Antigenicities of Fragments of Clostridium botulinum Type B Derivative Toxin

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Received for publication 31 December 1974

Two fragments with molecular weights of 110,000 and 60,000 were separated in a preparatory scale by gel filtration of the reduced Clostridium botulinum type B trypsinized derivative toxin on Sephadex G-200 with 0.05 M tris(hydroxy-methyl)aminomethane-0.38 M glycine buffer, pH 8.3, containing 5 mM ethylenediaminetetraacetate, 1 mM dithiothreitol, and 2 M urea as eluant. They were both antigenic, forming crossing precipitin lines against type B antitoxin in agar gel double diffusion tests.

Clostridium botulinum type A, B, E, and F progenitor toxins have been purified (7-9, 12). The molecule of the progenitor toxin of these types is composed of a toxic component of a fairly uniform molecular size of 7S and a nontoxic component(s) of different molecular sizes ranging from 7 to 13S (4, 7, 8, 12). The toxic component of type A, B, and trypsinized E and F progenitor toxins is made up of two polypeptide fragments with molecular weights of about 100,000 and 50,000; they are separable from each other by sodium dodecyl sulfate (SDS)-gel electrophoresis of the toxic component exposed to guanidine-hydrochloride or urea and β-mercaptoethanol (1) or dithiothreitol (DTT) (5). It was suggested that neither fragment was toxic (16), precipitable with antitoxin (15), or stimulated production of specific antitoxin or antibody in guinea pigs (15).

We succeeded in isolating the two fragments in a preparatory scale by gel filtration of the reduced type B derivative toxin on Sephadex G-200 with an eluant containing a reducing agent and in demonstrating distinct antigenicities possessed by these fragments.

MATERIALS AND METHODS

Type B derivative toxin. We purified type B progenitor toxin of C. botulinum type B Okra in two different forms (8); one was 12S and named “medium toxin” or “M toxin” and the other was 16S and named “large toxin” or “L toxin.” The purified M toxin was chromatographed on diethylaminoethyl-Sephadex at pH 8.0 to separate “the toxic component” or “derivative component” (7S) from “the nontoxic component” (75).

Type B antitoxin. Type B M toxin (0.2 mg/ml) was toxoided by dialysis against 0.1 M phosphate buffer, pH 7.0, containing formalin at 0.4%, for 6 days at 30°C. The toxoid was mixed with an equal volume of Freund complete adjuvant (Difco Laboratories, Detroit, Mich.). Three 1.0-ml doses of the toxoid emulsion were injected subcutaneously into two rabbits weighing 2.5 and 3.0 kg at 2- and 3-day intervals. Six weeks after the third injection, two booster injections were given, also subcutaneously, of 1.0-ml doses of M toxin (0.1 mg/ml of 0.05 M acetate buffer, pH 6.0) without adjuvant at a 2-day interval. Ten days after the last injection, the rabbits were exsanguinated from the cervical artery. Antitoxin titration was performed by passive hemagglutination with formalized sheep erythrocytes coupled with type B M toxin by the same procedures as those reported for type E antitoxin (13). Type B antitoxic horse plasma with a potency of 800 IU/ml, given by H. Kondo, Chiba Serum Institute, Ichikawa-shi, Chiba, was used as a reference. The potencies estimated were 510 and 260 IU/ml.

Chemicals. Diethylaminoethyl-Sephadex, A-50, medium, Sephadex G-200 (particle size, 40 to 120 μm), and Ficoll were products of Pharmacia Fine Chemicals, Uppsala, Sweden. Twice-crystallized trypsin was the product of Sigma Chemical Corp., St. Louis, Mo.; soy bean trypsin inhibitor was from Worthington Biochemical Corp., Freehold, N.J.; DTT was obtained from Pierce Chemical Co., Rockford, Ill.; SDS and urea were from Wako Pure Chemical Industries, Osaka; and chymotrypsinogen A, ovalbumin, bovine serum albumin, and human gamma globulin were from Schwarz/Mann, Orangeburg, N.Y.

Determination of toxicity. Toxicity was determined by the time-to-death method by intravenous injection into mice (2, 14).

