An Aqueous Suspension System for Phospholipase D-Mediated Synthesis of PS Without Toxic Organic Solvent

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ABSTRACT: Enzymatic synthesis of PS by phospholipase D (PLD)-mediated transphosphatidylation in an aqueous media was investigated. The purpose of this study was to establish a novel synthetic method where no toxic organic solvents were used. An attempt to react soybean lecithin (simply dispersed in an aqueous buffer) with an aqueous solution of L-serine and PLD was unsuccessful, giving only 20% of PS. By contrast, a suspension of lecithin adsorbed on fine powders such as silica was effectively converted into PS in an aqueous solution of L-serine and PLD. After screening various powders for use as the lecithin adsorbent, calcium sulfate was found to be the best with respect to lecithin conversion. In addition, calcium sulfate did not require prior adsorption of lecithin (i.e., the reaction proceeded effectively simply by adding the powder to an aqueous mixture of lecithin, L-serine, and PLD). With this "aqueous suspension system" of calcium sulfate, up to 180 mg/mL lecithin was completely converted, resulting in more than 80% PS in 24 h. The synthesized PS could easily be recovered from the powder by extracting with a mixture of *n*-hexane, ethanol, and diluted HCl.

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KEY WORDS: Lecithin, phosphatidylserine, phospholipase D, transphosphatidylation.

PS is a natural phospholipid that is especially abundant in animal brain tissue (1). Recently, PS has been shown to have therapeutic effects on several memory-related disorders. For example, Delwaide *et al.* (2) reported that oral administration of bovine brain cortex-derived PS (BC-PS) to patients with senile dementia improved their cognitive disorders. Another example is the report by Crook *et al.* (3) that patients with age-associated memory impairment treated with BC-PS had improved performance on tests related to learning and memory tasks of daily life.

One of the possible sources for preparing PS is animal organs such as bovine brain. In fact, many biochemical researchers have used BC-PS for clinical tests (2–5). However, these BC-PS preparations might not be suitable for human use as they may transmit infectious diseases such as bovine spongiform encephalopathy. In addition, animal organs themselves are not likely to be appropriate sources of PS for industrial-scale production because of their low availability.

An alternative for supplying PS is transphosphatidylation, in which the polar head group of lecithin (such as soybean lecithin) is exchanged with L-serine by phospholipase D (PLD) (6,7). The pharmacological effects of PS enzymatically synthesized from soybean lecithin were confirmed using several experimental animals (8–10).

Generally, transphosphatidylation is carried out in a biphasic system consisting of a water-immiscible organic solvent phase (e.g., diethyl ether or ethyl acetate) containing lecithin and an aqueous buffer phase containing the enzyme and L-serine (6,7). Regarding PS as food for human use, however, the use of such toxic organic solvents should be avoided in its production. In this aspect, Dittrich and Ulbrich-Hofmann (11) demonstrated synthesis of phosphatidylglycerol in purely aqueous media.

In this article, we report a novel reaction system for transphosphatidylation without using toxic organic solvents. The new system (named an "aqueous suspension" system) features use of a suspension of fine powder-adsorbed phospholipid in an aqueous buffer.

MATERIALS AND METHODS

Chemicals and enzyme. Soybean lecithin (SLP-PC70) was a gift from Tsuru Lecithin Industries Co. Ltd. (Mie, Japan). The phospholipid composition of the lecithin was: PC 83%, PE 6%, PA 8%, and others 3%.

Silica gel (Wako gel C-300), octadecylated silica gel (Wakogel 50C18) and activated carbon were from Wako Pure Chemical Industries (Osaka, Japan). Calcium sulfate powder (Type SF-CS) was from Mutsumi Chemicals Co. Ltd. (Mie, Japan). Calcium carbonate powder (Softon 3200) was from Shiraishi Calcium Co. Ltd. (Osaka, Japan). Calcium pyrophosphate powder was from Taihei Yogyo Yakuhin Co. Ltd. (Gifu, Japan). Diatomaceous earth (Celite, grade: Hyflo Super-Cel) was from Johns-Manville (Denver, CO).

