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Phosphatidylinositol-Specific Phospholipase C From *Bacillus cereus:* Improved Purification, Amino Acid Composition, and Amino-Terminal Sequence

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Phosphatidylinositol-specific phospholipase C was purified in a 27% yield from the culture medium of *Bacillus cereus* by a combination of ammonium sulfate precipitation and ion-exchange and hydrophobic interaction chromatography. The purified enzyme was free of other phospholipase C-type activities and exhibited a high specific activity of approximately 1,300 units/mg. Amino acid composition analysis and sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated a molecular weight of about 35 kDa. The sequence of the first 29 N-terminal amino acids was also determined.

Key words: N-terminal sequence, bacterial phospholipase, structure, isolation

Phosphatidylinositol-specific phospholipase C (PI-PLC) catalyzes the hydrolysis of the *sn*-3 phosphodiester bond of phosphatidylinositol,

$$\text{Phosphatidylinositol} \xrightarrow{\text{PI-PLC}} \text{Diacylglycerol} + \text{Inositol phosphate}$$

Abbreviations: BSA, bovine serum albumin; HPLC, high performance liquid chromatography; PC-PLC, phosphatidylcholine(ethanolamine)-hydrolyzing phospholipase C; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP_2, phosphatidylinositol 4,5-diphosphate; PI-PLC, phosphatidylinositol-specific phospholipase C; PTH, phenylthiohydantoin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SM-PLC, sphingomyelinase.

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cleaving the phospholipid molecule into a lipid-soluble (diacylglycerol) and a water-soluble (inositol phosphate) moiety. In mammalian systems PI-PLC is a ubiquitous enzyme that plays a key role in transmembrane signal transduction involving Ca^{2+}-mobilizing growth factors and hormones [1]. A number of recent reports describe complete or partial purification of PI-PLC from mammalian sources [e.g., 2–6]. So far only small quantities of mammalian PI-PLC have become available, which has limited the number of possible approaches to study the structure and function of this important enzyme.

It has been known for some time that certain bacteria secrete a soluble PI-PLC in the culture medium [7]. The bacterial enzymes differ from mammalian PI-PLC (for example, there is no metal ion requirement), but share the high specificity for phospholipids carrying inositol in the polar head group. Recent interest in bacterial PI-PLC has focused on their ability to release proteins that are tethered to the cell membrane via a glycosyl-phosphatidylinositol anchor [8,9]. Isolation of bacterial PI-PLC is facilitated by the fact that the protein is screted in a water-soluble form and several reports describing purification of the bacterial enzyme in milligram quantities have appeared [10–14]. So far, however, no structural data for bacterial PI-PLC have been published, while only one report [12] deals with some aspects of the enzymology of this enzyme.

In this paper we report an improved purification procedure for PI-PLC from *Bacillus cereus* culture medium together with data on the amino acid composition and the N-terminal sequence.

MATERIALS AND METHODS

Cells and Materials

Bacillus cereus (ATCC 6464) was obtained from the American Type Culture Collection (Rockville, MD). Ammonium sulfate (Ultra Pure) was from Schwarz/Mann Biotech (Cleveland, OH). Phosphatidylinositol (PI) was from Avanti (Birmingham, AL). Phosphatidylinositol 4,5-diphosphate (PIP_2) and phosphatidylinositol 4-monophosphate (PIP) were from Sigma (St. Louis, MO). Radiolabeled PI, PIP_2, PIP (all inositol-2-^{3}H), and [choline methyl-^{14}C]-phosphatidylcholine were obtained from NEN (Wilmington, DE). [N-methyl-^{14}C]sphingomyelin (bovine) was from Amersham (Arlington Heights, IL). BSA (fraction V) was from United States Biochemical Corp. (Cleveland, OH). CM-Sephadex and DEAE-cellulose (microgranular) were from Sigma (St. Louis, MO) and phenyl-Sepharose CL-4B was from Pharmacia (Piscataway, NJ). Spectrapor dialysis tubing (MW cutoff 6–8,000 or 12–14,000) was from Spectrum Medical Industries, Inc. (Los Angeles, CA). Molecular weight markers were from Biorad (Richmond, CA). All other reagents and materials were of the highest grade available.

