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COMPARATIVE GEL FILTRATION OF TOXIN PRECURSOR AND TRYPSIN-ACTIVATED TOXIN OF *CLOSTRIDIUM BOTULINUM* TYPE E

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ABSTRACT

SAKAGUCHI, GENJI (National Institute of Health, Tokyo, Japan), SUMIKO SAKAGUCHI, AND NOBUKO IMAI. Comparative gel filtration of toxin grecursor and trypsin-activated toxin of *Clostridium botulinum* type E. J. Bacteriol. **87**:401-407. 1964.—Precursor of type E botulinus toxin was highly purified from bacterial cells by extraction, ammonium sulfate precipitation, ribonuclease digestion, and chromatography on CM-Sephadex. The sample free from ribonucleic acid had a toxicity of 5.1×10^5 LD_{50} per mg of nitrogen before activation and 8.3×10^7 LD_{50} per mg of nitrogen after activation. This precursor and its activated product were subjected to gel filtration on a column of Sephadex G-200. No evidence for smaller fractions was obtained. Both precursor and trypsin-activated toxin were eluted in the void volume with 0.05 or 1 M acetate buffer (pH 6.0) or with 0.05 or 0.5 M phosphate buffer (pH 7.5). Intact trypsin and its degradation products were separated from toxin. The toxins eluted with the acetate buffers had potencies of 1.2×10^8 and 1.3×10^8 LD_{50} per mg of N, while those eluted with the phosphate buffers showed lower toxicities. Possible mechanisms involved in the activation process are discussed.

A markedly enhanced parenteral lethality results from trypsinization of toxin of *Clostridium botulinum* type E at a pH range of 5.5 to 6.5 (Duff, Wright, and Yarinsky, 1956). A partially purified precursor of type E toxin derived from bacterial cells contains ribonucleic acid (RNA), and trypsinization at pH 6.0 changes the electric charge from negative to neutral or positive (Sakaguchi and Sakaguchi, 1959). It was suggested that a group containing RNA may incompletely cover the toxicity, and the release of this chemical group by trypsin increases the toxicity of the molecule.

Gerwing, Dolman, and Arnott (1962) performed ultracentrifugal analyses and stated the mechanism of tryptic activation involves a fragmentation process of large molecules of nonactivated toxin ($S_{20,w} = 5.6$) into much smaller components not uniform in size ($S_{20,w} = <1$).

An attempt was made to compare the elution patterns of a highly purified precursor freed of RNA and the trypsin-activated toxin from a column of dextran gel. Sephadex G-200, consisting of cross-linked dextran chains with high porosity, separates substances with molecular weights smaller than about 200,000, which penetrate the swollen gel (Flodin, 1962). Should the molecules of trypsin-activated type E toxin be so small as to be dialyzable, as described by Gerwing et al. (1962), entirely different elution patterns between precursor and activated toxin should come out.

MATERIALS AND METHODS

Preparation of precursor sample. C. botulinum type E, strain VH (Dolman et al., 1950), was grown at 30 C for 4 days in a peptone medium of pH 6.3 (Sakaguchi and Sakaguchi, 1961). The bacterial cells were collected by centrifugation, washed twice with 0.05 M acetate buffer (pH 5.0), and extracted three times with 1 M acetate buffer of pH 6.0 at 37 C for 2 hr, followed by refrigeration overnight. Solid ammonium sulfate was added to the extract of 2,700 ml to 50% saturation, and the mixture was kept overnight in the cold. The precipitate was taken by filtration and dissolved in 0.05 M acetate buffer (pH 6.0). Salting out at 50% saturation of ammonium sulfate was repeated three times. The third ammonium sulfate precipitate was dissolved in 350 ml of 0.05 M acetate buffer (pH 6.0), and it was passed through a column of Sephadex G-25 (Sephadex and its derivatives were the products of Pharmacia, Uppsala, Sweden) with the same buffer as eluant to remove ammonium sulfate. Since the sample at this stage still contained RNA, it was digested with crystalline ribonu-

clease (Worthington Biochemical Corp., Freehold, N.J.) at a concentration of 50 μg/ml at 30 C for 4 hr in a cellophane casing, which was immersed in 0.02 M acetate buffer (pH 6.0). Dialysis was continued for another 48 hr in the cold against the same buffer.

