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Purification and Characterization of Toxin B from *Clostridium difficile*

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Toxin B from *Clostridium difficile* was purified to homogeneity and characterized. Purification of toxin B was achieved by gel filtration, chromatography on two consecutive anion-exchange columns, and chromatography on a high-resolution anion-exchange column in the presence of 50 mM $CaCl_2$. The molecular weight of toxin B was estimated to be 250,000 by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and 500,000 by gel filtration. No subunits were apparent when the toxin was reduced and analyzed by SDS-PAGE. The estimated molecular weight of native toxin B indicated that dimers may form in solution. Toxin B was homogeneous by SDS-PAGE, native PAGE, and high-resolution anion-exchange chromatography. No secondary sequences were detected when the amino terminus of the toxin was sequenced, which also indicated that contaminating peptides were absent from the preparation. The amino terminus of toxin B was determined to be NH_3-Trp-Leu-Val-Asn-Arg-Lys-Gln-Leu-Glu-Lys-Met-Ala-Asn-Val-Arg-Phe-Arg. One cytotoxic unit of toxin B was estimated to be 0.2 to 0.8 pg.

Clostridium difficile is the causative agent of pseudomembranous colitis. The disease is generally induced by antibiotic treatment and can result in a severe diarrhea with intestinal hemorrhaging. Pathogenic *C. difficile* has been found to produce at least two toxins, designated A and B. Toxin A has been described as an enterotoxin, since it causes enteric fluid accumulation and diarrhea. However, it also causes intestinal hemorrhage without affecting adenylate cyclase activity. These characteristics are not typical of true enterotoxins such as cholera toxin. Toxin B is a potent cytotoxin which has no apparent effect in the gut but is lethal when injected intravenously or administered intragastrically with sublethal amounts of toxin A (7). Apparently, once the colon is damaged by either toxin A or other trauma, toxin B enters the circulatory system via the damaged tissue and can cause widespread tissue deterioration. When applied to sensitive mammalian cells, toxin B causes the rounding and eventual death of the cell. The mechanism by which toxin B causes cell death is unknown. Several investigators have isolated toxin B from various *C. difficile* strains; however, there are incongruities in the descriptions of toxin B. The molecular weight reported for toxin B has varied by as much as 10-fold, while the specific activity attributable to toxin B has varied by as much as 10,000-fold (2, 6, 8–14). The reasons for these reported differences have not been sufficiently explained, although some investigators have suggested that at least some of the reported toxin B preparations are composed mainly of a contaminating protein.

In this report we present a procedure for the purification of toxin B to homogeneity from *C. difficile* 10463 and a description of the physical characteristics of the toxin. Also described are some characteristics of toxin B and a contaminating protein that may account for the observed differences in the description of toxin B.

MATERIALS AND METHODS

Bacterial strains and reagents. *C. difficile* 10463 was kindly supplied by Tracy Wilkins (Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg) and was used for the production of toxin B.

All reagents and chromatography gels were obtained from Sigma Chemical Co., St. Louis, Mo., except where noted. Media were obtained from Fisher Scientific Co., Dallas, Tex. The fast protein liquid chromatography system and associated columns were obtained from Pharmacia, Inc., Piscataway, N.J.

Protein determination. Protein concentration was determined by the method of Bradford (3) with the reagents supplied in the Bio-Rad Laboratories (Richmond, Calif.) protein kit. The protein standard used was bovine immunoglobulin G.

Cytotoxicity assay. Human foreskin diploid fibroblast cells (kindly supplied by D. Graves, University of Oklahoma Health Sciences Center, Oklahoma City) were used for the cytotoxicity testing. The cells were grown in Iscove medium containing 10% fetal calf serum and were maintained at 37°C in a humidified 5% CO_2 atmosphere. Cells were seeded into 96-well plates and, upon reaching confluency, were used for cytotoxicity testing within 24 h. Generally, serial 1:10 toxin dilutions were made in phosphate-buffered saline (50 mM sodium phosphate [pH 7.2]–150 mM NaCl) containing 100 μg of bovine hemoglobin per ml, and 10 μl of each toxin B dilution was added to the cells (each well contained 150 μl of medium). When a more exact estimate of the cytotoxic endpoint was necessary, 1:2 dilutions were made. One cytotoxic unit was defined as the amount (in grams) of protein needed to cause 100% of the cells in a well to become round within 24 h. There was an average of 15,000 to 30,000 cells per well.

