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Molecular Construction of *Clostridium botulinum* Type F Progenitor Toxin

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Molecular dissociation of purified type F progenitor toxin with an $s_{20,w}$ of 10.3 and a molecular weight of 235,000 into two components, toxic and atoxic, was demonstrated by ultracentrifugation, gel filtration, and diethylaminoethyl-Sephadex chromatography at pH 7.5. The ultracentrifugal analysis indicated that type F progenitor toxin dissociates into components of the same molecular size of 5.9S. The toxic component contained a toxicity of 2.5×10^8 50% lethal doses per mg of N. Much higher stability of progenitor toxin than that of derivative toxin, particularly at pH below 5, suggests that only progenitor toxin can act as an oral toxin.

A preceding paper (7) described the purification of *Clostridium botulinum* type F progenitor toxin. The purified toxin behaved as a homogeneous protein in ultracentrifugation at pH 6.0, polyacrylamide gel electrophoresis at pH 4.0, and Sephadex G-200 gel filtration of pH 4.3. It possessed a molecular weight of 235,000. The agar gel diffusion tests, however, demonstrated two precipitin lines between the purified progenitor toxin and the specific antitoxin, suggesting that type F progenitor toxin consists of antigenically distinct toxic and atoxic components as observed with types A, B, and E progenitor toxins (2, 3, 5). The postulated molecular construction was verified by results of ultracentrifugation, gel filtration, and diethylaminoethyl (DEAE)-Sephadex chromatography at pH 7.5 described in the present communication. The molecular construction of type F progenitor toxin was compared with those of other types.

MATERIALS AND METHODS

Type F progenitor toxin was purified by the method reported previously (7).

Sephadex G-200 (particle size, 40-140 μm), DEAE-Sephadex A-50 (medium), and Ficoll were the products of Pharmacia Fine Chemicals, Uppsala, Sweden. Yeast alcohol dehydrogenase (Miles-Seravac Ltd., Maindenhead, Berkshire, England), bovine serum albumin (fraction V, Armour Pharmaceutical Co., Chicago, Ill.), egg albumin (Pentex Inc., Kankakee, Ill.), and chymotrypsinogen A (Sigma Chemical Co., St. Louis, Mo.) were used for calibration of the Sephadex G-200 column.

Sedimentation experiments were performed in a Beckman model E ultracentrifuge equipped with ultraviolet absorption optics. The sedimentation constant was determined in a buffered 1.0 M NaCl solution by the band sedimentation method. Molecular weights were estimated by gel filtration on a column of Sephadex G-200 according to the method of Andrews (1). Protein contents were determined by the method of Lowry et al. (6) with bovine serum albumin as standard. The assay for toxicity and the agar gel double-diffusion test were carried out in the same way as described previously (7).

RESULTS

Ultracentrifugation of type F progenitor toxin at pH 6.0 and 7.5. Ultracentrifugation at either pH 6.0 or 7.5 of type F progenitor toxin showed a single boundary. The sedimentation constant, $s_{20,w}$, at pH 6.0 was calculated to be 10.3 and that at pH 7.5 to be 5.9. The results suggested that type F progenitor toxin had dissociated at pH 7.5 into smaller molecular-sized components of the same sizes.

Gel filtration at pH 4.0 and 7.5. Molecular dissociation was demonstrated also by gel filtration of type F progenitor toxin on a column of Sephadex G-200 (Fig. 1) at pH 4.0 and 7.5. The elution volume was used to calculate the molecular weights of type F progenitor toxin at pH 4.0 (elution volume = 208 ml) and type F derivative toxin at pH 7.5 (elution volume = 242 ml). These were estimated to be 230,000 and 128,000, respectively (Fig. 2).

