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Purification of *Clostridium botulinum* Type F Progenitor Toxin

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Clostridium botulinum type F progenitor toxin was purified to a homogeneous state as judged by gel filtration on Sephadex G-200, ultracentrifugation, and disc electrophoresis. The sedimentation constant, corrected to water at 20 C, of type F progenitor toxin was determined to be 10.3 and the molecular weight to be 235,000 by ultracentrifugation at pH 6.0. The purified toxin contained a toxicity of 1.2×10^{8} 50% lethal doses/mg of N. In agar gel double diffusion, it formed two precipitin lines at pH 6.0. The progenitor toxin of type F differs from that of type A in that it contains no hemagglutinin and from that of type E in that it is not activable.

Møller and Scheibel (17) isolated a then new type of *Clostridium botulinum* from an outbreak of human botulism which occurred in Langeland, Denmark. Dolman and Murakami (8) designated the strain as *C. botulinum* type F and reported that the strain was relatively low in toxigenicity and that its toxin was relatively unstable.

Of the seven types from A through G now known for *C. botulinum* toxin, progenitor toxins, implying a natural state in which the toxin appears in real life as an identifiable entity (15), of types A (1, 10, 13) and E (12) have been purified and characterized. Those of types B (9, 14), C (4), and D (5) have been partially purified. A smaller molecular-sized derivative toxin (15) of type B has been purified and characterized (2, 7). There has been no report dealing with purification of type F progenitor or derivative toxin.

The present investigation was undertaken to establish procedures for purifying type F progenitor toxin and to compare it with those of other types in molecular structure and other respects.

MATERIALS AND METHODS

Strain. *C. botulinum* type F, strain Langeland, was used. A suspension of approximately 1,000 viable spores/ml in 0.05 M acetate buffer, pH 5.0, was kept in a frozen state and inoculated directly into the medium for toxin production.

Toxin production. The medium for toxin production consisted of 1.0% glucose, 1.0% yeast extract (Oriental Yeast Kogyo Co., Osaka), 2.0% peptone (for toxin production; Mikuni Kagaku Sangyo Co., Tokyo), and 0.025% sodium thioglycolate, with pH 7.0 adjusted with a 10% NaOH solution before autoclaving. About 1,000 viable spores were inoculated into 5 liters of the medium in a flat-bottomed spherical flask. The culture was incubated at 30 C for 4 days.

Chemicals and reagents. SP-Sephadex, C-50, and Sephadex G-200, medium, were the products of Pharmacia Fine Chemicals, Uppsala, Sweden. Protamine sulfate (salmon sperm) and the reagents for polyacrylamide gel electrophoresis, i.e., acrylamide monomer, *N,N'*-methylenebisacrylamide, and ammonium persulfate, were obtained from Seikagaku Kogyo Co., Tokyo. The ultrafiltration apparatus and UM 10 membrane were purchased from Amicon Co., Lexington, Mass.

Determination of protein contents. Protein contents were determined by the method of Lowry et al. (16).

Determination of toxin potency. The time-to-death method, by intravenous injection of mice (3, 20), was applied. The linear correlation between the log dose of type F toxin and the log period in minutes from injection to death is shown in Fig. 1. Usually each sample was injected into three mice at 0.1-ml doses. When necessary, each of serial twofold dilutions of a material in 0.05 M acetate buffer, pH 6.0, was injected intraperitoneally into no less than four mice at 0.5-ml doses to calculate the 50% lethal dose (LD_{50}) in 4 days by using the method of Reed and Muench (18).

Agar gel double diffusion. The method reported by Kitamura et al. (12) was used.