Purification of toxin B. Brain heart infusion broth (50 ml) was inoculated with *C. difficile* 10463 and grown for 18 to 24 h at 35°C. This culture was used to inoculate approximately 100 ml of phosphate-buffered saline in a dialysis bag (12- to 14-kilodalton [kDa] exclusion limit; Fisher) that was suspended in 700 to 800 ml of brain heart infusion broth. Six to eight of these dialysis sac cultures which had been inoculated with *C. difficile* 10463 were grown anaerobically by the dialysis bag method (1) at 35°C for 72 h. The cells and spent

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medium from five to seven flasks (approximately 600 ml) were pooled, and the protease inhibitors phenymethysulfonyl fluoride (20 μg/ml) and tosylphenylchloromethyl ketone (60 μg/ml) were added. The temperature was decreased to 0 to 4°C and was maintained below 4°C for all purification steps. Cells and medium were separated by centrifugation at 16,000 × *g* for 30 min. The toxin B-containing supernatant was concentrated with a Minitan concentration system (Millipore Corp., Bedford, Mass.) equipped with eight filter packets (≈880 cm^2) with an exclusion limit of 100,000 daltons. The concentrated supernatant was dialyzed by constant-pressure dialysis in the Minitan apparatus against a 10-fold-greater volume of 150 mM NaCl–10 mM Tris hydrochloride (pH 8.0); the final volume was approximately 40 to 50 ml. This fraction was applied to a Sephacryl S-300 column (4.5 by 60 cm) which had been equilibrated with 10 mM Tris hydrochloride (pH 8.0) containing 100 mM NaCl. During all chromatographic steps, protein concentration was monitored by A_{280}.

The cytotoxic peak fractions were pooled and applied to a column (1.5 by 20 cm) packed with Accell QMA anion-exchange resin (Millipore) which was equilibrated with 10 mM Tris hydrochloride (pH 8.0) containing 50 mM $CaCl_2$ (buffer A). After the column was washed with 50 ml of buffer A, the bound protein was eluted with a linear NaCl gradient from 0 to 0.5 M in buffer A at a rate of 1 ml/min. The cytotoxic peak fractions were pooled, concentrated, and washed (to lower the NaCl concentration) in an Amicon 50-ml positive-pressure concentrator equipped with a 10-kDa exclusion limit filter (Millipore). Concentrated toxin was applied to a second Accell column (0.6 by 6 cm) which had been equilibrated with buffer A. After a 5-ml wash with buffer A, the bound protein was eluted with a linear NaCl gradient from 0 to 0.5 M in buffer A at a rate of 0.5 ml/min. The cytotoxic peak fractions were pooled and concentrated to 2 ml in a 10-ml-capacity Amicon positive-pressure concentrator equipped with a 10-kDa filter, diluted fivefold with 10 mM Tris hydrochloride (pH 8.0), and reconcentrated to 1 to 2 ml. This preparation was separated on a high-resolution Mono Q HR5/5 anion-exchange column (Pharmacia) with a linear gradient from 0 to 0.4 M NaCl in buffer A (40 ml). The cytotoxic fractions were concentrated, glycerol was added to a final concentration of 25%, and toxin B was stored at −20°C.

Gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) gels were prepared by the procedure described by Laemmli (5), employing 7.5% separating gels of 0.75-mm thickness. When necessary, sample desalting was accomplished by dialysis against 10 mM Tris hydrochloride (pH 8.0) in a microdialyzer (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Samples were made 25% in 4× SDS-PAGE sample buffer (pH 6.8 with 268 mM Tris hydrochloride–5% SDS–40% glycerol) with or without 5% mercaptoethanol.

Native gels were prepared as were the SDS-PAGE gels, except that SDS was left out of the running buffers, gels, and samples. The samples for the native gels were not denatured by heating.

Peptide mapping. Two-dimensional peptide maps of the various proteins were generated as described previously (15). Briefly, toxin B was radiolabeled with ^{125}I at pH 8.0 by the chloramine T method (4) and immediately subjected to SDS-PAGE. The gel was fixed, stained with Coomassie R-250, destained, and dried between two sheets of permeable cellulose. The toxin B band was excised from the gel, suspended with 0.5 ml of 50-μg/ml trypsin in 50 mM ammonium bicarbonate (pH 7.8), and rocked for 24 h at 37°C. The supernatant, which contained the eluted peptides, was lyophilized and suspended in 10 to 20 μl of electrophoresis buffer (15% acetic acid and 5% formic acid in water). The labeled peptides (approximately 1×10^6 to 2×10^6 cpm) were spotted onto a corner of a Kodak cellulose Chromagram sheet (20 by 20 cm; Fisher). The plate was wetted with the same buffer, and the peptides were subjected to electrophoresis until the pyronin tracking dye migrated 10 cm. Electrophoresis was carried out at a constant 500 V in a Pharmacia FBE-3000 flatbed electrophoresis unit cooled to 5°C. After electrophoresis, the plate was completely dried and subjected to chromatography in a chamber containing a mixture of butanol, pyridine, acetic acid, and water (65:50:10:40 [vol:vol:vol:vol]) until the solvent front was within 1 cm of the top of the plate. The plates were dried, wrapped in cellophane, and exposed to X-ray film for 1 to 3 days at −70°C. The positions of the peptides from each protein were determined by comparison of the two individual peptide maps with a mixed peptide map of the two proteins.

