Purification of *Clostridium difficile* Toxin A by Affinity Chromatography on Immobilized Thyroglobulin

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An efficient, single-step method for isolating highly purified toxin A from *Clostridium difficile* culture filtrates is described. The purification procedure was based on the affinity binding and release of toxin A to bovine thyroglobulin conjugated to agarose beads. The toxin strongly bound at 4°C to the carbohydrate binding determinant Gal α 1-3Gal β 1-4GlcNAc, a carbohydrate sequence which occurs on bovine thyroglobulin. Toxin bound to thyroglobulin at 4°C, allowing its separation from the culture filtrate and contaminating proteins during the purification scheme. The toxin was eluted by increasing the temperature to 37°C. The toxin-binding capacity was related to the amount of thyroglobulin immobilized on the gel: an affinity column containing 15 mg of bovine thyroglobulin per ml of gel bound 0.53 mg of toxin A per ml of gel. The percent recovery of purified toxin ranged from 56 to 80% and was inversely related to the amount of thyroglobulin coupled to the gel. The affinity-purified toxin was homogeneous as judged by crossed immunoelectrophoresis and gradient polyacrylamide gel electrophoresis and was immunologically identical to toxin A purified by conventional methods as determined by immunodiffusion analysis. The biochemical, hemagglutinating, and toxic properties of the toxin were preserved after affinity chromatography and were comparable with those of toxin A purified by conventional methods.

Clostridium difficile is now widely accepted to be the major etiological agent of pseudomembranous colitis. The pathogenic effect of the organism is related to the production of two toxins: an enterotoxin designated toxin A and a potent cytotoxin designated toxin B (3, 7, 10, 20). Both toxins are large, heat-labile, cytotoxic proteins that are lethal in animals. Unlike toxin B, toxin A elicits a hemorrhagic fluid response when injected into rabbit ileal loops (2, 12, 20, 21). Toxin B does not cause fluid accumulation in intestinal loops but is at least 1,000-fold more cytotoxic than toxin A (5, 15, 20, 21).

Toxin A can be separated from toxin B by anion-exchange chromatography or (NH₄)₂SO₄ precipitation and batch ion exchange and further purified to homogeneity by isoelectric precipitation at pH 5.5 to 5.6 (13, 20). Other methods reported for purifying toxin A have included gel filtration, hydrophobic interaction chromatography, preparative electrophoresis, and tangential flow filtration followed by fast-protein liquid chromatography (2, 13, 16, 18, 20). These methods, however, either require 2 to 3 days to obtain pure toxin A or require expensive equipment. We recently reported that the carbohydrate binding determinant for toxin A is a glycoconjugate containing the nonreducing terminal sequence Gal α 1-3Gal β 1-4GlcNAc (9). We showed that specific binding of toxin A to rabbit erythrocytes and to brush border membranes of hamsters was greatly enhanced at cold temperatures. The Gal α 1-3Gal β 1-4GlcNAc sequence also occurs as several branched copies in bovine thyroglobulin (17), and we showed that this glycoprotein inhibited specific agglutination of rabbit erythrocytes by the toxin (9). We concluded that the behavior of toxin A was similar to what has been observed with cold-agglutinating antibodies.

Studies on cold agglutinins and their carbohydrate receptors have indicated that binding is dependent on temperature and receptor density (22, 23). We have taken advantage of the binding of toxin A at cold temperatures to its carbohydrate binding determinant to purify large amounts of toxin using a thermal affinity chromatographic procedure. A method using immobilized bovine thyroglobulin for the purification of *C. difficile* toxin A from crude culture filtrate in one step is described here.

(A preliminary report of this work has been presented [H. C. Krivan, Annu. Meet. Am. Soc. Microbiol. 1987, B212, p. 60].)

MATERIALS AND METHODS

Bacterial strain and culture filtrate. A highly toxigenic strain of *C. difficile*, VPI 10463, was obtained from the culture collection of the Department of Anaerobic Microbiology at Virginia Polytechnic Institute and State University, Blacksburg. The organism was grown in brain heart infusion dialysis flasks similar to the method described by Sterne and Wentzel (19). After incubation at 37°C for 72 h, cells were removed by centrifugation. Culture filtrate was obtained by filtration of the culture supernatant fluid through 0.45- μ m (pore size) membranes.

Purification of toxin A by precipitation in acetate buffer. Toxin A was purified by anion-exchange chromatography and precipitation in acetate buffer at pH 5.5 as described by Sullivan et al. (20). This preparation was designated reference toxin A.

