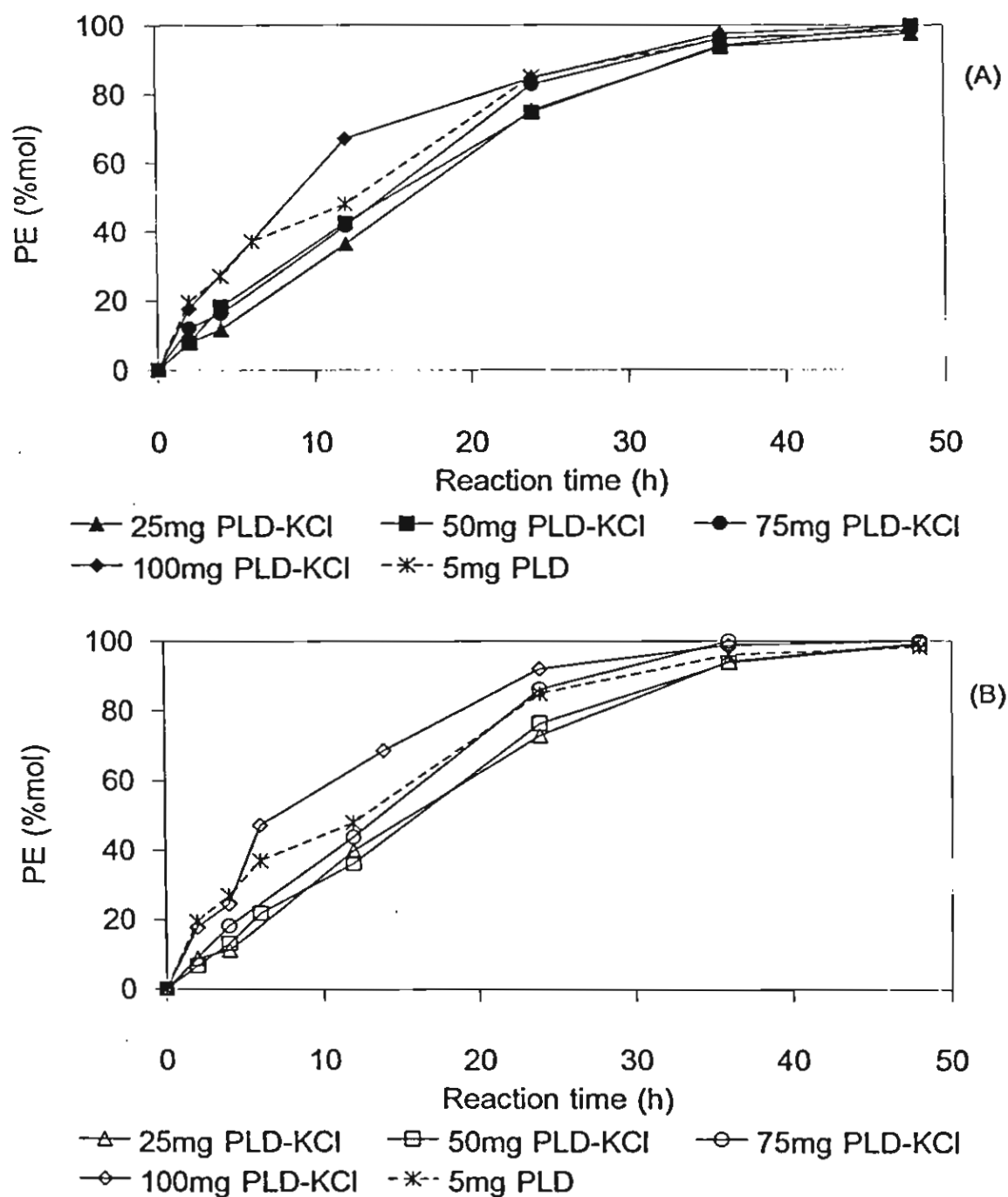


Figure 5 Effect of amount of salt-activated phospholipase D (PLD-KCI) on transphosphatidylation of *sn*1,2-dilauroyl-*sn*3-phosphatidylcholine and ethanolamine in anhydrous chloroform at 40°C.

(Phospholipase D was lyophilized in 0.1 M sodium acetate buffer containing 0.1 M CaCl₂ and KCl (A) or 0.2 M sodium acetate buffer containing 0.08 M CaCl₂ and KCl (B)).

