Synthesis of Phosphatidylglycerol from Soybean Lecithin with Immobilized Phospholipase D

XingGuo Wang, AiYong Qiu, WenYi Tao, and PeiYing Shen

School of Food Science, Wuxi University of Light Industry, 214036 P.R. China

ABSTRACT: Phosphatidylglycerol (PG) was synthesized from soybean lecithin with the help of phospholipase D (PLD) that was immobilized with calcium alginate gel-enveloped polyethyleneimine–glutarald ehyde. The immobilization conditions were optimized with response surface analysis: Ca–alginate 1.39%, polyethyleneimine 7.78%, glutaraldehyde 1.22%. The conversion of phosphatidylglycerol was 87%. The properties of the immobilized PLD were: optimal temperature 25–30°C, optimal pH 8.2, ratio of ether phase and water phase 1.5–2.5:1 (vol/vol), repeated 15 batches operating with stabilizing conversion percentage of phosphatidylglycerol.
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KEY WORDS: Enzyme immobilization, phosphatidylglycerol, phospholipase D, soybean lecithin.

Phospholipids are natural surfactants that have many applications in the food and pharmaceutical industries (1,2). Phosphatidylglycerol (PG) is a surfactant with a greater hydrogen bonding capacity than phosphatidylcholine (PC), leading to better alveolus-stabilizing properties (3). There are two methods, chemical and enzymatic, known for the conversion of phospholipids. Compared with chemical reactions, enzymes, especially immobilized enzymes, have many advantages, including higher yields of desirable products with fewer side reactions under milder conditions (4,5). In 1987 and 1989, Juneja *et al.* (6,7) described the synthesis of PG and phosphatidylserine with phospholipase D entrapped in octy-Sepharose Cl-4B. But the operational stability of the immobilized enzyme was poor, and its enzymatic activity decreased dramatically after reaction of five batches. In 1988, Tsunoda and Akira (8) entrapped phospholipase D with hydrophilic resin ENT and hydrophobic resin ENTP that were synthesized in the laboratory. A mixed resin of suitable hydrophobicity–hydrophilicity on phospholipase D had been obtained by adjusting proportion and molecular weight of both resins.

MATERIALS AND METHODS

Materials. Phospholipase D was prepared from a *Pseudomonas* sp. that was selected in our laboratory. Polyethyleneimine–cellulose (PEI–cellulose) and PEI were purchased from Tokyo Kasei (Tokyo, Japan). Amberlite XAD-2 was purchased from Rohm & Haas Corp. (Philadelphia, PA). Controlled-pore glass was from the Shanghai Institute of Biochemistry (Shanghai, China). Soybean lecithin was refined in the laboratory (PC 40%, phosphatidylethanolamine (PE) 31.2%, phosphatidylinositide (PI) 17.6%, phosphatidc acid (PA) 10.1%) (9).

Reactor. A glass bottle reactor (2.4 cm diameter and 4.5 cm high), placed in a jacket, was used in this study. The temperature was controlled by circulating water through the jacket. The reaction mixture was stirred at 1400 rpm with a stirrer bar (0.3 cm diameter and 1.4 cm long) and a magnetic stirrer. The reactor was tightly closed with a Teflon cap, covered with Teflon film, and the products were intermittently sampled with a syringe through a stainless pipe fitted in the cap. The top of the pipe was closed with Teflon tubing and a stop cock.

Preparation of Ca–alginate gel-enveloped PEI–glutaraldehyde. The sodium–alginate solution of desired concentration was injected into 0.1 M calcium chloride and stored for 1 h. The obtained gel was processed with PEI and glutaraldehyde of the desired concentration for 8 h at room temperature, and the calcium–alginate gel-enveloped PEI-glutaraldehyde was obtained.

Immobilization operation. A given volume supernatant of *Pseudomonas* fermentation was added to a given amount of Amberlite XAD-2,controlled-pore glass, PEI cellulose and calcium–alginate gel-enveloped PEI–glutaraldehyde, respectively, stirred for 0.5 h, then filtered, to obtain the immobilized enzyme preparations.

