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PURIFICATION OF PHOSPHOLIPASE C (ALPHA-TOXIN) FROM *CLOSTRIDIUM PERFRINGENS*

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SUMMARY

(1) Phospholipase C (EC 3.1.4.3) from *Clostridium perfringens* was produced by cultivation in a prereduced medium in a fermentor on a 10-l scale.

(2) The enzyme was purified by treatment with DEAE-Sephadex in a batch-wise process, Sephadex G-75 chromatography and isolectric focusing to a 200-fold increase in specific activity.

(3) The enzyme was found to be devoid of contaminating antigens as shown by immunodiffusion, immunoelectrophoresis and crossed electroimmunoassay against polyvalent antisera. No contaminating cytolytic toxins or enzymatic activities in the crude material were detected in the purified enzyme. Analytical electrophoresis on polyacrylamide and sodium dodecyl sulphate electrophoresis revealed two components.

(4) A molecular weight of 30 000 $\pm$ 2000 was calculated after gel chromatography and an isoelectric point of 5.7 $\pm$ 0.05.

INTRODUCTION

Phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) from *Bacillus cereus* was recently extensively purified and several enzymatic properties for the highly purified enzyme were reported[1]. The purified enzyme has been used for studies on degradation of phospholipids in erythrocyte membranes and was found to possess a substrate specificity different from the one previously reported for partially purified phospholipase C from *Clostridium perfringens* [1–3] (previously *Cl. welchii*. However, more recently Pastan *et al.*[4] reported that the extracellular fluid of *Cl. perfringens* contains one enzyme which requires calcium for hydrolysing lecithin and a second one which requires magnesium for hydrolysing sphingomyelin. Neither Sugahara and Ohsaka[5] nor Smyth and Arbuthnott[6] could resolve these two phospholipases by isoelectric focusing. In spite of the similar substrate specificities of phospholipase C from the two bacterial species mentioned above, as well as from *Pseudo-*

monas fluorescens[7] and *Acinetobacter calcoaceticus*[8], only the *Cl. perfringens* and the *A. calcoaceticus* enzyme seem to be haemolytic and cytolytic for other cells, while *B. cereus* and *P. fluorescens* produce other extracellular cytolytic toxins of protein nature[9,10].

The aim of this investigation was to obtain a highly purified phospholipase C from *Cl. perfringens* devoid of contaminating enzymes, such as glucosidases, and proteases and of other cytolytic proteins, such as theta-toxin, in order to permit characterization of some enzymatic properties in comparison with other bacterial phospholipase C and to enable future studies on the effect of this enzyme on different biological membranes. Many of the observations previously reported made with commercial, partially purified preparations must be interpreted with great caution, since contaminating proteins with membrane damaging activity probably interfered with most of the experiments performed[11].

MATERIALS AND METHODS

Production

Cl. perfringens, strain ATCC 13124, was obtained from American Type Culture Collection, Rockville, Md., U.S.A. The strain was grown in a prereduced proteose peptone medium supplemented with glucose, phosphate, cysteine and vitamins (Nord, C.-E., Möllby, R. and Wadström, T., unpublished). The enzyme was produced in a fermentor of 10-l volume (Biotec FL 110, Biotec, Stockholm, Sweden). The pH was kept constant at 7.2 with an automatic titrator by addition of 5 M NaOH. The temperature was controlled at 37° ± 0.1 °C and the impeller speed was 200 rev./min. An anaerobic atmosphere was maintained by bubbling of oxygen-free nitrogen gas through the culture (50 ml/min). Samples were taken from the culture during growth for determination of cell dry weight, glucose concentration and enzyme and toxin activities. The oxidation–reduction potential (Eh) was continuously recorded. An inoculum of about 1% of the final cell dry weight (1.7 g/l) was used and the culture was harvested after 3.5 h of growth when the glucose was depleted. The culture volume was rapidly cooled in an ethanol bath at −40 °C and centrifuged in a Sorvall RC2-B centrifuge at 4 °C (8000 × *g* for 40 min).

Purification

If otherwise not stated, all steps below were carried out at 4 °C. Crude culture supernatant (10 l) was dialysed by a continuous process in an artificial kidney (LKB Medical, Stockholm-Bromma, Sweden) against deionized water as recently described[12]. The final salt concentration was estimated with an LKB-conductolyzer and compared to a reference solution of NaCl.

The dialyzed material with a conductivity corresponding to less than 10 mM of NaCl was frozen on trays of stainless steel in a layer about 1-cm thick and freeze-dried for 64 h.

The dry material was dissolved in distilled water and dialyzed against distilled water in a cellophane bag for 18 h. The ionic strength was then estimated with the conductolyzer to be less than 10 mM.

Adsorption of impurities ("negative adsorption" of the enzyme) was performed by adding DEAE-Sephadex A-25 (2.5 g dry wt per l of starting culture supernatant)

to the dialyzed material. The pH was adjusted to 8.5 by addition of 4 M NaOH and the Sephadex was allowed to swell and adsorb for 18 h under slow stirring. The mixture was then centrifuged (8000 × *g* for 15 min) and the supernatant was decanted.

The nonadsorbed material (the effluent) was concentrated in a cellophane bag with polyethyleneglycol with a molecular weight of 20 000 (PEG 20 M) on the outside to reduce the volume to less than 300 ml.

The polyethyleneglycol-concentrated material of a 10-ml volume was subjected to Sephadex G-75 chromatography. The Sephadex column (4 cm × 100 cm) was equilibrated with 0.02 M Tris–HCl buffer, pH 7.0, containing 1 mM $CaCl_2$ and prepared as previously described[13]. The flow rate was 0.3 ml/min, 6-ml fractions were collected and the phospholipase C and haemolytic activities were determined in each fraction.

