Simplified Purification Method for \textit{Clostridium botulinum} Type E Toxin

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\textit{Clostridium botulinum} type E toxin was purified in three chromatography steps. Toxin extracted from cells was concentrated by precipitation and dissolving in a small volume of citrate buffer. When the extract was chromatographed on DEAE-Sephadex without RNase or protamine treatment, the first protein peak had most of the toxin but little nucleic acid. When the toxic pool was applied to a carboxymethyl Sepharose column, toxin was recovered in the first protein peak in its bimolecular complex form. The final chromatography step at 4°C on a DEAE-Sephacel column at a slightly alkaline pH purified the toxin (M, 145,000) by separating the nontoxic protein from the complex. At least 1.5 mg of pure toxin was obtained from each liter of culture, and the toxicity was 6 x 10^7 50% lethal doses per mg of protein. These values are significantly higher than those previously reported.

\textit{Clostridium botulinum} type E toxin is purified as an intact protein with an $M_r$ slightly below 150,000. It has a low specific toxicity that is increased up to a hundred or more times by trypsinization. Although the molecular change may not be responsible for the activation of toxicity, trypsinization cleaves a peptide bond so that the molecule, without detectably changing in size, becomes a two-subunit entity in which the larger subunit is covalently joined by disulfide linkage to the smaller subunit, which is about one-half the size of the larger subunit. The trypsinized type E toxin has the general molecular characteristics of the other botulinum toxin types that are activated by an endogenous enzyme(s) (11).

Purification of type E toxin, first reported in 1969 (7), starts with extracts made from vegetative cells (6). The nucleic acid, which interferes in the succeeding steps, is removed, and the resulting nucleic acid-free preparation is processed to obtain the toxin, which is noncovalently associated with a nontoxic protein of about the same size (bimolecular complex or M progenitor toxin). The toxin is then purified by removing the nontoxic protein by ion-exchange chromatography at a slightly alkaline pH.

Other laboratories have not recovered completely pure toxin with this original protocol and use additional treatments. The modifications make it possible to recover preparations of a desired purity (4, 15), but the added steps make purification cumbersome and lower the yield. High-performance ion-exchange chromatography (10) simplifies purification, but the special equipment is not yet available to most laboratories.

The present communication reports a simple purification protocol of three chromatographic treatments that recovers homogeneous toxin preparations in yields at least equal to the best previously reported yields.

\textbf{MATERIALS AND METHODS}

\textbf{Cell extract.} \textit{C. botulinum} type E 5545 (kindly supplied by C. L. Hatheway, Centers for Disease Control, Atlanta, Ga.) was grown for 4 days in tubes containing about 15 ml of cooked meat medium (Difco Laboratories, Detroit, Mich.) and stored at $-20^\circ$C as stock cultures. All incubations were done at 30°C.

Toxin production medium was 2.0% Trypticase-peptone (BBL Microbiology Systems, Cockeysville, Md.), 0.5% yeast extract (Difco), 1.0% glucose, and 0.025% sodium thioglycolate (BBL), adjusted to pH 6.5. When large volumes were used, a 12% glucose solution was autoclaved for 30 min and added to the broth portion, which was separately prepared and then autoclaved for 1 h.

A thawed stock culture was gently mixed, the large particles were allowed to settle, and about 0.5 ml was inoculated into a tube containing 30 ml of toxin production medium. After 25 h of incubation, the entire actively growing culture was transferred to 8 or 12 liters of toxin production medium in carboys. This culture was incubated for 55 h, at which time cells were collected and washed with 0.05 M sodium acetate buffer (pH 5.0), both by centrifugation (6).

The toxin in cells was extracted at 20°C rather than 30°C (6) because of the possibility of its being inactivated to a lesser degree at the lower temperature. The sedimented cells were dispersed in 800 ml of 0.2 M phosphate buffer (pH 6.0), adjusted to pH 6.0 with NaOH if necessary, and allowed to stand for 2 h with gentle stirring. This step was followed by standing overnight at 4°C and then for 2 h at 20°C with stirring. The resulting suspension was centrifuged, and the clarified supernatant fluid was saved as the first extract. The sedimented cells were reextracted with 400 ml of fresh buffer. The two extracts were combined, made 60% saturated with (NH$_4$)$_2$SO$_4$ (39 g/100 ml), and stored at 4°C.

