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Note

Fast protein purification of *Clostridium difficile* cytotoxin

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Clostridium difficile produces two toxins, an enterotoxin or toxin A and a cytotoxin or toxin B [1, 2], both of which are implicated in the etiology of antibiotic-associated colitis. This disease occurs after various antibiotic treatments and its incidence ranges from 10 to 20%. The cytotoxin or toxin B at very low concentrations has the property of rounding off all cultured cell lines through disorganization of the intermediate filaments [3].

This paper reports a fast method for the purification of *C. difficile* toxin B.

EXPERIMENTAL

Bacterial culture and cytotoxin production

C. difficile (strain No. 79685) was isolated in our laboratory from stools obtained from a patient presenting pseudomembranous colitis. This strain was grown in three 5-l flasks containing brain–heart infusion (Institut Pasteur Production, Paris, France) or synthetic basal medium according to Haslam et al. [4], supplemented with haemin (1 mg/l). After anaerobic incubation at 37°C for 72 h, the culture was filtered using a tangential flow system with an HVLP microporous filter (Millipore, St. Quentin, France) using a filter area of 1.40 m^2 and a recirculation rate of 860 ml/min·m^2. The filtrate was then ultrafiltered with the same system [PTHK filter, nominal relative molecular mass (M_r) limit of 100 000; Millipore] using a filter area of 1.40 m^2 and a recirculation rate of 1200 ml/min·m^2. During this step the retentate was equilibrated in 10 m*M* Tris–HCl buffer (pH 7.4).

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Enterotoxic and cytotoxic assays

Enterotoxic activity was determined by fluid accumulation by the isolated rabbit ileal loop assay according to the method described by Pierce and Wallace [5]. Cytotoxic activity was determined by the cytopathic effect, i.e., total rounding off of MacCoy cells. After trypsinization of monolayers from 75-cm^2 flasks, the cells were distributed into the 96 wells (5 mm diameter) of titration plates (Costar, Cambridge, MA, U.S.A.). Samples were introduced directly into each well (20 μl per well) after serial dilution in minimal eagle medium (MEM) (Intermed, Strasbourg, France). After exposure to the toxin for 24 h, each well was examined for cytotoxicity. The reciprocal of the highest dilution resulting in complete rounding off of the cells was defined as the number of cytotoxic units (CU) per 200 μl of sample. Specific activity was expressed as CU per milligram of protein. Protein concentrations were determined according to Bradford's method [6].

Analytical electrophoresis

Electrophoretic separations were performed on Phastgel IEF (pH range 3–10) and Phastgel gradient media (10–15 and 8–25% acrylamide) using a Phastsystem apparatus (Pharmacia, Uppsala, Sweden). Silver or Coomassie blue staining was performed as recommended by the manufacturer.

Chromatography

Chromatography was performed with a fast protein liquid chromatography apparatus (Pharmacia). This apparatus consists of two P-500 pumps, an LCC-500 gradient programmer, a UV-1 and a UV-2 monitor, a FRAC-100 fraction collector and an REC-482 recorder. Anion-exchange chromatography was performed on a Mono Q HR 10/10 column (Pharmacia). Sample injection, elution, collection and column regeneration were automated. During each analysis, 220 mg of proteins from retentate fractions were applied with a 50-ml injection loop. Fractions containing cytotoxic activity obtained from Mono Q analyses were pooled and incubated with 50 μg/ml ribonuclease (RNase) type III-A (Sigma, St. Louis, MO, U.S.A.) at 37°C for 30 min. After dialysis at 4°C against Milli-Q water (Millipore) and lyophilization, toxic fractions were resuspended in pH 5.2 buffer [50 m*M* sodium acetate–0.2 *M* sodium chloride–1 m*M* $ZnSO_4$–0.5% (v/v) glycerol] and incubated for 30 min at 37°C with three nucleases: nuclease P_1 from *Penicillium citrinum*, 160 U/ml final concentration (PL Biochemicals, Milwaukee, WI, U.S.A.), nuclease S_1 from *Aspergillus oryzae*, 2000 U/ml final concentration (Appligène, Strasbourg, France), and ribonuclease T_2 from *Aspergillus oryzae*, 5 U/ml final concentration (Serva, Heidelberg, F.R.G.). Amounts of 200 μg of RNase-treated fractions were then chromatographed on a Polyanion SI HR 5/5 column (Pharmacia) to purify toxin B further.

