# Thermophilic Phospholipase $A_2$ in the Cytosolic Fraction from the Archaeon *Pyrococcus horikoshii* [1]

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**ABSTRACT:** Hyperthermophilic archaeon *Pyrococcus horikoshii* produced phospholipase $A^2$ in a cytosolic fraction. The enzyme displayed optimal activity at 90°C and pH 7 and preferentially hydrolyzed *sn*-2 fatty acids in the following order: linoleoyl > oleoyl > arachidoyl phosphatidylcholine. Phospholipase $A_2$ had similar activities toward L-α-1-palmitoyl-2-arachidoyl derivatives of phosphatidylcholine and phosphatidylethanolamine. Phospholipase $A_2$ activity was unaffected by the addition of 0.0001–1 mM calcium, but was inhibited slightly by the addition of 2–10 mM calcium. The activity was enhanced more than 5–18-fold in the presence of 3–20% (vol/vol) glycerol. The activity was unaffected by the addition of 1–5 mM EDTA or 0.01–20 mM dithiothreitol. A caldarchaetidic acid derivative having a molecular weight of 1544 disappeared upon incubation of the cytosolic fraction with total lipid. The phospholipase $A_2$ was difficult to purify by general chromatography because it existed as an aggregate. Electrophoresis was carried out using 10–15% polyacrylamide gels containing sodium dodecyl sulfate (SDS-PAGE). No activity of phospholipase $A_2$ activity was observed in the absence of proteins less than 19 kD in size, as fractionated by SDS-PAGE.

Paper no. J9694 in *JAOCS 77*, 1147–1152 (November 2000).

**KEY WORDS:** Caldarchaetidic acid, hyperthermophile, phospholipase $A_2$, *Pyrococcus horikoshii*, thermostable enzyme.

The hyperthermophilic archaea *Pyrococcus horikoshii* and *Aeropyrum pernix* grow optimally at more than 90°C (1,2) and produce many interesting thermophilic enzymes (3–5). Total genome sequences have been analyzed (6–9), and base sequence data for them are available at http://www.mild.nite.go.jp. We are studying thermophilic phospholipase $A_2$ (PLA2), which is interesting to basic science as well as having technological applications for use in the degumming process of oil refineries. PLA2 plays a role in signal transduction, eicosanoid and platelet-activating factor formation, membrane remodeling, and general lipid metabolism in prokaryotes and eukaryotes (10). However, nothing is known about PLA2 from hyperthermophilic archaea.

The membrane of *P. horikoshii* contains predominantly tetraether lipids, with a small proportion of diether lipids (1). These ether lipids are *sn* glycerol-1-phosphate ethers, but the substrate of phospholipase is *sn* glycerol-3-phosphate esters. There is no motif of PLA2 in the total genome sequence from *P. horikoshii* (6,7), but PLA2 activity was observed in the broths from *P. horikoshii* and *A. pernix* (11). This study characterized PLA2 activity from *P. horikoshii*.

## MATERIALS AND METHODS

*Microorganism. Pyrococcus horikoshii* (JCM number 9974) was obtained from the Japan Collection of Microorganisms (Wako-shi, Japan) and cultured using the reported method (1) modified as follows. The medium, containing 13.5 g of NaCl, 4 g of $Na_2SO_4$, 0.7 g of KCl, 0.2 g of $NaHCO_3$, 0.1 g of KBr, 30 mg of $H_3BO_3$, 10.8 g of $MgCl_2{\cdot}6H_2O$, 1.5 g of $CaCl_2$, 25 mg of $SrCl_2$, 1 mL of Resazusin stock solution (0.2 g/L), 1 g of Bacto yeast extract (Difco, Detroit, MI), and 5 g of Bactotrypton (Difco) in 1 L, was autoclaved for 15 min. The organism was cultured at 95°C in a stationary culture in a 1-L medium bottle of Keio University Type (Ikemoto Rika Co., Tokyo, Japan). The working volume was 800 mL and the bottle was protected by plastic film to retain the hot medium in case of breakage. After autoclaving, 0.1% (wt/vol) powdered sulfur (sterilized by drying at 95°C for 3 d) was added. Carbon dioxide was passed through the medium for 3 min and finally, anaerobic conditions were completed by adding 10 mL of 10% (wt/vol) $Na_2S{\cdot}9H_2O$ solution until the color of the medium changed from pink to colorless and the pH of the medium changed to around 6.8. Then 1% of the seed culture was inoculated and cultured at 95°C for 2–4 d.

