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Complete purification and some properties of phospholipase C from *Bacillus cereus*

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SUMMARY

1. Phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) has been purified 450-fold over the growth medium of *Bacillus cereus*, with overall recovery of 23%.

2. Purification was performed using both $(NH_4)_2SO_4$ and ethanol precipitation, protamine sulfate fractionation, Sephadex G-100 gel filtration followed by ion exchange chromatography on DEAE- and CM-sephadex.

3. The preparation appeared to be pure on disc electrophoresis at pH 2.3 in 6 M urea, whereas a molecular weight between 21 000 and 25 000 could be derived from gel filtration on Sephadex G-100.

4. No hemolytic activity towards intact human erythrocytes could be established. Treatment of human red cell ghosts with pure phospholipase C resulted in a nearly complete degradation of the main phospholipid classes (except for sphingomyelin) up to 70% of total phosphorus. Similar results were obtained with liposomes derived from human erythrocyte total lipids.

In recent years, the ability of bacterial phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) to hydrolyze several phospholipids has led to increasing attention to its use as a tool in membrane investigations. Until now, however, phospholipase C has been only partially purified[1-7], whereas a high degree of purity would be desirable for application on membranes. In spite of this, different authors have used only partially purified preparations[8-13], making it doubtful whether the observed effects are caused by the action of the enzyme alone rather than by contaminations. In this report the complete purification of phospholipase C from *Bacillus cereus* is presented and some preliminary studies concerning the action of the enzyme on human erythrocytes and erythrocyte membranes are described.

For routine assays of enzymatic activity during purification, hydrolysis of egg yolk lipoprotein was measured by continuous titration of the acid produced with 0.02 M NaOH[5],

using a Radiometer PHM 26 connected to a titrator 11. The egg yolk lipoprotein substrate was prepared by homogenizing 4 egg yolks in 424 ml 0.15 M NaCl, 10 mM $CaCl_2$, 0.1 mM $ZnCl_2$, followed by centrifugation at 35 000 × *g* for 30 min at 0°. The supernatant could be used as substrate for a week if stored at +°. 7 ml of the supernatant were used per assay and 200 µl sodium deoxycholate solution (90 mg/ml) were added. The reaction was carried out at 37° for 5–10 min at pH 7.38. The consumption of 0.02 M NaOH was usually linear for at least 12–15 min. 1 unit of phospholipase C is defined as the amount of enzyme which liberates 1 µmole of titratable H^+ per min.

Alternatively, 10–15 mg of purified egg lecithin was dissolved in 2 ml diethyl ether and 0.5 ml of 0.1 M Tris buffer (pH 7.4) containing 10 mM $CaCl_2$ and aliquots of enzyme were successively added. The reaction tubes were capped and shaken at 37°. At 5 min intervals, samples were taken from the organic phase and analysed for phosphorus after destruction with 70% $HClO_4$ at 190° by a modification[14] of the procedure of Fiske-Subbarow. The amount of enzyme which causes the liberation of 1 µmole of choline phosphate/min appeared to be comparable (within 10%) with the number of units found in the acid production assay with egg yolk lipoprotein as substrate.

In order to determine specific activities, protein was measured according to Lowry *et al.*[15], using bovine serum albumin as a standard. Since part of the purification was carried out in 50% glycerol, making protein determinations according to Lowry less reliable[16], samples were dialysed and nitrogen was analyzed as ammonia by a micro-Kjeldahl method[17] and the amount of protein was calculated, assuming 16% nitrogen content.

Crude enzyme was obtained from log phase cultures of *B. cereus*, grown for 14 h at 37° by rotation in a medium containing per l: 10 g yeast extract, 10 g peptone, 5 g NaCl, 0.4 g Na_2HPO_4. The medium was adjusted to pH 7 with 1 M NaOH prior to the inoculation procedure. After removal of the cells by centrifugation, the supernatant was adjusted to 77% satn with solid $(NH_4)_2SO_4$ and allowed to stand for at least 48 h at 0–4°. The precipitate was usually collected by centrifugation in a Servall RC-2B centrifuge at 0°, equipped with the Szent-Györgyi continuous flow system, using a SS-34 rotor at 27 000 × *g*. The pellets were solubilized in water (1/100 × supernatant vol.) and dialysed for 3 h against running tap water, followed by dialysis over night against 250-fold vol. of distilled H_2O at 4°.