The fractions containing phospholipase activity from three Sephadex runs were pooled and concentrated in a cellophane bag against polyethyleneglycol as described above. The final volume was less than 50 ml.

Isoelectric focusing in a density gradient of glycerol (0–50%, v/v) and a pH gradient 5–7 of Ampholine (LKB-Produkter) was performed as earlier described[14,15]. Fractions of 2.5 ml were collected after 48 h and tested for phospholipase and haemolytic activity. Because of the low concentration of ions in the Tris-buffer used for gel chromatography, no dialysis step preceeding the isoelectric focusing was needed.

The fractions containing the highest activity of phospholipase C were pooled and dialyzed against 0.02 M Tris–HCl buffer, pH 7.0, containing 0.15 M NaCl and 1 mM $ZnCl_2$. The purified enzyme was divided into many small aliquots and kept at —20 °C. For long time storage the enzyme was freeze-dried in the same buffer.

Measurement of phospholipase C activity

Phospholipase C activity was measured by a modified titrimetric method according to Zwaal *et al.*[1]. One fresh egg yolk was suspended in 106 ml 0.15 M NaCl, supplemented with 10 mM $CaCl_2$ and 0.1 mM $ZnCl_2$, and centrifuged at 20 000 × *g* for 40 min at 4 °C. The opaque supernatant was quite stable and could be stored for five days at 4 °C.

0.5 ml of this substrate was mixed with 1.5 ml of the NaCl solution mentioned above, 0.05 ml of sodium deoxycholate in distilled water (90 mg/ml) and 0.05 ml of the enzyme solution, making a final volume of 2.1 ml. The release of titratable H^+ was followed in a titration unit ABU 13/TTA 31 in connection with a Titrigraph TT 11 (Radiometer, Copenhagen, Denmark) by instantaneous addition of 0.01 M NaOH. The reaction mixture was kept at 37 °C and constant pH between 7.0 and 7.2, depending on the starting pH. The mixture was continuously stirred with a teflon coated magnetic stirrer and kept under nitrogen atmosphere.

One unit of enzyme activity (U) was defined as the amount of enzyme, which liberated 1 μmole titratable H^+ per min. The specific activity is expressed as units per mg of protein. For screening purposes from a large number of fractions, those containing phospholipase C activity were recognized by their ability to cause a strong increase in turbidity of the substrate upon short time incubation in a test-tube at 37 °C (ref. 16).

Other enzyme and toxin assays

The haemolytic activities of phospholipase C (alpha-toxin) and theta-toxin were measured on erythrocytes of various species. If otherwise not stated sheep blood cells were used (1%, v/v). One haemolytic unit (HU) was defined as the amount of toxin in dilution which gave a 50% haemolysis after incubation at 37 °C for 1 h and 4 °C for 2 h[15]. Lipase, phosphatase, protease, deoxyribonuclease, ribonuclease, hyaluronidase and several glucosidases were assayed for on samples of the purified enzyme in a final concentration of 144 U/ml as previously described[11].

Other assays

Protein was determined according to Lowry *et al.*[17] after thorough dialysis against 0.01 M sodium phosphate buffer (pH 7.0) for 24 h to 36 h. Gel chromatography was followed by continuous recording of the changes in transmission at 280 nm by a Uvicord II (LKB-Produkter). Protein content in fractions after isoelectric focusing was estimated by $A_{280\ nm}$—$A_{310\ nm}$.

Disc electrophoresis on polyacrylamide

Analytical acrylamide electrophoresis was carried out in an alkaline discontinuous glycine–lutidine buffer system as recently described, and the proteins were stained with Coomassie brilliant blue[15].

Immunodiffusion, immunoelectrophoresis and electroimmunoassay

Immunodiffusion was carried out according to Ouchterlony[18] and immunoelectrophoresis according to Grabar and Williams[19]. Electrophoresis in agarose containing *Cl. perfringens* antiserum was carried out as described by Laurell[20] and crossed electroimmunoassay as described by Clarke and Freemann[21].

Immunizations

6-month-old sheep were immunized at 3-week intervals by a subcutaneous route. One part of crude enzyme (after Step 1) was mixed with an equal of volume of Freund's complete adjuvant and 2 ml of an emulsion, stable for at least 4 h at 20 °C, were injected in both buttocks of the animal. The antiserum used in this investigation was obtained by heart puncture 5 days after the fourth injection.

Molecular weight estimation

Polyacrylamide gel electrophoresis in a buffer system containing sodium dodecyl sulphate was performed as described by Weber and Osborne[22]. Sephadex G-75, Biogel P-60 and Biogel A-0.5 were packed in columns of 2 cm × 120 cm, and equilibrated in 0.02 M Tris–HCl buffer pH 7.0, containing 0.15 M NaCl. Cytochrome *c*, myoglobin, trypsinogen, ovalbumin, human serum albumin, human haemoglobin, malate dehydrogenase (calf intestine) and human gammaglobulin were used as reference substances for molecular weight estimation. The procedure has already been described in detail[12,13].

Spectrophotometry and pH determinations

Spectrophotometry and pH determinations were performed as earlier described[14].

