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Purification and some properties of phospholipase C from *Achromobacter xylosoxidans*

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ABSTRACT

A non-haemolytic phospholipase C (EC 3.1.4.3) was purified from the culture medium of *Achromobacter xylosoxidans* with a 5% yield and a purification factor of 330. A combination of ultrafiltration, acetone precipitation and two subsequent affinity chromatographic steps was used. The affinity chromatography is a new application of 2-(4-aminophenylsulphonyl)ethyl-cellulose, a sorbent that has previously been used for the purification of phospholipase C from *Bacillus cereus*. The purified enzyme gave four distinct bands on polyacrylamide gel electrophoresis, and each band was catalytically active. Under our experimental conditions, the phospholipids examined were hydrolysed in the following order: phosphatidylcholine, phosphatidylethanolamine, sphingomyelin. Neither the synthetic substrate *p*-nitrophenylphosphorylcholine nor phosphatidylinositol was hydrolysed under different experimental conditions. For maximal hydrolytic activity toward phosphatidylcholine, the enzyme required Triton X-100 and Ca^{2+} ions. EDTA was inhibitory, but the enzyme activity was almost completely restored by Zn^{2+}. The average molecuar mass of the phospholipase C, estimated by gel permeation, was 34 000 daltons.

INTRODUCTION

Little is known about the secretion of phospholipase C (PLC) in the culture broth of gram-negative microorganisms. At present, such an extracellular activity has been characterized for *Pseudomonas fluorescens, P. aureofaciens, P. aeruginosa, Acinetobacter calcoaceticus* and *Legionella pneumophila* [1–9].

The exact secretion mechanism of PLC is still unknown [10]. All virulent strains of *P. aeruginosa* produce the enzyme [7], but the role of haemolysins in the pathogenesis is not well understood [7,11].

P. aeruginosa produces two different PLCs, only one of which is haemolytic [12]. Berk *et al.* [13] have found that two isolated fractions of PLC from *P. aeruginosa* cause paralysis, death, dermonecrotic effects and increased vascular permeability in mice, and Meyers and Berk [14] reported a marked inflammatory response in mice that has been injected intraperitoneally with a purified enzyme.

Various procedures have been published for the purification of PLC from gram-negative bacteria, using either conventional methods [1,3,4] or affinity chromatographic techniques [7,11].

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In our previous reports [15,16] we have described a new non-haemolytic PLC isolated from the culture medium of *Achromobacter xylosoxidans* and have revealed some of its properties. This paper reports an affinity chromatographic method for the purification of this enzyme, and some more of its properties are described.

EXPERIMENTAL

Organism, medium and growth conditions

The producing strain *A. xylosoxidans* XC-1 has been characterized elsewhere [15]. The strain was grown on casein–soy broth (Institute of Parasitic and Infectious Diseases, Sofia, Bulgaria), containing additional 0.5% arabinose, 0.07 mg/l nicotinamide and 4 m*M* $ZnSO_4$, for 18 h at 24°C on a rotary shaker. These conditions corresponded to maximum PLC production in the medium [15].

Purification of phospholipase C

A 1-l culture solution was centrifuged for 20 min at 10 000 *g* and 2°C to remove the cells. The supernatant was then subjected to ultrafiltration through a Bulpore-9205 P membrane (Chemical Technology Institute, Burgas, Bulgaria) to a volume of 100 ml. The concentrate was saturated to 72% (v/v) with acetone (Merck, Darmstadt, Germany), whilst being kept at 0 to − 10°C under continuous stirring. The mixture was allowed to stand at −20°C for 1 h, and the precipitate was collected by centrifugation at 13 000 *g* for 15 min and resuspended in 10 ml of redistilled water. The preparation was dialysed against 1 l of 0.1 *M* $Na_2B_4O_7$–HCl (pH 8.0).