DEAE-Sephadex chromatography. The precipitate of the cell extract, collected by centrifugation, was dissolved in 35 ml of 0.05 M sodium citrate buffer (pH 5.5). The resulting solution was clarified by centrifugation and chromatographed on a DEAE-Sephadex A-50 gel (Pharmacia, Inc., Piscataway, N.J.) column that was equilibrated with the citrate buffer. After a sample was applied, the column was washed with the citrate buffer. Fractions with an $A_{260}/A_{278}$ ratio lower than 0.65, making up the first protein peak that
came in the void volume, were pooled as the sample for the next step.

**CM-Sepharose chromatography.** The toxic pool was applied to a carboxymethyl (CM)-Sepharose CL-6B (Pharmacia) column that was equilibrated with 0.025 M sodium citrate buffer (pH 6.0). After sample application, the column was washed with the citrate buffer. The first protein peak, coming through essentially unretarded, was collected.

**DEAE-Sephadex chromatography.** The toxic pool (ca. 150 ml) from the second chromatography step was dialyzed against 0.01 M phosphate buffer (pH 7.4). It was dialyzed for 1 to 2 h against 2 liters of buffer during the day, against a fresh 2 liters overnight, and for 1 to 2 h against another 2 liters of buffer the next morning. The DEAE-Sephacel (Pharmacia) column was equilibrated with the phosphate buffer, and chromatography was done at 4°C. After sample application, the column was washed with 50 ml of the phosphate buffer and then eluted with an NaCl gradient made with 80 ml of phosphate buffer and 80 ml of phosphate buffer containing 0.3 M NaCl.

**Polyacrylamide gel electrophoresis.** Toxin samples recovered by the purification steps were electrophoresed in polyacrylamide gels in the presence of 0.1% sodium dodecyl sulfate and 8 M urea. The method of Swank and Munkres (13) was modified. The gels were 5.0% acrylamide and 2.7% cross-linker that was made in a solution containing 7.2 mg of acrylamide and 0.6 g of N,N' -methylenebisacrylamide in 100 ml of water, and 5 μl of N,N',N,N'-tetramethylethylenediamine, brought to a final volume of 15 ml with water. The gels were then cast in tubes with a 5-mm diameter and a height of 70 mm.

Specimens to be electrophoresed were 20 μg of sample protein in 5 to 80 μl added to a mixture of 27 μl of buffer (0.01 M H₂PO₄-0.5% sodium dodecyl sulfate-8.0 M urea adjusted to pH 6.8 with Tris base) and 10 μl of 50% glycerol-1.0% bromphenol blue in water. When disulfide bond reduction was needed, the toxin sample was heated for 5 min at 100°C in the presence of 4.0% 2-mercaptoethanol. Molecular weight standards were from Sigma Chemical Co., St. Louis, Mo.

Electrophoresis was done at room temperature with a current of 6 mA per gel at 50 V for 4.0 h. The reservoir buffer was 0.1 M H₂PO₄-0.1% sodium dodecyl sulfate adjusted to pH 6.8 with Tris base. After electrophoresis, the gels were stained for 1.0 h at room temperature with 0.1% naphthol blue black (Sigma) in a 5:4:1 methanol-water-acetic acid mixture. Decolorization was done at 60°C with several changes of the dye-free solvent. The mobilities of the resolved protein bands were determined after rehydrating the gels in 10% acetic acid.

**Protein and toxicity assays.** Protein was determined spectrophotometrically by using an A₂₇₈ of 1.63 for a 1.0-mg/ml solution (4). It was also assayed by the method of Bio-Rad Laboratories, Richmond, Calif., which is based on the binding of a dye by proteins. Standards were made with serum albumin.