Further purification of precursor by ion-exchange chromatography. CM-Sephadex C-50 (medium) was buffered with 0.02 M acetate buffer of pH 6.0 and packed into a column (3.0 by 20 cm). The precursor sample (a total of 1.38 g of protein in 540 ml of the buffer) was applied on the column and eluted with 4,000 ml of the same buffer by gradient increase in concentration of sodium chloride up to 0.2 M. The elution pattern is shown in Fig. 1. The effluent in fractions 71 to 103 was pooled, and the precursor was precipitated in 50% saturated ammonium sulfate. The precipitate was dissolved in 100 ml of 0.02 M acetate buffer (pH 6.0) and subjected to gel filtration again on Sephadex G-25 with the same buffer as eluant. The precursor sample and the activated sample were kept in frozen state throughout the experimental period.

Activation of precursor. Trypsin (twice-crystallized; Nutritional Biochemicals Corp., Cleveland, Ohio) at pH 6.0 was used at a concentration of 0.05%. Incubation was at 37 C for 60 min.

Precursor and toxin assay. A sample of effluent of the precursor was added with an equal amount of 2% crude trypsin (E. Merck, Darmstadt, Germany) solution in 0.05 M acetate buffer (pH 6.0), and it was incubated at 37 C for 60 min to determine potential toxicity.

All the toxin materials were diluted twofold serially in 0.05 M phosphate buffer (pH 6.3) containing 0.2% gelatin. Groups of four mice per dilution were injected intraperitoneally with 0.5 ml of toxin. After 4 days, the LD_{50} was calculated according to Reed and Muench (1938).

Gel filtration. Sephadex G-200 (140 to 400 mesh, 4 g) was allowed to swell for 24 hr and packed into a column 3.0 cm in diameter to the height of 17 cm. The column was washed with an excess amount of each buffer before the experiment, and the same column was used throughout the experiments. The height of the Sephadex column changed from 17 to 18 cm according to the buffer used. The eluants used were 0.05 and 1 M acetate buffer (pH 6.0) and 0.05 and 0.5 M phosphate buffer (pH 7.5). Each sample consisted of the precursor of 8.8 mg of protein in 4 ml of buffer, with or without activation by 2 mg of trypsin. Gel filtration was run at room temperature (16 to 20 C). The effluent was collected in 5-ml portions with a fraction collector.

Protein determination. Protein content was determined by use of the Folin phenol reagent according to Lowry et al. (1951) with crystalline bovine serum albumin (Armour and Co., Chicago,

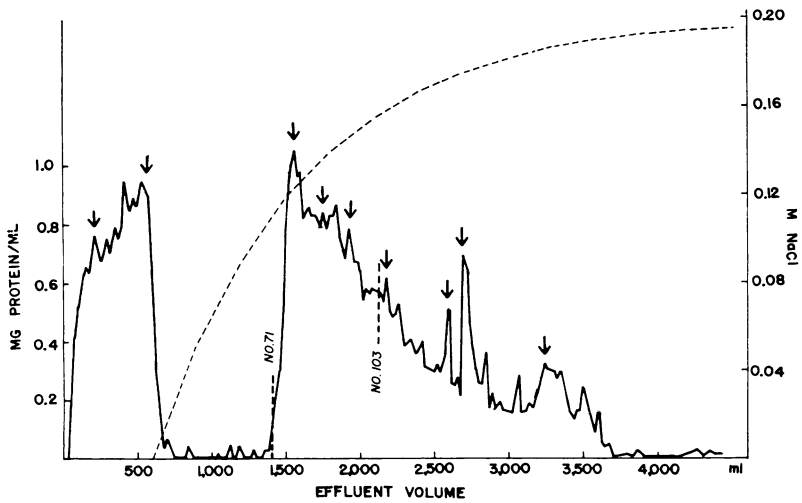

FIG. 1. *Fractionation of precursor sample on a CM-Sephadex C-50 column (3 × 19 cm) by gradient increase in ionic strength. Dotted line indicates the concentration of sodium chloride in addition to 0.02 M acetate buffer (pH 6.0). Arrows indicate the fractions which were assayed for toxicities after activation. From left to right: 11, 27, 78, 88, 96, 109, 130, 135, and 162.*

TABLE 1. *Toxicities of some precursor fractions eluted from CM-Sephadex*

Tube no.	Protein (μg/ml)	LD_{50} per ml × 10^6		LD_{50} per mg of N × 10^6	
		Before activation	After activation	Before activation	After activation
11	764	0.0026	0.33	0.021	2.8
27	948	0.0026	0.66	0.017	4.3
78	1,040	0.020	10	0.13	63
88	836	0.010	7.3	0.077	54
96	780	0.020	7.3	0.16	58
109	612	0.0051	1.0	0.052	10
130	508	—	0.16	—	2.0
135	692	—	0.16	—	1.4
162	320	—	0.010	—	0.2

Ill.) as a standard. Protein nitrogen was determined by dividing the protein values by 6.25. The value for trypsin was about 1.6 times as large as that determined by the Kjeldahl method and, for the precursor sample, about 1.2 times as large.

