

# Purification of phospholipase C from *Bacillus cereus* by hydrophobic chromatography on palmitoyl cellulose

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**Abstract** Phospholipase C (phosphatidylcholine choline-phosphohydrolase, EC 3.1.4.3) from *Bacillus cereus* (IAM-1208) was adsorbed to palmitoyl cellulose from a crude enzyme solution at pH 5–9. The adsorption was not influenced by ionic strength up to 2 M NaCl. The adsorbed enzyme was eluted almost completely by washing the cellulose with a suitable detergent, such as Triton X-100, Adekatol SO-120, Cation DT-205, or sodium deoxycholate. The enzyme was then purified by column chromatography on a palmitoylated textile (palmitoylated gauze) with an overall recovery of 91% and a 467-fold increase in specific activity over that of enzyme in the crude culture supernatant. Subsequent fractionation with acetone and chromatography on a Sephadex G-75 column separated two nearly homogeneous phospholipase C's. The enzyme adsorbed on palmitoyl cellulose was active, although its activity was about one-fourth that of free phospholipase C. Therefore, the enzyme appeared to be adsorbed to the cellulose through a hydrophobic site that was distinct from the catalytic site on the enzyme molecule. —Imamura, S., and Y. Horiuti. Purification of phospholipase C from *Bacillus cereus* by hydrophobic chromatography on palmitoyl cellulose. *J. Lipid Res.* 1979. **20**: 519–524.

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

Various purification methods of phospholipase C from bacteria have been reported by many investigators (1–13). The present paper reports a simple and effective procedure for large-scale purification of the enzyme based on the 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 by hydrophobic affinity chromatography with Pal-C (14, 15). Based on the activity of enzyme adsorbed on Pal-C, we have suggested a mode of adsorption of the enzyme to this material.

## MATERIALS AND METHODS

### Production of phospholipase C and preparation of crude enzyme sample

The culture medium contained 1 g of peptone, 1 g of meat extract, and 0.5 g of NaCl per 100 ml, and was adjusted to pH 7. Volumes of 100 ml of medium in 500-ml flasks were inoculated with *B. cereus* IAM-1208 and incubated for 20 hr at 30°C with continuous shaking on a rotary shaker. The cultures were then centrifuged at 5,000 g for 20 min at 5°C; the resulting supernatant was brought to 70% saturation with  $(\text{NH}_4)_2\text{SO}_4$ . The precipitate was collected after centrifugation at 5,000 g for 20 min at 5°C and dissolved in one-tenth of the volume of the initial supernatant with 10 mM Tris-HCl buffer (pH 7.5). This enzyme solution was stored at –20°C before use. For large-scale enzyme purification, cells were cultured for 16 hr at 30°C with forced aeration in a 30-l jar fermenter containing 20 l of the culture medium.

### Assay of phospholipase C activity

Phospholipase C was assayed using *E. coli* alkaline phosphatase by the method of Kurioka and Liu (16) with the following modifications. After the phospholipase C reaction had been stopped by adding laurylbenzene sulfonate (LBS), the phosphorylcholine (P-choline) liberated from lecithin in the phospholipase C reaction was hydrolyzed with the alkaline phosphatase. The resulting  $\text{P}_i$  was measured with molybdenum blue (17). The initial rate of lecithin hydrolysis, as measured by the amount of  $\text{P}_i$  liberated, was pro-

Abbreviations: Pal-C, palmitoyl cellulose; Pal-G, palmitoylated gauze; P-choline, phosphorylcholine; LBS, laurylbenzene sulfonate; SDS, sodium dodecyl sulfate; IAM, Institute of Applied Microbiology, University of Tokyo, Japan.

portional to the amount of enzyme, when enzyme catalyzing about 0–40% hydrolysis of the lecithin (5.2  $\mu\text{mol}$ ) was used.

