BEHAVIOUR OF TOXIN RELEASED FROM THE CELLS OF *CLOSTRIDIUM BOTULINUM* TYPE E

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SUMMARY

Clostridium botulinum type E toxin released by the cells to the medium was compared to the same type toxin retained within the cells. The released toxin, partially purified on a DEAE-cellulose column, had an $S_{20,w}$ value of 11-12. Both Eα and Eβ subunits were identified in this large molecular weight toxin by the immuno-agar-gel-diffusion test. This toxin had high toxicity, futher activatable by trypsin. Toxin retained in the cells, upon extraction, was found to be complexed with RNA and with an S-value of 15-19. Based on these findings, a possible release mechanism of the toxin is discussed.

Two forms of *C. botulinum* type E toxin have been identified and characterized, a "retained toxin" which can be extracted directly from the cells (1,2) and a "released toxin" excreted by the cells and collected from the extracellular medium (3,4). The retained form can be obtained as a large molecular weight prototoxin (1,5) of M.W. 350,000 (6) and 11.6 S (5,7), which consists of an activatable toxic proto-Eα and nontoxic proto-Eβ (2,5) each of which has a M.W. 150,000 (6) and 7.3 S (2). The toxin is retained in the cells when the cells are cultured at pH 6.3 (5,8). Cells cultured at pH 7.0 or above release toxin into the medium almost completely (3,9). It has been reported that this released toxin has a M.W. less than 19,000 (3,4). The large difference in the reported molecular weights for the retained and released forms indicated a drastic change in the toxin molecule during release from the cell. In the present study, a careful comparison of the two forms of toxin obtained from the same strain was undertaken, in order to gain an insight into the mechanism of release of toxin by the cells.

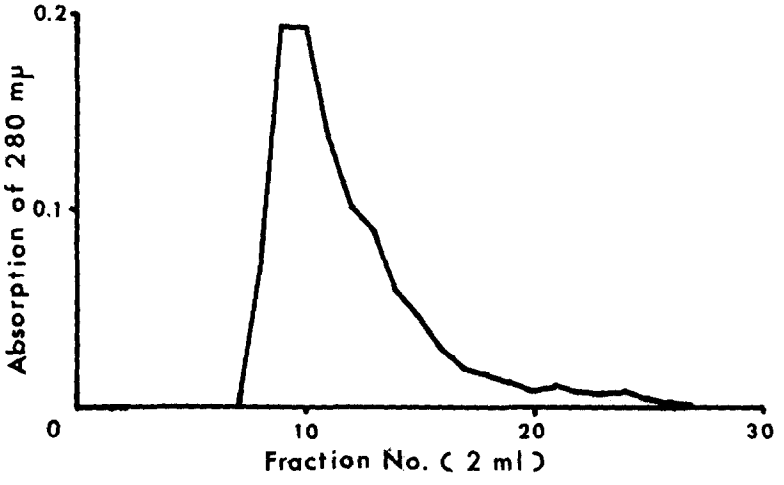

Fig. 1 DEAE-cellulose chromatography of released toxin.
The culture medium described by Emodi et al. (9) was 5% trypticase (B.B.L.), 0.5% peptone (Difco), 1% yeast extract (Difco), 0.2% sucrose and 0.02% sodium thioglycollate at pH 7.0 in distilled water. Two hundred ml of culture were autoclaved at 121°C for 20 min. After incubation at 30°C for 36 hrs, the cells were separated from the culture media by centrifugation at 5000 x g for 20 min. A pH of 6.4 was obtained for the supernatant which contained 80% of potential toxicity of the whole culture. The supernatant was precipitated overnight at 4°C with crystalline $(NH_4)_2SO_4$ at 60% saturation. The precipitate was then collected by centrifugation at 1000 x g at 4°C, dissolved in 0.05 M sodium phosphate buffer, pH 6.0 (Na_2HPO_4 - NaH_2PO_4), and dialyzed in the buffer for overnight at 4°C. The dialyzed toxic sample (A) was subjected to chromatography on DEAE-cellulose (BIO-RAD Labo.) which was equilibrated with sodium phosphate buffer pH 6.0 column of 1 x 20 cm. Two ml of sample (A) was eluted with 0.05 M sodium phosphate buffer, pH 6.0 at 4°C.