PLD was prepared in this laboratory from a recombinant strain of *Escherichia coli* bearing the PLD gene of *Streptomyces antibioticus* (12,13). PLD activity was measured using

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soybean lecithin as a substrate, as described previously (14). One unit of PLD activity was defined as the amount of enzyme that hydrolyzes 1 μmol PC in 1 min at 37°C.

Transphosphatidylation. Several reaction systems for transphosphatidylation were compared as follows.

(i) Biphasic system. A mixture consisting of 15 mg of lecithin dissolved in 1 mL of organic solvent (ethyl acetate or *n*-hexane), 0.9 mL of 3.3 M L-serine solution in 0.11 M acetic acid-sodium acetate buffer (pH 5.6), and 0.1 mL of 30 units/mL PLD solution was reacted at 30°C with vigorous stirring.

(ii) Homogeneous aqueous system. A mixture containing 7.2 mg/mL lecithin (simply dissolved by vigorous vortexing), 1.485 M L-serine, 50 mM acetic acid-sodium acetate buffer (pH 5.6), and 1.5 units/mL of PLD was reacted at 40°C.

(iii) Aqueous suspension system (with powder-adsorbed lecithin). Lecithin (0.2 g) was dissolved in 30 mL of ethanol. One gram of powder (e.g., silica) was added to the solution and mixed for 30 min at room temperature. The solvent was removed by evaporation, and the residual solid material was dried completely under vacuum. The powder-adsorbed lecithin (86.4 mg) thus obtained (corresponding to 14.4 mg of lecithin and 72 mg of the powder) was reacted in 2 mL of a solution containing 1.485 M L-serine (unless otherwise stated), 50 mM acetic acid-sodium acetate buffer (pH 5.6), and 1.5 units/mL of PLD at 40°C with stirring.

(iv) Aqueous suspension system (without preadsorption). A mixture consisting of 7.2 mg/mL of lecithin (not preadsorbed), 3.87 M L-serine, 50 mM acetic acid-sodium acetate buffer (pH 5.6), 36 mg/mL calcium sulfate powder, and 1.5 units/mL PLD was reacted at 40°C with stirring.

Any modifications in the reaction conditions made during the study are described in the text. All the experiments were run in duplicate, and the average value of each set of runs was presented with SE of 5%.

Analyses of phospholipid composition. Fifty-microliter portions of the reaction mixture (as a well-mixed emulsion or suspension in the case of the biphasic or the aqueous suspension system, respectively) were taken intermittently and mixed with 25 μL of 1 N HCl to inactivate the enzyme. Phospholipids were extracted with 100 μL of chloroform/methanol = 2:1. Two microliters of the phospholipid solution was spotted on a quartz rod coated with silica gel (Chromarod SIII; Iatron, Tokyo, Japan), and the rod was developed in chloroform/methanol/acetic acid (40:15:6, by vol). The rods were dried in an oven and then scanned by TLC/FID (Iatroscan MK-5, Iatron). Peak areas were calculated with an integrator (SIC Chromatocoder 12; System Instruments Co. Ltd., Tokyo, Japan). Results were expressed as percentage of the peak areas of phospholipid species. They were regarded as weight percentages and could vary slightly from the actual weight percentage (15).

Recovery of PS from the reaction mixture. PS synthesis was performed in a total volume of 20 mL under the following optimized conditions: 36 mg/mL of lecithin (not preadsorbed on the powder), 3.87 M L-serine, 50 mM acetic acid-sodium acetate buffer (pH 5.6), 36 mg/mL of calcium sulfate, and 1.5 units/mL of PLD at 40°C for 24 h. After the reaction, the calcium sulfate powder (on which the phospholipids were adsorbed) was collected by centrifugation. After washing with water, the precipitate was resuspended in 20 mL of *n*-hexane/ethanol (2:1 vol/vol), and then 5 mL of water and 0.5 mL of 1 N HCl were added. Subsequent centrifugation separated the mixture into three phases, i.e., an upper liquid phase, a lower liquid phase, and a precipitate (solid) phase. The upper liquid phase was recovered, and the solvent was evaporated to afford the synthesized PS.