The reaction mixture was prepared by sonicating a solution containing 5.2  $\mu\text{mol}$  of lecithin, 5  $\mu\text{mol}$  of  $\text{CaCl}_2$ , 0.72  $\mu\text{mol}$  of sodium deoxycholate, and 100  $\mu\text{mol}$  of Tris-HCl buffer (pH 7.5) per 1.9 ml at 9 kc and 180 W for 10 min at 15°C. The reaction was started by adding 0.1 ml of the enzyme solution to 1.9 ml of the reaction mixture; it was continued for 30 min at 37°C and stopped by adding 0.9 ml of 2.5% LBS solution. The resulting mixture was supplemented with 0.1 ml (4 units) of *E. coli* alkaline phosphatase and incubated for 30 min at 37°C; one unit of the phosphatase was defined as the amount that liberated 1  $\mu\text{mol}$  of *p*-nitrophenol per min at 37°C in the reaction mixture (2 ml) composed of 2  $\mu\text{mol}$  of *p*-nitrophenylphosphate and 100  $\mu\text{mol}$  of Tris-HCl buffer (pH 7.5). The incubation was terminated by adding 1 ml of 10% trichloroacetic acid. To the resulting mixture, 1 ml of 5% barium acetate solution was added to remove LBS and lecithin. The mixture was filtered through a filter paper, and 2 ml of the filtrate was used to determine  $\text{P}_i$  by the method of Allen (18) as modified by Nakamura and Mori (17). The precipitate (barium sulfate) formed was removed by centrifugation before measuring the absorbance at 720 nm of molybdenum blue. One unit of phospholipase C was defined as the amount that liberated 1  $\mu\text{mol}$  of  $\text{P}_i$  per min under the assay conditions.

#### Preparation of adsorbents and assay of adsorption capacity

Pal-C and Pal-G were prepared as described previously (14). CM-cellulose was regenerated by washing it with acid and alkali, and equilibrated with 10 mM acetate buffer (pH 6). Cellulose was washed with 10 mM acetate buffer (pH 6). The adsorption capacities of adsorbents were determined as described in previous papers (14, 15) using 2 ml of enzyme solution in 10 mM acetate buffer (pH 6).

#### Preparation and assay of phospholipase C adsorbed on Pal-C

Phospholipase C adsorbed on Pal-C (Pal-C–phospholipase C) was prepared as follows. One gram of Pal-C was vigorously stirred in 10 ml of 10 mM Tris-HCl buffer (pH 7.5) containing 15 units of phospholipase C (the crude enzyme sample) at 25°C for 5 min, and the mixture was filtered through a stainless steel filter cloth (SUS 316, 300 mesh). Pal-C on the filter was washed with 20 ml of the same buffer; about 1.8 g wet weight retained 5.4 units of phospholipase C per gram. This Pal-C–phospholipase C (0.1 g) was thoroughly mixed with 2.9 g of dry Pal-C for dilution,

and the resulting diluted Pal-C–phospholipase C was assayed for activity. The reaction was carried out for 30 min at 37°C in twice the volume (2 ml) of the standard reaction mixture for assay of phospholipase C activity, but without adding deoxycholate, in a Monod test tube (L-shaped test tube) with continuous shaking. The amount of P-choline liberated was determined as described above.

The activity assay of Pal-C–phospholipase C on repeated use was measured as follows. The first reaction was carried out in twelve test tubes, each containing the reaction mixture with 100 mg of the washed diluted-Pal-C–phospholipase C; one gram of the diluted Pal-C–phospholipase C had been washed by filtration on a glass filter with 10 ml of 10 mM Tris-HCl buffer (pH 7.5). After the reaction three of the mixtures were assayed as described above. The other nine were combined without adding LBS, rapidly filtered on a glass filter, and washed with 20 ml of 10 mM Tris-HCl buffer (pH 7.5). The material on the filter was then divided into eight portions (100 mg each) and subjected to a second reaction. After the second reaction, three of the mixtures were assayed, and the other five were filtered and washed as described above. The material on the filter was divided into three portions (100 mg each) and subjected to the third reaction.

#### Electrophoresis on a polyacrylamide gel column

Disc gel electrophoresis was carried out as described by Davis (19) at pH 4.0 in the presence of 6 M urea at 3 mA per column (5  $\times$  80 mm) for 3 hr at 20°C (3), or as described by Weber, Pringle, and Osborn (20) at pH 7.2 in the presence of 0.1% SDS at 8 mA per column for 4 hr at 25°C. The gel was stained with Amido Black 10B in the former case and with Coomassie Brilliant Blue R (20) in the latter case.

#### Determination of protein

Protein was determined by the method of Lowry et al. (21) with bovine serum albumin as a standard.

#### Materials

Alkaline phosphatase from *E. coli* was prepared according to the method of Torriani (22) as follows. *E. coli* W 3747 was grown in the medium of Torriani (22) on a rotary shaker for 20 hr at 30°C. Cells were harvested by centrifugation and washed twice with 10 times their weight of 10 mM Tris-HCl buffer (pH 7.5) with centrifugation. The washed cells were then suspended in about 3 times their weight of the same buffer and sonicated at 5°C for 10 min at 9 kc and 180 W. The disrupted cell suspension was centrifuged at 7,000  $g$  for 20 min at 5°C. The supernatant was heated for 10 min at 75°C in the presence of 10 mM  $\text{MgSO}_4$  and then allowed to stand at 0°C for 30 min.

