Purification and Characterization of Two Components of Botulinum C2 Toxin

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Two dissimilar proteins, designated as components I and II, of botulinum C2 toxin elaborated by strain 92-13 were purified to a homogeneous state. The molecular weights determined by sodium dodecyl sulfate gel electrophoresis were 55,000 for component I and 105,000 for component II. Whereas each component showed no or feeble toxicity even after being treated with trypsin, the toxicity was elicited when these two components were mixed and trypsinized. The toxicity of the mixture of components I and II at a ratio of 1:2.5 on a protein basis was 2.2 × 10^4 mouse intraperitoneal 50% lethal doses per mg of protein and increased by 2,000 times or more when treated with trypsin. These results indicate that the molecular characteristics of botulinum C2 toxin differ from those of the toxin of Clostridium botulinum types A through F in that C2 toxin is constructed with two separate protein components, which are not covalently held together, and that its toxicity is elicited by cooperation of the two components.

Clostridium botulinum type C and D strains produce three antigenically different toxins, C1, C2, and D. The C2 toxin is produced as a protoxin by certain strains of C. botulinum types C and D and those cured of thier prophages (3, 5, 12), so that toxicity can be demonstrated only after treatment with trypsin (3, 6). It has been reported that the trypsinized or endogenously nicked toxin molecules of C. botulinum types A through F are composed of two polypeptide chains (1, 10, 13, 18) which can be dissociated by treatment with a reducing agent in the presence of a detergent (8, 19). However, the association of these two chains is essential for eliciting the toxicity (19). In a previous paper, we reported that C2 toxin consists of two cooperative components which are resolved by ion-exchange chromatography or gel filtration (4). These two components individually show very feeble toxicity even after treatment with trypsin, but full toxicity can be attained simply by mixing these two components. This suggests that the molecular construction of botulinum C2 toxin differs from that of the toxin of C. botulinum types A through F. In the present paper, we describe the purification and characterization of these two components of C2 toxin and compare the molecular construction of C2 toxin with those of the other botulinum toxins.

MATERIALS AND METHODS

Bacterial strain and toxin production. Strain 92-13, resembling C. botulinum type C but producing only C2 toxin (11), was provided by S. Nakamura, Kanazawa University, Kanazawa, Japan. Medium for toxin production was the chopped-meat medium described previously (4). A 1-ml inoculum containing 10^7 refractile spores was inoculated into 5,000 ml of the medium in a flat-bottomed spherical flask, and the culture was incubated for 2 days at 37°C.

Activation and toxicity assay of C2 toxin. Tryptic activation was carried out in 0.05 M phosphate buffer, pH 8.0, for 30 min at 35°C unless otherwise indicated. Purified components I and II and C2 toxin were activated at a toxin-to-trypsin ratio of 2:1 on a protein basis. Crude materials were treated with trypsin at a final concentration of 200 μg/ml. Trypsinization was terminated by adding an amount of soybean trypsin inhibitor that was twice the weight of trypsin.

The toxicity in intraperitoneal mean lethal doses was determined in mice by the time-to-death method (4). When necessary, serial twofold dilutions of each sample were made in 0.05 M phosphate buffer, pH 8.0, containing 0.1% gelatin, and 0.1 ml was injected intraperitoneally or intravenously into separate groups of four mice. The 50% lethal dose was calculated from deaths within 4 days by the method of Reed and Muench (15).

Protein determination. Protein was determined by the method of Lowry et al. (9) with bovine serum albumin as the standard.

Polyacrylamide gel electrophoresis. Electrophoresis in a 7.5% polyacrylamide gel at pH 4.0 was carried out by the method of Reisfeld et al. (16). Neutral red was used as a marker dye to measure relative mobility. Sodium dodecyl sulfate gel electrophoresis and molecular weight determinations were performed in a 6.0% polyacrylamide gel by the method of Dunker and Rueckert (2). The molecular weight was determined in sodium dodecyl sulfate gel electrophoresis from the mobility rate relative to that of α-chymotrypsinogen A (molecular weight = 25,000) by using the following protein standards: gamma globulin (molecular weight = 150,000), phosphorylase a (molec-
ular weight = 94,000), bovine serum albumin (molecular weight = 66,000), and ovalbumin (molecular weight = 45,000).