Toxicity was assayed in mice of about 25 g. Routine assays were done by the intravenous method (3). Each of three or four mice was injected intravenously with 0.1 ml of sample, and the average time to death (in minutes) was determined. Mean lethal doses (LD₅₀) were read from a standard curve which showed the intravenous death times given by known intraperitoneal LD₅₀ of a relatively crude type E toxin preparation. Toxicities of purified preparations were also determined by the intraperitoneal method. Serial doubling dilutions made in 0.06 M phosphate-0.2% gelatin (pH 6.2) were injected intraperitoneally (0.5 ml) into separate groups of four mice. LD₅₀ were determined by the Reed-Muench calculation (9) with a criterion of death within 3 days.

Toxin was activated with trypsin (type XIII, tolensulfonl phenylalanl chloride methyl ketone treated; Sigma). Trypsin was added to the toxin in 0.03 M phosphate buffer (pH 6.0) so that the toxin/trypsin weight ratio was 2:1. The mixture was incubated for 30 min at 35°C, at which time soybean trypsin inhibitor (Sigma) at twice the enzyme weight was added to stop enzymatic action. Only activated toxicities are reported, but unactivated toxicities can be calculated from the activation factor, which is the toxicity of the trypsinized sample divided by the toxicity of the sample before trypsinization.

**RESULTS**

**Purification.** The culture fluid, freed of cells and then trypsinized, contained about 500 LD₅₀ per ml, an indication that most of the culture toxin is associated with cells (6). The intracellular toxin extracted from cells obtained from 8-liter cultures contained about 2 × 10⁶ LD₅₀ (Table 1). About 90% of this toxicity was recovered when the (NH₄)₂SO₄-precipitated material of the extract was dissolved in citrate buffer. Most of the toxin applied to the DEAE-Sephadex column was in the first protein peak, whose fractions came through essentially unretarded by the gel (Fig. 1). Those fractions with A₂₆₀/A₂₇₈ ratios of 0.5 to 0.65, indicative of low nucleic acid concentration, were pooled as the DEAE-Sephadex toxin. When this toxic pool was chromatographed on CM-Sepharose, most of the toxin was again unretarded and was recovered in the first protein peak (Fig. 2). The broad toxin peak resulted from the relatively high sample/gel volume ratio, deliberately chosen to save time and to avoid diluting the sample. The toxic pool contained about 50% of the applied protein but 80% of the toxicity. Protein adsorbed to the gel could be eluted with buffer containing 2.0 M NaCl.

All protein applied in the third chromatography step was bound by DEAE-Sephadex. Elution with an NaCl gradient recovered two protein peaks (Fig. 3), the first starting at about 0.05 M NaCl and the second starting at about 0.11 M NaCl. Almost all of the toxin was in the first of these two peaks.

**Polyacrylamide gel electrophoresis.** Figure 4, lane 1, shows the heterogeneity of the concentrated cell extract. The first (DEAE-Sephadex) chromatography step recovered toxin in a relatively impure state (lane 2). The CM-Sepharose toxin

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**TABLE 1. Purification by three chromatography steps of botulinum type E toxin from an 8-liter culture**

<table>
<thead>
<tr>
<th>Step</th>
<th>Total toxin recovered (mg)*</th>
<th>Total LD₅₀ (10⁶)</th>
<th>Specifc toxicity (10⁶ LD₅₀/mg)</th>
<th>Activation factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell extract</td>
<td>ND</td>
<td>1,890</td>
<td>ND</td>
<td>170</td>
</tr>
<tr>
<td>Concentrated extract</td>
<td>430</td>
<td>1,660</td>
<td>3.9</td>
<td>190</td>
</tr>
<tr>
<td>DEAE-Sephadex</td>
<td>75</td>
<td>1,360</td>
<td>18.1</td>
<td>240</td>
</tr>
<tr>
<td>CM-Sepharose</td>
<td>40</td>
<td>1,100</td>
<td>27.5</td>
<td>180</td>
</tr>
<tr>
<td>DEAE-Sephadex</td>
<td>13.5</td>
<td>810</td>
<td>60.0</td>
<td>200</td>
</tr>
</tbody>
</table>

