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Note

Fast protein liquid chromatography for the isolation of *Clostridium perfringens* type A α-toxin

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It is well known that *Clostridium perfringens* type A produces in culture medium twelve kinds of exotoxins. α-Toxin is phospholipase C (E.C. 3.1.4.3), which is lethal, necrotic and haemolytic. Several methods for the partial purification of α-toxin have been reported[1–7]. However, all the preparations obtained so far were not homogeneous on the basis of physiological activity and protein chemistry.

In this paper, we describe a purification procedure that involves three steps, one classical anion-exchange and two fast protein liquid chromatographic[8–10], for use in the development of immunological assays. The molecular weight and p*I* of α-toxin have been determined.

EXPERIMENTAL

Fast protein liquid chromatography (FPLC)

A Pharmacia (Uppsala, Sweden) FPLC system was used, consisting of a Mono Q HR 5/5 pre-packed column, two P-500 pumps, a GP 250 gradient programmer, a UV-1 monitor, a Frac 100 collector and a REC-482 recorder.

Electrophoresis

Isoelectric focusing was performed on a flat-bed apparatus (FBE 3000) and polyacrylamide gel electrophoresis on a vertical apparatus (GE 2/4 LS), all from Pharmacia.

Reagents

Clostridium perfringens ATCC 13124 as CCM 5744 and ATCC 8009 as CCM 5872 were kindly supplied by Professor Kocur (Brno, Czechoslovakia).

Brain heart infusion broth was obtained from Biomerieux, France, antigangrene serum from Institut Pasteur, France, and DEAE-trisacryl IBF and gel permeation polymer GF 05 IBF from LKB, France.

An electrophoresis calibration kit for low-molecular-weight proteins and an isoelectric focusing calibration kit for proteins in the range pH 3–10 were supplied by Pharmacia (Uppsala, Sweden).

All reagents were of analytical-reagent grade.

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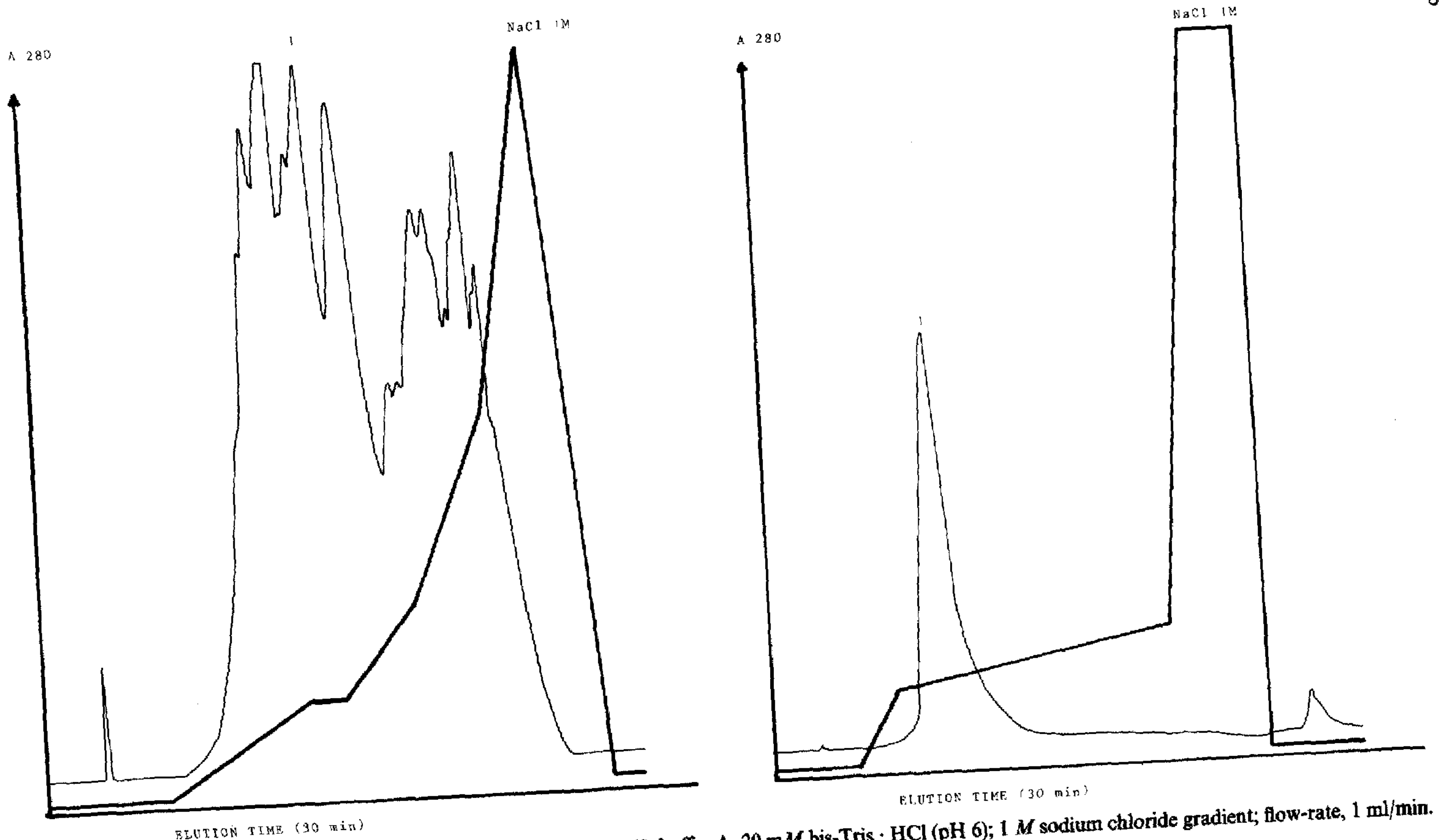