Anti-component I and II sera. Antiserum specific for each component was prepared. Purified component at 200 μg/ml was detoxified by dialysis against 0.05 M phosphate buffer, pH 8.0, containing 0.4% Formalin for 48 h at 30°C. A 0.5-ml (100 μg) portion of a toxoid was emulsified in an equal volume of Freund complete adjuvant (Difco Laboratories, Detroit, Mich.) and was injected subcutaneously into a rabbit. After 4 weeks, a 0.5-ml (100 μg) portion of each of the untreated and homologous components was injected subcutaneously. The animals were bled after 2 weeks.

Neutralization test and immunodiffusion. A mixture of antiserum and toxoid was held at room temperature (20 to 25°C) for 30 min and injected intravenously into mice to determine toxicity. Neutralized. The agar gel double-diffusion test was performed by the method reported previously (7).

Purification of botulinum type C and D toxins. Botulinum type C and D progenitor toxins were purified from the culture of C. botulinum type C strain CB19 and type D strain CB16, respectively (10, 14).

Chemicals. Phosphorylase a and ovalbumin (Pentex Inc., Kankakee, Ill.), bovine serum albumin (Fraction V, Armour Pharmaceutical Co., Chicago, Ill.), gamma globulin (Schwarz/Mann, Orangeburg, N.Y.), and α-chymotrypsinogen A (Sigma Chemical Co., St. Louis, Mo.) were purchased from Wako Pure Chemical Industries, Osaka, Japan. Soybean trypsin inhibitor was the product of Worthington Biochemicals Corp., Freehold, N.J.; trypsin (type III, twice crystallized) was from Sigma Chemical Co.

RESULTS

Purification of C2 toxin. All procedures were performed at 4°C unless otherwise stated.

Step 1: precipitation with ammonium sulfate. Solid (NH₄)₂SO₄ was added to the whole culture to 58% saturation (380 g/liter), and the mixture was adjusted to pH 7.5 with 4 N NaOH. The precipitate formed by standing the mixture overnight was collected by centrifugation for 20 min at 4,650 × g. The precipitate from a 5-liter culture was suspended in 80 ml of 0.1 M tris-(hydroxymethyl)aminomethane (Tri)-hydrochloride, pH 7.5, and the suspension was centrifuged for 20 min at 4,650 × g. This extraction procedure was repeated, and the supernatants were combined.

Step 2: acid precipitation. The supernatant was dialyzed against 3 liters of 0.05 M acetic acid buffer, pH 4.5, for 24 h. The precipitate formed during dialysis was collected by centrifugation for 10 min at 8,600 × g and extracted twice with 50 ml of acetic acid buffer containing 0.5 M NaCl. The toxic extract was dialyzed against 0.05 M Tris-hydrochloride, pH 7.5.

Step 3: diethylaminoethyl Sephadex chromatography. The precipitate formed during dialysis was removed by centrifugation for 10 min at 8,600 × g. The supernatant was applied to a column of diethylaminoethyl Sephadex A-50 (4 by 24 cm) equilibrated with the Tris-hydrochloride buffer. After sample application, the column was washed with 700 ml of the same buffer and then eluted with 1,000 ml of 0.3 M NaCl in 0.05 M Tris-hydrochloride buffer, pH 7.5. The eluted fractions were pooled, concentrated to 60 ml by ultrafiltration with a PM-30 membrane, and dialyzed against 0.01 M acetic acid buffer, pH 6.0.