The solution was made 40% (by vol.) with ethanol and allowed to stand for 30 min at −27°, after which the precipitate was centrifuged for 15 min at 27 000 × *g*. In contrast to the observations made by Ottolenghi[3,5], no significant activity was found in the supernatant. The pellet, showing a 2-fold increase in specific activity, was solubilized in glycerol–water (1:1, v/v) and all the following purification steps were performed in 50% glycerol (by vol.), in order to avoid extensive inactivation during purification. A 1.5% solution of protamine sulfate (Koch-Light) in 0.01 M Tris buffer was adjusted to pH 7 and added to the enzyme solution in 50% glycerol, up to a final concentration of 0.2 mg protamine sulfate/mg enzyme protein. The resulting mixture was stirred for 1 h at 0° and subsequently centrifuged for 15 min at 27 000 × *g*. All the activity was recovered in the supernatant with a 2-fold increase in specific activity (see Table I).

The supernatant was applied to a sephadex G-100 column, using 0.05 M Tris buffer (pH 7.6) containing 5 mM $CaCl_2$ and 50% (by vol.) glycerol as eluent. Two different sized columns were routinely employed. One 3.5 cm × 180 cm column was used for the scale

TABLE I

PURIFICATION OF PHOSPHOLIPASE C

Summary of purification starting with 13 l of *B. cereus* supernatant. Details described in the text.

Step	*Total activity (10^3 units)*	*Protein (mg)*	*Specific activity*	*% Recovery*	*Purification*
B. cereus - supernatant	130	57800	2–2.5	100	1
$(NH_4)_2SO_4$ - precipitate (77% satn.)	83.2	1140	73	64	32
Dialysis	75.4	1140	66	58	29
Ethanol - precipitate (40%; -27°)	58.5	462	127	45	56
Protamine sulfate supernatant (pH 7)	58.5	246	238	45	106
Sephadex G-100	46.8	62	754	36	336
DEAE - sephadex A-50	37.7	39.7	950	29	422
CM - sephadex C-50	29.9	29.6	1010	23	450

given in Table I and one 1.8 cm X 135 cm column for 1/5 (or less) the amounts given in Table I. The separation on the last column is shown in Fig.1. The resolution of the larger column is better, but in this case the effluent was scanned by LKB Uvicord only.

In order to achieve an appropriate flow rate, both gel filtration and the following ion exchange procedures were performed at room temperature. No significant loss of activity in 50% glycerol was observed at room temperature within the first week, after which the activity decreased 1% per day. This is in contrast to preparations which were stored at -20° which retained their full activity for at least 5 months. Usually, 95–100% of the activity which was applied to the column was regained during elution, but only 80% was suitable for further purification.

The pooled fractions from the sephadex column were adjusted to pH 8.5 with concentrated Tris and washed onto a DEAE-sephadex A-50 column. Elution was performed with a linear NaCl gradient (0–0.35 M NaCl) in 0.05 M Tris buffer (pH 8.5) containing 5 mM $CaCl_2$ and 50% (by vol.) glycerol (Fig.2). Under these conditions, about 90% of the applied activity was recovered, of which 80% was suitable for final purification on CM-sephadex C-50. The pooled fractions were adjusted to pH 7.2 and dialysed against the CM-sephadex elution buffer (0.05 M Tris, 5 mM $CaCl_2$, 50% glycerol (by vol.), pH 7.2) prior to application to the column. Elution was carried out with a linear NaCl gradient (0–0.2 M) in the elution buffer (Fig.3). About 80% of the applied activity was recovered in fractions having the same specific activities as high as 1000 μmoles/min per mg (see Table I).

