Activation of Clostridium botulinum Type E Toxin Purified by Two Different Procedures

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Clostridium botulinum type E toxin was purified from culture supernates and from cell extracts by two methods. The specific activity \([2 \times 10^4 \text{ mouse } LD_{50} \text{ (mg protein})^{-1}]\) of the toxin purified from cell extract under slightly acidic conditions was lower than that \([3 \times 10^5 \text{ LD}_{50} \text{ (mg protein})^{-1}]\) of the toxin purified from culture supernate under slightly alkaline conditions. Both toxin preparations were activated by trypsin treatment, but to different extents, the degree of activation of the toxin from cell extract being about 30-fold higher than that of the toxin from culture supernate. The two toxin preparations had the same electrophoretic mobility on SDS-polyacrylamide gels and antigenic specificity as revealed by agar gel double-immunodiffusion tests. The antigenic specificity of the two toxin preparations was unaltered by trypsin treatment. In SDS-polyacrylamide gel electrophoresis, a single band of \(M_r 144000\) was demonstrated before trypsin treatment and two bands of \(M_r 100000\) and \(55000\) appeared after trypsin treatment. The two toxin preparations were labelled with \(^{125}\text{I}\) and chymotryptic peptide maps were obtained before and after trypsin treatment. The two toxin preparations without trypsin treatment demonstrated many differences in their peptide maps, but the preparations after trypsin activation had similar peptide maps. These results indicate that the toxin obtained from culture fluid was a partially activated form, and that its molecular conformation was different from that of the toxin from cell extract. Differences in specific activity and activation ratio by trypsin treatment may be due to differences in the conformation of the toxin molecules.

INTRODUCTION

Clostridium botulinum strains are classified into seven groups (A through G) based on the antigenic specificity of the toxins produced. They are also divided into proteolytic or non-proteolytic groups depending upon whether they produce hydrolytic enzymes active against proteins such as casein, coagulated egg white (or albumin) or meat particles. The activity of the toxin produced by non-proteolytic strains of types B, E and F is significantly increased by treatment with trypsin, but the activity of toxin produced by non-proteolytic types C and D, and proteolytic types A, B and F, is not.

All toxin types except G have been purified. Although some investigators have reported relatively low-\(M_r\) toxins (Gerwing et al., 1965), the consensus view is that the \(M_r\) of all types of toxin (or neurotoxin) is approximately 150000 (Sugiyama, 1980). Type E toxin was purified from cell extracts by Kitamura et al. (1968), who obtained a 12S toxin \((M_r 300000)\) by column chromatography under acidic conditions. This 12S toxin was dissociated into a 7S neurotoxin \((M_r 150000)\) and a 7S non-toxic component by DEAE-Sephadex column chromatography under alkaline conditions. The neurotoxin thus obtained had a single-chain structure and was activated more than 100-fold by trypsin without changing its serological reactivity or \(M_r\).

Abbreviations: TLCK, tosyl-l-lysine chloromethylketone; QAE-Sephadex, quaternary aminoethyl Sephadex.
Trypsin treatment causes ‘nicking’ at at least one site of the toxin molecule (DasGupta & Sugiyama, 1972a), and the toxin is dissociated into two fragments, the heavy ($M_r$ 100,000) and the light ($M_r$ 50,000) chains, by reduction of the S-S bond which links these components.

Type C and D toxins show little activation by trypsin, even though the organisms are non-proteolytic. Toxins purified from culture supernates by anion-exchange chromatography under alkaline conditions have a dichain structure without trypsin treatment (Oguma et al., 1981; Syuto & Kubo, 1981; Murayama et al., 1984). This suggested to us that type C and D strains produce small but sufficient amounts of proteases to activate the toxins and cleave the peptide bonds. Here, we report attempts to purify type E toxin from culture supernate by the procedure used for type C and D toxins in addition to the procedure reported by Kitamura et al. (1968) to detect the activated form or dichain structure of the toxin. We also determined the $M_r$ of the toxin, because Gerwing et al. (1965) and Sacks & Covert (1974) obtained relatively low-$M_r$, toxin from culture supernates.

