Purification of phospholipase B from *Penicillium notatum* by hydrophobic chromatography on palmitoyl cellulose

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Abstract Phospholipase B (lysolecithin acyl-hydrolase, EC 3.1.1.5) from the mycelia of *Penicillium notatum* (Institute for Fermentation, Osaka, Japan; #4640) was adsorbed from a crude solution to palmitoyl cellulose. Adsorption was efficient at pH 4 at low ionic strength (10 mM buffer), and at pH 4–9 at higher ionic strength (1–2 M NaCl in 10 mM buffer). The adsorbed enzyme was eluted from the cellulose with a suitable detergent, such as Adekatol SO-120, Triton X-100, or deoxycholate. As an application of this procedure, the enzyme was purified from an extract of the mycelia by column chromatography on a palmitoylated textile (palmitoylated gauze) with an overall recovery of 59% and a 38-fold increase in specific activity. By subsequent column chromatographies on Amberlite XAD-2, Sephadex G-100 and G-150, and DEAE-Sephadex A-50, the enzyme was purified about 4,000-fold to a nearly homogeneous state from a mycelium extract with an overall recovery of 37%.—**Imamura, S., and Y. Horiuti.** Purification of phospholipase B from *Penicillium notatum* by hydrophobic chromatography on palmitoyl cellulose. *J. Lipid Res.* 1980. **21:** 180–185.

Supplementary key words hydrophobic adsorption · hydrophobic site · palmitoylated textile · detergent

Previously, phospholipase B of *P. notatum* was highly purified from a crude extract of mycelia by conventional procedures, such as precipitation with ammonium sulfate, electrophoresis, gel filtration, and ion-exchange chromatography (1, 2). The present paper reports an alternative procedure for enzyme purification utilizing the hydrophobic affinity of the enzyme for palmitoyl cellulose (Pal-C). The method was developed on the basis of our previous studies on the purification of *Chromobacterium* lipase (3, 4), *Streptomyces* phospholipase D (5), and *Bacillus* phospholipase C (6).

MATERIALS AND METHODS

Production of phospholipase B and preparation of crude enzyme sample

P. notatum (IFO-4640) was grown aerobically in culture medium containing 3.5 g of corn steep liquor, 5.5 g of lactose, 0.7 g of KH_2PO_4, 0.3 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of $CaCO_3$, and 0.25 g of soybean oil per 100 ml at pH 5.4 for 5 days at 26°C with continuous shaking on a rotary shaker. The mycelia were washed and homogenized with five times their weight of distilled water in a Waring blender for 10 min at 25°C. The homogenate was adjusted to pH 7 and filtered (Toyo filter paper, No. 82C). The filtrate was centrifuged at 8,000 *g* for 15 min at 5°C; the resulting supernatant was named the "mycelium extract." The supernatant was concentrated to one-third of its initial volume under reduced pressure at 40°C, mixed with two volumes of glycerol and stored at −20°C. The enzyme solution was dialyzed against 400 times its volume of distilled water for 2 hr at 0–5°C before use as the enzyme source (crude phospholipase B).

Assay of phospholipase B activity

Phospholipase B was assayed by the method of Kawasaki and Saito (1) with some modifications. The reaction was started by adding 0.1 ml of the enzyme solution to 0.9 ml of reaction mixture composed of 3.6 μmol of lysolecithin and 200 μmol of acetate buffer (pH 4). After incubation for 30 min at 37°C, the reaction was stopped by adding 2 ml of ethanol. Decrease in ester bonds was measured by the method of Augustyn and Elliot (7). One unit of phospholipase B was defined as the amount that caused a decrease of 1 μmol of ester bond per min.