Preparation of antisera and affinity purification of anti-toxin B antibody. New Zealand White rabbits were used for the production of anti-toxin B antibody. Two rabbits were given six intramuscular injections of toxin B made up in 1 ml of Freund complete adjuvant. The total amount of toxin B injected was approximately 100 μg. Booster inoculations of 100 μg of toxin B in Freund incomplete adjuvant were given after 3 and 6 weeks.

Antisera from these rabbits were affinity purified on a column of Affigel 10 (Bio-Rad) to which toxin B had been conjugated. The toxin B used in the coupling contained highly purified toxin B from step 6 of the purification. Before being used, the column was eluted with 100 mM glycine (pH 2.7) to remove any unconjugated toxin B. The column was then equilibrated with 10 mM Tris hydrochloride (pH 8.0). Toxin B antiserum was passed over the column, and all unbound protein was eluted with 10 mM Tris hydrochloride (pH 8.0) containing 200 mM NaCl. Bound antibody was eluted with 100 mM glycine (pH 2.7). The fractions were neutralized immediately by the addition of a sufficient amount of 0.5 M potassium phosphate buffer (pH 10) to bring the pH of the eluted antibody fraction to 7.0.

Amino acid sequence analysis. The amino-terminal sequence of toxin B was determined with a gas-phase amino-acid analyzer (model 470A; Applied Biosystems, Inc., Foster City, Calif.) equipped with an on-line model 120A phenylthiohydantoin (PTH) analyzer. All reactions were carried out according to the instructions of the manufacturers.

RESULTS

Toxin B purification. Approximately 50% of the total cytotoxic activity was lost during the concentration and dialysis of the culture supernatant. This loss was partially recovered by filtration on Sephacryl S-300. The apparent loss in activity appeared to be due to an inhibition of the toxin by high-molecular-weight DNA fragments which eluted at the void volume of the gel filtration column (Fig. 1A). The A_{260}/A_{280} ratio of the DNA contained in these fractions was approximately 1.8, and most of the DNA was more than 20 MDa in mass. Experiments in which this DNA was mixed with toxin B showed that the DNA could partially inhibit the activity of toxin B on cells (data not shown). Although other inhibitory substances could be present in the crude toxin preparation, the DNA appeared to at least contribute to the

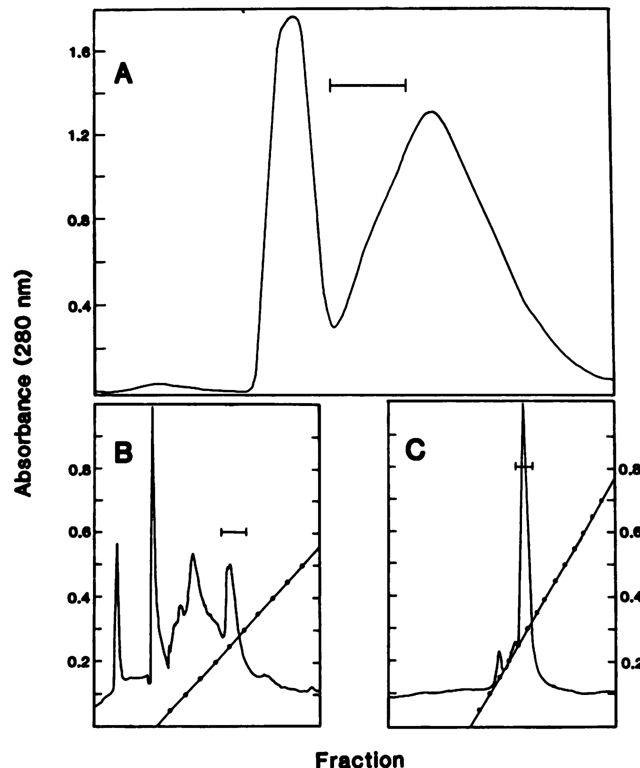

FIG. 1. Chromatographic steps in the purification of toxin B. Chromatography of toxin B over Sephacryl S-300 (A) and anion-exchange matrix Accell QMA (B and C). The bar indicates the fractions which contained the majority of the cytotoxicity and which were pooled for the succeeding step. Symbol: ●, NaCl (M).

