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Fast purification of *Clostridium sordellii* cytotoxin

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SUMMARY

We have developed a rapid method for the purification of proteins, combining titration curve analysis with a two-step column chromatographic procedure. We have used this approach to purify the cytotoxin (L toxin) from *Clostridium sordellii*. We have also determined the amino acid composition of this cytotoxin. This toxin has a p*I* value of 4.20 and an M_r of 260 000, reduction of which results in a band of M_r 43 000 on sodium dodecyl sulphate polyacrylamide gel electrophoresis. Since both the proteins of M_r 260 000 and 43 000 are recognized by the polyclonal anti-*C sordellii* L toxin, which neutralizes the L toxin cytotoxicity, we propose a hexameric structure for the protein of M_r 260 000, each subunit being M_r 43 000.

INTRODUCTION

Clostridium sordellii is one of the six *Clostridium* species capable of producing gas gangrene in humans [1]. This bacterium is also responsible for enteritis and enterotoxemia in cattle [2] and sheep [3]. *C. sordellii* was suspected to be the causal agent of pseudo-membranous colitis (PMC) in humans because cytotoxic extracts from stool filtrates of PMC patients were neutralized by *C. sordellii* antitoxins [4]. However, *C. sordellii* was never identified in stool filtrates from these PMC patients but *C. difficile* was present. In fact, *C. difficile* is the causal agent of PMC in humans [5,6] and there is a cross-neutralization of *C. difficile* toxins by *C. sordellii* antitoxins [7].

Two toxic proteins, A and B, have been isolated from culture supernatants of toxigenic strains of *C. difficile* [8–12]. Both toxins are cytotoxic but the B toxin is 1000-fold more cytotoxic that the A toxin, which is an enterotoxin that

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causes fluid accumulation and haemorrhage of the small intestine and caecum of animals [13–15]. Two toxic proteins, H and L, were also purified from *C. sordellii* strains [16]. The haemorrhagic (H) toxin is an enterotoxin that has physicochemical characteristics and biological activities similar to those of *C. difficile* toxin A [17]. The lethal (L) toxin is more cytotoxic and lethal than the haemorrhagic toxin and shows cross-reactivity with *C. difficile* B antitoxin [18]. The L toxin from *C. sordellii* has been purified previously in four steps: ammonium sulphate precipitation, anion-exchange chromatography, gel permeation and finally hydroxyapatite chromatography [18].

This paper describes a fast procedure for the purification of L toxin, based on electrophoretic titration curve analysis. Some physicochemical characteristics of this toxin are also presented.

EXPERIMENTAL

Bacterial strains, growth and toxin production

C. sordellii 6018 (Tox^+) was isolated from a patient in our clinical laboratory. The culture was grown anaerobically in 5 l of brain–heart infusion (Difco) for 60 h at 37°C. The culture was then filtered through HVLP (0.45 μm) and ultrafiltered through PTHK (molecular mass limit of 30 000) membranes (Millipore, St. Quentin, Yvelines, France) to eliminate bacteria and small proteins, respectively, and then equilibrated in 50 m*M* Tris–HCl (pH 7.6) [11].

Cytotoxicity and enterotoxicity tests

Cytotoxicity tests were performed on MacCoy cells [11]. The reciprocal of the highest dilution resulting in complete rounding of the MacCoy cells was defined as the number of cytotoxic units (C.U.) per 200-μl sample. Specific activity was expressed as C.U. per milligram of protein. Protein concentrations were determined using Bradford's method [19], and the nucleic acid concentrations were determined using the method of Warburg and Christian [20].

Chromatography

Chromatography was performed using a fast protein liquid chromatographic (FPLC) system (Pharmacia, Uppsala, Sweden) as described by Rihn et al. [11]. Anion-exchange chromatography was carried out using a Mono Q HR 10/10 column (100 mm × 10 mm I.D.) or a Mono Q HR 5/5 column (50 mm × 5 mm I.D.) (Pharmacia). After elution, collected fractions were desalted at a flow-rate of 6 ml/min on a fast desalting column HR 10/10 (100 mm × 10 mm I.D., Pharmacia), which was equilibrated with 50 m*M* Tris–HCl (pH 7.4) buffer.

