Homogeneity and Molecular Weight of Toxin of Clostridium botulinum Type B

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Gerwing et al. described the isolation and purification from culture filtrates of the toxin of Clostridium botulinum type B and characterized it as a homogeneous protein of less than 10,000 molecular weight. Analysis by various methods of samples of this toxin obtained from Gerwing et al., and preparations produced by their methods in our laboratories, furnished convincing evidence that neither her preparation nor ours was homogeneous. The molecular weight of the toxic component isolated from either of the preparations was 100,000 or greater and resembled, in a number of respects, the α component isolated by us from the crystalline toxin of C. botulinum type A.

One of the essential requirements for studying the structure of any protein is that the protein be homogeneous. It is also desirable that the protein have a small molecular weight. For these reasons, we felt that the isolation and purification of small molecular weight toxin of Clostridium botulinum from types A, B, and E by a group of investigators at the University of British Columbia (11) was an important advance in the studies of these substances. The toxins were described by the authors as homogeneous proteins of about 10,000 molecular weight for type A, 12,000 for type B, or 6,000 according to their latest revisions (11), and 18,000 for type E toxin.

In our laboratory we had succeeded in isolating, by column chromatography, two distinct components from the crystalline toxin of C. botulinum type A (5). One, which we named α, had five times the specific activity of the original toxin. The molecular weight and the homogeneity of α were established by sedimentation velocity and sedimentation equilibrium in the ultracentrifuge (3) by the method of Yphantis (16), and by gel filtration through G-200 Sephadex column by the method of Andrews (1). By these criteria, α appeared to be homogeneous with S20w 7 and of 128,000 to 150,000 molecular weight. The second component, β, with a molecular weight of 750,000 and essentially nontoxic, contained all the hemagglutinating activity usually associated with crystalline preparations.

Since we were interested in obtaining from C. botulinum type B a hemagglutinin-free toxic fraction similar to α component of type A crystalline toxin, and since there is a considerable advantage to studying material of 10,000 instead of 128,000 molecular weight, we attempted to isolate and examine this fraction from culture filtrates of type B organisms by using the procedures of Gerwing et al. Properties of the material obtained by us, as well as of the samples of type B botulinum toxin from Dr. Gerwing, are discussed in this report.

MATERIALS AND METHODS

Strains of C. botulinum type B (Lamanna) were obtained from George G. Wright (Fort Detrick, Frederick, Md.) and from J. Gerwing (University of British Columbia, Vancouver). Crystalline toxin type A, used as a standard for comparison with type B toxin, was kindly supplied by E. J. Schantz (Fort Detrick).

Two preparations of type B toxin described as homogeneous and of 10,000 molecular weight were kindly sent to us by Dr. Gerwing. In a 1-cm cuvette at 278 mλ, the optical density of one of these samples was 1.7 and toxicity was 3 X 106 minimal lethal dose (MLD)/ml for a 20-g mouse. This preparation was designated by us as "toxin I." The second sample was only slightly toxic, had an optical density of 0.15, and will be referred to as "toxin II." Type B toxin was also prepared in our laboratories. The organisms from a frozen culture were grown for 24 hr at 37 C in chopped-meat medium and seeded into a sac made from dialyzing tubing (Visking) containing saline (2). This sac was immersed into 2% casein hydrolysate broth containing 0.5% yeast extract, 0.075% cysteine hydrochloride, and 0.1% sodium-thioglycolate (10). Before seeding, the whole ensemble was autoclaved at 15 psi for 15 to 30 min, depending upon the volume of the medium. After rapid cooling, the organisms and sterile glucose solution (to 0.5% concentration) were added to the sac and to the broth. The pH
of the medium, after autoclaving, was 7.2. The cultures were incubated at 37 C until the maximal amount of toxin formed (5 to 8 days). This was ascertained by daily removal of a sample, and by testing the supernatant fluid by intravenous injection of 0.1 ml into mice. The maximal amount of toxin obtained by this method contained 2 to 3 x 10¹⁶ MLD/ml for a 20-g mouse.