inhibition. Fractions which contained the greatest level of cytotoxicity were eluted just after the fractions containing the DNA (Fig. 1A). Inclusion of 50 mM $CaCl_2$ in the elution buffers of the subsequent chromatographic steps increased the resolution of individual peaks. The $CaCl_2$ also decreased the number of contaminating proteins in the cytotoxic peak fractions compared with those in peak fractions after chromatography of the toxin B preparation in the absence of $CaCl_2$ (data not shown). The fractions from the Sephacryl S-300 column were applied to a column packed with Accell QMA anion-exchange resin to further purify the cytotoxic protein(s). As reported by others for anion-exchange separations (2, 4, 11, 12), there were two cytotoxic peak fractions, one which eluted at approximately 0.15 M NaCl and another which eluted at 0.3 M NaCl (Fig. 1B). The first cytotoxic peak apparently contained toxin A, as determined by reactivity on an immunoblot with a monoclonal antibody to toxin A (a generous gift of T. Wilkins). The second peak of cytotoxicity contained more than 85% of the total eluted cytotoxicity.

The fractions which contained the majority of the toxin B from the first anion-exchange column exhibited several protein bands when analyzed by SDS-PAGE (Fig. 2, lane 4). These fractions were pooled and applied to a second Accell column (Fig. 1C). After this step, only the 50- and 250-kDa proteins remained in the preparation (Fig. 2, lane 5). Final purification was achieved by the use of a high-resolution anion-exchange column (Mono Q) (Fig. 3). After this step, only the 250-kDa protein was present in the preparation (Fig. 4). The A_{280}/A_{260} ratio of the purified toxin B protein was 1.68. The solution of purified toxin B was made 25% in glycerol and stored at −20°C.

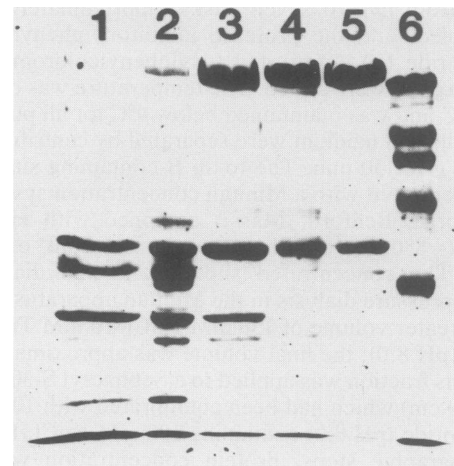

FIG. 2. SDS-PAGE analysis of proteins from various purification steps. The cytotoxic fractions from each step of the purification of toxin B were analyzed on a 7.5% SDS-PAGE gel. Lanes: 1, supernatant proteins after the removal of the *C. difficile* cells; 2, supernatant proteins after concentration; 3, concentrated cytotoxic fractions from step 3 (S-300 chromatography); 4, concentrated cytotoxic fractions after step 4 (anion-exchange chromatography); 5, concentrated cytotoxic fractions after step 5 (second anion-exchange chromatography). Molecular weight markers (from top to bottom) were 205,000, 116,000, 95,000, 66,000, and 45,000 (lane 6). Analysis of the protein from step 6 (Mono Q) is shown in Fig. 4.

Characterization of toxin B and determination of the amino-terminal sequence. Figure 3 shows the chromatograms obtained when freshly prepared toxin B from step 5 (Table 1) was chromatographed on the Mono Q column in the absence and presence of 50 mM $CaCl_2$. A single peak was detected in the absence of $CaCl_2$, whereas in the presence of $CaCl_2$, two

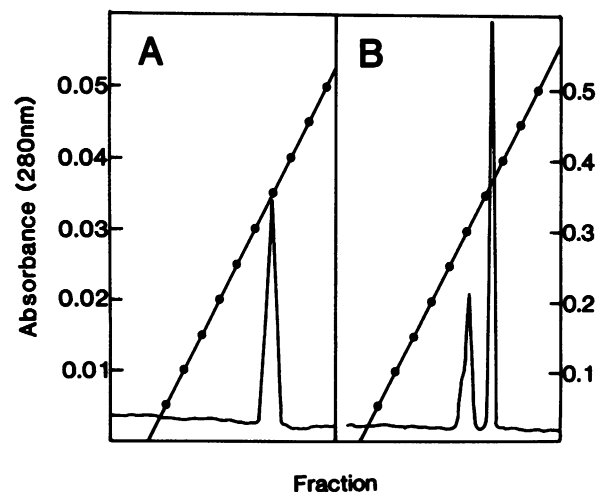

FIG. 3. Anion-exchange chromatography of toxin B in the absence and presence of $CaCl_2$. Toxin B from step 5 of the purification was separated by anion-exchange chromatography on the high-resolution Mono Q matrix in the absence (A) or presence (B) of 50 mM $CaCl_2$. In panel A, all of the applied cytotoxicity was present in the single eluted peak, whereas in panel B, all of the applied cytotoxicity was present in the second eluted peak. Symbol: ●, NaCl (M).