Antitoxin type F. The purified progenitor toxin was treated with 0.4% formalin at pH 6.0 and 30 C for 7 days. A 0.1-mg portion of the toxoid emulsified in an equal volume of aluminum phosphate gel was injected subcutaneously into each rabbit. The toxoid-adjuvant mixture was injected three times at 3-day intervals. Four weeks after the third injection, 0.1-mg doses of purified toxin were injected, also subcutaneously, twice at a weekly interval. The animals were bled 10 days after the final injection. The serum was fractionated with ammonium sulfate; the resulting immunoglobulin G fraction was further gel-filtered on a Sephadex G-200 column.

Polyacrylamide gel electrophoresis. Using the method reported by Reisfeld et al. (19), 4.5% gel at pH 4.0 was prepared. The gel columns were stained in an amido black 10B solution for about 18 h and then destained with 7% acetic acid. Neutral red was used as a marker dye to measure the relative mobility.

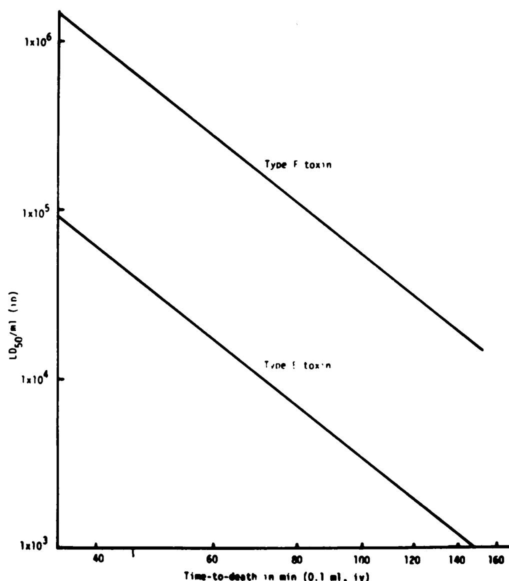

FIG. 1. *Relationship between the dose in intraperitoneal (ip) LD_{50} and the time from intravenous (iv) injection to death. Each dilution of type F toxin in 0.05 M acetate buffer, pH 6.0, was injected intravenously into 5 mice at a 0.1-ml dose.*

Ultracentrifugal analysis. A Beckman model E ultracentrifuge was used. The homogeneity of the purified toxin was proved by the band-sedimentation method with ultraviolet absorption optics. A sample (19 μg/5 μliters) in 0.1 M acetate buffer, pH 6.0, containing 1.0 M NaCl was centrifuged at 56,100 rpm at 20.0 C. The sedimentation equilibrium method (21) was employed to determine the molecular weight of the purified toxin. The toxin was centrifuged in 0.1 M acetate buffer, pH 6.0, containing 0.1 M NaCl at 5,227 rpm at 16.3 C in a double-sector cell.

RESULTS

Purification of type F progenitor toxin. (i) Step 1. The whole culture at pH 5.6 was acidified to pH 4.0 with 3 N H_2SO_4. The mixture was allowed to stand overnight at room temperature. The supernatant fluid was siphoned off, and the bottom fluid was centrifuged at 3,800 $\times$ g for 10 min. The packed precipitate was resuspended in 0.2 M phosphate buffer, pH 6.0.

(ii) Step 2. One-fourth volume of a saturated ammonium sulfate solution was added slowly to the suspension while the mixture was kept stirring on a magnetic stirrer. The mixture was allowed to stand at room temperature for 30 min and then was centrifuged. The supernatant fluid was added with solid ammonium sulfate (472 g/liter) to make 70% saturation. The precipitate formed was collected by centrifugation at 3,800 $\times$ g for 10 min and dissolved in 0.2 M phosphate buffer, pH 6.0. Ammonium sulfate fractionation was repeated again; the second precipitation was performed at 65% saturation (430 g/liter). The precipitate containing the most toxic activity was resuspended in 0.05 M acetate buffer, pH 4.5, and dialyzed against 20 volumes of the same buffer in the cold.