*Reagents.* L-α-1-Palmitoyl-2-[1-$^{14}C_1$]linoleoyl phosphatidylcholine (PLPC), L-α-1-palmitoyl-2-[1-$^{14}C_1$]oleoyl phosphatidylcholine (POPC), L-α-1-palmitoyl-2-[1 $^{14}C_1$]-arachidoyl phosphatidylcholine (PAPC), and L-α-1-palmitoyl-2-[1-$^{14}C_1$]arachidoyl phosphatidylethanolamine (PAPE) were

[1]Portions of this article were presented at the Biocatalysis Symposium at the 91st Annual Meeting and Expo of the American Oil Chemists' Society in San Diego, CA, April, 2000.

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supplied by NEN™ Life Science Products, Inc. (Boston, MA), 1-hexadecanoyl-2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoyl]-*sn*-glycero-3-phosphocholine (NBD-PC) and *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoic acid (NBD-acid) were obtained from Sigma-Aldrich, Japan (Tokyo, Japan). *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-hexadecanoyl-*sn*-glycero-3-phosphoethanolamine (NBD-PE) was supplied by Molecular Probes, Inc. (Eugene, The Netherlands). Crude extract of soybean was provided by Showa Sangyo (Kashima, Japan). The oil was extracted from soybean with hexane and not dewaxed. Lecitase 10L was provided by Novo Nordisk, Japan (Chiba, Japan) containing 10,000 IU/mL.

*Preparation and localization of PLA2*. Cells (wet weight 20 g) were suspended in 100 mL of 10 mM HEPES buffer (pH 7.5) containing 0.34 M sucrose, 0.1 mM dithiothreitol, 1 mM ATP, 1 mM phenylmethanesulfonyl fluoride, and 1 mM EDTA and disrupted by sonication for 10 min in an ice bath, using an ultrasonic sonifier 2N-700 (Toyo Industrial Manufacturer, Tokyo, Japan). The resulting mixture (cell lysate) was centrifuged at 6,000 × *g* for 20 min at 4°C. Maximal (80–90%) activity was detected in the supernatant. The supernatant was further centrifuged at 100,000 × *g* for 60 min at 4°C. The membrane-bound fraction precipitate was resuspended in 50 mM Tris-HCl, pH 8.0 to check the activity. The supernatant (cytosolic fraction) was concentrated using a Centriprep YM 10 (Nihon Millipore Ltd., Tokyo, Japan) and used as the PLA2 enzyme preparation.

*Enzyme assays.* Labeled phospholipid (3 nmol) was incubated at 90°C with 30 µg of the PLA2 enzyme preparation in a 250-µL reaction mixture for 60 min. The reaction was terminated by cooling the mixture in an ice bath. The labeled phospholipid and product were extracted by the method of Bligh and Dyer (12) and resolved on silica gel thin-layer chromatography (TLC) (Kieselgel 60 $F_{254}$, Merck, Tokyo, Japan) using a solvent system of chloroform/methanol/water (35:65:4, by vol). Reaction mixtures were visualized and quantified using PhosphorImagine (Nihon Bio-Rad Ltd., Yokohama, Japan). PLA2 activity was expressed as the difference between the activities in the presence and absence of the PLA2. Protein concentration was measured using a Nihon BioRad protein assay system.