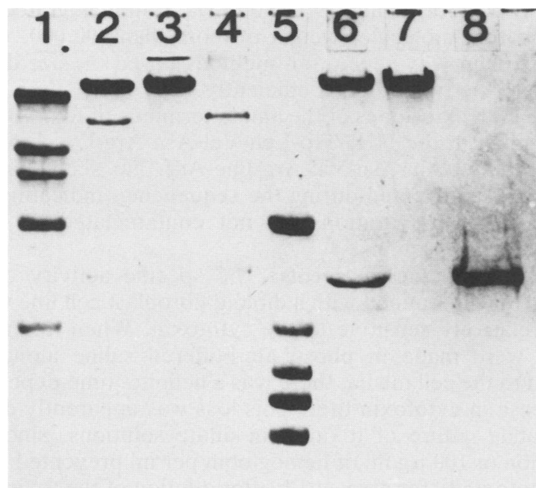

FIG. 4. SDS-PAGE analysis of toxin B separated by anion-exchange chromatography in the absence and presence of $CaCl_2$. Proteins from the peak fractions shown in Fig. 3 were subjected to SDS-PAGE analysis on a 7.5% gel. Proteins in lanes 2 through 4 were not reduced with β-mercaptoethanol, whereas proteins in lanes 6 through 8 were reduced with 5% β-mercaptoethanol. Lanes: 1, molecular weight markers (same as in Fig. 3); 2 and 6, proteins present in the single peak shown in Fig. 3A; 3 and 7, proteins contained in the larger peak shown in Fig. 3B; 4 and 8, proteins present in the fractions from the smaller peak in Fig. 3B. Low-molecular-weight markers were 66,000, 45,000, 36,000, 24,000, and 20,100. Da (lane 5).

peaks were clearly resolved. The protein contents of the peak fractions represented in Fig. 3A were analyzed by both nonreducing and reducing SDS-PAGE (Fig. 4, lanes 2 and 6, respectively). Both the 250- and 50-kDa proteins were present in the peak sample. The migration of the 250-kDa protein remained unchanged whether reduced or not; however, the migration of the 50-kDa protein was radically different. In the absence of reducing agent, it migrated predominantly as a 150-kDa protein; when reduced, it migrated as a 50-kDa protein. SDS-PAGE analysis of the protein content of the fractions which constituted the small and large peaks shown in Fig. 3B (Fig. 4, lanes 3 and 4 and lanes 7 and 8, respectively) revealed that the smaller peak contained exclusively the 50-kDa protein, whereas the larger peak contained only the 250-kDa protein. More than 99.5% of the recovered activity was present in the fraction which contained the 250-kDa protein, indicating that the 250-kDa protein was toxin B. A slight increase (Table 1) in the specific activity of toxin B occurred when the two proteins were separated by this method, suggesting that the 50-kDa protein was not required for the activity of toxin B.

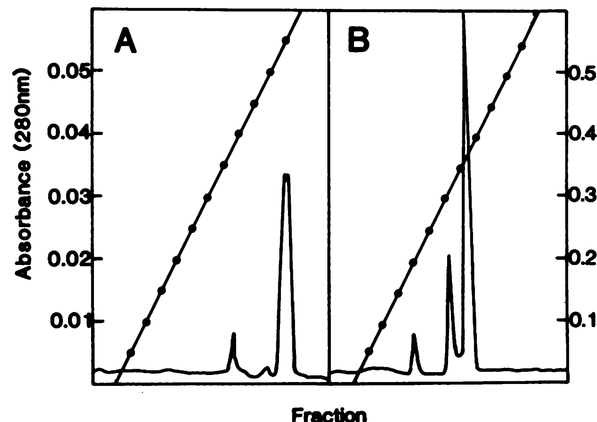

FIG. 5. Separation of toxin B after freezing in the absence and presence of glycerol. Toxin B from step 5 of the purification was frozen (−20°C) in the absence and presence of 25% glycerol, thawed, and separated by high-resolution chromatography on a Mono Q column in the presence of $CaCl_2$. (A) Toxin B frozen in 10 mM Tris hydrochloride (pH 8.0); (B) toxin B frozen in the same buffer plus 25% glycerol. Proteins present in each peak were evaluated by SDS-PAGE (Fig. 6).

