Purification and characterisation of intracellular toxin A of *Clostridium difficile*

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Summary. After sonic disintegration of *Clostridium difficile* cells, intracellular toxin A was purified to homogeneity by thyroglobulin affinity chromatography (TGAC) followed by anion-exchange (Mono Q) by fast protein liquid chromatography (FPLC). High haemagglutinating (HA) activity was detected in TGAC-unbound fractions ($2^0/50 \mu\text{l}$), but not in TGAC thermal eluates ($2^0/50 \mu\text{l}$). The low HA titre of the thermal eluates was markedly increased to $2^5/50 \mu\text{l}$ after dialysis against 0.02 M Tris-HCl (pH 7.5). A disparity in the position of the peaks containing cytotoxic and HA activity was observed in the first Mono Q-FPLC step. Intracellular toxin A without HA activity was obtained by a second Mono Q-FPLC step. The M_r of the intracellular toxin A was estimated by polyacrylamide gel electrophoresis (PAGE) to be 580 kDa under non-denaturing conditions. The minimum doses of the toxin causing cytotoxicity, mouse lethality and enterotoxicity were 0.83 ng, 8.7 ng and 5 μg , respectively.

Introduction

Among the various virulence factors proposed for *Clostridium difficile*, e.g., toxins A and B,^{1–4} factors related to mucosal association,⁵ tissue degradative enzymes,⁶ fimbriae⁷ and capsule,⁸ toxin A has been considered to be the most important because of its enterotoxicity. The M_r values of toxin A in non-denaturing and denaturing conditions were reported to be 400–600 kDa and 200–250 kDa, respectively.^{1–4, 9, 10} This toxin has various biological activities such as enterotoxicity, vascular permeability-increasing activity, mouse lethality, cytotoxicity and haemagglutinating (HA) activity.^{1–4, 9} These findings were mainly obtained from extracellular toxin A released into culture medium, and few studies on intracellular toxin A have been performed. In the present study, intracellular toxin A was purified, and characterised biochemically and biologically.

Materials and methods

Bacterial strains and preparation of cell extract

A 4-ml volume of *C. difficile* strain VPI 10463 cultured anaerobically at 37°C for 18 h in liver broth was inoculated into 4 L of Brain Heart Infusion Broth

(BHI; Becton Dickinson Microbiology System, Cockeysville, MD, USA) supplemented with Na_2HPO_4 0.2% w/v (m-BHI) as described by Nakamura *et al.*,¹¹ and incubated anaerobically at 37°C for 2 days. The culture was centrifuged and the sediment was washed twice with 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.0 (TBS). The washed sediment was suspended in 60 ml of TBS and sonicated for 7.5 min with a Tomy ultrasonic vibrator (model UR-200P, 20 kc) (Tomy Seiko, Tokyo, Japan). The bacterial debris was removed by centrifugation at 12000 *g* for 20 min, and the supernate was filtered through a 0.22- μm membrane filter Millex-GV (Nihon Millipore, Yonezawa, Japan).

Bovine thyroglobulin affinity chromatography (TGAC)

The method was based on that of Krivan and Wilkins.⁹ Briefly, 60 ml of cell extract was applied to the column at 4°C. After washing the column with 30 ml of TBS at 4°C, thermal elution was performed by washing with 40 ml of pre-warmed TBS at 37°C. Fractions (5 ml) were collected and monitored for absorbance at 280 nm (A_{280}), cytotoxicity and HA activity as described below.

Anion-exchange chromatography

An anion-exchange gel, Mono Q (Pharmacia LKB Biotechnology, Uppsala, Sweden), incorporated into a fast protein liquid chromatography (FPLC) apparatus (Pharmacia LKB Biotechnology) was used as de-

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scribed previously.¹⁰ The sample was filtered (0.22- μ m membrane) before being applied to the column, and elution by a 0–0.4 M NaCl linear gradient in 20 mM Tris-HCl (pH 7.5) was performed at a flow rate of 0.5 ml/min. Each fraction (0.5 ml) was examined for A_{280} , cytotoxicity and HA activity.

