INFECTION AND IMMUNITY, Dec. 1977, p. 741-745

Vol. 18, No. 3
Printed in U.S.A.

Purification of Beta-Toxin from *Clostridium perfringens* Type C

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Received for publication 26 July 1977

Beta-toxin was purified about 340-fold from culture supernatant fluid of *Clostridium perfringens* type C with a yield of about 24% in terms of biologically active beta-toxin. The purification involved ammonium sulfate fractionation, gel filtration through Sephadex G-100, isoelectrofocusing in a pH 3 to 6 gradient, and immunoaffinity chromatography. The purified beta-toxin gave a single band on polyacrylamide gel electrophoresis.

The predominant toxins produced by *Clostridium perfringens* type C are designated alpha and beta. The existence of delta-, theta-, and kappa-toxins has been reported in some strains of type C (2, 4, 5, 6, 8, 11, 12). Among these toxins, beta-toxin has been thought to be closely correlated with necrotic enteritis of animals and man, caused by this toxigenic type.

To study the role of beta-toxin in necrotic enteritis, it is desirable to obtain a highly purified toxin. Previous purification of this toxin has not been reported. We have previously investigated the optimal conditions of beta-toxin production by *C. perfringens* type C (J. Sakurai and C. L. Duncan), and in the present report, extensive purification of beta-toxin produced by *C. perfringens* type C is described.

MATERIALS AND METHODS

Culture medium and culture. The culture medium consisted of 2% proteose peptone, 2% yeast extract, 1.5% Casamino Acids, 1% dibasic sodium phosphate, and 1% glucose. The medium was inoculated with 5% by volume of a culture grown for 16 h at 37°C in fluid thioglycolate medium (Difco Laboratories, Detroit, Mich.). The pH of the culture was controlled at 7.5 as described by Labbe and Duncan (9).

Strain. The strain of *C. perfringens* type C used was CN 5386, isolated from a pig-bel case in New Guinea and kindly provided by P. D. Walker, Wellcome Research Laboratories, Beckenham, England.

Assay for beta-toxin activity. To estimate beta-toxin activity, 0.5 ml of the toxin sample was mixed with a 0.1-ml volume of *C. perfringens* type A diagnostic serum (Wellcome Research Laboratories) and 0.2 ml of saline. The mixture was left at room temperature for 30 min to allow neutralization of the alpha-toxin. A 0.4-ml amount of the mixture was serially diluted twofold with saline. Then 0.2 ml was injected intradermally into guinea pigs. Characteristic purple beta-toxin skin reactions were read after about 24 h.

Assay for alpha-toxin activity. To estimate alpha-toxin activity, 10 μl of the toxin sample was placed in a well (2-mm diameter) punched in an egg yolk agar (Difco) plate, and after 24 h at 37°C, the diameter of egg yolk turbidity was measured. The diameter caused by 0.05 unit of alpha-toxin (Sigma Chemical Co., St. Louis, Mo.) was 14 mm under our conditions.

Estimation of protein. Protein was estimated by the method of Lowry et al. with bovine serum albumin as a standard (10).

Isoelectrofocusing. Isoelectrofocusing with carrier ampholytes (pH 3 to 6) was performed in a gradient of sucrose by the method described in the 8100 Ampholine manual (LKB Instruments, Sweden). The apparatus used in our examination was the LKB 8101 column (110-ml capacity). A sample previously dialyzed against distilled water was applied in the less dense sucrose solution. Each electrophoretic run had a final potential of 500 V. Electrophoresis was continued at 4°C for 24 h or until the current became constant. After electrophoresis, 2.0-ml fractions were collected. The pH was measured, and the absorbance at 280 nm was determined. Fractions were assayed without prior dialysis to remove ampholine and sucrose. Each fraction was diluted 10- to 100-fold with 0.01 M phosphate buffer (pH 7.0), and toxin activities were determined.