Growth Conditions

The growth medium for *B. cereus* contained 40 g Bacto-peptone, 40 g yeast extract, 20 g NaCl, 1 g Na_2HPO_4, and water up to 4 L. The pH was adjusted to 7.0 with 1 M NaOH. A stock culture was prepared from a single colony isolate by inoculating 100 ml of medium and growing overnight on a shaker at 37°C. The stock culture was stored in 4-ml aliquots containing 50% glycerol at −20°C.

To obtain starting material for the purification of PI-PLC, *B. cereus* was grown in 4-L batches in a jar fermentor. A preculture was prepared by adding a 4-ml aliquot of stock culture to 100 ml of medium and incubation on a shaker at 37°C for 12 h. In a jar fermentor, 4 L medium was then inoculated with the preculture and incubated at 37°C with the stirrer set at 400 rpm and the airflow at 10 L/min. The pH was maintained at 7.0 by addition of NaOH and H_3PO_4. Incubation was for 3.5 h, at which point the culture was in the early stationary phase. As has been reported earlier [10], the increase of PI-PLC activity in the *B. cereus* culture medium closely paralleled cell growth and was optimal in the late logarithmic/early stationary phase.

Purification of PI-PLC

Ammonium sulfate precipitation and dialysis. A 4-L batch of *B. cereus* culture was cooled to 4°C and cells were removed by centrifugation for 30 min at 13,000*g*. The supernatant was collected, solid ammonium sulfate was added slowly to 90% saturation (576 g/L), and the solution was allowed to sit overnight at 4°C. The dark-brown precipitate was collected by centrifugation for 30 min at 13,000*g*, dissolved in the minimal volume of cold 5 mM Tris-maleate buffer (pH 6.5), and dialyzed against three changes of the same buffer at 4°C. All subsequent chromatography and dialysis steps were carried out at 4°C.

CM-Sephadex column chromatography. The dialysate was collected and a precipitate was removed by centrifugation for 30 min at 4,340*g*. The supernatant was applied to a column of CM-Sephadex (3.5 × 10 cm) and eluted with Tris-maleate buffer at a flow rate of 50 ml/h. The dark-colored breakthrough fractions, which contain the PI-PLC activity and other proteins not binding to the column, were collected and dialyzed against three changes of 20 mM Tris/HCl (pH 8.5).

DEAE-cellulose column chromatography. The dialysate was applied to a DEAE-cellulose column (3.5 × 10 cm) equilibrated in 20 mM Tris/HCl (pH 8.5) and eluted with a linear gradient (2 × 750 ml) from 0 to 0.3 M NaCl in the same buffer. The fractions containing PI-PLC activity were collected and concentrated to about 10 ml by ultrafiltration in an Amicon ultrafiltration cell with a YM10 membrane.

Phenyl-Sepharose column chromatography. The yellow concentrated DEAE-cellulose fractions were applied to a phenyl-Sepharose column (2 × 7 cm) equilibrated in 20 mM Tris/HCl (pH 7.5). The column was washed with at least 50 ml of the same buffer until all yellow material had been eluted. PI-PLC activity was then eluted with the same buffer containing 50% ethylene glycol (by volume). Fractions containing PI-PLC activity were collected, washed with pH 7.5 buffer, and concentrated by ultrafiltration. Enzyme solutions were stored in 20 mM Tris/HCl (pH 7.5) at 1–2 mg protein/ml at −20°C and were stable for several months.