Electrophoresis and isoelectric focusing

Native polyacrylamide gel electrophoresis (native PAGE), sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF) were performed using the Phast system according to the manufacturer's instructions (Pharmacia). Native PAGE was carried out on a PhastGel gradient 8–25, SDS-PAGE on a PhastGel gradient 10–15 and IEF on a PhastGel IEF 3–9. For SDS-PAGE analysis, samples were first reduced by boiling in 2.5% SDS and 5% β-mercaptoethanol for 15 min. All gels were silver-stained.

Electrophoretic titration curve analysis

A PhastGel IEF 3–9 was used to establish a pH gradient from 3.0 to 9.0 at 150 Vh and 3.5 W. The gel was then turned through 90°, and the protein sample was applied along the mid-line of the gel. A second electrophoresis separated proteins by their electrophoretic mobilities in the pH range 3.0–9.0. The gel was silver-stained according to Pharmacia's user manual. Under these conditions, each protein has a specific migration profile, which we call a titration curve.

Protein extraction from PhastGel gradient 8–25 after native PAGE

Two native PAGE, PhastGel gradient 8–25, were run in parallel using the Phast system. The cytotoxic fraction obtained after the second ion-exchange chromatography was applied to both gels. After migration, one gel was silver-stained and compared with the unstained gel in order to locate the protein band. The entire gel was cut into several bands, ensuring that the protein band revealed on the stained gel remained intact. Each gel band was passed through a 1-ml syringe to break the polyacrylamide gel, and 1 ml of 50 m*M* Tris–HCl (pH 7.5) was added to each sample and left overnight at 4°C. After centrifugation at 300 *g*, each supernatant was applied to an ultrafree-MC filter (Millipore), which retains proteins larger than M_r 10 000. The filters were then centrifuged at 2000 *g* with 2 ml of water to eliminate traces of acrylic acid. Each supernatant was then used for a cytotoxicity test.

Amino acid composition

The amino acid composition determination was performed on a high-performance liquid chromatography (HPLC) apparatus (Waters Assoc., Milford, MA, U.S.A.) after hydrolysis of the purified L toxin in 6 *M* hydrochloric acid at 110°C for 20 h [9].

Molecular mass estimation

Molecular mass was estimated by FPLC on a Superose 12 column (300 mm × 10 mm I.D., Pharmacia). After toxin elution, the molecular mass of the

L toxin was estimated by comparison of its retention time with those of proteins of known molecular mass.

Preparation of polyclonal L toxin antiserum

L toxin was inactivated by dialysis overnight in phosphate-buffered saline (PBS) (Biochrom K.G., Berlin, F.R.G.)–0.4% formaldehyde. A 20-μg amount of this inactivated L toxin with complete Freund adjuvant was injected into rabbits. A booster of 10 μg of protein was given every week for six weeks. Three days after the last injection, 20 ml of blood were collected and the polyclonal antiserum was obtained by centrifugation.

Immunoblotting test

Ultrafiltered supernatant of *C. sordellii* culture was used to perform native PAGE on a PhastGel gradient 8–25 and SDS-PAGE on a PhastGel gradient 10–15 using the Phast system. Proteins from each were then electrotransferred to BA 85 nitrocellulose sheets (Schleicher and Schuell, Dassel, F.R.G.) in a buffer consisting of 192 m*M* glycine and 25 m*M* Tris–HCl (pH 8.4)–methanol (4:1, v/v) at 60 V for 1 h. Nitrocellulose sheets were then saturated with 5% bovine serum albumin (BSA) in PBS, washed twice with 1% BSA in PBS and incubated with polyclonal L toxin antiserum (1:100) at 37°C for 2 h. After two washes with 1% BSA in PBS, the nitrocellulose sheets were incubated with peroxidase-conjugated anti-rabbit antibodies at 10 μg/ml at 37°C for 1 h. After two washes with PBS, a solution of 4-chloro-1-naphthol at 0.5 mg/ml in 0.3% hydrogen peroxide was used to reveal the proteins.

Cytotoxic neutralization test

A 10-μl volume of L cytotoxic solution, corresponding to 5 C.U., was incubated with 10 μl of different concentrations of polyclonal L toxin antiserum for 20 min at 20°C. The solution was deposited in a well containing 50 000 MacCoy cells and incubated overnight [11]. Next day, neutralization was considered to have occurred when the cells did not round. The 1:32 dilution of polyclonal antiserum was the highest dilution that neutralized cytotoxic activity. Therefore the titre of polyclonal antiserum was 16 000 antitoxic units per ml.