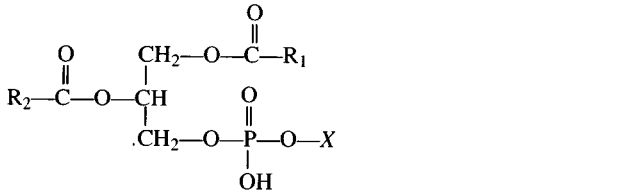
Reaction system on conversion of phosphatidylglycerol. Quantities of 0.5–1 g immobilized enzyme, 1 mg soybean lecithin, 0.4 mL pH 8.2 buffer, 0.4 mL 0.2 M $CaCl_2$, 0.16 mL 50% glycerol, 1.04 mL water, and 2 mL ether were introduced into the reactor. The mixture was stirred for 1 h at 25°C, then filtered and extracted twice with 2 mL ether. The ether phase was collected and concentrated for phospholipid analysis. The residual immobilized enzyme was added to fresh reactant, and the operation was repeated as above.

Phospholipids analysis (10). Chromarod thin-layer chromatography was performed with a flame-ionization detector (TLC/FID) (Iatroscan Analyzer TH-10, Iatron Laboratories Co., Ltd., Tokyo, Japan), connected to an integrator (Chromatorecorder SII integrator; System Instruments Co., Ltd.,

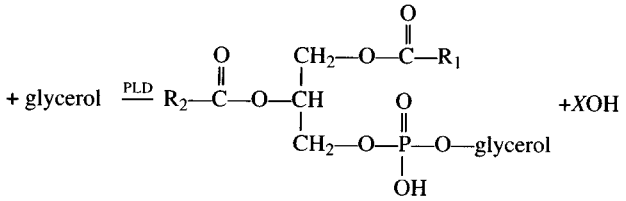
Tokyo, Japan), to analyze quantitatively the phospholipids in the reaction mixture. An aliquot (0.1 mL) of the reaction mixture was intermittently sampled and put into a test tube that contained 5 mL Folch solution (chloroform–methanol, 2:1) and 0.9 mL water. After mixing well, the solution was centrifuged for 2 min at 2500 rpm, the lower layer was collected, and the extraction was repeated twice with chloroform. The samples of the lower layers were concentrated with a vacuum rotary evaporator. The concentrated samples were spotted on the chromarod SII quartz rods coated with silica gel. Just before use, the blank rods were activated by passing them through the flame of the TLC/FID attached to the integrator. Disposable micropipettes were used for spotting. An aliquot (2 μL) of each sample, together with authentic samples, were spotted on the chromarods. The spotted rods, enclosed in a frame, were put into a glass tank that contained acetone as developing solvent. After development, the rods were activated (burned) again with chloroform–methanol–acetic acid–water (65:20:10:5). After redevelopment, the solvent was removed by putting the rods in an oven (120°C) for 15 min. The rods were then transferred to the instrument and scanned. The TLC/FID was conducted at a hydrogen gas flow rate of 180 mL/min, air flow rate of 2100 mL/min and a scan speed of 40 s per scan. The concentrations of PC, PA, PG, PE, and PI were calculated from their peak areas with the help of the integrator.

RESULTS AND DISCUSSION

Evaluation of reaction. The synthesis of PG follows:



[1]



where *X* represents the base group, such as choline or ethanolamine. The conversion of PG was calculated by the following equation:

$$\text{conversion percentage of PG} = [PG]/\{[PG] + [PC] + [PE] + \Delta[PA]\} \quad [2]$$

where [PC], [PG], and [PE] are the peak areas of PC, PG and PE, and Δ[PA] is the change in the PA peak area, in reactions measured by TLC/FID, which are proportional to their actual concentration values.