Assay of adsorption capacity of Pal-C

The enzyme activity adsorbed on Pal-C (the adsorption capacity) was determined by batchwise addition of the adsorbent (50 mg) to 0.8–1.2 units of enzyme in 10 mM buffer (2 ml). The enzyme solution was vigorously shaken with Pal-C at 25°C for 30 sec in a vibrator, and then the mixture was filtered through a Teflon filter-cloth (Teflon 501-B). The filtrate was

Abbreviations: Pal-C, palmitoyl cellulose; Pal-G, palmitoylated gauze; IFO, Institute for Fermentation, Osaka, Japan.

centrifuged (1,000 *g*, 10 min), and the resulting supernatant was assayed for enzyme activity. The difference between the activities of the original solution and of the supernatant was taken as the amount of activity adsorbed.

Determination of Adekatol SO-120

The amount of Adekatol SO-120 was determined on the basis of the spectral shift of the peak of Coomassie Brilliant Blue, from 550 nm to 600 nm. The increase in absorbance at 610 nm was proportional to the Adekatol SO-120 concentration up to 50 μg/ml of detergent ($A_{610\,nm} \approx 0.13$) under the assay conditions. The assay mixture was composed of 0.5 ml of 0.1 M acetate buffer (pH 5), 1 ml of 0.004% Coomassie Brilliant Blue, 2.5 ml of the test solution, and 1 ml of distilled water to give a total volume of 5 ml. The mixture stood for 10 min at 25°C, and the concentration of Adekatol SO-120 was calculated from the difference in absorbance at 610 nm of the sample and the control, using a standard curve for the absorbance difference versus the concentration of detergent. The control contained distilled water instead of the sample solution (2.5 ml).

Determination of protein

Protein concentrations were determined by the method of Lowry et al. (8) with bovine serum albumin as a standard.

Polyacrylamide disc gel electrophoresis

Disc electrophoresis on a column (5 × 80 mm) of polyacrylamide gel was carried out as described by Kawasaki and Saito (1). The gel was fixed and stained with Amido Black 10B in 7% acetic acid.

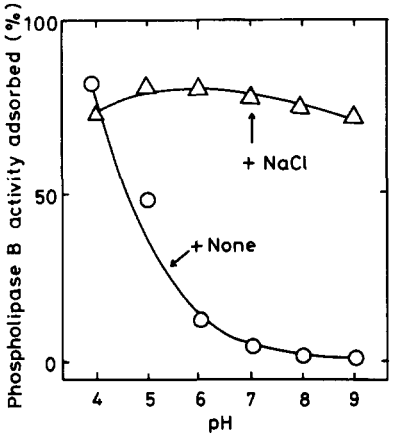

Fig. 1. Effect of pH on the adsorption of phospholipase B to Pal-C in the presence and absence of 1 M NaCl. Experimental conditions were as described in the text, except that enzyme solutions in buffers (10 mM) of various pH values were used in the presence and absence of 1 M NaCl. Acetate buffer was used for pH 4, 5 and 6; phosphate buffer for pH 7 and Tris-HCl buffer for pH 8 and 9.

Materials

Lecithin was prepared from fresh egg yolk by the method of Hanahan, Dittmer, and Warashina (9). Lysolecithin was prepared from the lecithin using venom of the Eastern cottonmouth moccasin (*Ansistrodon piscivorus piscivorus* from Sigma Chemical Co., St. Louis, MO) in the usual way (9, 10). Pal-C and Pal-G were prepared as reported previously (3). Amberlite XAD-2 was from Organo Co., Tokyo, and Topco Perlite and Radiolite #500 were from Toko Perlite Kogyo Co., Tokyo, and Showa Kagaku Kogyo Co., Tokyo, respectively. Adekatol SO-120 (a non-ionic detergent, the ethoxylate of linear *sec*-alcohols having chain lengths of 10–16 carbon atoms) was a product of Asahidenka Kogyo Co., Tokyo. Silicone KM-72 (anti-foaming agent) and Coomassie Brilliant Blue R were products of Shinetsu Chemical Industry Co., Tokyo, and E. Merck, Darmstadt, Germany, respectively. Teflon 501-B (filter cloth) was from Nakao Filter Kogyo Co., Osaka.

