DISSOCIATION OF CLOSTRIDIUM BOTULINUM TYPE-E TOXIN

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Received December 4, 1967

Clostridium botulinum type-E toxin is produced in the form of precursor, which is activated by trypsin (Duff, Wright, and Yarinski, 1956) producing up to several hundred-fold increase in toxicity. We have attempted purification of the precursor with the hope that the mechanism of the activation could be elucidated with the purified material. The precursor we isolated most recently appeared homogeneous below pH 6, with $S_{20,w}$ 12.3, which was in good agreement with the figure reported previously (Sakaguchi and Sakaguchi, 1967). Electrophoresis at pH above 7, however, resolved this material into two fractions and agar-gel diffusion demonstrated two distinct antigens in the material. These two components were separated by starch electrophoresis at pH 8. The potentially toxic component was named Eα and the nontoxic one Eβ. Each component, representing one of the two antigens, possessed an $S_{20,w}$ of 7.3.

Results and Discussion

To purify the precursor, the procedures reported by Sakaguchi, Sakaguchi, and Imai (1964) were applied with some modifications (to be published). The purified material appeared homogeneous when examined below pH 6 by ultracentrifugation ($S_{20,w}$ 12.3), electrophoresis or chromatography. The material contained 1 - 5 x 10^5 LD_{50}/mg N and 5 - 10 x 10^7 LD_{50}/mg N, before and after trypsinization, respectively. The material in 0.05 M acetate buffer, pH 6.0 named 12S precursor, was kept frozen until use.

Upon electrophoreses of 12S precursor on cellulose acetate membrane at different pHs (Fig. 1), a single band was observed at pH 6 and two above pH 7. At pH 7, the amount of the fast migrating band was more than that of the slow one; when the pH was raised to 8 or above, the amounts of the two

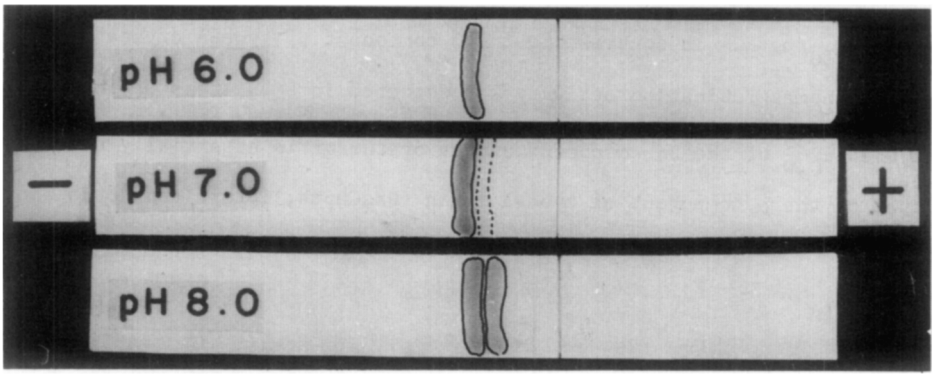

Fig. 1 Electrophoreses of 12S precursor on cellulose acetate membrane. Buffers (μ = 0.1), Na_2HPO_4-NaH_2PO_4-NaCl, pH 6.0 or pH 7.0 and sodium barbital-HCl-NaCl, pH 8.0; size of membrane, 1.4 x 11 cm; sample, 5 mg/ml applied at 4 cm from the anodic end of the membrane; current and other conditions, 1.2 mA for 60 min at 4 C; staining, with Amido Black 10B.

became equal as judged by color intensity. These results appeared to indicate the possibility of molecular dissociation.

The patterns of sucrose density gradient centrifugations performed at three different pHs are shown in Fig. 2. The higher the pH, the lower the sedimentation rate. The sedimentation pattern of the protein at pH 8 and 9 coincided with each other. Representative fractions obtained at different pHs formed two common precipitation lines in an agar-gel-diffusion plate.

The precursor sediments as a single boundary at pH 6.0 with $S_{20,w}$ 12.34 and at pH 8.0 with $S_{20,w}$ 7.30 (Figure 3 (1 and 3)). Electrophoreses on cellulose acetate membrane at either pH 6 or 8 of 12S precursor after adjustment to pH 8.0 by dialysis yielded two bands. The foregoing results proved the presence of two components differing in electric charge in the dissociation products.