Affinity chromatographic steps were carried out as described previously [17], with some modifications. A commercially obtained 2-(4-aminophenylsulphonyl) ethyl (APSE) derivative of cellulose containing 23 μmol/ml amino groups was used (Ostsorb AV, Spolchemie, Usti nad Labem, Czechoslovakia). Up to 600 mg of protein (500 U of PLC) were loaded onto a column (200 mm × 18 mm I.D.) of APSE-cellulose at a flow-rate of 18 ml/h and allowed to stand for 2 h at 4°C. The column was washed with 0.1 *M* sodium borate (pH 8.0), equal to about three total bed volumes, which contained additional 1 *M* NaCl, then the enzyme was eluted using 3 *M* Tris–HCl (pH 8.5) at a flow-rate of 30 ml/h. Fractions of 5 ml were collected throughout. All the procedures were carried out at 4–8°C.

The repeated affinity chromatography was carried out after extensive dialysis against 1-l portions of 0.1 *M* sodium borate (pH 8.0) under the conditions described above.

Enzyme assays

PLC activity was assayed by the method of Takahashi *et al.* [18] in 0.2 *M* sodium acetate–acetic acid (pH 6.2). The following substrates were used: L-α-phosphatidylcholine from egg-yolk or synthetic dilauroyl, synthetic L-α-phospha-

tidylethanolamine dipalmitoyl, L-α-phosphatidylinositol from soybean, and sphingomyelin from egg-yolk. All phospholipids were purchased from Sigma (St. Louis, MO, USA). In some assays, egg-yolk lipoprotein was used as a substrate. This was prepared as described by Little [19]. One unit of the enzyme was defined as the amount that would hydrolyse 1 μmol of substrate per minute, at pH 6.2 and 37°C. Reaction products were identified by thin-layer chromatography by (TLC), essentially as described by Sonoki and Ikezawa [4].

The hydrolysis of *p*-nitrophenylphosphorylcholine (NPPC) by PLC was assayed by the method of Kurioka and Matsuda [20], or by the modified procedure of Kamberov and Ivanov [17].

Other methods

Protein was determined by the method of Hartree [21] or Bradford [22] with bovine serum albumin (Serva, Heidelberg, Germany) as a standard.

Disc gel electrophoresis was run in 7.5% polyacrylamide in the system of Davis [23], at pH 8.3 and 2 mA per tube. Approximately 100 μg of protein were loaded into each gel. The gels were stained using Coomassie Brilliant Blue R 250 (Merck).

The molecular mass of PLC was determined by gel permeation of a Sephadex G 75 column (95 cm × 2.5 cm I.D.) in 0.05 *M* Tris–HCl (pH 7.8). The following standard proteins were used: RNAse II (13 700 daltons), PLC from *Bacillus cereus* (23 000 daltons), peroxidase (40 000 daltons) and human serum albumin (65 000 daltons), all from Fluka (Buchs, Switzerland).

The haemolytic activity of PLC was studied both on a blood agar (oxoid agar base with 3% sheep erythrocytes) or by the method of Tomita *et al.* [24] with a 5% suspension of fresh sheep erythrocytes in borate-buffered saline, containing 1 m*M* Mg^{2+}, 1 m*M* Ca^{2+} or 1 m*M* Ca^{2+} and 1 m*M* Mg^{2+}, or without added metal ions.

All other reagents used were of analytical grade or the highest purity available.

RESULTS

Purification of phospholipase C

A typical enzyme purification from *A. xylosoxidans* culture supernatant is summarized in Table I. The enzyme was purified 330-fold over the supernatant, with an overall recovery of 5%.

Although the precipitation gave an unsatisfactory degree of purification, the PLC thus obtained was quite stable (for at least six months) when stored at 4°C; therefore a precipitation step was included in the preparation of the crude enzyme.

In our preliminary experiments we have found that PLC from *A. xylosoxidans* showed a high affinity for APSE-cellulose. This adsorbent was first applied by Kamberov and Ivanov [17] for the purification of PLC from *B. cereus*.

