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STUDIES ON PHOSPHOLIPASE C FROM *PSEUDOMONAS AUREOFACIENS*

I. PURIFICATION AND SOME PROPERTIES OF PHOSPHOLIPASE C

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Summary

Phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) from *Pseudomonas aureofaciens* was purified 3600-fold from the culture filtrate with a recovery of 1.6%. Purification was performed with the use of $(NH_4)_2SO_4$ precipitation, Sephadex G-100 gel filtration and by ion-exchange chromatography on DEAE-Sephadex A-50 and CM-Sephadex C-50. The purified enzyme appeared to be homogeneous as revealed by polyacrylamide disc gel electrophoresis at pH 9.3. The molecular weight was estimated to be 35 000 by gel filtration on Sephadex G-75. Under our experimental conditions, phosphatidylethanolamine was more rapidly hydrolysed than phosphatidylcholine. Lyso forms of these two phosphatides were poor substrates. Phosphatidylserine, phosphatidylglycerol, phosphatidylinositol, cardiolipin and sphingomyelin were not hydrolysed. The enzyme activity with phosphatidylcholine as substrate was slightly stimulated by Ca^{2+}, Mg^{2+} and Mn^{2+}. However, these cations inhibited the activity with phosphatidylethanolamine as substrate. An anionic detergent, sodium deoxycholate, slightly enhanced the activity when phosphatidylcholine and phosphatidylethanolamine were used as substrates. A cationic detergent, cetyltrimethylammonium bromide, inhibited enzyme activity. EDTA and *o*-henanthroline inhibited the activity of the enzyme to a marked degree.

Introduction

MacFarlane and Knight [1] first identified the α-toxin of *Clostridium perfringens* type A as phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3), an enzyme catalyzing the hydrolysis of phosphatidylcholine into phosphorylcholine and 1,2-diacylglycerol. Shortly after, Chu [2]

found phospholipase C activity in the growth medium of *Bacillus cereus*, and many investigators have reported the existence of phospholipase C from bacteria such as *Clostridium sp.*, *Bacillus sp.*, *Pseudomonas sp.*, *Serratia sp.*, and *Acinetobacter* sp. [3—8]. The phospholipase C from these sources was a typical extracellular enzyme. Of these enzymes, the phospholipase C from *B. cereus* was completely purified by Zwaal et al. [9] and Otnaess et al. [10]. Recently, the enzyme from *Cl. perfringens* was extensively purified by affinity chromatography on agarose linked egg yolk lipoprotein by Takahashi et al. [11]. Furthermore, the enzyme from *Ps. schuylkilliensis* was purified by Arai et al. [12]. The enzymes from *Cl. perfringens* and *B. cereus* have been extensively investigated by many investigators. *Cl. perfringens* secretes an extracellular enzyme which has phospholipase C as well as lethal and hemolytic activities [13]. This enzyme hydrolyses phosphatidylcholine and sphingomyelin [11,13—15]. The enzyme from *B. cereus* strongly hydrolyses phosphatidylcholine, phosphatidylethanolamine and phosphatidylglycerol, but weakly hydrolyses their lyso forms and cardiolipin [7,16,17]. Both the enzymes from *Cl. perfringens* and from *B. cereus* are supposed to be metalloenzymes having Zn^{2+} in their molecules [18,19]. However, the former enzyme requires Ca^{2+} or divalent cation as an activator, whereas the latter does not. The enzymes purified so far have several differences in properties such as substrate specificities and metal ion requirements.

We have recently obtained some strains of *Pseudomonas sp.* which secreted relatively large amounts of phospholipase C into the growth media. The present paper describes the purification and some enzymatic properties of phospholipase C from *Ps. aureofaciens* and compares it with those of other microbial enzymes which have already been reported by various investigators.

Materials and Methods

Chemicals

Ethylenediaminetetraacetic acid (EDTA), *o*-phenanthroline, iodoacetic acid and *p*-chloromercuribenzoic acid were purchased from Katayama Chemical Industries, Ltd, Osaka, Japan. The reference proteins (Protein Calibration Kit) were supplied by Boehringer Mannheim GmbH (G.F.R.). Sodium deoxycholate and Tween 80 were obtained from Katayama Chemical Industries, Ltd., Osaka, Japan. Cetyltrimethylammonium bromide was a product of Wako Pure Chemical Industries, Ltd, Tokyo, Japan. All the materials or chemicals used were the purest commercial grade available.