The 12S precursor was subjected to starch electrophoresis at pH 8, with the results shown in Fig. 4. Two protein peaks of equal area were separated. One migrating toward the cathode was potentially toxic (Eα); the other migrating toward the anode was potentially nontoxic (Eβ). The ratio of the LD_{50}/mg N after activation to that before activation of Eα was about 100 and the potential toxicity was on the same level as 12S precursor, being 8×10^7 LD_{50}/mg N. In contrast to the β component of type-A crystalline toxin, no hemagglutination activity was detected in Eβ. It is of interest that the $S_{20,w}$ of the Eα component of the precursor is essentially the same as that of the α component of type-A toxin (DasGupta, Boroff, and Rothstain, 1966).

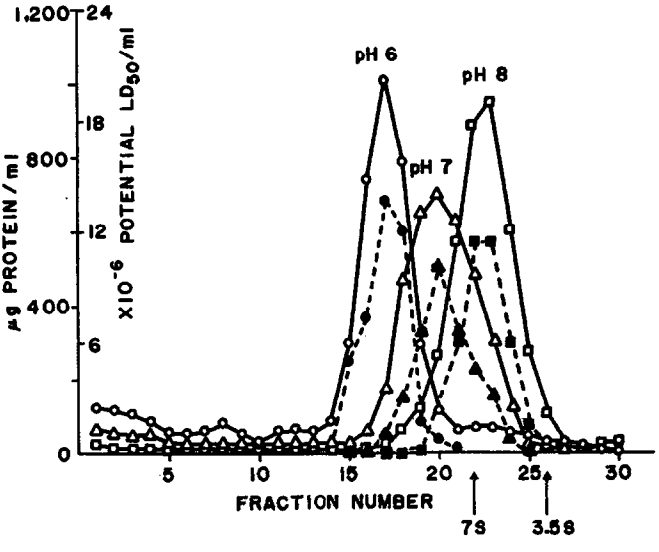

Fig. 2 Separation of 12S precursor in sucrose density gradient of 5 - 20% in a buffer of pH 6, 7 or 8. Sample, 400 μg in 0.2 ml; buffers, 0.05 M acetate, pH 6.0, and 0.05 M veronal, pH 7.0 and 8.0; centrifugation, at 39,000 rev/min for 7 hr at 5 C in an SW 39 rotor in a Beckman Ultracentrifuge model L_2; fractionation, 12 drops per tube; markers, a 7S globulin fraction of horse serum and egg albumin; protein determination, with Folin reagent; toxicity assay, by the mouse intravenous injection method with each fraction trypsinized at pH 6.0 and 37 C for 45 min. The time between injection and death was converted to mouse ip LD_{50}/ml. This assay method was used throughout the present investigation. ———, protein content; ....., potential toxicity.

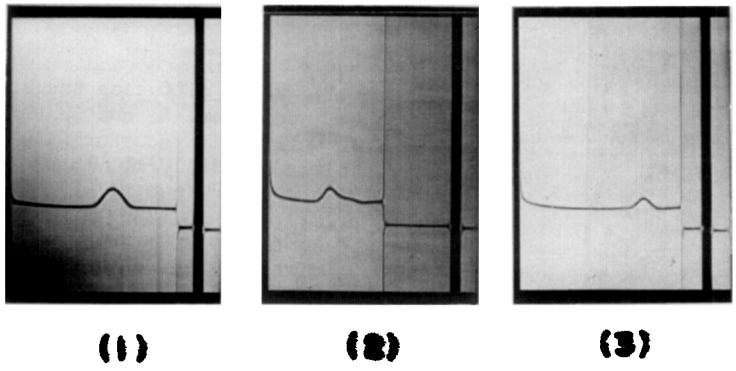

Fig. 3 Sedimentation of precursor samples. (1) the 12S precursor in 0.05 M acetate, pH 6.0, (2) the material dialyzed against 0.05 M phosphate, pH 7.0; (3) the material dialyzed against 0.05 M veronal, pH 8.0. Revolution, 56,100 rev/min; temperature, 20 C; time after reaching the indicated speed, 24 min.