RESULTS AND DISCUSSION

Comparison of the reaction systems. Figure 1 shows the time course of changes in phospholipid composition during PS synthesis in various reaction systems. As reported by Juneja *et al.* (6,7), the ethyl acetate/water biphasic system was very effective, generating 80% PS in 10 min (Fig. 1A). However, ethyl acetate is not suitable for food production owing to its toxicity. The use of *n*-hexane, which is allowed for food production, as the organic phase was unsuccessful, because neither transphosphatidylation nor hydrolysis occurred (Fig. 1B).

This inactivation of the enzyme in *n*-hexane can be explained according to studies performed by Ulbrich-Hofmann's research group (16,17). They found that addition of a small amount of aliphatic alcohol promoted the otherwise very slow reactions in the *n*-hexane/buffer system. After several physicochemical measurements, they concluded that the package density of the PC aggregates was one of the major causes of the solvent effects. In our case, although the reaction system contained a large amount of L-serine, a similar physicochemical state of PC may be the cause for the *n*-hexane-mediated inactivation.

The reaction in the homogeneous aqueous system showed slight productivity, generating 20% PS in 60 min (Fig 1C). However, prolonged incubation promoted hydrolysis of the product as well as the substrate, resulting in formation of PA to more than 50%. Compared to the homogeneous aqueous system, the aqueous suspension system using silica-adsorbed lecithin was more promising as the PS content reached more than 50%, although a considerable amount of PA was also formed (Fig. 1D).

Some examples for the use of solid support-adsorbed substrate in enzymatic reactions were reported by Berger *et al.* (18,19). They performed lipase-catalyzed esterification of glycerol in organic solvent and found that the creation of an "artificial interphase" between glycerol and organic media by adsorbing glycerol onto a solid support was essential for effective reaction. In our present study, the reason for the acceleration of the reaction by the use of powder-adsorbed lecithin may involve this "artificial interphase" between the solid and liquid phases.

Effect of L-serine concentration. The effect of L-serine concentration on PS synthesis was investigated in an effort to

