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PURIFICATION OF A PHOSPHOLIPASE C FROM *BACILLUS CEREUS*

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## SUMMARY

Phospholipase C activity present in the growth medium of *Bacillus cereus* was purified 20-fold by chromatography on polyethyleneimine-cellulose columns, or by treatment with protamine sulfate and subsequent chromatography on DEAE-cellulose columns. Purified enzyme preparations retained the ability to hydrolyze ethanolamine phosphoglycerides in the absence of choline phosphoglycerides. A typical preparation had a specific activity of about 9  $\mu$ moles/min per mg toward purified diacyl glycerophosphoryl ethanolamine and a specific activity of about 15–20  $\mu$ moles/min per mg toward diacyl glycerophosphorylmonomethylethanolamine and diacyl glycerophosphoryl choline. Monoacyl glycerophosphate was not hydrolyzed under similar conditions.

## INTRODUCTION

MACFARLANE AND KNIGHT<sup>1</sup> identified phospholipase C, an enzyme capable of hydrolyzing diacyl glycerophosphoryl choline (diacyl-GPC) to 1,2-diacylglycerol and phosphoryl choline, in preparations obtained from *Clostridium welchii*. Shortly thereafter, CHU<sup>2</sup> found cholinephosphohydrolase activity in the growth medium of *Bacillus cereus*. Because phospholipase C preparations obtained from *B. cereus* hydrolyzed a broader range of substrates than did the activity obtained from *Cl. welchii*, preparations from *B. cereus* have been used to aid conveniently in analyzing and identifying various phospholipids. Our studies reported here were undertaken to purify the phospholipase C activity found in *B. cereus* preparations, and to determine if that purification provided a more useful reagent.

## MATERIALS AND METHODS

*Substituted-cellulose ion exchangers*

Aminoethyl-cellulose(AE-cellulose), polyethyleneimine-cellulose(PEI-cellulose),

Abbreviations: GPE, glycerophosphoryl ethanolamine; GPI, glycerophosphoryl inositol; GPC, glycerophosphoryl choline; GP, glycerophosphate. AE-cellulose, aminoethyl-cellulose; PEI-cellulose, polyethyleneimine-cellulose; PAB-cellulose, *p*-aminobenzyl-cellulose.

and *p*-aminobenzyl-cellulose (PAB-cellulose) were purchased from Bio-Rad Laboratories, Richmond, Calif. DEAE-cellulose was obtained from Whatman. Prior to use, each adsorbant was treated with acid and alkali<sup>3</sup>, washed several times with distilled water, and finally resuspended in 0.01 M Tris-chloride buffer, pH 7.4.

### Enzyme assays

The extent of enzymatic hydrolysis of phospholipid compounds was monitored using either of two systems.

(I) Approx. 2  $\mu$ moles of substrate, egg lecithin was used routinely, was dissolved in 0.2 ml of diethyl ether. The tubes were then chilled in ice, and 2  $\mu$ moles of  $\text{CaCl}_2$ , 0.2 ml of 0.1 M Tris buffer (pH 7.4) and an aliquot of enzyme were successively added. The reaction was initiated by placing the capped tubes in a 37° water bath with constant agitation. Incubations were routinely allowed to proceed for 30 min. At the end of this time, the tubes were chilled in an ice bath, and the reaction permanently stopped by adding 4 ml of chloroform-methanol (2:1, v/v). The samples were agitated on a Vortex mixer, 0.4 ml of distilled water added, and the tube again thoroughly mixed. The phases were separated by brief centrifugation after which 250  $\mu$ l of the upper phase were transferred with a Hamilton syringe to a 125 mm  $\times$  15 mm test tube containing 0.6 ml of 70%  $\text{HClO}_4$ . The phosphoryl choline was converted to inorganic phosphate  $\text{P}_i$  by heating these samples for at least 45 min at 180°. Samples were then allowed to cool, and phosphate measured by the method of BARTLETT<sup>4</sup>.

(II) Alternatively, phospholipid substrate was emulsified in 0.1 M Tris buffer (pH 7.4) with a Bronson Sonicator to give an approximate concentration of 12.5  $\mu$ moles/ml.  $\text{CaCl}_2$  was added to the substrate to give a 1:1 molar ratio, and 0.2 ml of this emulsion used for each incubation. The tubes were chilled in an ice bath until enzyme aliquots had been added, and the capped reaction tubes incubated in a waterbath at 37° for 30 min with constant agitation. The remainder of the procedure was then effected as for the ether-water assay system.