Enzyme Assays

The enzyme assay routinely used in this study was essentially the one described by Ikezawa and Taguchi [13], except that radiolabeled PI, diluted with cold PI to a specific radioactivity of about 70,000 cpm/μmol, was used as the substrate. The extent of hydrolysis could therefore easily be determined by liquid scintillation counting of an aliquot of the aqueous phase (approximately 1 ml total) obtained after extraction of the assay mixture with 2.5 ml of chloroform-methanol-HCl, 66:33:1 (v/v/v) [13]. All activity data reported here are derived from the counts measured in 0.5 ml of the aqueous phase multiplied by a factor 2, thus representing the total

amount of substrate hydrolyzed in the assay mixture. A strict proportionality between the amount of enzyme added and the extent of hydrolysis was observed only when incubation times were limited to 10 min, and the amount of substrate hydrolyzed was kept below 10% of the total amount present in the assay mixture. Concentrated enzyme solutions were diluted in 0.1% BSA (pH 7.5) prior to assaying.

PI-PLC activity assays also were performed at pH 5.5 and 25°C as described by Sundler et al. [12]. PC-PLC activity was measured essentially as described by Ikezawa et al. [15] except that the buffer contained 10 mM $ZnCl_2$ and [^{14}C]-phosphatidylcholine (diluted to a specific radioactivity of 35,000 cpm/μmol) was used as substrate at a final concentration of 0.5 mM. The same method was used to measure sphingomyelinase (SM-PLC) activity except that the buffer contained 10 mM Mg acetate and [^{14}C]-sphingomyelin (diluted to a specific radioactivity of 35,000 cpm/μmol) was used as substrate. In all these assays the reaction was stopped by addition of the chloroform-methanol-HCl mixture and the amount of substrate hydrolyzed determined by liquid scintillation counting of the aqueous phase. The acetylcholinesterase releasing activity of the purified PI-PLC was determined as described by Ikezawa and Taguchi [13] using fresh human erythrocytes.

Protein Determination

Protein was routinely determined by the Bradford [16] microassay procedure using Biorad Dye Reagent. For comparative purposes the method of Lowry et al. [17] was also employed. For both methods BSA was used as the standard.

Amino Acid Analysis

Amino acid analyses were carried out by AAA Laboratories (Mercer Island, WA), using a Dionex Model D500 Mark II analyzer following company specifications (M. Pickering, Application Note #2). Hydrolysis was for 20 h in 6 N HCl at 115°C in the presence of 0.05% mercaptoethanol and phenol. Cysteine was determined in a separate run from the alanine/cysteic acid ratio after performic acid oxidation [19]. Analyses also were performed by the University of Oregon Biotechnology Laboratory using the phenylisothiocyanate precolumn derivatization method as described by Bidlingmeyer et al. [18], except that hydrolysis was for 20 h at 110°C. Derivatized amino acids were quantitated by HPLC using a Waters Pico-Tag™ column (3.9 × 150 mm) maintained at 38°C by a Waters temperature control module. The chromatography system consisted of a Beckman System Gold controller, a Beckman 126 pump with analytical heads, a Beckman 167 variable wavelength detector, and a Beckman 506 autosampler.

Amino Acid Sequencing

Amino acid sequencing was performed by the Protein Structure Laboratory, UC Davis (Davis, CA), using a Beckman 890M liquid-phase sequencer. Sequencing was done in the presence of 2 mg Polybrene using a 0.1 M Quadrol program. PTH amino acids were identified by two different HPLC methods as described by Bhown et al. [20] and Hunkapiller and Hood [21], respectively. Sequencing also was performed by the University of Oregon Biotechnology Laboratory on an Applied Biosystems Model 470A gas-phase protein sequencer using Edman degradation chemistry. In this case PTH amino acids were identified with an Applied Biosystems Model 120 PTH analyzer.

N-Terminal Analysis

Manual N-terminal analysis was performed using dimethylaminoazobenzene isothiocyanate as described by Chang [22,23].

SDS-PAGE

SDS-PAGE was performed using 12% acrylamide "mini" slab gels (Idea Scientific) with a stacking gel containing 6% acrylamide and the discontinuous buffer system of Laemmli [24]. Where necessary, samples were desalted using Centricon 10 microconcentrators (Amicon), dried by Speedvac, taken up in dissociation buffer, and bath sonicated for 5–10 min at room temperature.

