IMMUNOPURIFICATION OF PHOSPHOLIPASE C (α-TOXIN) FROM *CLOSTRIDIUM PERFRINGENS*

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1. Introduction

Recent use of this enzyme as a probe of aspects of phospholipid structures in membranes has stimulated a new interest in its purification and characterisation [1]. Here we report the isolation of phospholipase C from culture filtrates of *Clostridium perfringens* using disulphide-linked immunosorbents [2–4]. Where applicable, this technique offers a rapid, single column process for purification of antigenic materials. In this work, the requisite antisera were raised according to [5], i.e. without prior existence of purified antigen.

2. Materials and methods

Monoprecipitin anti-(α-toxin) sera were raised by injecting rabbits with minute quantities of toxin–antitoxin complex derived from immunoelectrophoretic gels [6] (1% agar, barbiturate buffer pH 8.6, I 0.05). Crude freeze-dried culture filtrate (1.0 mg) was electrophoresed and analysed using commercial polyvalent type A antiserum and extract of egg yolk (EEY, [7]) to identify the α-toxin–antitoxin arc [7]. After washing the gels with phosphate-buffered saline (PBS: 4°C, 3 days) the cathodic tip of the 'α-arc' was removed, as cleanly as possible, with a scalpel. In all, 74 'cuts' were pooled, suspended in 20 ml PBS, homogenized in a glass tissue-grinder and centrifuged (20 000 *g*, 4°C, 10 min); the pellet was resuspended in 10 ml PBS and divided into 2 equal portions. One portion was mixed with an equal volume of complete Freund's adjuvant (CFA) and this was used for footpad injections; the other portion was stored at −20°C until required for intravenous injection. Each of 20 rabbits received 2 separate footpad injections (the second 14 days after the first) of 0.2 ml CFA preparation. Four weeks later each rabbit was injected intravenously with 0.2 ml of suspension without CFA. Animals were bled 10 and 21 days after the last injection to yield 2 pools of sera, which gave a single precipitin line immunoelectrophoretic analysis using crude toxin.

Mark III-type immunosorbent columns were prepared and used as in [3] except that the disulphide-linked polymer was finally washed and the column equilibrated in borate-buffered saline (BBS; 0.1 M NaCl + 0.2 M $Na_2B_4O_7$, titrated to pH 7.3 with conc. HCl). Crude toxin was applied to the column in BBS and desorption was carried out with 0.2 M glycine–HCl, pH 2.2; the column could be recycled many times [3]. The principle advantage of using BBS was that the phospholipase C activity could be assayed directly from the column, by the egg yolk turbidity (EYT) assay [7].

Polyacrylamide gels were analysed for phospholipase activity by embedding the polyacrylamide gel in an agar gel containing egg yolk extract (EEY-agar). Phospholipase C diffused out from the polyacrylamide gel into the EEY-agar causing zones of turbidity which could be observed visually or traced using a densitometer.

3. Results

In several runs 50%–70% of adsorbed phospholipase C activity was recovered from the anti-α immunosorbent column (derived from the first pool of serum) in

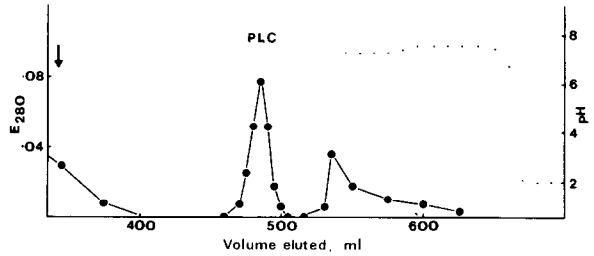

Fig. 1. A γ-globulin concentrate from monoprecipitin antisera (900 mg of protein; antitoxin equivalent to 800 LV units of α-toxin) was thiolated and polymerised [2] to give 480 mg of immunosorbent which was packed into a Mark III type column [3]. *Cl. perfringens* culture filtrate (43 mg; 755 LV units) was dissolved in 5 ml BBS, and loaded onto the column which was then eluted with BBS. Excess phospholipase C (42% of applied activity) was recovered in the excluded volume together with heterologous material; no excess activity was recovered if less than 400 LV units were loaded onto the column. When the absorbance at 280 nm approached zero (340 ml eluted; ↓) the column was desorbed with 70 ml 0.2 M glycine–HCl pH 2.2 and two further peaks were obtained. The first (PLC) contained the purified enzyme (69% of adsorbed activity); the second contained no phospholipase C activity. (-•-•-•) E_{280}; (. . . . .) pH.

the first peak (fig. 1). The material obtained in the second peak has not been characterised but was reproducibly well separated from the PLC peak and contained no active enzyme.

The purified enzyme hydrolysed both egg phosphatidyl choline and bovine brain sphingomyelin. It was lethal to mice and haemolysed human erythrocytes as shown (table 1). It showed approximately 100 times less haemolytic activity towards horse erythrocytes as compared with human. In addition the purified enzyme haemolysed sheep, pig and ox erythrocytes. It gave only one precipitin line on immunoelectrophoresis using polyvalent antisera and contained only traces of contaminants, on polyacrylamide gel (fig. 2). Traces of K-toxin (collagenase) but no θ-toxin (oxygen-labile haemolysin) activity was found in the purified material. The molecular weight of the phospholipase C was approximately 48 000 (fig. 2d). Isoelectric-focussing of both crude and purified material revealed 4 components (fig. 3a). When carried out in 6 M urea,