## EXPERIMENTAL PROCEDURES AND RESULTS

### Purification

The enzyme was obtained from the growth medium of a late log phase (20–24 h) static culture of *Bacillus cereus*, strain 7004. The cells were grown by Grain Processing, Inc. of Muscatine, Iowa, in 60 l of medium containing Casamino acids and 0.3% yeast extract<sup>5</sup>. After removal of the cells by centrifugation, the supernatant was brought to 70% saturation with about 240 pounds of  $(\text{NH}_4)_2\text{SO}_4$ . The resulting moist precipitate contained the desired phospholipase C activity and was stored at –20° until use. In a typical experiment, 20 g of frozen material were dissolved in 60 ml of ice-cold 0.1 M Tris-maleate buffer, pH 7.3. A cloudy solution was obtained, which was dialyzed for 4 h against running tap water at less than 10°. The solution of crude phospholipase C was then further dialyzed at 4° against 0.01 M Tris-maleate buffer (pH 7.4) for approx. 24 h, after which it was frozen in a dry ice-acetone bath and lyophilized. The resulting light amber powder was used as starting material for subsequent purification studies. It was stable in this form for at least 6 months when stored at 4°, and was capable of hydrolyzing pure diacyl-GPC in an aqueous emulsion at the rate of 1–2  $\mu$ moles/min per mg. From the original 20 g of frozen precipitate 2.4 g of lyophilized powder were obtained.

*Fractionation by further  $(\text{NH}_4)_2\text{SO}_4$  precipitation and chromatography on Sephadex G-100*

Initial attempts to purify phospholipase C focused on the use of  $(\text{NH}_4)_2\text{SO}_4$  precipitation. Essentially all phospholipase C activity was precipitated at  $(\text{NH}_4)_2\text{SO}_4$  concentrations of 50% or less, and no activity was observed in the 50–80%  $(\text{NH}_4)_2\text{SO}_4$  fraction. The 0–50% fraction was therefore subfractionated into a 0–25% fraction which showed no increase in specific activity, whereas that in the 25–50% fraction showed a 3–4-fold purification.

A loss of about 75% of the activity initially present in the 25–50%  $(\text{NH}_4)_2\text{SO}_4$  fraction occurred over a 5-day period. 75% of the remaining activity (as determined by assay of an unchromatographed control of the 25–50%  $(\text{NH}_4)_2\text{SO}_4$  fraction) was recovered from the void volume of a Sephadex G-100 column. This final fraction represented about 7% of the original crude activity and a 5-fold purification. Because the partially purified enzyme proved to be labile at 4°, the Sephadex G-100 step afforded no further purification over that obtained by the use of  $(\text{NH}_4)_2\text{SO}_4$  alone.

*Batch addition experiments using various anion-exchange celluloses*

In order to find suitable anion-exchange cellulose, a series of experiments involving batchwise addition of adsorbant to crude enzyme solutions was undertaken. Treatment of the enzyme solution with DEAE-cellulose led to 50% of both activity and protein bound to the adsorbant. Although 95% of the adsorbed protein was eluted with the salt wash, only 15% of the bound activity was recovered. Similar experiments using either AE-cellulose or PAB-cellulose likewise resulted in the binding of activity and protein to the ion-exchange cellulose. Only 50% of the activity bound to either adsorbant was eluted with the wash, and the recovered activity showed a decreased specific activity. In contrast, treatment with PEI-cellulose in this manner left 100% of the activity toward diacyl-GPC and 80% of the protein in the supernatant fraction. On the basis of these results, it was decided to chromatograph an enzyme preparation on a PEI-cellulose column.

*Chromatography of dialyzed, lyophilized phospholipase C on PEI-cellulose*

Chromatography of dialyzed, lyophilized phospholipase C on PEI-cellulose columns in a system of 0.01 M Tris buffer (pH 7.4) at 0°, showed that phospholipase C activity was not bound to the column, but was eluted with the solvent front. In contrast, the bulk of the non-active proteins, as well as the characteristic brown pigment present in the starting material, were bound to the column. These could, however, be eluted when NaCl was included in the buffer system (see Fig. 1). In various experiments, the recovery of phospholipase C activity ranged between 85 and 100%, with up to 22-fold purification achieved in peak fractions. The dialyzed, lyophilized crude phospholipase C had a specific activity of 1–2  $\mu\text{moles}/\text{min}$  per mg, while that obtained from PEI-cellulose chromatography had specific activities ranging as high as 38  $\mu\text{moles}/\text{min}$  per mg.

