Significance of 12S Toxin of Clostridium botulinum Type E

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The pathogenesis of type E botulism is discussed as an aspect of the physicochemical and biological properties of 12S toxins (prototoxin and trypsin-activated 12S toxin) and the Eα and Eβ components of each 12S toxin. A molecular weight of 350,000 was determined for each 12S toxin and 150,000 for Eα and Eβ. Owing to the structure comprising the subunits Eα and Eβ, 12S toxins are much more stable than Eα at low pH values and high temperatures. Such was also the case with type A 19S toxin and its α component. The Eα component alone accounts for the total toxicity of type E toxin. The toxic substance detected in the blood of the animals administered 12S toxins orally or parenterally was identified as Eα from the molecular size and the chromatographic pattern. Prototoxin escaping from detoxification in the stomach owing to the subunit structure may undergo dissociation in the intestine to release the Eα component. After absorption, the activated Eα appeared in the circulating blood without any further signs of dissociation or enzymatic digestion.

Clostridium botulinum type E toxin is obtainable in a form whose parenteral toxicity is enhanced by activation with trypsin (5, 16). We succeeded in purifying the toxin and its trypsin-activated product (“trypsin-activated 12S toxin” or “act. 12S toxin”; 12). To the active toxin, which possesses the same molecular size as act. 12S toxin and had been called precursor by us, the term “type-E prototoxin” was proposed (12). The molecules of 12S toxins, prototoxin and act. 12S toxin, consist of activable or fully toxic “Eα” and nontoxic “Eβ” components; the two components have the same S20,w of 7.3 and exist in equimolecular ratio (11, 12). Below pH 6, 12S toxins behaved as homogeneous protein and possessed the same S20,w of 11.6 (12, 15). When exposed to pH 8, both of them dissociated; Eα was separated from Eβ by starch-block electrophoresis (11, 12).

With respect to types A and B toxins, toxic 7S molecules analogous to Eα have been purified (3, 4). These toxic molecules were derived from the macromolecular toxins consisting of the toxic component and the nontoxic component analogous to Eβ. Such macromolecular toxins have been demonstrated to occur naturally in cultures and in foods (17, 18).

In the present paper we discuss the significance of the subunit structure of 12S toxin in the pathogenesis of type E botulism as suggested by our recent findings on physicochemical and biological properties of 12S toxins and their individual components.

MATERIALS AND METHODS

Toxins. Type E 12S toxins were purified by the method described previously (12). The prototoxin preparations contained a mean toxicity of $9 \times 10^4 \text{LD}_{50}/\text{mg}$ of N, which increased to $4.7 \times 10^7 \text{LD}_{50}/\text{mg}$ of N when trypsinized; the act. 12S toxin preparations, freed of trypsin by filtering the trypsin-activated materials through a Sephadex G-200 column, contained a mean toxicity of $5.7 \times 10^7 \text{LD}_{50}/\text{mg}$ of N. The preparations in 0.03 M acetic buffer, pH 6.0, were kept at −20°C until use.

The type A crystalline toxin in 0.9 M (NH₄)₂SO₄, provided by E. J. Schantz, Fort Detrick, Frederick, Md., was kept at 4°C until use.

A specimen of rabbit serum taken 4 hr after an oral administration of a crude prototoxin contained about 500 mouse LD₅₀/ml. The serum was provided by T. Ono, Hokkaido Institute of Public Health, Sapporo. Trypsinization did not increase the toxicity of the serum.

Toxic sera were obtained from mice after they had developed signs of botulism after intraperitoneal (ip) or intravenous (iv) injection with either kind of 12S toxin or “act.-Eα” (Eα from act. 12S toxin).

Determination of toxicity. The ip and iv injection methods described previously (12) were used for determination of toxicity. The iv injection method was applied to 12S toxins and Eα only after tryptic activation. Identical LD₅₀ values were obtained by direct ip injection and by converting the death time following
iv injection into ip LD50 value with act. 12S toxin, act.-E\textalpha and "act. proto-E\textalpha" (proto-E\textalpha activated with trypsin; unpublished data). Usually each of three mice was injected with 0.1 ml of the specimen chilled in an ice bath.