(iii) Step 3. The precipitate formed during dialysis was collected by centrifugation at 8,600 $\times$ g for 10 min and extracted with 0.25 M NaCl-0.1 M acetate buffer, pH 4.5. The extract recovered little toxicity and was therefore discarded. The residue was extracted again with 0.5 M NaCl-0.1 M acetate buffer, pH 4.5. The toxic extract was added dropwise with a freshly prepared 2% protamine solution. The amount of protamine to be added was estimated from the absorption of the extract at 260 nm (A_{260}); addition with 2.0 ml of the 2% protamine solution to 100 ml of extract with an A_{260} of 1.0, containing about 3 mg of protein/ml, gave a satisfactory result. The precipitate formed was removed by centrifugation at 6,800 $\times g$ for 10 min. The supernatant was adjusted to pH 4.2 with 0.5 M NaCl-0.1 N acetic acid and allowed to pass through a column (1.5 by 10 cm) of SP-Sephadex, C-50, equilibrated with 0.5 M NaCl-0.1 M acetate buffer, pH 4.2.

(iv) Step 4. The percolate from the SP-Sephadex column was diluted with 0.1 M acetate buffer, pH 4.2, to make the NaCl concentration to 0.1 M. It was applied to a column of SP-Sephadex, C-50 (1.5 by 10 cm), and equilibrated with 0.1 M NaCl-0.1 M acetate buffer, pH 4.2. The toxin adsorbed onto the column was eluted at an NaCl concentration of approximately 0.35 M (Fig. 2). The toxic fractions were pooled and concentrated by ultrafiltration through a UM 10 membrane.

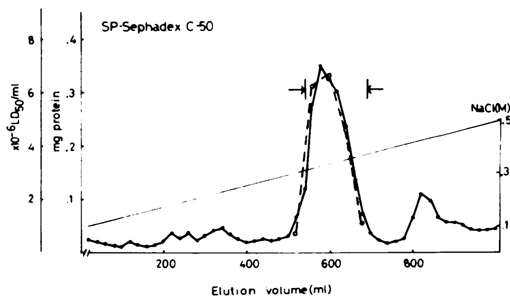

FIG. 2. *SP-Sephadex chromatography of type F toxin. The toxin fraction after protamine treatment was applied to an SP-Sephadex, C-50, column and eluted by linear gradient in NaCl from 0.10 to 0.5 M in 500 ml of 0.10 M acetate buffer, pH 4.5. Ten-milliliter fractions were collected. Symbols: protein content, ●; toxic activity, O; NaCl concentration, ——. Fractions indicated by arrows were pooled.*

(v) Step 5. The concentrated toxic fraction was applied to a column of Sephadex G-200 (2.5 by 190 cm). A major protein peak was eluted after a minor one (Fig. 3). The front peak was resolved into two bands in disc electrophoresis; the retarded peak formed a single band. The toxic fractions of the major peak showing a single band in disc electrophoresis were pooled and concentrated by ultrafiltration through a UM 10 membrane.

All overall purification of about 50-fold was accomplished from the acid precipitate of the whole culture (Table 1).

Examinations of the purified type F progenitor toxin for homogeneity and some other properties. (i) Gel filtration. The purified material was eluted from a column of Sephadex G-200 with 0.1 M acetate buffer-0.3 M NaCl, pH 4.2, as eluant in a single symmetrial peak. The toxicities per milligram of nitrogen of representative fractions were on the same level throughout the peak (Fig. 4). The elution volume of the purified toxin from the column coincided to that of the toxin in the culture supernatant.