Immunoaffinity chromatography. Two grams of dry gel of activated CH-Sepharose 4B (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) was used per column. The dry gel was swollen and washed on a sintered glass filter with 400 ml of 1 mM HCl. Commercial *C. perfringens* type A diagnostic serum was fractionated by the method of Hebert et al. (7). The immunoglobulin fraction obtained (40 mg) and the gel (2 g) were mixed in $NaHCO_3$ solution (0.1 M, pH 8.0). The mixture was put in a screw-cap test tube and rotated end-over-end for 1 h at room temperature. The gel was washed with distilled water until the absorbance at 280 nm of the wash was negligible. The washed gel was suspended in 10 ml of tris(hy-

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droxymethyl)aminomethane buffer (0.1 M, pH 8.0) and then rotated end-over-end in a test tube for 1 h at room temperature. The gel was washed with 0.05 M tris(hydroxymethyl)aminomethane buffer (pH 8.0) containing 0.5 M NaCl and then with 0.05 M acetate buffer (pH 4.0) containing 0.5 M NaCl. The gel was mixed with 0.02 M phosphate buffer (pH 7.0) containing 0.5 M NaCl and poured into a Pharmacia K 9/15 column (Pharmacia). The column was equilibrated with the same buffer.

Discontinuous gel electrophoresis. Polyacrylamide gel electrophoresis was carried out as described by Davis (3).

RESULTS AND DISCUSSION

Ammonium sulfate fractionation of beta-toxin. Strain CN 5386 was cultured in a medium containing 1% glucose with the pH controlled at 7.5 and was centrifuged just after the growth reached maximum (ca. 5 h). The culture supernatant fluid obtained was used as the starting material for purifying beta-toxin.

The culture supernatant fluid was fractionated at 4°C in narrow steps with increasing amounts of ammonium sulfate, and each fractional precipitate was assayed for protein content and beta-toxin. The data for the total activity and the toxin recovery in each fraction are given in Table 1. About 80% of the beta-toxin was precipitated between 30 and 50% saturation, and its specific activity was about 10 times higher than that of the culture supernatant fluid.

Sephadex G-100 column chromatography of beta-toxin. The ammonium sulfate fraction (30 to 50%) was dissolved in 0.01 M phosphate buffer (pH 7.0), and about 100 mg of protein was applied to a Sephadex G-100 column (2.5 by 80 cm), previously equilibrated with 0.01 M phosphate buffer, pH 7.0. Elution of the column was done with the same buffer. Eight-milliliter fractions were collected. A plot of absorbance at 280 nm and alpha- and beta-toxin activities are shown in Fig. 1. The protein applied to the column was separated into two peaks, based on absorbance at 280 nm, and both alpha- and beta-toxin activities were eluted simultaneously between the two peaks. The data show that the molecular weight of beta-toxin is obviously very close to that of alpha-toxin. On the other hand, discontinuous gel electrophoresis of the pooled toxin fractions showed that the sample contained at least six protein bands (data not shown).

Isoelectrofocusing of beta-toxin. For further fractionation of the Sephadex G-100 eluted

TABLE 1. *Yield of beta-toxin on fractional ammonium sulfate precipitation of culture supernatant fluids*

Saturation with ammonium sulfate (%)	Total protein[a] (mg)	Total beta-toxin activity[b] (U × 10^{-3})	Sp act (U/mg)	Yield (%)
0	1252.5	60	47.9	100
0–30	1.1	0.08	72.7	0.13
30–40	37.0	16	432	27
40–50	57.0	32	561	53
50–60	35.0	0.8	22.9	1.3
60–70	10.0	0	0	0

[a] Protein content was measured by the method of Lowry et al. (10).

[b] Beta-toxin activity was measured as described in the text.