PAGE

The method was based on that of Kamiya *et al.*¹⁰ The gels were silver stained (BioRad Laboratories, Richmond, CA, USA), according to the manufacturer's instructions (BioRad Bulletin 1089). An electrophoresis calibration kit (Pharmacia LKB Biotechnology) was used for estimation of M_r . Native PAGE in non-denaturing conditions was performed in a 4–15% gel. Samples were electrophoresed at 125 V for 18 h after initial electrophoresis at 8 mA for 30 min.

Cytotoxicity assay

Cytotoxicity was determined with baby hamster kidney (BHK-21/WI-2) cells as described by Nakamura *et al.*¹¹ The cytotoxic titre (CU)/50 μ l was expressed as the dilution that induced a 100% cytopathic effect after incubation at 37°C for 48 h. CU_{50} was calculated according to the results of six cytotoxic titrations of one specimen.

HA assay

This assay was based on the method described by Krivan *et al.*¹² with a slight modification in that a rabbit erythrocyte 1.5% suspension was used instead of 2.5%. Two-fold serial dilutions of toxin preparations (50 μ l) were performed with 0.05 M Tris-HCl, 0.1 M NaCl, pH 7.2, in the wells of U-bottom microtitration plates (Greiner, Nürtingen, Germany); 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 HA.

Mouse lethality

Serial two-fold diluted toxin preparations in saline (0.5 ml) were injected intraperitoneally into a mouse. The activity was expressed as 50% lethal dose (LD_{50}).

Rabbit ileal loop response test

Ligated intestinal loops (seven loops/rabbit with each loop 7–10 cm in length) were prepared in Japanese white rabbits (1.7–2.0 kg) according to the method of De and Chatterje.¹³ One-ml samples were injected into ligated ileal loops. The fluid which accumulated in each loop was examined 16 h after the

injection. The intensity of the response was expressed as the ratio of fluid volume (ml) accumulated to the length (cm) of the loop.

Protein assay

Protein was quantitated by the dye-binding method of Bradford¹⁴ with the BioRad protein assay kit (BioRad Laboratories). Bovine serum albumin (Wako Pure Chemical, Osaka, Japan) was used as a standard.

Results

Purification of intracellular toxin A

The extract of sonic disintegrated cells was applied to TGAC at 4°C followed by washing with 30 ml of TBS at 4°C, and thermal elution at 37°C of toxin A was performed (fig. 1.). The TGAC-unbound fractions (fr 2–13) had 2^{15} CU/50 μ l of cytotoxicity and 2^9 /50 μ l of HA activity, showing that almost no HA activity substance of the cell extract bound to thyroglobulin. The first fraction (fr 19) of the thermal eluates was shown to have high cytotoxicity (2^{11} CU/50 μ l) but low HA activity (2^0 /50 μ l). The thermal eluates (fr 19–22) were dialysed at 4°C overnight against 0.02 M Tris-HCl (pH 7.5) before Mono Q-FPLC. Interestingly, the HA titre of the thermal eluates was markedly increased to 2^5 /50 μ l after dialysis.

The thermal eluates (21 ml) were applied to Mono Q-FPLC and a 0–0.4 M NaCl gradient elution was performed (fig. 2). The fr 30–32 eluted at 0.24–0.26 M NaCl showed high cytotoxicity (2^{10-12} CU/50 μ l) coincident with the peak of A_{280} (0.40). In contrast, the highest HA titre (2^6 /50 μ l) was detected in fr 35 eluted at 0.28 M NaCl in spite of a very low A_{280} value, indicating a disparity in the position of the peaks displaying cytotoxic and HA activity. Cytotoxicity (2^6 CU/50 μ l) and relatively high HA activity (2^3 /50 μ l) were also detected in fr 37–39 eluted at 0.30–0.31 M NaCl, forming a shoulder in the profile of each activity. These fractions were followed by a coincidental decrease in cytotoxicity and HA activity. The cytotoxic fractions without HA activity (fr 30–32, 1.5 ml) were dialysed and applied again to Mono Q-FPLC (fig. 3). A peak showing cytotoxicity in fr 30–34 eluted at 0.24–0.27 M NaCl, had no HA activity at all. Highly purified cytotoxic fractions (fr 30–32) without HA activity were designated intracellular toxin A and analysed further.