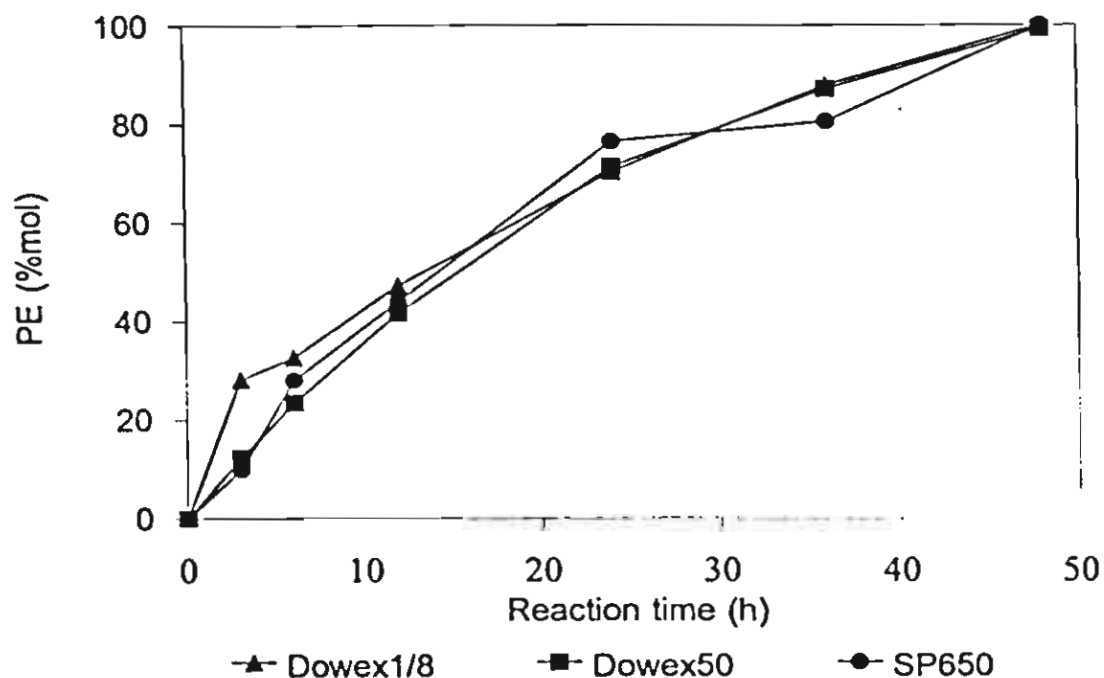


Figure 6 Effect of types of cation exchange resin on transphosphatidylation of *sn*1,2-dilauroyl-*sn*3-phosphocholine and ethanolamine catalyzed by salt-activated phospholipase D in anhydrous chloroform at 40°C.

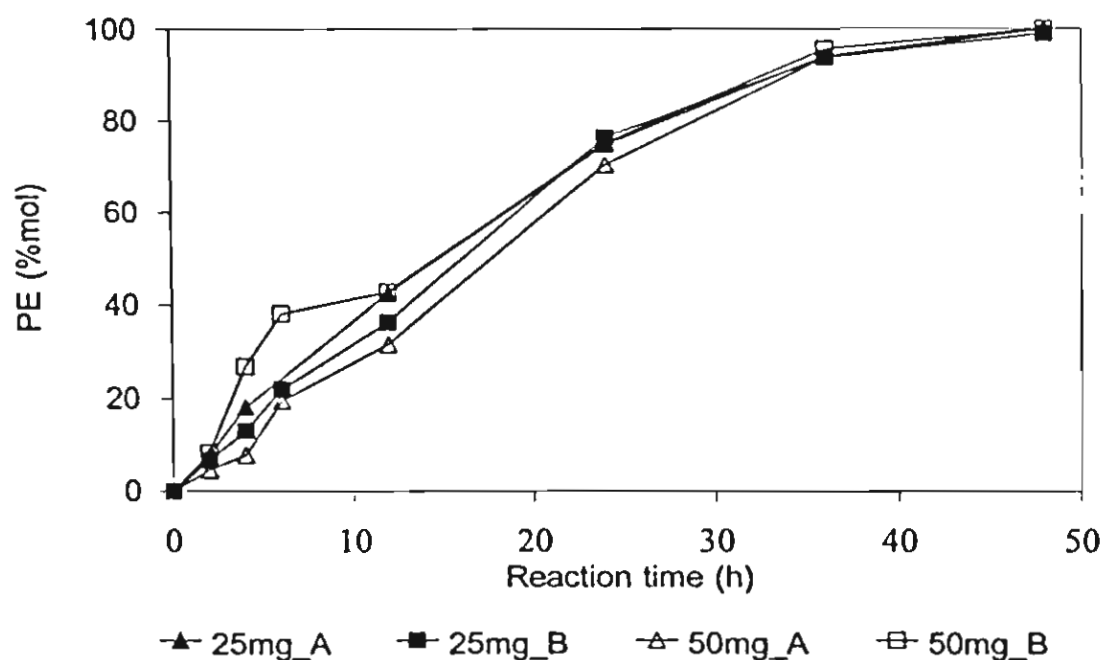


Figure 7 Effect of amount of cation exchange resin (Dowex 1x8) on transphosphatidylation of *sn*1,2-dilauroyl-*sn*3-phosphocholine and ethanolamine catalyzed by salt-activated phospholipase D in anhydrous chloroform at 40°C.
(Phospholipase D was lyophilized in 0.1 M sodium acetate buffer (A) or 0.2 M sodium acetate buffer (B)).