Activation of "proto-E\textalpha" (E\textalpha from prototoxin) was accomplished with trypsin (twice crystallized; Nutritional Biochemical Corp., Cleveland, Ohio); the trypsin had (TU) E\textalpha = 2.03 × 10^4 as determined by the Kunitz method (13). Preliminary experiments showed that proto-E\textalpha at 5 µg/ml yielded the highest toxicity when the trypsin concentration was 5, 25, or 125 times that of the substrate. The toxin was not maximally activated at lower trypsin concentration; higher enzyme concentrations gave maximum toxicity which dropped rapidly. In the present investigation, the proto-E\textalpha at 1 to 30 µg/ml was treated with trypsin at 100 µg/ml. Incubation was for 15 min at 33 C.

The toxicities of type A toxin and its \alpha component (4) were determined by the iv injection method (1).

Sucrose density gradient centrifugation. An 0.2-ml sample was layered on top of 4.8 ml of Veronal hydrochloride buffer (pH 8.0, \mu = 0.1) having a linear sucrose density gradient of 5 to 15% or on a similar gradient made with NaH2PO4-Na2HPO4 buffer (pH 6.0, \mu = 0.1). The tubes were centrifuged at 200,000 X g (SW50 L rotor) for 5 hr at 5 C in a Beckman L2 ultracentrifuge.

Estimation of molecular weight. The molecular weights of E\textalpha and E\beta were estimated by gel filtration on Sephadex G-200 with the type A \alpha component as a marker. Toxins were dissolved in 0.05 M KH2PO4-Na2HPO4 buffer of pH 8.0 at which they would dissociate. A 4-ml sample containing 3.7 mg of type E prototoxin or act. 12S toxin or 6 mg of type A toxin was filtered through a Sephadex G-200 column (2.5 by 95.6 cm) at 20 C; elution was with the same buffer at a constant flow of 26 ml/hr. Those of 12S toxins were determined by the sedimentation equilibrium method (20).

Diethylaminoethyl (DEAE)-Sephadex chromatography. DEAE-Sephadex A-50 (Pharmacia Fine Chemicals, Uppsala, Sweden) was equilibrated with 0.01 M KH2PO4-Na2HPO4 buffer, pH 8.0. All toxins were made to pH 8.0 by passing through a Sephadex G-25 column equilibrated with the same buffer. Prototoxin, act. 12S toxin, or type A toxin (3 mg in 6 ml) was applied onto a column (1 by 14 cm) and eluted at 4 C by linear gradient from 0 to 0.5 M NaCl in the starting buffer.

A 6-ml specimen of buffered toxic serum was chromatographed at room temperature in the same way as before.

Stabilities of toxins at different pH values and temperature. Type E act. 12S toxin (5 µg/ml), act.-E\textalpha (4.3 µg/ml) separated chromatographically, type A crystalline toxin (1.2 µg/ml), and the type A \alpha component (6.1 µg/ml) separated by Sephadex G-200 gel filtration were exposed to 33 C for 30 min at different pH values from 1.0 to 12.0. The toxic potencies before and after the exposure were compared by the iv injection method. The buffers used were: 0.2 M sodium acetate-hydrochloride (pH 1, 2, 3, and 4); sodium acetate-hydrochloride (pH 4 and 5, \mu = 0.1); NaH2PO4-Na2HPO4 (pH 6 and 7, \mu = 0.1); Veronal-hydrochloride (pH 8 and 9, \mu = 0.1); 0.1 M borate-sodium hydroxide (pH 10, 11, and 12). One volume of each toxin sample was mixed with nine volumes of each buffer added with NaOH or HCl to adjust the final pH to the indicated values.

Effect of temperature on stabilities of act. 12S toxin (10.0 µg/ml) and act.-E\textalpha separated chromatographically (4.3 µg/ml) in NaH2PO4-Na2HPO4 buffer (pH 6.0, \mu = 0.1) was tested at 0, 10, 20, 33, 37, and 50 C with an incubation period of 30 min.

Other methods. Protein content was determined by the method of Lowry et al. (14). The agar-gel-diffusion test has been described elsewhere (12).

**RESULTS**

Dissociation of 12S toxins and consequent change in molecular weight. Upon gel filtration of either of the type E 12S toxins on Sephadex G-200 at pH 8, the dissociated components were eluted in a single peak at a position more retarded than that at pH 6; type A toxin was resolved into two peaks, of which the late-eluted peak alone was toxic. Since the mixture of E\textalpha and E\beta was eluted at the position comparable to that where the type A toxic component was eluted (Fig. 1A and B), the molecular weights of E\textalpha and E\beta should be 150,000 (2, 4), as reported for the type A \alpha component.