Selection of support for immobilization of phospholipase D. In the reaction system of phospholipase D catalysis, the substrate phospholipid was insoluble in water, and hydrophobic organic solvent was used. The supports with hydrophobic groups were examined with a view to improving the dispersibility of the gels. Among the supports tested for phospholipase D immobilization, such as controlled-pore glass, Amberlite XAD-2, PEI–cellulose, and calcium–alginate gel-enveloped PEI–glutaraldehyde, the calcium–alginate gel-enveloped PEI–glutaraldehyde was the most efficient for the transphospholipid reaction. Effect of immobilized support material on conversion percentage of PG—immobilized support material and conversion of PG (%), respectively: controlled-pore glass, 18; Amberlite XAD-2, 23; PEI cellulose, 48; Ca–alginate gel-enveloped PEI–glutaraldehyde, 59. The hydrophobic interaction between phospholipase D and support seemed to result in efficient immobilization. The dispersibility in the gels was also satisfactory in the reaction mixture. All further experiments were therefore carried out with phospholipase D that was immobilized in the calcium-alginate gel-enveloped PEI–glutaraldehyde.

Optimization of immobilization conditions. Conditions for phospholipase D immobilization with calcium–alginate gel-enveloped PEI–glutaraldehyde were optimized by the response surface methodology (RSA). The variable range for calcium–alginate was 1–2%, for PEI 4–8%, and for glutaraldehyde 1–2%. The test design and response values are shown in Table 1, and the results of regression and variance analysis are shown in Table 2. The regression equation is:

$$y = 54.33 + 6.00x_1 + 4.63x_2 - 2.88x_3 + 3\ .83x_1^2$$

$$- 2.42x_2^2 + 5.08x_3^2 - 1.75x_1x_2 + 0.75x_1x_3 + 6.50x_2x_3 \quad [3]$$

The variance analysis shows that the *F* value is greater than that of F0.05(9,5), so the regression equation is remarkable.

If $y/x_1 = 0$, $y/x_2 = 0$, and $y/x_3 = 0$, the regression equation is solved, and the optimal immobilizing condition is as fol-

TABLE 1
Optimal Test Design and Response Value of Immobilized PLD[a]

Number of experiment	Ca–alginate (x_1)	PEI (x_2)	GA (x_3)	Response value
1	−1 (1.0%)	−1 (4%)	0 (1.5%)	42
2	−1	0 (6%)	−1 (1.0%)	64
3	−1	0	1 (2.0%)	59
4	−1	1 (8%)	0	61
5	0 (1.5%)	−1	−1	72
6	0	−1	1	47
7	0	1	−1	54
8	0	1	1	63
9	1 (2.0%)	−1	0	59
10	1	0	−1	83
11	1	0	1	70
12	1	1	0	78
13	0	0	0	58
14	0	0	0	56
15	0	0	0	62

[a]PLD, phospholipase D; PEI, polyethyleneimine; GA, glutaraldehyde.

TABLE 2
Regression Analysis and Variance Analysis on Conversion Percentage of PG[a]

	Coefficient	Standard error	*t* value
a_0	54.33	3.88	14.02
a_1	6.00	2.37	2.53
a_2	4.63	2.37	1.95
a_3	–2.88	2.37	– 1.21
a_{11}	3.83	3.49	1.10
a_{22}	–2.42	3.49	– 0.69
a_{33}	5.08	3.49	1.46
a_{12}	–1.75	3.36	0.22
a_{13}	0.75	3.36	

lows: calcium–alginate, x_1 = 1.39%; PEI, x_2 = 7.78%; and glutaraldehyde, x_3 = 1.22%.

Effect of temperature on relative conversion percentage of phosphatidylglycerol. Figure 1 shows the effect of temperature on the relative conversion percentage of PG with immobilized phospholipase D. When the reaction time is 0.5 h, the optimal temperature is 30°C, and when the reaction time is 1 h, the optimal temperature is 25°C.

Heat stability of immobilized phospholipase D. The immobilized phospholipase D was stored for 30 min in the range of 20–60°C, and then the residual conversion percentage of PG was measured. Figure 2 is a heat-stabilizing curve of immobilized phospholipase D. The stabilizing temperature is below 40°C.