Materials

All chemicals were of analytical grade if otherwise not stated. Ammonium sulphate, 1,10-phenanthroline, 2,2-bipyridin, magneson, cupferron, EDTA, Titriplex V (3-Aza-3-(carboxymethyl)-pentamethylendinitrilotetraacetic acid), glycine, sodium dodecyl sulphate and all salts and buffer substances were purchased from Merck AG, Darmstadt (Germany). Dithiothreitol, cholesterol, ovalbumin (5 times crystallised), trypsinogen, myoglobin, malate dehydrogenase (horse heart, salt free), sphingomyelin, synthetic DL-lecithin, crude soy bean lecithin (Grade III-S) and ethyleneglycol-bis (β-aminoethyl ether) *N,N'*-tetraacetic acid (EGTA) were purchased from Sigma Chem. Comp., St. Louis, Mo., (U.S.A.). Cytochrome *c* was purchased from Nutritional Biochem. Corp., Cleveland, Ohio, (U.S.A.), human serum albumin and gammaglobulin were gifts from KABI, Stockholm, (Sweden). Polyethyleneglycol (PEG 20 M) of technical grade was purchased from KEBO, Stockholm, (Sweden). Proteose peptone and Freund's complete adjuvant were purchased from Difco Labs, Detroit, Mich. (U.S.A.).

Carrier ampholytes (Ampholine) were obtained from LKB-Produkter, Stockholm-Bromma (Sweden), Sephadex and Blue dextran 2000 from Pharmacia, Uppsala, (Sweden) and the Bio-gel products from Biorad. Labs, Richmond, Calif. (U.S.A.). Acrylamide and *N,N'*-methylene bisacrylamide were purchased from British Drug House, Poole (England) and Coomassie brilliant blue from ICI, Manchester (England). A polyvalent antiserum against *Cl. perfringens* type A was obtained from the Pasteur Institute, Paris (France).

RESULTS

Cl. perfringens strain ATCC 13124 was chosen out of 10 different type culture collection strains for production of phospholipase C. These strains were all grown in a fermentor and strain ATCC 13124 was found to produce two to five times more

TABLE I

Step	*Purification scheme procedure*
1	dialysis in an artificial kidney
	freeze-drying
	dialysis in cellophane bag
2	"negative adsorption" on DEAE-Sephadex
	concentration against PEG 20 M
3	gel chromatography on Sephadex G-75
	concentration against PEG 20 M
4	isoelectric focusing
	dialysis in cellophane bag
	(freeze-drying)

enzyme than the others. This strain was also grown in some different media supplemented with different carbohydrates to optimize the production of the enzyme. These data will be presented in detail elsewhere (Nord, C.-E., Möllby, R. and Wadström, T., unpublished). Purification was performed as presented in Table I.

Dialysis and concentration (Step 1)

Dialysis of crude culture supernatant in an artificial kidney followed by freeze-drying was found to be a convenient method to reduce the initial volume of 10 l to about 500 ml and to remove most of the constituents of the growth medium. The freeze-dried material was dissolved in distilled water and dialysed in a cellophane bag over night. This step gave a 50% recovery of the enzymatic activity (Table II).

TABLE II

	Purification step	*Volume (ml)*	*Protein (mg/ml)*	*Total protein (mg)*	*Activity (U/ml)*	*Total activity (U)*	*Recovery (%)*	*Specific activity (U/mg)*	*Increase in specific activity*
	Supernatant	10 000	2.7	27 000	5	50 000	100	1.85	1
1	Freeze-drying	425	32	13 000	72	30 600	61	2.25	1.22
2	DEAE-Sephadex A-25	27	71.9	1 940	1 120	30 200	61	15.6	8.4
3	Sephadex G-75	74	1.3	96	144	10 700	22	111	60
4	Isoelectric focusing	36	0.39	14	144	5 200	10.4	370	200

DEAE-Sephadex adsorption (Step 2)

The dialysed material ($I < 0.01$) was mixed with dry DEAE-Sephadex A-25 and pH was adjusted to 8.5. Many acidic compounds were adsorbed on the ion exchanger by this batch process, while the phospholipase was not ("negative adsorption", Table II). The DEAE-Sephadex was then removed by centrifugation, and the supernatant was concentrated by dialysis against polyethyleneglycol (PEG 20 M) to a volume of less than 30 ml. This purification step gave a total recovery of the enzymatic activity and a 7-fold increase in specific activity.

Sephadex chromatography (Step 3)

Sephadex gel chromatography gave a recovery of 40% of the enzymatic activity. Several attempts to change the order of this purification step with the next, *i.e.* isoelectric focusing, were all in vain, since the recovery after gel chromatography, performed in several different buffer systems, was only around a few percent. Neither did supplementing the eluting buffers with calcium and zinc ions increase the yield upon this chromatography. Fig. 1 shows elution of the haemolysin just after the void volume. The haemolysin was shown to be identical with theta-toxin by the following criteria: (a) a molecular weight of 60 000–65 000, (b) an isoelectric point of 6.8, (c) activation by reducing agents, such as dithiothreitol, (d) inhibition by cholesterol[23,24].

The second peak containing haemolytic activity coincided with the elution of the phospholipase C activity. Since a sample of 10 ml was found to be optimal to apply onto the Sephadex column to obtain the best resolution, and a further concentration of the material of Step 2 caused precipitation of proteins, three separated runs were necessary for the 30 ml of the material in Step 2. The fractions containing the phospholipase C activity were pooled and concentrated against PEG 20 M before isoelectric focusing. As is seen in Table II, this chromatography step gave another 7-fold increase in specific activity.