The precipitate formed was removed by centrifugation, and the resulting supernatant was brought to 70% saturation with  $(\text{NH}_4)_2\text{SO}_4$  and centrifuged at 7,000 *g* for 20 min at 5°C. The precipitate obtained was dissolved in 1.5 times the volume of the starting cell suspension of 10 mM Tris-HCl buffer (pH 7.5) and stored as the preparation of *E. coli* alkaline phosphatase.

Lecithin was prepared from fresh egg yolk by the method of Hanahan, Dittmer, and Warashina (23). Peptone and meat extract were products of Kyokuto Seiyaku Co., Tokyo, and Wako Pure Chemical Industries Co., Osaka, respectively. Adekatol SO-120 was from Asahidenka Kogyo Co., Tokyo, and Cation DT-205 from Nippon Oils & Fats Co., Tokyo; *p*-nitrophenyl phosphate was from Nakarai Chemical Co., Kyoto, and Amido Black 10B from Merck Darmstadt; Silicone KM-72 (anti-foaming agent) was from Shin-Etsu Chemical Industry Co., Tokyo. CM-cellulose and cellulose powder (40 mesh) were products of Brown Co., Berlin, New Hampshire, and Toyo Kagakusangyo Co., Tokyo, respectively. The reference proteins used for molecular weight determination were bovine serum albumin, RNA polymerase, and trypsin inhibitor from Boehringer Mannheim GmbH, Mannheim.

## RESULTS

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

When Pal-C was added batchwise to a crude enzyme solution in 10 mM acetate buffer (pH 6), the total activity present (0.4 units) was completely adsorbed on the adsorbent by addition of over 100 mg of Pal-C (Fig. 1); unmodified cellulose adsorbed about 15% of

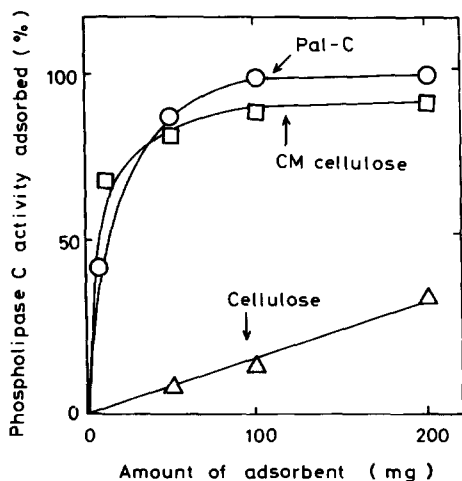


Fig. 1. Adsorptions of phospholipase C on cellulose, CM-cellulose, and Pal-C. Experimental conditions were as described in the text, except that the indicated amounts of adsorbents and crude enzyme sample containing 0.4 units of enzyme were used.

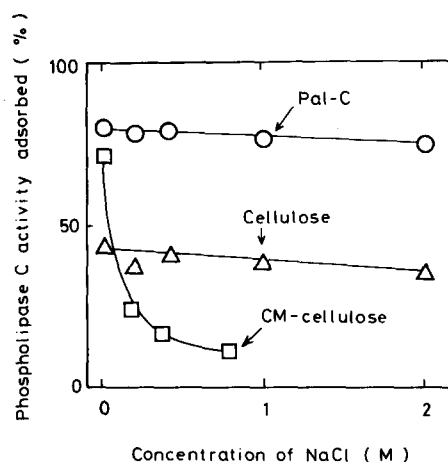


Fig. 2. Effects of NaCl concentration on the adsorption of phospholipase C to cellulose, CM-cellulose, and Pal-C. Experimental conditions were as described in the text, except that the enzyme solutions (0.4 units) contained NaCl at the concentrations indicated. Circles, adsorption on Pal-C (50 mg); squares, adsorption on CM-cellulose (50 mg); triangles, adsorption on cellulose (500 mg).