Screening of organisms

23 strains of *Pseudomonas sp.* were examined for production of phospholipase C with plates of egg yolk agar [6]. In particular, *Ps. aeruginosa*, *Ps. fluorescens*, *Ps. aureofaciens* and *Ps. chlororaphis* showed a typical phospholipase C reaction on egg yolk agar plate. These strains were cultured for 16 h at 33°C in a liquid medium on a rotary shaker. Enzyme assays (see Enzyme assays) were carried out on the culture broth. These strains were strong producers of phospholipase C. Finally, *Ps. aureofaciens* IFO-3521 was selected for

further experiments with this enzyme because this strain is nonpathogenic [20] and a good producer of the enzyme.

Enzyme assays

(1) Phospholipase C

(1.1) Method using the egg yolk reaction. 0.4 ml of egg yolk solution prepared according to the modified method of van Heyningen [21,22] was added to 0.2 ml of enzyme solution and the reaction mixture was incubated for 20 min at 37°C. After incubation, the reaction was stopped by the addition of 2 ml of saline which had been precooled in an ice bath. Enzyme activity was determined by measuring A_{450nm} with a Shimazu Bausch and Lomb spectrophotometer, Spectronic 70. This method was used for routine assays.

(1.2) Method involving inorganic phosphate analysis. Unless otherwise stated, the incubation mixture contained 0.1 ml of 0.02 M phosphatidylcholine in water and 0.4 ml of enzyme solution diluted with 0.2 M $Na_2B_4O_7$/HCl buffer (pH 8.0). The incubation was carried out for 20 min at 37°C and the reaction was stopped by the addition of 2.5 ml of chloroform/methanol (2 : 1, v/v). The mixture was then vigorously shaken with a Vortex mixer for 10 s. The resulting emulsion was centrifuged for 10 min at 2000 rev./min. A 0.2 ml aliquot was withdrawn from the water layer, and subjected to phosphate analysis according to the method of Eibl and Lands [23], after decomposition of organic phosphate by the method of Fiske and SubbaRow [24]. One unit of the enzyme activity is defined as the amount of enzyme which catalyzes the hydrolysis of 1 μmol of phosphatidylcholine per min at pH 8.0 and at 37°C.

(2) Lipase

The incubation mixture contained 0.02 ml of Ediol (50% emulsion of coconut oil, Calbiochem.), 30 mg of bovine serum albumin, 0.1 M phosphate buffer (pH 6.8) and culture broth in a total volume of 1 ml. The incubation was carried out for 20 min at 37°C. After incubation, the enzyme activity was assayed according to the procedure described by Dole [25].

Estimation of protein

Protein contents were estimated by the method of Warburg and Christian [26].

Preparation of substrate

Phosphatidylcholine and phosphatidylethanolamine were extracted from egg yolk according to the procedure described by Bligh and Dyer [27] and were purified by silicic acid (Mallinckrodt Chemical Works) column chromatography [28]. Lyso analogues were prepared from phosphatidylcholine and phosphatidylethanolamine as follows: 6 mmol of each phosphatide, 180 ml of ethyl ether and 120 ml of 0.2 M $Na_2B_4O_7$/HCl buffer (pH 7.0) were mixed with 10 mg of snake venom from *Crotalus adamanteus*. The incubation was carried out at 25°C for 45 min with stirring. Then, to the reaction mixture, 40 ml of methanol and 100 ml of petroleum ether/ethyl ether (1 : 1, v/v) were added. The water layer containing the resultant lysophosphatide was collected,

and from this, lipids were extracted and purified by silicic acid column chromatography. Sphingomyelin, phosphatidylglycerol and cardiolipin were prepared respectively from beef brain, *Micrococcus lysodeikticus* and beef heart.

Results

Correlation between bacterial growth and phospholipase C production

The growth of *Ps. aureofaciens* was followed with the production of phospholipase C. Fig. 1 shows that the release of enzyme into the culture broth began at early log phase of bacterial growth and the maximum enzyme release was observed at the middle log phase. After that period, the enzyme activity in the culture broth decreased gradually as the period of cultivation was extended. This decrease seemed to be due to a rise in pH or proteolysis. Mid-log phase cells were also examined for activity. Wet cells obtained at the mid-log phase were washed twice with saline and suspended in 0.05 M Tris/HCl buffer (pH 7.5). The cells were sonicated and dialyzed against 0.01 M Tris/HCl buffer (pH 7.5) containing 30% glycerol, and the enzyme activity was measured with the dialyzed sonicate. The intracellular enzyme activity was one-tenth as much as the activity in the culture filtrate.

