Purification and characterisation of *Clostridium difficile* toxin A by bovine thyroglobulin affinity chromatography and dissociation in denaturing conditions with or without reduction

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Summary. Highly purified toxin A of *Clostridium difficile* was obtained by bovine thyroglobulin affinity chromatography followed by two sequential anion-exchange chromatography steps on Q Sepharose FF and Mono Q. After Q Sepharose FF chromatography of a thyroglobulin affinity-purified toxin A preparation, two major peaks of cytotoxicity representing toxins A and B were detected. The homogeneity of the final toxin A preparation obtained from Mono Q anion-exchange fast protein liquid chromatography was ascertained by gel electrophoresis developed by silver stain. The mol. wt of toxin A in non-denaturing conditions was estimated to be 520–540 Kda by native polyacrylamide gel electrophoresis (PAGE) developed by silver stain. In contrast, with sodium dodecyl sulphate (SDS)-PAGE under reducing or non-reducing conditions, a major band of 240 Kda and 10 minor and 27 faint bands (non-reduced conditions), or four minor and 31 faint bands (reduced conditions) were detected after silver staining. In two-dimensional PAGE, the seven minor bands of > 240 Kda obtained by non-reducing SDS-PAGE migrated to the 240-Kda position after reduction with β -mercaptoethanol.

Introduction

It is firmly established that *Clostridium difficile* is the major aetiological agent of pseudomembranous colitis and many cases of antibiotic-associated diarrhoea (George *et al.*, 1978; Larson *et al.*, 1978; Borriello and Larson, 1981). It produces at least two toxins, designated toxin A (enterotoxin) and toxin B (cytotoxin). Despite the similarity of the two toxins with respect to cytotoxicity, mouse lethality and the ability to increase vascular permeability, toxin B is a much more potent cytotoxin than toxin A and only toxin A is enterotoxic (Sullivan *et al.*, 1982; Banno *et al.*, 1984; Lyerly *et al.*, 1988).

Toxin A has been reported to haemagglutinate rabbit erythrocytes (Krivan *et al.*, 1986). This haemagglutination is temperature-dependent, occurring at 4°C but not at 37°C. A similar association between toxin A and bovine thyroglobulin, which is also temperature-dependent, was reported by the same investigators (Krivan *et al.*, 1986). This type of binding is thought to be due to the affinity of the

toxin for the sugar sequence Gal α 1-3Gal β 1-4GlcNAc, located on the surface of rabbit erythrocytes and bovine thyroglobulin (Krivan *et al.*, 1986). Krivan and Wilkins (1987) have taken advantage of the binding of toxin A to its carbohydrate-binding determinant at low temperature to purify toxin A by a thermal affinity chromatographic procedure. We have reported recently that the method yields an impure product containing, in addition to toxin A, trace amounts of toxin B and a few other proteins at low level (Kamiya *et al.*, 1988).

The purpose of the present study was to modify the thyroglobulin affinity chromatography method to obtain highly purified toxin A, to determine the mol. wt of the toxin in its native and denatured forms, and to estimate the effect of sodium dodecyl sulphate (SDS), under reducing and non-reducing conditions, on the toxin molecule.

Materials and methods

Bacterial strains and culture filtrates

Toxigenic *C. difficile* strain VPI 10463 and non-toxigenic *C. difficile* strain M-1 were grown separately in

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dialysis tubing and used for preparation of culture filtrates as described by Kamiya *et al.* (1988).

Bovine thyroglobulin affinity chromatography

The method was based on that of Krivan and Wilkins (1987) and was described by Kamiya *et al.* (1988). Briefly, 250 mg of bovine thyroglobulin (Sigma, St Louis, MO, USA) dissolved in 50 ml of 0.1 M morpholinepropane-sulphonic acid buffer (pH 7.0) was allowed to react with 10 ml of activated Affi-Gel 15 (Bio-Rad Laboratories) overnight at 4°C with shaking; 95% of the thyroglobulin was bound to the beads. The remaining active sites on the gel were blocked by treatment with 0.1 M ethanolamine for 30 min at 4°C. The coupled beads were packed into a column (Pharmacia, Uppsala, Sweden; column c 10/10, 10 × 100 mm) and washed at 37°C with 20 bed volumes (120 ml) each of pre-warmed basic buffer (0.1 M glycine-NaOH containing 0.5 M NaCl, pH 10.0) and acidic buffer (0.1 M glycine-HCl containing 0.5 M NaCl, pH 2.0). The column was equilibrated at 4°C with 20 bed volumes of 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.0 (TBS). A 100-ml volume of *C. difficile* culture filtrate was applied to the column. After washing the column with 25 ml of TBS at 4°C, thermal elution was performed by washing with 50 ml of pre-warmed TBS at 37°C. Fractions (5 ml) of the washings at 4°C and the eluates obtained at 37°C were collected and monitored for absorbance at 280 nm (A_{280}), cytotoxicity and haemagglutination (HA) activity as described below.