2.4 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 8).

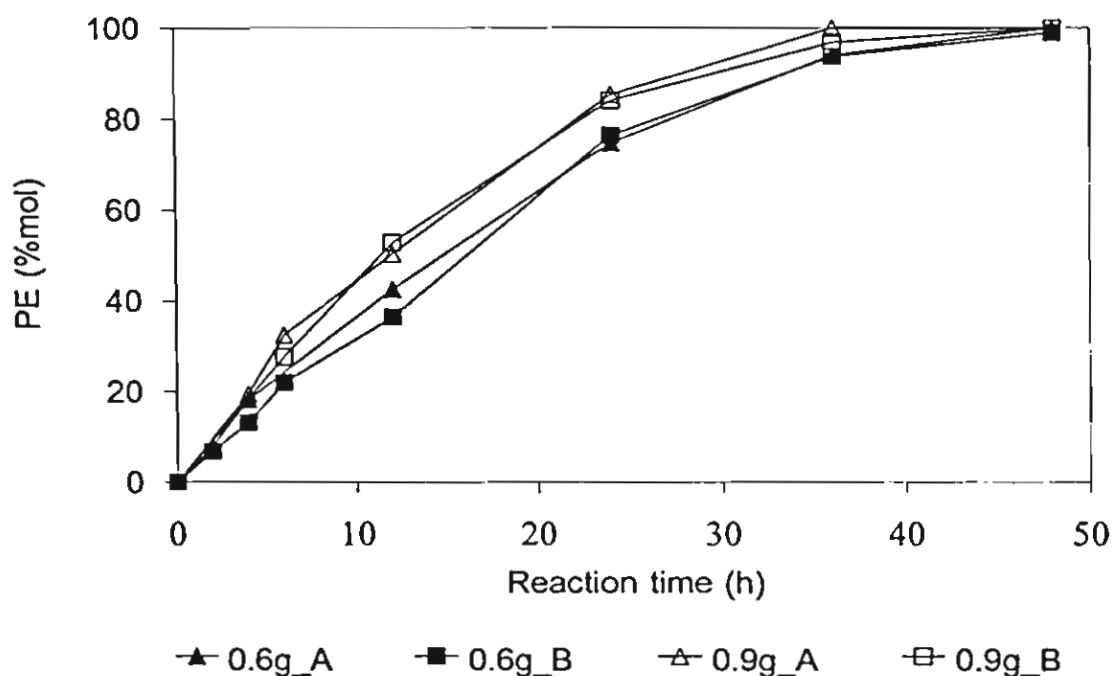


Figure 8 Effect of ethanolamine concentration on transphosphatidylation of *sn*1,2-dilauroyl-*sn*3-phosphocholine catalyzed by salt-activated phospholipase D in anhydrous chloroform at 40°C.

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

2.5 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 9). The transphosphatidylation reaction carried out in anhydrous system at 60°C was completed in 12 h.