Yield of purified intracellular toxin A

The yield of purified intracellular toxin A is shown in the table. The protein concentration of the purified toxin A preparation (1.5 ml) was 30 μ g/ml, showing specific activity of 1.21×10^6 CU/mg of protein. From TGAC thermal eluates, 28.7% of the cytotoxicity was

Fig. 1. Bovine thyroglobulin affinity chromatography (TGAC) of a cell extract of *C. difficile* after sonic disintegration. Cell extract (60 ml) was applied to the affinity column, and 5-ml fractions were collected at 4°C. From fraction 19 (↓), thermal elution was performed at 37°C. ●—●, cytotoxicity; ▲—▲, HA activity; —, A_{280} .

Fig. 2. Mono Q anion-exchange chromatography profile of the thermal eluates from TGAC; 21 ml of the eluates was applied to the Mono Q column. Each 0.5-ml fraction collected was examined for A_{280} (—), cytotoxicity (●—●) and HA activity (▲—▲); ----, molar concentration of NaCl.

Fig. 3. Mono Q anion-exchange chromatography profile of the intracellular toxin A preparation obtained by the first Mono Q-FPLC. Cytotoxic fractions (fr 30–32) without HA activity in the first Mono Q-FPLC were applied again to a Mono Q column after dialysis. Symbols are the same as in fig. 2.

Table 1. Purification yield of intracellular toxin A of *C. difficile*

Step	Volume (ml)	Protein content (mg/ml)	Total protein (mg)	Cytotoxicity (CU/ μ l)	Total cytotoxicity (CU)	Specific activity (CU/mg)	Fold purification	Percentage yield
Sonication	60	3.6	216	1.16×10^5	1.39×10^8	6.44×10^5	—	—
TGAC	21	0.020	0.420	4.50×10^3	1.89×10^5	4.50×10^5	1.00	100
First Mono Q	3	0.042	0.126	1.81×10^3	1.09×10^5	8.65×10^5	1.92	57.7
Second Mono Q	1.5	0.030	0.045	1.81×10^3	5.43×10^4	1.21×10^6	2.69	28.7

Cytotoxicity to BHK-21/WI-2 cells.

Fig. 4. Analysis of intracellular and extracellular toxin A by native PAGE (4–15%) developed by silver stain. Lane 1, mol. wt markers: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), bovine serum albumin (67 kDa); 2, purified intracellular toxin A (2.5 μ g); 3, extracellular toxin A (2.5 μ g) purified by the methods of Kamiya *et al.*¹⁰

recovered and 2.69-fold purification was achieved in the final preparation.

PAGE

The final preparation of intracellular toxin A was analysed by native PAGE to check the purity and M_r in non-denaturing conditions (fig. 4). The intracellular toxin A preparation exhibited one band (580 kDa) developed by silver stain, indicating that the toxin was highly purified. The M_r of extracellular toxin A was 40 kDa lower than that of the intracellular form.

Biological activity of purified intracellular toxin A

The biological activity of purified intracellular toxin A was examined. CU_{50} cytotoxicity assays and LD_{50} in mouse lethality assays were 0.83 ng and 8.7 ng, respectively; 5 μ g of the toxin induced fluid accumulation with haemorrhage in the ligated ileal loop but 1 μ g had no effect.

The purified toxin A had no HA activity even at a

concentration of 15 μ g/ml. Expression of HA activity of the toxin *in vitro* with ileal loop contents was attempted. A 50-cm long ileal loop was washed thoroughly with 10 ml of sterile saline. The toxin (50 μ g/ml) was mixed with the same volume of the washed contents, incubated at 37°C for 0.5, 1, 2, 4 and 8 h, and then subjected to HA assay. Cytotoxicity remained but HA activity was not detected in any of the mixtures.

Discussion

On the basis of the presence of the trisaccharide receptor (Gal α 1-3Gal β 1-4GlcNAc) for toxin A on bovine thyroglobulin,¹² TGAC was developed for the purification of toxin A released into the culture medium (extracellular toxin A).^{9,10} This is an excellent single step method which can be further improved if followed by anion-exchange chromatography.¹⁰ In this study, toxin A in the sonic extract of cells (intracellular toxin A) was purified by TGAC followed by successive anion-exchange chromatography.