From the appearance of disc electrophoresis in polyacrylamide under depolymerizing conditions at pH 2.3 in 6 M urea, the final preparation is estimated to be approximately 98% pure (Fig.4). The most extensive purification published heretofore appears to have been that of Diner[7], who purified phospholipase C from *Clostridium welchii* for 60–70%, with a specific activity of 72. Although we have used a similar assay as Diner, our preparation is approximately 10 times as active. An explanation for this might be that Diner did not include glycerol during the purification steps, which could result in a partially inactivated preparation. It has been shown by Kleiman and Lands[6] that the more the enzyme is purified, the less stable it becomes in aqueous buffers; an observation which we confirm and which made us decide to use glycerol in the purification process.

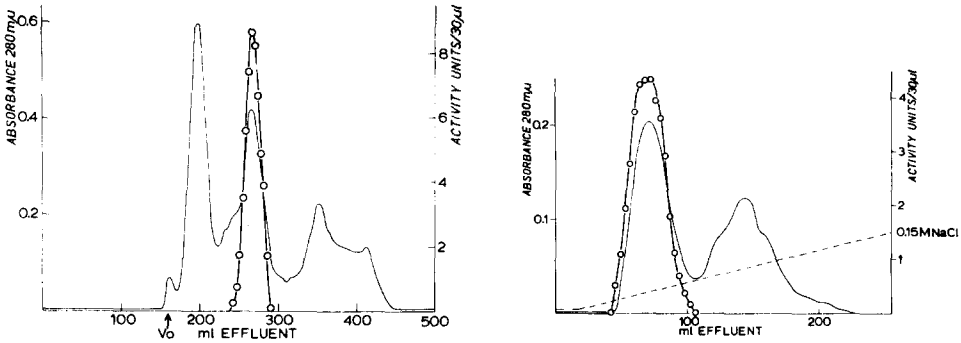

Fig.1. Sephadex G-100 gel filtration at 20° in 0.05 M Tris–HCl (pH 7.6) containing 5 mM $CaCl_2$ and 50% (by vol.) glycerol. The column (1.8 cm x 135 cm) was packed and eluted under reduced pressure (40 cm) and 4 ml-fractions were collected at a flow rate of 8 ml/h. The void volume, V_0, was 160 ml. Activity (o–o); absorbance at 280 mμ (—).

Fig.2. DEAE-sephadex A-50 chromatography at 20° in 0.05 M Tris–HCl (pH 8.5) containing 5 mM $CaCl_2$ and 50% (by vol.) glycerol. Elution was performed with a linear NaCl gradient extending from 0–0.35 M; elution volume equals 10 times the bed volume of the column. The flow rate was adjusted to 12 ml/h and 4 ml-fractions were collected. Activity (o–o); absorbance at 280 mμ (—); salt gradient (- - -).