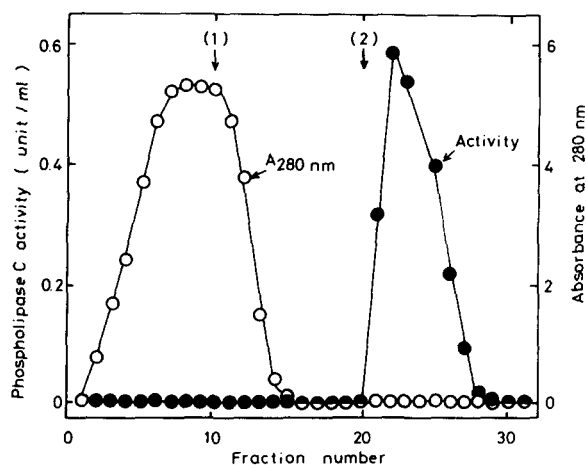
the total activity upon addition of 100 mg. CM-cellulose, 100 mg, adsorbed about 90% of the total activity, but increasing the amount did not result in further adsorption.

The adsorption to Pal-C was hardly influenced by pH in the range from pH 5 to 9 and was only slightly affected by NaCl concentration up to 2 M NaCl. On the other hand adsorption to CM-cellulose was largely

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

Detergent	Elution of Phospholipase C	
	Concentration <i>w/v</i> %	%
None		0
Triton X-100	0.003	11
	0.01	82
	0.03	100
Adekatol SO-120	0.003	15
	0.01	66
	0.03	93
Cation DT-205	0.003	17
	0.01	91
	0.03	100
Deoxycholate	0.003	0
	0.01	15
	0.03	75
	0.1	100

Pal-C (100 mg) was shaken for 30 sec in a vibrator in 1.8 ml of 10 mM Tris-HCl buffer (pH 7.5) containing 0.4 units of crude enzyme. Detergent (0.2 ml) was added to the final concentration indicated, and the mixture was shaken for 30 sec, filtered through a Teflon filtercloth (Teflon 501-B), and centrifuged. The resulting supernatant was assayed for enzyme activity. The enzyme activity eluted with detergent was expressed as the "percentage elution" relative to the total activity adsorbed on Pal-C. The latter was calculated as the difference between initial activity and that found in the supernatant recovered from the suspension.



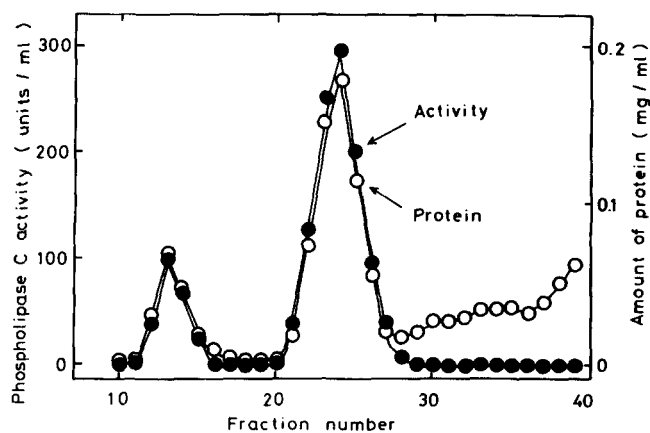
**Fig. 3.** Chromatography of phospholipase C on a palmitoylated textile column. Pal-G (2 kg dry weight) was packed into a column (15 × 50 cm) with the aid of 50% ethanol and washed with deionized water. The supernatant (16 l) of the culture medium was applied to the column. The column was washed with 5 mM Tris-HCl buffer (pH 8) until the absorbance at 280 nm became low, and the column was eluted with the same buffer (20 l) containing 0.1% (w/v) Adekatol SO-120. The times when washing (1) and elution (2) were started are indicated by arrows. Fractions of 2 l were collected at a flow rate of 660 ml per min. All procedures were carried out at 20–30°C. Other experimental conditions are described in the text.

prevented by the presence of as little as 0.5 M NaCl (Fig. 2). The mode of adsorption of unmodified cellulose was similar to that of Pal-C, although the adsorption capacity of cellulose was less than one-twentieth of that of Pal-C (Fig. 2).

The enzyme adsorbed on Pal-C could be eluted from the adsorbent with various detergents (Table 1). Cation DT-205 (hexadecyltrimethyl ammonium chloride), Triton X-100, and Adekatol SO-120 (a nonionic detergent, the ethoxylate of linear *sec*-alcohols having chain lengths of 10–16 carbon atoms) were the most effective: 0.03% (w/v) solutions of these detergents caused almost complete elution, whereas 0.1% (w/v) deoxycholate was required for complete elution.

