

Production of Lysophospholipid Using Extracellular Phospholipase A₁ from *Serratia* sp. MK1

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For the efficient production of lysophospholipid the hydrolysis of phospholipid using phospholipase A₁ from *Serratia* sp. MK1 was studied in an aqueous-solvent, a two-phase and an emulsion system. Judged on the basis of productivity and the degree of hydrolysis, the yield of lysophospholipid in a two-phase system was found to be better than that obtained in an emulsion system. Among the 13 organic solvents tested phospholipase A₁ showed the most efficient catalytic activity and stability in butyl acetate. When 20% phospholipid was used it was completely hydrolyzed in this two-phase system.

Lysophospholipids have been widely studied as novel emulsifying agents, pharmaceutical agents and food preservatives. Since lysophospholipids have several physiological functions, such as fat digestion in the animal duodenum, cell fusion in eucaryotic organisms, cell autolysis by solubilizing the bacterial membrane (7), natural biosurfactants with low toxicity, gentle solubilizing agents (2) and synergistic effects with other surfactants (3), and the inhibition of prostaglandin (6), the research on them has been of special interest.

For the synthesis of biologically useful lysophospholipids with a desired fatty acid, an easy and simple synthetic approach is needed. In conventional synthetic methods of lysophospholipids, the use of high pressure and temperature for the fat hydrolysis results in high cost and undesired side reactions.

Several enzymes are currently used for the synthesis of lysophospholipid with a desired fatty acid. Until recently, because lipases are rather less expensive than phospholipases, and have a broad substrate specificity they have often been used to hydrolyze phospholipid. Phospholipases, however, have site specificity and fatty acid specificity and are actually preferable to lipases. It is difficult to obtain phospholipase A₁ and A₂ in large quantities. Attempts have been made to screen mi-

croorganisms which produce phospholipase A₁ and/or A₂.

Our laboratory recently reported the isolation of a microorganism, *Serratia* sp. MK1 (5) which produces extracellular phospholipase A₁.

In this study, the feasibility of phospholipid hydrolysis was examined using the extracellular phospholipase A₁ from *Serratia* sp. MK1 in a two-phase system and an emulsion system. A comparative study was carried out in these two systems.

MATERIALS AND METHODS

Chemicals

Phosphatidylcholine (PC, 99% purity), lysophosphatidylcholine (LPC, 99% purity) and fatty acid (oleic acid, 99% purity) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and used as standards. Phospholipon 90G (90% phosphatidylcholine) which was used as a substrate for the enzyme reaction was supplied by Natterman Chemie (Köln, Germany). All other chemicals were of reagent grade.

Microorganism

The microorganism used in this study was *Serratia* sp. MK1, isolated from soil by our group (5). *Serratia* sp. MK1 was cultured on a medium composed of 15.12 g Na₂HPO₄·12H₂O, 3.0 g KH₂PO₄, 1.143 g (NH₄)₂HPO₄, 0.5 g NaCl, 0.246 g MgSO₄·7H₂O, 0.0147 g CaCl₂·2H₂O, 0.556 mg FeSO₄, 5.0 g fructose per 1-liter water at 30°C for 14 h with reciprocal shaking (170 rpm).

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Enzyme Preparation

The culture supernatant was obtained by centrifugation ($5,600 \times g$, 4°C for 15 min) of the culture broth of *Serratia* sp. MK1 and this was used as a catalyst for the production of lysophospholipid.

Two-phase System

The hydrolysis reaction was carried out in a two-phase aqueous-solvent system, using a 50 ml glass bottle reactor with stirring (400 rpm). The temperature was controlled at 40°C by a thermomixer and the pH of the aqueous phase was adjusted to pH 8.0 by using 0.1 M Tris-HCl buffer. The reaction mixture was composed of phospholipase A₁ (32 Units), CaCl₂ solution (10 mM final concentration) in 10 ml aqueous phase and phospholipid (Phospholipon 90G) in 10 ml solvent phase. The reactor was tightly closed with a Teflon plug.

Emulsion System

Phospholipid emulsion was prepared by emulsifying Phospholipon 90G, sodium deoxycholate (2.6 mM final concentration) in 0.1 M Tris-HCl buffer using a sonicator. The reaction was started by the addition of CaCl₂ and the enzyme solution. Other reaction conditions were the same as those for the two-phase system.