In one experiment, phospholipase C purified by PEI-cellulose chromatography was lyophilized, dissolved, and then passed over a Sephadex G-100 column. Only 25% of this activity was recovered from the Sephadex G-100 column. The loss in activity associated with this step may be due to intrinsic lability of the partially purified enzyme, to loss of an essential cofactor, or to a combination of these and other as yet unknown factors.

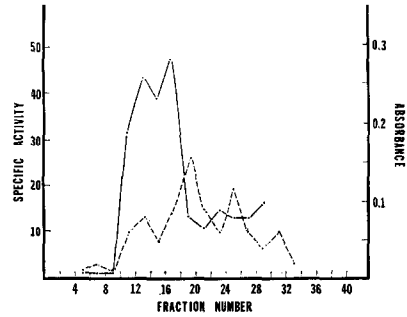
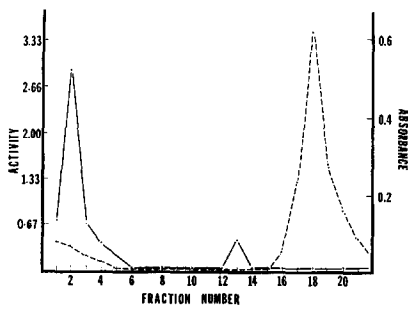


Fig. 1. Chromatography of phospholipase C activity on PEI-cellulose. 275 mg of dialyzed, lyophilized phospholipase C was dissolved in 14 ml of 0.01 M Tris buffer (pH 7.4) and applied to a column of PEI-cellulose (2.5 cm  $\times$  21 cm) which had previously been equilibrated with this buffer. The column was eluted with the starting buffer, 9-ml fractions were collected, and the flow rate was 18 ml/h. Activity is expressed in units of  $\mu$ moles of phosphate released per min (—). Absorbance, measured at 280  $\mu$ , is indicated on the right (---). All operations were performed at 0°.

Fig. 2. Purification of phospholipase C from *B. cereus* by protamine sulfate treatment and DEAE-cellulose chromatography. 54 mg of dialyzed, lyophilized phospholipase C were dissolved in 2.5 ml of 0.01 M Tris buffer, pH 7.4. The supernatant from protamine sulfate treatment (see text) was then applied to a column of DEAE-cellulose which had been equilibrated with 0.01 M Tris buffer (pH 7.4) at 0°. The column was washed with 2 vol. of starting buffer, and then a linear concentration gradient of KCl (final concentration, 1.0 M; total gradient volume, 100 ml) in 0.01 M Tris buffer (pH 7.4) was begun. Column flow rate was about 10 ml/h, and fractions of approx. 5 ml were collected. Specific activities are expressed as  $\mu$ moles of phosphate released per min per mg of protein (—). Absorbance was measured at 280  $\mu$  (---).

#### *Protamine sulfate treatment of phospholipase C*

A 2% solution of protamine sulfate (Sigma) in 0.01 M Tris buffer was prepared, and the pH adjusted to 6.7. To a solution of dialyzed, lyophilized phospholipase C (10 mg/ml, dry wt./vol), protamine sulfate solution was slowly added with mixing to give a final ratio of 0.2 mg protamine sulfate per mg of enzyme protein. After completing the addition of protamine sulfate, the resulting cloudy mixture was allowed to stand for 30 min at 0°, centrifuged in a laboratory centrifuge, and the supernatant decanted. This resulted in a 2–3 fold increase in specific activity in the supernatant, with complete preservation of total starting activity. Phospholipase C activity in this enzyme preparation was labile to freezing and thawing, and showed a 50% decrease in its initial activity when stored at  $-10^{\circ}$  for 24 h and then thawed.