Preparation of immobilized thyroglobulin. Bovine thyroglobulin (500 mg; Sigma Chemical Co., St. Louis, Mo.) was dissolved in 100 ml of 0.1 M MOPS buffer (morpholinepropanesulfonic acid; pH 7.0), centrifuged (8,000 $\times$ g) to remove insoluble particulate matter, and filtered through a 0.22- μ m (pore size) membrane filter. The glycoprotein solution was then reacted with 20 ml of activated Affi-Gel 15 (Bio-Rad Laboratories, Richmond, Calif.) overnight at 4°C on a shaker. The remaining active sites on the gel were

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FIG. 1. Elution profile obtained after thermal affinity chromatography of toxin A from *C. difficile* culture filtrate. Culture filtrate was passed through a bovine thyroglobulin-Affi-Gel 15 column (15 by 50 mm) prepared as described in Materials and Methods. Elution was effected by increasing the temperature to 37°C. Fractions (5 ml) were collected and monitored for A_{280} (○) and cold-hemagglutinating activity (●).

blocked with 0.1 M ethanolamine for 30 min at 4°C. Approximately 90% of the bovine thyroglobulin coupled to the gel, as judged by estimating the protein content of washings obtained during the preparation procedures.

The coupled beads (ca. 20 mg of thyroglobulin per ml of gel) were packed in a glass column (15 by 50 mm) and washed at 37°C with 20 bed volumes each of prewarmed 0.1 M glycine-sodium hydroxide buffer containing 0.5 M NaCl, pH 10.0 (basic buffer), and 0.1 M glycine hydrochloride buffer containing 0.5 M NaCl, pH 2.0 (acid buffer), to ensure that free thyroglobulin did not remain ionically bound to the gel. The washing cycle was then repeated at 4°C with TBS (0.05 M Tris buffer containing 0.15 M NaCl, pH 7.0) to equilibrate the column before affinity chromatography. For binding capacity experiments, various amounts of bovine thyroglobulin, bovine serum albumin (BSA), and human orosomucoid (Sigma) were coupled to Affi-Gel 15 by the same procedure.

Affinity chromatography. Crude *C. difficile* culture filtrate cooled to 4°C was applied to the column at 4°C. The unbound protein (effluent) was monitored for A_{280} and cold-hemagglutinating activity. After being washed with 10 to 20 bed volumes of cold TBS, the column was warmed to 37°C and toxin A was eluted with 3 to 4 bed volumes of prewarmed TBS at 37°C. Eluted toxin was concentrated by ultrafiltration through an XM-300 membrane (Amicon Corp., Lexington, Mass.) and filtered through a 0.22- μ m (pore size) membrane filter (Millipore Corp., Bedford, Mass.). To reuse the column, the gel was washed with 10 bed volumes of acid buffer at room temperature followed by 10 bed volumes of TBS, pH 7.0, at 4°C to equilibrate the column.

Assays. (i) **Protein assay.** Protein concentration was estimated as described by the method of Bradford (4) by using the Bio-Rad protein assay kit. Bovine gamma globulin was the standard.

(ii) **Cytotoxicity assay.** The cytotoxicity of affinity-purified toxin A preparations was determined with Chinese hamster ovary cells (CHO-K1) as described previously (6). The minimum dose yielding cell rounding in 100% of the cell population after 24 h was defined as the 100% tissue culture infective dose.

(iii) **Enterotoxigenicity assays.** Affinity-purified toxin preparations (1 ml) in TBS were tested for enterotoxigenicity by

using rabbit ileal loops as described previously (12). Enterotoxigenicity was also tested by giving the toxin intragastrically to hamsters as previously described (14). Enterotoxigenicity was expressed as the minimum dose of toxin necessary to cause an intestinal hemorrhagic fluid response.

(iv) **Mouse lethality assay.** Serial twofold dilutions (0.5 ml) of affinity-purified toxin A in TBS were injected intraperitoneally into male ICR outbred mice (8 to 10 weeks old; Dominion Laboratories, Dublin, Va.) as described previously (20). The minimum lethal dose of toxin which caused death in mice by 18 h was defined as the 100% lethal dose.

(v) **Hemagglutination assay.** The cold-hemagglutinating activity of toxin A with rabbit erythrocytes (Hazelton Dutchland, Inc., Denver, Pa.) was determined as described previously (9). Titers were expressed as the reciprocal of the highest dilution of toxin A which caused agglutination in microtiter wells.

Immunological methods. Crossed immunoelectrophoresis was performed on glass plates (5 by 5 cm) in 1.2% low electroendosmotic agarose (Sigma) in 125 mM Tris-Tricine buffer, pH 8.6, according to the method of Axelsen et al. (1). The agarose used for the second dimension contained 0.1 ml of goat antiserum produced against crude culture filtrate of *C. difficile* VPI 10463 (6). Ouchterlony double-immunodiffusion analysis was done in 1% low electroendosmotic agarose in borate-EDTA buffer (20 mM sodium borate-150 mM NaCl-5 mM EDTA-15 mM NaN₃), pH 8.2. The gels were incubated 48 h in a moist chamber at 4°C.