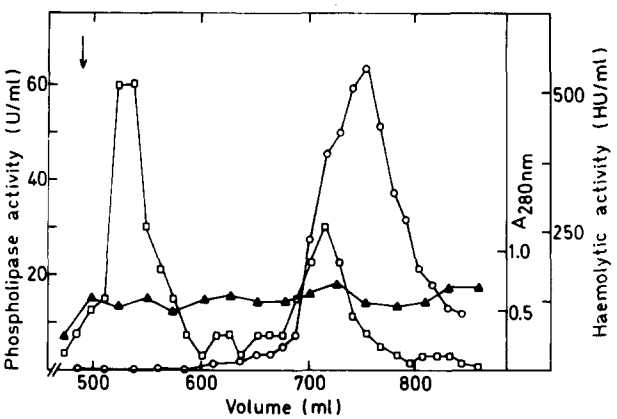

Fig. 1. Chromatography on Sephadex G-75. Phospholipase C was chromatographed at 4 °C on a 4 cm × 100 cm column of Sephadex G-75 in 0.02 M Tris–HCl, pH 7.0, containing 1 mM $CaCl_2$. Elution rate was 0.3 ml/min and 6-ml fractions were collected. ○—○, phospholipase C activity; □—□, haemolytic activity; ▲—▲, $A_{280\ nm}$. Arrow indicates void volume (484 ml).

Isoelectric focusing (Step 4)

Preparative electrofocusing is generally preceeded by dialysis against 1% (w/v) of glycine or distilled water in order to decrease the ionic strength and to permit focusing at a high voltage in a relatively short period of time. However, dialysis was not necessary after Sephadex chromatography due to the low molarity of the eluting buffer. Furthermore, dialysis against glycine completely inhibited the enzymatic activity, a remarkable fact which has not been further investigated.

Phospholipase C was focused in one single peak (Fig. 2), and the isoelectric point (pI) was shown to be 5.7 ± 0.05. A considerable degree of purification of the enzyme was obtained in this step (Table II) and the small amount of theta-toxin (pI 6.8) in the material of Step 3 was completely removed in this step.

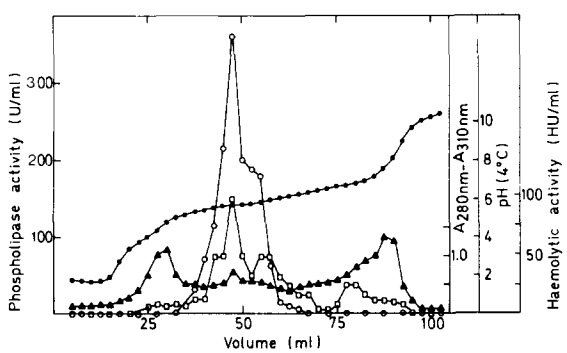

Fig. 2. Isoelectric focusing. Phospholipase C was focused in an isoelectric column (110 ml) at 4 °C and 5–600 V in a pH gradient of Ampholine 5–7. After focusing for 36–48 h fractions of 2.5 ml were collected and pH (●—●) was measured at 4 °C. ○—○, phospholipase C activity; □—□, haemolytic activity; ▲—▲, $A_{280\ nm} - A_{310\ nm}$.

Some properties of the purified phospholipase

The purified phospholipase C is still haemolytic and this activity seems not to be affected by reducing agents and cholesterol as in the case for the haemolytic

activity of purified theta-toxin indicated above. The purified phospholipase was lethal for mice ($LD_{50} = 2.5\,\mu g$; $125\,\mu g/kg$ mouse) and dermonecrotic upon injection of $0.6\,\mu g$ in the shaved skin of a rabbit. All these properties were thus shown to be caused by the purified phospholipase (alpha-toxin) and not by contaminating theta-toxin.

Stability

Ampholine and glycerol were removed after isoelectric focusing by extensive dialysis against 0.02 M Tris–HCl, pH 7.0, containing 0.15 M NaCl, and the purified enzyme was then stored at −20 °C or freeze-dried. Dialysis against a volatile buffer, such as ammonium acetate or bicarbonate[12,15] before freeze-drying gave a lower recovery (Table III). Supplementing zinc ions (1 mM) to the Tris-buffered saline was shown to stabilize the enzyme upon dialysis and storage at −20 °C (Table III). For storage for several months this product was freeze-dried after dialysis.

TABLE III

EFFECT OF DIALYSIS AND FREEZE-DRYING ON PHOSPHOLIPASE C (STEP 4, TABLE I)

Treatment	*U/ml*
Before treatment, pool of most active fractions after isoelectric focusing	165
After dialysis at 4 °C for 18 h against	
TBS* + 1 mM $ZnCl_2$	130
TBS* + 10 mM $CaCl_2$	90
Ammonium acetate buffer, 0.1 M, pH 7.0	85
Ammonium acetate buffer, 0.1 M, pH 7.0 +1 mM $ZnCl_2$	83
Ammonium acetate buffer, 0.1 M, pH 7.0 +1 mM $ZnCl_2$ + 1 mM $CaCl_2$	82
After freeze-drying** in	
TBS* + 1 mM $ZnCl_2$	108
Ammonium acetate buffer, 0.1 M, pH 7.0	60
Ammonium acetate buffer, 0.1 M, pH 7.0 +1 mM $ZnCl_2$	52

* 0.15 M NaCl, buffered with 0.02 M Tris–HCl, pH 7.0.

** Freeze-dried in the denoted buffer system and then dissolved in the same volume of distilled water.

The highly purified enzyme was stored at −20 °C in the Tris–buffered saline and showed a half-life of about 30 days. However, upon freezing and thawing of the enzyme the stability decreased considerably; *e.g.* repetition of this procedure 9 times during 3 weeks gave a 50% decrease in enzymatic activity, while storage at 4 °C for the same period of time only gave a loss of 25% of the activity. Storage in some other buffers or in the Tris-buffered saline supplemented with albumin did not stabilize the enzyme significantly. For long time storage the enzyme was freeze-dried in ampoules (Table III). The recovery was about 75%.