Determination of trypsin activity. Trypsin activity of each fraction was determined by the method of Kunitz (1946) with casein (Hammarsten; E. Merck) as the substrate, and was expressed in terms of equivalent micrograms of a crystalline trypsin (Nutritional Biochemicals Corp.).

RESULTS

The elution pattern of ion-exchange chromatography of the ribonuclease-digested precursor on CM-Sephadex C-50 is shown in Fig. 1; toxicities before and after activation of some fractions are given in Table 1. Toxicities of precursor at some purification steps are given in Table 2. Each sample of precursor (4 ml) contained 8.80 mg of protein, 2.6 × 10^5 LD_{50} without activation, and 5.8 × 10^7 LD_{50} after activation as determined after defrosting; trypsin-activated toxin (4 ml) contained 11.9 mg of protein and 5.8 × 10^7 LD_{50}.

The elution patterns of gel filtration on a column of Sephadex G-200 are shown in Fig. 2 to 5. The results of toxicity assay on some of the eluted fractions with or without activation are shown in Table 3. In every set of gel filtration experiments, precursor and toxin were eluted in the first peaks in a same effluent volume. The main peak of a preparation of urease (molecular weight, 480,000; Tokyo Kasei Industry) was eluted in the same effluent volume as precursor or toxin.

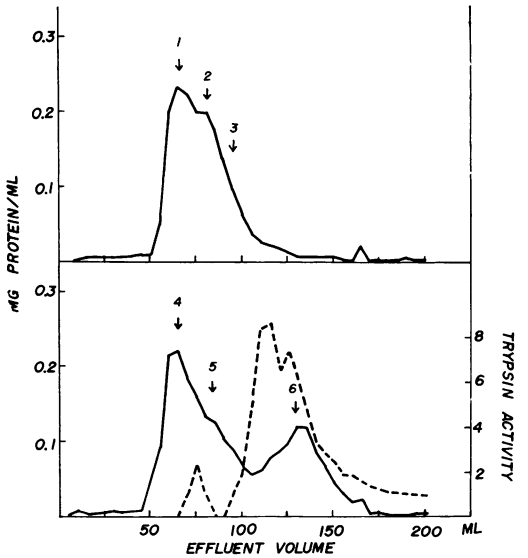

FIG. 2. *Elution patterns of precursor (upper) and trypsin-activated toxin (lower) from a Sephadex G-200 column. Eluant: 0.05 M acetate buffer (pH 6.0). In Fig. 2 to 5, dotted line represents trypsin activity. Numbered arrows indicate the fractions assayed for toxicities.*

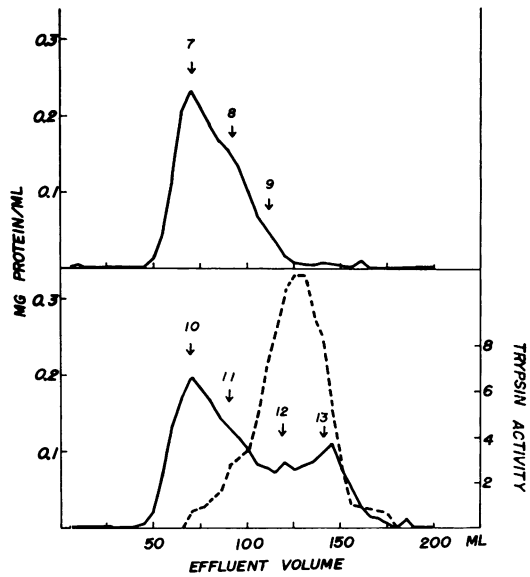

FIG. 3. *Elution patterns of precursor (upper) and trypsin-activated toxin (lower) from a Sephadex G-200 column. Eluant: 1.0 M acetate buffer (pH 6.0).*

The peak fraction of precursor or toxin had the highest specific toxicity, indicating that the first peak is not homogenous.