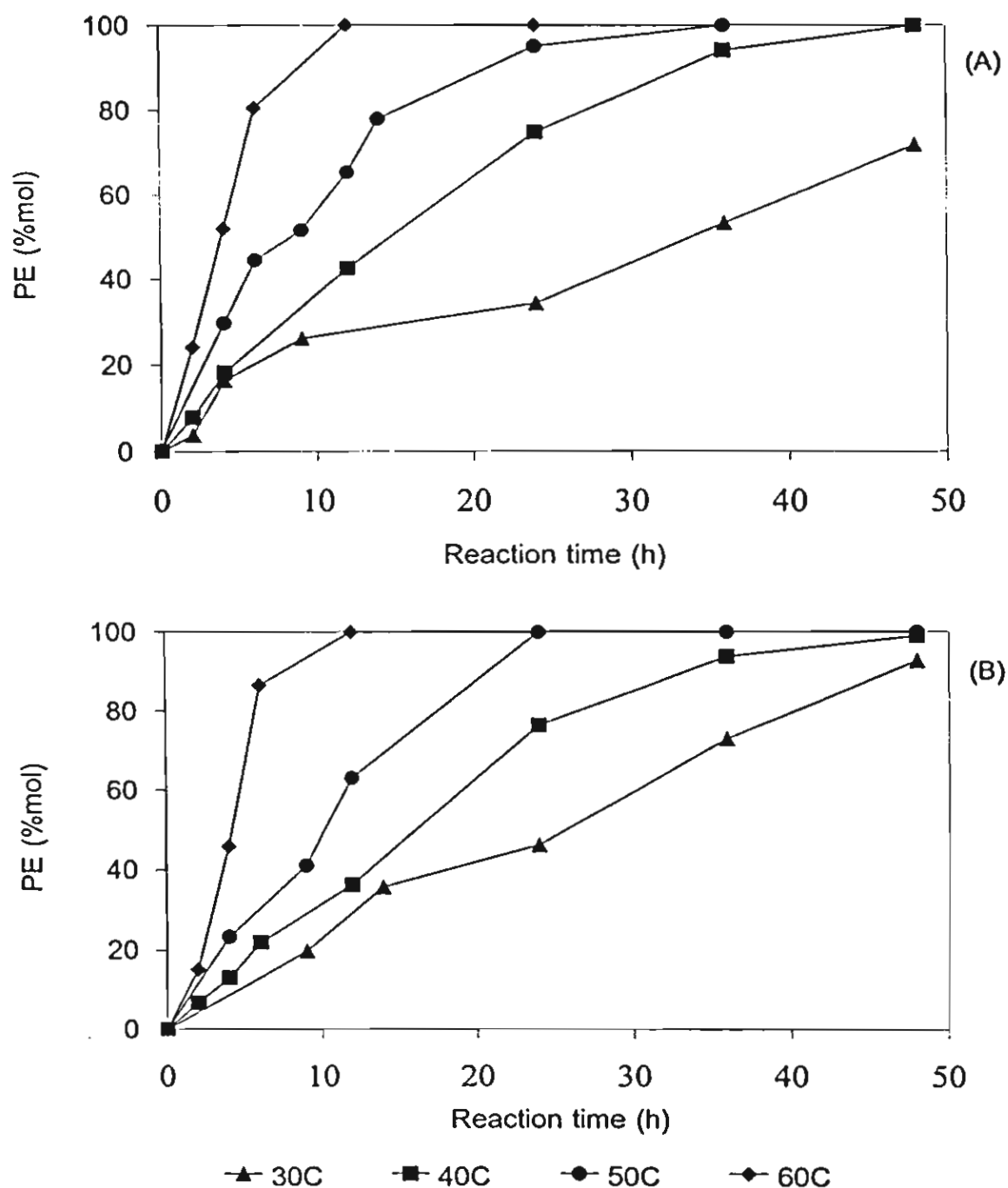


Figure 9 Effect of reaction temperature on transphosphatidylation of *sn*1,2-dilauroyl-*sn*3-phosphocholine and ethanolamine catalyzed by salt-activated phospholipase D in anhydrous chloroform. (Phospholipase D was lyophilized in 0.1 M sodium acetate buffer (A) or 0.2 M sodium acetate buffer (B)).

2.6 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 9). 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 6), 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.

Conclusion

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 perform in anhydrous organic solvent. With the help of a salt-activation technique and addition of cation exchange resin, a 100% conversion of phosphocholine to phosphoethanolamine was achieved within 24 h.

Suggestion

The suggestion for further studies is transphosphatidylation of phosphocholine with other head groups in anhydrous solvent system.

Output

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Appendix

Reprint "Lipase-catalyzed acidolysis and phospholipase D-catalyzed transphosphatidylation of phosphocholine"

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Lipase-catalyzed acidolysis and phospholipase D-catalyzed transphosphatidylation of phosphocholine

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. First, DLPC was chemically synthesized from glycerophosphocholine and lauric acid. Next, 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. For the acidolysis of DLPC with oleic acid, among the four lipases studied (CAL-B, Lipozyme TL IM, Lipozyme RM IM and lipase D immobilized on Accurel EP-100), 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: Acidolysis, lipases, phosphocholine, phospholipase D, salt-activation, transphosphatidylation.

1 Introduction

Phospholipids from natural sources contain several fatty acids and their proportion depends on the source [1]. Most phospholipids contain an appropriate amount of saturated fatty acids having a chain length between 12–26 carbon atoms. In general, stearic acid and palmitic acids serve as major fatty acids constituents of mammalian phospholipids. It has been shown that unsaturated fatty acids are usually located preferentially at the *sn*2-position in phosphocholine (PC) [2]. By exchanging fatty acids asymmetrically in the phospholipid molecule, a desired phospholipid can be synthesized. 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 [3]. It is also desirable to have phospholipids containing specific fatty acids for scientific purposes and possibly for practical applications.

One promising approach towards their synthesis is the use of natural phospholipids as starting material and replacement of the existing fatty acid with the desired one

[1, 3, 4]. Synthesis of phospholipids is difficult by chemical means since control of regio- and also stereo-selectivity must be ensured. Phospholipids containing the same fatty acids at the *sn*1- and *sn*2-positions could be synthesized by chemical reaction [5]. However, biologically active polyunsaturated fatty acids (PUFA) are chemically unstable, which explains why chemical synthesis of phospholipids containing PUFA proceeds with low yield. Alternatively, lipases, phospholipase A₁ (PLA₁) and phospholipase A₂ (PLA₂) have been used for the targeted incorporation of fatty acids to the *sn*1- or *sn*2-positions of phospholipids [6, 7].

