Enzymatic Method of Increasing Phosphatidylcholine Content of Lecithin[1]

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An enzymatic method was established to increase the phosphatidylcholine (PC) contents of soybean and egg lecithins. Other phospholipids of lecithin were phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidic acid (PA). Seven preparations of phospholipase D (PLD), PLD-1 to PLD-6 of *Streptomyces* origin and PLD-7 of cabbage origin, were tested for their ability to increase PC by transphosphatidylation in the presence of choline chloride (CC). The reactions were carried out at 30 C in a biphasic system that consisted of an aqueous phase containing PLD along with a buffer (optimum pH) having desired concentration of CC and Ca^{2+} and an ethyl acetate phase containing lecithin phospholipids. Intermitttent samples were extracted and analyzed by HPLC. Four of six PLD's of *Streptomyces* origin showed good transphosphatidylation (increase of PC contents of soybean lecithin from approximately 35% to 60-70% on a phospholipid basis) at 2.5 M CC, but the other two microbial PLD's completely hydrolyzed the phospholipids to PA. Cabbage PLD-7 showed poor transphosphatidylation. PLD-3 gave the highest PC contents (70%) at 1.75 M CC. One hundred percent transphosphatidylation of pure PE to PC was achieved with PLD-3. PI was inert to the attack of most PLD preparations examined with the exception that PLD-3 hydrolyzed PI significantly. Purified PI could not be transphosphatidylized to PC; 100% PA was formed. Soybean lecithin containing about 80% PC and purified egg yolk lecithin with 75% PC could be converted to products having 95% PC and almost 100% PC, respectively, by PLD-3 at 1.75M CC.

Vegetable oils such as soybean, corn, cottonseed, linseed, peanut, rapeseed, safflower and sunflower have been used as lecithin sources (1). Soybean oil is the primary and largest source of commercial lecithin, with phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) as the principle phospholipids. Other components are phosphatidic acid (PA), glycolipids, phosphatidylserine (PS), triglycerides, carbohydrates and sterols, etc. (1,2).

The demand for lecithin having a high PC content has been increasing in the cosmetic, pharmaceutical, food and other industries (1.2). High-PC lecithin and its lyso derivatives have the capability to function as surfactants in wide pH ranges (3). Because phospholipids occur naturally in skin and other biological membranes (4), they have been used for cosmetic formulations where modified and specially refined lecithins having superior characteristics are desired. These modified forms often have additional functionality unavailable from commercial-grade lecithin. For example, liposomes made of pure PC are more effective than egg or soybean lecithins for softening or restoring dry, aged skin (5).

[1]Studies on Enzymatic Conversion of Phospholipids (v).
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The industrial production of pure phospholipids has become more important. Previously, we prepared almost pure phosphatidylglycerol (6,7) and PE (8) by transphosphatidylation of pure PC using phospholipase D (PLD) with minimal PA formation. PS containing almost no PA was obtained by the same method (unpublished data).

Other workers have reported the conversion of PC to other phospholipids by PLD in low yield (9). The conversion of choline plasmalogen from ethanolamine plasmalogen was about 40% via transphosphatidylation by cabbage PLD in diethyl ether (10). PI was thought to be inert to the attack of PLD (11,12); however, one report shows the transphosphatidylation of PI to bis-(phosphatidyl) inositol by PLD from cauliflower florets in the presence of free inositol, diethyl ether and Ca^{2+} (13). Apparently, the enzyme did not attack a specific hydroxyl on the acceptor PI molecule because more than one isomer of bis-(phosphatidyl) inositol was formed (13).

A complex and expensive purification procedure is usually required to obtain individual phospholipids of more than 50-60% purity. As a different approach, we have investigated an enzymatic method to increase PC contents in soybean lecithin and in egg lecithin. PLD produced by several microorganisms has been found to catalyze the hydrolysis of a wide range of phospholipids (12), but specificity is thought to depend on the origin of the enzyme. It is known that enzymes from bacteria had restricted substrate specificities while those from ***actinomyces*** had broader specificities (14). In the present article, we describe the action of several PLD preparations obtained from various sources on the PE and the PI in the lecithin and on their pure forms in a biphasic reaction mixture with or without choline chloride (CC) acceptor.