TABLE 2. *Purification of precursor from bacterial cells*

Sample	Volume (ml)	Protein (mg/ml)	LD_{50} per ml × 10^6		LD_{50} per mg of N × 10^6	
			Before activation	After activation	Before activation	After activation
Whole culture	60,000	0.258*	0.00051	0.16	0.012	4.0
Washed-cell suspension	1,100	17.6	0.032	11	0.011	3.7
Cell extract	2,655	1.82	0.021	8.5	0.71	29
3rd $(NH_4)_2SO_4$ precipitate (after desalting)	550	3.35	0.082	15	0.15	27
Ribonuclease digest	572	2.51	0.026	9.0	0.064	22
Effluent (no. 71–103) from CM-Sephadex	650	0.60	0.020	6.6	0.21	69
$(NH_4)_2SO_4$ precipitate of CM-Sephadex effluent (after desalting)	150	2.02	0.16	26	0.51	83
Precursor after defrosting (sample for gel filtration on Sephadex G-200)	150	2.20	0.065	14	0.18	41

* Determined on the precipitate by 5% trichloroacetic acid.

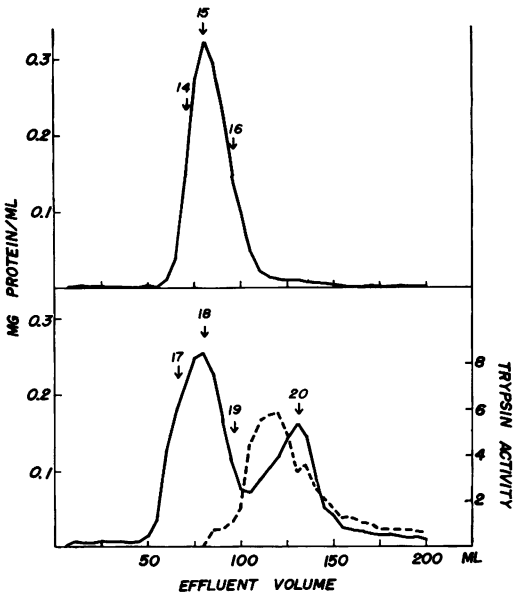

FIG. 4. *Elution patterns of precursor (upper) and trypsin-activated toxin (lower) from a Sephadex G-200 column. Eluant: 0.05 M phosphate buffer (pH 7.5).*

When an activated sample was subjected to gel filtration, two protein peaks were eluted. The second peak was sufficiently retarded, but it was shown to be only slightly toxic. The highest trypsin activity was demonstrated between the first and the second protein peaks. The amount of protein at the peak in trypsin activity was very small, and the total trypsin activity recovered was very low, indicating that a large part of trypsin had decomposed during the processes of activation or during the subsequent storage, or both. No appreciable difference in amount of protein was shown between the precursor and

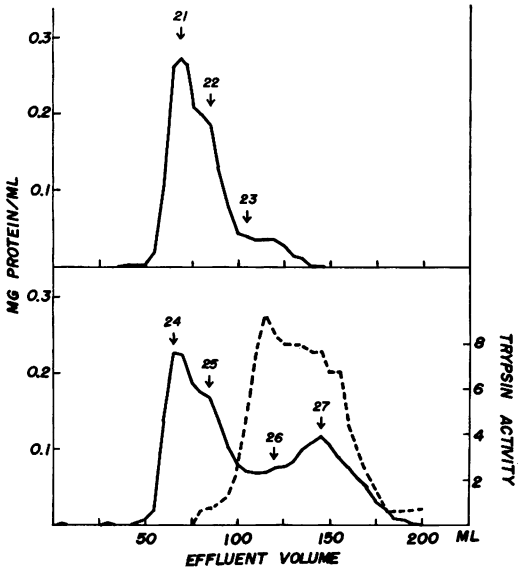

FIG. 5. *Elution patterns of precursor (upper) and trypsin-activated toxin (lower) from a Sephadex G-200 column. Eluant: 0.5 M phosphate buffer (pH 7.5).*

TABLE 3. *Toxicities of precursor and toxin eluted from Sephadex G-200 column*

Eluant	Sample	Fraction*	Protein (μg/ml)	LD_{50} per ml × 10^6		LD_{50} per mg of N × 10^6	
				Before activation	After activation	Before activation	After activation
Acetate (0.05 M, pH 6.0)	Precursor	1	232	0.016	2.9	0.44	77
		2	200	0.0036	0.71	0.11	22
		3	96.8	—	0.16	—	11
	Toxin	4	217	4.5		130	
		5	126	0.49		24	
		6	121	0.041		2.1	
Acetate (1 M, pH 6.0)	Precursor	7	233	0.029	3.1	0.77	83
		8	156	0.0032	0.42	0.13	17
		9	52.8	0.0020	0.16	0.24	19
	Toxin	10	200	3.7		120	
		11	134	0.66		31	
		12	88.8	0.016		1.1	
		13	99.2	0.0051		0.32	
Phosphate (0.05 M, pH 7.5)	Precursor	14	151	0.0065	0.46	0.27	19
		15	322	0.015	1.3	0.28	26
		16	146	0.0033	0.19	0.014	8.1
	Toxin	17	178	0.33		12	
		18	256	2.6		64	
		19	112	0.12		6.9	
		20	160	0.00049		0.019	
Phosphate (0.5 M, pH 7.5)	Precursor	21	276	0.041	1.8	0.93	41
		22	189	0.0065	0.24	0.21	7.8
		23	43.2	0.0018	0.082	0.26	12
	Toxin	24	231	1.9		56	
		25	170	0.23		8.5	
		26	77.5	0.00032		0.026	
		27	118	0.00012		0.0061	
	Original precursor (4 ml)		2,200	0.065	14	0.18	41
	Original toxin (4 ml)		2,980	15		30	