Purification of phospholipase C from Ps. aureofaciens

Ps. aureofaciens IFO-3521 was grown in a polypeptone medium (pH 7.0) containing meat extract and NaCl. After a cultivation for 9 h at 32°C (±2°C) below pH 8.0, the organisms were removed by centrifugation and the culture broth was precipitated with $(NH_4)_2SO_4$ at 80% saturation. The resulting precipitate was collected by continuous centrifugation at 14 000 rev./min. The precipitate was redissolved in a minimal volume of 0.01 M $Na_2B_4O_7$/HCl buf-

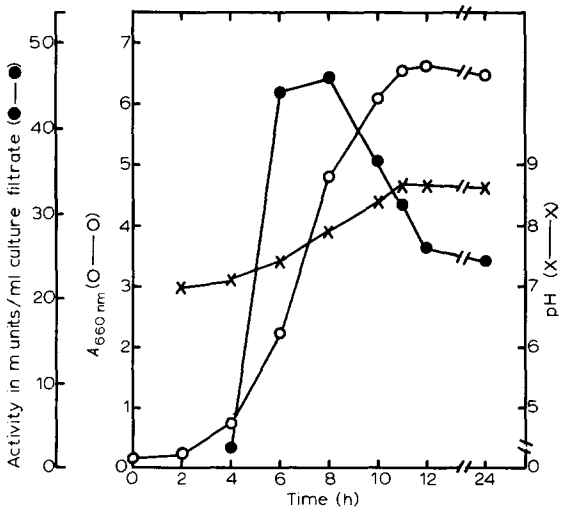


Fig. 1. Production of phospholipase C during the growth of *Ps. aureofaciens*. The growth was followed for 24 h by measuring A_{660nm}. At the same time, 10 ml of culture medium was withdrawn and the supernatant assayed for phospholipase C activity. ○———○, A_{660nm}; ●———●, phospholipase C activity; ×———×, pH.

fer (pH 7.5) containing 40% (v/v) of glycerol and was preserved at −20° C. This crude preparation was used as starting material for the purification of phospholipase C. This fraction was essentially free from lipase activity.

(1) Batch treatment with DEAE-Sephadex A-50. DEAE-Sephadex A-50 was washed and equilibrated with 0.01 M Tris/HCl buffer (pH 7.5). Equal volumes of this gel and crude enzyme (80% $(NH_4)_2SO_4$ ppt.) were mixed by stirring for 2 h at 5° C. The supernatant containing the enzyme was collected through a glass filter, and then concentrated through a Diaflo membrane UM 10 (Amicon Corp., Lexington, Mass., U.S.A.).

(2) Sephadex G-100 gel filtration. 20 ml of the concentrated supernatant described above was placed on a column of Sephadex G-100 (3.0 × 93 cm) and eluted with 0.05 M Tris/HCl buffer (pH 7.5) containing 0.3 M NaCl. Fractions of 5 ml were collected at a flow rate of 20 ml/h. A peak of enzyme activity appeared immediately after the peak of high molecular weight proteins. The active fractions were pooled, concentrated to 375 ml through a Diaflo membrane UM 2 and dialyzed for 16 h against 0.01 M Tris/HCl buffer (pH 8.7) containing 5 mM $CaCl_2$ and 30% (v/v) glycerol.

(3) Chromatography on DEAE-Sephadex A-50 column. 400 ml of the dialyzed solution was placed on a column (4.2 × 25 cm) of DEAE-Sephadex A-50 which had been previously equilibrated with 0.01 M Tris/HCl buffer (pH 8.7) containing 5 mM $CaCl_2$ and 30% (v/v) glycerol. The elution from the column was carried out by using a linear gradient from 0 to 0.2 M of NaCl. Fractions of 8.8 ml were collected at a flow rate of 53 ml/h. Most of enzyme was adsorbed to the gel and eluted at the salt concentration of approx. 0.1 M. The major active fractions were pooled, concentrated to 300 ml through a Diaflo membrane UM 2 and dialyzed for 16 h against 0.01 M Tris-maleate buffer (pH 6.0) containing 5 mM $CaCl_2$ and 30% (v/v) glycerol.