A gel electrophoresis experiment designed to localize the three different phospholipase C-type activities of *B. cereus* was carried out as follows: Ammonium sulfate precipitate was collected, dialyzed, and centrifuged as described above. The dialysate was desalted and concentrated to 4.25 mg protein/ml by centrifugation through a Centricon filter. To each lane 10 μl of this solution was added after mixing with an equal volume of dissociation buffer and bath sonication for 5 min. Several lanes were cut from the gel with a razor blade, washed in distilled water for 2 × 10 min, and equilibrated for 2 × 10 min in buffer. The buffers used were 0.125 M Tris, pH 6.8; 0.1 M borate, 10 mM $ZnCl_2$, pH 7.0; and 0.1 M borate, 10 mM Mg acetate, pH 7.0, for the lanes assayed for PI-PLC, PC-PLC, and SM-PLC activity, respectively. All washes were performed at 4°C. A representative lane was stained for protein with Coomassie Blue R-250. Individual lanes were then cut into 1-mm slices with a Hoefer Scientific gel slicer and slices were incubated in the premixed assay solutions. Assays were started by addition of radioactive substrate and proceeded for 20 min (PC-PLC and SM-PLC) or 10 min (PI-PLC) at 37°C. Extraction and liquid scintillation counting was as described above under Enzyme Assays.

RESULTS

B. cereus has been reported to secrete three different PLC-type activities in its growth medium [15]. All hydrolyze the phosphodiester bond between the polar head group and the diacylglycerol moiety of phospholipid molecules but show phospholipid-specificity and differ in their optimal reaction conditions. PC-PLC is a Zn^{2+}-dependent enzyme hydrolyzing phosphatidylcholine and phosphatidylethanolamine, SM-PLC (sphingomyelinase) is Mg^{2+}-dependent and is specific for sphingomyelin, whereas PI-PLC has no metal ion requirement and specifically hydrolyzes phosphatidylinositol. It was therefore of interest to examine whether these activities could be separated and identified using a protein separation technique with a high resolving power such as SDS-PAGE.

The result of this experiment is shown in Figure 1. The protein sample consisted of a 90% saturation $(NH_4)_2SO_4$ cut of *B. cereus* culture medium after dialysis and removal of proteins precipitated during dialysis. Although the gel was run under denaturing conditions, it was possible to achieve partial renaturation of all three enzymes sufficient for unambiguous localization in the gel pattern. All three activities are clearly separated. PI-PLC and SM-PLC run close together with apparent molecular weights of around 35 kDa and PC-PLC with an apparent molecular weight of around 28 kDa. The PI-PLC and SM-PLC activities appear to coincide with a weakly stained doublet of bands in the Coomassie-stained lane, whereas the PC-PLC activity