Criteria of purity

(i) Immunodiffusion of material from the different steps of purification and the purified theta-toxin revealed a single precipitin line with purified phospholipase C which showed reaction of non-identity with purified theta-toxin (Fig. 3). Immuno-

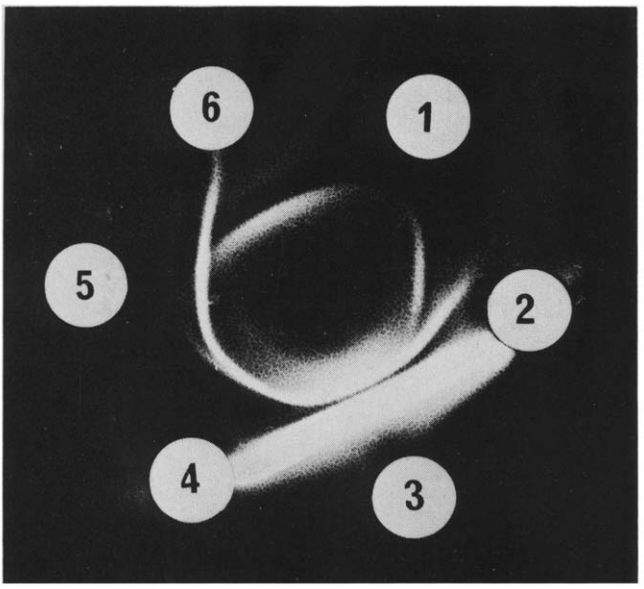

Fig. 3. Immunodiffusion. Immunodiffusion of phospholipase C after different steps of purification against a polyvalent antiserum (central well). Samples were applied as follows: 1, crude supernatant; 2, freeze-dried crude material (Step 1); 3, enzyme after DEAE-Sephadex adsorption (Step 2); 4, enzyme after Sephadex G-75 chromatography (Step 3); 5, enzyme after isoelectric focusing (Step 4); 6, purified theta-haemolysin (first peak, Fig. 1). All wells contain 10 μl.

electrophoresis of purified phospholipase C against the same polyvalent antiserum from sheep immunized with the crude Step 1 material, also revealed only one band (Fig. 4). Crossed electroimmunoassay resolved more than 15 precipitin lines with

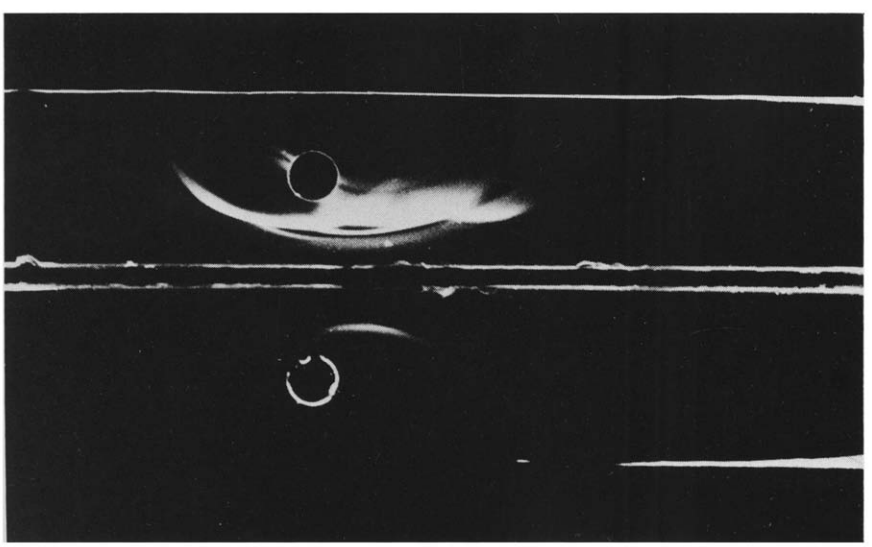

Fig. 4. Immunoelectrophoresis. 10 μl of material from the purification Step 2 and from Step 4 (Table I) were applied in the upper and lower wells and subjected to electrophoresis at 100 V, 12 mA for 60 min. Polyvalent clostridial antiserum (0.15 ml) was applied in the centre well.