(4) First chromatography on CM-Sephadex C-50 column. 310 ml of the dialyzed solution was placed on a column (4.2 × 22 cm) of CM-Sephadex C-50, which had previously been equilibrated with 0.01 M Tris-maleate buffer (pH 6.0) containing 5 mM $CaCl_2$ and 30% (v/v) glycerol. The proteins were eluted from the column using a linear gradient from 0 to 0.2 M NaCl. Fractions of 4.4

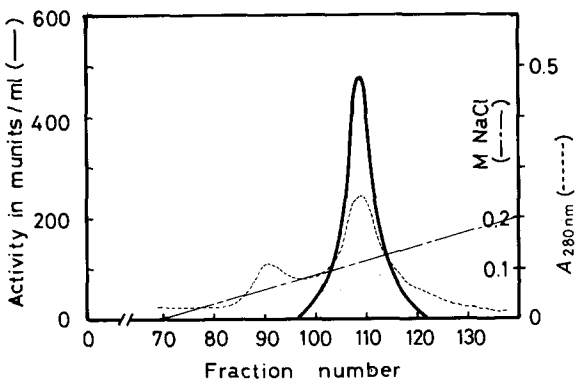


Fig. 2. Second chromatography on CM-Sephadex C-50 column. 30 ml of enzyme solution (0.09 mg/ml) applied to a column (4.2 × 15 cm). The chromatographic conditions are shown in the text, ------, A_{280nm}; ———, enzyme activity; ———·———, NaCl gradient.

ml were collected at a flow rate of 53 ml/h. The enzyme activity was eluted at the salt concentration of approx. 0.1 M. The elution pattern of the enzyme was symmetrical. However, the pooled enzyme solution proved to be contaminated by some other protein(s) as revealed by analytical polyacrylamide disc gel electrophoresis. The purity of the enzyme at this stage was 80%. This solution was dialyzed for 16 h against 0.01 M Tris-maleate buffer (pH 6.0) containing 5 mM $CaCl_2$ and 30% glycerol.

(5) Second chromatography on CM-Sephadex C-50 column. 30 ml of the above enzyme solution was placed again on a column (4.2 × 15 cm) of CM-Sephadex C-50. The column was eluted by the same experimental procedure as that of Step 4. The peak of the enzyme activity coincided with that of protein (A_{280nm}). The elution pattern of the enzyme was also symmetrical (Fig. 2). The active fractions were pooled and concentrated to 2 ml with a Collodion Bag (Sartorius-Membrane filter GmbH, Göttingen, Germany). Finally, at this step, the specific activity increased about 3600-fold and the recovery of the phospholipase C activity was 1.6%, from the culture filtrate. A summary of the purification procedure is given in Table I.

Disc gel electrophoresis

The purity of the final enzyme preparation was investigated by analytical polyacrylamide gel electrophoresis in Mitsumi disc electrophoresis apparatus (Mitsumi Industrial Corp., Tokyo) according to the method of Davis and Ornstein [29,30]. As shown in Fig. 3, the final enzyme preparation obtained after second chromatography on CM-Sephadex C-50 was shown to be apparently pure.

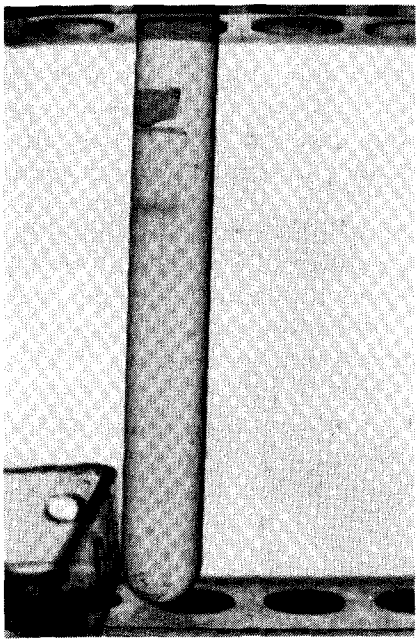
Identification of the reaction products

The reaction mixture containing 0.2 ml of 0.02 M phosphatidylcholine or phosphatidylethanolamine, 0.2 ml of 0.2 M $Na_2B_4O_7$/HCl buffer (pH 8.0) and 0.1 ml of enzyme was incubated for 120 min at 37°C. After incubation, the reaction was stopped by the addition of 2.5 ml of chloroform/methanol (2 : 1, v/v). After centrifugation at 2000 rev./min for 10 min, the water and the chloroform layers were subjected to paper and thin-layer chromatography respectively. Thin-layer chromatography on Silica Gel G plates with chloroform / methanol/water (65 : 25 : 4, by vol.) as solvent revealed the presence of diacyl-