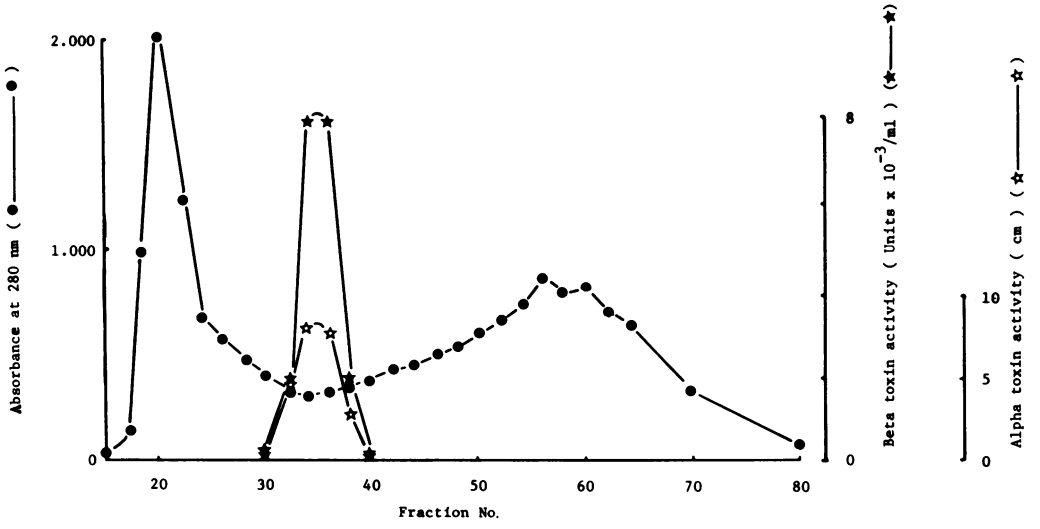

FIG. 1. *Sephadex G-100 column chromatography of ammonium sulfate fractionated beta-toxin.*

beta- and alpha-toxin mixture, isoelectrofocusing with carrier ampholyte (pH 3 to 6) was used. About 43 mg of protein was applied to the column followed by isoelectrofocusing for 24 h. Most of the beta-toxin resided in fractions from pH 5.3 to 5.8, with the peak of activity at an isoelectric point (pI) of 5.53 (Fig. 2). A minor peak of beta-toxin was detected with a pI of 5.06. The minor peak was reproducible under our experimental conditions. The pI measurements of alpha-toxin showed a main peak with a pI of 5.28 and a shoulder at a pI of about 5.49. From the data, it is clear that the beta-toxin fraction obtained by isoelectrofocusing still contained alpha-toxin. Discontinuous gel electrophoresis of the fraction indicated that the sample contained only two bands (data not shown).

Immunoaffinity chromatography of beta-toxin. Immunoglobulin fractionated from commercial *C. perfringens* type A diagnostic serum by ammonium sulfate was coupled to activated CH-Sepharose 4B. This immunoglobulin contained antibody against alpha-toxin but not beta-toxin, since the toxigenic type A strain does not produce beta-toxin. The beta-toxin fractions (5 mg of protein) pooled from the isoelectrofocusing column were applied to the affinity column, and the column was eluted with 0.02 M phosphate buffer (pH 7.0) containing 0.5 M NaCl and then with 0.2 M glycine-hydrochloride buffer (pH 2.8) containing 0.5 M NaCl. Figure 3 shows a typical elution profile of beta- and alpha-toxin from the column. When eluted with the phosphate buffer, beta-toxin passed through the column, but alpha-toxin was adsorbed to the column. Alpha-toxin was eluted with the glycine-hydrochloride buffer to disrupt antigen-antibody bonds. The data indicate that alpha-toxin can be effectively removed from beta-toxin by this method. If beta-toxin from the affinity column contained a small amount of alpha-toxin due to column overloading, re-chromatography was done on the same column.

Purity of beta-toxin obtained by affinity chromatography. Purity of the toxin was tested by polyacrylamide gel electrophoresis. A 50-μg portion of the purified toxin gave a single band (Fig. 4A). A similar gel was cut into strips (2 mm wide), and the toxin was extracted from each piece with 0.5 ml of 0.01 M phosphate buffer, pH 7.0. Coincidence of the toxin activity with this band was demonstrated (Fig. 4).

The purification results are summarized in Table 2. The purification achieved from the culture supernatant fluid of *C. perfringens* type C was about 340-fold, with a yield of about 24%