**METHODS**

**Bacterial strain and culture.** Clostridium botulinum type E strain, Iwanai, was isolated in Japan from human botulism caused by consumption of fermented fish (Nakamura et al., 1956). Seed culture was obtained by growing organisms in cooked meat medium for 1 d at 30°C. Larger-scale cultures were prepared by two different procedures. One was a dialysis sac method to obtain the toxin released into culture fluid (Syuto & Kubo, 1972, 1977). The other involved extraction of toxin from bacteria cultured in Erlenmeyer flasks (Kitamura et al., 1968). The medium used in the dialysis sac method was 1% (w/v) polyethylene, 1% (w/v) lactalbumin hydrolysatle, 0-5% (w/v) peptone, 0-5% (w/v) yeast extract, 1% (w/v) glucose, 0-2% (w/v) NaCl and 0-05% (w/v) cysteine. HCl, pH 7.8. Glucose, NaCl and cysteine. HCl were inside the dialysis tube. For the purification of intracellular toxin, bacteria were cultured in a medium consisting of 2% (w/v) peptone, 0-5% (w/v) yeast extract, 1% (w/v) glucose and 0-05% (w/v) cysteine. HCl, pH 6-3.

**Toxin assay.** Toxin was diluted serially with 60 mM-phosphate buffer (KH$_2$PO$_4$/Na$_2$HPO$_4$, pH 6-0) containing 0-2% gelatin. Five mice weighing approximately 20 g were injected intraperitoneally with 0-5 ml dilute toxin and LD$_{50}$ was determined 4 d after injection, by the method of Reed & Muench (1938). The minimum lethal dose (MLD) was obtained by injecting two mice with serially twofold diluted toxin preparations. Bovine pancreas trypsin (twice crystallized, Sigma) was added to toxin dissolved in 160 mM-phosphate buffer (KH$_2$PO$_4$/Na$_2$HPO$_4$, pH 6-0) at a final concentration of 25 µg ml$^{-1}$ and the mixture was incubated at 37°C for 30 min. The reaction was terminated by addition of soybean trypsin inhibitor (Sigma) to a final concentration of 250 µg ml$^{-1}$.

**Purification of toxin from culture fluid.** The method of Syuto & Kubo (1972, 1977) was used, with slight modifications. After 5-6 d incubation, the contents of the dialysis sac were centrifuged (12,000 g, 30 min) and toxin was precipitated from the supernate by adding 2 vols saturated ammonium sulphate solution. The resulting precipitate was dissolved in 26-2 mM-borax/41.6 mM-sodium phosphate buffer (pH 8.0), and then serially applied to two columns, Sephadex G-75 (6.5 x 48 cm) and DEAE-cellulose (2-8 x 50 cm), both of which had been equilibrated with the same buffer. Unadsorbed fractions from the two connected columns were pooled, and applied to a column of QAE-Sephadex A-50 (2.2 x 15 cm), previously equilibrated with the borax/phosphate buffer (pH 8.0). After the column had been washed with 4 column volumes of borax/phosphate buffer, elution was carried out with an exponential gradient of NaCl generated by mixing 500 ml of the initial buffer and 250 ml of the same buffer containing 200 mM-NaCl. Active fractions were pooled and dialysed against saturated ammonium sulphate solution. The precipitate was collected by centrifugation, dissolved in the borax/phosphate buffer (pH 8-0), and placed on a column of Sephadex G-200 (2.2 x 90 cm) previously equilibrated with the same buffer containing 100 mM-NaCl. The column was eluted with the same buffer. A single peak appeared at about 165 ml and the constituent fractions were pooled and assayed for their toxicity in mice and their purity by SDS-gel electrophoresis.