The PLA2 assay using NBD-PC was done by modified methods of Moreau (13) and Kleuser *et al.* (14). The substrate, 100 µL of NBD-PC (100 µg/mL of chloroform) was placed in a 2-mL vial and the solvent was removed in a stream of nitrogen. Then, 0.5–1 mL of the cell lysate and 0.1 mL of 25 mM sodium phosphate buffer at pH 7.0 were incubated at 90°C for 40–500 min in the vial. The reaction was terminated by chilling at 0°C. The product was extracted with 3 mL of ethyl acetate/acetone (2:1, vol/vol) and 10 µL of NBD-PE by vigorous shaking. After centrifugation for 10 min at 2,600 × *g*, the upper phase was collected. The solvent from the upper phase was removed by nitrogen bubbling and the residue was dissolved in 150 µL of methanol/chloroform (1:1, vol/vol). The dissolved samples (100 µL) were used for high-performance liquid chromatography (HPLC) analysis. The samples were eluted with the solvent chloroform/methanol/water/acetic acid (500:250:45:45, by vol) at a flow rate of 1.5 mL/min on Partisil-5 (4.6 × 250 mm) and LiChrosorb SI-100 (4.6 × 150 mm) columns obtained from GL Science Inc. (Tokyo Japan). Fluorescence was monitored at 450 nm excitation and 510 nm emission using a fluorescence HPLC monitor (Shimadzu RF535; Kyoto Japan). The retention times of NBD-acid, NBD-PE, and NBD-PC were 3.5, 5.5, and 8.5 min, respectively.

The phospholipase $A_1$ (PLA1) activity was measured by quantitating the amount of labeled lysophosphatidylcholine produced. Lysophospholipase activity was measured using L-1[palmitoyl-1-$^{14}$C]-lysopalmitoyl phosphatidylcholine (NEN™ Life Science Products, Inc.).

*Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE ) fraction of PLA2.* Approximately 60 µg of cell lysate was fractionated by 10–15% SDS-PAGE. The gel was then cut into 3- to 5-mm gel pieces, and proteins were eluted from the gel by a modified Hager-Burgess method (15). The eluted proteins were precipitated with acetone and redissolved without adding 6 M guanidine hydrochloride. The activity of the fractions was analyzed by the omission method; one piece of gel was omitted, and the activity of the eluted protein from all other parts of the gel was assayed.

*Total lipids from* P. horikoshii. Total lipids were extracted from cells (50 g) by processing with alkaline solvents and sonication (16). The cells were washed with distilled water and suspended in 100 mL of methanol. After addition of 50 mL of methanol, the mixture was sonicated for 20 min and centrifuged at 4,500 × *g* for 20 min. The supernatant was transferred to a recovery flask. The precipitated cell debris was suspended in 300 mL of chloroform/methanol/1 M $NH_4OH$ (1:2:0.5, by vol) and sonicated for 20 min. Each suspension was centrifuged and the supernatant was combined with those obtained previously. The extraction was repeated three times. Solvent was removed from combined supernatants with a rotary evaporator at 40°C. The residual lipids were dissolved by adding 50 mL of chloroform/methanol (2:1, vol/vol) and 5 mL of water, and methanol was added to the mixture until the solution was clear. The mixture ("total lipids") was used as the substrate of PLA2 and spotted on Silica gel 60, 1 mm (Merck) TLC plates. The developing mixture was chloroform/methanol/2-propanol/triethylamine/0.25% KCl (30:9:25:18:4.5). First, the chromatogram was sprayed with the lipophilic fluorochrome 1,6-diphenyl-1,3,5-hexatriene. Then, specific visualization of phospholipid was achieved as a clear band by phospholipid staining with 1,6-diphenyl-1,3,5-hexatriene and molybdeum blue (17). The polar phospholipid fraction was eluted from the thin-layer plate with chloroform/methanol (1:1, vol/vol) and used for mass spectrometry analysis.

*Mass spectrometry analysis.* Phospholipids were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and post-source decay (PSD) fragment spectra in MALDI-TOF-MS. A KOMPACT

MALDI IV instrument with a curve field reflection (Shimadzu) and 2,5-dihydroxybenzoic acid matrix were used as reported previously (18). In the positive mode measurements, NaCl solution was added to the sample to assist the formation of sodium adducts. PSD fragment spectra were measured under the positive mode condition. The molecular ion $[M + Na]^+$ was selected as the precursor ion.

*Oil degumming using PLA2.* Degumming was carried out in a well-sealed Erlenmeyer flask with shaking at 70°C for 48 h. After the reaction, 0.1 g of activated clay was added, the mixture was centrifuged at 2,500 × *g* for 20 min, and 0.5 g of $Mg(NO_3)_2$ was added to the supernatant in a crucible. The oily substance was burned and heated in an electric oven maintained at 800°C for 1 h. The phosphorus content was assayed according to the American Oil Chemists' Society official method Ca 12-55 (19).