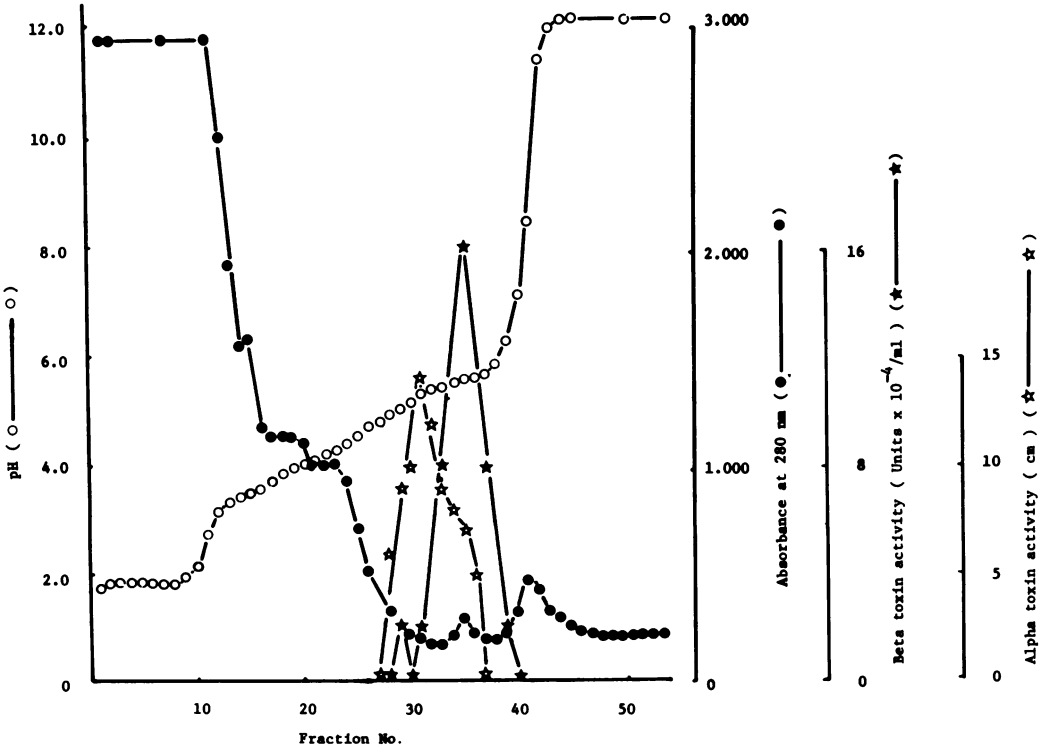

FIG. 2. *Isoelectrofocusing of Sephadex G-100 eluted beta-toxin fractions in a pH 3 to 6 gradient.*

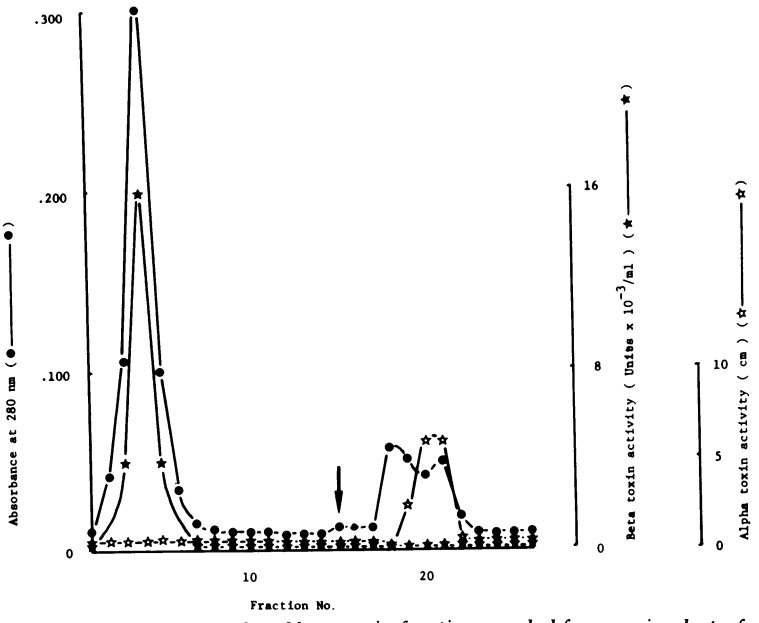

FIG. 3. *Immunoaffinity chromatography of beta-toxin fractions pooled from an isoelectrofocusing column. The column was eluted with 0.02 M phosphate buffer (pH 7.0) containing 0.5 M NaCl, followed by elution (indicated by the arrow) with glycine-hydrochloride buffer (0.2 M, pH 2.8) containing 0.5 M NaCl.*