Fig. 1. FPLC trace of 20 mg of crude toxin. Column, Mono Q HR 5/5; buffer A, 20 m*M* bis-Tris · HCl (pH 6); 1 *M* sodium chloride gradient; flow-rate, 1 ml/min. Peak 1 shows a hot cold haemolytic activity (toxin).

Fig. 2. FPLC trace of 20 mg of second-step fractions 1. Chromatographic conditions as in Fig. 1, except the gradient.

Preparation of crude toxin

A 1-l volume of oxygen-free brain heart infusion broth was seeded with 50 ml of a 24-h culture of the bacteria in the same medium and incubated for 6 h at 35°C. The culture fluid was centrifuged at 4000 *g* for 15 min and the supernatant fluid was used as the starting material.

Purification

First step. For anion-exchange chromatography with a DEAE-trisacryl column, the supernatant fluid was diluted 1:5 with 0.025 *M* Tris · HCl buffer (pH 8), then mixed in a batch with 500 ml of gel and the gel was equilibrated in a column (700 × 35 mm I.D.) with the same buffer. Elution was carried out by a linear gradient from 0 to 1 *M* sodium chloride, with a total volume of 1000 ml and at a flow-rate of 80 ml/h. The collected tubes were tested and desalted on Sephadex GF-05 and then freeze-dried.

Second step. For anion-exchange FPLC, buffer A was 20 m*M* bis-Tris · HCl (pH 6), buffer B was 1 *M* sodium chloride and the gradient applied was 0% B for 2 min, 0–12.5% in 8 min, held for 2 min at 12.5%, then 12.5–100% in 10 min. The flow-rate was 1 ml/min and the sample size was 20 mg. The collected tubes were desalted and then freeze-dried.

Third step. This was the same as the second step, except that the gradient applied was 0% B for 2 min, 0–10% in 2 min, 10–17% in 16 min and 17–100% in 1 sec.

Protein determination

Protein was determined by the method of Lowry *et al.*[11] using bovine serum albumin as a standard.

Haemolytic activity

This was determined by loading 0.05 ml of sample into wells bored in a gel containing 3% of sheep red cells and keeping the mixture for several hours at 37°C and subsequently at 4°C.