Determination of protein. Protein was determined by the colorimetric method with the Folin phenol reagent (10). DTT, if contained in samples, was removed by dialysis against 0.05 M tris(hydroxy-methyl)aminomethane (Tris)-hydrochloride buffer, pH 8.0, or 0.05 M phosphate buffer, pH 7.2.

SDS-gel electrophoresis. Type B derivative toxin (1 mg/ml of 0.05 M acetate buffer, pH 6.0) was added...
with one-tenth volume of a trypsin solution (10 to 50 μg/ml of 0.05 M Tris-0.38 M glycine buffer, pH 8.3). The final pH was 8.1. The mixture was incubated at 25 C for 60 min, when soy bean trypsin inhibitor in twice the quantity of trypsin was added to terminate hydrolysis. Toxicity increased by two- to 10-fold, which was equivalent to the activation ratio attained with 20 μg of trypsin per ml at pH 6.0 and 33 C for 20 min.

Electrophoresis in 5% polyacrylamide gel was performed in principle according to Weber and Osborn (17). Each sample contained 50 μl of a derivative toxin or fragment solution, 50 μl of 0.02 M phosphate buffer, pH 7.2, containing SDS at 2.0% and glycerol at 50%, and 20 μl of a 0.5 M DTT solution or distilled water. The mixture was incubated at 37 C for 2 h. A current of 7 mA was applied per column. After electrophoresis, the gel column was stained in a 0.2% Coomassie brilliant blue solution for 30 to 60 min.

Separation of two fragments on Sephadex G-200. A 2-ml portion of derivative toxin (1.7 mg/ml) was treated with 0.2 ml of trypsin (0.1 mg/ml of 0.05 M Tris-0.38 M glycine buffer, pH 8.3) at 25 C for 60 min and then added with 0.2 ml of soy bean trypsin inhibitor (0.2 mg/ml). A toxicity of 1.65 × 10^4 50% lethal doses/ml was furnished. The trypsinized derivative toxin was incubated with 0.35 g of urea and 0.48 ml of 0.5 M DTT at 25 C for an additional 60 min. This was applied to a Sephadex G-200 column (2.5 by 39 cm) equilibrated with 0.05 M Tris-0.38 M glycine buffer, pH 8.3, containing 5 mM ethylenediaminetetraacetate, 1 mM DTT, and 2 M urea, and eluted with the same buffer.

Agar gel double diffusion. Agar gel (1%) in 0.075 M phosphate buffer (pH 7.2)-0.075 M NaCl was used. Seven wells of 2 mm in diameter, six at each angle of a hexagon (7 mm on one side) and the other one at the center, were made in agar gel on a glass plate (5 by 5 cm). The center well received type B antitoxin (260 IU/ml), and the lateral wells appropriately diluted derivative toxin and fragment solutions (159 to 269 μg/ml). After incubation of the filled plates in a moist chamber for 2 days at 20 C, the precipitin lines were stained with a 0.1% thiazine red solution.

RESULTS

In SDS-gel electrophoresis, the nonreduced derivative toxin with or without trypsinization migrated in one band at the same mobility rates, and the trypsinized and reduced toxin migrated in two bands at different mobility rates, both of which were higher than that of nonreduced derivative toxin. The untrypsinized but reduced toxin migrated in three bands, one with the same mobility rate as that of the untrypsinized and unreduced toxin being the major one (Fig. 1). With human gamma globulin (molecular weight, 160,000), bovine serum albumin (molecular weight, 67,000), ovalbumin (molecular weight, 45,000), and chymotrypsinogen A (molecular weight, 25,000) as references, the approximate molecular weights of type B derivative toxin and its two fragments were estimated to be 170,000, 110,000, and 60,000, respectively.

In Sephadex G-200 gel filtration of the reduced type B derivative toxin, three peaks were

Fig. 1. Polyacrylamide gel electrophoresis in the presence of SDS; a 42-μg sample each of (A) derivative toxin, (B) DTT-treated derivative toxin, (C) trypsinized derivative toxin, and (D) trypsinized and DTT-treated derivative toxin. The arrows in Fig. 1, 3, and 4 indicate the starting points.
eluted (Fig. 2). Fractions of each peak were pooled, concentrated by dialysis against Ficoll, and dialyzed against 0.05 M phosphate buffer, pH 7.2. Little or no toxicity was demonstrated in any concentrate. The first peak recovered 262 μg of protein and no detectable toxin in 0.9 ml, the second peak recovered 538 μg and 29,000 50% lethal doses in 1.0 ml, and the third peak recovered 159 μg and 16,000 50% lethal doses in 0.9 ml.

