PURIFICATION BY AFFINITY CHROMATOGRAPHY OF PHOSPHOLIPASE C FROM *BACILLUS CEREUS*

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1. Introduction

Phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) is a bacterial enzyme widely used in membrane and phospholipid studies. For such studies the highest possible degree of purity is required. Both the *Bacillus cereus* and the *Clostridium perfringens* enzymes have been purified to apparent homogeneity using conventional techniques [1–3]. However, using a phospholipase C-hyperproducing strain of *B. cereus* [4], homogeneous preparations of enzyme were not consistently obtained using the purification scheme described [2] (C. Little and A. B. Otnaess, unpublished observation). The *Cl. perfringens* enzyme has also been purified by a method involving affinity chromatography on agarose-linked egg yolk lipoprotein [5] and we have applied this technique to the purification of the *B. cereus* enzyme. The purification scheme here presented is simpler and more convenient and gives higher yields of enzyme than do the previously published methods.

2. Materials and methods

2.1. *Preparation of affinity gel*

Sepharose 4B (Pharmacia) was activated by cyanogen bromide as described by Marsh et al. [6]. Following activation, the gel (150 ml settled vol) was washed with 600 ml of 0.1 M sodium bicarbonate buffer (pH 9.5), 1.5 litre water and 1.5 litre of 0.1 M sodium borate buffer (pH 9.6). The activated gel was then coupled to egg yolk lipoprotein as described by Takahashi et al. [5]. After coupling, the gel was washed with 1.5 litre portions of the following: de-ionized water, 0.05 M Tris–HCl buffer (pH 7.5) containing 0.5 M NaCl, 0.05 M Tris–HCl buffer containing 30% glycerol and finally veronalbuffered saline [7] containing 1 mM $ZnSO_4$.

2.2. *Enzyme assays*

Phospholipase C activity was routinely assayed by continuous titration of the acid produced in egg yolk lipoprotein substrate as described by Zwaal et al. [1] with the following modifications; the reaction vol was 3.6 ml and the reaction temperature was 23°C. The assays were carried out on Radiometer automatic tritration equipment SBR2c/ABU12/TTT2 using 0.02 M NaOH as titrant. One unit of phospholipase C activity is defined as the amount of enzyme releasing 1 μmol of titratable H^+/min at 23°C and pH 7.35.

The purified enzyme was also assayed by measuring the rate of release of acid-soluble phosphate from pure lecithin. One mg/ml dipalmitoyl-DL-3-lecithin (Koch Light) in 0.05 M veronal buffer (pH 8.2) containing 0.1% deoxycholate, 0.05 M KCl and 0.23 M mannitol was sonicated until clear (8–12 30 sec sonications were required). The enzyme (0.11 μg/ml) was incubated at 37°C with the sonicated liposomes. Aliquots (1–5 ml) were withdrawn every 15 min and the reaction was stopped by the addition of cold 70% perchloric acid. The solution was filtered through 0.22 μm millipore filters and the acid-soluble phosphate in 1 ml filtrate measured [8].

Sphingomyelinase activity was measured by replacing lecithin with pure sphingomyelin (Koch-Licht) in the above assay system.

Haemolytic activity was tested by incubating

enzyme samples (0.01 mg/ml) with a 2.5% suspension of human erythrocytes in veronal-buffered saline (pH 7.4) at 37°C for up to 1 hr.

2.3. *Polyacrylamide gel electrophoresis*

Polyacrylamide gel electrophoresis studies were carried out both in the discontinuous Tris–Cl/Tris–glycine buffer system of Davis [9] using 7.5% gels and in the sodium dodecyl sulphate system of Laemmli [10] using 10% gels. The gels were subsequently stained using Coomassie Brilliant Blue R-250 [11] and examined both by inspection and at 540 nm using a Gilford 2400-S spectrophotometer with gel scanning attachment.

Protein was assayed as described by Lowry et al. [12] with bovine serum albumin as standard.

Unless otherwise stated, all operations were carried out at 0–4°C.

3. Results and discussion

3.1. *Purification*

3.1.1. Growth of bacteria

Bacillus cereus (ATCC 10987 AB-1) [4] was grown in the medium described by Zwaal et al. [1]. After inoculation from an overnight shaker culture maintained at 37°C, the medium (1 litre batches in 2 litre conical flasks with cotton wool plugs) was incubated at 37°C with vigorous shaking (200 rev/min). The cultures were periodically tested for enzyme activity and harvested when the latter had reached a maximum value. This usually corresponded to a growth time of 4–5 hr at which point the culture had an absorbance at 600 nm of around 1.9. The cultures were harvested by centrifugation (16 000 *g* for 10 min).