TABLE 1. Binding capacity and recovery of *C. difficile* toxin A on agarose gels containing different amounts of bovine thyroglobulin or other proteins

Ligand (amt bound, [mg]/4 ml of resin) ^a	Binding capacity (mg) ^b	Amt eluted (mg)	% Recovery
No protein (0)	0	0	0
Bovine TG (6)	0.60	0.48	80
Bovine TG (60)	2.12	1.49	70
Bovine TG (140)	5.68	3.20	56
Orosomucoid (100)	0	0	0
BSA (100)	0	0	0

^a TG, Bovine thyroglobulin.

^b Calculated by analyzing effluent by hemagglutination at 4°C.

FIG. 2. Analysis of toxin preparations by crossed immunoelectrophoresis. The upper portion of the gel in each plate contained 0.1 ml of goat antiserum to *C. difficile* VPI 10463 culture filtrate. (Panel 1) The well contained 60 μ g of 10463 culture filtrate. The toxin A immunoprecipitin arc is indicated by the arrow. (Panel 2) The well contained 50 μ g of 4°C effluent from the thyroglobulin Affi-Gel 15 column. (Panel 3) The well contained 50 μ g of toxin A eluted at 37°C.

Gradient polyacrylamide gel electrophoresis. Analysis by gradient polyacrylamide gel electrophoresis was done in 4 to 30% concave precast gels (Isolab, Inc., Akron, Ohio) in Tris-borate buffer (90 mM Tris–80 mM boric acid–2.5 mM disodium EDTA), pH 8.4. Gels were stained with Coomassie blue R-250. High-molecular-weight standards were purchased from Pharmacia Fine Chemicals, Piscataway, N.J.

RESULTS

Affinity chromatography on immobilized thyroglobulin. Culture filtrate (100 ml, 1.2 mg of protein per ml) was passed at 4°C through the thyroglobulin affinity column (15 by 50 mm), and then the column was washed with cold buffer (TBS, pH 7.0). The column was subsequently warmed to

37°C and eluted with buffer (TBS, pH 7.0) at the same temperature. Under these conditions all of the toxin A appeared to be removed from the culture filtrate as indicated by the absence of hemagglutination activity in the unbound column effluent (Fig. 1).

Most of the toxin was eluted at 37°C (remaining A_{280} , <0.01) with approximately 3 bed volumes of prewarmed TBS (Fig. 1). A small amount of residual toxin A slowly eluted from the column (hemagglutination titer of 4) and was removed by treating the column with acid buffer. Repeated cycles on the affinity thyroglobulin column gave very similar results, and the toxin-binding capacity of the column was not influenced by multiple acid washings.

Binding capacity and toxin recovery. Homogeneous preparations of toxin A (0.1 mg/ml) purified by precipitation in acetate buffer (reference toxin A) were passed through columns containing various amounts of immobilized thyroglobulin to determine the relationship between the ligand concentration and the capacity of the column to bind toxin A (Table 1). Toxin A did not bind to the gel in the absence of thyroglobulin or to gels coupled with other proteins. The capacity of each thyroglobulin column to bind toxin A increased as the concentration of thyroglobulin immobilized in the gel increased and reached a maximum of 5.68 mg/4 ml of resin. The estimated recovery varied between 56 and 80%. The recovery of toxin A was inversely related to the con-

FIG. 3. Analysis of toxin preparations by gradient polyacrylamide gel (4 to 30%) in Tris-borate buffer, pH 8.2. Samples included the following (lanes): 1, *C. difficile* VPI 10463 culture filtrate (120 μ g); 2, culture filtrate effluent at 4°C (110 μ g); 3, thermal eluted toxin A (15 μ g); 4, reference toxin purified by precipitation in acetate buffer (15 μ g); and 5, the high-molecular-weight markers (12.5 μ g) thyroglobulin (M_r , 669,000), ferritin (440,000), catalase (232,000), lactate dehydrogenase (140,000), and BSA (67,000).

FIG. 4. Analysis of toxin preparations by Ouchterlony double diffusion. The center well contained 20 μ l of antiserum to *C. difficile* VPI 10463 culture filtrate. Well 1 contained 30 μ g of reference toxin A purified by precipitation in acetate buffer; well 2 contained 30 μ g of toxin A purified by thermal affinity chromatography.

centration of bovine thyroglobulin coupled to the gel (Table 1).

