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Affinity Chromatography Purification of Type A Botulinum Neurotoxin from Crystalline Toxic Complex

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Type A botulinum neurotoxin was purified from toxic crystals by adsorption to *p*-aminophenyl-β-D-thiogalactopyranoside coupled to CH-Sepharose 4B. At pH 6.3, the toxic complex was held by the binding between the ligand and the hemagglutinin of the complex; the toxin is eluted selectively by dissociating the complex with buffer-saline of pH 7.9. The single-step affinity chromatography recovered 50 to 60% of applied toxicity as preparations of greater than 99% purity.

Clostridium botulinum type A neurotoxin is usually purified by ion-exchange methods (2, 7, 9) which separate it from the toxic crystals in which the toxin is associated noncovalently with a nontoxic hemagglutinin (2) and probably a second nontoxic protein (8). The methods are time consuming and give low recoveries because of the recyclings needed to obtain preparations of acceptable purity.

The toxin and hemagglutinin (HA) constituting the crystals are distinct antigens that dissociate in alkaline conditions (2). This laboratory tried to purify the toxin by affinity chromatography wherein the entire toxic complex of the crystals was fixed to an adsorbent and then to elute sequentially HA and toxin. The adsorbent was type A antitoxin (immunoglobulin G fraction of a rabbit immunized with toxoid of pure toxin) immobilized on a solid phase (CNBr-activated Sepharose 4B or via the 6 carbon spacer arm of CH-Sepharose 4B). Within the capacity limits of the adsorbent columns, essentially all applied toxicity was bound. However, all elution methods used recovered only a small fraction of the bound toxin (unpublished results).

The present report considers the isolation of toxin in the crystals by affinity chromatography in which HA instead of toxin is bound by the ligand. Although anti-HA immunoglobulin G was used with some success, a more suitable ligand was suggested by the observation that galactose and some of its derivatives are inhibitors of the hemagglutinin (1). When coupled to CH-Sepharose 4B by a peptide bond formed in the carbodiimide reaction, *p*-aminophenyl-β-D-thiogalactopyranoside (pAPTG), which is an analog of one of the most potent HA inhibitors, binds HA apparently irreversibly (5).

MATERIALS AND METHODS

Toxic crystals. Toxic crystal preparations were obtained from *C. botulinum* type A Hall strain by the method of Duff et al. (6) or by a modification of that method (10).

Affinity chromatography. CH-Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N.J.) was swollen in 0.5 M NaCl and washed with water. For each 1.5 g of freeze-dried gel stock being processed, separate aqueous solutions containing 42 mg of pAPTG (Calbiochem, La Jolla, Calif.) or 60 mg of 1-ethyl-3(3-dimethyl-aminoethyl)-carbodiimide·HCl (Sigma Chemical Co., St. Louis, Mo.) in 1.0-ml volumes were prepared. These solutions were added to a small beaker containing the washed gel in 10 ml of water. While stirring, the pH was adjusted to 4.5 with 0.1 N NaOH and maintained between 4.5 to 6.0 during the first hour. The slurry was poured into a screw-capped tube and gently mixed, end-over-end, on a shaker for 20 h at room temperature (20 to 23°C). The gel was washed with water and then with 100 ml of 0.025 M phosphate buffer (pH 6.3). Storage was at 4°C after adding NaN_3 to 0.02% as preservative.

The batch chromatography procedure was used. The desired amount of pAPTG-gel (drained) was placed in a screw-capped tube and mixed with the crystals dissolved in 0.025 M phosphate buffer (pH 6.3). Toxin concentration was 2 to 3 mg of crystals per ml. After gently mixing for 2 h at room temperature, the slurry was transferred to a sintered glass plate filter and washed with 200 ml of buffer. The fluids of the reaction mixture and the wash were pooled as toxin that was not bound by the adsorbent.

The drained gel was placed in a 20-ml beaker and suspended in 5.0 ml of 0.1 M phosphate buffer–1.0 M NaCl (pH 7.9). The slurry was adjusted to pH 7.9 with 0.2 N NaOH and after 15 min was packed for chromatography in a column (0.9-cm inside diameter). The solution coming through during the packing was saved as part of the toxin fraction. The column was then eluted with additional buffer-saline while collecting 2.0-ml fractions. Fractions with significant protein content (absorbance at 278 nm [A_{278}] = 0.2 or greater) were combined with the sample from the column packing step. Pool total was 10 to 12 ml.

Tests of purity. Neurotoxin preparations were subjected to electrophoresis in polyacrylamide gels

with sodium dodecyl sulfate (3). In this procedure, the toxin shows only the protein band formed by units of molecular weight 145,000. When subjected to electrophoresis after reduction of its disulfides, the toxin is resolved into its heavy (molecular weight, 97,000) and light (molecular weight, 53,000) subunits (4).

Preparations were also examined by Ouchterlony serology. Antitoxin was the serum from a rabbit that had been immunized with the toxoid resulting from treating once-crystallized type A toxic crystals with 0.6% Formalin. This anticrystalline serum gave three immune precipitate lines when reacted against antigen comparable to the immunizing antigen (10). Anti-HA serum was from a rabbit immunized with a completely nontoxic type A hemagglutinin sample derived from the crystals.