3.1.2. First affinity column

After centrifugation of the bacterial cultures, the medium supernatant was allowed to percolate through a column of agarose-linked egg yolk lipoprotein (4.7 × 5 cm) at a flow rate of approximately 8 ml/min. Afterwards the column was washed with 500 ml of veronal-buffered saline (pH 7.4) containing 1 mM $ZnSO_4$. Enzyme activity was eluted from the column using 5 M urea freshly dissolved in the saline–zinc buffer at a flow rate of 2–3 ml/min. The column eluate was collected in 10 ml fractions and fractions containing enzyme activity were pooled and then centrifuged (20 000 *g* for 10 min) to remove a slight turbidity. The clear supernatant was then concentrated to 30–40 ml by ultrafiltration in an Amicon ultrafiltration cell using a UM-2 membrane. The concentrate was then dialyzed twice against 2 litre batches of the saline–zinc buffer (one 3 hr dialysis and one overnight). At this stage, the enzyme preparation was strongly haemolytic to human erythrocytes.

3.1.3. Heat treatment

After dialysis, the enzyme preparation was maintained at 69–70°C for 5 min, during which time a precipitate formed. The enzyme solution was then cooled rapidly and filtered to remove the precipitate.

3.1.4. Second affinity column

The enzyme preparation was once more loaded onto a column of agarose-linked egg yolk lipoprotein (3.0 × 3.3 cm) at a flow rate of 2–3 ml/min. The column was then washed with 50 ml of the saline–zinc buffer. Enzyme activity was then eluted from the column using a freshly prepared solution of urea (5 M) in the saline–zinc buffer. Five ml fractions of column eluate were collected and fractions containing enzyme activity were pooled, concentrated to 20–30 ml by ultrafiltration and dialyzed three times against 2 litre batches of saline–zinc buffer (two 3 hr dialyses and one overnight).

3.1.5. DEAE-Sephadex treatment

Occasionally the above purification scheme yielded a preparation that was slightly brown. In these cases, the enzyme was dialyzed against 1 litre 10 mM phosphate buffer (pH 8) for 2 hr and then passed through a column of DEAE-Sephadex A-25 (1.6 × 2 cm) previously equilibrated against the above phosphate buffer. The brown material bound to the column whereas the enzyme passed straight through and was collected, dialyzed three times against 2 litre batches of saline–zinc buffer and stored at −20°C.

3.2. *Comments on purification*

A typical purification starting with 5 litre bacterial culture yielded 27 mg enzyme with an overall recovery of 73% (table 1). The enzyme was purified some 970-fold over the bacterial supernatant. Freshly

Table 1
Purification of phospholipase C

Step	Total vol (ml)	Total activity (units)	Protein (mg)	Specific activity (u/mg)	Recovery (%)
B. cereus					
Supernatant	5040	55.450	36.290	1.53	100
1st affinity column	98	49.350	60.4	817	89
Heat	98	49.350	38.6	1279	89
2nd affinity column	58	42.510	29	1466	77
DEAE-Sephadex	63	40.320	27.1	1488	73

prepared 5 M urea was found the most suitable medium for eluting phospholipase C activity from the affinity column. Very high recoveries (table 1) and sharp elution profiles (fig.1) were obtained. Older solutions of 5 M urea (maintained at 4°C for 2 weeks) gave sharp elution profiles but the recoveries of activity and the specific activities of the final enzyme product were less than half of the values reported in table 1. Since the use of 2-week old solutions of urea led to an apparently homogeneous final enzyme product, it seems likely that the formation of cyanate ions in the urea solutions led to enzyme modification and inactivation. Guanidine HCl (1 M) pH 7.4 also gave sharp elution profiles and high recoveries of activity (83% from the first affinity column) when used as elution medium. However, the enzyme was generally less stable in guanidine solutions than in urea. Incubation at 23°C for 2 hr at pH 7.4 of enzyme (0.08 mg/ml) in guanidine (3 M) caused 36% inactivation, whereas urea (7 M) caused 7% inactivation. The use of 1 M guanidine resulted in somewhat lower yields of enzyme activity in the purification scheme than did 5 M urea and so the latter is used in our standard procedure. Lower concentrations of guanidine or urea (0.5 M and 2 M respectively) were ineffective as elution media.

Five litre culture supernatant appeared to be near to the maximum capacity of an affinity column of dimensions 4.7 × 5 cm. Using larger volumes of supernatant resulted in considerable leakage of activity from the column during the later stages of loading.