In this paper, phospholipase D-catalyzed transphosphatidylation of 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC) with ethanolamine in anhydrous organic solvent was investigated. In addition, lipase-catalyzed incorporation of oleic acid into the *sn*1-position of DLPC was also studied as a model reaction.

2 Materials and methods

2.1 Materials

Lipase (triacylglycerol hydrolases, EC 3.1.1.3) of *Rhizopus delemar* (lipase D, D-EP100; immobilized on polypropylene (EP100)) was from Unilever (The Netherlands).

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Immobilized lipases of *Thermomyces lanuginosa* (Lipozyme TL IM) and *Rhizomucor meihei* (Lipozyme RM IM) were from Novozymes (Bagsvaerd, Denmark). Non-specific immobilized lipase from *Candida antarctica* B (CAL-B) was from Roche Molecular Biochemicals (Penzberg, Germany). Phospholipase D (EC 3.1.4.4; PLD) was from peanut (*Sigma*, Germany) or produced recombinantly from *Escherichia coli* containing the PLD-encoding gene from *Streptomyces antibioticus* (plasmid provided by the Laboratory of Molecular Biotechnology, Nagoya University, Japan) [8]. PLA₂ was from porcine pancreas (*Sigma*). All chemicals and solvents used were reagent grade and purchased from common commercial suppliers, with the exception of EP 100 (particle size 200–400 µm; Akzo, Obernburg, Germany) and glycerophosphocholine (GPC) or glycerophospho-ethanolamine (GPE) (*Euticals S.P.A.*, Lodi, Italy).

2.2 Chemical synthesis of 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC)

DLPC was synthesized according to the method of *Pisch* et al. [5]. Briefly, 2.75 mmol fatty acid and 3 mmol 1,1'-carbonyl diimidazol were dissolved in 20 mL dichloromethane (dried over 3 Å 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 further carbon dioxide was formed. Then 1 mmol glycerophosphocholine and 2.75 mmol 1,8-diazabicyclo[5.4.0]undec-7-en 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 vol/vol) 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.

2.3 Production of recombinant PLD

The recombinant PLD from *Streptomyces antibioticus* was synthesized according to the method of *Iwasaki* et al. [8]. Synthetic medium containing (per liter) 5 g glucose, 2 g glycerol, 5 g K₂HPO₄, 5 g KH₂PO₄, 11 g Na₂PO₄·12H₂O, 3 g (NH₄)₂SO₄, 3 g MgSO₄·7H₂O, 40 mg FeSO₄·7H₂O, 4 mg CaCl₂, 1 mg MnSO₄·7H₂O, 1 mg ZnSO₄·7H₂O and 0.05 mg Na₂MoO₄·7H₂O was used to cultivate the recombinant *E. coli* containing the PLD-encoding gene of *Streptomyces antibioticus*. 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 OD₆₆₀ reached 3.0 (approx. 15 h), isopropyl-β-D-thiogalactopyranoside (IPTG) was added to give a final concentration of 1 mM and cultivation was continued for 24 h.

Culture supernatant was collected by centrifugation and concentrated by membrane filtration (Centricon) followed by lyophilization to obtain a powder of PLD.

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

The PLD enzyme was prepared by lyophilization from sodium acetate buffer (pH 5.6) containing potassium chloride (KCl). According to the method of *Fu* et al. [9], 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) to achieve a final dry preparation of 98 wt-% KCl. The buffer pH was adjusted to 5.6 using 100 mM HCl solution. The PLD content of the resulting PLD-KCl powder preparations from 0.1 M and 0.2 M sodium acetate buffer was approximately 0.68 and 0.56 mg per 100 mg PLD-KCl, respectively.

2.5 Transphosphatidylation of DLPC in a biphasic system

According to the method of *Pisch* et al. [5], 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. The reaction was started by adding 9 mL PLD solution (0.05 mg/mL of 0.1 M sodium acetate buffer containing 0.1 M CaCl₂, 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 phospholipids 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 vol/vol). 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 sulphate and solvent was evaporated under vacuum to obtain the crude phosphoethanolamine (PE).

2.6 Transphosphatidylation of DLPC in anhydrous organic solvent

The reaction was carried out using a method modified from *Rich and Khmel'nitsky* [10]. To a 4-mL screw-capped vial, 25 mg DLPC and 0.6 g ethanolamine dissolved in 5 mL chloroform (dried over 3 Å 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 exchange resin (Dowex 1 × 8, 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 phospholipid composition by

TLC-FID. The reaction was stopped by centrifugation to separate the enzyme and resin from the reaction mixture. Crude phospholipid was isolated by evaporation of solvent under vacuum.