Separation of the two components by DEAE-Sephadex chromatography. Ultracentrifugation and gel filtration indicated the molecular dissociation of type F progenitor toxin at pH 7.5, but neither procedure separated one

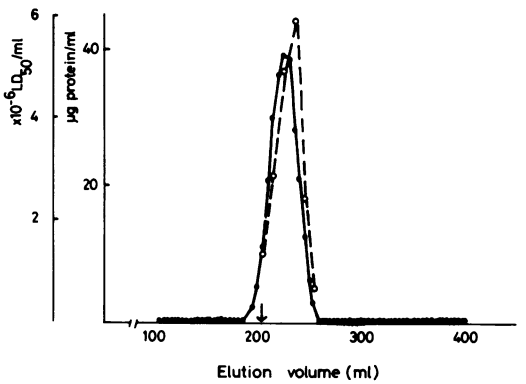

FIG. 1. *Gel filtration of type F progenitor toxin on Sephadex G-200. Purified type F progenitor toxin (1.2 mg in 5 ml) was applied to a Sephadex G-200 column (2.5 by 98 cm) equilibrated with 0.05 M phosphate buffer, pH 7.5. Fractions of 5.2-ml amounts were collected at a flow rate of 15 ml/h. Protein contents,* ●——●*; toxicity,* O- - -O. *The arrow indicates the elution peak obtained with 0.1 M acetate buffer, pH 4.2, containing 0.3 M NaCl.*

component from the other. Purified type F progenitor toxin was chromatographed on a column of DEAE-Sephadex A-50 at pH 7.5. The elution profile (Fig. 3) indicates two protein peaks of approximately the same area; one was toxic and the other atoxic. The toxic component possessed a toxicity of 2.5×10^8 50% lethal doses per mg of N or twice as high as that of the progenitor toxin.

Agar gel double-diffusion tests with the dissociated components. Each of the two components separated by DEAE-Sephadex chromatography was concentrated by dialysis against Ficoll and subjected to agar gel double-diffusion tests. The line formed by the toxic component coalesced with one of the two lines formed with the progenitor toxin and that formed by the atoxic component with the other one (Fig. 4).

Stablities of type F progenitor and derivative toxins at different pH values. Type F progenitor and derivative toxins were incubated at different pH values at 35 C for 30 min. The remaining toxicity was assayed. The results demonstrated that at pH 5.0 or below derivative

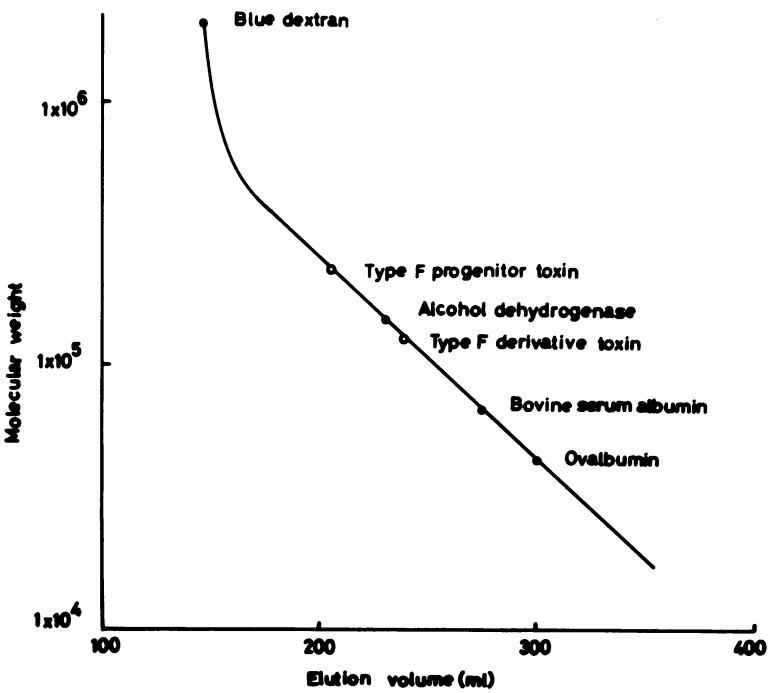

FIG. 2. *Molecular weight determination of type F progenitor toxin by gel filtration on a Sephadex G-200 column. Each of purified type F progenitor toxin, alcohol dehydrogenase (molecular weight = 150,000), bovine serum albumin (molecular weight = 69,000), ovalbumin (molecular weight = 45,000), and chymotrypsinogen A (molecular weight = 23,000), dissolved in 0.05 M phosphate buffer, pH 7.5, was applied to a Sephadex G-200 column (2.5 by 98 cm) equilibrated with the same buffer. The toxin was eluted also with 0.1 M acetate buffer, pH 4.2, containing 0.3 M NaCl.*