Step 4: carboxymethyl sephadex chromatography. The dialyzed fraction was applied to a column of carboxymethyl sephadex C-50 (2 by 10 cm) equilibrated with 0.01 M acetic acid buffer, pH 6.0. The column was washed with 60 ml of the buffer and eluted with 150 ml of the same buffer containing 0.3 M NaCl (Fig. 1). Each of the nonadsorbed and the eluate fractions had a toxicity of less than 1% of that applied. However, most toxicity was recovered when the two fractions were combined. Therefore, the nonadsorbed and the eluate fractions were designated as components I and II, respectively. These two fractions were concentrated separately by ultrafiltration through a PM-30 membrane.

Step 5: gel filtration on Sephadex G-100. The concentrated components were applied to separate Sephadex G-100 columns (2.5 × 95 cm) equilibrated with 0.05 M Tris-hydrochloride buffer, pH 7.5, and eluted with the same buffer (Fig. 2); the component I was rechromatographed on the Sephadex G-100 column. High toxicity was demonstrated when components I and II were mixed and trypsinized, whereas each component showed a very low toxicity.

The overall purification is summarized in Table 1. The recovery of toxicity was 20% of that in the culture as determined by the toxicity obtained by trypsinizing a mixture that was made by combining components I and II in the ratio of their concentrate volumes. The degree of the purification on a toxicity basis was 95-fold relative to the toxic preparation obtained by precipitation with (NH₄)₂SO₄.

Polyacrylamide gel electrophoresis. Purified components I and II each showed a single band in disc electrophoresis at pH 4.0. Electrophoretic mobilities relative to the marker dye were 0.51 and 0.42, respectively (Fig. 3). In sodium dodecyl sulfate gel electrophoresis, the purified components before and after treatment with 2-mercaptoethanol each showed a single band (Fig. 4). The molecular weights estimated by sodium dodecyl sulfate gel electrophoresis were 55,000 for component I and 105,000 for component II.
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Toxicities. The toxicities of C2 toxin were studied in a mixture made by adding various amounts of component I to a fixed amount of component II and by treating with trypsin (Fig. 5). The maximum toxicity was obtained when the ratio of component I to component II was from 1:2.0 to 1:2.5. Mixtures of these ratios increased 2,000-fold or more in toxicity when treated with trypsin. From these results, C2 toxin was defined in the present experiments as a mixture of components I and II at a ratio of 1:2.5 on a protein basis. Component II showed a very low toxicity after treatment with trypsin, whereas component I before or after trypsin treatment was not lethal to mice when 100 μg was injected intravenously (Table 2).

The toxicities of different combinations of trypsinized and untrypsinized component I or II were examined. A mixture of trypsinized component I and untrypsinized component II was not lethal to mice, whereas that of untrypsinized component I and trypsinized component II showed 82% of the toxicity of trypsinized C2 toxin (Table 3).

The toxicity of purified C2 toxin to mice by the intravenous route was higher than that by the intraperitoneal route; one 50% lethal dose required 5.4 ng of protein by the intravenous route and 49 ng by the intraperitoneal route.

Activation conditions. The purified C2 toxin

Elution pattern of the second gel filtration of component I; (b) elution pattern of gel filtration of component II.
**Table 1. Purification of components I and II of botulinum C₂ toxin**

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Potential toxicity ($\times 10^{-4}$ I.p. LD₅₀)$^a$</th>
<th>Recovery (%)$^b$</th>
<th>Specific toxicity ($\times 10^{-2}$ I.p. LD₅₀/mg of protein)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole culture</td>
<td>5,000</td>
<td>24,600</td>
<td>472</td>
<td>100</td>
<td>1.9</td>
</tr>
<tr>
<td>Ammonium sulfate precipita-</td>
<td>100</td>
<td>460</td>
<td>2.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extract of ammonium sulfate</td>
<td>160</td>
<td>4,570</td>
<td>2.6</td>
<td>84</td>
<td>8.6</td>
</tr>
<tr>
<td>precipitate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extract of acid precipitate</td>
<td>100</td>
<td>4,570</td>
<td>2.6</td>
<td>84</td>
<td>8.6</td>
</tr>
<tr>
<td>Diethylaminoethyl effluent</td>
<td>60</td>
<td>400</td>
<td>2.6</td>
<td>84</td>
<td>8.6</td>
</tr>
<tr>
<td>Carboxymethyl Sephadex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonabsorbed (component I)</td>
<td>120</td>
<td>82</td>
<td>—$^c$</td>
<td>NS$^d$</td>
<td></td>
</tr>
<tr>
<td>Eluate (component II)</td>
<td>150</td>
<td>166</td>
<td>3.3</td>
<td>0.7</td>
<td>2.0</td>
</tr>
<tr>
<td>Mixture of I and II’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Component I</td>
<td>45</td>
<td>4.8</td>
<td>$^*^f$</td>
<td>NS$^d$</td>
<td></td>
</tr>
<tr>
<td>Component II</td>
<td>50</td>
<td>48.5</td>
<td>3.1</td>
<td>0.7</td>
<td>6.4</td>
</tr>
<tr>
<td>Mixture of I and II’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ i.p. LD₅₀, Intraperitoneal 50% lethal dose.