RESULTS

Preliminary experiments on batch adsorption of phospholipase B to Pal-C

The adsorption of phospholipase B to Pal-C was significantly affected by both the ionic strength and pH of the enzyme solution. At lower ionic strength (10 mM buffers), the extent of adsorption was maximal at pH 4 and decreased with increase from pH 4 to 9, indicating that adsorption is most near the isoelectric point (pH 4) of the enzyme (2) (**Fig. 1**). However, the poor adsorption at higher pH values (pH 7–9) was restored by increase in the ionic strength by addition of up to 2 M NaCl, as illustrated in **Fig. 2,** for which 10 mM phosphate buffer of pH 7 was used (Fig. 2). At higher ionic strength (1 M NaCl in 10 mM buffers), increase in pH from 4 to 9 hardly influenced the extent of adsorption: about 0.6 unit of enzyme was adsorbed to 50 mg Pal-C at all pH values from pH 4 to 9 (Fig. 1). Since the adsorption capacity of untreated cellulose was less than one-sixth of that of Pal-C at pH 4 or at higher ionic strength, the palmitoyl ligand may be necessary for enzyme adsorption.

The phospholipase B adsorbed to Pal-C was partially recovered in the presence or absence of 1 M NaCl by washing the Pal-C with various detergents, such as Triton X-100, Adekatol SO-120 (a non-ionic detergent, the ethoxylate of linear *sec*-alcohols having chain lengths of 10–16 carbon atoms) or deoxycholate

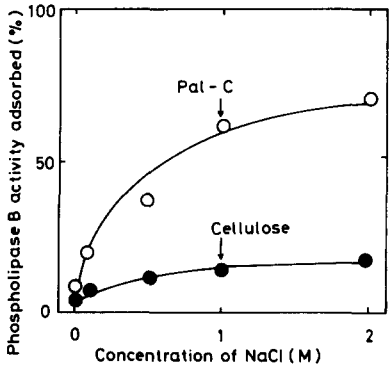

Fig. 2. Effect of NaCl concentration on adsorptions of phospholipase B to Pal-C and cellulose. Experimental conditions were as described in the text, except that the enzyme solutions in 10 mM phosphate buffer (pH 7) contained NaCl at the concentrations indicated. Open circles, ○, adsorption on Pal-C (50 mg); closed circles, ●, adsorption on cellulose (100 mg).

(**Table 1**). The extent of elution was nearly maximal with 0.3 (w/v)% detergent, and upon further addition of detergent the maximal elution was 50–60% of the total activity adsorbed on the adsorbent.

Purification of phospholipase B

On the basis of the preliminary experiments with Pal-C, attempts were made to purify the enzyme from the mycelia by a procedure involving chromatography on a palmitoylated textile (palmitoylated gauze:Pal-G) column. Mycelia were cultured for 3 days in a 30-liter jar fermenter containing 20 liters of culture medium and then homogenized under the conditions described under "Materials and Methods." The mycelial homogenate (6 liters) prepared from 1.2 kg wet weight of mycelia was adjusted to pH 4 and allowed to stand for 40 hr at 5°C with addition of 25 ml of toluene. This aged homogenate was centrifuged at 4,000 *g* for 10 min at 5°C, and the resulting supernatant was filtered (Toyo filter paper, No. 82C) on a Büchner funnel covered by a layer (ca. 1 cm depth) of Radiolite #500. The filtrate (6 liters) was applied to a column containing 2 kg of Pal-G. The column was washed with 30 liters of 1 mM phosphate buffer containing 0.2 mM EDTA and eluted with 20 liters of the same buffer containing 0.1% Adekatol SO-120 (**Fig. 3A**). The eluate showed scarcely any absorbance at 280 nm, indicating that the enzyme had been well separated from the bulk of proteins. The portion of the eluate with activity (14 liters) was concentrated to 1.4 liters at 50°C under reduced pressure with 0.001% Silicone KM-72 as an anti-foaming agent. The precipitate formed was removed by centrifugation at 3,000 *g* for 20 min at 5°C. The recovery of the enzyme in this supernatant was 59%, with 38-fold increase in specific activity over that of enzyme in the filtrate. The Adekatol SO-120 in the supernatant was removed on an Amberlite XAD-2 column: the supernatant was passed through the column (6 × 25 cm), and the column was washed with 1 mM phosphate-0.2 mM EDTA buffer (pH 7) until the enzyme activity could no longer be detected in the washing fluid. The effluent was then concentrated by lyophilization, and the lyophilizate was dissolved in 23 ml of distilled water and centrifuged at 5,000 *g* for 10 min at 5°C. The resulting supernatant was carefully separated, and the precipitate was repeatedly washed with 13 ml of distilled water to dissolve most of the enzyme present. The combined supernatant (36 ml) was applied to a Sephadex G-100 column. As shown in Fig. 3B, most of the impurity had been removed, with an 18-fold increase in specific activity. The fractions with enzymatic activity (Nos. 36–50) were combined and lyophilized, and the lyophilized powder (370 mg) was dissolved in 4 ml of distilled water and applied to a Sephadex G-150 column (Fig. 3C). The fractions containing most of the activity (Nos. 20–26) were combined and again lyophilized (yield, 75 mg; protein content, 9 mg).