It was found that if the toxin B preparation was frozen in the absence of glycerol, the separation of the 50- and 250-kDa proteins could not be achieved, even in the presence of $CaCl_2$. The same toxin preparation was frozen in the absence (Fig. 5A) and presence (Fig. 5B) of glycerol and then chromatographed on the Mono Q column in the presence of 50 mM $CaCl_2$. Toxin B (from step 5 of the purification) which had been frozen in buffer (10 mM Tris hydrochloride [pH 8.0]–100 mM NaCl) could not be resolved into the 50- and 250-kDa proteins, as was shown by SDS-PAGE analysis of the proteins in those fractions (Fig. 6, lane 4). In contrast, the same toxin preparation frozen in the presence of glycerol could still be separated into the 250- and 50-kDa proteins, as confirmed by SDS-PAGE analysis (Fig. 6, lanes 1 and 2, respectively). A minor protein of 45 kDa, which was present in the toxin preparation, sometimes contaminated the toxin preparation and was also resolved from the 250- and 50-kDa proteins in the presence of $CaCl_2$ (Fig. 6, lane 3).

Purified toxin B and the 50-kDa protein were also separated by nondenaturing gel electrophoresis (Fig. 7). The 50-kDa protein exhibited a mobility similar to that of toxin B under reduced conditions (Fig. 7, lanes 1 and 2). Identical results were obtained when the proteins were not reduced (data not shown).

Although the 50-kDa protein was not cytotoxic, the possibility existed that it was proteolytically derived from the larger protein. Therefore, the tryptic peptides from each

TABLE 1. Purification table for *C. difficile* toxin B

Fraction (purification step)	Vol (ml)	Amt of protein (mg)	Total CU[a]	CU/mg	Recovery[b] (%)	Purification (fold)
1. Supernatant	510	388	2.6×10^{10}	6.6×10^{7}	100	1
2. Concentrate	50	161	1.3×10^{10}	7.8×10^{7}	49	1
3. S-300	90	40.5	2.3×10^{10}	5.6×10^{8}	88	8
4. Accell column 1	8	20.3	1.6×10^{10}	7.8×10^{8}	62	12
5. Accell column 2	1.3	8.0	7.5×10^{9}	9.4×10^{8}	29	14
6. Mono Q	1.0	4.0	5.1×10^{9}	1.3×10^{9}	20	19

[a] CU, Cytotoxic units.
[b] The change in the recovery in steps 2 and 3 reflects the concentration of high-molecular-weight DNA, an inhibitor of toxin B, in fraction 2 and its removal in fraction 3.

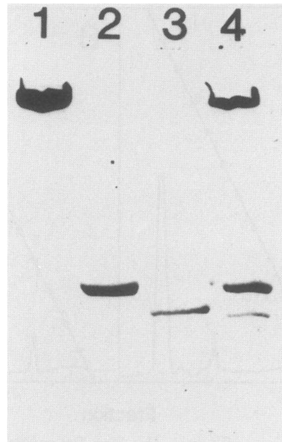

FIG. 6. SDS-PAGE analysis of fractions obtained from anion-exchange analysis of toxin B frozen in the presence and absence of glycerol. The peak fractions shown in Fig. 5 were analyzed for the proteins present in each. Lanes show the proteins present in the fractions of the major peak in Fig. 5B (lane 1), the middle peak in Fig. 5B (lane 2), the smallest peak in Fig. 5B (lane 3), and the major peak in Fig. 5A (lane 4).

protein were analyzed by high-resolution, two-dimensional peptide mapping. Little or no peptide homology existed between the 250-kDa toxin B (Fig. 8A) and the 50-kDa protein (Fig. 8B), indicating that the 50-kDa protein was not derived from the 250-kDa toxin B. Also, affinity-purified antibody specific for the 250-kDa toxin B did not react with the 50-kDa protein or reveal multiple forms of toxin B in the culture supernatants (data not shown). This indicated that the 50-kDa protein was not being proteolytically removed from the toxin B, since it would have resulted in more than one size of toxin B.