*FIG. 1. Sephadex G-200 gel-filtration patterns of type E 12S toxin (A) and type A toxin (B) at pH 8. (A) •, Prototoxin; ○, act. 12S toxin. (B) ●, Protein content; △, toxicity; ○, hemagglutinating activity against chicken red blood cells expressed in reciprocal of the highest dilution showing positive agglutination. No activity was detected with fractions of type E 12S toxin.*
The molecular weights of 12S toxins were calculated to be 349,000, with fiducial limits of 10.6% at 95% level of probability.

Chromatographic patterns of Eα and Eβ. DEAE-Sephadex chromatography resolved each 12S toxin into two peaks of approximately the same protein content (Fig. 2A). Most of the apparent or potential toxicity was demonstrated in the first peak.

Agar-gel-diffusion tests showed that the toxicogenic peak corresponded to Eα and the nontoxicogenic one to Eβ. Each peak formed a single precipitation band against either of the anti-12S toxins (Fig. 3).

The proto-Eα fraction contained 5.7 × 10^6 and 1.3 × 10^6 LD50/mg of N before and after trypsin activation, respectively; the act.-Eα fraction contained 1.3 × 10^6 LD50/mg of N. The potential toxicity of the proto-Eα fraction and the apparent toxicity of the act.-Eα fraction were the same, being twice that of the parental 12S toxins.

As for type A toxin, the toxic α component was eluted at a position relatively later than that for Eα and the nontoxic β component (4) at the same position as Eβ (Fig. 2A and B). The α fraction contained a toxic potency five times that of the parental toxin as measured by the time to death titration.

**FIG. 2.** DEAE-Sephadex chromatographic patterns of type E 12S toxin (A) and type A toxin (B). (A) ○, Prototoxin; ©, act. 12S toxin; dashed line, NaCl concentration.

**FIG. 3.** Agar-gel-diffusion tests with 12S toxin and its components. (A) Anti-prototoxin, 420 international units (IU)/ml; (B) anti-act. 12S toxin, 420IU/ml; (1) proto-Eα, 200 µg/ml; (2) act. 12S toxin, 400 µg/ml; (3) proto-Eβ, 200 µg/ml; (4) act.-Eα, 200 µg/ml; (5) prototoxin, 400 µg/ml; (6) act.-Eβ, 200 µg/ml.

Protection of mice with anti-proto-Eα. Mice injected ip with 0.25 ml of anti-proto-Eα or anti-type A rabbit serum were challenged 90 min later by oral administration of 1 ml of type E cell suspension containing a potential toxicity of 3.5 million ip LD50 with or without trypsin activation. All mice receiving the anti-proto-Eα survived at least 2 hr longer (Table 1).

Dissociation and consequent change in stability of 12S toxins. The toxic components of both types E and A in the dissociated form were much more unstable at lower pH values than in the undissociated form. Act.-Eα lost its toxicity nearly completely in 30 min when exposed to pH 4, as did the type A α component at pH 3 (Fig. 4A and B).

When incubated for 30 min at pH 6, act.-Eα lost a large part of the toxicity at 37 C and all the toxicity at 50 C; act. 12S toxin retained about half at 50 C and practically all of the toxicity at 37 C or below (Fig. 5).

Under the same conditions of trypsin activation, the highest toxicity was attained more readily by trypsin-Eα than with prototoxin.

Resistance of Eα and Eβ to trypsic digestion at pH 8. Prototoxin (1.3 mg/ml) was incubated with trypsin (750 µg/ml or higher concentrations) at pH 8 and 37 C for as long as 120 to 180 min. A little toxicity remained in the digest. An agar-gel-diffusion test demonstrated that the antigenicity of Eα was not affected but that of Eβ was destroyed.

Sucrose density gradient centrifugation resolved the digest into two peaks; a small part sedimented to a position of about 5.3S and the remaining large part was left unsedimented. In the agar-gel-diffusion test, the fractions within the smaller
a All mice died within 3 to 4 hr after challenge.  
\( ^{b} \) All mice died in 2 to 3 hr after challenge.  
\( ^{c} \) One mouse died in 5 hr and the other in 24 hr.

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Fig. 1. Stabilities of type E act. 12S toxin and act.-E\(\alpha\) at different temperatures. (●) Act. 12S toxin; (○) act.-E\(\alpha\). The toxicity of each toxin sample mixed with the buffer at 0°C was taken as 100.