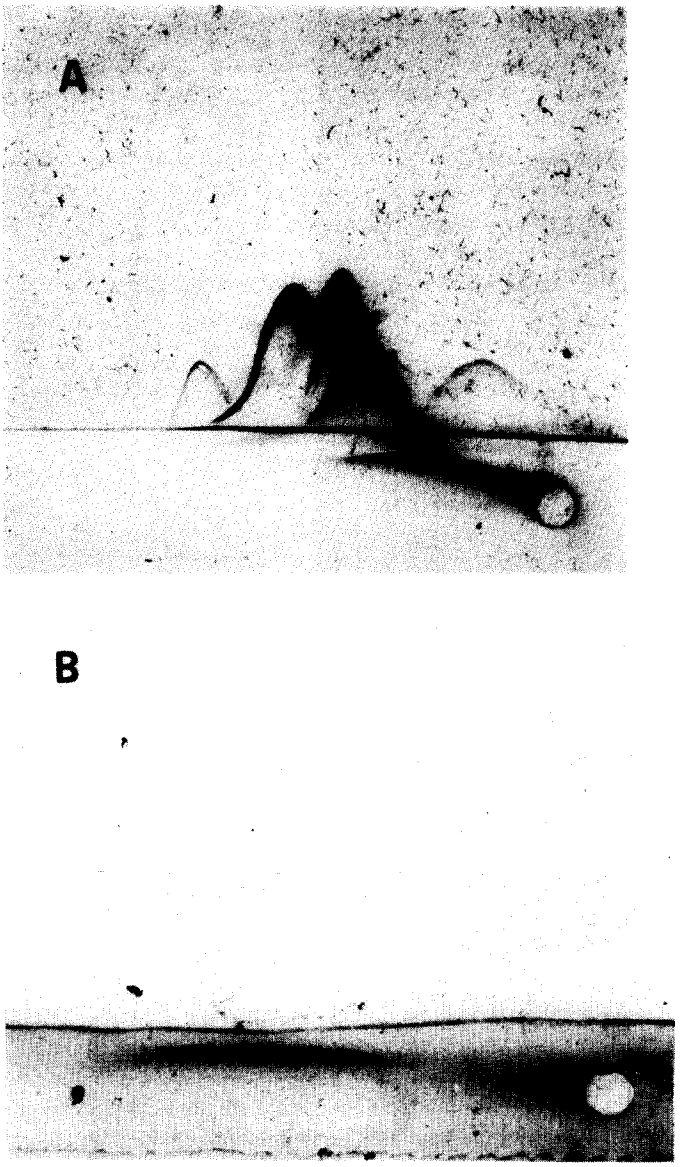

Fig. 5. Crossed electroimmunoassay of phospholipase C. 10 μl of material from purification Step 2 (Fig. 5A) and from Step 4 (Fig. 5B) (Table I) were applied in the wells and subjected to electrophoresis at 10 V/cm for 90 min with the anode to the left. After that agarose containing 2 μl polyvalent antiserum per cm^2 was cast on the upper part of the glass plate and electrophoresis at 3–4 V/cm was performed overnight (12 h) (anode at the upper part of the gel).

the crude material, but only one single band with the purified enzyme (Fig. 5). Very similar results were obtained with the polyvalent sheep antiserum produced in this laboratory and the commercial one from the Pasteur institute. The flat precipitin line (Fig. 5B) could be converted into a rocket-like line when the buffer of the antibody containing gel was increased one pH unit in comparison with the standard

procedure (25 mM Veronal buffer, pH 8.6). This was probably partly due to a less pronounced electroendosmotic flow in the gel.

(ii) Electrophoresis on polyacrylamide gel revealed two distinct bands well separated in the highly purified material (Fig. 6). Two bands were also resolved in sodium dodecyl sulphate electrophoresis (Fig. 7).

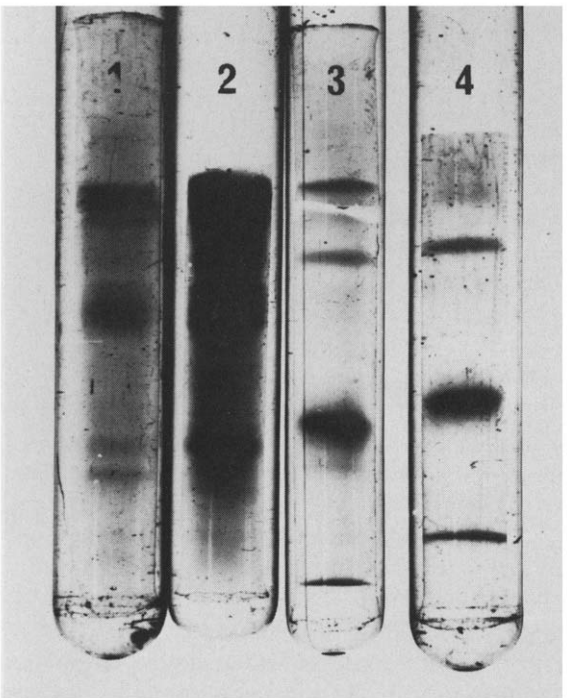

Fig. 6. Polyacrylamide disc electrophoresis. Polyacrylamide disc electrophoresis was run at 1.5–2.5 mA per tube for 80 min at 6 °C. The gels were stained with Coomassie blue and the excess of stain was removed by soaking the gels in destaining solution over night. Bromophenol blue was used as a tracking dye and can be seen as the lowest band in Tubes 3 and 4. 75 μl of sample mixed with 25 μl of glycerol were applied on top of each gel. 1, Purification Step 1 (Table I); 2, Purification Step 2; 3, Purification Step 3; 4, Purification Step 4.

(iii) However, none of the different enzymes or toxins found in the crude material were detected in the purified preparation. Neuraminidase was not assayed for but should have been removed upon isoelectric focusing, since the isoelectric point is in the range of 5.2–5.5 (Smyth, C. J., personal communication).

Molecular weight estimation

The molecular weight of crude and purified phospholipase C was determined by chromatography on Sephadex G-75 and Biogel A-0.5 (Fig. 8). From these experiments a molecular weight of 30 000 $\pm$ 2000 was calculated. Experiments on Biogel P-60 and Sephadex G-50 with the purified enzyme confirmed this value. However, two protein bands were separated on sodium dodecyl sulphate electrophoresis with molecular weights of 22 000 and 55 000 (Fig. 7).