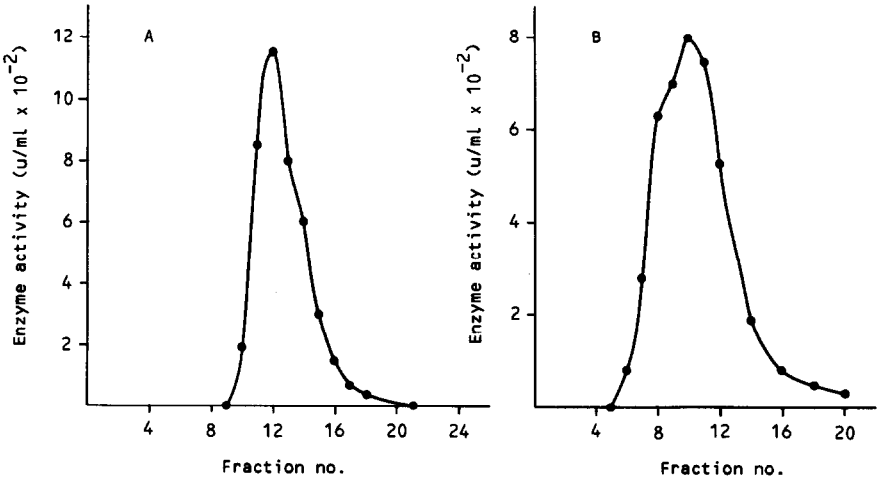

Fig.1. Elution profiles for phospholipase C from affinity column. Freshly prepared solutions of urea (5 M) in veronal-buffered saline (pH 7.4) containing 1 mM zinc sulphate were used as elution medium. A) Elution from first affinity column, 10 ml fractions were collected. B) Elution from second affinity column, 5 ml fractions were collected.

In addition, the first column was not reusable. Presumably phospholipase C and the various lipolytic and proteolytic exoenzymes of *B. cereus* degradation of the column lipoprotein during the overnight loading. The second affinity column, however, could be used at least 4 times with apparently undiminished effectiveness. Between each run, the column was well washed with 5 M urea and saline–zinc buffer and stored in the latter.

3.3. *Purity of the final enzyme product*

An attempt was made to analyze the final enzyme preparation by N-terminal amino acid analysis [13]. Tryptophan appeared to be the N-terminal amino acid and the usual degradation products of tryptophan made a quantitative assessmant of enzyme purity on this basis extremely difficult (R. Wallin, personal communication). The final enzyme preparation was also examined by polyacrylamide gel electrophoresis. The enzyme appeared as a single band under non-denaturing conditions and also when studied in the presence of sodium dodecyl sulphate following exposure to β-mercaptoethanol (fig.2). Such apparently homogeneous preparations of enzyme possessed specific activities of 1400–1500 μmol/mg when measured at 23°C using the continuous titration method. When measured at 37°C, specific activities of around 2900 μmol/mg were obtained, representing values almost three-times higher than those reported by Zwaal et al. [1] for the pure enzyme. When enzyme activity was measured in terms of the rate of hydrolysis of pure lecithin, the final preparations had specific activities of approximately 700 μmol phosphate released/min/mg protein. These preparations were totally inactive when sphingomyelin replaced lecithin in the phosphate release assay. In addition, the final enzyme preparation showed very little haemolytic activity to human erythrocytes when tested as described in Materials and methods. At 37°C, 0.01 mg/ml enzyme caused approximately 2% haemolysis after 1 hr.

The present purification scheme is simpler and more convenient than the 8 step scheme of Zwaal et al. [1] and avoids the need to lyophilize large volumes of culture supernatant in the scheme of Otnaess et al. [2]. In addition, the overall recovery of activity is superior to the recoveries obtained using the other two purification methods and is also superior to the recovery reported for the *Cl. perfringens* enzyme purified by a scheme involving affinity chromatography [5].

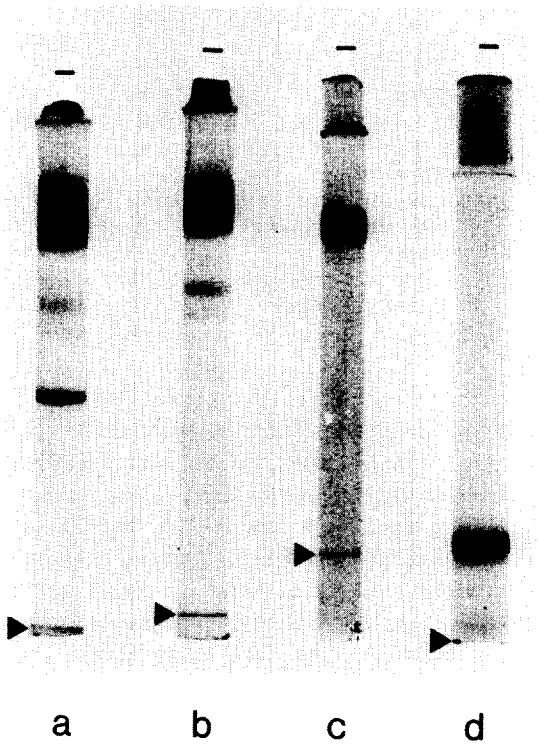

Fig.2. Polyacrylamide gel electrophoretic patterns after the various purification steps. a) After the first affinity column, b) after heat treatment, c) and d) final enzyme preparation. a), b) and c) were run in the system of Davis [9]. d) Was run in the system of Laemmli [10] after reduction with β-mercaptoethanol. Approx. 80 μg protein was loaded onto each gel.

Acknowledgements

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