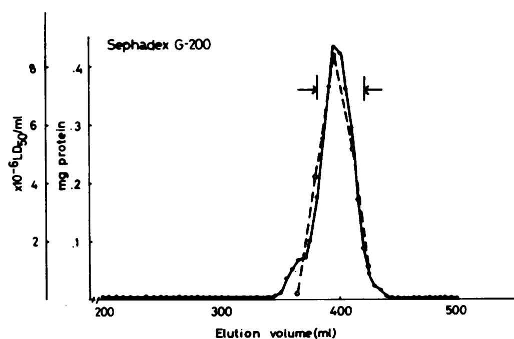

FIG. 3. *Gel filtration of type F toxin on Sephadex G-200. The toxic fraction eluted from SP-Sephadex and concentrated to 10 ml was applied to a Sephadex G-200 column (2.5 by 189 cm) equilibrated with 0.1 M acetate buffer, pH 4.2, containing 0.3 M NaCl and eluted with the same buffer. Five-milliliter fractions were collected at a flow rate of 18 ml/h. Symbols: protein content, ●; toxic activity, O. Fractions indicated by arrows were pooled.*

(ii) Disc electrophoresis. The purified type F toxin showed a single band with a relative mobility of 0.48 to the marker dye.

(iii) Agar gel diffusion. Two distinct precipitin lines were formed with any of the serial dilutions of the purified toxin and the antipurified type F toxin rabbit immunoglobulin G (Fig. 6).

(iv) Toxin potency. The toxicity of the purified material determined by the intraperitoneal injection method was 1.2×10^8 LD_{50}/mg of N. No increase in the toxicity resulted from the tryptic treatment of the purified toxin at pH 6.0.

(v) Direct hemagglutinin. No hemagglutinin activity was detected with the purified type F toxin at a concentration of 0.12 mg/ml against chicken erythrocytes at either 20 or 6 C; the crude culture supernatant showed slight activity (Table 2).

(vi) Ultraviolet absorption spectrum. The ultraviolet absorption spectrum of the purified toxin is shown in Fig. 7. The maximal absorption was at 278 nm with small shoulders at 253, 259, and 283 nm; the minimum was at 250 nm. The A_{278}:A_{250} ratio was 2.86. The A_{278} (1%) was 12.0 from the protein content in the bovine serum albumin equivalent.

(vii) Ultracentrifugation. The sedimentation velocity method demonstrated the homogeneity of the purified toxin with a sedimentation coefficient corrected to water at 20 C ($s_{20,w}$) of 10.3. From an assumed partial specific volume of 0.749 ml/g, the molecular weight of type F progenitor toxin was calculated to be 235,000.

DISCUSSION

Purification of *C. botulinum* type F progenitor toxin was accomplished for the first time. Since

TABLE 1. *Purification of C. botulinum type F progenitor toxin*

Step	Vol (ml)	Total		$\times 10^6$ LD_{50}/mg of N[a]	Recovery (%)
		Protein (mg)	Toxicity ($\times 10^7$ LD_{50})		
Whole culture	10,000	ND[b]	222	ND	100
Acid precipitate	700	4,641	200	2.7	91
Ammonium sulfate precipitate	50	521	189	2.7	86
Extract in 0.5 M NaCl	60	183	150	51.2	68
Protamine supernatant	75	63	85	84.2	43
SP-effluent	15	30	57	118	26
G-200 effluent	6	13.5	29	133	13

[a] LD_{50} per milligram of protein $\times$ 6.25.
[b] ND, Not determined.

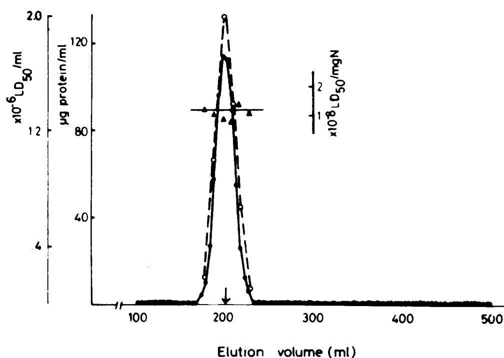

FIG. 4. *Sephadex G-200 gel filtration patterns of purified type F toxin. A sample of 3.3 mg in protein was applied to a column (2.5 by 98 cm) of Sephadex G-200 and eluted with 0.3 M NaCl-0.1 M acetate buffer, pH 4.2. Fractions (5.2 ml) were collected at a flow rate of 15 ml/h. Symbols: protein content, ●; toxic activity, ○; specific activity, ▲. The arrow indicates the elution position of the toxin in the culture supernatant from the same column.*