Fig. 2. Sucrose density gradient centrifugation at p\(H\) 8 of prototoxin exposed to p\(H\) 8 with or without trypsin and agar-gel-diffusion test with each fraction. The upper and lower rows of wells contained anti-prototoxin and prototoxin, alternately.

Identification of the toxic substance appearing in blood. The toxic substance appearing in blood after oral or parenteral administration of either 12S toxins or act.-E\(\alpha\) was determined to be about 7S by sucrose density gradient centrifugation at p\(H\) 6 (Fig. 7) and 8. Toxicity was demonstrated only with the fractions sedimenting to the same position as E\(\alpha\), regardless of the route of administration of 12S toxins or E\(\alpha\).

DEAE-Sephadex chromatography of the toxic serum specimens carried out under conditions identical to those for separating E\(\alpha\) from E\(\beta\) (Fig. 2A) demonstrated that the elution pattern for the toxic substance in the blood was the same as that for E\(\alpha\) (Fig. 8). The serum of the rabbit fed crude prototoxin was slightly toxic. The toxic substance behaved in the same way as E\(\alpha\), being sedimented to the same relative position and eluted in the same volume in chromatography.

DISCUSSION

Both type E 12S toxins, prototoxin and act. 12S toxin, possess an \(S_{20, w}\) of 11.6 (12, 15) and a molecular weight of 350,000 and consist of equimolar toxic E\(\alpha\) and nontoxic E\(\beta\) components,
each with an $S_{20,w}$ of 7.3 (11) and molecular weight of about 150,000. Although Eα and Eβ are separable from each other by starch-block electrophoresis (11, 12), DEAE-Sephadex chromatography separated them more completely and rapidly. In the chromatography, Eα behaved as a more basic protein than the type A α component, whereas Eβ and the type A β component were eluted in the same elution volume. Tryptic activation did not involve any change in the electric charge, molecular size, or antigenicity possessed by prototoxin (15) and by proto-Eα (12).

The dissociated toxic components of types E and A toxins were much more unstable than the undissociated toxins. The different stabilities were particularly pronounced below pH 4 or 3. The significance of the Eβ component in the complex is, therefore, probably that it stabilizes the Eα component. Dissociation may involve a conformational change in the Eα molecules, as the possible conformational transition was demonstrated at pH 7 (11).

Eα appeared to be more resistant to tryptic hydrolysis than Eβ, since tryptic treatment of prototoxin at pH 8 resulted in the formation of a 5.3S product having an antigenicity identical to Eα and in the complete loss of the antigenicity of Eβ.

The facts that Eβ is nontoxic, that Eα contains a specific apparent or potential toxicity twice as high as the parental 12S toxin, and that anti-proto-Eα protects mice against the challenge with type E whole cells show that it is Eα alone that accounts for the total toxic action. If Eα alone, however, was ingested, the low pH of the gastric juice would detoxify it very quickly at body temperature. When developed in foodstuffs, the type E toxin is in the 12S form (17) and type A possibly in the 19S form (18). Only such undisassociated toxins may pass through the stomach without detoxification. In the intestine, prototoxin
may be activated with trypsin at slightly acidic pH; act. 12S toxin and also type A 19S toxin may undergo dissociation in a lower part of the intestine, where the reaction is more alkaline. The freed Ee component does not appear to be rapidly digested by trypsin or other proteinases.

The toxic substance detected in blood after oral or parenteral administration of 12S toxin or Ee possessed the same molecular size and electrical charge as Ee itself. It is therefore considered that Ee or 12S toxin may be absorbed in its intact form through the intestinal barrier, and that Ee then appears in the circulating blood. The toxic material appearing in the lymph of the rats orally exposed to type A 19S toxin was found by Heckly et al. (10) to be about 7S (4 to 11S).

The toxic materials with a molecular weight of 150,000 of types E, A (4), B (3), and probably of all other types may be of pathogenetic importance, playing the active role in toxin action. Consequently, nontoxic Ee and analogous materials may look insignificant. Nevertheless, the macro-molecular toxin, including Ee, or the analogue could be of pathogenetic significance before absorption. The subunit structure of type E 12S toxin, which has been isolated and studied in this laboratory for many years, is important in food hygiene. Since such unusually small molecular toxins as those reported (6, 8, 9, 19) have not been found to exist naturally (17, 18), they cannot be considered as significant in the present discussion.

LITERATURE CITED