Table 1
Comparative phospholipase C, haemolytic and lethal activities of *Cl. perfringens* culture filtrate and purified enzyme

	LV/mg N	HU × 10^3/ mg N	LU × 10^3/ mg N
Culture filtrate	179	12.5	3
Purified enzyme	3400	222	50

Phospholipase C activities were measured by the egg yolk turbidity assay [7]. Standard curves were constructed with a culture filtrate of known antitoxin combining capacity. 1 LV unit is that amount of antitoxin which in the presence of 1 IU. of antitoxin will just cause turbidity in an egg yolk solution. 1 HU is that amount of activity causing 50% haemolysis of a 0.75% (v/v) suspension of human erythrocytes after 30 min at 37°C followed by 30 min at 0°C. Lethality to mice was estimated by injecting 0.3 ml of serial (2^{-n}) dilutions; 1 pair of mice per dilution was used [1]. 1 LU is the amount of activity killing one or both mice in 24 hr.

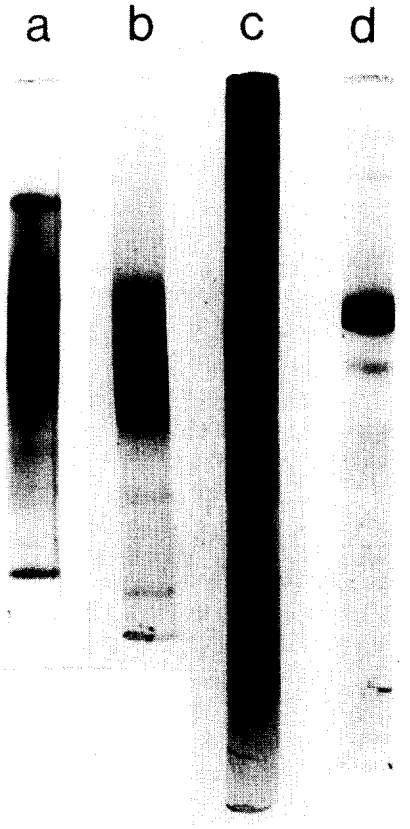

Fig. 2. Polyacrylamide gel electrophoresis of *Cl. perfringens* culture filtrate and purified phospholipase C. Gels a) and b) were run in the absence of SDS [9], gels c) and d) in the presence of SDS [10]. Gels were loaded with: a) and c) culture filtrate (10 LV units); b) purified phospholipase C (20 LV units); d) purified phospholipase C (10 LV units).

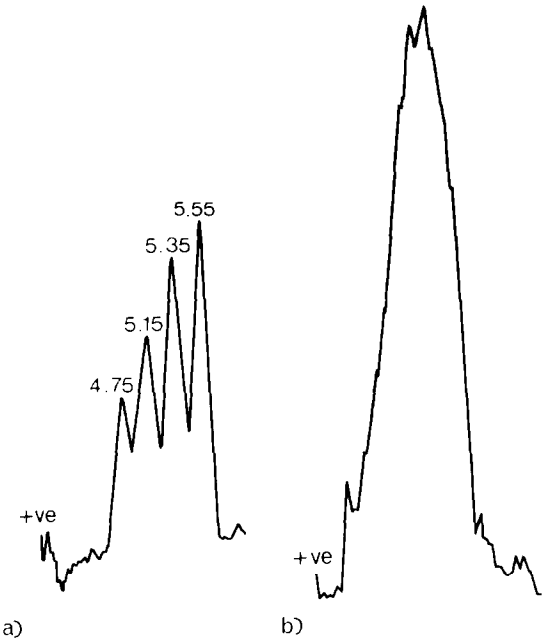

Fig. 3. Electrofocussing of *Cl. perfringens* culture filtrate. Both gels a) and b) contained 7% polyacrylamide, 2% Ampholine® (pH 5–8) and 500 μg culture filtrate; gel b) in addition contained 6 M urea. The gels were electrofocussed for 4 hr [11], washed in water for an hour and embedded in tubes of EEY-agar. When the agar had set, the tubes were incubated at 37°C and the pattern of turbidity produced in the agar was recorded on a microdensitometer. The pI of each peak is shown in the figure and was estimated from the pH gradient determined in duplicate gels.

the latter technique revealed one broad band of activity (fig. 3b).

4. Discussion

The immunosorbent technique described here rapidly and efficiently purified phospholipase C (α-toxin) from crude culture filtrates of *Cl. perfringens*. Such material should be useful for definitive experiments on the role of α-toxin in *Cl. perfringens* infections of muscle [8], and, as a probe with which to explore aspects of the phospholipid structure of membranes. Smyth and Arbuthnott [1] have discussed the origin and significance of the varying number of components observed upon electrofocussing and in ordinary gel electrophoresis. Using the EEY-agar technique to analyse polyacrylamide gels we have demonstrated one broad band of phospholipase activity by electrophoresis and 4 distinct components by electrofocussing (fig. 3a). Electrofocussing in 6 M urea did not produce a single sharply-focussed component as might have been predicted by [1]. Only one diffuse band of activity was observed, which was not sharpened when the period of electrofocussing was increased to 8 hr, (fig. 3b). Perhaps some caution should thus be exercised in accepting the effect of urea on electrofocussing as evidence for interconvertible conformers [1] until more is known about the detailed effect of urea on the production of pH gradients using ampholines and on the proteins themselves. The combined effects could lead to reductions in pI differences such as cause the individual proteins to run together and this could be missed in preparative sucrose-density gradient columns.

Acknowledgements

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