Experimental Procedures

Materials. Soybean lecithins (SLP-White and SLP-PC 70 grades) and purified egg yolk lecithin were obtained from True Lecithin Co., Ltd. (Mie prefecture, Japan) and Asahi Kasei Co., Ltd. (Ibaraki prefecture, Japan), respectively. CC and chemicals for chromatography were procured from Wako Pure Chemical Industries Ltd. (Osaka, Japan). PE was purchased from Avanti Polar Lipids (Birmingham, Alabama), and all other chemicals used in the present study including phospholipids were from the sources mentioned in our previous report (6). PI (ammonium salt) was purified from soybean lecithin (SLP-White) according to Colacicco and Rapport (15). Unisil (activated silicic acid 100-200 mesh, Clarkson Chemical Co., Inc., Williamsport, Pennsylvania) was used for PI purification. Purified PI contained 7% PA.

Enzymes. Seven types of PLD were obtained from different sources as shown in Table 1 (8). All of the enzymes used were of microbial (***Streptomyces***) origin except PLD-7, which was prepared by the authors from cabbage leaves (16).

Reactor. A glass bottle reactor (1.6 cm i.d. and 3.1 cm high) placed in a jacket was temperature-controlled by

TABLE 1

Reaction Conditions for Various PLD Enzymes Examined (8)

Enzyme origin (PLD)		Manufacturer[a]	Buffer	pH	Ca^{2+} (mM)
1	*Streptomyces chromofuscus*	TJ	0.1 M tris-HCl	8.0	40
2	*Streptomyces* sp.	TJ	0.2 M acetate	5.6	-
3	*Streptomyces* sp.	SC	0.2 M acetate	5.6	80
4	*Streptomyces* sp.	SC	0.2 M acetate	5.6	40
5	*Streptomyces* sp.	KM	0.2 M phosphate	7.0	40
6	*Streptomyces prunicolor*	YK	0.2 M phosphate	7.0	-
7	cabbage	SP	0.2 M acetate	5.6	80

[a]TJ, Toyo Jozo Co., Tokyo; SC, Sugiyama Chemical and Industrial laboratory, Yokohama; KM, Kyowa Medex Co., Tokyo; YK, Yakult Central Institute for Microbiological Research, Tokyo; SP, self prepared.

circulating 30 C water through the jacket. The reaction mixture was stirred at 1200 rev. min^{-1} by a magnetic stirrer. The reactor was tightly closed with teflon plug and a stopcock.

Reaction mixture and conditions. Reactions were performed in a buffer-ethyl acetate biphasic system. Lecithin (SLP-White or SLP PC-70 or purified egg yolk lecithin) or each pure phospholipid, 30 mg, was added to 2 g of ethyl acetate, and the mixture was sonicated (Sonicator, Ohtake Works, Tokyo, Japan; 50 Watt, continuous pulse for 1 min) under ice cold conditions in a sonication vessel (1.4 cm i.d and 9.5 cm high). One g of buffer (optimum pH) containing the desired concentration of CC and enzyme was put into the reactor and the lecithin (or pure phospholipid)-ethyl acetate was added. The reaction was carried out at 30 C for three to four h under the continuous mixing. For hydrolytic reactions, all other conditions were the same except that no CC was supplied.

Assay of enzyme activity. Activity units of the respective enzymes were calculated by hydrolytic reactions as mentioned above. One unit of hydrolytic activity of enzyme is defined as that amount of enzyme which hydrolyzes one μmol of pure PC/min at 30 C. In this study, one unit of PLD was used, except for PLD-3 (0.2 unit of the soluble state).

Measurement of phospholipids. Phospholipids were extracted from an aliquot (0.1 ml) intermittently drawn from the reaction mixture as reported (6), and the concentrated extract was analyzed by HPLC. A Japan Spectroscopic Co., Ltd. (Tokyo, Japan) (JASCO BIP-1) was attached to a JASCO UVIDEC-100 V detector which was operated at 205 nm and 0.32 ABU. The column and precolumn were Wakosil 5 SIL (4.6 × 150 mm)and Wakosil 5 SIL (4 × 30 mm) from Wako Pure Chemical Industries, Ltd., Osaka, Japan. The eluting solvent was n-hexane/isopropyl alcohol/25mM potassium phosphate buffer (pH = 7.0)/ethanol/acetic acid (367:490:62:100:1.0, v/v) (17). The solvent mixture was filtered through Whatman chromatography paper 3 mm Chr and then in vacuum using a Dismic-25 JP disposable syringe filter unit 0.2 μm(hydrophobic) (Tokyo Roshi Co., Ltd., Tokyo, Japan) before the acetic acid was added. The elution solvent was degasified with a Branson Yamato 3200 ultrasonic cleaner. Ten μl samples were injected at a flow rate of one ml/min under 80 Kg/cm^2 pressure at room temperature and peaks were recorded by a Sic Chromatocorder 11 (System Instruments Co., Ltd,. Tokyo, Japan). Each run took 60 to 65 min.