TABLE 1. Elution of phospholipase B from Pal-C with various detergents

Detergent	Concentration	Relative Elution	
		Buffer	Buffer + 1 M NaCl
	w/v, %	%	
None		14	0
Triton X-100	0.003	26	30
	0.03	44	51
	0.3	48	57
Adekatol SO-120	0.003	35	24
	0.03	44	43
	0.3	51	51
Deoxycholate	0.003	36	31
	0.03	48	53
	0.3	53	54

Pal-C (3 g) was vigorously stirred in 40 ml of 10 mM phosphate–1 M NaCl buffer (pH 7) containing 26 units of enzyme (crude enzyme sample) at 25°C for 5 min, and the mixture was filtered through a glass filter. Pal-C on the filter was washed with 20 ml of the same buffer and recovered in a wet weight of 4.6 g. The amount of enzyme adsorbed was determined from the difference between the activities of the original solution and the filtrate. Results showed that the Pal-C retained 5.4 units of enzyme per gram. For the test, portions of the Pal-C (200 mg each) were shaken for 30 sec in a vibrator in 2 ml of 10 mM phosphate buffer (pH 7) containing detergent at the final concentration indicated in the presence and absence of 1 M NaCl. The mixtures were filtered and centrifuged as described in the text for assay of adsorption capacity. The enzyme activity eluted with detergent was expressed as the "percentage elution" relative to the total activity adsorbed on Pal-C; the total activity was also corrected for change in activity of free enzyme on addition of detergent.