**Purification of toxin from cell extract.** Toxin was purified according to the procedures reported by Kitamura et al. (1968) and DasGupta & Rasmussen (1983), except that CM-Sepharose was used instead of CM-Sephadex C-50. After 48 h incubation, cells were collected by centrifugation (12,000 g, 30 min). Cells (20 g wet weight) obtained from 10 l of culture were treated with 50 mM-sodium acetate buffer (pH 5-0) and the toxin extracted with 200 mM-phosphate buffer (KH$_2$PO$_4$/Na$_2$HPO$_4$, pH 6-0) at 37°C for 2 h and then at 4°C overnight. The cell extract was precipitated with 60% saturated ammonium sulphate and the precipitate redissolved in 20 mM-acetate buffer (pH 6-0). After dialysis against the same buffer, the extract was applied to a column of CM-Sepharose (55 ml bed volume) previously equilibrated with 20 mM-acetate buffer (pH 6-0). Unadsorbed fractions were collected and treated with pancreatic ribonuclease at a final concentration of 7-5 µg ml$^{-1}$ at 30°C for 5 h and successively at 8°C overnight. After removal of insoluble material by centrifugation (1000 g, 10 min), the resulting supernate was applied to a second column of CM-Sepharose (180 ml bed volume) previously equilibrated with 20 mM-acetate.
buffer (pH 6-0). The column was washed with the equilibration buffer and eluted with an exponential gradient of NaCl (30-120 mM). Toxic fractions were pooled and precipitated by the addition of solid ammonium sulphate to a final concentration of 60% saturation. The precipitate was collected by centrifugation, dissolved in 20 mM-acetate buffer (pH 6-0), and this solution was applied to a column of Sephadex G-200 (2.2 × 90 cm) previously equilibrated with the same buffer containing 100 mM-NaCl. A symmetrical portion of the main peak was pooled, dialysed against 50 mM-acetate buffer (pH 6-0), and then loaded onto the third column of CM-Sepharose (120 ml bed volume) previously equilibrated with the same buffer. After the column had been washed with the same buffer, the toxin was eluted with an exponential gradient of NaCl (20-100 mM). Fractions exhibiting toxic activity were pooled, dialysed against 10 mM-phosphate buffer (pH 7-4) and applied to a column of DEAE-Sephadex A-50 (50 ml bed volume) previously equilibrated with the same buffer. Neurotoxin was first eluted with a linear NaCl gradient between the initial buffer and the same buffer containing 200 mM-NaCl, and the nontoxic component was then eluted with the same buffer containing 400 mM-NaCl.

**Polyacrylamide gel electrophoresis.** This was done in 7% (w/v) polyacrylamide gel in the presence of 0.1% (w/v) SDS according to the method of Laemmlu (1970). Proteins (20-30 μg) were treated with 2% (v/v) 2-mercaptoethanol in the presence of 1% (w/v) SDS at 100 °C for 5 min and subjected to electrophoresis (4 mA per gel, 4 h). For M, calibration, the standard proteins (Bio-Rad) used were myosin (M, 200000), β-galactosidase (116250), phosphorylase (92500), bovine serum albumin (66200) and ovalbumin (45000).

**Agar gel double immunodiffusion test.** Agar gel (7 ml, 1%, w/v) dissolved in 10 mM-phosphate-buffered saline was poured into Petri dishes (9 cm diam.) and wells (5 mm diam.) were cut 4 mm apart. Each well received 10 μl of sample and the plates were then incubated for 2 d at room temperature in a moist chamber. After washing with phosphate-buffered saline, the plates were stained with 0.002% Coomassie brilliant blue R250 for several hours. Antiserum against type E crude toxin was produced as follows. The toxin was precipitated with ammonium sulphate from culture supernate and applied to a DEAE-cellulose column. The unadsorbed fractions were collected and toxoided by dialysis against 20 mM-sodium phosphate buffer (pH 6-0) containing 0.4% formalin for 7 d at room temperature. After mixing with an equal volume of Freund's incomplete adjuvant, the resulting emulsion was injected subcutaneously into rabbits.