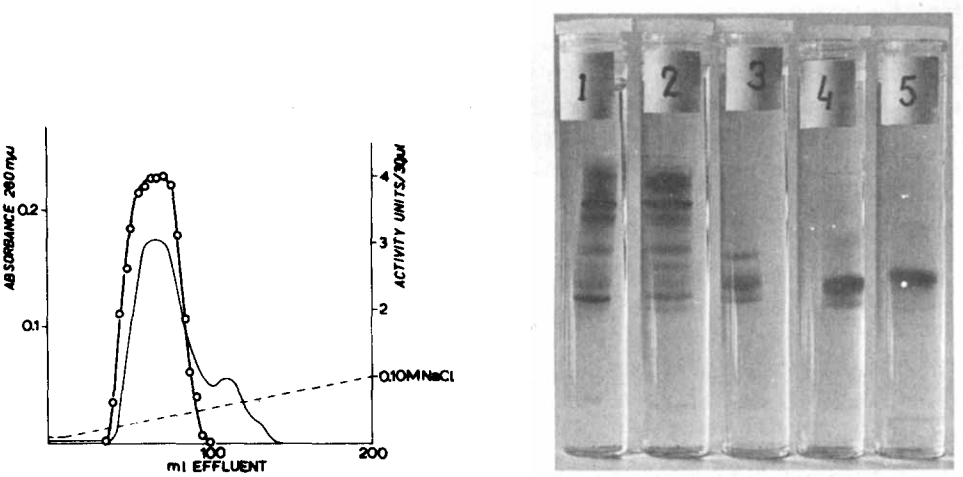

Fig.3. CM-sephadex C-50 chromatography at 20° in 0.05 M Tris HCl (pH 7.2) containing 5 mM $CaCl_2$ and 50% (by vol.) glycerol. Elution was performed with a linear NaCl gradient extending from 0–0.2 M; elution volume equals 10 times the bed volume of the column. The flow rate was adjusted to 10 ml/h and 4-ml fractions were collected. Activity (o–o); absorbance at 280 mμ (—); salt gradient (- - -).

Fig.4. Disc electrophoretic patterns after different purification steps. Electrophoresis in 7.5% polyacrylamide at pH 2.3 was carried out with 60–80 μg protein/tube in the Shandon disc electrophoresis apparatus (Shandon Scientific Company, Ltd., London) in the presence of 6 M urea (100–120 V; 5 mA/tube; 2.5 h at 20°; stained with Amido Black 10B). 1. $(NH_4)_2SO_4$ precipitate; 2. protamine sulfate supernatant; 3. Sephadex G-100; 4. DEAE-sephadex A-50; 5. CM-sephadex C-50. (Compare Table I). The front band is caused by glycine from the electrode buffer and is also present in gels to which no protein was applied.

The smallest sephadex column was calibrated with bovine serum albumin, pepsin, and cytochrome *c*. The different elution volumes in 50% glycerol were compared with the elution volume of phospholipase C, according to the method of Determann and Michel[18]. From these values, the mol. wt. of phospholipase C was estimated to be 23 000 (± 2000), assuming a spherical shape of the molecule. From the behavior on DEAE- and CM-sephadex columns, an isoelectric point between pH 7.2 and 8.5 is concluded.

No hemolytic activity towards intact human erythrocytes could be established either in isotonic phosphate buffer (pH 7.4) or in isotonic saline containing 10 mM $CaCl_2$. 40 enzyme units did not cause more than 2% hemolysis to 0.25 ml packed cells in 4 ml buffer for 1 h at 37°. Incubations with human red cell ghosts with pure phospholipase C either with or without extra Ca^{2+} resulted in a complete degradation of lecithin and phosphatidylethanolamine, whereas phosphatidylserine was only partially converted. However, no significant amount of spingomyelin was degraded★. These observations are responsible for the fact that only 70% of the total lipid phosphorus is attacked in erythrocyte ghosts. With respect to the individual phospholipid classes essentially the same results were obtained with liposomes derived from human erythrocyte total lipids, while purified egg lecithin dispersed as liposomes was completely degraded. A value of 68–74% phosphorus release from erythrocyte ghosts after treatment with crude phospholipase C from *B. cereus* was first reported by Lenard and Singer[9] and later confirmed by other groups[10,11]. However, these groups did not investigate the degree of degradation of individual phospholipids. From our observations it cannot be concluded, as has been done by Glaser *et al.*[11], that "25 to 30% of the phospholipids are in a physical state different from the remainder of the lipids, perhaps involved in a more tightly coupled interaction with the membrane proteins". Our results clearly demonstrate that the amount of lipid phosphorus released from red cell ghosts after treatment with pure phospholipase C from *B. cereus* primarily depends upon the chemical nature of the phospholipids involved. Furthermore, more caution should be exercised in extrapolating the results obtained with erythrocyte ghosts (or other isolated membrane preparations) to the structure of the native membrane.

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★It should be noted that crude phospholipase C from *Clostridium perfringens* acts on sphingomyelin[13].

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