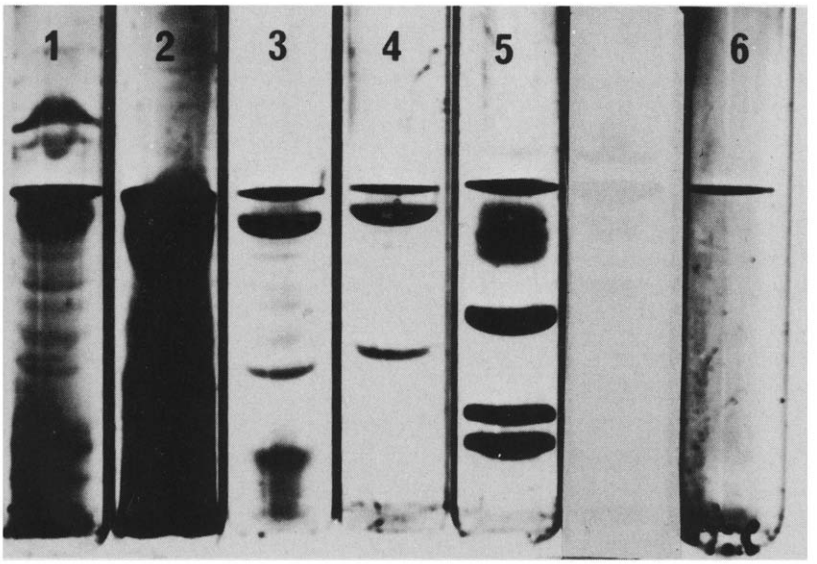

Fig. 7. Sodium dodecyl sulphate acrylamide electrophoresis. Gels contained 11.7% (w/v) acrylamide and 0.153% (w/v) bisacrylamide. Buffer system contained 0.05 M Tris–glycine buffer, pH 8.3, and 0.1% (w/v) sodium dodecyl sulphate. Samples were treated with 1% (w/v) sodium dodecyl sulphate for 1 h at 37 °C. 100 μl of each sample diluted 1:1 in 40% (w/v) sucrose were applied. The electrophoresis was run at 2 mA per tube at 6 °C until the tracking dye bromophenol blue was 5–10 mm from the bottom of the gel. 1, Purification Step 1 (Table I); 2; Purification Step 2; 3, Purification Step 3; 4, Purification Step 4; 5, marker proteins, 15 μg of each, from the top of the gel: serum albumin, ovalalbumin, trypsinogen, myoglobin, cytochrome *c*; 6, artifact band in control gel.

Phospholipase C activity

The highly purified enzyme was shown to release water-soluble phosphorus from synthetic DL-lecithin, from crude soy bean lecithin, and from the egg yolk substrate in the titrimetric assay (Möllby, R., unpublished). One phospholipase C

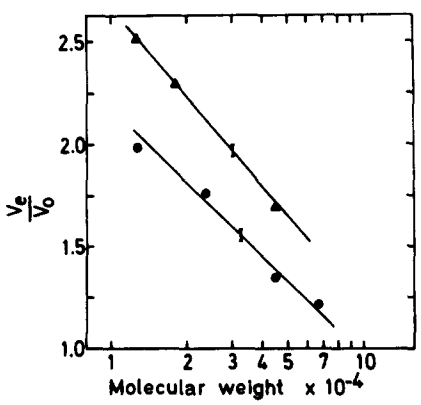

Fig. 8. Molecular weight determination. Molecular weight was determined by gel chromatography on Sephadex G-75 (●—●) (5 experiments), and on Biogel A-0.5 (▲—▲) (2 experiments). Standard proteins plotted in the figure (from left to right): cytochrome *c*, myoglobin, trypsinogen, ovalbumin and human haemoglobin. Range of phospholipase C activity is marked by I.

unit, as defined by the titrimetric assay, was shown to release about 1 μmole of water-soluble phosphorus from the egg yolk suspension. There was no significant change in the specific activity towards lecithin and sphingomyelin during the purification procedure.

DISCUSSION

It is generally agreed that phospholipases are important tools in studies of membranes structure[25]. Topological studies on the distribution of antigenic sites and plant agglutin binding sites revealed that partially purified phospholipase C from *Cl. perfringens* affected the surface of erythrocytes and other cells[26]. Similar studies were also of fundamental importance for the creation of the fluid mosaic structure membrane model in which the membrane matrix is formed by a discontinuous lipid bilayer interrupted by intercalated proteins[27].

However, the purity of the commercial products of the phospholipases used was not reported in these studies, and it was not until very recently that the first phospholipase C was obtained in a highly purified state[1]. Two bacterial enzymes, one from *B. cereus* and one from *Cl. perfringens*, have been used in almost all these studies. However, it is well-known that both these bacterial species produce many extracellular proteins, some of which might also alter the membrane structure. Such proteins are *e.g.* proteases, glucosidases including neuraminidase and cytolytic toxins without known enzymatic activity such as cereolysin and theta-haemolysin[9,23,24]. It is thus of utmost importance that the purity of the purified phospholipases is investigated before such studies are started and that this also includes enzyme and toxin assays to exclude a contamination with any of these proteins. It is also probable that several of the biological effects reported to be caused by the *Cl. perfringens* phospholipase C on different cells will later be shown to have been caused by contaminating theta-toxin and/or protease activity.

It is interesting to point out that most well-known extracellular enzymes and toxins of *Cl. perfringens* type A are acidic proteins with isoelectric points between 4.5 and 6.8, *e.g.* a collagenase, a second protease, glucosidases, neuraminidase, hyaluronate-lyase, theta-toxin and others[11]. At least some of these proteins seem to have similar molecular weights between 20 000 and 60 000 which makes the purification difficult and emphasizes the importance of testing the purity of the final product by different biological assays.