$^b$ Toxicity of whole culture was taken as 100%.

$^c$ —, Mice survived for 100 min but not 6 h after intravenous injection of 4 μg of trypsinized protein.

$^d$ NS, Not significant.

$^e$ Toxicity after trypsinizing a mixture made of components I and II in a ratio corresponding to volumes of the two fractions.

$^f$ Mice survived after intravenous injection of 4 μg of trypsinized protein.

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**Fig. 3. Polyacrylamide gel electrophoresis of purified components I and II. A 10-μg portion of purified component I or II was applied to a gel column and electrophoresed for 210 min at 3 mA per column. (a) Component I, (b) component II, and (c) a mixture of I and II.**

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**Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of components I and II. A 5-μg portion of purified component I or II was applied on a gel column (4 by 70 mm) and electrophoresed for 240 min at 8 mA per gel. (a) Component I before and (b) after reduction; (c) component II before and (d) after reduction.**

was treated with trypsin at different pH’s for 30 min at 35°C, and the toxicities were determined. The optimum condition of activation was pH 1.0. Maximum activation of C₂ toxin was attained by incubation for 30 min at a trypsin-toxin ratio of 1:2 (Fig. 6). Toxicity persisted on the same level for 180 min.

**Immunodiffusion and neutralization tests.** In agar gel double-diffusion tests, purified components I and II each gave a single precipitin line against a mixture of rabbit anti-component I and II sera that crossed each other. The C₂ toxin, a mixture of components I and II, formed two precipitin lines, of which one fused in line of identity with the line of component I, and the other fused with that of component II (Fig. 7). Neither anti-component I nor II serum formed a precipitin line with 100 μg of the progenitor toxins of *C. botulinum* types C or D. A 10-μl
portion of anti-component I or II serum completely neutralized 20 mouse intraperitoneal 50% lethal doses of activated C2 toxin but did not neutralize the same amount of type C or D toxin.

**DISCUSSION**

The evidence of the present study shows that the two dissimilar protein components, designated as components I and II, are requisite for the lethality of C2 toxin in mice; a markedly higher toxicity was obtained when components I and II were mixed and treated with trypsin, whereas the components individually show a very low toxicity even after trypsinization. In a previous paper, we reported that these two components in culture supernatant can be separated by ion-exchange chromatography or gel filtration without treatment with detergent, reducing agent, or protease (4), indicating that components I and II exist as separate forms in culture fluid. This was confirmed in the present study.

![Graph](image)

**FIG. 5.** Toxicity of C2 toxin in mixture made with various ratios of components I and II. A 0.25-ml portion of component I containing 2 to 400 µg of protein per ml was added to 0.25 ml of component II containing 80 µg of protein per ml. Mixtures were treated with trypsin at 200 µg/ml. Toxicity in mixture made with the ratio of 0.4 (1:2.5 on a protein basis) was taken as 100%.