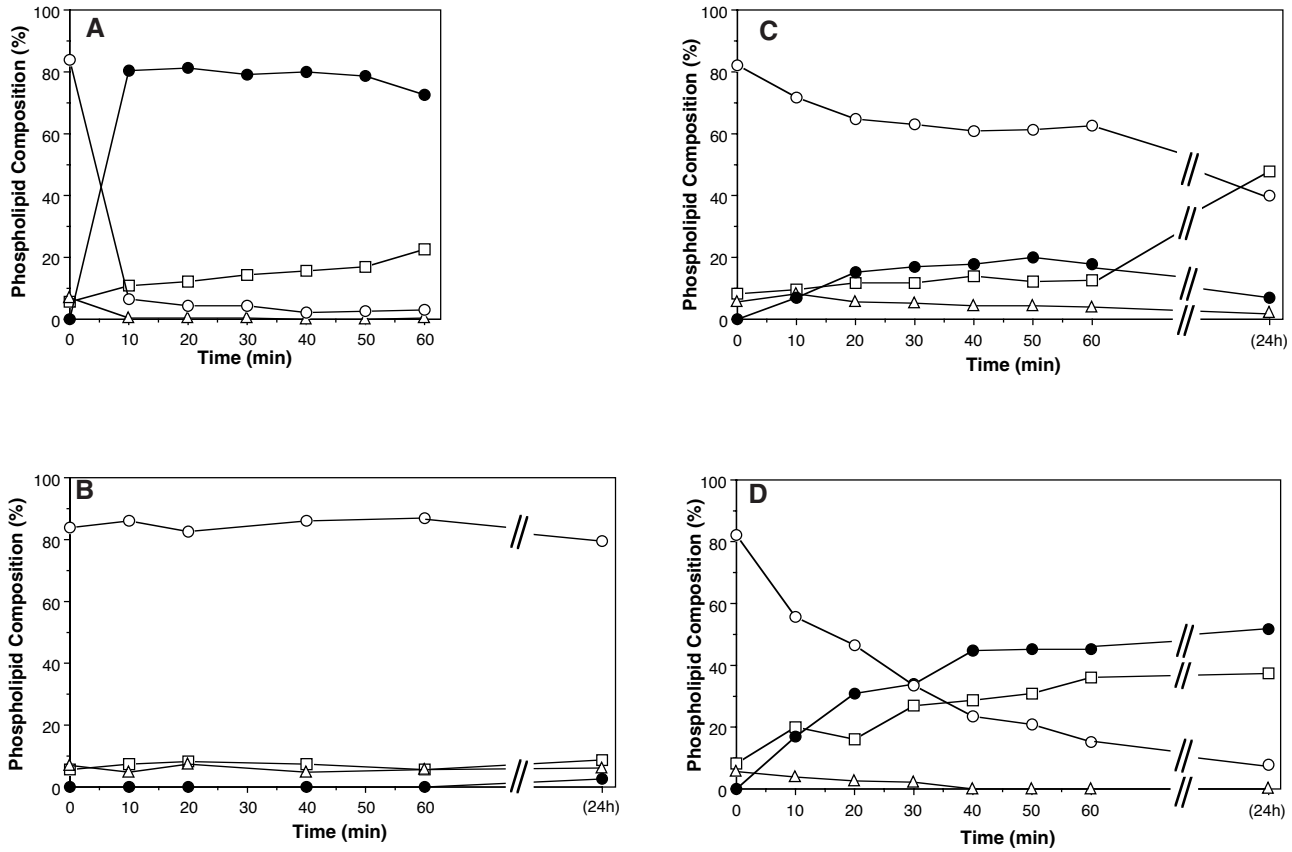

FIG. 1. Comparison of several reaction systems for PS synthesis. (A) Ethyl acetate-buffer biphasic system, (B) *n*-hexane-buffer biphasic system, (C) homogeneous aqueous system, and (D) aqueous suspension system using silica-adsorbed lecithin. Symbols: closed circles = PS, open circles = PC, open squares = PA, open triangles = PE.

reduce the formation of PA. The experiments were done using the aqueous suspension system with silica-adsorbed lecithin. As shown in Table 1, increasing the L-serine concentration from 1.485 to 3.87 M suppressed the formation of PA from 37.5 to 10.3% (the soybean lecithin contained approximately 8% of PA from the beginning). Therefore, 3.87 M of L-serine was used for subsequent experiments. It should be noted that an L-serine concentration of 3.87 M is a little less than its solubility in the buffer (approximately 4.3 M).

Screening of powders. Various water-insoluble powders were screened as the lecithin adsorbent in the aqueous suspension system. Calcium sulfate powder was found to be very effective, resulting in complete consumption of the substrate (Table 2). PS content reached more than 85% in 40 min, with almost no increase of PA (Fig. 2). We are not sure why calcium sulfate showed the best performance. A possible speculation might be that the "artificial interface" formed between calcium sulfate and water phases was the most suitable for the reaction.

The reactions performed so far were done with powder-adsorbed lecithin that was prepared prior to the reaction. However, this adsorption step is time-consuming; therefore, if possible, it is preferable to omit it. To simplify the operation (i.e., to omit the prior adsorption step), the reaction was tested in a mixture containing 7.2 mg/mL of lecithin (simply

TABLE 1
Effect of L-Serine Concentration on Suppression of PA Formation[a]

	Phospholipid composition after 24-h reaction (%)		
L-Serine conc. (M)	PC	PS	PA
1.485	7.9	52.6	37.5
2.97	11.6	64.2	24.1
3.87	10.1	77.8	10.3

[a]The reaction was performed in an aqueous suspension system using silica-adsorbed lecithin.