Some samples were also analyzed by a TLC/FID Iatroscan Analyzer as previously reported (6).

RESULTS

Composition of lecithin. The initial compositions of lecithin substrates, SLP-White, SLP-PC 70 and purified egg yolk lecithin, as analyzed by HPLC and TLC/FID, are given in Table 2. Only four major components, PC, PE, PI and PA, were considered in this study. SLP-PC 70 and purified egg yolk lecithin were partially purified lecithins and contained only phopholipids.

TABLE 2

Initial Composition of Lecithins Studied

Phospholipids	Composition[a] (%)		
	Soybean lecithin		Purified egg yolk lecithin
	SLP-White	SLP-PC 70	
PC	35.5	80.1	74.5
PE	29.5	15.4	25.5
PI	19.0	2.5	—
PA	16.0	2.0	—

[a]Calculated on a phospholipid basis.

Transphosphatidylation of lecithin (SLP-white). Transphosphatidylation of lecithin (SLP-White) was carried out with seven PLD preparations (Table 1) in the presence of CC. PLD-1 and PLD-5 showed almost negligible transphosphatidylation activity, and no increase of PC contents was observed at varying concentrations of CC (Table 3). The original PC was hydrolyzed to PA within 15 min. PE was hydrolyzed but not as efficiently as PC, and there was little hydrolysis of PI. In the case of PLD-1, after four h of reaction, there were 80% of PA and no PC (Fig. 1, PLD-1), Almost the same type of time-course profile was obtained with PLD-5. In our previous paper on conversion of PC to PE, PLD-1 and PLD-5 showed poor

TABLE 3

Increase of PC Content of Lecithin by Different PLD Enzymes

Enzyme (PLD)	Enzyme (units)	CC concentration (M)	Maximum PC[a] (%)	Time (min)
1	1	2.5	35.9[b]	—
2	1	2.5	60.8	45
3	0.2	1.75	69.9	60
4	1	2.5	61.9	60
5	1	2.5	36.0[b]	—
6	1	2.5	66.0	60
7	1	2.5	44.9	10

[a]PC concentration is calculated on a phospholipid basis.
[b]Almost no increase of PC content.

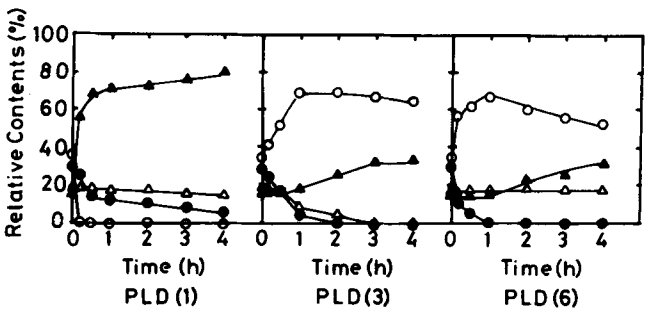

Fig. 1. Time-course profiles of tranphosphatidylation of lecithin using PLD-1, 3 and 6. ○, PC; ●, PE; △, PI; ▲, PA. Lecithin (SLP-White), 15 g/l; PLD-1, 1 unit at 2.5 M CC; PLD-3, 0.2 unit at 1.75 M CC; PLD- 6, 1 unit at 2.5 M CC; other conditions as listed in Table 1.

transphosphatidylation activity and the product formed was gradually subjected to hydrolysis (8).

The maximum PC with PLD-2, PLD-4 and PLD-6 were obtained at 2.5 M CC (Table 3). None of the three enzymes could attack PI but PLD-6 showed a higher transphosphatidylation rate of PE to PC than PLD-2 and PLD-4. PE was completely transphosphatidylized to PC within 60 min with PLD-6. There was no increase of PA within the first 60 min but PC was later subjected to hydrolysis (Fig. 1, PLD-6).

PLD from cabbage (PLD-7) showed poor transphosphatidylation, and the maximum PC obtained was 44.9% within 10 min (Table 3); soon the PC was subjected to hydrolysis.

Among the seven PLD enzymes examined, PLD-3 gave the highest PC (69.9%) at 1.75 M CC (Table 3). PLD-3 was able to transphosphatidylize PE to PC completely and also attacked PI (Fig.1, PLD-3).