Anion-exchange chromatography

Two kinds of anion-exchange gel, Q Sepharose FF (Pharmacia) and Mono Q (Pharmacia), incorporated into a fast protein liquid chromatography (FPLC) apparatus (Pharmacia) were used as described previously for the analysis of *C. difficile* toxins by Mono Q (Borriello *et al.*, 1987). The sample was filtered (0.2- μ m membrane) before applying to the column via a 10-ml super-loop (Pharmacia). Elution by a 0–1.0 M NaCl gradient in 20 mM Tris-HCl (pH 7.5) was performed. Each fraction was examined for A_{280} , cytotoxicity and HA activity. The cytotoxic and haemagglutinating fraction (toxin A) from Q Sepharose FF chromatography was dialysed against 20 mM Tris-HCl (pH 7.5) overnight at 4°C before Mono Q chromatography.

Cytotoxicity assay

Cytotoxicity was detected with African green monkey kidney (Vero) cells as described previously (Kamiya *et al.*, 1988). The cultured cells were used for the assay within 1 day. Samples (100 μ l) of two- or ten-fold serial dilutions of toxin preparations in phosphate-buffered saline (PBS, pH 7.2) were added to the cells in the presence of 100 μ l of maintenance medium (Eagle's MEM containing calf serum 2%). The titre (cytotoxic unit; CU/100 μ l) was expressed as the highest dilution

that induced a 100% cytopathic effect (CPE) after incubation for 24 h.

Haemagglutination assay

This assay for toxin A was based on the method described by Krivan *et al.* (1986) with the slight modification that a 1% rabbit erythrocyte suspension was used instead of 2.5%. Two-fold serial dilutions of toxin preparations (50 μ l) were performed with an isotonic buffer (0.1 M Tris, pH 7.2 containing 50 mM NaCl) in the wells of V-bottom microtitration plates; 50 μ l of blood-cell suspension was added to each well and allowed to react for 3 h at 4°C. The HA titre was expressed as the highest dilution of the specimen that caused macroscopic haemagglutination.

Gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed, as described by Laemmli (1970), on samples prepared in the presence or absence of β -mercaptoethanol 5% on a 5% resolving slab gel, 1.5 mm thick; the gels were silver stained (Bio-Rad Laboratories), according to the manufacturer's instructions (Bio-Rad Bulletin 1089), or stained with Coomassie blue R-250 (Bio-Rad Laboratories). High mol.-wt standards (Bio-Rad Laboratories) were used for estimation of mol. wts. Analytical PAGE was done in a 4–30% gradient gel in Tris-glycine buffer, pH 8.8, and in non-denaturing conditions. The gradient gels were silver stained or stained with Coomassie blue R-250. Mol. wts were estimated by comparison with high mol.-wt standards (Pharmacia).

Two-dimensional gel electrophoresis. For the first dimension, SDS-PAGE loaded with non-reduced samples was performed at 30 mA for 3 h at room temperature. The reducing agent, β -mercaptoethanol, was omitted from the sample buffer for electrophoresis in the first dimension. The gel track was excised and equilibrated for 4 h in 63 mM Tris-HCl, β -mercaptoethanol 5% v/v, SDS 1% w/v, pH 6.8, at room temperature as described by Rautenberg and Stender (1986). Electrophoresis in the second dimension was performed as described above.

Protein assay

Protein content was determined by the dye-binding method of Bradford (1976) with the Bio-Rad protein assay kit. Bovine serum albumin was used as a standard.

Analysis of non-toxigenic C. difficile strain M-1 filtrate

For all of the above procedures, fractions corresponding to those exhibiting toxin A biological properties yielded by the toxigenic strain were collected and analysed.