2.7 Acidolysis of DLPC with oleic acid

Reaction media and different enzyme preparations were adjusted to a given water activity (a_w , 0.11–0.53) by pre-equilibration over saturated salt solution for at least 48 h at room temperature. The salts used were LiCl (a_w = 0.11), CH₃COOK (a_w = 0.23), MgCl₂ (a_w = 0.33), K₂CO₃ (a_w = 0.43) and Mg(NO₃)₂ (a_w = 0.53). The reaction medium consisted of 1 mM DLPC and 2 to 10 mM oleic acid in hexane. The reaction was started by adding 50 mg immobilized lipase to 1 mL of reaction medium in a Teflon-lined screw-capped vessel, which was shaken by a thermomixer at 1.200 rpm, 40 °C. Samples were periodically withdrawn and applied to TLC plates using chloroform:methanol:water (65:24:5 vol/vol/vol) as developing system [11]. The band corresponding to phosphocholine (PC), visualized under iodine vapor, was scraped off and methylated, followed by GC analysis.

2.8 Analytical methods

Changes in phospholipid compositions during a reaction were quantitatively determined by TLC-FID analysis (Iatroscan, Iatron Laboratories, Tokyo, Japan) as described by Pisch et al. [5]. To 60 µL sample, 0.3 mL Folsh's solution and 0.3 mL distilled water were added, followed by vigorous shaking for 30 s and centrifugation. The organic layer (1–2 µL) was applied to a Chromarod and developed in a system of chloroform:methanol:water (40:20:1 vol/vol/vol) for 40 min. The Chromarod was air dried for 2 min, followed by TLC-FID analysis (hydrogen flow rate = 160 mL/min; air flow rate = 2 L/min; 30 s/scan). The fatty acid compositions of phospholipids were determined by converting all fatty acids into the corresponding fatty acid methyl esters followed by GC analysis as described previously [12].

2.9 Regiospecific analysis of phospholipids

The regiospecific analysis of phospholipids was carried out using the methods modified from Aldercreutz [13]. The modified phospholipid was subjected to either Lipozyme-catalyzed ethanolysis in the *sn*1-position or to PLA₂-catalyzed hydrolysis in the *sn*2-position. The phospholipids and the lyso-phospholipids (LPL) products were then analyzed for their fatty acid compositions. Phospholipid (6 mg) was dissolved in 500 µL ethanol or 500 µL diethyl ether-ethanol mixture (4:1 vol/vol) and Lipozyme

RM IM (20 mg) or immobilized PLA₂ (10 mg) was added. The reactions were complete after 26 or 3 h, respectively. The samples before and after reaction were collected for fatty acid analysis by GC as described above.

3 Results and discussion

3.1 Chemical synthesis of phospholipids

The incorporation of one type of fatty acid to the *sn*1- and *sn*2-positions of glycerophosphocholine (GPC) was performed by chemical synthesis starting from GPC (Tab. 1). A high yield of the desired PC was only achieved using lauric acid yielding 82% DLPC. Purification by crystallization from acetone:chloroform (9:1 vol/vol) at –20 °C gave more than 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, glycerophosphoethanolamine (GPE) was used as alternative starting material, but only 10% yield of phospholipids was observed in the incorporation of lauric acid.

Tab. 1. Products from chemical synthesis of phospholipids^{*}.

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

^{*} GPC – glycerophosphocholine, GPE – glycerophosphoethanolamine, PX – phospholipid, LPX – lysophospholipid, GPX – glycerophosphocholine or glycerophosphoethanolamine, FA – fatty acid.

3.2 Transphosphatidylation of DLPC

One alternative for the synthesis of phospholipids bearing different alcohols or nucleophiles, is PLD-catalyzed head group exchange. Since most PLD utilize alcohols rather than water, it is possible to accomplish transphosphatidylation reaction even in the presence of high amounts of water, such as in a biphasic (buffer/solvent) system [5]. However, the considerable amount of water present results in an undesirable hydrolytic side-reaction. Thus, an anhydrous organic solvent system has been recently suggested using a non-aqueous scavenger, *i.e.* a cation exchange resin, which would be able to efficiently trap the positively charged choline, which exerts strong inhibitory action on PLD [10]. In this study, transpho-

sphatidylation (or head group exchange) of DLPC and ethanolamine was carried out in both, a conventional biphasic system and in an anhydrous solvent system composed of chloroform.