TABLE 2
Effects of Various Water-Insoluble Powders on PS Synthesis[a]

	Phospholipid composition at 24 h (%)		
Powder	PC	PS	PS
Silica	10.1	77.8	10.3
Octadecylated silica	8.9	37.1	53.2
Calcium carbonate	10.0	33.5	55.6
Calcium sulfate	0	85.1	11.9
Calcium pyrophosphate	20.3	57.7	20.2
Celite	37.5	40.5	13.5
Activated carbon	60.8	13.2	18.4

[a]Transphosphatidylation was carried out in 3.87 M L-serine, 50 mM acetic acid-sodium acetate buffer (pH 5.6), 1.5 units/mL phospholipase D, and 43.2 mg various powder-adsorbed lecithin/mL (corresponding to 7.2 mg lecithin/mL lecithin and 36 mg powder/mL).

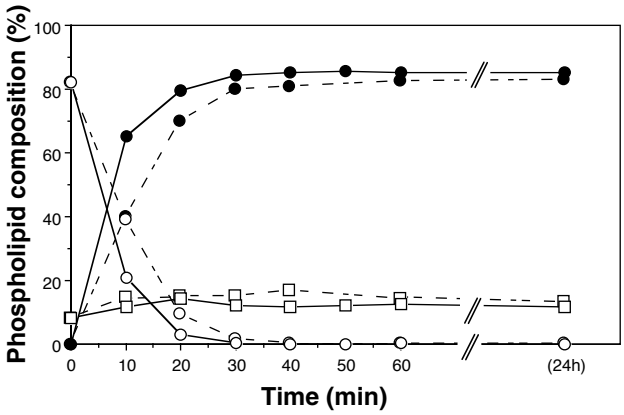

FIG. 2. PS synthesis in the aqueous suspension system using calcium sulfate powder. Reactions were performed in the aqueous suspension system with (solid lines) or without (dashed lines) prior adsorption of lecithin onto the powder. In both cases, the reaction mixture contained 7.2 mg/mL lecithin, 36 mg/mL calcium sulfate, 3.87 M L-serine, 1.5 units/mL of phospholipase D (PLD) and 50 mM acetic acid-sodium acetate buffer (pH 5.6). Symbols: closed circles = PS, open circles = PC, open squares = PA.

dissolved by vortex mixing), 3.87 M L-serine, 50 mM acetic acid-sodium acetate buffer (pH 5.6), 36 mg/mL calcium sulfate and 1.5 units/mL of PLD. As indicated in Figure 2 (dashed lines), the reaction proceeded in a similar manner as that of the lecithin preadsorbed on the powder (Fig. 2, solid lines). Therefore, the adsorption step was omitted in subsequent investigations.

Figure 3 shows the time course of PS formation at various initial substrate concentrations in the calcium sulfate-assisted aqueous suspension system. At any initial concentration tested (up to 180 mg/mL), PS content reached approximately 80% within 24 h, although the reaction rate became slower at

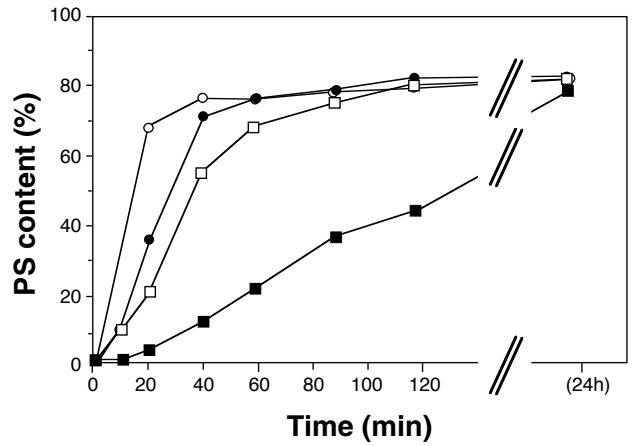

FIG. 3. Effect of initial substrate concentration on PS synthesis in the calcium sulfate powder-assisted aqueous suspension system. The reactions were performed in the presence of 3.87M L-serine, 36 mg/mL of calcium sulfate powder, 50 mM acetic acid-sodium acetate buffer (pH 5.6), and 1.5 units/mL PLD with 13.5 (open circles), 36 (closed circles), 50 (open squares), and 180 (closed squares) mg/mL of lecithin. For abbreviation see Figure 2.