Effect of choline chloride concentration. The effect of varying CC concentrations (0.75-5.0 M) was examined and a 2.5 M CC concentration was found to be optimal in most reactions. For example, with PLD-2, the maximum PC and the highest initial rate of transphosphatidylation (r_{trans}) of PC were obtained at 2.5 M CC. There was little increase in transphosphatidylation at a 5 M concentration of CC. Although this effect may be due to substrate inhibition (Fig. 2), a 5 M CC concentration was not as inhibitory to PLD-3 as the other enzymes. The maximum r_{trans} for PC was at 1.75 M CC with PLD-3 (Fig. 2).

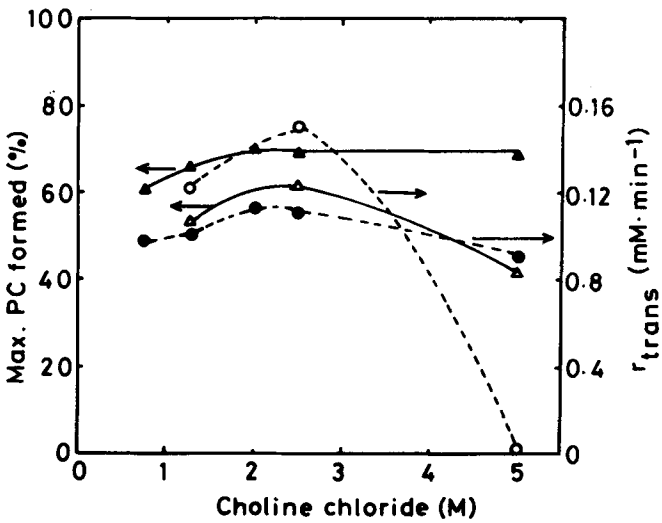

Fig. 2. Effect of CC concentration on the initial rate of transphosphatidylation of PC (r_{trans}) and maximum PC contents by PLD-2 and 3. ○···○, r_{trans} of PLD-2; ●···●, r_{trans} of PLD-3; △—△, Max. PC by PLD-2; ▲—▲, Max. PC by PLD-3. Lecithin (SLP-White), 15 g/l; PLD-2, 1 unit; PLD-3, 0.2 unit; other conditions as listed in Table 1.

Effects of temperature and pH. The substrate specificity of PLD-3 was investigated under various temperatures (25-35 C) at constant pH (5.6) and under various pH's (4.0-8.0) at constant temperature (30 C) with lecithin (SLP-White) in the presence of 1.75 M CC. The transphosphatidylation rate of PC was highest at pH 5.6 and 30 C; and the substrate specificity pattern did not change at different pH's and temperatures.

Hydrolysis and transphosphatidylation of pure PE and PI. One hundred percent conversion of PE to PC with 100% selectivity, i.e., no byproduct PA formation, was obtained using both PLD-3 and PLD-6.

PI seemed to be almost inert to the hydrolytic attack of all PLD enzymes examined except PLD-3. Reaction of PI with 1.75 M CC by PLD-3 did not give PC; the PI was

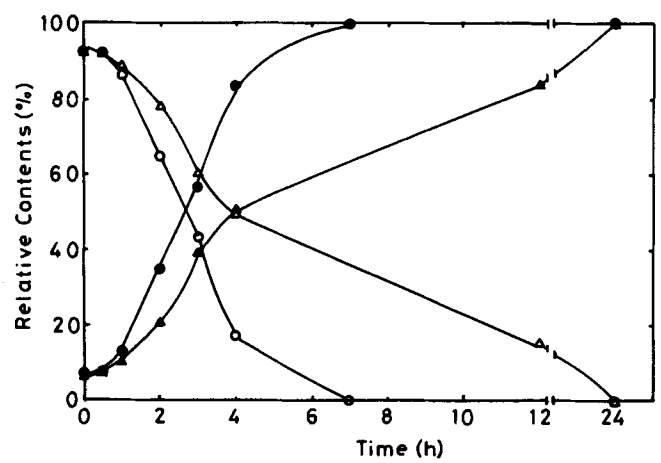

Fig. 3. Hydrolysis and transphosphatidylation of PI by PLD-3. ○, PI (in the absence of CC); ●, PA (in the absence of CC); △, PI (in the presence of 1.75 MCC); ▲, PA (in the presence of 1.75 MCC). PI, 15 g/l; PLD-3, 0.2 unit; other reaction conditions as listed in Table 1.