TABLE I

PURIFICATION OF PHOSPHOLIPASE C FROM *PS. AUREOFACIENS*

Step	Total activity (units)	Protein (mg)	Specific activity (munits/mg)	Recovery (%)	Purification
Start (culture broth)	900	186900	4.8	100	1
80% $(NH_4)_2SO_4$ ppt	680	26000	26	76	5
DEAE-Sephadex A-50 (batch)	480	9522	50	53	10
Sephadex G-100	185	693	260	21	54
DEAE-Sephadex A-50	110	50	2200	12	458
CM-Sephadex C-50	32	2.8	11500	4	2395
CM-Sephadex C-50	14	0.8	17500	1.6	3645



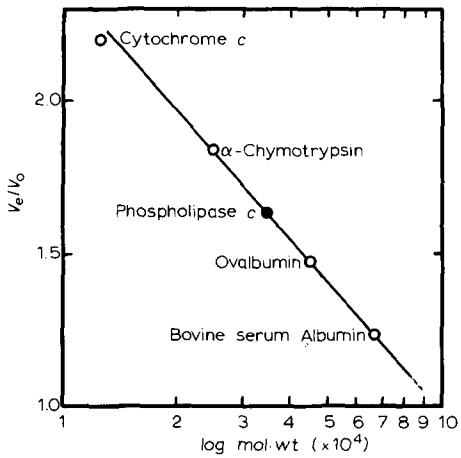


Fig. 3. Disc gel electrophoretic pattern after second chromatography on CM-Sephadex C-50 column. Electrophoresis in 7.5% polyacrylamide at pH 9.3 was carried out with 50 μg protein tube for 100 min at 20°C (4 mA/tube; stained with Amidoschwarz 10B).

Fig. 4. Molecular weight determination of phospholipase C by gel filtration of Sephadex G-75 column (3.0 × 92 cm). The conditions of gel filtration and the calculation of molecular weight are shown in the text.

glycerols and unreacted substrate in the lipid extract. Phosphatidylcholine or phosphatidylethanolamine from egg yolk, diolein and oleic acid were used as reference lipids. The chromatograms of the water-soluble fractions were developed with a mixture of methanol/formic acid/water (80 : 13 : 7, by vol.) on a strip of Whatman chromatography paper (W & R Balston Ltd, England). The components were stained with the Hanes-Isherwood reagent [31]. Each spot on the paper coincided with that of an authentic sample of phosphorylcholine or phosphorylethanolamine (Tokyo Kasei Kogyo Co., Ltd, Tokyo, Japan). A spot corresponding to inorganic phosphate was not observed. The result indicated that the enzyme was indeed a phospholipase C which catalyzed the hydrolysis of glyceride-phosphate bond of phosphoacylglycerols.

Enzyme properties

Molecular weight. The molecular weight of this enzyme was estimated by gel filtration with Sephadex G-75 columns (3.0 × 92 cm) (Fig. 4) [32]. Gel filtration was carried out in 0.05 M Tris/HCl buffer (pH 7.5) containing 0.3 M NaCl. Elution volumes of the six reference proteins indicated in Fig. 4, V_e/V_o (V_o; void volume, V_e; elution volume), were plotted against molecular weight on a log scale. From the standard curve thus obtained, the molecular weight of the purified phospholipase C was calculated to be 35 000.

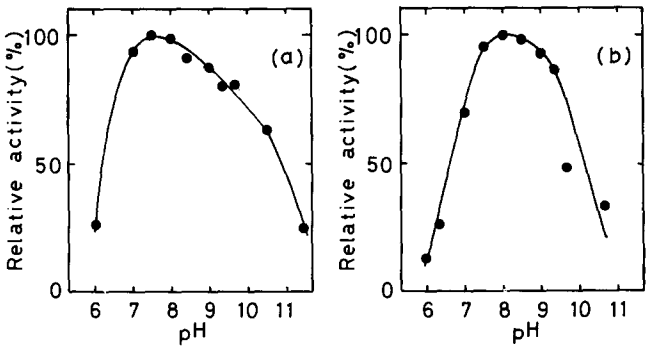


Fig. 5. Effect of pH on the activity of phospholipase C for degradation of phosphatidylcholine (a) and phosphatidylethanolamine (b). The activity measurements were carried out after 20 min incubation at 37°C according to Methods section described in the text. The activity units of enzyme used were $3.0 \cdot 10^{-2}$ in (a) and $1.5 \cdot 10^{-2}$ in (b).