RESULTS

Protein concentrations, total activities and specific activities obtained after each step are given in Table I.

Tangential flow filtration of bacterial cultures allowed the bacteria to be sep-

TABLE I

PURIFICATION OF TOXIN B

Purification step	Protein (μg/ml)	Activity (CU/ml)	Total cytotoxic units (CU)	Specific activity (CU/μg)	Percentage of starting material*	Purification**
Cell-free filtrate	8000	4000	$60 \cdot 10^5$	0.5	100.0	1.0
Ultrafiltration	3000	5000	$58 \cdot 10^5$	1.7	96.6	3.3
First anion-exchange chromatography	126	10000	$38 \cdot 10^5$	79.4	63.3	158.8
Second anion-exchange chromatography	56	2000	$12 \cdot 10^5$	35.7	20.0	71.4

*Percentage of starting material is based on total CU in the purification step/total CU in starting material $\times$ 100.

**Purification is based on specific activity in the purification step/specific activity of starting material.

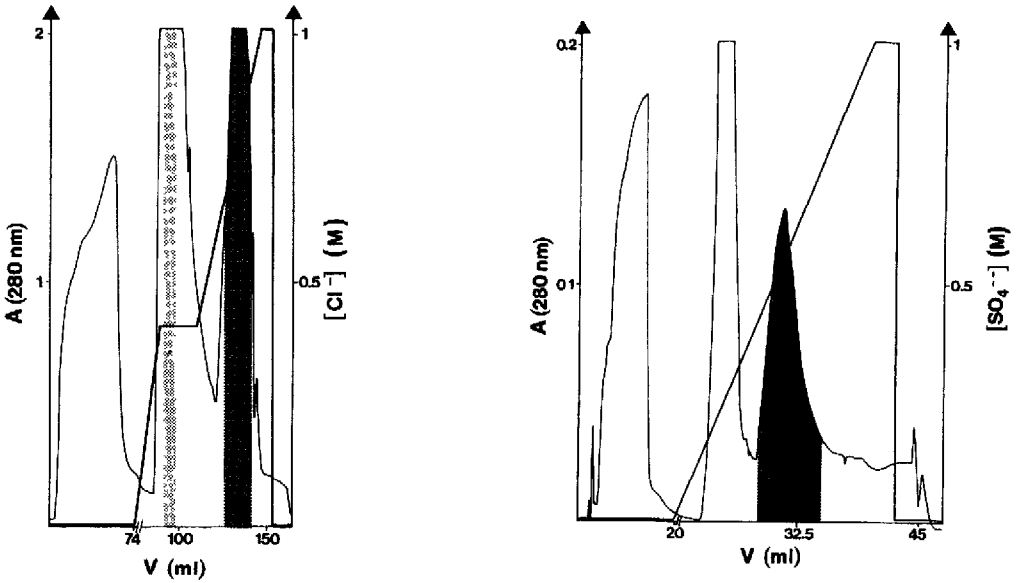

Fig. 1. First anion-exchange chromatography. Buffer A, 10 m*M* Tris-HCl (pH 7.4); buffer B, buffer A + 1 *M* sodium chloride, discontinuous gradient from 0 to 1 *M* sodium chloride; flow-rate, 4.0 ml/min; sample, 220 mg of retentate fraction protein; detection at 280 nm (2 absorbance units full scale). *V* = Elution volume; grey area = enterotoxic fractions according to ileal rabbit loop assay; black area = cytotoxic fractions according to MacCoy cell test.

Fig. 2. Second anion-exchange chromatography. Buffer A, 10 m*M* Tris-HCl (pH 7.4)-1 m*M* β-mercaptoethanol; buffer B, buffer A + 1 *M* magnesium sulphate, continuous gradient from 0 to 1 *M* magnesium sulphate; flow-rate, 0.5 ml/min; sample, 200 μg of nuclease-treated cytotoxic fractions diluted four-fold with buffer A, detection at 280 nm (0.2 absorbance units full scale). *V* = Elution volume; black area = cytotoxic fractions (CU > 10^3).