TLC-FID Analysis

In order to analyze the phospholipid, lysophospholipid and free fatty acid, the reaction mixture was extracted with Folch solution (chloroform: methanol = 2:1, v/v) and the extract was analyzed using a thin layer chromatography-flame ionization detector (TLC-FID, Iatron MK-5; Iatron Laboratories, Tokyo, Japan). The solvent extract was applied to the chromarod SIII quartz rods (Iatron Laboratories, Tokyo, Japan). It was developed with chloroform: methanol: acetic acid: water (25:15:4:2, v/v/v/v). After development, each separated lipid component was analyzed. Results are expressed as a percentage of peak areas. Air flow rate and hydrogen flow rate were adjusted to 2.0 l/min and 160 ml/min, respectively.

pH-stat Method

Phospholipase activity was assayed by titrating the free fatty acids released by hydrolysis of the substrate (Modification of the method of Dennis (1)) with 10 mM NaOH using a Fisher Titration set consisting of a Model 380 pH meter, Model 383 titrator and Model 395 burette/dispenser. Egg yolk lecithin emulsion was prepared by emulsifying 2.68% (w/v) egg yolk lecithin for 5 min at maximum speed in a Waring blender. Before enzyme assay, 1 ml of 0.3 M CaCl₂ and 78 mM sodium deoxycholate solution were added to 28 ml of lecithin emulsion. For the enzyme assay, emulsion was vigorously mixed with the enzyme solution with stirring in a 50 ml glass vessel for 10 min at 40°C , pH 8.0. One unit of phospholipase activity was defined as 1 μmole of fatty acid equivalent released per min under assay conditions.

RESULTS AND DISCUSSION

Comparison of a Two-phase System, with an Emulsion System

The culture supernatant of phospholipases was harvested at an early stationary phase because the pattern of phospholipase production from *Serratia* sp. MK1 showed growth associated, as is characteristics of many bacterial exoenzymes (4, 5). According to our optimization procedure, culture medium was optimized, the culture supernatant was directly used as an enzyme source and the enzyme activity was 32 units/ml; that was sufficient to act as reaction catalyst (5).

For the detection of reaction products, standard materials (99% purity) of PC, LPC and oleic acid were assayed by the TLC-FID method. When the phospholipid as a substrate was completely hydrolyzed, the peak area of lysophospholipid reached 60% and the rest of the peak area, 40%, was fatty acid.

To compare the production of lysophospholipid of the two-phase system with that of the emulsion system, the reaction was carried out in a reactor containing 250 mg phospholipid for 90 min. As shown in Fig. 1, the phospholipid in the two-phase system was more rapidly hydrolyzed by the enzyme than that dispersed in the emulsion system. In 15 min the peak area of lysophospholipid reached 52% in the two-phase system but only 15% in the emulsion system. One of the reasons for this difference is that the phospholipid is more soluble in the two-

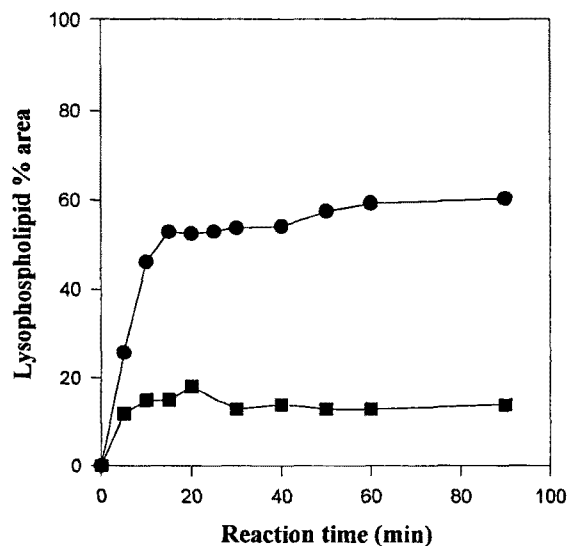


Fig. 1. Comparison of a two-phase system and an emulsion system for the production of lysophospholipid. (●), Two-phase system; (■), Emulsion system. Phospholipid quantity was 250 mg in a two-phase system and an emulsion system, respectively. Butyl acetate was used as a solvent phase in the case of two-phase system.

phase system than in the aqueous system. The other reason is the phospholipid molecule at the interface of the two phase system is more accessible to the enzyme than in the emulsion system. Consequently, the two-phase system was better than the emulsion system in terms of the productivity and degree of hydrolysis.