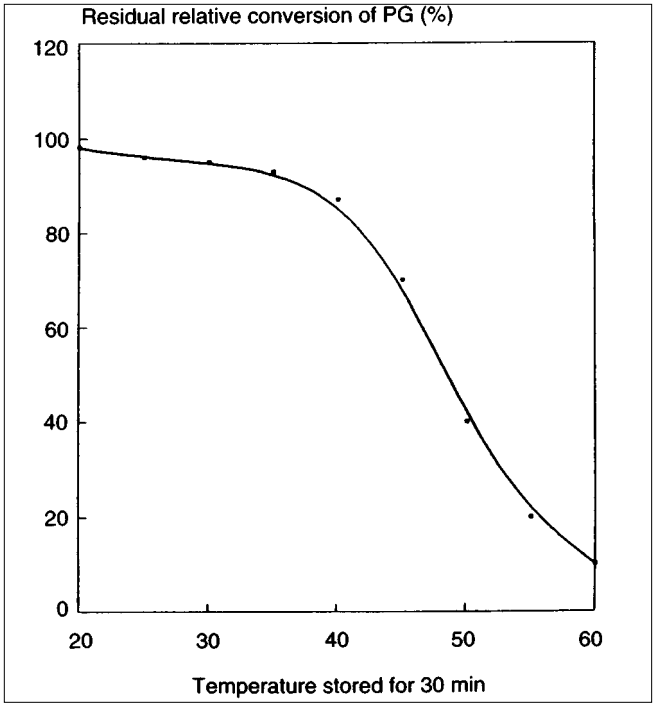


FIG. 2. Heat stability of immobilized phospholipase D. See Figure 1 for abbreviation.

Effect of pH on relative conversion percentage of PG. Figure 3 shows the effect of pH on relative conversion of

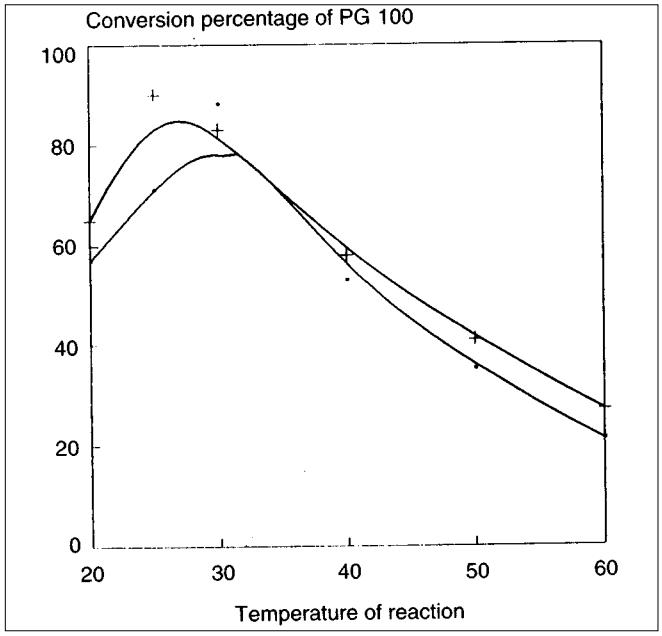


FIG. 1. Effect of temperature on conversion percentage of phosphatidylglycerol (PG); ●, reaction time 0.5 h; +, reaction time 1.0 h.