RESULTS

Purification of the L toxin

The L toxin has been previously described as an acidic protein [18] and it was therefore possible to purify it on a Mono Q HR 10/10 column, which is a strong anion exchanger charged with quaternary amine groups. The first step of purification was chromatography of an ultrafiltered solution of *C. sordellii* culture on a Mono Q column at pH 7.5. After elution, the collected fractions

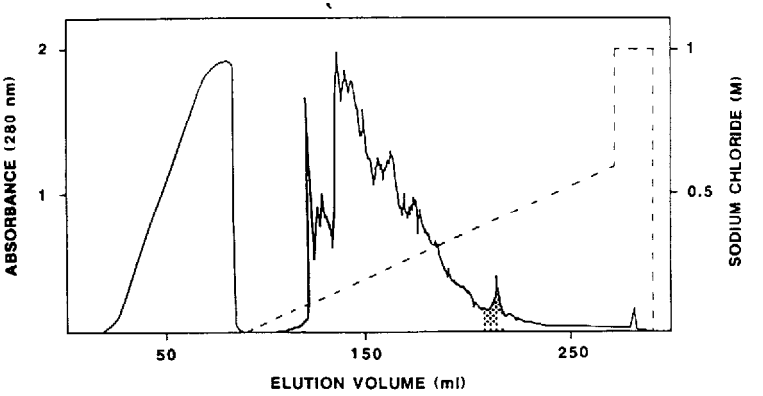

Fig. 1. First ion-exchange chromatography: 180 mg of ultrafiltered protein in solution were applied to the Mono Q HR 10/10 column Starting buffer was 50 m*M* Tris-HCl (pH 7.4), elution buffer was 50 m*M* Tris-HCl-1 *M* sodium chloride (pH 7.4). The gradient was continuous from 0 to 0.6 *M* sodium chloride for 45 min at a flow-rate of 4.0 ml/min. The shaded area represents the cytotoxic elution volume determined by the MacCoy cell test.

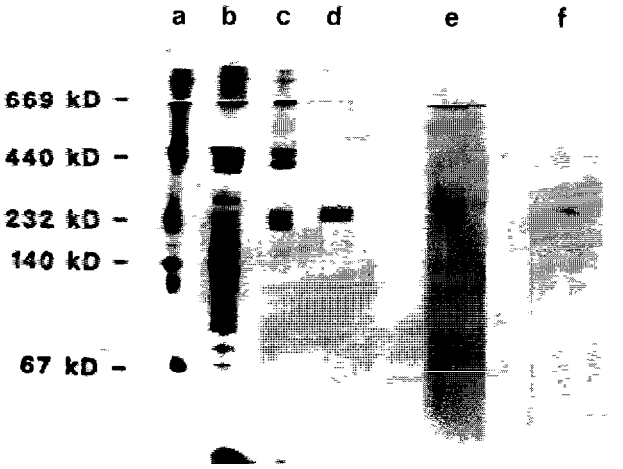

Fig. 2. Silver-stained native PAGE and immunoblotting. Proteins were separated on PhastGel gradient 8-25 at 280 Vh and 2.5 W. Lane a, molecular mass protein standards (3 μg); lane b, fraction obtained after ultrafiltration (8 μg); lane c, cytotoxic fraction obtained after the first ion-exchange chromatography (3 μg); lane d, cytotoxic fraction obtained after the second ion-exchange chromatography (2 μg); lane e, cytotoxic supernatant obtained after protein extraction from native PAGE (1 μg); lane f, immunoblot corresponding to native PAGE performed with a crude fraction obtained after ultrafiltration (5 μg).

were tested on MacCoy cells for the presence of L toxin. Fig. 1 shows that the L toxin was eluted at between 0.39 and 0.42 *M* sodium chloride. Fractions with cytotoxic activity were desalted and used for electrophoretic analysis. On native PAGE (Fig. 2, lane c) several bands are present, with a major band of M_r