Another method for culturing the organisms, i.e., seeding the organisms directly into the medium, was suggested by Dr. Gerwing. While providing a greater volume of toxin, this method yielded only 10³ to 2 x 10⁶ MLD/ml. An additional advantage in the sac method was the absence of some black, nontoxic material which, in the cultures without the dialyzing casing, precipitated with the toxin upon the addition of a 3.0 M NaCl solution. At the end of the incubation period, the cultures were freed from the organisms and debris by centrifugation and filtration through Millipore filters. To bring the salt concentration of the culture filtrate to 50% saturation, solid (NH₄)₂SO₄ (Baker Analyzed Reagent) was added at 31.3 g/100 ml (12). A precipitate appearing in 24 to 48 hr at 4 C was re-dissolved in one-fiftieth of the original volume of the culture filtrate in 0.067 M citrate phosphate buffer, pH 5.6. Residual turbidity was removed by centrifugation with no loss of toxicity. Nearly 100% of toxicity found in the culture filtrate was recovered on 50-fold concentration. This is in agreement with Gerwing's experience. The concentrated material will be referred to as “crude toxin.”

The preparation and use of diethylaminoethyl (DEAE)-cellulose and Sephadex G-100 column was described previously (6). Citrate phosphate buffer (0.067 M, pH 5.6) was prepared by titrating Na₂HPO₄ with citric acid, both of 0.067 M, to pH 5.6. For gel filtration experiments, this buffer was adjusted to 0.1 M guanidine acetate by adding the solid salt. Chromatography with 0.15 M Tris(hydroxymethyl)aminomethane (Tris)-chloride buffer (pH 8.0) and generation of linear gradient elution with Cl⁻ was carried out as reported previously (6). All chromatography was done at 24 ± 2 C. Effluents were collected in 2.5 to 3.0 ml/tube at a flow rate of 30 to 35 ml/hr with a Gilson fraction collector.

Toxicity was determined by intravenously injecting the toxin solution into mice and noting their survival time (4). Fluorescence and optical density measurement of the protein samples were described previously (6). Ouchterlony gel double-diffusion tests were performed by the usual method in 1.0% Noble agar (Difco). Antisera for type B toxin were obtained from Porton Microbiological Establishment (Porton, Wilts., England). Sucrose gradient centrifugation was performed in a gradient of 5 to 20% sugar concentration in an SW-39 swinging bucket rotor at 100,000 x g for 16 hr. The contents were removed through a puncture at the bottom of the tube.

RESULTS

Chromatography of “crude toxin” on DEAE-cellulose column at pH 5.6. After dialysis against 200 ml of pH 5.6 buffer at 4 C for 16 hr, 2 ml of “crude toxin” was applied on a DEAE-cellulose column (0.9 x 30 cm) equilibrated with 0.057 M citrate phosphate buffer (pH 5.6) and eluted with the same buffer. Immediately upon voidance of one column volume, the elution profile showed the presence of a large sharp peak. The early part of the elute in this region was water-clear; thereafter, increasing amounts of colored materials appeared. Most of the activity of the “crude toxin” applied on the column was found in this peak with the maximum of 10² MLD/ml in the apex tube of the peak. The material from the peak will be referred to as “pH 5.6 toxin.” This preparation resembled the material obtained by Gerwing et al. (8), which they described as homogeneous and of 10,000 or less molecular weight.

Homogeneity and purity of “pH 5.6 toxin” and “toxins I and II.” Portions of “pH 5.6 toxin” and “toxins I and II” were brought to pH 8 by dialysis against 10 volumes of 0.15M Tris-chloride buffer, pH 8.0. The outside buffer was changed four times during the 16 hr of dialysis at 4 C. Application of “pH 5.6 toxin” on DEAE-cellulose column (0.9 x 30 cm) equilibrated with pH 8 buffer resulted in the emergence of a protein immediately after void volume (Fig. 1, peak 1). Extensive washing of the column with the equilibrating buffer failed to elute any more material. At this point, a linear gradient elution was generated by mixing 130 ml of 0.15 M Tris-chloride buffer (pH 8.0) containing 0.5 M NaCl with 130 ml of the equilibrating buffer. This resulted in the elution of three additional peaks (Fig. 1). The second peak contained most of the toxicity of the starting material, with highest concentration in the 17th tube from the point of start of the gradient. The other peaks were only slightly toxic. Chromatography of “toxin I” gave similar results (Fig. 2). The largest peak was only slightly toxic, its toxicity appearing to be due to trailing of the toxic component emerging in the region of the 17th tube. “Toxin II,” chromatographed similarly on a DEAE-cellulose column, yielded a small nontoxic peak before the gradient. After commencement of the gradient, maximal toxic activity was again obtained in the 17th tube. The peak in this region was much smaller than that obtained with “toxin I” and more irregular (Fig. 3). Rechromatography of the toxic peaks from all three preparations, at pH 8.0 on DEAE-cellulose column (not shown here), but with a shallower Cl⁻ gradient, resulted in the elution of the toxic peak with greater irregularity of the profile, which further indicated the presence of more than one component.