### Purification of phospholipase C

On the basis of the above experiments with Pal-C, the enzyme was purified from the culture medium



**Fig. 4.** Chromatography of phospholipase C on Sephadex G-75. A solution (1 ml) of the lyophilized powder (36.6 mg) was chromatographed on a Sephadex G-75 column (2 × 37 cm) at 20°C in 10 mM Tris-HCl buffer (pH 8). Fractions of 2.5 ml were collected at a flow rate of about 10 ml per hr. Other experimental conditions are described in the text.

using a column of palmitoylated textile (Pal-G) as an affinity adsorbent.

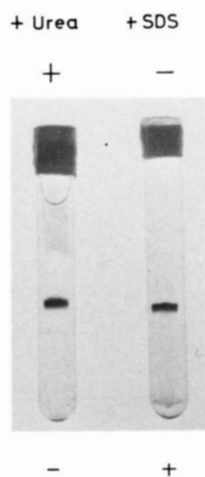
The supernatant (16 l) obtained by centrifugation of cultures was applied to a column containing 2 kg of Pal-G. The column was washed with 5 mM Tris-HCl buffer (pH 8) and then eluted with the same buffer containing 0.1% Adekatol SO-120 (Fig. 3).

The portion of eluate with activity (14 l) was concentrated to 900 ml at 40°C under reduced pressure with 0.001% Silicone KM-72 as an anti-foaming agent. The concentrated solution was then centrifuged, mixed with acetone to 60% (v/v) and kept for 30 min at 5°C. The precipitate formed was collected by centrifugation at 3,000 *g* for 20 min, dissolved in 15 ml of 10 mM Tris-HCl buffer (pH 8), and dialyzed against 2 l of the same buffer for 5 hr at 10°C. This dialyze was concentrated by lyophilization. The lyophilized material was dissolved in 1 ml of distilled water and chromatographed on a Sephadex G-75 column. Activity was eluted in two peaks, as shown in Fig. 4.

The eluate containing the first peak (fraction 1) (nos.

TABLE 2. Summary of purification of phospholipase C

Step	Total Activity	Protein	Specific Activity	Recovery
	units		units/mg	
Culture supernatant	$5.6 \times 10^3$	69,800	0.09	100
Pal-G eluate	5.1	120	42	91
Concentrate	4.6	117	40	82
60% Acetone precipitate	2.8	18	155	50
Dialyze	2.8	18	155	50
Lyophilized powder	2.7	17	160	48
Sephadex G-75				
Fraction 1	0.48	0.41	1,170	9
Fraction 2	2.2	1.66	1,320	39



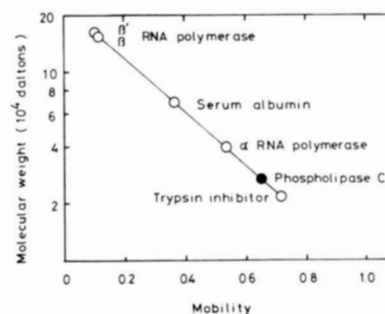
**Fig. 5.** Polyacrylamide disc gel electrophoresis of the purified enzyme. Experimental conditions were as described in the text, except that 40  $\mu$ g and 15  $\mu$ g of the enzyme protein were applied in the presence of 6 M urea and 0.1% SDS, respectively.

12–15) was slightly turbid. Most of the activity was found in the second peak (fraction 2) (nos. 21–27). The fractions of eluate constituting each peak were separately pooled and lyophilized. These fractions had almost equal specific activities: 1,170 units/mg for fraction 1 and 1,320 units/mg for fraction 2. The purification procedure is summarized in **Table 2**.

Fraction 2 gave a single protein band on polyacrylamide disc gel electrophoresis in the presence of 6 M urea or 0.1% SDS, as shown in **Fig. 5**, but suitable conditions for electrophoresis of fraction 1 could not be found. The molecular weight of the enzyme in fraction 2 was determined to be about 27,000 by the SDS–polyacrylamide disc gel electrophoresis (**Fig. 6**). This value is similar to those for the molecular weights of purified phospholipase C's from other strains of *B. cereus* (3, 4).