TABLE I

PURIFICATION OF PHOSPHOLIPASE C FROM *A. XYLOSOXIDANS* STARTING WITH 1 l OF CULTURE SUPERNATANT

Step	Total activity (U)	Protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification (*n*-fold)
Culture supernatant	1000	3500	0.29	100	–
Ultrafiltration	770	550	1.4	77	4.8
Acetone precipitate	600	220	2.7	60	9.1
Affinity column I	170	3.5	48.5	17	167
Affinity column II	48.5	0.5	97	4.8	334

In the present study the chromatographic purification was carried out at different pH values, *i.e.* 5.5, 7.0, 8.0 and 9.0. The best results were obtained at pH 8.0 in 0.1 *M* sodium borate. Sodium chloride was added to the buffer at 1 *M* final concentration to reduce a non-specific binding. A low temperature was also required for optimal affinity adsorption of PLC. All chromatographic procedures were carried out at 4–8°C.

Chaotropic agents, such as urea or guanidine chloride, which were shown to be excellent eluents for *B. cereus* PLC [17], were not suitable for desorption of PLC of *A. xylosoxidans* from the APSE-cellulose column. Although the enzyme was stable after elution with urea and a sharp elution profile was obtained (not shown), it was drastically inactivated during the subsequent dialysis or desalting by gel permeation. Other substances, such as NaCl (up to 3.5 *M*), sorbitol (30%, w/v), or Tris (0.5–3 *M*, pH 7.2–9.0), were tested for the desorption of PLC. The best results were achieved using 3 *M* Tris (pH 8.5) (Fig. 1). Sodium chloride and sorbitol did not desorb the enzyme at all. On the other hand, when Tris was applied at 0.5–1.5 *M*, the elution zone was broadened.

In both affinity chromatographic steps a significant inactivation of PLC was observed, causing a low recovery of enzyme activity (Table I). Our attempt to stabilize PLC by adding gelatin, bovine serum albumin or glycerol to the preparation at 0.05, 0.02 and 20% (w/v), respectively, did not improve the yield during the chromatographic purification. The addition of $ZnCl_2$ at 5–15 m*M* final concentration, had also no effect on enzyme stability.

In a previous report [16] we have shown that PLC from *A. xylosoxidans* consisted of at least four isozymes, as revealed by chromatography on CM Sephadex and disc gel electrophoresis. The purified preparation in this study appeared as four distinct bands with different mobility on disc electrophoresis (Fig. 2). All these bands were catalytically active when unstained gels were sliced and tested for activity (data not shown).

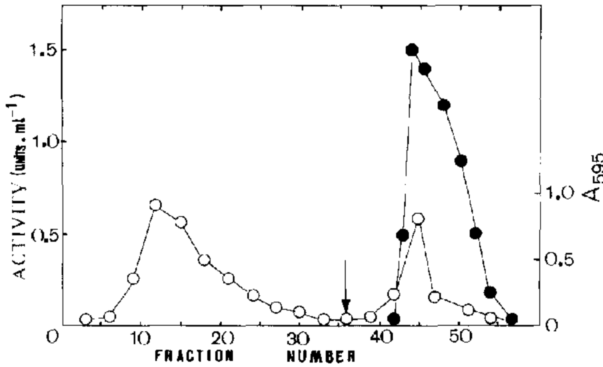

Fig. 1. Elution profile of phospholipase C from the affinity column. The column (200 mm × 18 mm I.D.), packed with APSE-cellulose gel, was washed and equilibrated with 0.1 *M* sodium borate–HCl buffer (pH 8.0) containing 1 *M* NaCl. The acetone–precipitate preparation was loaded on the column at a flow-rate of 18 ml/h. After washing with three column volumes of the same buffer, the enzyme was eluted with 3 *M* Tris–HCl (pH 8.5) at a flow-rate of *ca.* 30 ml/h. Fractions of 5 ml were collected throughout. Enzyme activity was determined by the method of Takahashi *et al.* [18] (see Experimental). (●) Enzyme activity in U/ml; (○) protein content by the method of Bradford [22]. The arrow indicates the beginning of elution.