The Effect of Various Organic Solvents in a Two-phase System

In order to select a suitable organic solvent for the two-phase system, various organic solvents were examined. The effect of the solvent on the phospholipase A₁ for lysophospholipid production in a two-phase system was studied by measuring phospholipase catalytic activity and stability of the enzyme.

As shown in Fig. 2, hydrophilic solvents showed a higher degree of hydrolysis; butyl acetate in particular completely hydrolyzed substrate within 60 min. This result also indicates that hydrophobicity of the solvents affects the production of lysophospholipid. Especially at log P values above 2.90, lysophospholipid production was particularly severely prohibited mainly because of the insolubility of the phospholipid.

The stability of phospholipase A₁ in solvent was determined by measuring the residual activity of the enzyme after thoroughly mixing in solvent at 40°C. Phospholipase A₁ was added to 0.1 M Tris-HCl buffer (pH 8.0) and solvent (40 ml), and agitated at 400 rpm at 40°C

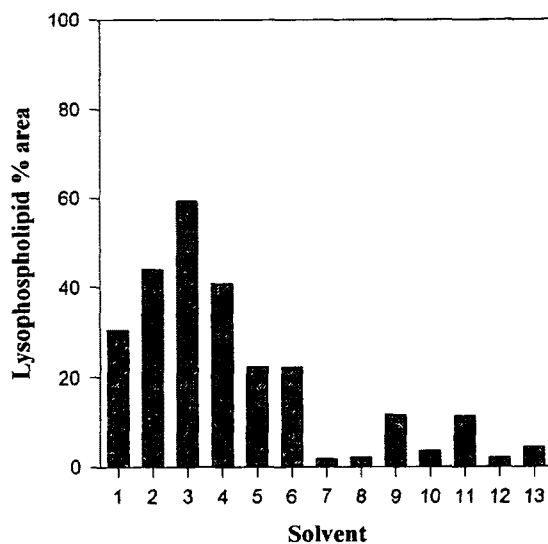


Fig. 2. Effect of various organic solvents in a two-phase system.

Phospholipid concentration was 2.5% in solvent phase. Reaction accomplished within 60 min. 1, Ethyl acetate (0.64); 2, Diethyl ether (0.88); 3, Butyl acetate (1.70); 4, Isopropyl ether (1.90); 5, Benzene (2.00); 6, Chloroform (2.14); 7, Butyl ether (2.90); 8, Cyclohexane (3.20); 9, Hexane (3.52); 10, Heptane (4.05); 11, Isooctane (4.80); 12, Decane (5.60); 13, Dodecane (6.60). Numbers in parentheses designates log P value.

in a stirred reactor. After stopping the agitation at predetermined time intervals, 100 μ l of the phospholipase A₁ solution was taken out from the aqueous phase. This enzyme solution was added to an emulsion solution of the phospholipid. The residual activity of phospholipase A₁ was determined by the pH-stat method.

As shown in Fig. 3, of the solvents tested, butyl acetate showed the best residual activity. Most of the solvents, however, deactivated the phospholipase A₁ below the 40% level within 60 min.

Considering the above results, it appears that hydrophilic solvents such as butyl acetate and diethyl ether dissolved the phospholipid very well, because phospholipid has amphiphilic properties; the reaction was therefore faster in the hydrophilic than in the hydrophobic solvents. Taking into consideration of substrate solubility and enzyme stability, butyl acetate was decided upon as the best solvent for batchwise hydrolysis of phospholipid in a two-phase system.

Optimal Substrate Concentration in the Organic Solvent Phase

In phospholipid hydrolysis, it is natural that the degree of hydrolysis and substrate concentration should be increased in order to make the separation step easy and to get a better quality product. The hydrolysis was therefore measured. The substrate concentration, which maximizes the degree of hydrolysis is shown in Fig. 4. At constant speed of agitation, pH, temperature, and ratio of solvent to aqueous phase, the hydrolysis of phospholipid by the