**Iodination and peptide mapping.** Activated toxin was prepared as follows. Purified toxin (300 μl, 160 μg) dissolved in 100 mM-phosphate buffer (pH 6-0) was mixed with 100 μl bovine pancreas trypsin (250 μg ml⁻¹, Sigma) and incubated at 37 °C for 45 min in order to activate the toxin. Trypsin was removed by adsorbing it to a column (0.5 ml bed volume) of lima bean trypsin inhibitor-immobilized Sepharose (prepared in our laboratory). Unadsorbed protein was pooled, lyophilized, and used as activated toxin. Radioiodination, subsequent chymotrypsin digestion, and two-dimensional separation of peptides on silica gel-coated thin layer plates were done as described by Elder et al. (1977), with some modifications. Protein (15 μg) dissolved in 200 μl 500 mM-phosphate buffer (pH 7-5) was labelled with 0.2 mCi (7-4 kBq) ¹²⁵I for 60 min at room temperature by the chloramine T method. Free ¹²⁵I was removed by dialysis against 50 mM-NH₄HCO₃ (pH 7-5) at 4 °C overnight using Spectropor membrane tubing (M, cut-off 1000; Spectrum Medical Industries). The labelled sample was digested with 45 μg TLCK-treated bovine pancreas chymotrypsin (Sigma) for 20 h at 37 °C. Digested material was lyophilized and then dissolved in 10 μl TLE buffer (acetic acid/formic acid/H₂O, 15:5:80, by vol.). A portion (1-2 μl) of the digest was spotted on a silica gel G thin-layer plate (Merck), and peptides were resolved by electrophoresis in the first dimension and by ascending chromatography in the second dimension. Electrophoresis was carried out at 500 V for 140 min in TLE buffer using a Pharmacia flat-bed apparatus (FBE3000) with cooling at 4 °C. Chromatography in the buffer (n-butanol/pyridine/acetic acid/H₂O, 32.5:25.5:20, by vol.) was performed for about 4 h until the buffer front reached the top of the plate. The plate was then dried and examined by autoradiography using X ray film (Fuji Photo Film).

**RESULTS**

**Purification of toxins**

The results of the purification of type E toxin from culture supernate and from cell extract are summarized in Table 1.

When the culture was incubated for 5 d using the dialysis sac procedure, more than 70% of the toxic activity was recovered from the culture supernate. The toxin was not adsorbed to the column of QAE-Sephadex A-50 in borax/phosphate buffer (pH 8-0) (Fig. 1), on which type C toxin was reported to be adsorbed (Syuto & Kubo, 1972, 1977). Large amounts of non-toxic proteins, the major component of which was a protein of M, 86000, were adsorbed to the column (Fig. 1; Fig. 3a, lane 1). The unadsorbed fractions (neurotoxin) thus obtained, however, were slightly contaminated with a non-toxic protein of M, 86000. This contaminant was removed by gel filtration on Sephadex G-200 in the presence of 100 mM-NaCl.
Table 1. Purification of C. botulinum type E toxin

The toxin was purified four times from culture fluid and three times from cell extract. A representative purification is shown in each case.

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Before activation</th>
<th>After activation</th>
<th>Specific activity (10^-6 x LD₅₀)</th>
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<td>After activation</td>
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<td></td>
<td></td>
<td></td>
<td>(LD₅₀ x 10^-6)</td>
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<td>Toxin from culture fluid</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td>Ammonium sulphate precipitate</td>
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<td>330</td>
<td>2.1</td>
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<td>3-9</td>
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<td>0-06</td>
<td>21-7</td>
<td>1-97</td>
<td>680</td>
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</table>

* The elution patterns of these columns are shown in Figs. 1 and 2, respectively.