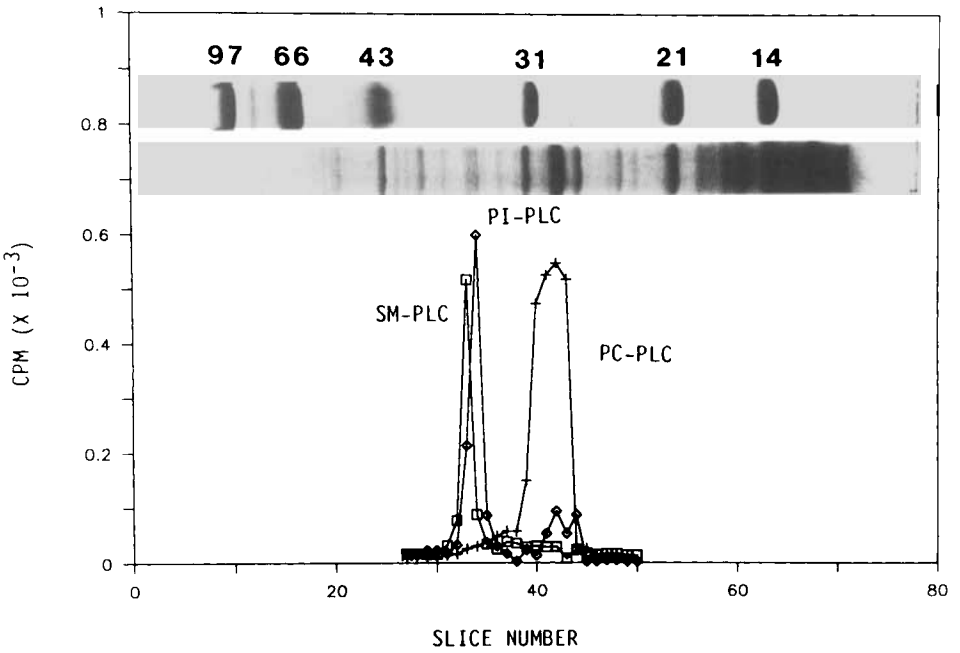

Fig. 1. Separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and identification of the three phospholipase C-type activities secreted in the *B. cereus* culture medium. Crude ammonium sulfate precipitate of the culture medium was subjected to SDS-PAGE and different lanes were analyzed for PI-PLC (◇), PC-PLC (+), and SM-PLC (□) activity as described in Materials and Methods. Background-level counts were obtained for slices 1–27 and 51–80. For convenient representation the actual CPM numbers for the PC-PLC activity were reduced by a factor 10. A sample lane and a lane containing molecular weight markers, both stained with Coomassie Blue, are shown on top. Numbers indicate the molecular weights of the molecular weight markers in kDa.

TABLE I. Purification of PI-PLC From *B. cereus* (ATCC 6464)

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification factor
$(NH_4)_2SO_4$ 90% saturated	194	6,900	36	100	1
CM-Sephadex	73	5,920	80	86	2.2
DEAE-cellulose	3.8	2,180	580	32	16
Phenyl-Sepharose	1.44	1,850	1,280	27	36

coincides with a heavily stained band (Fig. 1). As was found earlier by isoelectric focusing [15], our data show that at least three different proteins are responsible for the three phospholipase C-like activities secreted by *B. cereus*.

A typical purification of PI-PLC from *B. cereus* growth medium is summarized in Table I, and an SDS-polyacrylamide gel of the material obtained at the various stages of purification is shown in Figure 2. These data are representative of more than ten different preparations obtained by this procedure. A highly purified enzyme preparation (Fig. 2) is obtained in only four steps with a good overall recovery. The

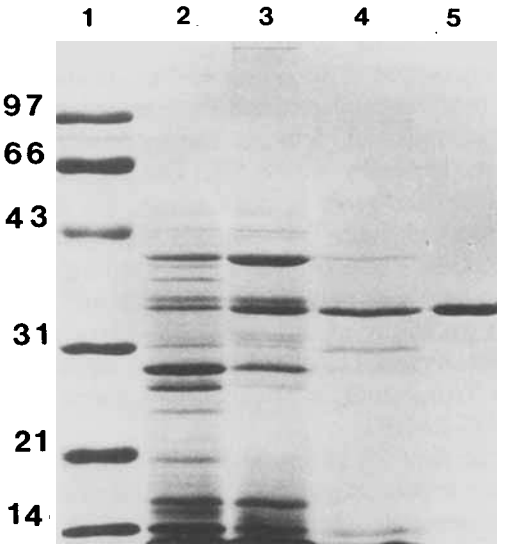

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel of PI-PLC during the various stages of purification. **Lane 1:** Molecular weight markers. **Lane 2:** Ammonium sulfate precipitate. **Lane 3:** After CM-Sephadex chromatography. **Lane 4:** After DEAE-cellulose chromatography. **Lane 5:** After phenyl-Sepharose chromatography. Numbers indicate the molecular weights of the molecular weight markers in kDa.

purity of the final enzyme preparation was generally better than 95%, with the specific activity ranging from 1,200 to 1,400 units/mg. Some trace contaminants, running in the 35–30-kDa range, could be observed on an overloaded gel. In some of our preparations purity, as judged by SDS-PAGE, could be improved slightly by repeating the DEAE-cellulose chromatography on a small column (2 × 7 cm) in 20 mM Tris/HCl (pH 7.5) and elution with a linear gradient (2 × 100 ml) in the same buffer. In some cases it was necessary to repeat the phenyl-Sepharose step to obtain a completely colorless enzyme preparation. All preparations tested gave a single N-terminus (Ala) in about 50% yield. The whole purification procedure could be upscaled with the same overall recovery of activity and purity of the final product by combining several ammonium sulfate cuts prior to the chromatography steps.