* Numbers correspond to those above the arrows in Fig. 2 to 5.

the toxin peaks. These facts indicate that the second peak of the trypsin-activated sample was not derived from the precursor but from the trypsin added.

To eliminate the possible interaction between molecules of toxin due to hydrogen bonding, 0.1 M phosphate buffer (pH 7.0) containing 8 M urea was also used as eluant. The effluent, however, was completely nontoxic as was the case with type A toxin (Schantz, Stefanye, and Spero, 1960).

DISCUSSION

By the culture method for *C. botulinum* type E presented here, a large part of precursor of toxin was recovered in bacterial cells, which are easier to handle than is culture supernatant. In the purification steps, extraction of cells by 1 M acetate buffer (pH 6.0) and ion-exchange chromatography on CM-Sephadex C-50 accomplished a high degree of purification. It was shown that RNA contained in the precursor extracted from bacterial cells can be removed by ribonuclease digestion without changing the toxicity before and after activation. Therefore, the ribonucleic acid itself has no role in covering the toxicity as pointed out by Gerwing et al. (1961).

The present study showed that neither toxin precursor nor trypsin-activated toxin of *C. botulinum* type E penetrates swollen gel of Sephadex G-200. The results may be indicative of large molecular weights (200,000 or larger) possessed by both precursor and trypsin-activated toxin.

If so, the hypothesis proposed by Gerwing et al. (1962) that trypsin activation involves a fragmentation process of large toxin molecules ($S_{20,w}$ = 5.6) into much smaller molecules ($S_{20,w}$ = <1) is not valid. Of course, possible protein-protein interaction should be taken into consideration before drawing a decisive conclusion on molecular weight from gel filtration. The possibility, however, would not be large, because the results were similar with eluants of different ionic strength and different pH values, whereby interaction due to electrostatic bonding may have been excluded. Besides, dissociation of crystalline type A toxin ($S_{20,w}$ = 20) into small fragments ($S_{20,w}$ = 5) reported by Wagman and Bateman (1953) does not involve any activation phenomenon. Therefore, fragmentation into such small molecules as to be dialyzable is not likely to be a prerequisite to the activation process. Fiock, Yarinsky, and Duff (1961) observed two components of $S_{20,w}$ = 12.5 and 4.7 by ultracentrifugal analysis of a partially purified trypsin-activated toxin. Although Gerwing et al. (1962) ascribed the discrepancy in sedimentation constant of trypsin-activated type E toxin to the difference in starting material and purification procedures, Fiock's toxin was derived from whole culture and should contain toxins of both intracellular and extracellular origin.

The present investigation did not demonstrate any fragment in appreciable amount, whether toxic or not, released from the precursor molecule as a result of activation that can penetrate into gel of Sephadex G-200. The amount of protein eluted in the first peak of precursor and that of the activated toxin were not appreciably different. Therefore, if activation by trypsin should involve a fragmentation process, the molecular weight of the resulting fragments may still be larger than 200,000.

If activation should involve an unmasking process, that is, releasing inhibiting substance(s) from the precursor molecule, the masking group would not be much larger than the toxin, because toxin of 1.3×10^8 or 1.2×10^8 LD_{50} per mg of N was eluted in the peak with acetate buffers of pH 6.0. If type E toxin had the same toxicity as type A crystalline toxin (2.2×10^8 LD_{50} per mg of N), the peak might contain inert protein at 40% or less. There remains another possibility that no peptide fragment is released by activation, or that the fragment, if there was any, might be too small to be detected by the present method.

The potencies of the toxin and those achieved by activation of the precursor eluted by phosphate buffers of pH 7.5 were lower than those obtained with acetate buffers of pH 6.0. This may reflect a lower degree of stability of both toxin and precursor at pH 7.5.

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