## RESULTS AND DISCUSSION

*Occurrence of PLA2 in the cytosolic fraction.* A cell lysate of *P. horikoshii* containing PLA2 was prepared by sonication, followed by centrifugation at 6,000 × *g* to remove cellular debris. The bulk (98%) of the total activity was in the supernatant. When the supernatant was centrifuged at 100,000 × *g* for 1 h to obtain a membrane fraction and a cytosolic supernatant, most of the activity (87.2%) was in the cytosolic supernatant.

Labeled phospholipid was incubated at 90°C in the absence and presence of the cell lysate (Fig. 1). The main product of enzyme action was a labeled free fatty acid, the predicted product of PLA2 action. Labeled lysophospholipid, which is a product of PLA1, was also formed in very small quantities. This pattern of activity is consistent with the presence of a PLA2. Note that with our methods it is not possible to rule out the presence of an enzyme possessing joint PLA1 and PLA2 activity.

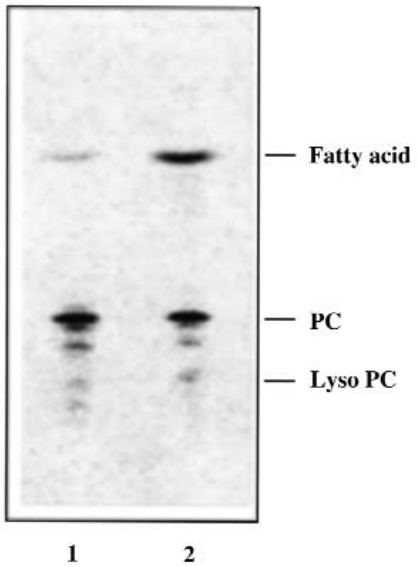


**FIG. 1.** Phospholipase activity of cell lysate from *Pyrococcus horikoshii*. The substrate, L-α-1-palmitoyl-2-[1-$^{14}C_1$]linoleoyl phosphatidylcholine (3 nmol), and cell lysate from *P. horikoshii* (lane 1: 0 μg; lane 2: 90 μg) were incubated at 90°C for 60 min. The products were separated by silica gel thin-layer chromatography by using chloroform/methanol/water (35:65:4, by vol) and detected by PhosphorImagine (Nihon Bio-Rad Ltd., Yokohama, Japan). The positions of phosphatidylcholine (PC), lysophosphatidylcholine (lyso-PC), and fatty acid are indicated.

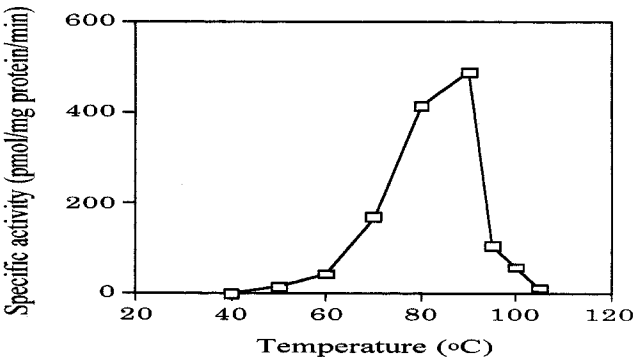


**FIG. 2.** Effect of temperature on hydrolysis of 1-hexadecanoyl-2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoyl]*sn*-glycero-3-phosphocholine (NBD-PC) in 40 mM phosphate buffer at pH 7 for 40 min.