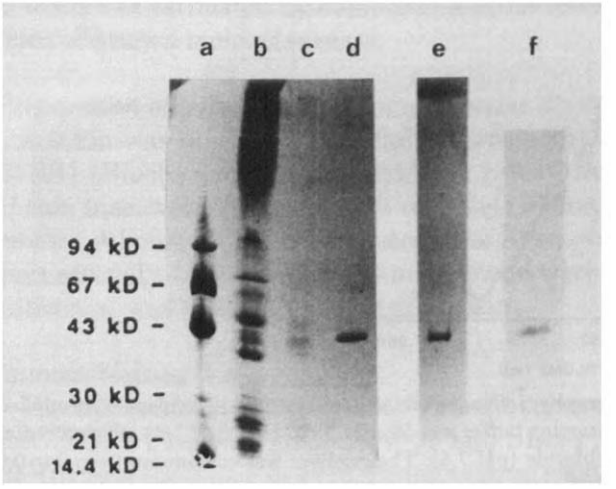

Fig. 3. Silver-stained SDS-PAGE and immunoblotting. Proteins were separated on PhastGel gradient 10–15 at 70 Vh and 3 W. Lane a, molecular mass protein standards (3 μg); lane b, fraction obtained after ultrafiltration (2 μg); lane c, cytotoxic fraction obtained after the first ion-exchange chromatography (0.5 μg); lane d, cytotoxic fraction obtained after the second ion-exchange chromatography (1.5 μg); lane e, cytotoxic supernatant obtained after protein extraction from native PAGE (1 μg); lane f, immunoblot corresponding to SDS-PAGE performed with fraction obtained after ultrafiltration (2 μg).

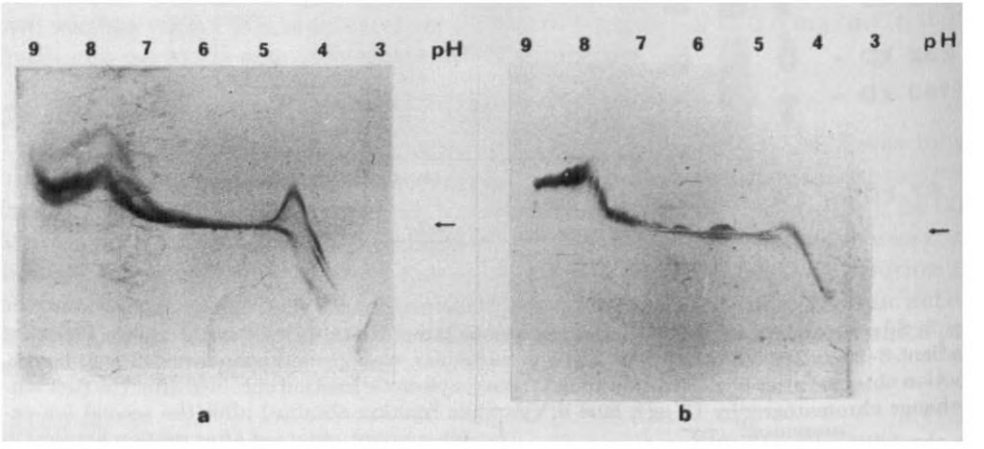

Fig. 4. Electrophoretic titration curve analysis. The arrow indicates the line of deposition of the sample. Proteins were separated at 60 Vh and 0.2 W. (a) Fraction obtained after the first ion-exchange chromatography (10 μg). (b) Fraction obtained after the second ion-exchange chromatography (5 μg)

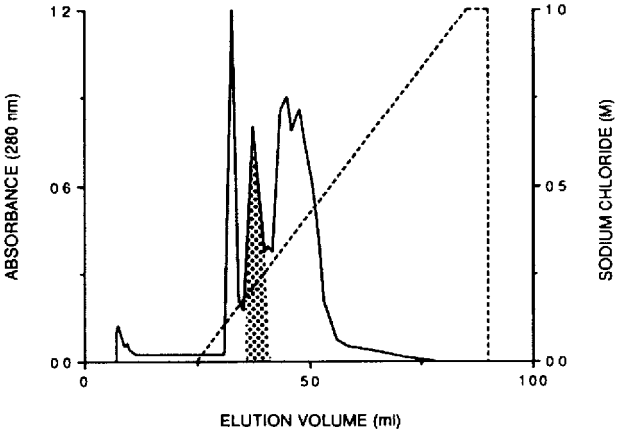

Fig. 5. Second ion-exchange chromatography: 20 mg of cytotoxic fraction protein from the first ion-exchange chromatography were applied to the Mono Q HR 5/5 column. Starting buffer was 50 m*M* sodium acetate (pH 4.55), elution buffer was 50 m*M* sodium acetate–1 *M* sodium chloride (pH 4.55). The gradient was continuous from 0 to 1 *M* sodium chloride for 30 min at a flow-rate of 2.0 ml/min. Just after elution, each fraction was adjusted to pH 7.00 with 3 *M* Tris base. The shaded area represents cytotoxic elution volume determined by the MacCoy cell test