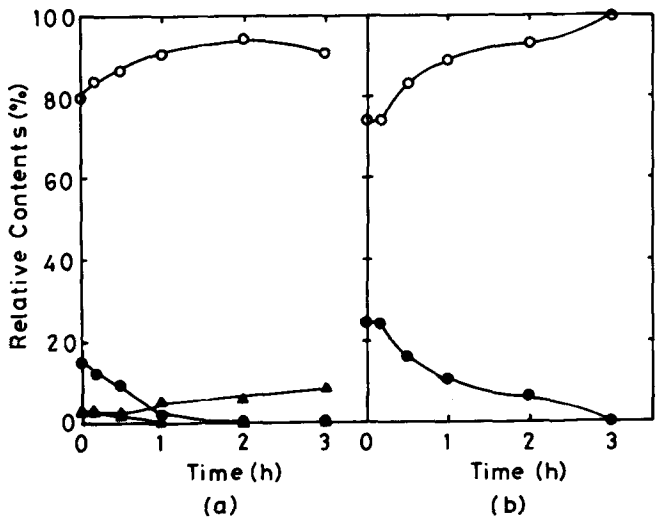

Fig. 4. Transphosphatidylation of (a) SLP-PC 70 lecithin, and (b) purified egg yolk lecithin by PLD-3 in the presence of 1.75 M CC. ○, PC; ●, PE; △, PI; ▲, PA. Lecithin (SLP-PC 70 or purified egg yolk lecithin), 15 g/l; PLD-3, 0.2 units; other reaction conditions as listed in Table 1.

completely hydrolyzed to PA. The rate of formation of PA was, however, lower in the presence of CC than the hydrolytic reaction in the absence of CC (Fig. 3).

Mixtures of enzymes. Different sets of mixtures of PLD enzymes, i.e., PLD-2 & PLD-6 and PLD-3 & PLD-6, were used for transphosphatidylation of lecithin (SLP-White). The above reactions were conducted at different pH levels including the optimum pH of each enzyme in the mixture and in the presence of 1.75 M or 2.5 M CC. The maximum PC could not be increased further than that obtained by PLD-3 alone; rather, some inhibitory action on PI was observed. The maximum PC obtained with a mixture of PLD-3 and PLD-6 was 67.9% at pH 7.0.

Transphosphatidylation of SLP-70 and purified egg yolk lecithins. SLP-PC 70 and the purified egg yolk lecithin (Table 2) were transphosphatidylized in the presence of 1.75 M CC by PLD-3. Products formed from SLP-PC 70 included 95% PC and 5% PA after two h of reaction. Longer reaction times increased PA contents and decreased PC contents (Fig. 4a). Purified egg yolk lecithin after three hr of reaction gave almost 100% PC (Fig. 4b).

DISCUSSION

Comparing the time-course profiles of the transphosphatidylation of the seven PLD preparations, PLD-1 and PLD-5 had strong activitiy for hydrolysis of PC rather than transphosphatidylation. These two enzymes also could not transphosphatidylize PE and PI to PC. In the case of PLD-3 and PLD-6, transphosphatidylation activity of PE to PC was efficient; almost complete conversion of PE to PC was obtained without any significant byproduct formation. In the case of PE, the hydrolytic activity of PLD-3 was poorer than its transphophatidylic activity. A similar substrate specificity pattern was observed in our previous study on conversion of PC to PE (8).

It has generally been assumed that PLD preparations will not attack PI in spite of their action toward other phospholipids (12). We found that, of the enzymes used in this study, only PLD-3 attacks PI significantly. However, hydrolysis was predominant rather than transphosphatidylation. This result could be explained by the structures. Only PI has a bond of phosphate with a secondary hydroxyl group; other phospholipids have bonds of phospate with primary hydroxyl groups. Reaction of purified PI with PLD-3 with or without CC resulted in PA formation. PA cannot be transphosphatidylized to PC except by converting PA to PC by a synthetic procedure (18).

Substrate specificity can sometimes be broadened if the experimental conditions are altered (12). In our experiments, variation of temperature and pH did not affect the substrate specificity but did affect reaction rates. Mixtures of enzymes under various conditions did not increase PC content.

This method has a significant value for modification of lecithin as well as for purification of phospholipids. On an industrial scale, the overall recovery of PC depends upon the composition of the lecithin. A lecithin substrate containing no PI and PA could give almost pure PC. Lecithin preparations of high PC (95% PC) and almost pure PC could be obtained from SLP-PC 70 and purified egg yolk lecithin, respectively. The main limitation of this method is the cost of the enzyme. This problem can be solved to some extent by running the reaction continuously or in a repeated batch mode using immoblized PLD (7).

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