*Properties of PLA2 from* P. horikoshii. The PLA2 activity was measured using NBD-PC, which is a sensitive fluorescent substrate that is stable at 95°C (11). Released NBD-acid was separated from the substrate NBD-PC and an internal standard of NBD-PE using HPLC. PLA2 from *P. horikoshii* reacted optimally at pH 7 and 90°C (Figs. 2 and 3). PLA2 retained more than 95% activity for 80 min at 80°C, pH 7.5, and more than 85% activity at 90°C, pH 7.5. Substantial inactivation (65%) occurred at 100°C, pH 7.5. No fatty acid was produced by the cell lysate when trimyristin, tristearin, or triolein was incubated at 90°C overnight. Therefore, no thermostable lipase activity was observed in the PLA2 from *P. horikoshii*. PLA2 activity at 90°C was unaffected by the addition of 0.0001–1 mM calcium, and the activity at 90°C was inhibited slightly by the addition of 1–10 mM calcium (Fig. 4). PLA2 activity at 90°C was enhanced more than 5–18-fold in the presence of 3–20% (vol/vol) glycerol (Fig. 4). PLA2 activity

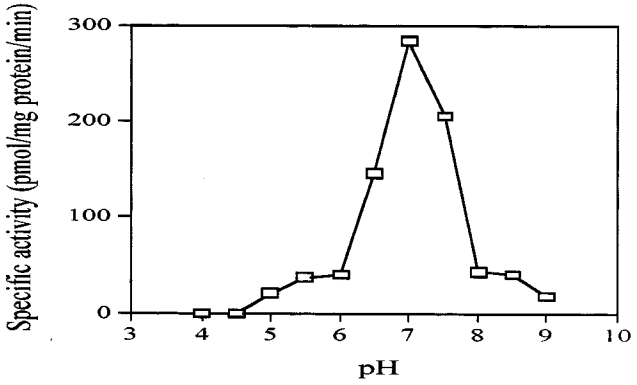


**FIG. 3.** Effect of pH on hydrolysis of NBD-PC at 90°C for 40 min. See Figure 2 for abbreviation.

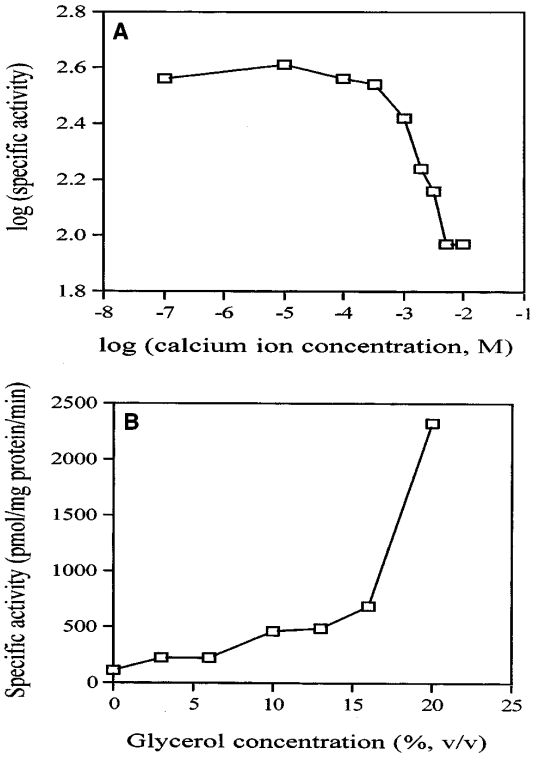


**FIG. 4.** Effect of (A) calcium on the hydrolysis of NBD-PC in 50 mM Tris buffer at pH 8 and 90°C and (B) glycerol in 40 mM phosphate buffer at pH 7 and 90°C. See Figure 2 for abbreviation.