The TGAC profile of the cell extract was different from that of extracellular toxin A reported before;¹⁰ in cell extracts, toxin A with poor HA activity was eluted at 37°C and HA activity was present in TGAC-unbound fractions eluted at 4°C. In considering that toxin A binds through binding sites in the HA-activity component to the trisaccharide receptor in TGAC, it would be expected, theoretically, that the minimum numbers of binding sites needed for binding to thyroglobulin and for causing HA would be one and two, respectively. Therefore, we considered that the toxin A with poor HA activity in thermal eluates would express binding sites sufficient to bind to thyroglobulin but not to cause HA by its configuration, and that HA activity in TGAC-unbound fractions may have no relationship to toxin A. When the TGAC-unbound fractions were analysed by Mono Q column chromatography, HA activity without cytotoxicity was eluted faster than toxin A, with high cytotoxicity shown by fractions at the position of toxin B, supporting the postulate outlined above. We are now attempting to purify the HA-positive substance. Recently, Kamiya and Borriello¹⁵ obtained a highly cytotoxic but weakly haemagglutinating variant

of toxin A, which they designated toxin A', from TGAC-unbound fractions of culture filtrate. In culture filtrates, due to the presence of larger amounts of haemagglutinating form of toxin A, poor or non-haemagglutinating forms would pass more readily through TGAC.

The increase in HA activity of the thermal eluates after dialysis against 0.02 M Tris-HCl (pH 7.5) was demonstrated. It is possible that small molecules inhibiting HA activity might be removed by dialysis. It is also possible that a significant decrease of NaCl concentration might induce configurational change of the intracellular toxin A, resulting in the increase of HA titre.

On Mono Q-FPLC, the toxin was eluted with 0–1.0 M NaCl gradient at a flow rate of 1 ml/min (each fraction, 1.0 ml). Under these conditions, the peaks of HA and cytotoxic activities were different, indicating that there might exist conditions whereby these two peaks can be separated more distinctively. Therefore, we examined several chromatographic conditions on Mono Q-FPLC, and, as shown in this study, could clearly separate two peaks, when the toxin was eluted with 0–0.4 M NaCl gradient at a flow rate of 0.5 ml/min (each fraction, 0.5 ml). On the second Mono Q-FPLC under these conditions, the highly purified intracellular toxin A which had no detectable HA activity but was highly cytotoxic (2^{12} CU/50 μ l) was obtained. However, it should be noted that there were fractions with cytotoxicity and HA activity and in these fractions cytotoxicity and HA activity decreased concomitantly on the first Mono Q-FPLC. These findings seem to indicate that, although a non-haemagglutinating form represents most of the intracellular toxin, a haemagglutinating form might also be present. As toxin A with poor or no HA activity and the toxin with high

HA activity are present in culture filtrate,¹⁵ and this study has shown that toxin A without HA activity is present in relatively large amounts in cell extracts, it might be speculated that toxin A is synthesised in cells as the non-haemagglutinating form and then processed to express HA activity.

The M_r of the intracellular toxin A with non-detectable HA activity in non-denaturing conditions was estimated to be 580 kDa which is slightly larger than that of extracellular toxin A; both toxin A with potent HA activity and toxin A with poor or non-detectable HA activity have a M_r of 540 kDa.^{10, 15} The difference was ascertained when intracellular toxin A and extracellular toxin A with potent HA activity were analysed simultaneously by native PAGE. This finding suggests that a small molecule (40 kDa) may act as an HA inhibitor and be cleaved during release of toxin A from the cells into the culture medium.

The minimum dose (CU₅₀, 0.83 ng) of intracellular toxin A causing cytotoxicity was within the range of previous reports.^{1, 3, 16} The minimum dose (LD₅₀, 8.7 ng) causing mouse lethality was less than that reported for extracellular toxin A.^{1–3} This may be due partly to a difference in the unit between LD₅₀ and minimum lethal dose (MLD). The intracellular toxin A (5 μ g) without HA activity induced fluid accumulation with haemorrhage in the ileal loop test. We could not activate the toxin to show HA activity by contact with ileal loop contents *in vitro*. However, these findings do not mean that enterotoxicity and HA are unrelated. It seems that toxin A without HA activity possesses sufficient binding sites to bind to intestinal epithelial cells but not to cause HA.

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