It was found that the initial reaction rate was approximately four times higher in a biphasic system compared to anhydrous chloroform although the same amount of enzyme and substrate concentration was used (Tab. 2). In the anhydrous system, also longer reaction times (48 vs. 24 h) were needed for complete conversion (100% vs. 96.2–98.6%). However, the high conversion observed here is in contrast to other work, in which a PLD suspended in a dry or nearly dry non-aqueous solvent has been reported to have very low or zero activity compared to a biphasic system [10]. PLD activity in an anhydrous organic solvent system offers the advantage of easier product recovery. In addition, the enzyme is only suspended and can also be isolated more easily for subsequent batch reactions. Therefore, further studies focused on the increase of the PLD activity in the anhydrous organic solvent system. In contrast to literature data, the addition of different cation exchange resins (Dowex 1 × 8, Dowex 50 and Toyopearl SP-650) had no measurable positive effect on the transphosphatidylation in the anhydrous solvent system (data not shown).

Tab. 2. Transphosphatidylation of 1,2-dilauroyl-*sn*-glycero-3-phosphocholine and ethanolamine in biphasic (chloroform and water) and anhydrous solvent (chloroform) systems catalyzed by phospholipase D at 40 °C.

System	Phospholipase D sources	Reaction time [h]	PE [†] [%]	Initial reaction rate [% PE/h]
biphasic	peanut	24	98.6	16.6
biphasic	recombinant <i>E. coli</i>	24	96.2	15.8
anhydrous	peanut	48	100	4.4
anhydrous	recombinant <i>E. coli</i>	48	100	3.9

[†]PE – phosphoethanolamine.

Another alternative, the use of a salt activation technique was found to be much more effective. It was suggested that a highly polar salt matrix may help to maintain the native structure of an enzyme in organic media [14]. Thus, recombinant PLD from *Streptomyces antibioticus* produced in *E. coli* was lyophilized from sodium acetate buffer pH 5.6 in the presence of different amounts of KCl to produce a salt-activated preparation (PLD-KCl).

It was found that, similarly to the transphosphatidylation using free enzyme, a quantitative head group exchange was possible within 48 h using the PLD-KCl preparations (Fig. 1). Conversion was moderately increased when

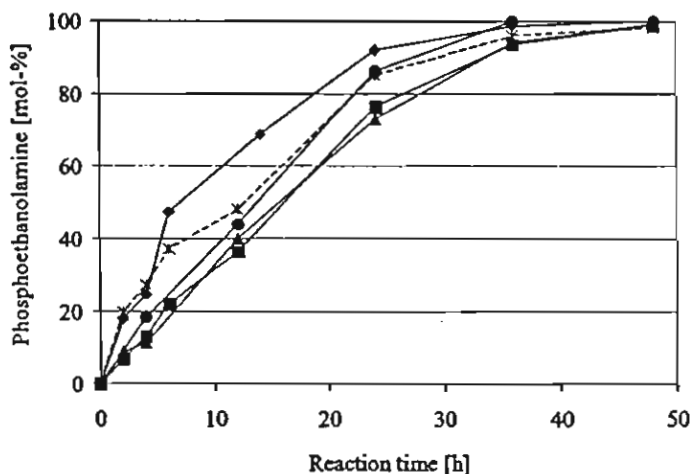


Fig. 1. Effect of salt-activated phospholipase D (PLD) on transphosphatidylation of DLPC and ethanolamine in anhydrous chloroform at 40 °C. Amount of PLD-KCl used; 25 mg (▲) 50 mg (■), 75 mg (●) and 100 mg (◆). Free PLD (5 mg) was used as a control (x).

increasing amounts of PLD-KCl. Thus, at a first glance, 75 mg PLD-KCl gave comparable reaction profiles as 5 mg of free PLD. However, by re-calculating the actual amount of PLD/mg dry powder of PLD-KCl, it was found that the salt-activation technique lead to a 10–12 folds higher activity of PLD based on the same amount of enzyme.

Variation of the reaction temperature revealed that the initial reaction rate substantially increased at higher temperatures and a complete transphosphatidylation could be achieved within 12 h at 60 °C (Fig. 2).

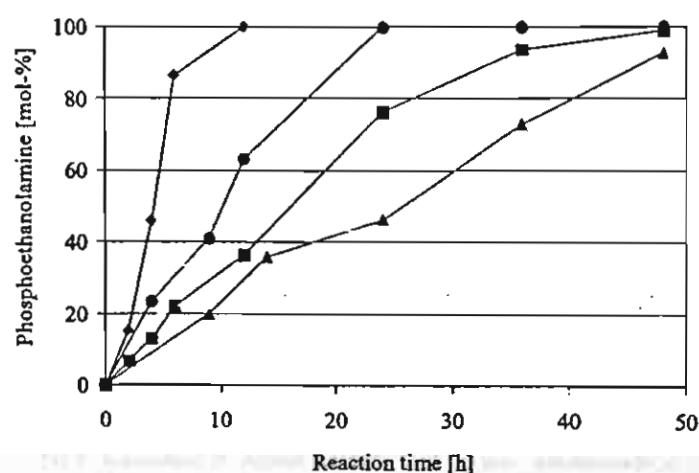


Fig. 2. Effect of reaction temperature on transphosphatidylation of DLPC and ethanolamine catalyzed by salt-activated phospholipase D lyophilized from 0.2 M sodium acetate buffer in anhydrous chloroform at 30 °C (▲), 40 °C (■), 50 °C (●) and 60 °C (◆).