Results

Thyroglobulin affinity chromatography

A 100-ml sample of culture filtrate with a protein concentration of 1.55 mg/ml (cytotoxicity titre 6.7×10^7 ; HA titre 64) was applied to the thyroglobulin affinity column. Fractions (fr) 1–20 were unbound proteins, including toxin B. Peaks of cytotoxicity and A_{280} were observed in these fractions (fig. 1) with a maximum cytotoxic titre of 10^7 and an $A_{280} > 2.0$. No HA activity was detected in these fractions. The column was washed with 25 ml of TBS at 4°C, and then moved to 37°C for thermal elution with 50 ml of pre-warmed TBS. Both cytotoxicity and A_{280} were present in fr26, the first fraction to elute at 37°C; the cytotoxic titre was 10^5 and the A_{280} was 0.6–0.7. Similarly, a remarkable increase in HA titre (512) was detected in this fraction. By washing with TBS at 37°C, the A_{280} and the titres of each activity gradually decreased. The first four fractions after thermal elution, fr 26–29, were collected and used for further purification.

Q Sepharose FF anion-exchange chromatography

An 18-ml sample of thyroglobulin (TG) affinity-purified toxin A was applied to a Q Sepharose FF

column (bed volume 24 ml) and a 0–1.0 M NaCl linear gradient elution was performed (fig. 2). One minor and two major peaks of cytotoxicity were observed. The first cytotoxic fraction, fr 8, was of low titre and probably represented unbound toxin. The second peak of cytotoxicity, fr 33–42, eluted at 0.46–0.64 M NaCl and exhibited a maximum HA titre of 16. In fr 49–56, eluted at 0.78–0.92 M NaCl, a final peak of cytotoxicity of maximum titre 8192 was observed but no corresponding significant increase of A_{280} . These fractions showed no HA activity, indicating that the last cytotoxic fraction represented contaminating toxin B originating from the TG-affinity “purified” toxin A sample, in keeping with our previous observations of the purity of these preparations (Kamiya *et al.*, 1988). The fractions with HA activity, fr 33–38, were collected and used for the last step of purification of toxin A.

Mono Q anion-exchange chromatography

A 12-ml sample of TG- and Q Sepharose FF-purified toxin A were applied to a Mono Q column (bed volume 1 ml) and a 0–1.0 M NaCl gradient elution was performed (fig. 3). At 0.15 M NaCl, a 5-ml plateau (fr 15–19) was introduced into the gradient to ensure complete elution of a large

Fig. 1. Bovine TG-affinity chromatography of *C. difficile* culture filtrate; 100 ml of filtrate was applied to the affinity column, and 5-ml fractions were collected at 4°C. From fr 26 (arrowed) thermal elution was performed at 37°C: —, A_{280} ; ○, cytotoxicity; ▲, HA; ND, below level of detection.

Fig. 2. Q Sepharose FF anion-exchange chromatography profile of the toxin-A containing fractions from the TG-affinity column; 18 ml of the thermal eluents from the TG column (fr 25–29) was applied to the Q Sepharose FF column. Each 2-ml fraction collected was examined: ○, cytotoxicity; ▲, HA; —, A₂₈₀; --, molar concentration of NaCl; ND, below level of detection.

Fig. 3. Mono Q anion-exchange chromatography profile of the toxin-A fraction obtained by Q Sepharose FF chromatography; 12 ml of the Q Sepharose FF-purified toxin A (fr 33–38) was applied to the Mono Q column. Each 1-ml fraction collected was examined: ○, cytotoxicity; ▲, HA; —, A₂₈₀; --, molar concentration of NaCl; ND, below level of detection.

protein component. These fractions showed neither cytotoxicity nor HA activity. In fr 25, which eluted at 0.30–0.34 M NaCl, the highest A_{280} reading was recorded. The cytotoxicity and HA activities of this fraction were at maximum titres of 2.6×10^5 and 512, respectively. Unlike the cytotoxicity profile following Q Sepharose FF chromatography, no other cytotoxic fractions were detected following Mono Q chromatography. Fr 25, representing highly purified toxin A, was collected and analysed further.

Yield of purified toxin A

The purification yield of toxin A from a culture filtrate is shown in the table. The loss of cytotoxicity after the first purification step is a reflection of the loss of toxin B, which is a much more potent cytotoxin than toxin A. Q Sepharose FF chromatography did not give an effective recovery of toxin A, based on the percentage yield of cytotoxicity. However, the contaminating toxin B was removed. On the other hand, Mono Q anion-exchange chromatography gave a good recovery of toxin A. The protein concentration of the final purified toxin A preparation was 590 $\mu\text{g}/\text{ml}$, showing specific activity of 4.4×10^6 CU/mg of protein with a HA titre of 512.