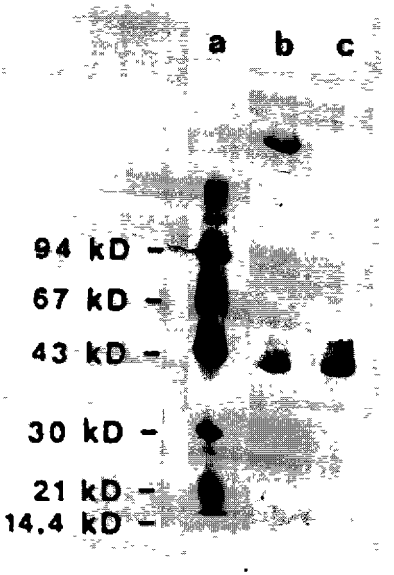

Fig. 6. SDS-PAGE with and without boiling. Proteins were separated on PhastGel gradient 10–15 at 70 Vh and 3 W. Lane a, molecular mass protein standards (3 μg); lane b, unboiled fraction (1.5 μg) obtained after the second ion-exchange chromatography mixed with 2.5% SDS and 5% β-mercaptoethanol; lane c, the same sample as lane b, but boiled for 15 min (1.5 μg).

TABLE I

PURIFICATION OF L TOXIN

Purification step	Total protein (mg)	Volume (ml)	Protein (μg/ml)	Activity (C.U./ml)	Total cytotoxic units (C.U.) ($\times 10^6$)	Specific activity (C.U./μg)	Percentage of starting material[a]	Purification[b] (fold)	Percentage of nucleic acids[c]
Cell-free filtrate	33 600	4800	7000	3000	14.4	0.4	100.0	1	14.5
Ultrafiltration	1790	1120	1600	12 500	14.0	8.0	97.2	18	12.6
First ion-exchange chromatography	66	225	294	42 000	9.4	143.0	65 3	332	4 6
Second ion-exchange chromatography	10	50	205	50 000	2.5	244.0	17 0	570	0 8

[a]Percentage of starting material is based on the total C.U. in the purification step divided by the total C.U. in starting material, multiplied by 100.
[b]Purification is based on the specific activity in the purification step divided by the specific activity of starting material.
[c]Determined by measuring the ratio $A_{260\,nm}/A_{280\,nm}$ [20].

TABLE II

AMINO ACID COMPOSITION OF L TOXIN

Amino acid	Percentage	
	This study	Popoff [18]
Aspartic acid[a]	8.37	13.45
Glutamic acid[b]	10.82	10.33
Proline	4.19	2.94
Glycine	9.87	8.35
Serine	5.81	6.06
Threonine	6.79	5.92
Alanine	9.45	7.39
Lysine	7.56	6.93
Arginine	4 07	2.75
Histidine	1.08	1.78
Isoleucine	7.67	7.88
Leucine	7.00	6.70
Methionine	1 11	2.57
Valine	7 50	6.15
Phenylalanine	5.02	4.96
Tyrosine	3 57	4.50
Tryptophan	N.D.[c]	N.D.[c]
Cysteine	N.D.[c]	1.32

[a]Includes aspartic acid and asparagine.
[b]Includes glutamic acid and glutamine.
[c]N D. = not determined.

240 000. On SDS-PAGE there are also several bands with a major band of M_r 43 000 (Fig. 3, lane c).

An electrophoretic titration curve analysis was performed to determine the optimum pH for the separation of the L toxin from contaminating proteins. This method uses a pH gradient of 3 to 9 to separate the proteins depending on their electrophoretic mobilities in this gradient. On the gel (Fig. 4a) at least three different titration curves are present, suggesting contamination with other proteins after the Mono Q chromatography step. As shown in Fig. 4a, at pH 5.0–7.5 all the proteins have the same electrophoretic behaviour, but below pH 5.0 and above pH 7.5 the titration curves are different. Therefore, we tried chromatography on a Mono Q HR 5/5 column with buffers at pH 4.55. Fig. 5 shows this second step of purification: the L toxin was eluted between 0.15 and 0.25 *M* sodium chloride. After desalting, fractions containing the L toxin were subjected to electrophoretic analyses. On native PAGE (Fig. 2, lane d) a single band is present at M_r 240 000, likewise, one band is present on SDS-PAGE at M_r 43 000 (Fig. 3, lane d). A cytotoxicity test performed with the protein of M_r 43 000 was negative. SDS-PAGE was also performed with L toxin mixed