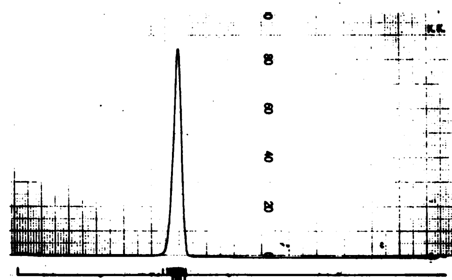

FIG. 5. *Disc electrophoretic pattern of purified type F toxin. A sample of 80 µg in protein was electrophoresed at pH 4.0 for 210 min. The current applied was 2.5 mA per tube. The stained protein bands were monitored by a densitometer (type FDA IV, Fujiriken Co., Tokyo) at 600 nm.*

types A and E progenitor toxin are known to be composed of two components, toxic and atoxic, and to dissociate into the two components under slightly alkaline conditions (7, 13), in the present investigation, aiming at isolating the intact type F progenitor toxin, care was taken to avoid possible molecular dissociation during purification. All the procedures were performed under acidic conditions to isolate the same natural toxin as that appearing in culture. As the purified toxin was demonstrated by gel filtration to have a molecular size identical to that of the toxin in the culture supernatant, none of the procedures employed altered the molecular size during purification.

The purified toxin behaved as a homogeneous protein in all the procedures for testing the homogeneity, but agar gel diffusion with serial dilutions of the preparation gave two precipitin lines. The fact that the two lines were formed at any dilution of the toxin may suggest that type F progenitor toxin is also composed of two components, probably toxic and atoxic components, like types A and E progenitor toxin (15).

Trypsinization of type F toxin at pH 6 did not induce any increased toxicity. This must have been due to spontaneous activation during the 4 days of incubation with the protease(s) produced by the organisms themselves. The toxins of proteolytic *C. botulinum* are not activable, except a type B strain Okra which produces both activable toxin (11) and an activating enzyme (6). This may not necessarily reflect different proteases produced by these organisms, but may suggest different molecular structures of these toxins.

The purified type F progenitor toxin had an $s_{20,w}$ of 10.3 and a molecular weight of 235,000, which is significantly smaller than those of types A (900,000 [15]) and E (350,000 [12]) toxin. The specific toxicity of type F progenitor toxin was 1.2×10^8 LD_{50}/mg of N, which is higher than that of type E (8×10^7 LD_{50}/mg of N [12]), but lower than that of type A (2.4×10^8

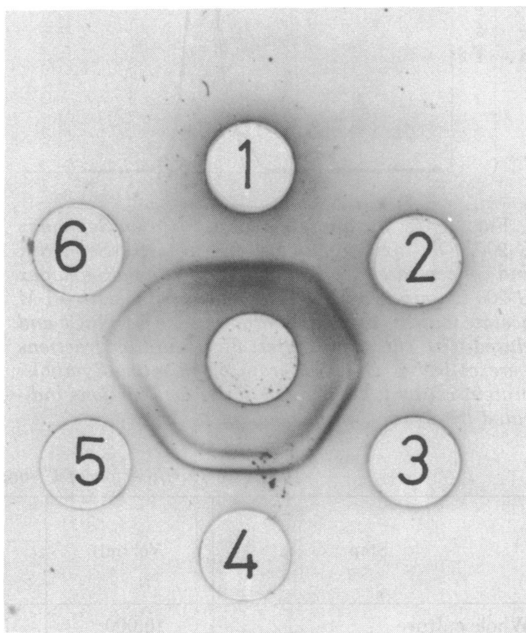

FIG. 6. *Agar gel double diffusion tests with purified type F toxin. Center well: anti-type F toxin globulin; lateral wells: purified type F toxin; 1: 625 µg/ml; 2: 312 µg/ml; 3: 156 µg/ml; 4: 78 µg/ml; 5: 39 µg/ml; 6: 19.5 µg/ml. The gel was washed with phosphate-buffered saline, pH 7.3, and the precipitin lines were staind with thiazine red.*