Polyacrylamide gel electrophoresis (PAGE)

To check the purity of the purified toxin-A preparation and estimate its mol. wt under non-denaturing conditions, native PAGE was performed. The highly purified toxin A exhibited only one band of 520–540 Kda when developed by silver stain (fig. 4). This shows that the modified method for the purification of toxin A removes contaminating toxin B and other proteins present following TG-affinity chromatography.

For estimation of the mol. wt of the denatured form of toxin A, SDS-PAGE of β -mercaptoethanol reduced or non-reduced toxin was performed (fig.

Fig. 4. Analysis of toxin A preparation by native gradient PAGE (4–30%) in Tris-glycine buffer, pH 8.8, developed by silver stain: lane 1, high mol. wt markers—thyroglobulin (669 Kda), ferritin (440 Kda), catalase (232 Kda), lactate dehydrogenase (140 Kda), bovine serum albumin (67 Kda, Pharmacia); lane 2, highly purified toxin A (5 μg).

5). In addition to a major band of 240 Kda, 10 minor and 27 faint bands ranging from 38 Kda to 440 Kda were detected by silver staining after electrophoresis in non-reducing conditions (fig. 5, lanes 2 and 5). The sizes of the 10 minor bands were estimated to be 345, 340, 330, 320, 310, 295, 255, 180, 155 and 38 Kda. Among these bands, that of 255 Kda, which was clearly distinguished from the

Table I. Purification yield of toxin A from *C. difficile* culture filtrate

Purification step	Volume (ml)	Cytotoxicity (CU/100 μl)	Total cytotoxicity (CU)	Protein concentration (mg/ml)	Total protein (mg)	Specific activity (CU/mg of protein)	Fold purification	Percentage yield	HA titre
None	100	6.7×10^7	6.7×10^{10}	1.55	155	4.3×10^8	—	—	64
Thyroglobulin	18	3.3×10^4	5.9×10^6	0.16	2.9	2.0×10^6	1	100	128
Q Sepharose FF	12	1.6×10^4	1.9×10^6	0.11	1.32	1.4×10^6	0.70	32	16
Mono Q	1	2.6×10^5	2.6×10^6	0.59	0.59	4.4×10^6	2.2	44	512

major band of 240 Kda when gels were loaded with 1 μ g of toxin A (fig. 5, lane 5), was most intensely silver stained. On the other hand, the SDS-PAGE profile after electrophoresis in reducing conditions with β -mercaptoethanol exhibited a different pattern (fig. 5, lanes 1 and 4). In addition to the major band of 240 Kda, four minor bands, 345, 175, 155 and 38 Kda, and 31 faint bands, ranging from 46 Kda to 440 Kda, were detected. Unlike the pattern from non-reducing conditions, the 255-Kda band was not detected after reduction. The bands of 340, 330, 320, 310 and 295 Kda were very faint, compared to those without reduction. Among 38 bands obtained in non-reducing conditions and 36 bands in reducing conditions, 31 bands migrated to common points. In contrast to these findings, Coomassie blue staining demonstrated only one band of 240 Kda in both non-reducing and reducing

Fig. 5. Analysis of toxin-A preparation by SDS-PAGE, with or without β -mercaptoethanol, developed by silver stain: lanes 1 and 2, highly purified toxin A (5 μ g) with (lane 1) or without (lane 2) β -mercaptoethanol 5%; lane 3, mol. wt markers—myosin (200 Kda), β -galactosidase (116 Kda), phosphorylase B (92.5 Kda), bovine serum albumin (66 Kda; Bio-Rad), ovalbumin (45 Kda); lanes 4 and 5, highly purified toxin A (1 μ g) with (lane 4) or without (lane 5) β -mercaptoethanol 5%.

conditions (data not shown). In parallel, 100 ml of culture filtrate of a non-cytotoxicigenic *C. difficile* isolate, strain M-1, was applied to the TG affinity column and treated by the method used for purification of toxin A. Thermal elution at 37°C was followed by Q Sepharose FF and Mono Q anion exchange chromatography. The final fraction, corresponding to the one in which toxin A would be expected to elute, was analysed by the gel-electrophoresis methods. No bands were detected by silver staining (data not shown), confirming that the material detected by silver stain of the denatured toxin-A preparation represents components of toxin A and not material from any other source.