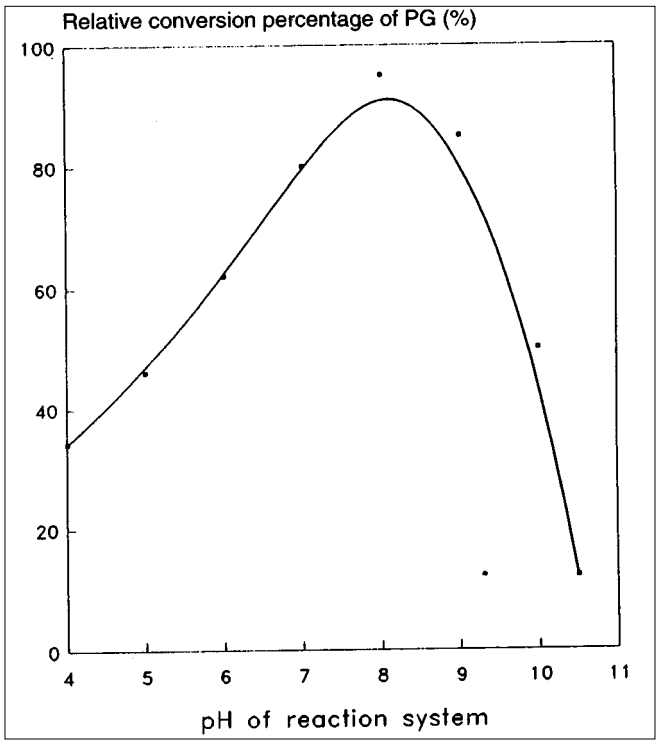


FIG. 3. Effect of pH on conversion percentage of PG. See Figure 1 for abbreviation.

PG with immobilized phospholipase D. The optimal pH is 8.2.

pH stability of immobilized phospholipase D. The immobilized phospholipase D was stored for 30 min in the range of pH 4.0–10.0, and residual conversion of PG was measured. Figure 4 shows a pH stabilizing curve of immobilized phospholipase D. Within the pH range of 6.0–10.0, the immobilized enzyme is quite stable.

Effect of organic solvent on conversion of PG. Some organic solvents were tried to evaluate their effect on the transphosphatidylation reaction with immobilized phospholipase D. The results are: kind of solvent and conversion of PG (%), respectively—ether, 85; ethyl acetate, 46; benzene, 13; hexane, 7; chloroform, 6. Ether is the most effective among tested organic solvents. Figure 5 shows the effect of ether concentration on the conversion of PG with immobilized phospholipase D. In the range of 1.5–2.5 for the ratio of ether phase and water phase, the conversion of PG is the most effective.

Repeated-batch operational stability of immobilized phospholipase D. Figure 6 shows a repeated-batch operational stabilizing curve of immobilized phospholipase D. The results show that, up to 15 batches, there was no significant decrease in the activity of immobilized phospholipase D, and the half-life of conversion is 25 batches. The significant fall in conversion of PG in the repeated-batch operation could be due to loss of some gel during removal of the spent reaction mixture, and also due to gradual inactivation of the phospholipase D.

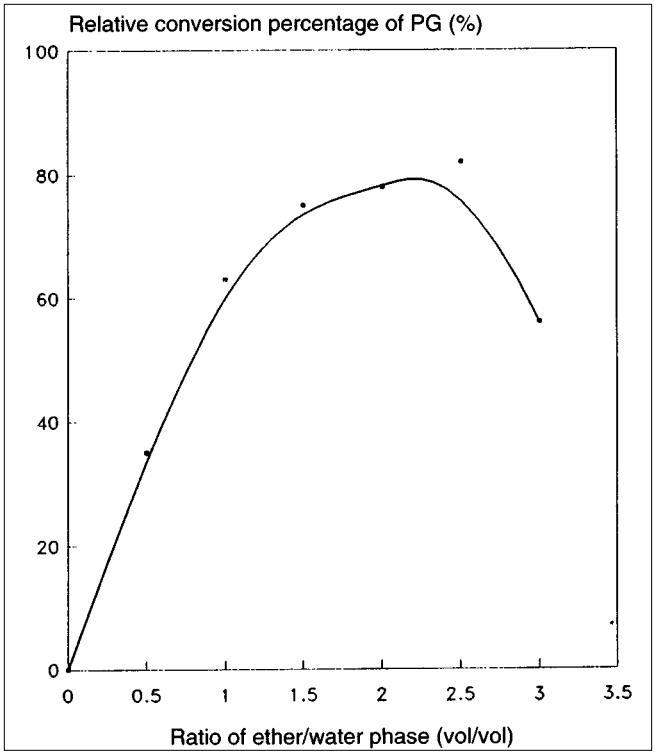


FIG. 5. Effect of ether concentration on relative conversion percentage of PG. See Figure 1 for abbreviation.