TABLE 2. *Hemagglutinin activities of types A and F progenitor toxin*[a]

Toxin type	Temp (C)	Concn (μg of protein/ml)										
		120	60	30	15	7.5	3.8	1.9	0.94	0.47	0.23	0.12
A[b]	6			+	+	+	+	+	+	+	+	−
	20			+	+	+	+	+	+	+	−	−
F[c]	6	−	−	−	−	−	−	−	−	−	−	−
	20	−	−	−	−	−	−	−	−	−	−	−
Supernatant[d]	6	+ (2×)[e]	+ (4×)	+ (8×)	+ (16×)	+ (32×)	+ (64×)	− (128×)	− (256×)	− (512×)	− (1,024×)	− (2,048×)

[a] The reaction mixture contained 0.5 ml of a sample diluted in 0.075 M NaCl-0.075 M phosphate buffer, pH 7.3, containing 0.25% bovine serum albumin and 0.05 ml of a 2.5% suspension of chicken erythrocytes.
[b] Crystalline type A toxin.
[c] Purified type F progenitor toxin.
[d] Supernatant of a 98-h culture of *C. botulinum* type F.
[e] Numbers in parentheses give dilution.

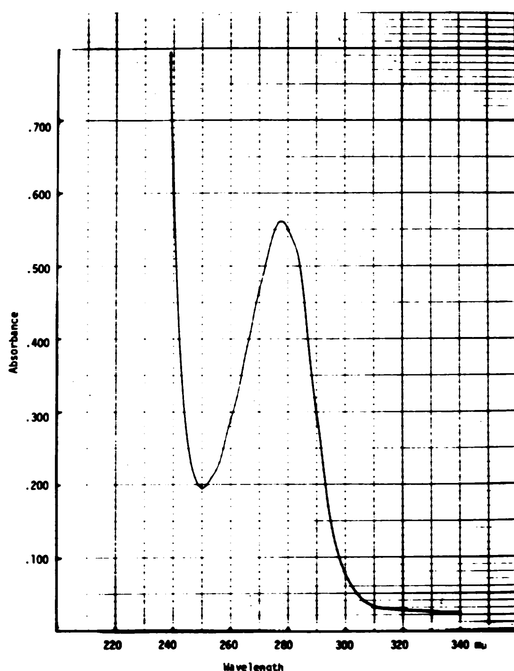

FIG. 7. *Ultraviolet absorption spectrum of purified type F toxin. A solution of 466 μg/ml in 0.1 M acetate buffer, pH 4.2, containing 0.3 M NaCl was examined in a Hitachi spectrophotometer, type 124.*

LD_{50}/gm of N [15]) progenitor toxins. Type F progenitor toxin, like type E, contains no hemagglutinin, although the culture supernatant contains some activity.

To perform ion exchange chromatography of crude toxin at an acidic reaction, it was necessary to remove such strongly acidic substances as nucleic acids, since the presence of such substances caused precipitation of the toxin when equilibrated to pH 4.5 or below at a low salt concentration. Attempts were made, therefore, to remove such substances from the toxin material by treating with streptomycin, ribonuclease, diethylaminoethyl-Sephadex, or protamine. The treatment with protamine appeared to be most practicable for the purpose, as its effect was not significantly influenced by either the salt concentration or the pH value. The quantity of protamine to be added, however, should have been determined preliminarily in a small scale, because the acidic substance contents varied from one preparation to another and the quality of protamine also varied from lot to lot.

In the final Sephadex G-200 gel filtration, the column length was crucial for separation of the major toxin peak from the minor contaminant peak. It seems possible to separate these materials more efficiently by the recycling method of gel filtration.

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