Two-dimensional (2D) PAGE

To examine the effect of reduction with β -mercaptoethanol on each band present in non-reducing conditions, 2D-PAGE was performed (fig. 6). After reduction by β -mercaptoethanol for 4 h, the seven minor bands which were larger than the major 240-Kda band migrated to the same position as the 240-Kda major band. 2D-PAGE also confirmed that the major 240-Kda band dissociated into smaller bands after treatment with β -mercaptoethanol. Other minor bands or spots (arrow), estimated as 175-, 155- and 38-Kda bands in reducing conditions, appeared to originate from three minor bands of 180, 155 and 38 Kda, respectively, with only the 180-Kda band being obviously affected by reduction.

Discussion

Purification of toxin A has been performed by several investigators. Some groups have used an acetic acid buffer (pH 5.5) precipitation method after separation by anion-exchange chromatography (Taylor *et al.*, 1981; Sullivan *et al.*, 1982; Lysterly *et al.*, 1986b), others have used affinity chromatography on an agarose gel and elution of toxin A with galactose (Lönnroth and Lange, 1983). However, the effectiveness of this affinity chromatography method has not been confirmed by other investigators (Lysterly and Wilkins, 1988). Other methods based upon preparative electrophoresis (Stephen *et al.*, 1984) or chromatofocusing (Rihn *et al.*, 1984) have also been reported for toxin A purification.

Krivan *et al.* (1986) reported that toxin A binds to hamster brush-border membranes, indicating that there is a receptor of toxin A on the surface of the membrane. The binding to the membrane is temperature dependent. At 4°C, large amounts of toxin A bind to the membrane, but the toxin does

Fig. 6. 2D-PAGE of denatured toxin A. Highly purified toxin A (7 μ g) was first electrophoresed under non-reduced conditions. The track was excised and, after reduction with β -mercaptoethanol, SDS-PAGE was performed in the second dimension. Arrows—minor bands estimated as 175, 155 and 38 Kda after reduction.

not bind as well at 37°C. They also showed that toxin A binds to rabbit erythrocytes in a temperature dependent manner, although higher concentrations of toxin A are required for haemagglutination to be detected. The receptor in each case is considered to involve the trisaccharide Gal α 1-3Gal β 1-4GlcNAc. Bovine thyroglobulin (TG) was shown to inhibit haemagglutination at concentrations of ≥ 0.5 μ g/ml, and to bind toxin A in a temperature-dependent manner (Krivan *et al.*, 1986). Krivan and Wilkins (1987) utilised this temperature-dependent binding capability of toxin A to bovine TG for purification of toxin A, reporting that TG-affinity chromatography was a single, effective and gentle method for its purification. However, as reported in our previous study (Kamiya *et al.*, 1988), the fraction thermally eluted at

37°C contains not only toxin A but also trace amounts of toxin B and other proteins. Therefore, we tried to establish a modified purification method based on TG-affinity chromatography.

In the present study, a low HA titre of toxin A eluted from the affinity column even at 4°C (fig. 1). This is probably due to the loading of a large amount of toxin A-rich crude culture filtrate on to the column. Unlike the profile of the TG-affinity chromatogram in our previous report (Kamiya *et al.*, 1988), a significant increase of A₂₈₀ was observed after thermal elution. This is partly because, in this experiment, we did not wash the column at 4°C to the same degree as in the previous experiments. These results indicate that, even at 4°C, small amounts of toxin A might be eluted from the bovine TG-affinity column. Thermal elution

from fr 26 yielded a good recovery of toxin A. The HA titre of this fraction was 512, which is much higher than that of the thermal eluent after washing with 250 ml of TBS in the previous study (Kamiya *et al.*, 1988).

Q Sepharose FF chromatography of the TG-purified toxin A yielded three peaks of cytotoxicity. The last cytotoxic peak, eluting at 0.78–0.92 M NaCl, is presumably due to contaminating toxin B because these fractions showed no HA activity, confirming our previous demonstration of contaminating toxin B following TG-chromatography (Kamiya *et al.*, 1988). The Q Sepharose FF chromatography purification step did not give a good recovery, as determined by the percentage yield of cytotoxicity, although the contaminating toxin B and other proteins in the TG-affinity-purified "toxin A" specimens were removed. However, despite the loss of some toxin A, this purification step was retained as preliminary experiments showed that application of thermal eluents at 37°C from the TG column directly onto a Mono Q anion-exchange column caused high back pressure and a shortening of column life. Separation of toxin A by Mono Q anion-exchange chromatography seemed to be complete, because even by silver staining, which is a much more sensitive stain than Coomassie blue, only one band of 520–540 Kda was detected in the final toxin-A preparation. This estimation of mol. wt of toxin A is in general agreement with other reports (Sullivan *et al.*, 1982; Lysterly *et al.*, 1986b; Krivan and Wilkins, 1987). However, Rihn *et al.* (1984) reported that toxin A in native form is smaller—52 Kda. Their estimated mol. wt of toxin A has not been confirmed by other investigators. Furthermore, the recent report (Johnson *et al.*, 1988) that all of the 6.1 kb cloned segment of the toxin A gene is in open reading frame, supports the large size estimates of toxin A.