3.3 Acidolysis of DLPC and oleic acid

Lipase-catalyzed acidolysis of DLPC was performed in organic solvents at controlled water activity in order to incorporate oleic acid to the *sn*1-position of DLPC. The effects of types of commercial immobilized lipases, fatty acid concentration and initial water activity were studied.

Among the four lipases studied, a non-regiospecific lipase from *Candida antarctica* type B (CAL-B) and three *sn*1,3-specific lipases, Lipozyme TL IM showed the highest activity (Fig. 3). The incorporation of oleic acid into DLPC at 48 h was 59.6%, 54.0%, 50.0% and 35.4% using Lipozyme TL IM, CAL-B, lipase D immobilized on Accurel EP-100, and Lipozyme RM IM, respectively. The result was in accordance with the work of Peng et al. [15]. Consequently, Lipozyme TL IM was used in subsequent studies.

Variation of the initial water activity using Lipozyme TL IM in *n*-hexane revealed that the highest final incorporation of oleic acid to DLPC (64.2%) was obtained at a_w 0.11 at a relatively high initial rate. Increasing water activity above 0.33 lead to a decrease in initial rate, because the reaction equilibrium was shifted to the hydrolytic side (Tab. 3).

Tab. 3. Incorporation of oleic acid to DLPC catalyzed by Lipozyme TL IM in *n*-hexane using various reaction conditions (48 h).

Initial water activity	DLPC:Oleic acid [mol/mol]	Temperature [°C]	Initial reaction rate	Incorporation at 48 h [%]
0.11	1:2	40	1.18	52.6
0.11	1:4	40	2.99	54.2
0.11	1:4	60	1.55	48.6
0.11	1:6	40	2.68	81.9
0.11	1:8	40	2.40	100.0
0.11	1:8	60	2.76	66.4
0.11	1:10	40	1.54	91.0
0.23	1:4	40	0.85	35.3
0.33	1:4	40	5.67	51.0
0.43	1:4	40	1.09	57.5
0.53	1:4	40	0.43	26.0

It was also found that the incorporation increased with increasing oleic acid concentration and quantitative incorporation was found at a DLPC to oleic acid ratio of 1:8 (mol/mol). At the ratio of 1:10 (mol/mol), however, the initial rate was dramatically decreased and the final incorporation dropped to 91.0%, which could be due to an inhibitory effect of a too high concentration of the fatty acid [16].

An increase of the reaction temperature to 60 °C resulted in less incorporation of oleic acid. The regiospecific analysis showed that no oleic acid was incorporated into the *sn*2-position of the products (data not shown).

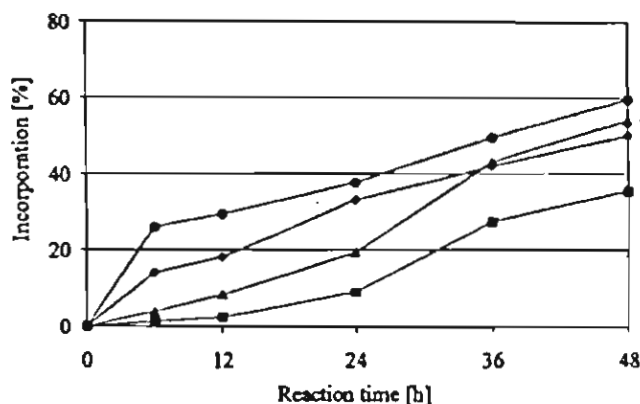


Fig. 3. Acidolysis of DLPC and oleic acid (1:4 mol/mol) catalyzed by CAL-B (▲), Lipozyme RM IM (■), Lipozyme TL IM (●), and immobilized lipase D (D-EP100, ◆) in *n*-hexane at a_w 0.11, 40 °C.

4 Conclusion

DLPC was enzymatically modified using lipase to afford non-symmetrical phospholipids or PLD to afford the corresponding ethanolamine derivative. Oleic acid could be quantitatively incorporated into DLPC using Lipozyme TL IM under optimized reaction conditions. PLD-catalyzed head group exchange was possible with recombinant enzyme in biphasic and anhydrous organic solvent systems. The lower activity in pure chloroform could be substantially enhanced by applying a salt activation technique, which allowed for quantitative head group exchange from DLPC to DLPE.

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