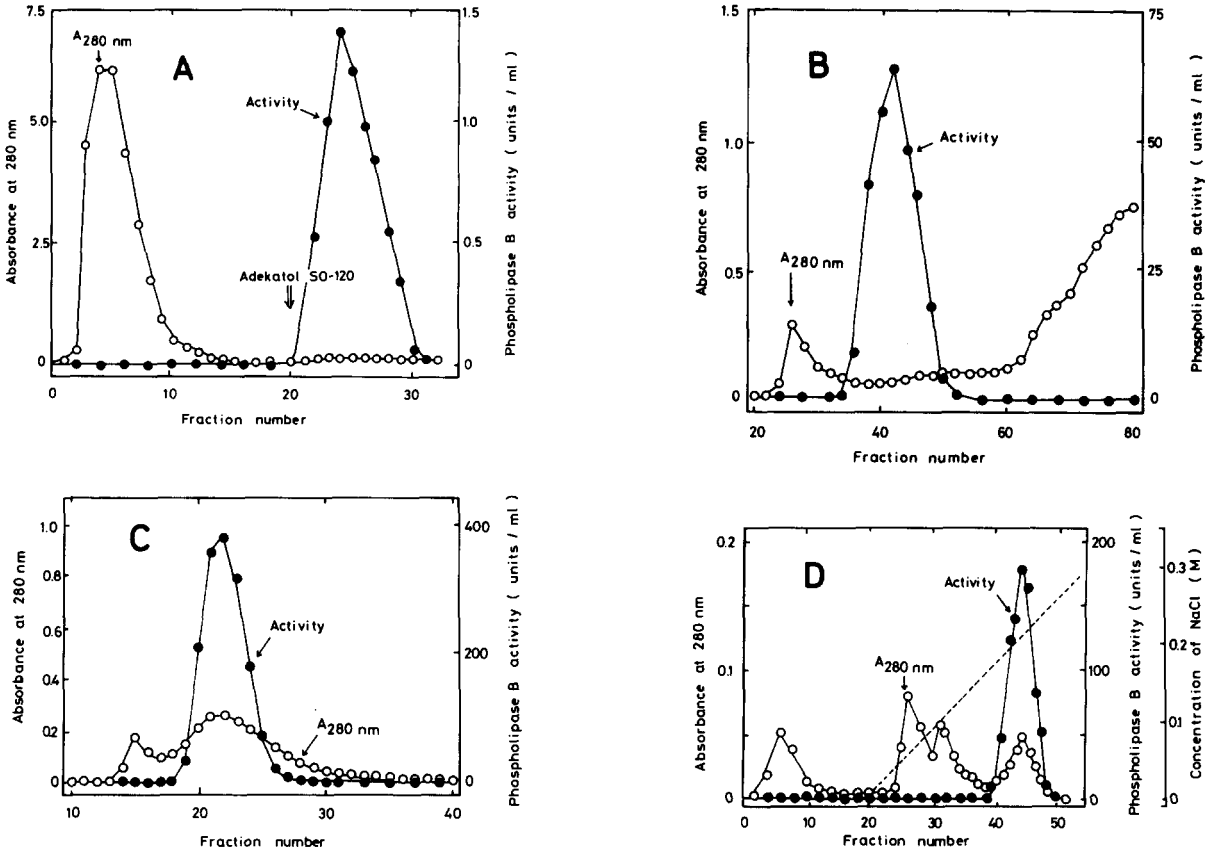

Fig. 3. Column chromatography. A: on Pal-G; Pal-G (2 Kg dry weight) was packed into a column (diameter, 15 cm; length, 50 cm) with the aid of 50% ethanol and washed with water. The filtrate (6 liters) of the mycelium extract (pH 4) was applied to the column. The column was washed with 1 mM phosphate–0.2 mM EDTA buffer (pH 7) (30 liters) and then eluted with the same buffer (20 liters) containing 0.1 (w/v)% Adekatol SO-120. The time when elution was started is indicated by a double arrow. Fractions of 2 liters were collected at a flow rate of 650 ml per min.

B: on Sephadex G-100; the supernatant (36 ml) of the solution containing the lyophilizate was chromatographed on a Sephadex G-100 column (5.5 × 68 cm) in 5 mM phosphate-2 mM EDTA buffer (pH 7). Fractions of 15 ml were collected at a flow rate of about 80 ml per hr.

C: on Sephadex G-150; a solution (4 ml) of the lyophilizate (370 mg) was chromatographed on a Sephadex G-150 column (2.2 × 60 cm) in 5 mM phosphate-2 mM EDTA buffer (pH 7). Fractions of 7.2 ml were collected at a flow rate of 31 ml per hr.

D: on DEAE-Sephadex A-50; a solution (13 ml) of the lyophilized powder (13 mg) was applied to a DEAE-Sephadex A-50 column (1 × 10 cm) previously equilibrated with 10 mM phosphate-2 mM EDTA buffer (pH 7). The column was washed with 20 ml of the same buffer and then eluted with 70 ml of a linear gradient of 0 to 0.3 M NaCl in the same buffer at a flow rate of about 10 ml per hr, and fractions of 2 ml were collected. All procedures were carried out at 20°C. Other experimental conditions are described in the text.