To investigate the possible sub-unit structure of toxin A, we examined it under reducing and non-reducing conditions by SDS-PAGE followed by silver staining. The size of the major band was estimated to be 240 Kda, which is similar to that reported by other investigators (Sullivan *et al.*, 1982; Lysterly *et al.*, 1986b); in addition ten minor and 27 faint bands under non-reducing conditions, and four minor and 31 faint bands under reducing conditions, of 38–440 Kda, were detected.

Although many workers have dissociated toxin A into one major sub-unit of c. 240 Kda, there has been relatively little work on, and few demonstrations of, other possible sub-unit components of toxin A. The work of Rihn *et al.* (1984) showing two sub-units of 41.5 and 16 Kda is difficult to

interpret as the parent molecule of 52 Kda does not correspond with the much larger protein described by all other workers. Lysterly *et al.* (1986a) reported the presence of one major band and numerous minor bands after PAGE in denaturing and reducing conditions. Some minor bands were demonstrated by Coomassie-blue staining (the only report to have noted these fragments with this staining procedure) but additional bands were seen after immunoblot analysis with a monoclonal antibody (PCG-4) which recognises the toxin-A binding site thought to be involved in adherence to mammalian cells and which neutralises the enterotoxic and HA properties of the toxin. Rautenberg and Stender (1986) used monospecific antibodies to the native toxin for immunoblotting and recognised one or two break-down products in the 35-Kda area after PAGE in denaturing and reducing conditions. Our own work clearly demonstrates that multiple fragments of the major sub-unit of toxin A (c. 240 Kda) are produced in denaturing and reducing conditions, and that these can be readily detected by silver staining. This may have advantages over immunoblotting techniques in that antibodies do not have to be available and non-immunogenic or non-exposed fragments should be detected. This latter point is exemplified in the findings of Rautenberg and Stender (1986) where few additional bands were detected in immunoblot experiments with monospecific antibody to toxin A.

We have also shown for the first time that, in denaturing but non-reducing conditions, a different pattern emerges. Among ten minor bands present in these conditions, the seven bands which are larger than the 240-Kda major band migrated to the position of the 240-Kda major band after reduction. The 255-Kda band also migrated to this position after reduction. The structural and biological relationship between those non-reduced fragments and those obtained under reduced conditions is unknown.

It is unlikely that the additional bands seen after denaturation and reduction are contaminants introduced by these procedures because fractions from a non-toxigenic strain corresponding to those in which toxin A would be expected yielded no detectable protein. It is also unlikely that the fragments detected resulted from trace amounts of contaminating proteases, because Lysterly *et al.* (1986a) have demonstrated that serine, metallo-, and sulphhydryl protease inhibitors do not prevent the appearance of multiple minor protein bands after denaturation and reduction.

Our findings support the hypothesis that toxin A

is composed of sub-units, and furthermore, we have shown for the first time that fragments apparently larger than the major sub-unit of 240 Kda can be detected. If the 240-Kda band is the major sub-unit, as proposed, it is possible that the larger fragments are composed of combinations of the major sub-unit with some of the smaller fragments.

Our results confirm that toxin A of *C. difficile* is a large complex molecule composed of sub-units. The structure-function relationship between the sub-unit component, and the three biological activities of the toxin, i.e., binding and HA, cytotoxicity, and enterotoxicity, are unknown, although there is good

evidence for the presence of multiple repeat units involved in binding (Lyerly *et al.*, 1988). Various combinations of these units with each other and with other functional units would explain many of the bands seen. Our detection and purification of HA rich-cytotoxin rich and HA poor-cytotoxin rich 'variants' of toxin A (unpublished) may help us to more readily assign a function to some of the sub-units.

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