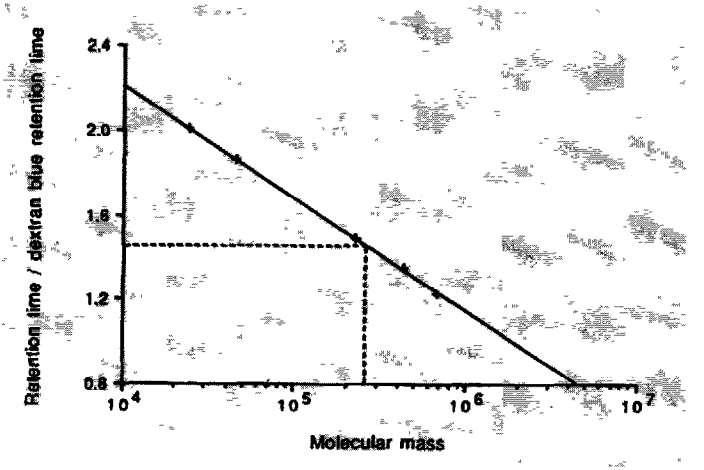

Fig. 7. Molecular mass estimation of L toxin. The calibration proteins of known molecular mass were chymotrypsinogen A (M_r 25 000), ovalbumin (M_r 45 000), catalase (M_r 232 000), apoferritin (M_r 440 000) and thyroglobulin (M_r 669 000). Retention times of these standard proteins and L cytotoxin on a Superose 12 HR 10/10 column were used to calculate the ratio of retention time to dextran blue retention time.

with 2.5% SDS and 5% β-mercaptoethanol but without heating. When the sample was not heated, two bands were present on the gel: one of M_r 260 000, corresponding to the unreduced L toxin, and one of M_r 43 000, corresponding to the reduced L toxin (Fig. 6, lane b). Yet, when the sample was boiled for 15 min, a single band of M_r 43 000 was detected (Fig. 6, lane c). The absence of contaminants after the second ion-exchange chromatography step was confirmed with a new titration curve, a single curve being present on the gel (Fig. 4b). Extraction of protein from native PAGE was carried out as described in Experimental. The supernatants corresponding to the different bands from native-PAGE were tested for cytotoxicity. Only one supernatant, corresponding to the band at M_r 240 000, was cytotoxic. Moreover, native PAGE and SDS-PAGE performed with this supernatant showed one band at M_r 240 000 (Fig. 2, lane e) and one at M_r 43 000 (Fig. 3, lane e), respectively. Immunoblotting tests were performed as described in Experimental with polyclonal L toxin antiserum, which neutralizes the cytotoxic activity of L toxin. In the fraction obtained by ultrafiltration, only one band was detected on the nitrocellulose sheet, after transfer from both PAGE and SDS-PAGE, at M_r 240 000 (Fig. 2, lane f) and M_r 43 000 (Fig. 3, lane f), respectively. Protein concentrations, total activities and specific activities obtained after each step of purification are presented in Table I; the percentage of nucleic acid present at each stage of purification is also noted.

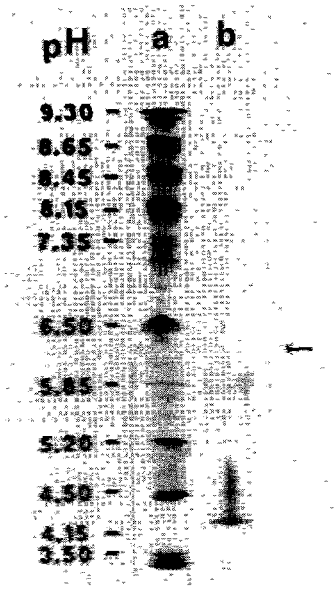

Fig. 8. Isoelectric focusing. A pH gradient from 3.0 to 9.0 was set up with a Phast IEF Gel at 500 Vh and 3.5 W. The arrow indicates the point of deposition of the samples. Lane a, p*I* protein standards (2 μg); lane b, cytotoxic fraction obtained after the second ion-exchange chromatography (1 μg).