The molecular weight of the native 250-kDa toxin B was estimated by nondenaturing gel filtration on a Pharmacia high-resolution Superose 6 column. The 250-kDa toxin migrated as a single protein with an apparent molecular weight of 500,000 relative to gel filtration standards, suggesting that

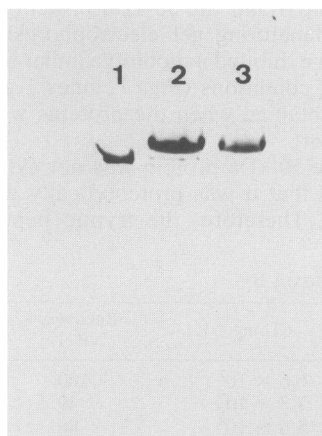

FIG. 7. Analysis of toxin B by native PAGE. Purified toxin B and the 50-kDa protein were separated by native PAGE under reduced conditions. Lanes: 1, purified 50-kDa contaminant; 2, toxin B with some 50-kDa protein contaminating it; 3, purified toxin B. Identical results were obtained under unreduced conditions (not shown).

toxin B may form dimers. The 50-kDa protein migrated with an apparent molecular weight of more than 400,000, which may explain why separation methods based on size do not separate the two proteins efficiently.

The first 20 residues of the amino terminus of toxin B were determined to be NH_3-Trp-Leu-Val-Asn-Arg-Lys-Gln-Leu-Glu-Lys-Met-Ala-Asn-Val-Arg-Phe-Arg. No secondary sequence was detected during the sequencing, indicating that the toxin B preparation was not contaminated by other peptides and proteins.

Cytotoxicity measurements. The specific activity of the toxin was determined with a diploid fibroblast cell line which was relatively sensitive to the cytotoxin. When toxin dilutions were made in phosphate-buffered saline alone and added to the cell media, there was a definite, time-dependent decrease in cytotoxin titer. This loss was apparently due to the labile nature of toxin B in dilute solutions, since the addition of 100 μg/ml of hemoglobin per ml prevented a loss of cytotoxicity for at least 1 h after dilution of the toxin. The purified 250-kDa toxin B caused 100% of the cells to become round at between 0.2 and 0.8 pg per well (approximately 15,000 to 30,000 cells per well). Due to the inherent error associated with the cytotoxic assay (because of pipetting errors and different cell numbers and ages), exact values for the minimum cytotoxic dose are difficult to obtain; however, similar values were obtained with six different preparations of purified toxin.

DISCUSSION

The central questions of this study were whether the 250-kDa protein, the 50-kDa protein, or both were required for cytotoxicity and whether physical methods could be used to separate the proteins. There have been a variety of reports concerning the purification of toxin B which have presented different descriptions of toxin B. Some investigators have reported that toxin B was quite large (200 to 300 kDa) when analyzed by SDS-PAGE (2, 8) or native PAGE (7, 14), while others have described a cytotoxin which was 150 to 185 kDa which, when reduced with a thiol reagent, migrated as a 50-kDa monomer (9–11). In this study, much of the 50-kDa protein was eluted earlier in the salt gradient during anion-exchange chromatography and was found in fractions containing little cytotoxicity, suggesting that this protein alone was not cytotoxic.

It was necessary to separate these two proteins to evaluate their relative contributions to cytotoxicity. The approaches used here to purify toxin B were similar to those used by other investigators and were basically gel filtration and anion-exchange chromatography. However, these methods did not yield homogeneous toxin B, and further steps were necessary. Three conditions were found to be necessary for separation of the 50- and 250-kDa proteins. First, the toxin could not be frozen in the absence of glycerol; second, it was necessary to keep $CaCl_2$ in the elution buffers; and third, it was necessary to chromatograph the toxin on a high-resolution anion-exchange column. The results showed that the cytotoxicity resided with the 250-kDa protein and that the 50-kDa protein was not cytotoxic. The 250-kDa toxin B was found to be homogeneous by SDS-PAGE, native PAGE, and high-resolution gel filtration. Also, the amino-terminal sequence analysis revealed no secondary sequences which might have arisen from contaminating proteins; this provided additional evidence that this toxin B preparation was homogeneous. The 50-kDa protein did not appear to be a necessary subunit of the toxin, since the specific activity of