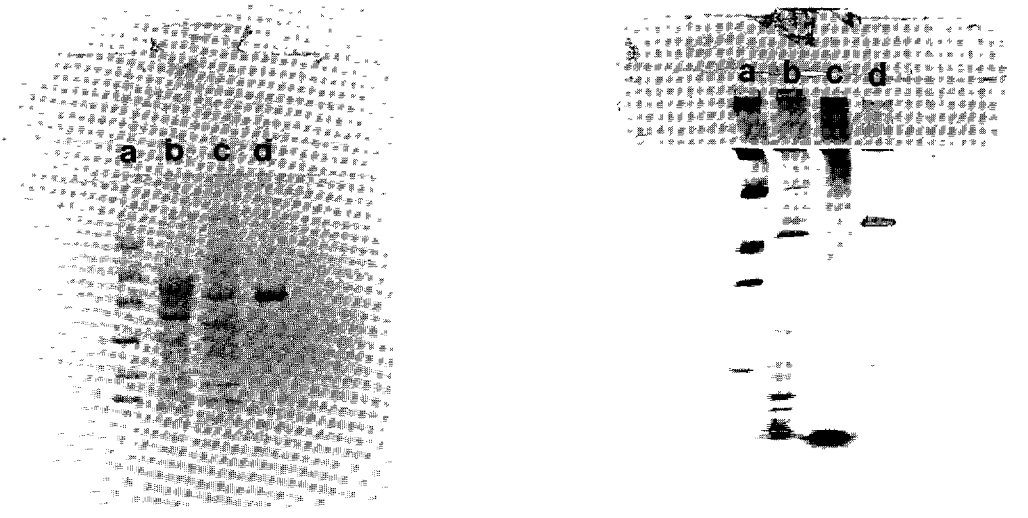

Fig. 3. SDS-PAGE. Proteins separated on Phastgel 10–15 (10–15% acrylamide) at 70 V h and 3 W. The samples were reduced with SDS to 2.5% and β-mercaptoethanol to 5%. The gel was stained with Coomassie blue. Samples were deposited starting from the left; lane a, molecular mass calibration proteins (2.9 μg), phosphorylase (M_r=94 000), bovine serum albumin (M_r=67 000), ovalbumin (M_r=43 000), carbonic anhydrase (M_r=30 000), soybean trypsin inhibitor (M_r=21 000), α-lactalbumin (M_r=14 400); lane b, fraction obtained after ultrafiltration (3.0 μg); lane c, cytotoxic fraction after the first ion-exchange chromatography (2.0 μg); lane d, cytotoxic fraction after the second ion-exchange chromatography (1.5 μg).

Fig. 4. Native PAGE. Proteins were separated on Phastgel 8–25 (8–25% acrylamide) at 280 V h and 2.5 W; the gel was stained with silver. Samples were deposited starting from the left; lane a, protein test mixture (2.5 μg), thyroglobulin (M_r=669 000), ferritin (M_r=440 000), catalase (M_r=232 000), lactate dehydrogenase (M_r=140 000), bovine serum albumin (M_r=67 000); lane b, fraction obtained after ultrafiltration (2.0 μg); lane c, fraction obtained after the first ion-exchange chromatography (1.0 μg); lane d, fraction obtained after the second ion-exchange chromatography (1.0 μg).

arated from the culture medium in 10 min. The filtrate was then concentrated more than 19-fold by ultrafiltration in 30 min. During this step the toxic fraction was equilibrated with the first ion-exchange buffer. Only 3% of the total toxic activity was lost during this step. The first anion-exchange chromatography allowed a good separation of the enterotoxic and cytotoxic fractions in 45 min. This separation was checked by the ileal rabbit loop assay and the MacCoy cell test. As described previously [7], the enterotoxic fractions were eluted with 0.4 *M* sodium chloride and cytotoxic fractions between 0.67 and 0.91 *M* sodium chloride; the plateau with 0.4 *M* sodium chloride improved the separation of both activities (Fig. 1). Cytotoxic fractions obtained after this step had a higher absorbance at 260 than at 280 nm, which suggests that nucleic acids were eluted with the toxic protein. In order to confirm this, toxic fractions were treated with different ribonucleases and desoxyribonucleases. Only ribonucleases caused a decrease of absorbance at 260 nm, which confirms the presence of RNA-bound toxin B.