Little toxic activity was detected in the culture supernate which had been incubated for 48 h under the conditions described by Kitamura et al. (1968). Toxin was extracted from the cells and purified by column chromatography. Sephadex G-200 yielded a major and a minor protein peak (Fig. 2). The major peak eluted at a Kᵥ 0.11, and the minor peak at Kᵥ 0.2. The former had a toxic activity of 1 × 10⁴ MLD ml⁻¹ and the latter of 1 × 10³ MLD ml⁻¹, corresponding to the 12S and 7S toxins, respectively. Therefore, the fractions comprising the 12S peak was collected and then applied to CM-Sepharose. After the third CM-Sepharose column elution, a single peak of 12S toxin coincident with the peak of protein concentration was obtained. After dialysis against 20 mM-Tris/HCl buffer (pH 7.4), a neurotoxin (or 7S toxin) was separated from a non-toxic protein by ion-exchange chromatography on a column of DEAE-Sephadex A-50.

As summarized in Table 1, the specific activities of the toxin preparations purified from culture fluid and cell extract were different: the former (2.9 × 10⁵ LD₅₀ mg protein⁻¹) was 14-fold higher than the latter (2 × 10⁴ LD₅₀ mg protein⁻¹). The specific activity of each toxin preparation was similar after trypsin activation.

SDS-polyacrylamide electrophoresis of purified toxins and non-toxic components

The purity and Mₑ values of the toxins and the non-toxic protein thus obtained were analysed by SDS-polyacrylamide gel electrophoresis. Extracellular toxin obtained from Sephadex G-200 fractionation showed a single band of Mₑ 144000 (Fig. 3a, lane 2). Extracellular non-toxic protein obtained from QAE-Sephadex A-50 showed multiple bands but a major band of Mₑ 86000 (Fig. 3a, lane 1). The 12S toxin obtained from the third CM-Sepharose column showed two bands (Fig. 3b, lane 1), corresponding to neurotoxin (Mₑ 144000) and 7S non-toxic protein (Mₑ 123000). Neurotoxin and 7S non-toxic protein were isolated from 12S toxin by DEAE-Sephadex chromatography (Fig. 3b, lanes 2 and 3). Our final toxin preparations, obtained from culture fluid and from cell extract, showed the same mobilities on SDS-polyacrylamide gel electrophoresis under reducing conditions (Fig. 4a). After trypsic activation both toxin preparations showed two bands corresponding in Mₑ to 100000 (heavy chain) and 55000 (light chain) (Fig. 4b).

Antigenic specificity

Although the non-toxic protein (Mₑ 86000) purified from culture fluid showed similar chromatographic behaviour to toxin on QAE-Sephadex A-50, its precipitin line crossed with
Activation of botulinum type E toxin

Fig. 1. QAE-Sephadex A-50 chromatography. The unadsorbed fractions of the DEAE-cellulose column were layered on the column, which was washed with the equilibration buffer (arrow A) and eluted with an exponential gradient (0–200 mm) of NaCl (arrow B). The flow rate was 35 ml h⁻¹ and 7 ml fractions were collected. ●, A₂₈₀; ○, toxic activity; ———, NaCl gradient. The toxin recovered in fractions 23–70 was pooled (370 ml, A₂₈₀ 0·06).

Fig. 2. Sephadex G-200 gel filtration. The toxin fractions obtained from the CM-Sepharose column were concentrated with ammonium sulphate, and redissolved in 20 mm-acetate buffer (pH 6·0) containing 100 mM-NaCl. A sample (5 ml) was loaded on a Sephadex G-200 column with flow rate of 6 ml h⁻¹ and 1·6 ml fractions were collected. ●, A₂₈₀; ○, toxic activity. The toxin recovered in fractions 76–97 was pooled (35 ml, A₂₈₀ 0·45).

that of the toxin (Fig. 5a, wells 1 and 6). Precipitin lines of toxin preparations purified from culture fluid and cell extract fused with each other, both before (Fig. 5a, wells 3 and 4) and after activation by trypsin (Fig. 5). The non-toxic 7S component of 12S toxin obtained from the DEAE-Sephadex column exhibited different antigenic specificity from that of the non-toxic protein of M₄, 86000 and the two toxin preparations (Fig. 5a).