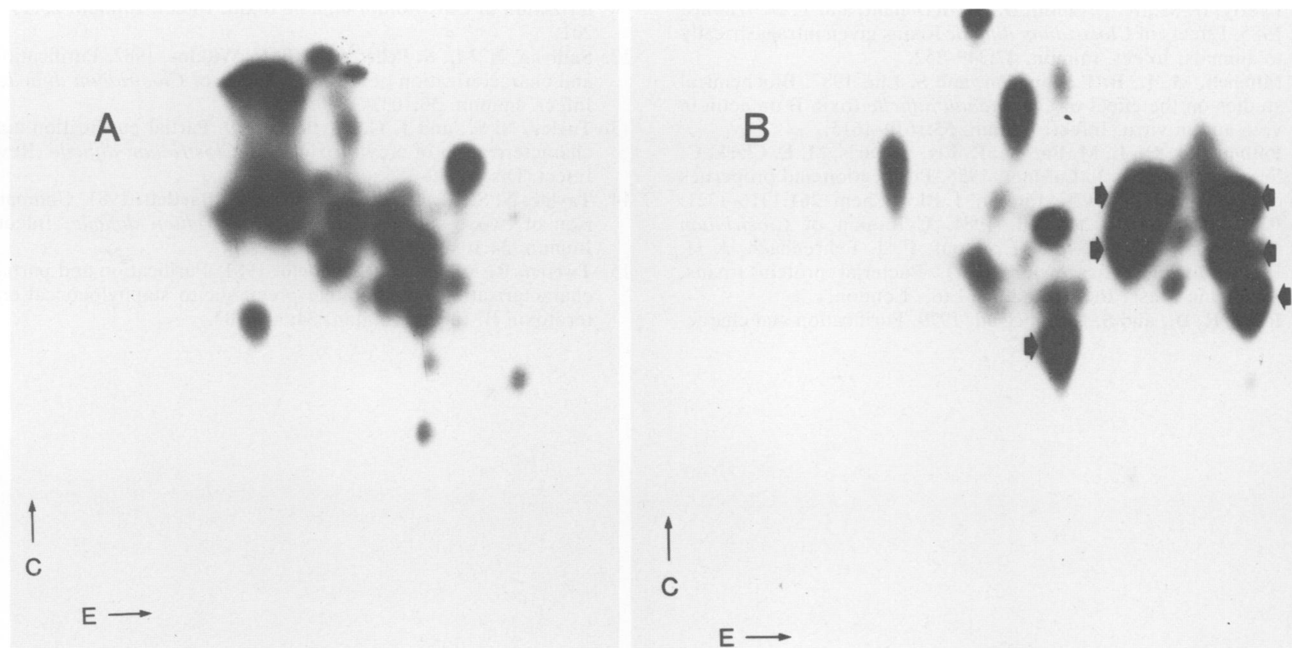

FIG. 8. Analysis of tryptic peptides from toxin B and the 50-kDa protein contaminant. The two-dimensional tryptic maps of the 250-kDa toxin B (A) and the 50-kDa contaminating protein (B) are shown. E and C indicate the directions of the electrophoresis and chromatography steps, respectively, and the heavy arrows in panel B indicate the major peptides which are unique to the 50-kDa protein.

toxin B increased slightly after the 50-kDa protein was removed. Also, the 50-kDa protein was not derived from the 250-kDa toxin by proteolytic cleavage. The fact that the 50-kDa protein copurified with toxin B was due to similarities in the physical characteristics of the native 50-kDa protein and toxin B. A weak interaction between toxin B and the 50-kDa protein, however, cannot be ruled out.

Analyses of toxin B and the 50-kDa protein by SDS-PAGE and native PAGE reveal a significant difference in the mobility of the 50-kDa protein in these two gel methods. Separation of the 50-kDa protein from toxin B was easily achieved by SDS-PAGE under either reducing or nonreducing conditions. In contrast, the migrations of both proteins were nearly identical on native PAGE. This was not surprising, since they appear to be similar in their charge and native molecular weight as judged by their retention characteristics on the high-resolution anion-exchange and gel filtration columns. In view of these observations, native PAGE is not appropriate for the comparison of toxin B preparations, since toxin B and the 50-kDa protein have nearly identical mobilities in this system.

Analysis of purified toxin B by high-resolution gel filtration revealed that the apparent native molecular weight was about 500,000. One possible explanation of this result is that toxin B normally forms dimers in solution. Whether dimerization is actually responsible for the apparent molecular weight of the native toxin B is currently unknown, although this confirms the observation of workers who have reported the molecular weight of toxin B to be between 400,000 and 600,000 (2). Toxin B prepared in this study was highly cytotoxic when applied to sensitive cells. One cytotoxic unit was estimated to be 0.2 to 0.8 pg of purified toxin B. This value is similar to that obtained by Banno et al. (2) and Lyerly et al. (6) for their preparations of toxin B.

The findings of this study help resolve some of the discrepancies in the reported description of toxin B from *C. difficile*. The 50-kDa protein exhibited the properties of the protein isolated by some investigators (9–11) as toxin B. This protein is a contaminant, but under nondenaturing conditions it has physical properties similar to those of toxin B. We also provide a standard purification scheme for the preparation of highly active, homogeneous toxin B and the amino-terminal sequence of toxin B. The availability of homogeneous toxin B will be important in studies which are aimed at understanding the mechanism and structure-function relationships of toxin B.

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