Haemolytic activity titration was carried out by the method of Möllby *et al.*[12] on a micro titration plate. The titre was expressed in haemolysis units 50% (HU 50%). One haemolytic unit 50% was defined as the amount of haemolysin which gave 50% haemolysis of a 1% sheep red cell suspension.

Polyacrylamide gel electrophoresis (PAGE)

PAGE was performed according to the method of Laemmli[13]. Aliquots (25 μl) of the fraction were dissociated by heat for 4 min then applied on ready-made 4–30% gradient polyacrylamide gels (PAA 4/30) equilibrated in 90 m*M* Tris–borate buffer (pH 8.3). After electrophoresis for 180 min at 125 mV, the gels were fixed and stained with Coomassie Brilliant Blue R 250 according to standard procedures[14].

Isoelectric focusing

Isoelectric focusing was carried out using a 0.5 mm thin layer of agarose gel, following the LKB technique with Pharmalyte pH 3–10 and focusing according to the instructions supplied with the Pharmacia apparatus. The gel after fixation was stained with silver following the method of Goldman and Merril[15].

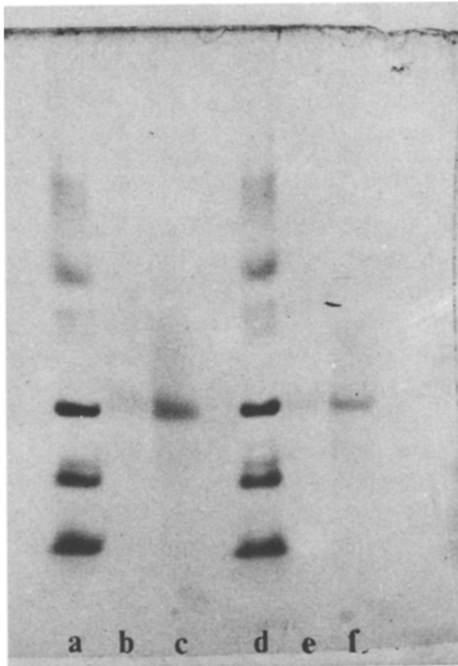

Fig. 3. Polyacrylamide gel analysis. Lanes a and d = calibration kit; b and c = peak 1 of first FPLC (sample amounts, 1 and 3 mg, respectively); e and f = peak of second FPLC (sample amounts 1 and 2 mg, respectively).

Immunological protein test

This was performed by counter-current immunoelectrophoresis (CCIE)[16].

RESULTS AND DISCUSSION

The supernatant fluid contained 11.043 mg of protein and 203 HU 50% per mg.

First purification step

The initial aim was to eliminate peptones contained in the supernatant and classical anion-exchange chromatography allowed maximal use of starting material.

TABLE I

IMMUNOLOGICAL AND PHYSICO-CHEMICAL VALUES OF FRACTIONS OBTAINED AFTER EACH STEP OF PURIFICATION

Fraction	*Protein (mg)*	*Haemolytic activity (HU 50% per mg)*	*Yield (%)*	*Mol. wt.*	*pI*	*CCIE (lines)*
Supernatant	11043	203	100			
One step	475	300				3
Second step	68	495		41000	4.95	1
Third step	48	1089	2.33	41000	4.95	1

Fractions that showed haemolytic activity were eluted between 0.35 and 0.60 *M* sodium chloride and were pooled. The amounts of crude toxin obtained was 475 mg with 300 HU 50% per mg.

Second purification step

Samples of 20 mg can be fractionated on a Mono Q HR 5/5 column. The pool of peaks 1 had a haemolytic activity of 495 HU 50% per mg (Fig. 1). It was tested by CCIE, PAGE and isoelectric focusing and gave one line.

Third purification step

The chromatogram showed only one peak (Fig. 2). The purity of 48 mg of protein with 1089 HU 50% per mg was measured by PAGE and isoelectric focusing, which showed the α-toxin to be free from several impurities. Its p*I* was 4.95 $\pm$ 0.1 and its molecular weight was 41,000 (Fig. 3).

All results are reported in Table I and were similar to those obtained by other workers[3,5,7].

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