Affinity Chromatography Purification of *Clostridium perfringens* Enterotoxin

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Anti-enterotoxin immunoglobulins immobilized on CH-Sepharose or CNBr-Sepharose were used for affinity chromatography purification of *Clostridium perfringens* enterotoxin. Cell extracts containing enterotoxin or partially purified toxin preparations were applied to the column and nonspecifically-bound protein was eluted. NaOH was used to elute specifically bound toxin. The purity of enterotoxin purified by Sephadex G-100 chromatography followed by affinity chromatography appears similar to toxin highly purified by conventional means. The procedure can be used successfully for the rapid (less than 2 h) purification of small amounts of enterotoxin.

*Clostridium perfringens* type A is one of the most common causes of human food poisoning (11, 25). The enterotoxin responsible for *perfringens* food poisoning is synthesized only during sporulation (15, 16) and is released into the medium upon lysis of the sporangium (12). Frieben and Duncan (18) have shown that the enterotoxin is a structural component of the spore coat.

The current procedure used in our laboratory for purifying the enterotoxin of *C. perfringens* involves sequential elution of a crude cell extract from a Sephadex G-100 column, a Cellex-T anion exchange column, and an hydroxyapatite column (28). Immunodiffusion to identify toxin fractions and concentration and dialysis prior to application of toxin on subsequent columns are required.

Affinity chromatography (5–7, 23, 29) has been used successfully for the rapid purification of a large number of proteins. This paper reports the purification of *C. perfringens* enterotoxin using anti-enterotoxin immunoglobulins immobilized on a Sepharose matrix.

**MATERIALS AND METHODS**

**Antiserum and immunoglobulin preparation.** Antiserum was prepared, using previously purified enterotoxin (28), in 2.0- to 2.5-kg female New Zealand white rabbits. Rabbits were injected intramuscularly with 0.25 mg of enterotoxin in either Freund complete adjuvant (first injection) or Freund incomplete adjuvant (subsequent injections). A total of four injections were made at 6-week intervals. Rabbits were bled every 2 weeks from the ear vein or artery using a bleeding apparatus (Belloco) and a pump to apply suction. Blood was allowed to clot for 1 h at 37°C and then incubated at 7°C for several hours. Serum was removed, clarified by centrifugation, and frozen at -20°C. Immunoglobulins were fractionated from sera by ammonium sulfate precipitation using the procedure of Hebert et al. (21).

**Cell extracts.** *C. perfringens* strain NCTC 8339 was routinely kept frozen in cooked meat medium (Difco) at -20°C. Thawed, cooked meat cultures were used to inoculate 10 ml of fluid thiglycollate medium (Difco). The cultures were then heat shocked for 20 min at 75°C and incubated at 37°C for 8 h. These tubes were used to inoculate six 50-ml tubes of fluid thiglycollate, which were incubated at 37°C for another 8 h. Each 50-ml culture was used to inoculate 5 liters of Duncan-Stang sporulation medium (14). After incubation for 8 h at 37°C, the sporulating cells were harvested at 22,400 x g using a Beckman J-21B centrifuge equipped with continuous flow apparatus. Cells were resuspended to about 1% of their original volume and sonified for 10 min with a Biosonic IV Sonifier (Bronwill). The sonic extract was centrifuged at 17,300 x g for 30 min and the supernatant containing the enterotoxin was stored frozen at -20°C in 2-ml volumes.

**Gel chromatography.** Twenty grams of Sephadex G-100 (Pharmacia) was swollen for 3 days in 0.05 M tris(hydroxymethyl)aminomethane buffer (pH 7.6) with 0.1 M KCl and 0.02% azide as a preservative. The gel was poured into a K26/70 column (Pharmacia) and equilibrated with the same buffer. Four milliliters of cell extract (20.8 mg of protein/ml) in 30% sucrose was added to the column. Protein was eluted from the column using the same buffer at a 10-cm pressure head; fractions of 7.5 ml were collected using a fraction collector. Toxin-containing fractions, as determined by immunodiffusion, were pooled and constituted Sephadex G-100-purified enterotoxin.