* A 1.0-mg/ml solution with an A₂₇₈ of 1.63 was used.
A After trypsinization.
ND, Not determined.
resolved into two protein bands (lane 3), indicating that the bimolecular complex of the toxin had been purified. The final chromatography step on DEAE-Sephacel at a slightly alkaline pH recovered pure toxin (lane 4) with an $M_r$ of 145,000. When this toxin was trypsinized and treated with a disulfide-reducing agent, it resolved in electrophoresis into a large subunit with an $M_r$ of 105,000 and a small subunit with an $M_r$ of 50,000 (lane 5).

**Toxicity and recovery.** Recoveries of protein and toxin by the several chromatography steps are summarized in Table 1. In this run, pure toxin was recovered at the rate of 1.7 mg/liter of culture.

The specific toxicity of the bimolecular complex determined by intravenous titration was slightly below $3.0 \times 10^7$ LD$_{50}$/mg of protein, and that of the purified toxin was about $6.0 \times 10^7$ LD$_{50}$/mg of protein. The purified toxin had essentially the same toxicity when determined by the intraperitoneal titration method. When protein was assayed by the Bio-Rad assay, the specific toxicity was 10 to 15% higher, since this assay gave lower protein values. About 200-fold toxicity activation occurred when the purified toxin was trypsinized.

The purified toxin did not lose toxicity over a 2-month
The purification of type E botulinum toxin without the use of specialized equipment has been time-consuming, primarily because of the treatments used to obtain the extracted toxin in a solution with a low nucleic acid concentration. High-performance ion-exchange chromatography avoids many of the difficulties, but other steps are needed for use with open columns (10). The method described in the present report simplifies the purification by adapting the nucleic acid-removing chromatography technique used in the purification of type B (5) and A (12, 14) botulinum toxins. When this citrate buffer–DEAE-Sephadex chromatography technique is used, it becomes unnecessary to use the several treatments of the nucleic acid-free extracts reported to be essential for purifying type E toxin (4, 6). The second chromatography step now recovers the toxin in the form of a bimolecular complex, and the third chromatography step recovers pure toxin.

The first chromatography step works most efficiently if the (NH₄)₂SO₄-precipitated cell extract is dissolved in a small volume of buffer so that a concentrated extract can be applied. The extract is applied directly to the column, since the residual (NH₄)₂SO₄ is removed by the molecular sieving property of the gel. When the dimensions of the chromatographic columns are those described in the figure legends, all the extract from an 8- or 12-liter culture can be processed in one run.

The toxin purified in the present study was determined to be a 150,000-Mr unit composed of a large subunit with an Mr of 105,000 and a small subunit with an Mr of 50,000. These Mr's are within the ranges expected from the values recently reported for toxin purified by a different procedure: 147,000, 102,000, and 50,000 in one laboratory (4) and 144,000, 100,000, and 55,000 in another laboratory (15), respectively. The Mr of the high-performance ion-exchange chromatography-purified toxin was reported to be 137,000, but those of the subunits were not stated (10).

The minimum of 1.5 mg of toxin purified from each liter of culture compares favorably with the 1.0-mg/liter yield recently reported (4), the best yield so far. The specific toxicity of 6 × 10⁷ LD₅₀'s per mg of protein is higher than the previously reported LD₅₀'s of 1 × 10⁸ (10) and 2 × 10⁷ (15) per mg of protein.

The present study was started because of our interest in the molecular nature of the toxin that caused two illnesses which were clinically diagnosed as infant botulism, the form of illness in which *Clostridium botulinum* colonizes the intestinal tract (1). The toxin that caused these illnesses was neutralized by the antitoxin used to identify type E botulinum toxin but was produced by organisms whose other properties were those of *Clostridium butyricum* (2, 8). A preliminary study strongly suggested that the toxin described here also purified the toxin of these unusual *C. butyricum* strains. In this case, *C. butyricum* and type E botulinum toxins purified by an identical procedure can be compared.

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LITERATURE CITED