In SDS-gel electrophoresis, the main band of the second peak had the same relative mobility as that of the fragment having a molecular weight of 110,000 (Fig. 3B), and the main band of the third peak as that of the fragment having a molecular weight of 60,000 (Fig. 3C). The concentrate of the first peak freed of DTT hardly migrated in the gel and remained on top of the gel column (Fig. 3A). Upon reduction again with DTT, however, the concentrate of the first peak migrated in the gel column in two major bands (Fig. 4B) with the same relative mobility rates as those of the 110,000- and 60,000-molecular-weight fragments (Fig. 4A).

The results of agar gel double diffusion tests are shown in Fig. 5. Each of type B derivative toxin and its two fragments formed a single precipitin line against type B antitoxin. The precipitin lines of the two fragments fused with that of the derivative toxin, but they crossed each other. It appeared that the concentrate of the first peak hardly diffused into the agar gel.

**DISCUSSION**

Beers and Reich (1) and DasGupta and Sugiyama (5) reported that *C. botulinum* derivative toxins produced by proteolytic type A, B, and F strains are in the form of "nicked toxin" in the sense analogous to that used for diphtheria toxin (3, 6). Upon SDS electrophoresis of the reduced nicked toxin, two polypeptide fragments are separable from each other. To the contrary, the active component of progenitor toxin produced by nonproteolytic type E strains is in the form of "intact toxin," which requires trypsinization to be separated into the two fragments in SDS-gel electrophoresis under the same conditions.

We found that type B derivative toxin obtained in the present investigation from the proteolytic Okra strain contained both intact and nicked toxins at various proportions. For molecular weight estimation of the fragments by SDS-gel electrophoresis and for separation of one fragment from the other by gel filtration, therefore, trypsinization of the toxic component under carefully controlled conditions was always necessary to assure the complete transformation of intact toxin into nicked toxin.

We succeeded in separating two fragments in
a preparatory scale by gel filtration of the trypsinized, DTT- and urea-treated toxic component on a Sephadex G-200 column with a DTT-, ethylenediaminetetraacetate-, and urea-containing buffer, pH 8.3, as eluant. Neither fragment was toxic, and the molecular weights estimated for them were 110,000 and 60,000, which were in good accordance with the results reported by Beers and Reich (1) and DasGupta and Sugiyama (5). In spite of the observation by Sugiyama et al. (15) that the toxic component of type A crystalline toxin lost its ability to precipitate with the antitoxin, we demonstrated in agar gel double diffusion that both fragments form precipitin lines against type B antitoxin. Both lines fused with that of the toxic component, but they crossed each other. Both fragments are antigenic, and their antigenicities are distinct.

In gel filtration of the reduced derivative toxin, a protein peak emerged earlier than the two fragments. SDS-gel electrophoresis of this protein indicated that it was representing aggregates made up of the two fragments. Partial aggregation may have resulted from such a low concentration of DTT as 1 mM in the eluant. The aggregate formed no clear precipitin line in agar gel diffusion. The failure in forming precipitin lines may not have necessarily been due to the loss of the antigenicities, but perhaps was due to the size of the aggregate being too large, as a result of the removal of DTT, to diffuse into the agar gel. It is highly possible that the failure to detect the precipitin lines of the fragments of the toxic component of reduced type A crystalline toxin (15) was due to the possible formation of such larger molecular-sized aggregates of the fragments in the wells in the agar gel, as the reducing agent diffused away into the agar gel, or to the impaired sensitivity of the antitoxic immunoglobulin to react with the fragments caused by the diffused reducing agent (11), or both.

The present results substantially support the findings by Beers and Reich (1) and DasGupta and Sugiyama (5) in that the toxic component of *C. botulinum* type B-activated progenitor toxin is composed of two fragments of different molecular sizes, which are connected with at least one disulfide bond. Being different from their observations (15), ours proved that both fragments are antigenic and their antigenicities are distinct. The present results indicate that separation of one fragment from the other is a prerequisite of investigation of the antigenicity and other biological properties of each fragment.
LITERATURE CITED