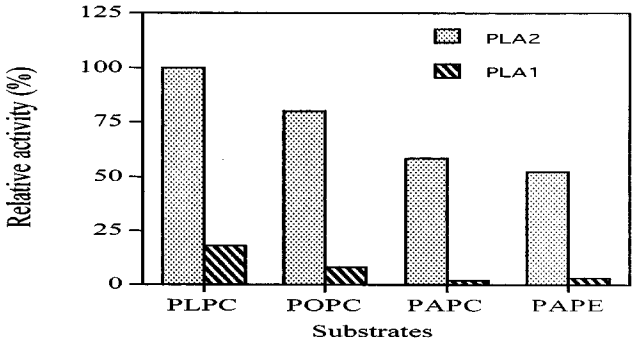


**FIG. 5.** Substrate specificity of phospholipase $A_2$ (PLA2) from *Pyrococcus horikoshii*. The reaction mixture contained 40 mM of phosphate buffer at pH 7.0, 3 nmol of substrate, and 30 μg of PLA2 in a total volume of 0.25 mL. After incubation at 90°C for 40 min, radioactive labeled products were assayed. Radioactivity in the fatty acid fraction or lysophospholipid fraction was expressed as a percentage of total radioactivity recovered. All data are corrected for nonenzymatic release of total label, which was less than 3% in all experiments. L-α-1-Palmitoyl-2-[1-$^{14}C_1$]linoleoyl phosphatidylcholine (PLPC), L-α-1-palmitoyl-2-[1-$^{14}C_1$]oleoyl phosphatidylcholine POPC, L-α-palmitoyl-[1-$^{14}C_1$]arachidoyl phosphatidylcholine (PAPC), and L-α-1-palmitoyl-[1-$^{14}C_1$]arachidoyl phosphatidylethanolamine (PAPE) are the labeled substrates indicated in the Materials and Methods section. PLA2 activity was detected by radioactivity in the fatty acid fraction and phospholipase $A_1$ (PLA1) activity was detected by radioactivity in the lysophospholipid fraction.

was unaffected by the addition of 1–5 mM EDTA or 0.01–20 mM dithiothreitol. Hydrolysis of phosphatidylcholine by the PLA2 from *P. horikoshii* occurred preferentially in the following order: PLPC > POPC > PAPC (Fig. 5). PLA2 had similar activities toward PAPC and PAPE. PLA1 activity from *P. horikoshii* had the same fatty acid specificity (Fig. 5). Lysophospholipase activity of *P. horikoshii* was also observed at an optimum pH of 6–7. These specificities are different from the previously reported cytosolic PLA2, which selectively hydrolyzes arachidonic acid esters (20).

*Reaction with lipid from* P. horikoshii. The total lipid from *P. horikoshii* cells was extracted with a weak alkaline solvent using sonication (16). The total lipid was incubated for 12–24 h with *P. horikoshii* cytosolic fraction and fractionated by TLC. A polar lipid (polar lipid M) at $R_f$ value 0.6 in the thin-layer chromatogram was consumed by the concentrated cytosolic fraction. Polar lipid M also was consumed in a Tris-HCl buffer only, but the reaction product pattern was different (data not shown). In negative mode MALDI-TOF mass spectrometry of polar lipid M eluted from the TLC plates, a single peak at 1543 Da of the compound $[M–H]^-$ was observed. In positive mode MALDI-TOF-MS of polar lipid M, two peaks at 1567 Da and 1589 Da were observed. The lipids in the positive mode correspond to the chemical species of single and double sodium adducts; $[M + Na]^+$; $Na^+$ (23 Da) and $[M – H + 2Na]^+$ ; $2Na^+$ (46 Da). These results indicate that the molecular weight of polar lipid M was 1544 Da, and the positive mode MALDI-MS confirmed that the compound had a negative charged group such as $O=P(O^-)O^-$. Fragments of polar lipid were observed at 178 Da, 239 Da, and 62 Da in positive mode PSD fragment MALDI-TOF-MS, corresponding to inositol ($C_6H_{12}O_6$; 180 Da), inositol phosphate ($C_6H_{12}O_6$-PO-OH; 244 Da), and phosphate (-PO-OH; 64 Da). The above-mentioned mass fragment peaks are coincident with these molecular parts, within the spectral experimental error. Polar lipid M may be a derivative of sugar alcohol and caldarchaetidic acid [phosphate derivative of tetraether or tetra-*O*-di(diphytanyl) diglycerol phosphate]. The major lipid in the *Pyrococcus* genus is 2,3-di-*O*-phytanyl-*sn*-glycero-1-phosphoryl-1-*myo*-L-inositol (21). The core lipid of *P. horikoshii* is 1299 Da and is estimated to be a caldearchaeol [tetraether core lipid or tetra-*O*-di(diphytanyl) diglycerol phosphate] derivative having inositol phosphate on both sides of the lipid backbone (16).