Gel filtration of “pH 5.6 toxin” and “toxins I

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FIG. 1–3. Dissociation of various purified preparations of the toxin of Clostridium botulinum type B on DEAE-cellulose column. Columns (0.9 × 30 cm) were equilibrated and eluted with 0.15 M Tris-chloride buffer, pH 8.0. Gradient of Cl− at constant pH was commenced at points marked by arrows. Flow, under gravity, at 30 ml/hr, 24 ± 2 °C, 3 ml/tube. Elution of protein was monitored by fluorescent intensity.

Fig. 1. Chromatography of "pH 5.6 toxin." First peak after the gradient, apex at 17 tubes, contained maximal toxicity per unit of fluorescence. Material emerging before the gradient was not toxic. Farther away from the 17th tube, the toxicity became minimal, although amount of the eluted protein increased.

Fig. 2. Chromatography of "toxin I." First peak after the gradient, apex at 17 tube, showed maximal toxicity per unit of fluorescence. Trailing edge of this peak suggested inhomogeneity.

Fig. 3. Chromatography of "toxin II." Although no sharp and clear peaks were obtained, tube 17, after generation of gradient, contained maximal toxicity per unit of fluorescence.

and II." Portions of 1.0 ml of each of the preparations were eluted through a Sephadex G-100 column (1.5 × 60 cm) equilibrated with 0.067 M citrate phosphate buffer (pH 5.6) containing 0.1 M guanidine acetate. For the calibration of the column, samples of blue dextran and horse heart cytochrome c were also filtered through the same column under identical conditions (Fig. 4). All toxin samples resolved into two peaks. One component from each of the toxins emerged in the void volume, thus indicating that this component was either 100,000 molecular weight or larger (Fig. 5–7). This material will be referred to as the "large molecular weight protein." The second peak from "pH 5.6 toxin" and "toxin I" appeared ahead of cytochrome c (Fig. 5, 6), showing their molecular weights to be larger than 12,000. The second peak from "toxin II" emerged after cytochrome c and thus was less than 12,000 molecular weight (Fig. 7). The second component of these toxins will be referred to as "small molecular weight protein." It was evident that the "large molecular weight protein" component was present in all preparations. The smaller molecular weight components in "pH 5.6 toxin" and "toxin I" appeared similar but were different from the one in "toxin II."

Samples from every fourth tube of the experiments shown in Fig. 5–7 were assayed for toxicity. Maximal toxicity was coincidental with maximal protein concentration in the large peak; thereafter, toxicity decreased with the increase in the distance from this peak and was not enhanced by the emergence of the "small molecular weight protein." Therefore, the second peak eluting from G-100 Sephadex column was either nontoxic or of very low toxicity. After chromatography of the "pH 5.6 toxin" at pH 8.0 on DEAE-cellulose column, when 1.0 ml of the eluate containing highest toxicity was applied on Sephadex G-100 column, only one peak eluted and at the void volume, indicating that the toxin fraction was either equal to or higher than 100,000 molecular weight.