Ribonuclease-treated fractions were then injected on to a second anion-ex-

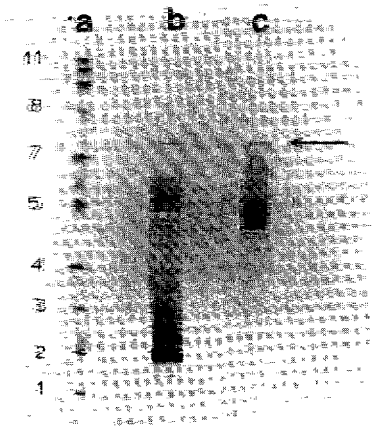

Fig. 5. Isoelectrofocusing. A gradient from pH 3 to 9 was performed with Phastgel IEF 3–9 at 500 V h and 3.5 W. Samples: lane a, protein test mixture (2 μg), from bottom to top, 1 = amyloglucosidase (3.50); 2 = soybean trypsin inhibitor (4.55); 3 = β-lactoglobulin A (5.20); 4 = bovine carbonic anhydrase B (5.85); 5 = human carbonic anhydrase B (6.55); 6 = horse myoglobulin (6.85); 7 = horse myoglobulin (7.35); 8 = lentil lectin (8.15); 9 = lentil lectin (8.45), 10 = lentil lectin (8.65); 11 = trypsinogen (9.30); values in parentheses indicate p*I* of standard proteins; lane b, fraction obtained after ultrafiltration (5 μg); lane c, toxic fraction after the second ion-exchange chromatography (5 μg). The arrow on the right indicates the point of deposition of samples.

change column and chromatographed in 90 min. The purified toxin eluted with 0.6 *M* magnesium sulphate as a single protein peak (Fig. 2). The other peaks corresponded to nucleotides with few protein contaminants, as shown by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Under denaturing conditions, toxin B revealed a single protein band with $M_r = 52\,200 \pm 1500$ (Fig. 3). Under native conditions with gradient gel PAGE according to the method of Margolis and Wrigley [8], the M_r of toxin B was calculated to be $290\,000 \pm 15\,000$ (Fig. 4). Isoelectrofocusing (IEF) showed at least three protein bands between pH 6.25 and 6.45, indicating microheterogeneity of the purified toxin B (Fig. 5).

DISCUSSION

The purification scheme included ultrafiltration, ammonium sulphate precipitation, gel permeation and anion-exchange chromatography [9–11]. M_r values of native protein range between 130 000 and 600 000 according to different authors [1, 2, 12].

Using native gradient gel PAGE we obtained $M_r = 290\,000 \pm 15\,000$, compatible with a hexameric structure of toxin B. The large differences reported for the M_r values of proteins in the literature could correspond to multiple associations of a single subunit. Moreover, only Rolfe and Finegold [13] and Pothoulakis et al. [14] obtained a subunit of about 50 000 using SDS-PAGE. We have also obtained the same result [3, 15] using a different purification procedure. The other workers did not use such a denaturing electrophoresis procedure and hence were not

able to obtain evidence of subunits. Lyerly et al. [16] detected a major band at $M_r = 300\,000$ and numerous faster migrating minor bands, as described previously by Rothman et al. [17]. The ability of some *C. difficile* strains to produce proteases might modify the size of the molecule secreted by the bacteria. As Pothoulakis et al. [14] did not determine the M_r values in native gels, a comparison with our results is not possible.

IEF of purified toxin, without any RNA contamination, indicated three isotoxins with p*I* values of 6.25, 6.40 and 6.45, respectively. These values can be compared with those obtained by Thelestam and Brönnegard [18], who purified toxin B by preparative IEF only. The presence of RNA bound to toxin B has not been previously reported. This RNA is not removed by conventional physicochemical methods (ammonium sulphate precipitation and chromatography with buffers containing 8 *M* urea). Only enzymatic degradation allowed an RNA-free protein to be obtained but, as shown in Table I, this treatment decreased the specific activity (by 55%) of the final toxin preparation, perhaps because RNA has a protective role for toxin B.

To rule out the possibility of RNA provided by the culture medium, we cultured *C. difficile* in a synthetic medium. Under these conditions, clostridial RNA was still found associated with the partially purified toxin. Florin and Thelestam [19] reported that the action of toxin B was delayed after toxin pre-incubation with ATP and polyphosphates. Their toxin B preparation was able to bind nucleotides in addition to polyphosphates. The rapidity of our purification process could explain why toxin B was eluted with endogenous bacterial RNA. However, further characterization of this RNA is still in progress. The role, if any, of the toxic activity is also being investigated.

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