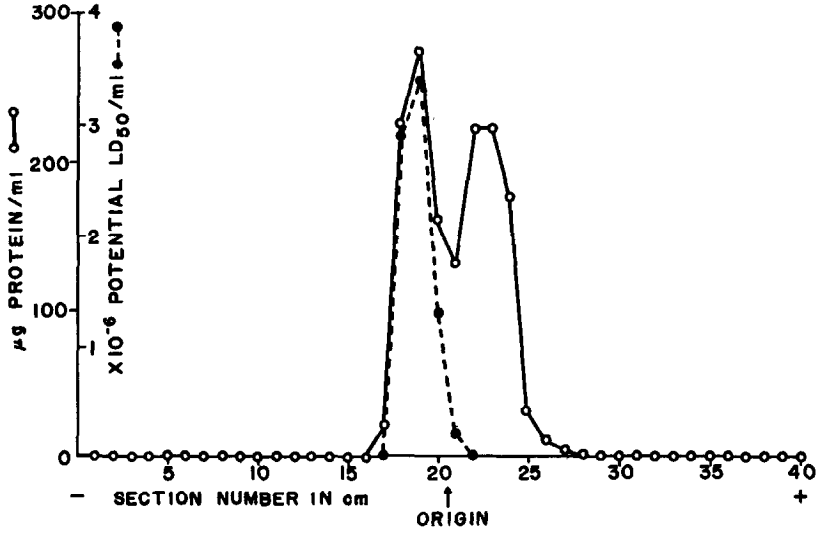

Fig. 4 Starch electrophoresis of 12S precursor at pH 8. Sample, 4 mg; trough size, 2 x 1.5 x 40 cm; buffer, 0.05 M veronal, pH 8.0; current and other conditions, 6 mA, 200 V, for 24 hr at 6 C; extraction, each 1-cm segment with 1.0 ml of 0.05 M veronal, pH 8.0.

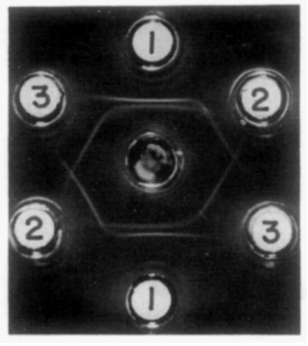

Fig. 5 Agar-gel diffusion tests performed with 12S precursor, Eα and Eβ components. Central well, antiprecursor with an antitoxin titer of 485 IU/ml; lateral wells, (1) 12S precursor (600 μg/ml), (2) Eα (280 μg/ml), (3) Eβ (230 μg/ml). Agar gel, 1% agar in 0.05 M acetate buffer, pH 6.0; distance between antigen and antibody wells, 7 mm; incubation, at 4 C for 2 days.

The single precipitation line of Eα fused with one of the two lines of 12S precursor; that of Eβ with the other one (Fig. 5). The 12S precursor may consist of Eα-Eβ complex since Eα and Eβ have different electric charges. The two precipitation lines of 12S precursor (Fig. 5, well 1) can be interpreted as indicating the presence of Eα-Eα and Eβ-Eβ complexes, however, a more plausible explanation for the two lines may be spontaneous dissociation in agar gel.

To clarify the peculiarity of the sedimentation pattern at pH 7 (Fig. 2), 12S precursor adjusted to pH 7 by dialysis was analyzed by ultracentrifugation. Two boundaries, the major one with $S_{20,w}$ 10.3 and the minor one with $S_{20,w}$ 6.9, were observed (Fig. 3 (2)). Sedimentation in sucrose density gradient at pH 6.0 of 12S precursor after exposure to pH 8.0 for 30 min at 6 C gave a single peak with 10.8S, which was calculated by the method of Martin and Ames (1961). When the exposure to pH 8.0 was prolonged, a slower sedimenting peak at 7S became visible. A critical change in the molecular shape possibly resulting in the reduction of S value from 12 to 10 must have preceded the molecular dissociation into the 7S components. An immediate drop in potential toxicity by about 50% was often observed when 12S precursor was exposed to pH 7 or 8. The potential toxicity of the precursor,

therefore, seemes to be related to the three dimensional structure of the molecules.

Further studies are being made to clarify the effect of ionic strength and other factors on the molecular dissociation of the precursor and also the activated toxin and to examine for the possibility of dissociation of E_{α} into still smaller subunits with molecular sizes as small as reported by Gerwing et al. (1964).

Acknowledgements

This investigation has been supported in part by U. S. Public Health Service Research Grant UI-00123, from National Center for Urban and Industrial Health. The authors wish to thank the cooperation of Mrs. I. Ohishi of this Department in agar gel diffusion tests and of Mr. K. Suzuki of this Institute in ultracentrifugal analyses.

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