For further purification, a portion of the lyophilized powder (13 mg) was dissolved in 13 ml of distilled water and applied to a column of DEAE-Sephadex A-50 (Fig. 3D). The column was washed with 20 ml of 10 mM phosphate-2 mM EDTA buffer (pH 7) and then eluted with a linear gradient of NaCl (0–0.3 M) in the same buffer. The fractions constituting the enzyme peak (Nos. 41–47) were collected, dialyzed against 500 ml of 10 mM phosphate-2 mM EDTA buffer for 5 hr at 5°C and lyophilized. The purification procedure is summarized in **Table 2.** The lyophilized powder, having a specific activity of 3,430 units per

TABLE 2. Summary of purification of phospholipase B

Step	Total Activity	Specific Activity	Recovery
	units	*units/mg protein*	%
Mycelium extract	22.8×10^3	0.85	100
Pal-G	13.8		60
Concentrate	13.2	32.4	59
Amberlite XAD-2	13.2	40.4	59
Sephadex G-100	10.9	734.0	48
Sephadex G-150	10.4	1160.0	46
Lyophilized powder	10.3	1150.0	45
Lyophilized powder	1.87×10^3	1150.0	45
DEAE-Sephadex A-50	1.47	3430.0	37

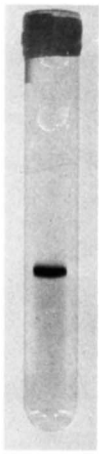

Fig. 4. Polyacrylamide disc gel electrophoresis of the purified enzyme. Experimental conditions were as described in the text, except that 32 μg of the enzyme protein was applied to the column.

mg protein, gave a single protein band on disc gel electrophoresis (**Fig. 4**).

DISCUSSION

The adsorption of phospholipase B to Pal-C was influenced by the ionic strength and pH of the enzyme solution. The adsorption was maximal at the isoelectric point of the enzyme (pH 4) or at higher ionic strength, such as 2 M NaCl, suggesting that this adsorption was due to a hydrophobic interaction (11–13). The enzyme adsorbed to Pal-C or Pal-G was partially recovered in the eluate (50–60% of the total activity adsorbed) by elution with a suitable detergent.

Our previous reports (3–6) showed that lipase from *Chromobacterium*, phospholipase D from *Streptomyces*, and phospholipase C from *Bacillus* have a hydrophobic site distinct from the catalytic site in their enzyme molecule; these enzymes can be purified on a Pal-G column due to a hydrophobic interaction between the palmitoyl group in the adsorbent and the hydrophobic site in the enzyme molecules; and these enzymes are active when adsorbed on Pal-C or Pal-G. Furthermore, we demonstrated with the lipase (4) and the phospholipase D (5) that a hydrophobic substance(s), such as fatty acid, binds to a hydrophobic site on the purified enzyme molecule and thus stimulates the enzyme activity. In the native enzymes in cell cultures, the hydrophobic substance(s) appear to occupy the hydrophobic site completely.

Because of the similarities in findings on the present enzyme, phospholipase B, and on above enzymes, such as its behavior on adsorption to and elution from Pal-C (Pal-G), it seems likely that the enzyme may also have a hydrophobic site and a hydrophobic substance in the native enzyme molecule; the activity of phospholipase B adsorbed on Pal-C could not be measured due to elution of the enzyme from the adsorbent as a result of the detergent effect of the substrate lysolecithin. The hydrophobic substance on the hydrophobic site that increases the enzyme activity appears to be removed from the native enzyme during the process of adsorption to and elution from the adsorbent, because the adsorption-elution process was accompanied with the poor recovery of the enzyme activity.

The phospholipase B was found to be a glycoprotein by Kawasaki & Saito (1). However, the presence of a hydrophobic site in the enzyme molecule has not yet been reported. Thus the present type of experiments using the hydrophobic adsorbent may represent a new experimental approach to detection of a hydrophobic binding site and also a hydrophobic substance(s) affecting enzyme activity or enzyme stability.

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