Compared with controlled-pore glass, Amberlite XAD-2 and PEI cellulose, the phospholipase D immobilized with calcium–alginate gel-enveloped PEI–glutaraldehyde was the

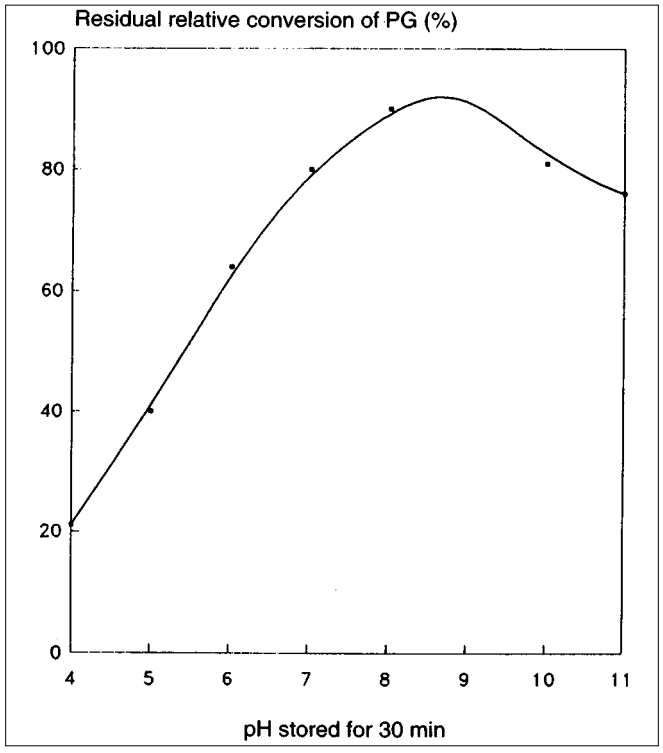


FIG. 4. The pH stabilitfy of immobilized phospholipase D. See Figure 1 for abbreviation.

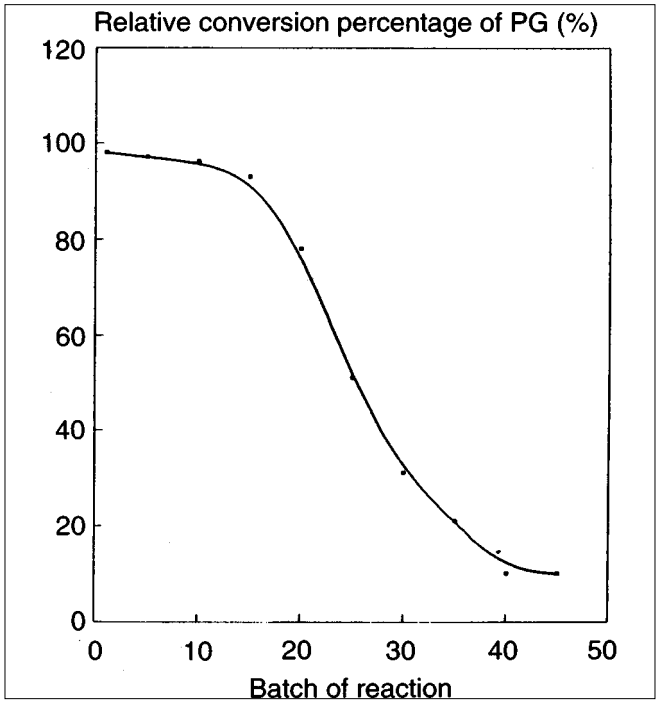


FIG. 6. Repeated batch operation stability of immobilized phospholipase D. See Figure 1 for abbreviation.

most effective in the transphospholipid reaction. The optimal conditions for the immobilized enzyme is calcium–alginate 1.39%, PEI 7.78%, and glutaraldehyde 1.22%. The reaction specifications for the immobilized enzyme are as follows: temperature 25–30°C; pH 8.2; and ratio of ether phase and water phase 1.5–2.5:1 (vol/vol).

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