Optimum pH for substrate degradation. The activity measurements were carried out after 20 min incubation at 37°C with the buffer ranging from 6 to 11 (pH 6—9; $Na_2B_4O_7$/HCl, pH 9—11: $Na_2B_4O_7$/NaOH). The optimal pH for the degradation of phosphatidylcholine or phosphatidylethanolamine was 7.5—8 or 8—8.5, respectively (Fig. 5). Below pH 7, the enzyme had little activity. When Tris buffer was used instead of borate buffer, a slight inhibition was observed in the alkaline range.

Time course of substrate degradation. The time courses of enzymic degradation of phosphatidylcholine and phosphatidylethanolamine at pH 8.0 are given in Fig. 6. At this pH, the initial rate of degradation of phosphatidylethanolamine was about two-fold greater than that of phosphatidylcholine. The degradation of each substrate ceased, when approximately 30% of the substrate originally present was hydrolysed. This seems to be due to enzyme inactivation or product inhibition.

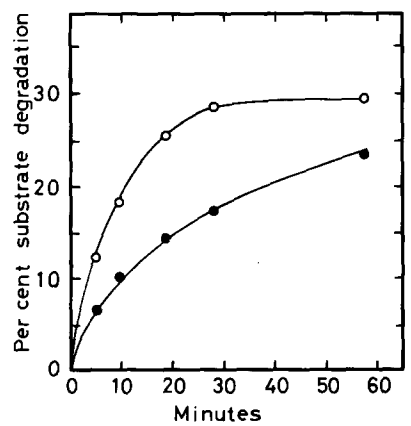


Fig. 6. Time-course of substrate degradation. 0.4 ml of 0.15 M $Na_2B_4O_7$/HCl buffer (pH 8.0) containing $3.0 \cdot 10^{-2}$ unit of enzyme, was mixed at zero time with 0.1 ml of 0.02 M phosphatidylcholine (●———●) or phosphatidylethanolamine (○———○). At the indicated time intervals, the enzyme reaction was stopped and the enzyme activity was determined, according to the methods section described in the text.

Stability of the enzyme. Incubation of the enzyme for 20 min at 37°C in various buffers (pH 4—11) prior to addition of the substrates indicated that this phospholipase C was relatively stable between pH 6.5 and 9. Heat treatment for 10 min at temperatures ranging from 40 to 100°C in 0.02 M borax/HCl (pH 6 and 8.5) indicated that the activity of this enzyme was completely lost at 70°C. At lower temperatures, the enzyme was more stable in the alkaline region than in the acidic one. The purified enzyme was very unstable on storage and dialysis, but was protected by addition of glycerol. No appreciable loss of activity occurred even after about 6 months when the enzyme, after $(NH_4)_2SO_4$ precipitation, was preserved at −20°C in a solution containing 40% (v/v) glycerol.

Effects of metal ions and several inhibitors. The effects of various divalent cations on the enzyme activity were examined. At a concentration of 4 mM, Zn^{2+}, Fe^{2+} and Cu^{2+} were inhibitory whereas Ca^{2+}, Mg^{2+} and Mn^{2+} at the same concentration slightly stimulated the enzyme activity (10—30%) when phosphatidylcholine was used as the substrate (Table II). The inhibitory effects of these metal ions on the activity with phosphatidylethanolamine became more pronounced as the concentration increased (Fig. 7). This enzyme was different from those of *Cl. perfringens* [13] and *Ps. fluorescens* [7] due to the effects of divalent cations. EDTA and *o*-phenanthroline were also inhibitory, as shown in Table II. These inhibitory effects suggest that this enzyme may be a metalloenzyme. *p*-Chloromercuribenzoic acid and iodoacetic acid had no effect on the enzyme activity.

Effects of detergents. The effects of an amphipathic cation, such as cetyltrimethylammonium, and of an amphipathic anion, such as deoxycholate, on the enzyme activity were examined. At a concentration of 0.1%, sodium deoxycholate stimulated the hydrolysis of phosphatidylcholine (about 30%), as shown in Fig. 8. However, when phosphatidylethanolamine was used as the substrate, the stimulation effect was observed only at a higher concentration of this detergent. The difference in the optimal concentration for activation seems to be based on the different solubility of both substrates. Cetyltrimethylammonium bromide was inhibitory with either substrate.