Chymotryptic peptide mapping

Toxins from culture supernate and cell extract were ¹²⁵I-iodinated before and after tryptic activation and then chymotryptic peptide mapping was carried out (Fig. 6). In the case of the toxin from culture fluid, 22 chymotryptic peptides were detected. When this toxin was treated with trypsin, 11 peptides were lost and 8 new chymotryptic peptides were formed. In the case of the toxin from cell extract, 32 spots were detected. After tryptic activation 21 spots were lost and 8 new spots were formed. Before activation by trypsin, the toxin preparations from culture
supernate and from cell extract had different chymotryptic peptide maps, although some of the spots were common to both maps. The toxin from cell extract had 12 spots which were absent in the toxin from culture supernate, whereas the toxin from culture supernate had 2 spots which were absent in the toxin from cell extract. On the other hand, after trypsin treatment, both
Fig. 6. Chymotryptic peptide mapping of $^{125}$I-iodinated toxins purified from culture fluid and from cell extract, with and without trypsin activation. Chymotryptic peptides were separated by electrophoresis in the first dimension, followed by thin-layer chromatography in the second dimension. (a, b) Toxin from culture fluid (a) and from cell extract (b) without trypsin activation; (c, d) toxin from culture fluid (c) and from cell extract (d) with trypsin activation; (e)-(h), traced figures of (a)-(d), respectively (solid spots are those common to the two toxins without activation; stippled spots are those generated after trypsin activation).

preparations demonstrated 19 spots, and 17 of the spots were common. These results indicate that after trypsin activation the toxin from culture supernate had a very similar molecular structure to the toxin from cell extract, although they differed before activation.

DISCUSSION

Type E strains of C. botulinum are reported to be non-proteolytic or non-ovolytic, and it has generally been thought that toxin from these strains was not activated by endogenous enzyme. We purified the toxin from both culture fluid and cell extract by different procedures. The toxin preparations obtained showed the same $M_r$ of 144000, and no toxic protein of a smaller size could be found. In the culture fluid there were large amounts of a protein of $M_r$ 86000, a size similar to that of the toxin reported by Sacks & Covert (1974). However, this protein had little lethal activity in mice and its antigenic specificity was entirely different from that of the purified toxins.

The antigenic specificity shown in double gel-immunodiffusion and the mobility on SDS-polyacrylamide gel electrophoresis of the two toxin preparations were the same with or without trypsin treatment. Before trypsin treatment, both toxin preparations were single-chain molecules. The differences between the two toxin preparations were in their specific activities and in their chymotryptic peptide maps before trypsin treatment. After trypsin treatment, however, the two preparations had similar specific activities and peptide maps; the specific activities were consistent with the value reported by DasGupta & Rasmussen (1983). Both toxin preparations were converted to a dichain structure by trypsin treatment.
These results indicate that toxin obtained from culture fluid is a partially activated form of the toxin from cell extract. Since the activation was partial and nicking did not occur during activation, this finding is similar to the activation phenomenon with the trypsin-like enzyme (TLE) reported by DasGupta & Sugiyma (1972b). The mechanism of partial activation is not yet clear, but the chymotryptic peptide maps indicated that partial activation is closely related to a conformational change in the toxin molecules. As to the cause of the conformational change, two possibilities may be considered: (1) type E cultures may produce small amounts of proteases like TLE even though they are 'non-proteolytic'; and these endogenous enzymes may cleave a peptide bond(s) at the tail of the toxin molecule, making changes undetectable on SDS-polyacrylamide gel electrophoresis, but which represent the molecular change responsible for partial activation; (2) the dialysis sac procedure or purification of toxin under alkaline conditions may induce the conformational changes without cleavage of peptide bonds. In any case, the purification procedure affects the specific activity and activation ratio of type E toxin, so the method used should be selected according to the purpose. We have demonstrated the existence of small amounts of proteases in culture fluid by using peptidyl cumarylamide, and are now trying to purify them to demonstrate the relationship between partial activation and enzyme activity.

REFERENCES