Toxicity. Titrations used an intraperitoneal assay procedure in which separate groups of six to eight mice were injected with 0.5 ml of the appropriate serial twofold dilutions (10). Specific toxicities were based on $E^{1\%}_{1cm}$ at 278 nm of 16.6 for crystals and 16.3 for neurotoxin (7).

RESULTS AND DISCUSSION

Table 1 shows the pertinent data of representative runs. Each test was performed with a different pAPTG-gel preparation, and three different crystal lots were processed.

The method consistently recovered 50 to 60% of the starting toxicity. With the gel-to-crystal ratios used, 3% or less of the applied toxicity was lost from not binding to the adsorbent. Since the specific toxicity of the crystal lots was about 3.5×10^7 50% lethal doses per mg, adsorbent sufficient to form a 6.0- by 0.9-cm column bound at least 50 mg of crystals. Since the protein not held by the adsorbent during sample application was only slightly more than would be expected from the toxicity in the fraction, the 40 to 50% of starting toxicity not accounted for in Table 1 remained with the adsorbent. Attempts to elute this toxin have not been successful.

The recovered toxin samples had A_{260}/A_{280} of 0.5. The specific toxicities were consistently near the high end or even slightly greater than the 9.2×10^7 to 1.18×10^8/mg found for toxin purified by ion-exchange chromatography and titrated by the intraperitoneal method (7).

The preparations from all runs were of comparable purity. When analyzed by polyacrylamide gel electrophoresis at 30 μg per gel, only the toxin band was seen. When 60 μg per gel was subjected to electrophoresis, a faint band below that of toxin indicated the presence of units of 130,000 molecular weight. The color intensity of this band relative to that of toxin suggested the faster-moving material to comprise less than 1% of the total protein. The band was not seen in polyacrylamide gel electrophoresis of reduced toxin.

In serological tests using anti-HA serum, no immune precipitate line developed with toxin quantities up to 240 μg per well. Against anticrystalline serum, 100 μg of toxin per well developed one line, but at 240 μg per well a faint second line became visible. The second line was probably due to the 130,000-molecular-weight material but was different from the third precipitate line given by crystals reacting with anticrystalline serum (10). The best neurotoxin preparations obtained by ion-exchange methods have this material; its presence is normally not recognized because the purity tests are done with relatively smaller sample amounts. The protein persisted when toxin from affinity chromatography was subjected to the diethylaminoethyl-Sephadex chromatography that is used to isolate toxin from crystals (9).

TABLE 1. *Affinity chromatographic purification of type A botulinum toxin from crystalline type A toxic complex*[a]

Column (cm[c])	Toxicity (total LD_{50}[b])		
	Applied	Unbound	Recovered
3.0	5.44×10^8	0.5×10^7 (1)[d]	3.18×10^8 (58)
3.0	5.44×10^8	0.5×10^7 (1)	3.39×10^8 (62)
3.0	6.75×10^8	1.9×10^7 (3)	4.94×10^8 (74)
3.0	4.00×10^8	1.0×10^7 (3)	2.25×10^8 (56)
6.0	1.04×10^9	1.0×10^7 (1)	6.08×10^8 (58)
6.0	1.80×10^9	2.0×10^7 (1)	9.56×10^8 (53)

[a] Ligand is pAPTG coupled to CH-Sepharose 4B.
[b] LD_{50}, 50% lethal dose.
[c] Length of 0.9-cm-diameter columns.
[d] Number in parentheses indicates percentage of applied toxicity.

Attempts were made to purify toxin directly from culture fluids. Considerable purification could be obtained, but the binding of toxicity by the adsorbent was less than desirable and the eluted toxic preparations often contained several other proteins.

The strong binding between HA and pAPTG makes difficult the dissociation of the complex (5). However, spent gels have been regenerated and reused. The bound HA was first denatured by treating the gel with 6 M guanidine·HCl during 2 h of holding at room temperature with occasional stirring. The gel was then washed exhaustively to remove HA and denaturant and then equilibrated with the pH 6.3 phosphate buffer. Gel lots have been reused up to four times without noticeable loss in usefulness.

The affinity chromatography described here differs from the usual approach in that the immobilized ligand has no intrinsic affinity for the protein being purified; the ligand is complementary to a second distinct component with which the desired protein is noncovalently associated in certain buffers. The neurotoxin is, therefore, bound indirectly to the adsorbent via the second

component. A change to conditions that dissociate the toxic complex selectively elutes the neurotoxin because of the strong hemagglutinin-adsorbent binding.

The procedure has several advantages over ion-exchange methods. The pAPTG gel is stable at 4°C; preparations have been used after 6 months of storage. When both adsorbent and crystals are available, ≥10 mg of neurotoxin can be obtained in about 2 h instead of the several days needed for the ion-exchange methods.

The amount of toxin that can be recovered from a given amount of crystals is significantly greater. Because of the several repeat chromatographic treatments needed to obtain high-purity toxin, the ion-exchange methods recover at best about 20% of the starting toxicity. This recovery contrasts with the 50 to 60% from the single affinity chromatography step.

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