TABLE II

EFFECTS OF METAL IONS AND SOME OTHER COMPOUNDS ON THE ACTIVITY OF PHOSPHOLIPASE C

Metal salt and other compounds	Concentration (mM)	Relative activity* (%)
None		100
$CaCl_2$	4	110
$MgCl_2$	4	120
$MnCl_2$	4	125
$ZnCl_2$	4	71
$FeCl_2$	4	39
$CuCl_2$	4	5
EDTA	4	5
o-Phenanthroline	0.1	15
Monoiodo acetic acid	0.1	100
PCMB**	0.1	95

* Expressed as % of the activity with no addition.

** PCMB, *p*-chloromercuribenzoate.

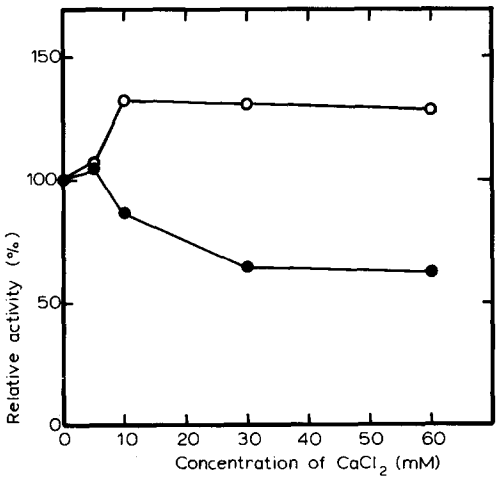


Fig. 7. Effect of $CaCl_2$ concentration on the activity of phospholipase C for phosphatidylcholine (○———○) and phosphatidylethanolamine (●———●). The incubation system and assay method are the same as those described in the text except that $CaCl_2$ was added at various concentrations to the incubation mixture.

Substrate specificity. The action of phospholipase C on several phospholipids was examined. This enzyme hydrolysed phosphatidylethanolamine more rapidly than phosphatidylcholine at pH 8.0 (Fig. 6). The initial rate of degradation of lysophosphatidylcholine was nearly the same as that of phosphatidylcholine, but the degradation ceased when approximately 10% of the substrate originally present was hydrolysed. Acidic glycerophospholipids such as phosphatidylserine, phosphatidylglycerol and cardiolipin were not hydrolysed, nor was sphingomyelin. This enzyme resembles the enzyme from *Ps. fluorescens* in that it rapidly hydrolyses phosphatidylethanolamine.

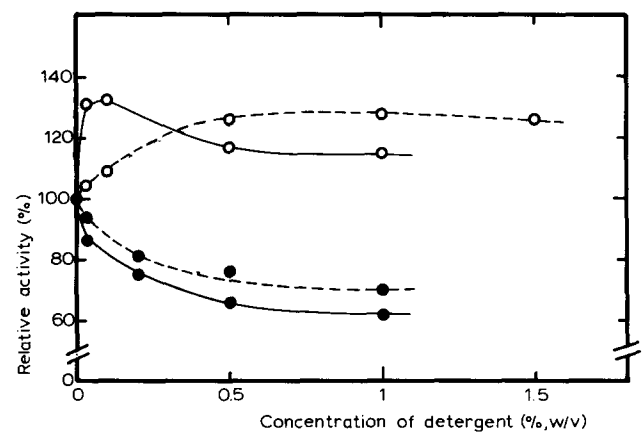


Fig. 8. Effects of detergents on the activity of phospholipase C, for phosphatidylcholine (———) and phosphatidylethanolamine (- - - - - -). (○) sodium deoxycholate; (●) cetyltrimethylammonium bromide. The incubation system and assay method are the same as those described in the text except that each detergent was added at various concentrations to the incubation mixture.