*Oil degumming by PLA2.* Thermostable PLA2 has attracted much attention for industrial use, such as in the degumming process of oil refineries to reduce wastewater problems and running costs. However, the present PLA2 from porcine pancreas requires the degumming process to be performed at 65°C. After the reaction, the separation of lysophosphatide from the oil is done at 75°C (22). The total operational costs for neutralization and soapstock splitting are estimated to be $9.19 per metric ton and the costs of the enzymatic degumming process are estimated to be $5.27 per metric ton (23). Therefore, $452 million in refining costs for soybean oil may be saved if total refining of world production [115,424 thousand metric ton/yr in 1997 (24)] is con-

**TABLE 1**
**Degumming of Crude Soybean Oil at 70¡C**

| Reaction mixture | Phosphorus |
|---|---|
| Crude extract of soybean (10 g) | 460 ppm |
| Crude extract of soybean (10 g) and water (1 mL) | 227 ppm |
| Crude extract of soybean (10 g), water (1 mL), and citric acid (10 mg) | 95.2 ppm |
| Crude extract of soybean (10 g), water (1 mL), and lecitase (1 mL) | 12.4 ppm |
| Crude extract of soybean (10 g), water (1 mL), and phospholipase $A_2$ (1,500-fold concentrated cytosolic fraction) from *P. horikoshii* (1 mL) | 204 ppm |

verted to the enzymatic refining process. However, pancreatic PLA2 is not thermostable and is expensive. Thermophilic PLA2 from *P. horikoshii* was not effective for the degumming, in spite of the 1,500-fold concentrated cytosolic fraction used (Table 1). Cytosolic PLA2 activity was as weak as 0.2–0.6 pmol/mL/min. It is possible that microbial PLA2 could be engineered to increase reactivity and enzyme yield.

*Purification of PLA2.* PLA2 was difficult to purify by general chromatographic methods such as gel filtration, ionic exchange, hydrophobic chromatography, etc., because PLA2 existed as aggregates. The size of the aggregates was increased by adding mild detergents such as Triton X-100 and *n*-octyl-β-D-glucoside. Electrophoresis was carried out by SDS-PAGE, the gel was cut into pieces, and its proteins were extracted. No PLA2 activity was detected in any single fraction, but 10% of the original activity could be recovered from the recombined protein mixture from the entire gel. Ten percent of the original PLA2 activity was also recovered when fractions containing proteins of <10 kD were combined with the fraction containing 28.5–34 kD proteins. The protein in the <10 kD fraction was named subunit 1. No PLA2 activity was observed in the absence of subunit 1. PLA2 activity increased more than 150% (compared to the eluted activity of total fractions) when an excess amount of subunit 1 (Fig. 6) was added to the fractions containing proteins >19kD. The $K_m$ value for NBD-PC of the mixture of subunit 1 with all fractions containing proteins >19kD was 28.5 μM.

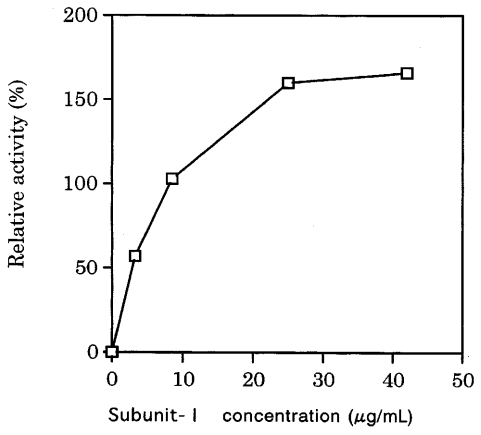


**FIG. 6.** Effect of the fraction of less than 10 kD (<10 kD) molecular mass on PLA2 activity. Subunit-I was mixed with the fractions eluted from gel slices containing proteins greater than 19 kD (>19 kD bands) and the PLA2 activity of the mixture was compared to the activity eluted from the entire gel. See Figure 5 for abbreviation.

## ACKNOWLEDGMENTS

The authors thank Dr.Yutaka Kawarabayasi at National Institute of Technology and Evaluation, Dr. Anisur Rahman Kahn of University of Dhaka, and Professor Yaeko Masuchi of Tokyo University for their great assistance with this study. The authors are grateful to Showa Sangyo and Novo Nordisk, Japan, for donating degumming substrate and lecitase.

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[Received July 10, 2000; accepted October 2, 2000]