higher substrate concentrations. It should be noted that in terms of absolute amount of PS, higher initial substrate concentrations gave larger amounts of PS. For example, after 120 min, the reaction with an initial substrate concentration of 50 mg/mL resulted in a PS content of 85.8%, corresponding to 42.5 mg/mL of PS in the reaction mixture ($50 \times 0.858 = 42.5$), whereas the run starting with 180 mg/mL of substrate gave a PS content of 46.2%, which equals 83.2 mg/mL of PS ($180 \times 0.462 = 83.2$).

Recovery of the product. From the results described so far, the reaction conditions were optimized as follows: 36 mg/mL of lecithin (not preadsorbed on the powder), 3.87 M L-serine, 50 mM acetic acid-sodium acetate buffer (pH 5.6), 36 mg/mL of calcium sulfate powder, and 1.5 units/mL of PLD at 40°C. In a separate experiment, we found that PS was mostly adsorbed on the surface of calcium sulfate particles (data not shown). Therefore, it was possible to separate PS by simple centrifugation. However, it was then necessary to recover the PS from the ones adsorbed on the precipitated calcium sulfate particles. To check the yield of PS under these conditions, the reaction was performed on a 20-mL scale for 24 h. After the reaction, the synthesized PS was recovered as described in the Materials and Methods section. From the 20-mL scale reaction (starting with 720 mg of lecithin), 675 mg of phospholipid was recovered. Figure 4 shows the TLC/FID chromatogram of the initial soybean lecithin (Fig. 4A) and the recovered product (Fig. 4B). The PS content in the final product was approximately 90%, which was slightly higher than that in the reaction mixture before the recovery operation. This was probably because a part of the impurities (e.g., PA) was selectively removed during the recovery steps. Consider-

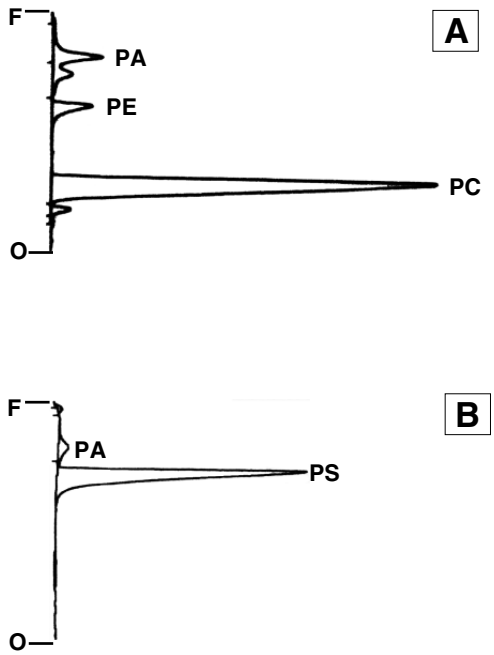

FIG. 4. Thin layer chromatogram of initial soybean lecithin (A) and the synthesized PS (B). "O" and "F" represent the origin and front of the development, respectively.

ing the average M.W. of the PC, PE, and PS (estimated from the FA composition), the overall yield of PS from the soybean lecithin was calculated to be approximately 89%.

In addition to the recovery of PS, the excess L-serine that remains in the reaction mixture should be recovered and recycled. In fact, our preliminary experiments revealed that the L-serine (which was recovered from the reaction mixture by centrifugation) could be reused several times for subsequent reactions with freshly added lecithin, calcium sulfate, and PLD.

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