Discussion

Recently, phospholipase C has attracted the attention of many investigators, because this enzyme can be used as a tool for membrane research. In the present paper, we report the purification and some properties of phospholipase C from *Ps. aureofaciens*. An approx. 3600-fold purification was achieved starting from the culture filtrate with a yield of about 1.6%. This purified enzyme was shown to be apparently homogeneous by analytical disc electrophoresis. Phospholipase C, from the sources investigated so far seems to be a typical extracellular enzyme. Also in our experiments, almost all of the enzyme from *Ps. aureofaciens* was secreted into the growth medium, but the cells did contain about one-tenth of the enzyme of the culture filtrate at the mid-log phase of growth. The existence of some intracellular enzyme activity has been similarly reported in the cases of *B. cereus* [33] and *Cl. perfringens* [34]. From this observation, the following possibilities are suggested; (I) some active form of enzyme, identical with or somewhat different from that secreted from the cell, exists within the cell; (II) phospholipase C may exist in a form firmly bound to the membrane, not within the cell. Further detailed study must be made to solve this problem. Various enzymatic properties of phospholipase C from *Ps. aureofaciens* have been investigated. The estimated molecular weight of this enzyme, 35 000, is smaller than the values for the enzyme from *Cl. perfringens*, 43 000—51 200 reported by Takahashi et al. [11], Shemanova et al. [35], Stahl [36] and Mitsui et al. [37], but is slightly larger than those for the enzyme from *B. cereus*, 20 000—25 000 reported by Ottolenghi [38], Zwaal et al. [9] and Otnaess et al. [10]. From the chromatographic behavior on DEAE-Sephadex A-50 and CM-Sephadex C-50 columns, the isoelectric point of *Ps. aureofaciens* phospholipase C is a pH range between 6 and 8.7. Under our experimental conditions, phosphatidylethanolamine was hydrolysed at an initial velocity about twice that for phosphatidylcholine, but sphingomyelin and acidic phospholipids were not hydrolysed. On the contrary, phosphatidylcholine was hydrolysed more rapidly than phosphatidylethanolamine by the partially purified enzyme from *B. cereus* (unpublished data), and this is in accord with the results reported by Kleiman et al. [39] and Doi et al. [7] Takahashi et al. [11] have reported the hydrolysis of sphingomyelin by the enzyme purified from *Cl. perfringens*. Lehmann [40] has reported the hydrolysis of various species of phospholipids by the enzyme from *Acinetobacter calcoaceticus*. As shown above, the differences in substrate specificities are observed with the enzymes from organisms of different species. Probably the substrate specificity of phospholipase C depends, at least partly, on the micellar nature of the substrate. The physical state of substrate micelles may be influenced by the impurities in the enzyme preparation. The degradation of phosphatidylcholine by the enzyme from *Ps. aureofaciens* was not markedly influenced by the divalent metal ions, but the degradation of phosphatidylethanolamine was inhibited (Table II, Fig. 7.). Ottolenghi [19] reported that the enzyme from *B. cereus* was not activated by Ca^{2+}. However, the enzyme from *Cl. perfringens* or *Ps. fluorescens* is activated by some divalent metal ions. On the basis of an activation by Ca^{2+} and a stimulation by amphipathic cations such as cetyltrimethylammonium bromide, Bangham and Dawson [41] suggested that

the enzyme from *Cl. perfringens* has a negative charge and reacts with substrate micelles bearing a positive charge conferred by amphipathic cations or divalent metal ions. In contrast with this interpretation, Diner [42] suggested from kinetic analyses that Ca^{2+} was associated not only with lipid micelles but also with the enzyme. The enzyme activity from *Ps. aureofaciens* was inhibited by cetyltrimethylammonium bromide, but slightly activated by amphipathic anion such as sodium deoxycholate (Fig. 8). Differences in the affinity of each enzyme for the lipid micelle are probably responsible for this variation. Also, it must be considered that amphipathic compounds may affect the size of micelle as well as their charge. Because metal-chelating agents such as EDTA and *o*-phenanthroline were inhibitory for the enzyme activity, the enzyme from *Ps. aureofaciens* may be a metalloenzyme. Further studies are needed to demonstrate the presence of a metal-containing active site of the phospholipase C from *Ps. aureofaciens*. The apparent K_m values for phosphatidylcholine (egg yolk) and for phosphatidylethanolamine (egg yolk) were 0.6 mM and 6.5 mM, respectively (unpublished data). The apparent V for phosphatidylethanolamine was about 10-fold larger than that for phosphatidylcholine. Various K_m values for phosphatidylcholine of the enzyme from *Cl. perfringens* have been reported [42—44]. The discrepancies in these K_m values can be explained on the basis that different experimental conditions used influence the physical state of the lipid micellar substrates which in turn affect the kinetic properties of the enzyme.

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