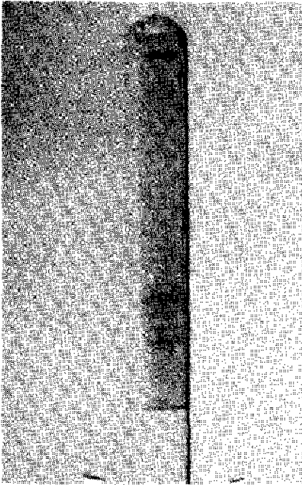

Fig. 2. Electrophoretic patterns of the purified phospholipase C. Conditions are described in the text.

Molecular mass

The molecular mass of the enzyme estimated by gel permeation on Sephadex G 75 was calculated to be 34 000 ± 1500 (n = 7).

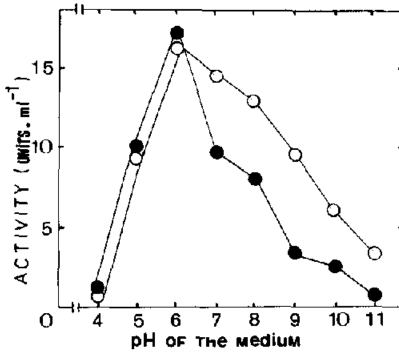

Fig. 3. pH optimum for substrate hydrolysis. Enzyme activity was measured in buffers ranging from pH 4.0 to pH 11.0 (see text), using phosphatidylcholine (●) or egg-yolk lipoprotein (○) as a substrate (three experiments in triplicate).

Optimum pH for substrate hydrolysis

The assays were performed in the following buffers, ranging from pH 4 to pH 11: 0.2 *M* sodium acetate–acetic acid, pH 4–6; 0.2 *M* sodium maleate–NaOH, pH 5–6; 0.2 *M* sodium borate–HCl, pH 7–9; 0.2 *M* Tris–HCl or Tris–NaOH, pH 8–11. The optimal pH for the degradation of both L-α-phosphatidylcholine and egg-yolk lipoprotein was *ca.* 6.0 (Fig. 3). The parallel assays in acetate and maleate buffers at pH 5–6, and Tris and borate at pH 7–9, showed no significant influence of the kind of the buffer used on the rate of enzymic hydrolysis.

Substrate specificity

PLC from *A. xylosoxidans* hydrolysed phosphatidylcholine more rapidly than phosphatidylethanolamine at pH 6.0 (Table II). As shown, the enzyme also hy-

TABLE II

SUBSTRATE SPECIFICITY OF PHOSPHOLIPASE C FROM *A. XYLOSOXIDANS*

The activity toward phosphatidylcholine (from egg-yolk) was taken as 100% (this corresponds to 97 U/mg). The reaction mixture (0.5 ml) contained 1 μg of PLC and 2 μmol of the corresponding substrate (two experiments in triplicate).

Substrate	Activity (% of control)
Phosphatidylcholine (egg-yolk)	100
Phosphatidylcholine (dilauroyl)	98
Phosphatitdylethanolamine (dipalmitoyl)	31
Spingomyelin (egg-yolk)	5
Egg-yolk lipoprotein	140

TABLE III

EFFECT OF DIVALENT METAL IONS ON THE ACTIVITY OF PHOSPHOLIPASE C FROM *A. XYLOSOXIDANS*

Activity is expressed as a percentage of the control, *i.e.* the activity with no additions. Metal ion concentrations were 1 m*M* or 4 m*M* (three experiments in triplicate).

Ion	Activity (% of control)			
	Phosphatidylcholine (from egg-yolk)		Lipoprotein (from egg-yolk)	
	1 m*M*	4 m*M*	1 m*M*	4 m*M*
Ca^{2+}	200 ± 7	315 ± 8	100 ± 1.6	100 ± 2.8
Mg^{2+}	150 ± 5	210 ± 5	100 ± 2.3	100 ± 1.4
Zn^{2+}	177 ± 4	198 ± 5	38 ± 2	22 ± 1.5
Mn^{2+}	180 ± 3.5	204 ± 4	90 ± 4	90 ± 3.5
Co^{2+}	90 ± 2	91 ± 2.5	42 ± 1.5	27 ± 1.5
Cu^{2+}	54 ± 2	25 ± 1.5	0	0

drolysed sphingomyelin at a rate of 5% of that of phosphatidylcholine. This activity was not influenced by divalent metal ions, such as Mg^{2+}, Ca^{2+} or Zn^{2+}, at concentration of 1–10 m*M*. On the other hand, phosphatidylinositol was not hydrolysed, even after incubation for 24 h. The synthetic substrate NPPC was not hydrolysed at two different reaction conditions (see Experimental).