Ouchterlony gel double-diffusion tests on the eluted materials are shown in Fig. 8. The central well contained rabbit antiserum prepared against type B toxin; well 1, the "crude toxin"; well 2, "toxin I"; well 3, "large molecular weight protein"; and well 4, "small molecular weight protein." "Toxin II" did not react with the antiserum. There was a line of identity in all three antigen samples associated with the "small molecular weight protein" component. Although the number of precipitation lines decreased after chromatography of "crude toxin," indicating some degree
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FIG. 4-7. Demonstration of a large (toxic) and small (nontoxic) molecular weight protein in various preparations of the toxin of Clostridium botulinum type B by gel filtration. A Sephadex G-100 column (1.5 x 60 cm), calibrated with blue dextran and cytochrome c and used for the toxin analysis, was eluted with 0.067 M citrate phosphate buffer (pH 5.6) containing 0.1 M guanidine acetate. Eluate was collected at 15 ml/hr in 2.8 ml/tube at 24 ± 2 °C, by gravity flow. Elution of toxin (solid line) was monitored by fluorescent intensity (F.I.).

Fig. 4. Calibration of column with 1.0-ml samples of blue dextran (○) and cytochrome c (X); elution was monitored by absorbance at 620 and 412 m,u, respectively. For blue dextran, the apex was in the 12th tube and for cytochrome c, in 28th tube. Arrows in Fig. 6 and 7 mark these reference points.

Fig. 5. Resolution of 1.0 ml of “pH 5.6 toxin.” Major peak eluted at the position of blue dextran; second peak eluted three tubes ahead of cytochrome c.

Fig. 6. Resolution of 1.0 ml of “toxin I.” Elution profile was qualitatively identical with toxin in Fig. 5.

Fig. 7. Resolution of 1.0 ml of “toxin II.” Major peak, as in the case of “pH 5.6 toxin” and “toxin I,” eluted at the position of blue dextran; second peak eluted four tubes after cytochrome c.

of purification (Fig. 8, well 2), it was nonetheless clear that it still contained more than one component. “Toxin I,” obtained from Gerwing, and the “crude toxin” purified on DEAE-cellulose at pH 5.6 [a procedure which Gerwing et al. (8) employed as the final step in purification and which was claimed to yield a homogeneous toxin] were shown to be quite heterogeneous by the Ouchterlony test (Fig. 8, wells 3 and 4).

A search for small molecular weight toxin was
Absorption and amino acid composition of type B toxin. On amino acid analysis, type B toxin of 10,000 molecular weight was reported to show 4 moles of tyrosine and 4 moles of phenylalanine per mole of toxin, but no tryptophan (8). The extinction coefficient of this protein (1 mg/ml at 277 mμ) was 1.2; thus 1 mg of protein of molecular weight 10,000 should have contained 0.4 μmole of tyrosine and 0.4 μmole of phenylalanine. The absorbance of this protein at 278 mμ, attributable primarily to these two aromatic amino acids, appeared to us to be excessive. Therefore, we compared the absorbance of ribonuclease (five times crystallized; Calbiochem, Los Angeles, Calif.) and free tyrosine with the reported absorbance of the toxin. The enzyme contains six residues of tyrosine and three of phenylalanine per mole, but no tryptophan. Thus, 1,052 μg/ml of enzyme (0.0767 μmole, molecular weight 13,700) contained 0.460 μmole of tyrosine and 0.230 μmole of phenylalanine. Absorbance of the enzyme in 0.075 M phosphate buffer (pH 6.8), at 278 mμ, was 0.678. Absorbance of 0.4 μmole of free tyrosine per ml in the same buffers at 274 mμ was 0.577. Therefore, it follows that such concentrations of tyrosine and phenylalanine cannot fully account for the extinction coefficient of 1.2 reported for the type B purified toxin. When "toxins I and II," as well as "pH 5.6 toxin," were examined in the Aminco-Bowman Spectrophotofluorometer for fluorescence, the preparations fluoresced in the activation and fluorescent wavelengths characteristic of tryptophan.

**FIG. 8.** Ouchterlony gel double-diffusion test with *Clostridium botulinum* type B toxin at various stages of purification. Central well, rabbit antiserum against type B toxin (Portion); well 1, (NH₄)₂SO₄ precipitate of toxic culture filtrate reconstituted in citrate phosphate buffer (pH 5.6) one-fiftieth of the original volume ("crude toxin"); well 2, toxin obtained after chromatography on DEAE-cellulose at pH 5.6 ("Toxin I"); well 3, "large molecular weight protein" obtained from "toxin I" after gel filtration on G-100 Sephadex column (see Fig. 6); well 4, "small molecular weight protein" obtained from "toxin I" after gel filtration on G-100 Sephadex column (see Fig. 6).