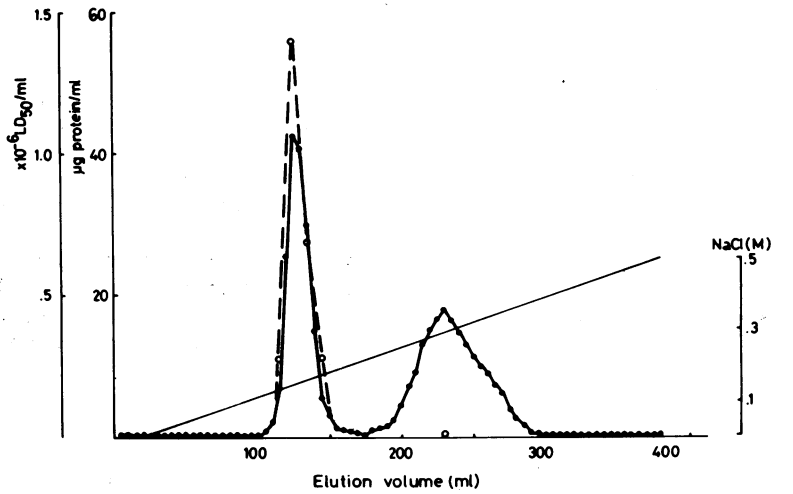

FIG. 3. *DEAE-Sephadex chromatography of type F progenitor toxin. Purified type F progenitor toxin was dialyzed against 0.01 M phosphate buffer, pH 7.5, and applied to a DEAE-Sephadex A-50 column (0.9 by 7 cm) equilibrated with the same buffer. Elution was performed with a linear gradient increase in NaCl concentration from 0 to 0.5 M in 400 ml of the buffer. Five-milliliter fractions were collected. Protein content,* ●——●*; toxic activity,* O- - -O*; NaCl concentration,* ——.

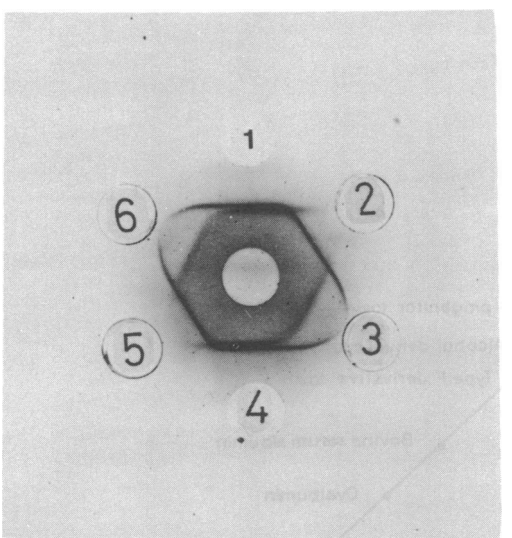

FIG. 4. *Agar gel double-diffusion tests with type F progenitor and derivative toxins and the atoxic component. Center well, anti-type F progenitor toxin globulin. Lateral wells: (1) and (4) progenitor toxin (505 μg/ml), (2) and (5) toxic component (300 μg/ml), and (3) and (6) atoxic component (246 μg/ml). Agar gel, 1% agar in 0.05 M acetate buffer, pH 6.0; distance between every two wells, 7 mm; incubation at 4 C for 2 days.*