Effect of metal ions and EDTA

To distinguish between the effect on the physical state of substrate liposomes and on the enzyme itself, we examined various divalent cations and EDTA using two different substrates, *i.e.* phosphatidylcholine liposomes and egg-yolk lipoprotein suspension, respectively. As shown in Table III, the hydrolysis of phosphatidylcholine was stimulated by Ca^{2+}, Zn^{2+}, Mg^{2+} and Mn^{2+}, and was inhibited by Co^{2+} and Cu^{2+} at 1 and 4 m*M*. Indeed, Ca^{2+} at 4 m*M* exhibited the most potent stimulating effect on the hydrolysis of phosphatidylcholine liposomes, which was equal to 315 ± 8% with respect to the control. This effect was not further increased at concentrations greater than 6 m*M* (data not shown). When egg-yolk lipoprotein was used as a substrate, Zn^{2+}, Co^{2+}, Mn^{2+} and Cu^{2+} inhibited the PLC activity, whereas Ca^{2+} and Mg^{2+} had no effect at the concentrations tested (Table III). When the purified PLC (0.2 mg) was incubated with 10 m*M* EDTA for 3 h at 4°C, 97% of its activity was lost. However, when the enzyme was dialysed twice against 0.1 *M* sodium borate (pH 7.2) to remove EDTA, and subsequently treated with Ca^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+} or Co^{2+}, at 5 m*M* final concentration, its activity was restored to 6.7, 90, 2.6, 5.0 and 1.8%, respectively.

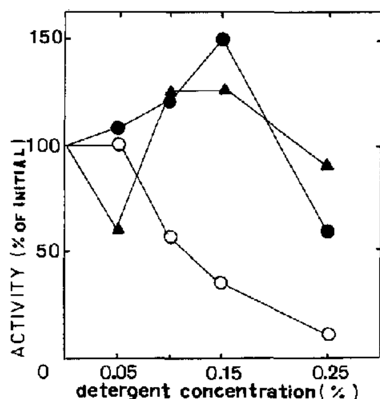

Fig. 4. Effect of detergents on the activity of phospholipase C. Detergents were applied at the concentrations indicated, after substrate sonication (egg-yolk phosphatidylcholine). (●) Triton X-100; (▲) sodium deoxycholate; (○) cetyltrimethylamonium bromide; three experiments in triplicate.

Effect of detergents

As shown in Fig. 4, 0.05% sodium deoxycholate inhibited by 40% the rate of hydrolysis of phosphatidylcholine. When higher concentrations were applied, a slight stimulating effect was observed, up to 30% at a concentrtion of 1.0%. Triton X-100 stimulated the hydrolysis by *ca.* 50%, at a concentration of 0.15%. On the other hand, the cationic detergent cetyltrimethylamonium bromide (CTAB) was inhibitory at concentrations greater than 0.05% (Fig. 4).

Other effects

Dithiothreitol ad *p*-chloromercuribenzoate, when applied in concentrations up to 0.1 m*M*, had no effect on the purified enzyme.

When PLC was incubated for 20 min at different temperatures (from 40 to

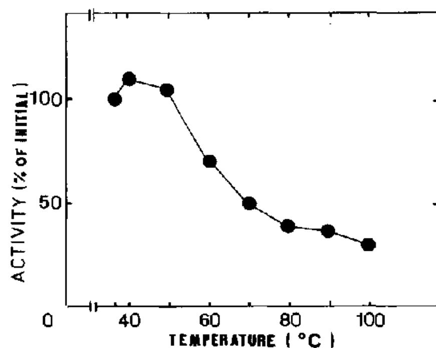

Fig. 5. Effect of temperature on phospholipase C from *A. xylosoxidans*. Aliquots of the purified enzyme (0.05 mg of protein) were incubated for 20 min in 0.2 *M* sodium acetate–acetic acid (pH 6.2) at various temperatures, and the activity was measured at 37°C (four experiments in triplicate).