Attempts to obtain a small molecular weight type B toxin of 10,000 molecular weight described in the literature (8) did not prove successful. This does not deny the possible existence of such subunits. That such molecules may exist, perhaps in the form of subunits of larger aggregates, was observed by Wagman (15), who occasionally obtained dialyzable toxin after extensive dialysis of crystalline toxin. We also observed toxicity in some of our dialysates, but could not proceed with further isolation because this activity was low and very labile. Schantz and Lauffer (13) demonstrated toxic substances in type A crystalline preparations which diffused upward through an agar-gel column. From the diffusion rates, they calculated the substance to be of about 5,000 or less molecular weight. It is conceivable that the α component isolated by us from type A crystalline preparations of 128,000 to 150,000 molecular weight, and homogeneous by several criteria, is not a single peptide chain but is an aggregate of subunits which may also be toxic.

Also made by density gradient sedimentation. Tubes of 5-ml capacity were layered with successive concentrations of 1 ml of 20 to 5% sucrose in 0.067 M citrate phosphate buffer (pH 5.6) and allowed to equilibrate at 4 C for 16 hr. On the top of the sucrose solution in two of these tubes, 0.5 ml of the "pH 5.6 toxin" was layered. The third tube was layered with 0.5 ml of cytochrome c solution (12,000 molecular weight) as a marker and control. The tubes were placed in an SW-39 swinging bucket rotor and centrifuged at 100,000 × g in a Spinco model L ultracentrifuge. Toxicity was minimal at the level of sedimentation of cytochrome c. The toxin in all cases, however, could be recovered from the bottom portion of the centrifuged solutions, indicating that no toxic protein of 12,000 molecular weight or less was present in any of the preparations tested.
To date, however, following Gerwing's methods of culturing, isolation, and purification of type B botulinum toxin as closely as possible, we were unable to obtain a toxic component of 10,000 molecular weight. We did observe small molecular weight proteins which coprecipitated with the heavier toxin at 50% (NH₄)₂SO₄ saturation. The small molecular weight proteins were either nontoxic or only slightly toxic. Whatever toxicity they possessed could be traced to trailing of the high molecular weight peaks. If any of the 10,000 molecular weight toxin were present on centrifugation in sucrose gradient, it would be reasonable to expect that this toxin would be found in the region occupied by cytochrome c (12,000 molecular weight). Instead, this portion of sucrose solution was either inactive or only slightly toxic. That botulinum toxins in cultures of C. botulinum exist as large molecular weight substances and do not aggregate into heavier moieties on purification (7) was demonstrated by Schantz and Spero (14). No evidence has been presented by Gerwing et al. to demonstrate that the slow component with Sₘ₉₉ 0.9 seen by them on ultracentrifugation was indeed toxic, because this component was neither isolated nor assayed after centrifugation. On the contrary, the material that eluted from a DEAE-cellulose column at pH 5.6, although emerging as a single peak, when rechromatographed on a similar column at pH 8.0 with a salt gradient, resolved into at least three peaks. One of these, the toxic peak, resembled the component α obtained from type A botulinum toxin; it eluted at the same ionic strength of the buffer as did α and appeared to be about the same molecular weight. In addition, this component from type B toxin emerged just ahead of the larger peak of little toxicity, which resembled the β component of type A toxin.

We therefore conclude that, although demonstration and isolation of a small molecular weight botulinum toxin is most desirable for further studies of the chemistry of the toxin, we find no evidence in the examination of our toxin preparation, and none in the samples of type B toxin received from Gerwing et al., to justify the belief that the isolation of such subunits has been successful. Furthermore, preparations obtained from these workers and examined in our laboratories, as well as the type B toxin prepared by us according to their methods, proved to consist of a number of components. These observations cast doubt on the validity of their conclusions concerning the amino acid analysis (8), the terminal amino acid (8), sequence of amino acid adjacent to the active site (9, 10), and, finally, the value they have reported for the molecular weight of C. botulinum type B toxin.

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