The effect of chromatography of protamine sulfate-treated phospholipase C on columns of DEAE-, PEI- and CM-cellulose was studied. After washing the enzyme solution onto the column with about 15 ml of starting buffer, stepwise elution was effected using 0.1, 0.25 and 0.5 M KCl in the Tris buffer. Under these conditions, phospholipase C was inactivated by CM-cellulose. PEI-cellulose afforded recovery of approx. 50% of the activity applied, and additional 4-fold purification over the protamine sulfate step. Activity was eluted with the starting buffer, ahead of the bulk of other proteins. DEAE-cellulose chromatography produced results similar to PEI-cellulose chromatography, with the important exception that it allowed full recovery of the initial enzyme activity which had been applied to the column. After the enzyme was partially purified by  $(\text{NH}_4)_2\text{SO}_4$  fractionation and subsequent chromatography on columns of Sephadex G-100, activity bound to DEAE-cellulose columns at pH 7.4 was not eluted, even in the presence of 2 M NaCl. In contrast, treatment of crude

phospholipase C preparations with protamine sulfate yielded an enzyme preparation in which the activity was not adsorbed to DEAE-cellulose columns at pH 7.4 in the presence of 0.01 M Tris buffer (see Fig. 2). Specific activities of greater than 25  $\mu$ moles/min per mg were obtained when diacyl-GPC was used as a substrate. Elution of DEAE-cellulose columns with larger volumes of starting buffer prior to initiating the KCl gradient allowed complete separation of the desired activity from the bulk of the other proteins. Phospholipase C with specific activities as high as 45  $\mu$ moles/min per mg was then obtained (see Table I). Fractions having high specific activities accounted for up to 45% of the initial total crude activity.

TABLE I

## PURIFICATION OF PHOSPHOLIPASE C BY DEAE-CELLULOSE OR PEI-CELLULOSE CHROMATOGRAPHY

Summary of purification effected by chromatography of phospholipase C on columns of DEAE-cellulose and PEI-cellulose. Experimental conditions described in text and Figs. 1 and 2.

Step	Purification	Specific activity ( $\mu$ moles/min per mg)	Recovery (%)
Crude, dialyzed, lyophilized	1	1-2	100
Protamine sulfate supernatant	3	6	110
DEAE-cellulose	23	47	47*
Crude, dialyzed, lyophilized	1	1-2	100
PEI-cellulose	19	38	34**

\* 47% of the initial activity was recovered in the fraction having this specific activity. Total recovery of activity was greater than 70%, with at least 5-fold purification in peripheral fractions.

\*\* Total recovery of activity was 87%. Other fractions, representing 31 and 22% of the initial activity had respective specific activities of 18 and 14  $\mu$ moles/min per mg.

*Stability and preservation of activity**Stability of partially purified enzyme preparations*

In contrast to the apparent stability of dialyzed, lyophilized phospholipase C solutions, enzyme which has been partially purified exhibits marked lability. A 25-60%  $(\text{NH}_4)_2\text{SO}_4$  fraction demonstrated a 70% loss of activity when stored at a concentration of about 1 mg/ml for 2 days at 4°, but no loss of activity when similarly stored at a protein concentration of 20 mg/ml.

Prolonged (17-day) storages at 4° of a PEI-cellulose-purified phospholipase C preparation of high specific activity (27  $\mu$ moles/min per mg) resulted in retention of 35-40% of the initial enzyme activity. The protein concentration of these samples was less than 0.1 mg/ml. It was subsequently observed that essentially all of this decrease in activity occurred within the first several days following elution from the column. A sample of crude enzyme which was treated first with protamine sulfate, and then chromatographed on DEAE-cellulose was concentrated by rotary evaporation under vacuum at room temperature. When subsequently stored at 4° at a protein concentration of about 0.1 mg/ml, 50% of the activity was stable for over 1 month (37 days).

In order to study the effect of various methods of enzyme concentration on stability, aliquots of phospholipase C purified by protamine sulfate treatment and DEAE-cellulose chromatography were tested immediately after elution from the column. The effects of rotary evaporation, collodion-bag filtration,  $(\text{NH}_4)_2\text{SO}_4$ , and lyophilization were examined. The preservation of activity was compared to that initially present in the pooled fractions, as well as to that measured in an untreated

control stored at 4°, and to crude starting and protamine sulfate-supernatant activities. The results are summarized in Table II.

The activity of phospholipase C preparation toward phosphoglyceride substrates was observed. Activities in a two-phase ether-water system were assayed. The results of these experiments are summarized in Table III.