100°C) in 0.2 *M* acetate buffer (pH 6.0), its activity decreased above 50°C. However, 30% of activity remained after incubation at 100°C (Fig. 5).

Haemolytic acitivity

No haemolysis was observed when the strain *A. xylosoxidans* XC-1 was grown on blood agar. The purified PLC did not lyse sheep erythrocytes in either blood agar or 5% erythrocyte suspension with Ca^{2+} or Mg^{2+} or both ions being added.

DISCUSSION

In this paper a new application of APSE-cellulose is described for the purification of PLC isolated from the culture broth of *A. xylosoxidans*. This adsorbent was first applied to purify PLC from *B. cereus* [17]. In the present study a commercially obtained batch of the sorbent was used, which indicates the possibility of future applications on a larger scale, and makes it a universal tool for the purification of different types of bacterial PLCs.

Despite the improvement of the purity following each purification step, the recovery of PLC from *A. xylosoxidans* declined significantly during the chromatographic purification. Such an inactivation process also took place when the enzyme was chromatographed on ion-exchange columns (unpublished data). Although several attempts were made to stabilize the enzyme (see Results), we did not succeed in improving the final yield of PLC.

Finally, Tris was the best eluting agent for PLC. Other amines applied at high concentrations, such as triethanolamine, gave even more complete desorption and also a very sharp elution profile (unpublished data).

In a previous study [16], we have shown that PLC from *A. xylosoxidans* is not a phosphor-repressive protein, unlike the enzyme from *P. aeruginosa* [7], and its synthesis is extremely sensitive to temperature changes. This may reflect the existence of a temperature-sensitive mechanism of gene expression of PLC in *A. xylosoxidans*, as already shown for other psychrophilic microorganisms [25].

From its substrate specificity, PLC from *A. xylosoxidans* is closer to the enzyme from *P. aeruginosa* [7], than to those from other *Pseudomonas* species, which preferentally hydrolyse phosphatidylethanolamine [1,4]. On the other hand, it did not hydrolyse NPPC [16], thus indicating probable differences in the substrate binding site, and maybe in the structure of catalytically active site, compared with other known bacterial PLCs.

The stimulation of PLC activity by Ca^{2+} (Table III) is most probably due to an influence on the substrate liposomes, rather than to an effect on the enzyme molecule itself, since Ca^{2+} has no effect on the egg-yolk lipoprotein reaction. It is very likely that PLC from *A. xylosoxidans* is a metalloenzyme, considering the inhibition by EDTA (see Results). Since its activity was 90% restored by 5 m*M* $ZnCl_2$, it may contain Zn as a cofactor. One fact that argues against such a hypothesis is that Zn^{2+} inhibits the hydrolysis of the egg-yolk lipoprotein (Table

III). More detailed experiments are needed to elucidate this problem. Such a study using atomic adsorption spectroscopy of the purified enzyme is now in progress in our laboratory.

From the range of detergents examined, the highest degree of stimulation of phosphatidylcholine hydrolysis was elicited by Triton X-100, and the largest inhibitory effect by CTAB. On the other hand, a biphasic curve was obtained with sodium deoxycholate: two close concentrations (*i.e.* 0.05 and 0.1%) give opposite effects (Fig. 4). One plausible explanation for this result could be the existence of critical molar ratios, which induce drastic changes in the reciprocal orientation of the enzyme and the substrate.

p-Chloromercuribenzoate and dithiothreitol had no effect on PLC activity, suggesting that it is not a sulphydryl-dependent enzyme.

In conclusion, PLC from *A. xylosoxidans* XC-1 is a non-haemolytic enzyme with some unique properties among those other known bacterial PLCs, in that it has (i) temperature-sensitive expression control, (ii) isoenzyme forms, which are easily separated by ion-exchange chromatography and native electrophoresis, and (iii) a lack of hydrolysis of the synthetic substrate NPPC.

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