#### Activity of phospholipase C adsorbed on Pal-C

The activity of phospholipase C adsorbed on Pal-C (Pal-C–phospholipase C) was measured in a reaction mixture without deoxycholate to avoid elution of the enzyme. To compare the activities of the adsorbed and free forms, we varied the amounts of the two, as shown in **Table 3**. An activity of free enzyme of 0.005 units, 0.67  $\mu$ mol of  $P_i$  liberated/30 min, was nearly equivalent to that of Pal-C–phospholipase C that adsorbed 0.02 units of free enzyme, 0.66  $\mu$ mol of  $P_i$  liberated/30 min. Therefore, the activity of adsorbed enzyme is about one fourth that of free enzyme. The adsorbed enzyme could be used for repeated reactions without loss of the enzymatic activity: 100 mg of Pal-C–phospholipase C, which retained 0.007 units of free enzyme, showed an activity of 0.26  $\mu$ mol of  $P_i$  liberated/30 min in the



**Fig. 6.** Determination of the molecular weight of the enzyme in fraction 2 by SDS–polyacrylamide disc gel electrophoresis. The purified enzyme (15  $\mu$ g protein) in fraction 2 was subjected to electrophoresis, and the reference proteins were run under similar conditions. Other experimental conditions are described in the text.

first reaction, 0.25  $\mu$ mol in the second reaction, and 0.25  $\mu$ mol in the third reaction.

## DISCUSSION

Methods for purification of phospholipase C's from other strains of *B. cereus* have been reported by Zwaal et al. (3), Otnaess et al. (4), and Ikezawa et al. (12). In their methods the enzymes were purified to a homogeneous state as judged by disc gel electrophoresis with a recovery of 23%, 50%, and 8.4%, respectively. In comparison with their methods, our present method (recovery, 48%) seems efficient and simple for large-scale purification of phospholipase C as in the case of the purification of *Chromobacterium* lipase (14).

Takahashi, Sugahara, and Ohsaka (7) purified phos-

**TABLE 3.** Comparison of activities of free and adsorbed phospholipase C

Enzyme Preparation		unit <sup>a</sup>	Phospholipase C Activity $\mu$ mol of $P_i$ Liberated per 30 min
Free phospholipase C		0.005	0.67 <sup>e</sup> $\pm$ 0.020
		0.010	1.23 $\pm$ 0.040
Pal-C–phospholipase C	100 mg <sup>b</sup>	0.010	0.34 $\pm$ 0.010
	200	0.020	0.66 $\pm$ 0.020
	100 <sup>c</sup>	0.007	1st <sup>d</sup> 0.26 $\pm$ 0.015 2nd 0.25 $\pm$ 0.015 3rd 0.25 $\pm$ 0.015

<sup>a</sup> Amount of enzyme in the free or adsorbed state expressed as unit that was determined with the standard reaction mixture for phospholipase C activity in the text.

<sup>b</sup> Weight of the diluted Pal-C adsorbing phospholipase C.

<sup>c</sup> Weight of the diluted and washed Pal-C adsorbing phospholipase C.

<sup>d</sup> Time of use for the activity assay.

<sup>e</sup> Mean values of three runs.

The experimental conditions were as described in the text.

phospholipase C from *Clostridium perfringens* by affinity chromatography on a column containing egg-yolk lipoprotein linked to Sepharose 4B with a recovery of about 60% and a 200-fold increase in specific activity. This affinity chromatography was applied to the purification of phospholipase C from *Bacillus cereus* by Little, Aurebekk, and Otnaess (8). However, the chromatography appears unsuitable for large-scale preparation of a purified enzyme due to the low capacity of the affinity adsorbent, as pointed out by Yamakawa and Ohsaka (13); they reported an alternative method for large-scale purification of the *Clostridium* enzyme involving conventional procedures, such as precipitation with ammonium sulfate, ion-exchange chromatography, and gel filtration. In addition, it is uncertain whether both the *Clostridium* enzyme and the *Bacillus* enzyme are adsorbed through a catalytic site due to affinity for its substrate analogue (the adsorbent) or are adsorbed through a hydrophobic site in its molecule distinct from the catalytic site.

The present chromatography with Pal-C (Pal-G) was due to a hydrophobic interaction between the palmitoyl group in the adsorbent and some hydrophobic site on the enzyme molecule other than its catalytic site, based on the following evidence. The adsorption of the enzyme to Pal-C was not influenced appreciably by ionic strength or pH, the enzyme adsorbed on Pal-C could be recovered by elution with a suitable detergent, and the enzyme adsorbed on Pal-C was active.

Preliminary experiments showed that crude preparations of phospholipase C from various sources were also adsorbed on Pal-C. Therefore, we consider that Pal-C (Pal-G) should be useful for purifications of phospholipase C and possibly for purification of other phospholipases. ■■

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