**TABLE 2.** Toxicities of components I and II and C2 toxin before and after trypsinization.

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Toxicity (i.p. LD₅₀/100 µg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trypsinized</td>
</tr>
<tr>
<td>Component I</td>
<td>*</td>
</tr>
<tr>
<td>Component II</td>
<td>50</td>
</tr>
<tr>
<td>C2 toxin*</td>
<td>2,200</td>
</tr>
</tbody>
</table>

*a* i.p. LD₅₀, Intraperitoneal 50% lethal dose.

*b* | Mice survived after intravenous injection.

*c* Components I and II were mixed at the ratio of 1:2.5 on a protein basis.

*d* —, Mice survived for 100 min but not 6 h.

**TABLE 3.** Toxicity in mixtures made with different combinations of trypsinized and untrypsinized components I and II.

<table>
<thead>
<tr>
<th>Mixture</th>
<th>Toxicity (i.p. LD₅₀/ml)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-I + T-II</td>
<td>17.9</td>
<td>1.0</td>
</tr>
<tr>
<td>T-I + UT-II</td>
<td>—/</td>
<td>—/</td>
</tr>
<tr>
<td>UT-I + T-II</td>
<td>14.6</td>
<td>0.82</td>
</tr>
<tr>
<td>UT-I + UT-II</td>
<td>—/</td>
<td>—/</td>
</tr>
</tbody>
</table>

*a* Equal volumes of trypsinized (T) and untrypsinized (UT) component I (2.3 µg/ml) and component II (5.8 µg/ml) were mixed, and the toxicity was determined. Trypsinization of each component was carried out as described in the text.

*b* i.p. LD₅₀, Intraperitoneal 50% lethal dose.

*c* Toxicity of T-I + T-II taken as 1.0.

*d* Both components trypsinized.

**FIG. 6.** Activation of C2 toxin with trypsin at different pH values. A 0.25-ml portion of C2 toxin containing 5 µg of component I and 12.5 µg of component II was mixed with an equal volume of each of the buffer solutions at different pH values containing 400 µg of trypsin. The buffers used were 0.05 M phosphate buffer, pH 6.3 and 7.1, and 0.1 M Tris-hydrochloride pH 7.9, 8.8, and 9.4.

by the successful purification of components I and II without such treatments. It has been reported that the trypsinized or endogenously nicked toxic components of types A through F progenitor toxins consist of two fragments with approximate molecular weights of 100,000 and 50,000 linked together with a disulfide bridge(s) (1, 10, 13, 17, 18). The fragments of type B and C toxins can be resolved by chromatography only when treated with both a detergent and a reducing agent (8, 19). The toxicity is lost comitant to dissociation; it is restored on reassociation of these two fragments by removing
the detergent and the reducing agent (19). The toxicity of botulinum C2 toxin, however, was regenerated simply by mixing components I and II, indicating that the in vitro binding of component I and II molecules is not required for toxicity. The molecular construction of C2 toxin is entirely different from that of the toxic component of progenitor toxins of C. botulinum types A through F in that its components are not covalently held together, so the components exist naturally as separate molecules.

Gel filtration, polyacrylamide gel electrophoresis, and sucrose density ultracentrifugation were tried in attempts to see whether purified components I and II of C2 toxin form a complex in vitro. But all attempts to demonstrate the complex failed. As we reported previously, the separation of components I and II of botulinum C2 toxin was observed with 1- or 2-day cultures of C. botulinum types C and D and strains producing only C2 toxin (4). It was also demonstrated in sodium dodecyl sulfate gel electrophoresis that components I and II each gave a single band even after reduction. These results indicate that C2 toxin intrinsically constitutes two separate polypeptides, although this does not rule out the possibility that these two components form a complex at the site where biological activity occurs.

In agar gel diffusion, purified components I and II each formed a single precipitin line; they crossed each other. This shows that components I and II are immunologically distinct protein molecules. Anti-component I or II serum gave no precipitin line with the progenitor toxin of C. botulinum types C and D and did not neutralize both of these toxins. This indicates that the components of C2 toxin have no antigenic relation with these botulinum toxin types.

ACKNOWLEDGMENTS

This work was supported by a grant-in-aid for scientific research from the Ministry of Education, Science and Culture.

LITERATURE CITED