**Affinity columns.** Columns were prepared using either CH-Sepharose 4B or CNBr-Sepharose 4B (Pharmacia). One gram of dry gel was used per column, giving a bed volume of 4 ml for CH-Sepharose and 3.5 ml for CNBr-Sepharose. For CH-Sepharose columns the dry material was swollen and washed on a sintered glass filter with 200 ml of 0.5 M NaCl. Forty milli-
grams of 1-ethyl-3-(3-dimethylamino propyl)carbodiimide-hydrochloride (Bio-Rad) was dissolved in 4 to 5 ml of water and the pH was adjusted to 4.5 with 0.5 N HCl. The gel, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-hydrochloride, and immunoglobulins (10 to 35 mg) were mixed in a beaker and the pH was maintained between 4.5 and 6 for 1 h. The mixture was then put in a screw-cap test tube and rotated end over end for 24 h at room temperature. The gel was washed with distilled water until the absorbance at 280 nm of the wash was negligible. The wash was dialyzed against distilled water and quantitated for any immunoglobulins not coupled. The amount of protein coupled was calculated as the difference between protein applied and noncoupled protein. The coupling procedure was repeated with 10 ml of 1 M ethanolamine in place of immunoglobulins; this was done to block any remaining carboxyl groups on the column that might later result in nonspecific protein adsorption. The gel was then washed with distilled water followed by 10 mM phosphate buffer containing 0.5 M NaCl and 0.02% azide (equilibration buffer). The gel was then washed with this buffer and poured into a K 9/15 column (Pharmacia). The column was equilibrated with 10 mM phosphate buffer containing 0.5 M NaCl and 0.02% azide prior to each run.

CNBr-Sepharose was swollen and washed with 1 mM HCl and the immunoglobulins (24 mg) in NaHCO₃ (0.1 M, pH 8.3) were coupled by mixing end over end for 2.5 h. The gel was washed with NaHCO₃ (0.1 M containing 0.5 M NaCl, pH 8.3) until absorbance at 280 nm was negligible. Ethanolamine (1.0 M, pH 8) was added and the mixture was rotated end over end for 2.5 h. The gel was then washed five times each with carbonate buffer and acetate buffer (0.1 M, pH 4), both containing 0.5 M NaCl. The gel was mixed with the equilibration buffer, poured into a K 9/15 column, and equilibrated. A peristaltic pump (Gilson Minipuls II) was used to elute protein from the column at a flow rate of 0.3 ml/min. One-milliliter fractions were collected using a Gilson Race Track fraction collector equipped with an ultraviolet monitor and a recorder to monitor and record absorbance at 280 nm.

Cell extract or G-100-purified toxin was loaded on the column and washed through with equilibration buffer until the recorder pen reached the base line. Eluant (usually 5 to 7 ml of 0.01 N NaOH) was added, followed by equilibration buffer. Eluted fractions were collected into 0.5 ml of 0.2 M phosphate buffer (pH 7.0) to expose the toxin to alkali for a minimum length of time.

Immunodiffusion. Toxin-containing fractions were identified by precipitin reactions in agar gels as previously described (27). Reactions were observed after incubation overnight, 24 and 48 h at room temperature.

Disc gel electrophoresis and gel immunodiffusion. Gel electrophoresis was performed on 7% acrylamide gels with pH 9.5 tris(hydroxymethyl)amino- methane glycine as the running buffer. Gels were run in duplicate with and without stacking gels. One gel was stained with Coomassie blue G-250 and the other was subjected to immunodiffusion against anti-enterotoxin serum as described by Stark and Duncan (28).

Stained gels were scanned on a Zeineh soft laser densitometer (Biomed Instruments) using either the laser beam or white light.