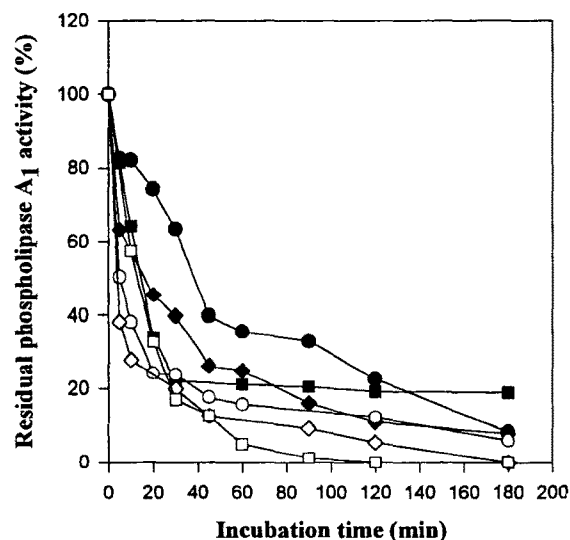


Fig. 3. Stability of phospholipase A₁ in the organic solvents. After incubation of phospholipase A₁ with each of the solvents for 5, 10, 20, 30, 45, 60, 90, 120, 180 min at 40°C, residual activity was determined by pH-stat method. Each symbols represented as follows; (●), Butyl acetate; (◆), Benzene; (■), Diethyl ether; (○), Ethyl acetate; (◇), Cyclohexane; (□), Butyl ether.

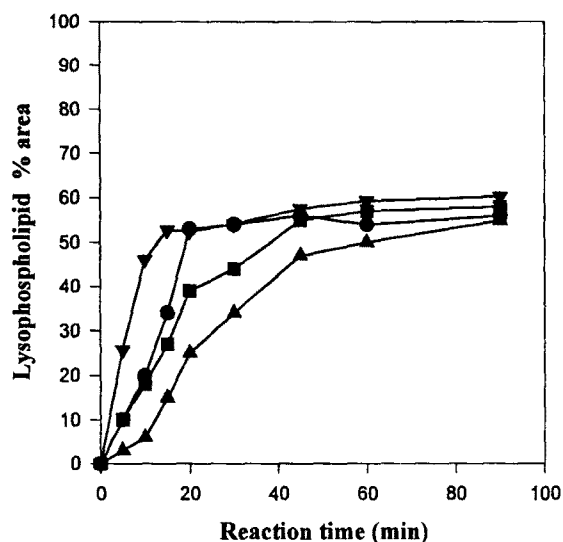


Fig. 4. Effect of phospholipid concentration in butyl acetate on the lysophospholipid production. Phospholipid concentration was as follows; 2.5% (▼), 6% (●), 10% (■), 20% (▲) in solvent phase.

phospholipase A₁ was measured at various phospholipid concentrations in 10 ml butyl acetate. We tried to use different concentration of substrate in the range of 2.5~30%; above 20%, it was very difficult to dissolve the substrate completely. We therefore decided to take 20% as the maximum. In this two-phase system, a large yield of pro-

duct could be obtained using a high concentration of phospholipid.

REFERENCES

1. Dennis, E. A. 1973. Kinetic dependence of phospholipase A-2 activity on the detergent triton X-100. *J. Lipids Res.* **14**: 152-159.
2. Dufour, J. and A. Goffeau. 1978. Solubilization by lysolecithin and purification of the plasma membrane ATPase of the yeast *Schizosaccharomyces pombe*. *J. Biol. Chem.* **253**: 7026-7032.
3. Fujita, S. and K. Suzuki. 1990. Surface activity of the lipid products hydrolyzed with lipase and phospholipase A₂. *JAOCS.* **67**: 1008-1014.
4. Givskov, M., L. Olsen, and S. Molin. 1988. Cloning and expression in *Escherichia coli* of the gene for extracellular phospholipase A₁ from *Serratia liquefaciens*. *J. Bacteriol.* **170**: 5855-5862.
5. Kim, M. K., J. K. Kim, and J. S. Rhee. 1996. Isolation of a phospholipase A₁-producing microorganism. *J. Industrial Microbiol.* **16**: 171-174.
6. Shier, W. T. 1977. Inhibition of prostaglandin synthesis by lysolecithin. *Biochem. Biophys. Res. Commun.* **78**: 1168-1174.
7. Tsuchido, T. 1994. Induction of cell autolysis of *Bacillus subtilis* with lysophosphatidylcholine. *Appl. Microbiol. Biotechnol.* **41**: 106-109.

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