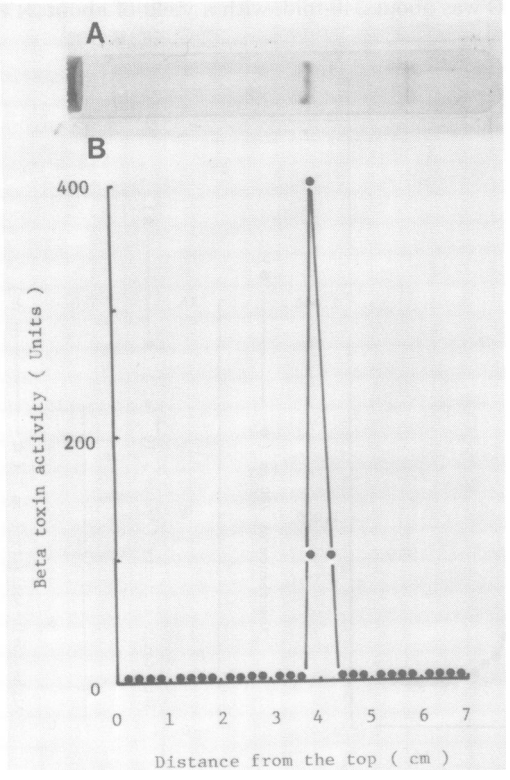

FIG. 4. *Polyacrylamide gel electrophoresis of beta-toxin obtained from immunoaffinity chromatography. (A) The gel was stained by Coomassie brilliant blue G-250. (B) Beta-toxin activity in sections (2 mm) of gel. Minor band towards cathodal end of gel represents tracking dye.*

TABLE 2. *Summary of purification of beta-toxin of C. perfringens type C*

Step	Total protein[a] (mg)	Total activity[b] (U × 10^{-4})	Sp act (U/mg)	Yield (%)
Supernatant fluid	18,880	127.4	67.5	100
Ammonium sulfate fraction	1,155	87.8	760	69
Sephadex G-100 fraction	183.6	72.5	3,950	57
Isoelectrofocusing fraction	18.8	32.0	17,000	25
Affinity chromatography fraction	13.1	30.1	23,000	23.6

[a] Protein content was measured by the method of Lowry et al. (10).

[b] Beta-toxin activity was measured as described in the text.

with respect to beta-toxin activity. As little as 0.04 μg of the purified toxin had necrotic activity in the skin of guinea pigs.

To our knowledge, the results presented here are the first published report of the purification of beta-toxin produced by *C. perfringens* type C. The molecular weight and pI of beta-toxin are obviously very close to that of alpha-toxin. In other experiments we have conducted, when beta-toxin was applied to a diethylaminoethyl-

cellulose column, the specific activity of beta-toxin decreased remarkably, but that of alpha-toxin did not. However, the two toxins were separated by the column. By using immunoaffinity chromatography, both separation of the two toxins and preservation of biological activity were achieved.

Smith and Arbuthnott have reported previously that the pI values of alpha-toxin produced by *C. perfringens* type A were 5.25 and 5.49 and that the pI value of the major electrophoretic component of alpha-toxin was 5.49 (13). Bernheimer and Grushoff reported pI values of 5.2 and 5.5 (1). Our observed pI values for alpha-toxin produced by *C. perfringens* type C were 5.28 and 5.49 and are thus similar to those reported.

Our data showed pI values of 5.06 and 5.53 for beta-toxin. A major peak was at a pI of 5.53, and a minor peak was at a pI of 5.06. We do not know whether this strain produces two distinct electrophoretic forms of the toxin or whether one of the two forms represents merely aggregated beta-toxin.

Purified beta-toxin activity was neutralized by commercial *C. perfringens* type B or C diagnostic serum, but not by type A, D, or E diagnostic serum. This is in agreement with the fact that only type B and C strains produce beta-toxin.

ACKNOWLEDGMENTS

This research was supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison, by Public Health Service research grant AI-11865-06 from the National Institute of Allergy and Infectious Diseases, research grant FD-00203-07 from the Food and Drug Administration, and by contributions to the Food Research Institute by member industries. C.L.D. is the recipient of Public Health Service Research Career Development Award AI-70721-03 from the National Institute of Allergy and Infectious Diseases.

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