TABLE II

EFFECT OF METHOD OF CONCENTRATION ON STABILITY OF PURIFIED PHOSPHOLIPASE C

Results expressed as % of activity initially recovered from DEAE-cellulose chromatography purification step. 100% activity = 26  $\mu$ moles/min per mg.

Sample	Original activity (%)
1. Control—stored at 4° without concentration after elution from DEAE-cellulose	46
2. Rotary evaporated at room temperature	41
3. Collodion bag (porosity 70 000)	0
4. Lyophilized	54
5. (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> precipitated	22
6. Crude enzyme solution	100
7. Protamine sulfate supernatant	100

TABLE III

SPECIFICITY OF PHOSPHOLIPASE C PREPARATION PURIFIED BY PEI-CELLULOSE COLUMN CHROMATOGRAPHY

Using the two-phase ether-water assay system, activities of the phospholipase C preparation were observed. Each incubation contained approx. 2  $\mu$ moles of substrate. Two amounts of enzyme were incubated with each substrate, and the extent of hydrolysis at 0, 20, and 40 min determined.

Substrate	Activity ( $\mu$ moles/min per mg protein)
Diacyl glycerophosphoryl choline	14.8; 20.7
Diacyl glycerophosphoryl ethanolamine	8.8
Diacyl glycerophosphoryl monomethylethanolamine	15.3; 20.8
Diacyl glycerophosphate	No reaction

### Effects of ions and inhibitors

The effect of several sulfhydryl reagents and Zn<sup>2+</sup> on phospholipase C activity was studied. Aliquots of enzyme which had been purified by protamine sulfate treatment followed by chromatography on DEAE-cellulose were pretreated with dithiothreitol for 30 min at room temperature. Emulsified lecithin substrate containing CaCl<sub>2</sub> in a 1:1 molar ratio was then added, and the enzyme activities determined. Dithiothreitol showed an inhibitory effect at concentrations of 1 mM or higher. This effect was marked at 10 mM. *p*-Chloromercuribenzoate was not inhibitory at concentrations around 0.1 mM, nor was 5,5'-dithio-bis-(2-nitrobenzoic acid) over a range of concentrations from 5000 to 5  $\mu$ M. Zn<sup>2+</sup> added as the acetate salt showed little effect at the level of 0.1 mM. In the presence of 0.25% glutathione and 0.1 mM Zn<sup>2+</sup>, 25% inhibition relative to control values was observed. Although Ca<sup>2+</sup> was routinely added to the assay system to give an approx. 1:1 molar ratio with the lecithin substrate, under the conditions of the assay, omission of Ca<sup>2+</sup> did not alter the observed activities.

## DISCUSSION

The ability of phospholipase C from bacterial sources to hydrolyze different phospholipids has been studied by several workers. MACFARLANE AND KNIGHT<sup>1</sup> found that *Cl. welchii* produced a phospholipase C. Recently, phospholipase C from *Cl. welchii* has been purified about 240-fold<sup>6</sup>. Additional studies<sup>7</sup> on the phospholipase C produced by *Cl. welchii* revealed that in some but not all enzyme preparations, a second enzyme with cholinephosphohydrolase activity existed. This enzyme hydrolyzed sphingomyelin about 10 times more rapidly than lecithin. PASTAN *et al.*<sup>7</sup> purified this sphingomyelin-hydrolyzing enzyme 190-fold. Although crude phospholipase preparations from *Cl. welchii* readily hydrolyzed emulsions of lecithin, they did not possess activity toward pure ethanolamine phosphoglycerides<sup>8</sup>. It was noted, however, that the enzyme from *Cl. welchii* hydrolyzed ethanolamine phosphoglycerides which were present in red blood cell membranes or were emulsified in the presence of choline phosphoglycerides<sup>9</sup>.

Shortly after the recognition of phospholipase C production by *Cl. welchii*, the existence of a phospholipase C in the growth media of *B. cereus* cultures was reported by CHU<sup>2</sup>. OTTOLENGHI<sup>10</sup> studied some properties of the phospholipase C from *B. cereus*, and purified it about 4-fold by means of ethanol fractionation and gel filtration. By means of protamine sulfate treatment and DEAE-cellulose chromatography at pH 8.7, SLEIN AND LOGAN<sup>11</sup> separated several phospholipase fractions from the growth medium of *B. cereus*. Using a mixture of diacyl-GPC, diacyl glycerophosphoryl ethanolamine (diacyl-GPE), diacyl glycerophosphoryl inositol (diacyl-GPI), monoacyl-GPC, and sphingomyelin, they showed the existence of at least two types of phospholipase C activity, one capable of degrading diacyl-GPC and diacyl-GPE, and the other capable of hydrolyzing diacyl-GPI and sphingomyelin.