For futher characterization we also compared the specific activity of our preparation with that of Sundler et al. [12] using their enzyme activity and protein assay procedures. We find a specific activity of 540 units/mg for our preparation compared to 395 units/mg reported by Sundler et al. for their nearly homogeneous preparation. The purified enzyme was free of PC-PLC and SM-PLC activities, and diacylglycerol was the only product detectable in the organic phase after prolonged incubation of phosphatidylinositol with the enzyme preparation. Under the standard assay conditions and with ten times the amount of PI-PLC added, there was no significant hydrolysis of either PIP or PIP_2. At the detergent/PI molar ratio of 2:1 used in the standard assay, the detergents cholate, Triton X-100, and n-octylglucoside gave 30%, 18%, and 12% of the activity observed with the standard detergent deoxycholate, respectively. Incubation of human erythrocytes with pure PI-PLC under conditions

previously described for bovine erythrocytes [13], resulted in rapid release of membrane-bound acetylcholinesterase. Treatment of purified PI-PLC with the reducing agent dithiothreitol or with the sulfhydryl reagents iodoacetamide or N-ethylmaleimide had no effect on enzyme activity.

The amino acid composition of *B. cereus* PI-PLC is shown in Table II. These data are the averages of two independent determinations using two different methodologies as described in Materials and Methods. The agreement between data obtained by the two techniques was generally within 5%. The presence of a single cysteine residue was indicated after performic acid oxidation, but this result needs to be confirmed by an independent method. The relatively high abundance of acidic amino acids correlates with the acidic isoelectric point of 5.4 reported for this enzyme [15]. The protein contains a rather high percentage of apolar amino acids (about 35%), which may be related to the ability of this enzyme to interact with aggregated lipid substrates. The molecular weight calculated from the amino acid composition is 35,227 Da (assuming 2 Trp), which is in excellent agreement with the molecular weight estimated from SDS-PAGE.

The sequence of the first 29 N-terminal amino acids of *B. cereus* PI-PLC determined by liquid-phase sequencing of the native intact protein is shown in Figure 3. The sequence of the residues in boldface was confirmed by gas-phase sequencing

TABLE II. Amino Acid Composition of PI-PLC From *B. cereus***

Amino acid	Nearest integer (residues/mol)
Asp + Asn	44
Glu + Gln	34
Ser[a]	24
Gly	20
His	6
Arg	10
Thr[a]	18
Ala	14
Pro	16
Tyr	19
Val	15
Met	7
Cys[b]	1
Ile	22
Leu	22
Phe	13
Lys	23
Trp[c]	⩾2

*Average of the results from two independent methods described in Materials and Methods. The two data sets agreed within 5% except for Met.

[a]Values were increased by 10% (Ser) and 5% (Thr) to correct for loss during acid hydrolysis.

[b]Determined from the Ala/cysteic acid ratio after performic acid oxidation.

[c]So far two Trp residues have been identified in the sequence (see Fig. 3).

Fig. 3. Sequence of the N-terminus of *B. cereus* PI-PLC determined by liquid-phase sequencing of the native protein. The sequence of the residues in boldface was confirmed by gas-phase sequencing of the native protein.

of native intact PI-PLC. The sequence of residues 12–17 was selected to construct an oligonucleotide probe that will be used in recombinant DNA studies.