L toxin characteristics

The amino acid composition of L toxin was determined and is presented in Table II. The M_r of L toxin was estimated by comparison with the reference curve (Fig. 7). The ratio of the retention time of the L toxin to that of dextran blue corresponds to an M_r of 260 000. On native PAGE, the M_r of this protein was estimated at 240 000, since migration depends on two parameters: molecular mass and charge.

IEF was performed with the purified toxin (Fig. 8). The isoelectric point (p*I*) of L toxin was determined as 4.20.

DISCUSSION

The new, faster purification procedure for *C. sordellii* L toxin described here consists of two steps, compared with four steps in the previously described procedure [18]. The new method employs a titration curve to determine the purification strategy for the L toxin after the first ion-exchange chromatography step. Proteins are electrophoresed through a pH gradient. From the curves obtained one can select the pH to be used for the greatest separation of the required protein from contaminating proteins. Moreover, the complexity of the titration curve explains why several attempts to purify the L toxin in

the pH range 5.0–7.5 (e.g. hydrophobic interaction chromatography or weak anion-exchange chromatography) failed, since at pH 5.0–7.5 all the proteins present have the same electrophoretic behaviour (Fig. 4a). The titration curve is followed by chromatography at the determined pH (here pH 4..55), and is then followed by a second titration curve to check the purity (Fig. 4b). Thus, the L toxin can be purified in only two steps and in only five days, culture included, without the need for dialysis or ammonium sulphate precipitation. The chromatographic steps alone are achieved in half a day.

The amino acid composition of the L toxin of *C. sordellii* determined here (Table II) closely resembles that determined by Popoff [18] with one exception. We have a value of 8.37% for aspartic acid compared with 13.45% found by Popoff. The composition obtained here for *C. sordellii* L toxin also compares favourably with that of *C. difficile* B toxin, except for isoleucine and leucine [9]. *C. sordellii* L toxin appears to be an atypical protein since the proportion of isoleucine residues present (7.67%) is higher than for leucine residues (7.00%). Generally the inverse occurs as is seen in the case of the *C. difficile* B toxin, which has 5.42% isoleucine residues and 9.18% leucine residues [9].

The L toxin purified here has a p*I* value of 4.20, comparing favourably with a p*I* of 4.55 determined by Popoff [18]. The smear present on the IEF gel (Fig. 8) near the band at pH 4.20 is probably due to the nucleic acids present in the sample. Indeed, the presence of nucleic acids (0.85%), which are charged molecules, can interfere with the determination of the L toxin p*I*, which is based on the charge of the protein. Rihn and co-workers [9,11] have previously observed this phenomenon when determining the p*I* of *C. difficile* B toxin, their sample containing 5% of nucleic acid. We found an M_r of 260 000, which is similar to the previously found value of 240 000 [18]. The slight difference is probably due to the different methods used to estimate the molecular mass. We have used a chromatographic technique based specifically on the size of the protein (Fig. 7), whereas Popoff [18] simply used PAGE for his determination on which the migration of a protein is modified slightly by the charge on the protein. We also found an M_r of 240 000 on native PAGE. Two different tests confirm that the purified protein at M_r 260 000 is the L toxin: (1) extraction of protein from native PAGE shows that the protein present at M_r 240 000 is the only protein that is cytotoxic (Fig. 2, lane e); (2) on immunoblotting after native PAGE, the polyclonal L toxin antiserum which neutralizes the cytotoxicity only recognizes the protein at M_r 240 000 (Fig. 2, lane f).

The cytotoxic supernatant, obtained after protein extraction from native PAGE, was subjected to SDS-PAGE and revealed a single band at M_r 43 000 (Fig. 3, lane e). Likewise, SDS-PAGE performed with and without boiling showed that the L toxin must be boiled in the presence of β-mercaptoethanol to be completely dissociated into subunits (Fig. 6, lane c), which would suggest that the subunits are linked by disulphide bonds. Moreover, immunoblotting shows that the band at M_r 43 000 is immunologically recognized by polyclonal

L toxin antiserum, which neutralizes the L toxin cytotoxicity (Fig. 3, lane f). Yet, this subunit of M_r 43 000 is not cytotoxic. Hence, we propose a hexameric structure for the L toxin with an M_r of ca. 260 000, each monomer having an M_r of ca. 43 000. Only the hexameric structure is cytotoxic. A hexameric structure has also been suggested for the cytotoxin of *C. difficile* with a total M_r of ca. 300 000, each (non-toxic) monomer having an M_r of ca. 50 000 [11].

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