Characterization of affinity-purified toxin A. Unbound and eluted fractions were pooled separately and concentrated before analysis by crossed immunoelectrophoresis and gradient gel electrophoresis. These analyses showed that only toxin A bound to the column and that it was eluted at 37°C in pure form (Fig. 2 and 3). This was also confirmed by the absence of hemagglutination activity before thermal elution (Fig. 1).

To determine whether the affinity-purified toxin A was identical to toxin A purified by conventional methods, we analyzed a reference toxin A preparation (20) with our thermal eluted toxin A. Ouchterlony double-immunodiffusion analysis (Fig. 4) showed a reaction of identity.

The enterotoxic, cytotoxic, and lethal activities of the thermal eluted toxin were within the range of values previously reported for toxin A (2, 12–14, 20, 21). Purified toxin A injected into rabbit ileal loops (5 µg) or administered intragastrically (75 µg/kg of body weight) elicited an intestinal hemorrhagic fluid response. The 100% tissue culture infective (CHO-K1) and lethal (mouse) doses were 10 and 75 ng, respectively. The affinity-purified toxin A also showed no difference in the titer of agglutination of rabbit erythrocytes when compared with the reference toxin A preparation.

DISCUSSION

A simple one-step purification of *C. difficile* toxin A was achieved on an immobilized bovine thyroglobulin column by affinity chromatography. The procedure is based on the presence of terminal Galα1-3Galβ1-4GlcNAc, a carbohydrate sequence that occurs as a highly branched structure in bovine thyroglobulin and which also is found on the cell surface of rabbit erythrocytes (8, 17). We have previously shown that toxin A has a temperature-dependent affinity for this carbohydrate sequence in bovine thyroglobulin and on rabbit erythrocytes and that specific agglutination of rabbit erythrocytes by the toxin was inhibited by bovine thyroglobulin (9). Toxin binding is enhanced and stable at 4°C, but the toxin dissociates from its carbohydrate-binding site at 37°C in a manner similar to what has been observed with cold agglutinins. The unusual behavior of the toxin at 4 and 37°C allowed us to purify the toxin by a similar procedure with rabbit ghost membranes (H. C. Krivan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, B60, p. 34).

It has been reported that toxin A could be affinity purified by using agarose gels and that the toxin could be eluted with galactose (11). We have not been able to confirm these experiments. Our findings indicate that toxin A does not bind to agarose beads or other proteins (BSA and orosomucoid) coupled to Affi-Gel 15 agarose unless the glycoconjugate contains the carbohydrate sequence Galα1-3Galβ1-4GlcNAc.

The thyroglobulin thermal affinity purification procedure offers several advantages over other purification methods for toxin A: (i) it is an efficient, single-step method for isolating highly purified toxin A; (ii) the column is reusable; (iii) thermal elution is a gentle process—i.e., all of the biochemical, immunological, and toxic properties of the toxin are preserved; and (iv) the thyroglobulin column offers an advantage over the use of rabbit erythrocytes or ghosts in that there is no possibility that the final product will be contaminated with hemoglobin or membrane components; however, as with any affinity chromatography procedure, ligand leak-

age does occur, and occasionally a small amount of bovine thyroglobulin may leak from the gel.

There are two important factors to be considered when preparing the affinity column. First, the source of the thyroglobulin is critical and must be of bovine origin. We have previously shown that human thyroglobulin does not bind toxin A (9) because of the complete absence of terminal α-galactosyl residues present in this glycoprotein (17). Thyroglobulin from other species contain various amounts of terminal α-galactosyl residues (17), but bovine thyroglobulin contains the highest amount. Also, the concentration of bovine thyroglobulin immobilized on the agarose beads can influence the percent recovery of toxin A. Although the toxin-binding capacity is related to the amount of coupled thyroglobulin in the gel, the percent recovery appears to be inversely related (Table 1). This observation may be related to the density of the carbohydrate binding sites in the gel. It has been postulated for cold agglutinins that receptor density and multivalency influence their thermal amplitude because multivalent interactions are inherently cooperative (22, 23); i.e., when the receptor density is high enough, cold agglutinins can bind at 37°C. The lower percent recovery of toxin A from our columns containing a high concentration of thyroglobulin may be due to the same phenomenon, and hence less toxin was eluted off the column at 37°C. We therefore recommend the use of 15 to 20 mg of bovine thyroglobulin immobilized per ml of gel. Our highly toxigenic strain of *C. difficile* yields high concentrations of toxin A (ca. 0.1 mg/ml of culture filtrate), and by using such columns containing 10 ml of gel we can purify approximately 0.07 mg of toxin A per ml of culture filtrate.

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