The purification scheme shown in Table I was chosen after several preliminary experiments trying also other methods such as salt fractionation and QAE-Sephadex chromatography. Since there are few laboratories that have the possibility to perform dialysis and freeze-drying on a large scale, it should be pointed out that Step 1 (Table I) can be exchanged for ammonium sulphate fractionation or concentration of the supernatant by rotary evaporation[12–15]. It was previously shown that the optimal recovery after ammonium sulphate precipitation was obtained at a final saturation of 40 or 50%. However, the crude enzyme in the culture fluid was precipitated with the highest yield (around 70%) at 66% final saturation at 4 °C. The recovery in several experiments upon concentration of the fluid by rotary evaporation was between 80 and 90%. However, studies by isoelectric focusing in the gel, in combination with a zymogram technique for detection of phospholipase activity,

revealed two to four "isoenzymes" in the culture fluid. It seems as if the last two concentration methods might increase the heterogeneity, an observation which is now being further studied (Möllby, R., Smyth, C. J. and Wadström, T., unpublished).

Separation of a commercial phospholipase C of *Cl. perfringens* by isoelectric focusing resolved two components[11]. This is in agreement with previous reports by Bernheimer *et al.*[28], Sugahara and Ohsaka[5] and Smyth and Arbuthnott[6]. More recent experiments on these two forms revealed that the acidic form (pI 4.5) could be converted to the alkaline form (pI 5.7) by performing the experiments in columns with 4 or 6 M urea. Similar observations were also recently reported by Smyth and Arbuthnott[6] and seem to indicate that the acidic form is probably caused by aggregation or complex formation with some unknown substance(s).

Addition of calcium and zinc ions after electrofocusing increased the recovery, probably by counteracting the chelating effect of Ampholine[11]. When the electrofocusing column was loaded with larger quantities of partially purified enzyme (Step 4, Table I), the recovery was higher and no reactivation was observed upon addition of Ca^{2+} or Zn^{2+} (Table III).

Both the *B. cereus* and the *Cl. perfringens* phospholipase C were reported to be zinc-requiring metalloenzymes[29,30] but the data presented are not conclusive. Both enzymes are inactivated by EDTA and 1,10-phenanthroline and the inactivation is reversed by addition of Zn^{2+} but not by Ca^{2+}. In the case of the *Cl. perfringens* enzyme the inactivation by EDTA was dependent on the enzyme concentration[30]. Enzyme in a higher dilution was more rapidly inactivated. The chelating properties of Ampholine might cause rapid inactivation of small quantities of enzyme but did not affect the yield upon electrofocusing in Step 4. This fact might also explain the previous report that isoelectric focusing was not suitable for purification of phospholipase C since the purification was performed on a rather small scale and with a very low final yield of purified enzyme[31].

Very similar isoelectric points have been reported for the main component of the clostridial phospholipase C[5,6,11,28], while there are great discrepancies in the value of the molecular weight. Meduski and Volkova[32] and Casu *et al.*[33] presented the highest values up to now (106 000 and 90 000) but other groups have later found values between 26 000 and 35 000 by different gel chromatographic techniques[34–36]. A disparity in sedimentation coefficients from 3.8 S up to 7.9 S was also reported[32,34–35]. It is thus still probable that different molecular forms of the enzyme exist, or that different strains might produce different enzymes. Studies along this line are now under way and investigations are also made to reveal if the two bands, separated by analytical acrylamide electrophoresis and sodium dodecyl sulphate electrophoresis, might be caused by formation of a polymer under certain conditions. The high molecular form obtained in sodium dodecyl sulphate electrophoresis on 55 000 is in very close agreement with the value obtained in another laboratory[6], while the value on 30 000 is in perfect agreement with a previous determination by Sephadex chromatography[37].

Some properties of the purified enzyme that are of importance for biological studies are reported in this communication. A more extensive study on the properties of the enzyme and its substrate specificity will soon be published (Möllby, R. and Norenius, L.). However, it should be already mentioned that the purified enzyme degrades both lecithin and sphingomyelin as was recently reported by Sugahara and

Ohsaka[5]. Pastan *et al.*[4] reported that a second enzyme specific for the degradation of sphingomyelin could be purified from a crude culture filtrate of *Cl. perfringens*. However, it should be emphasized that the convenient and inexpensive assay used during purification of the *Cl. perfringens* enzyme as well as of the *B. cereus* enzyme[1] could not detect sphingomyelinase activity. A similar ratio of lecithin to sphingomyelin hydrolysis of the crude enzyme and the highly purified enzyme of Step 4 seems to rule out the possibility that strain ATCC 13124 produces an enzyme which degrades sphingomyelin as well.

The different substrate specificities of the highly purified *Cl. perfringens* enzyme and the *B. cereus* enzyme[2,3] as well as a phospholipase C from *Staphylococcus aureus*, with a specificity for sphingomyelin[15] will probably make them very valuable for studies on membrane structure and function. Their effects on human diploid fibroblasts, frog muscle properations and on release of histamine from rat mast cells are already being investigated and have revealed great dissimilarities between the effects of the enzymes (Möllby, R., Strandberg, K., Thelestam, M. and Wadström, T., unpublished).

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A report on a biologically and physiochemically homogeneous preparation of phospholipase C from *Cl. perfringens* was very recently published (Mitsui, K., Mitsui, N. and Hase, J. (1973) *Japan. J. Exp. Med.* 43, 65–80). The enzyme was purified by repeated $(NH_4)_2SO_4$ fractionations, acetone fractionations and gel filtrations and shown to possess the same biological activities as earlier reported upon. However, the molecular weight of 49 000, as determined by gel filtration, is not in accordance with the findings reported in this or earlier publications.

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