Biological activity. Biological activity was assayed as erythema units using guinea pigs, as previously described (27).

Electroimmunodiffusion. Electroimmunodiffusion as described by Duncan and Somers (13) was used for quantitation of enterotoxin.

Protein determination. Protein was quantitated using the method of Lowry et al. (24).

Concentration of column fractions. Column fractions were concentrated under nitrogen using an Amicon standard cell 12 and a PM 10 membrane.

RESULTS

Figure 1 shows a typical elution profile of enterotoxin from a CH-Sepharose 4B affinity column loaded with cell extract. The first peak represents cell extract proteins that are not adsorbed to the column. The arrow indicates the point at which NaOH is applied; the second peak is purified toxin.

The elution profile of toxin eluted from the same column loaded with G-100-purified toxin is shown in Fig. 2.

CNBr affinity columns produced similar profiles except that the second (toxin) peak was broader and flatter.

The appearance of toxin in the first peak, as evidenced by immunodiffusion precipitin lines, was used as an indication that the capacity of
the column had been exceeded. The amount of material loaded on a column was adjusted accordingly to avoid overloading the column.

Re-equilibration of the above column with 0.1 M phosphate buffer (pH 7.2) and elution of toxin from G-100-purified toxin by addition of 0.2 M NaCl, followed by elution with NaOH, resulted in the elution curve shown in Fig. 3. Immunodiffusion revealed toxin in peaks 1 and 3 but not in peak 2, although peak 2 showed slight erythemal activity in the guinea pig; this erythemal activity was neutralizable by anti-enterotoxin serum. A CNBr affinity column equilibrated with 0.02 M phosphate buffer (pH 6.9) and eluted with the same buffer containing 0.5 M NaCl, as described by Cukor et al. (8), followed by NaOH, showed similar properties: toxin was detected serologically in peaks 1 and 3 but not in peak 2. However, a very faint band in the proper location for toxin protein was detected when peak 2 was examined by gel electrophoresis.

Eluted toxin from a single column was examined by disc gel electrophoresis to assess purity. Figure 4a shows a typical gel and a laser scan of the gel; Fig. 4b shows the gel immunodiffusion pattern.

Toxin fractions from several column runs were pooled, concentrated, and examined on disc gels (Fig. 5). The two upper bands seen in gel A were routinely observed when cell extracts were used as the source of enterotoxin. G-100 column chromatography apparently removes these proteins, as the bands were not seen in gels of toxin from G-100-purified toxin. These upper bands do not produce precipitin lines against anti-enterotoxin serum and appear to be contaminants.

Recovery of enterotoxin as biologically active toxin and total enterotoxin protein is shown in Table 1. The results indicate that a great deal of biological activity is lost on exposure to NaOH. Decreasing the pH of the NaOH from 11 to 9 had completely abolished its ability to elute active protein from the column.

It can also be seen in Table 1 that all the enterotoxin applied to the column is not recovered. Increasing the amount of NaOH to elute more toxin resulted in decreased biological activity. Recovery of enterotoxin protein can be increased by running 2 volumes of NaOH, separated by a volume of equilibration buffer, through the column.

A comparison of protein patterns on disc gel loaded with crude cell extract, G-100-purified toxin, highly purified toxin from hydroxylapatite columns, and affinity chromatography-purified toxin (Fig. 6) indicates the relative purity of the various preparations. The purity of enterotoxin purified by Sephadex G-100 chromatography followed by affinity chromatography appeared similar to highly purified toxin from hydroxylapatite columns (28).