DISCUSSION

The purpose of the work presented here was to obtain a highly purified preparation of phosphatidylinositol-specific phospholipase C from a bacterial source in sufficient quantity to begin biochemical and biophysical studies on the structure and function of this enzyme. A number of publications report methods for purification of bacterial PI-PLC. Ikezawa and coworkers have published procedures for purification of PI-PLC from the culture media of the bacilli *B. cereus* and *B. thuringiensis* [10,11,13]. Low [14] describes a procedure for purification of PI-PLC from *Staphylococcus aureus*, while Sundler et al. [12] describe purification to near homogeneity of PI-PLC using commercially obtained crude "phospholipase C" (*B. cereus*) as the starting material. For practical reasons we selected the nonpathogenic bacillus *B. cereus* as the enzyme source for PI-PLC, and adopted the purification scheme of Ikezawa and Taguchi [13], since it appeared to result in high quantities of pure protein.

We find that the procedure developed by Ikezawa and Taguchi [13] is a good initial strategy for purifying PI-PLC. However, during the course of this work we observed some significant differences between our results and those reported previously [13]. Our procedure results in a higher recovery of total activity (1,800 units versus 400 units per 4-L culture) with a lower final protein recovery (1.5 mg versus 9 mg per 4-L culture). Furthermore, we find that an additional purification step following the three-step purification procedure described earlier [13] is needed to obtain a highly purified PI-PLC preparation that is free of colored contaminants. Although the bulk of strongly colored material, which is present in the growth medium and coprecipitates during the ammonium sulfate treatment, is removed during the DEAE-cellulose chromatography, the preparation still contains yellow contaminations. At this stage the preparation appears relatively clean by SDS-PAGE (see Fig. 2). Removal of the yellow material by phenyl-Sepharose chromatography, however, results in a significant increase of the specific activity (Table II). This is because the yellow contamination, which does not form a distinct band in SDS-PAGE but is sometimes visible as a faint smear in the low molecular weight range, contributes strongly in the protein assays (see below). Several attempts were made to circumvent this problem by selecting less strongly colored growth media. Although *B. cereus* appeared to grow well in other selected media, secretion of PI-PLC activity was lower and less consistent than observed with the standard medium used here.

Another important difference is that we see a consistently higher specific enzyme activity at all stages of the purification, with the specific activity of the final

preparation about 20-fold higher than previously reported by Ikezawa and Taguchi [13]. (For this comparison to be meaningful we have multiplied their values with a factor 1/0.4 so that both their and our values for the specific activity reflect the total amount of substrate hydrolysed in the assay mixture.) The specific activity of our preparation (540 units/mg) also compares favorably to that reported by Sundler et al. [12] (395 units/mg) when measured using their assay conditions and protein determination.

Several factors other than increased purity may contribute to the rather large difference in specific enzyme activity between our preparation and the one reported earlier [13]. One factor is the enzyme activity assay. The assay used in our study, however, is identical with the one used in the previous report [13], except that we use radioactive substrate combined with liquid scintillation counting to determine the inositol phosphate formed, rather than nonradiolabeled substrate combined with a phosphate determination.

A second factor is the protein determination. We find that protein recoveries and therefore specific activities are strongly dependent on the method used for protein determination. This is especially true in the early stages of purification, where the colored material present in the preparation contributes strongly, but not equally, to various colorimetric protein determinations. For example, the yellow material eluting in the breakthrough of the phenyl-Sepharose column gives a 4–5 times higher signal in the Lowry protein assay [17] compared to the Bradford assay [16]. Since the Bradford assay more closely estimates the true amount of protein in the sample, we selected this method as the standard protein assay in our study. The difference between the specific activity of our PI-PLC preparation and that of Ikezawa and Taguchi [13] may therefore in part reflect a difference in the amount of protein estimated by the different protein determination methods used. This may be compounded by the presence of a component in their final preparation that contributes strongly to the protein determination, but that is absent from our final preparation. There may be other factors contributing to the difference in specific activity as well. For example, the intrinsic specific activity of PI-PLC may be dependent on the strain of bacteria used or there may be inhibitors present that are removed more completely in our procedure.

In conclusion, the procedure described here yields a highly purified preparation of *B. cereus* PI-PLC with a high specific activity that is free of contaminating phospholipase C activities. The quality of this preparation is sufficient to initiate structure-function analysis as is exemplified by the amino acid composition and N-terminal sequence data reported here.

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