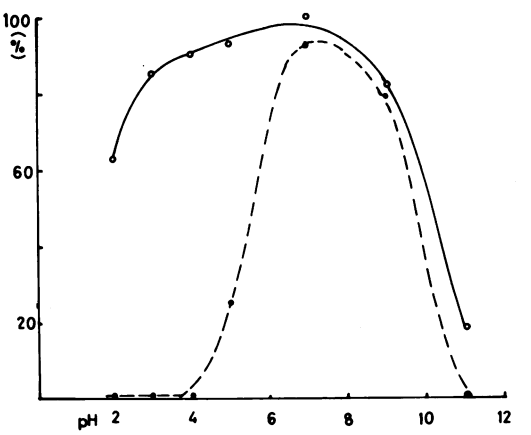

FIG. 5. *Stabilities of type F progenitor and derivative toxins at different pH values. Progenitor and derivative toxins separated chromatographically were exposed to 35 C for 30 min at different pH values. Progenitor toxin,* O——O*; toxic component,* ●- - -●*. The buffers used were: 0.2 M sodium acetate-hydrochloride buffer, pH 2.0 and 3.0; 0.2 M acetate buffer, pH 4.0 and 5.0; 0.1 M phosphate buffer, pH 7.0; 0.2 M boric acid-NaOH buffer, pH 9.0 and 11.0. The toxicity of each toxin in 0.1 M acetate buffer, pH 6.0, was taken as 100.*

toxin was much more unstable than progenitor toxin, which retained 60% or more of the toxicity at a pH range between 2.0 and 9.0 (Fig. 5).

DISCUSSION

Molecular dissociation of type F progenitor toxin into two components was suggested by agar gel double-diffusion tests (7). It was verified decisively by ultracentrifugation, gel filtra-

tion, and DEAE-Sephadex chromatography at pH 7.5 in the present investigation. In the first two procedures, molecular dissociation occurred but the components were not separated; they were separated from each other only by DEAE-Sephadex chromatography. In agar gel double-diffusion tests, the lines formed with the separated components gave reaction of identity with the different lines of the two formed with progenitor toxin.

Ultracentrifugal analysis at pH 7.5 demonstrated that the two dissociated components were of the same molecular size, and that the molecular dissociation occurred very rapidly, as no boundary representing the progenitor toxin was observed before the speed of revolution reached the maximum. The elution position of the peaks of protein and toxicity were slightly different in gel filtration of the progenitor toxin at pH 7.5, which may have been caused by possible interaction of one or both components with Sephadex gel particles. The specific activity of the toxic component or derivative toxin was twice as high as that of the progenitor toxin, indicating that toxicity of type F progenitor toxin is entirely dependent upon the toxic component. All these results indicate that the molecule of type F progenitor toxin with an $s_{20,w}$ of 10.3 consists of one molecule each of toxic and atoxic components with the same $s_{20,w}$ of 5.9.

The results obtained in this investigation are very similar to those obtained with type A, B, and E progenitor toxins (2, 3, 5), all of which are composed of two components, toxic and atoxic, no matter how large or small the molecular size. The crystalline toxin (molecular weight, 900,000 [2]) and type B-L toxin (molecular weight, 500,000 [5]) are composed of the toxic and nontoxic components of different molecular sizes, whereas type B-M toxin (molecular weight, 350,000 [5]), type E progenitor toxin (molecular weight, 350,000 [3]) and type F progenitor toxin (molecular weight, 230,000) are composed of the two components of the same molecular size. It may be noteworthy that the molecular sizes of the components of type F progenitor toxin are significantly smaller than those of type B-M and E progenitor toxins, and hence the molecule of type F progenitor toxin is appreciably smaller than those of types B-M and E. It seems justifiable to generalize that progenitor toxins of other types may also consist of toxic and atoxic components that could dissociate in alkalinity.

We confirmed that type F derivative toxin is much more labile than the progenitor toxin, particularly at pH values below 5. The atoxic component may act to stabilize type F toxic component as suggested with type E toxins by Kitamura et al. (4). Only the progenitor toxin, therefore, may constitute the material toxic via the oral route in the sense that it resists inactivation by gastric juice and digestive enzymes.

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