**DISCUSSION**

Previous attempts in our laboratory to use affinity chromatography for purification of enterotoxin had been unsuccessful; none of the reagents used eluted the antigen (enterotoxin). These reagents included thiocyanate (9, 10), formic acid (17, 20), glycine-hydrochloride buffer (19, 22), HCl (4), acetic acid (5), and iodide (10). Yanagida (30) had been unsuccessful in eluting antigen from an immunoadsorb-
FIG. 4. (a) Disc gel electrophoresis and gel scan (laser) of toxin eluted from an affinity column loaded with 0.5 mg of G-100-purified toxin. (b) Gel immunodiffusion of a duplicate gel described in (a).
ent column with several of the same reagents but had succeeded with 0.01 N NaOH. Thus far 0.01 N NaOH has been the only reagent to successfully desorb enterotoxin from affinity columns prepared in this laboratory. Since this reagent causes some loss of biological activity it will be necessary to investigate additional reagents to find a more suitable eluant. Coupling of immunoglobulins to CH-Sepharose should be done in distilled water; buffers with amino, carbonate, or phosphate groups should not be used since these groups compete with the coupling reaction (1) and introduce charged groups onto the matrix which can lead to nonspecific binding. Azide should not be present in coupling buffers for CNBr- or CH-Sepharose columns, since it effectively inhibits ligand binding (26).

It was necessary to dialyze all toxin fractions before determining protein by the Lowry method; eluted fractions were collected into tubes containing 0.2 M phosphate buffer, and it was found that any concentration of phosphate buffer greater than 0.05 M caused a white precipitate to form upon addition of the phenol reagent. Interference in the Lowry procedure has been noted by other authors (2, 3).

Recently we have become aware of another group attempting to purify C. perfringens enterotoxin by affinity chromatography. This group has used a column equilibrated with phosphate buffer and has eluted toxin by simply increasing the ionic strength of the buffer with NaCl (H. M. Barnhart et al., Abstr. 35th Annu. Meet. Inst. Food Technol., p. 76, 1975). This same method was used by Cukor et al. (8)
for the purification of diphtheria toxin. We have found this method unsatisfactory. The appearance of toxin in the first elution peak indicated that toxin was not binding completely even though the amounts loaded on the column did not exceed column capacity. Column runs prior and subsequent to these runs using our own methods satisfactorily bound similar amounts of toxin from crude preparations. A high ionic strength equilibration buffer is known to decrease nonspecific adsorption (29) which probably interfered with toxin binding. It is not clear what this nonspecific adsorption is. Elution of the columns by adding NaCl to the buffer produced an absorption peak at 280 nm but gel electrophoresis of the material revealed only an occasional faint protein (toxin) band. It is possible that nucleic acids are adsorbing nonspecifically and could be eliminated with ribonuclease or deoxyribonuclease. Only a very small amount of toxin was eluted by raising the ionic strength of the buffer; this toxin was undetectable by immunodiffusion but had slight erythematous activity in guinea pigs (neutralizable by anti-enterotoxin serum) and could be observed as a faint band on gels. The majority of the toxin remained on the column and could be removed by elution with NaOH.

Affinity chromatography of crude cell extract results in toxin contaminated with at least two other proteins. This problem can be overcome by chromatography of crude cell extract on a Sephadex G-100 column prior to affinity chromatography. One G-100 run provides enough material for 20 affinity chromatography runs. The toxin purified by affinity chromatography appears to be at least as pure as that purified by conventional means. Affinity chromatography can successfully be used for the rapid (less than 2 h) purification of small amounts of toxin. It results in 5.9- to 8.5-fold purification of the protein. This and the specific activity of the toxin purified are lower than values reported for conventionally purified toxin (28). This may be due to partial inactivation of toxin by elution with NaOH; once this difficulty has been solved the values should be much higher.

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FIG. 6. Disc gel electrophoresis of various enterotoxin preparations. The gels were loaded with (A) crude cell extract, (B) G-100-purified toxin, (C) highly purified toxin from hydroxylapatite columns, (D) toxin purified by affinity chromatography from cell extract, and (E) toxin purified by affinity chromatography from G-100-purified toxin. Arrow indicates faint bands of contaminating proteins.

LITERATURE CITED
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