An important characteristic of the phospholipase C from *B. cereus*, however, was the enzyme's broader substrate specificities. Most notable was its intrinsic ability to hydrolyze diacyl-GPE compounds<sup>2</sup> not bound to natural membranes without the obligatory additions of choline-containing phosphatides. This ability is in marked distinction to the activity of *Cl. welchii* phospholipase C which required added choline-containing phospholipids for full activity. The observation that crude phospholipase C bound to DEAE-cellulose at pH 7.4 was not eluted at even high salt concentrations, and that this binding was prevented by pre-treatment of the enzyme with protamine sulfate, suggested that the enzyme had a strong negative charge, and possibly was reversibly complexed to nucleic acids. BANGHAM AND DAWSON<sup>12,13</sup> related the rate of enzymic hydrolysis of phospholipids by a phospholipase C preparation from *Cl. welchii* to the charge interaction between the enzyme and substrate. They claimed that for hydrolysis to occur, a net positive charge had to exist on the surface of the substrate micelles. At pH values where the phospholipase C catalyzed lecithin hydrolysis, phosphatidyl ethanolamine micelles were regarded to have a negative surface and were not hydrolyzed to a substantial degree. This hydrolysis was explained by the observation of a net positive surface charge on diacyl-GPC micelles under the conditions in which hydrolysis occurred. The broad substrate specificity demonstrated by phospholipase C preparations from *B. cereus* may be indicative of a different charge distribution on the enzyme molecule relative to that existing on the enzyme obtained from *Cl. welchii*.

The broad specificity of phospholipase C preparations from *B. cereus* makes it a valuable reagent for studying the structures of certain phosphoglycerides. A preparation from *B. cereus* has been used to confirm the structure of cardiolipin<sup>14</sup>, phosphatidyl glycerol isolated from bacteria and higher plants<sup>15,16</sup>, and a racemic *O*-alanyl ester of phosphatidyl glycerol<sup>17</sup>. DE HAAS AND VAN DEENEN<sup>18</sup> using synthetic acyl glycerophosphoryl cholines found that the phospholipase C acted on isomers with the phosphoryl choline and fatty acid moieties at either the 1, 2 or 3 positions of glycerol, but that the isomers with an unesterified 3-hydroxyl were not effective substrates. By utilizing a *B. cereus* phospholipase C preparation, HILL *et al.*<sup>19</sup> converted purified diacyl-GPE to corresponding diglycerides, and then used thin-layer chromatography to separate the resulting diglycerides according to the degree of unsaturation of the fatty acids esterified to the glycerol moiety.

The study reported here was undertaken to purify and characterize the phospholipase C from *B. cereus*. We felt it important to ascertain whether the phospholipase activity toward pure ethanolamine derivatives was separable from the activity toward choline phosphoglyceride, and whether a purified enzyme preparation would retain the ability to hydrolyze pure diacyl-GPE compounds. Column chromatography of crude phospholipase C from *B. cereus* afforded a 20-fold purification. Enzyme fractions purified in this manner retained the ability to hydrolyze either purified diacyl-GPC or purified diacyl-GPE and the rate of hydrolysis of choline phosphoglycerides was approximately twice that for ethanolamine phosphoglyceride. Also, hydrolysis of the diacyl monomethylethanolamine derivative was observed to proceed at a rate essentially equal to that for diacyl-GPC. No appreciable activity toward sphingomyelin has been demonstrated in the crude material or in either of these purified enzyme preparations.

Because purification of the phospholipase C activity from *B. cereus* resulted in substantial lability, and because our experience as well as that of a number of other workers demonstrated the usefulness of this preparation even in its crude form, it does not seem obligatory to purify the enzyme prior to its use as an analytical tool. However, a comparison of the amino acid structures of phospholipase C activities derived from *B. cereus* and *C. welchii* might provide valuable insight into the relationship between enzyme structure and function, particularly in view of the different specificities exhibited by both enzymes as well as their mutual general function as choline phosphohydrolases. Such studies will be possible as phospholipase C preparations from both sources become available in increasingly pure forms.

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