RESULTS AND DISCUSSION

Clostridium botulinum type E VH was grown for released toxin production in the medium described by Emodi et al. (9). Toxin from the medium was concentrated and dialyzed with 0.05 M sodium phosphate buffer pH 6.0. The dialyzed toxic sample (A) was subjected to chromatography on a DEAE-cellulose column. The column was equilibrated and the sample was eluted with the 0.05 M sodium phosphate buffer of pH 6.0. The major peak was eluted fast (Fig. 1) and pigment remained on the column. The first peak had high toxicity. Potential toxicity per mg protein of this fraction was 9.8×10^4 LD50. This elution result was the same as that of Emodi et al. (4). The DEAE-cellulose major fraction was concentrated with Ficoll (Pharmacia) at 4° C. This concentrated toxic

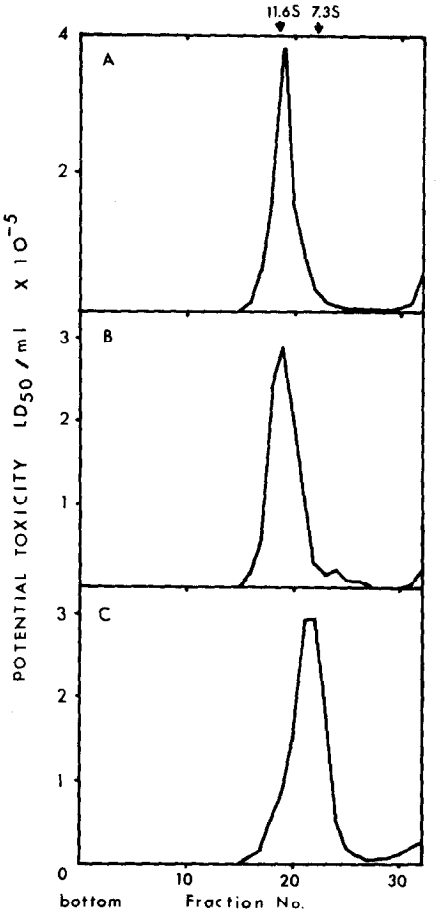

Fig. 2 Sucrose-density-gradient centrifugation of DEAE-cellulose toxic fraction (A) and RNase treated DEAE-cellulose toxic fraction (B) at pH 6.0, and of DEAE-cellulose toxic fraction at pH 8.0 (C). A 0.2 ml sample was placed on top of the sucrose-density-gradient of 5-20% (w/w) in 0.05 M sodium phosphate buffer, pH 6.0 or 0.05 M phosphate buffer, pH 8.0. Centrifugation was performed at 39,000 rev/min for 7 hrs at 5°C in SW 39 rotor in a Beckman centrifuge model L_2-65B.

Two tenths ml portion of DEAE-cellulose toxic fraction was treated with 0.05 ml of RNase (EC 2.7.7.16) (6 mg/ml; N.B.C. solution) at 37°C for 20 min at pH 6.0. Toxin potencies were determined by the intravenous injection method (5). The toxin samples were activated with trypsin (EC 3.4.4.4.) (1 mg/ml; N.B.C.) at 37°C for 20 min before injection.

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sample (B) was run to estimate molecular size by sucrose-density-gradient centrifugation at pH 6.0 along with a purified sample of 11.6 S prototoxin as a comparison indicator. All toxicity sedimented as a single boundary very close to the position of the prototoxin e.g. with a value of 11-12S (Fig. 2-A). The sample (A) yielded the same results as that of sample (B). The result obtained

is definitely larger than the reported size of the released toxin (3,4).

It is possible that this toxic fraction having 11-12 sedimentation constant included prototoxin, activated form of the 12S toxin or RNA-proto-Eα having 12-13 S (10). To check for the presence of RNA-proto-Eα , the DEAE-cellulose fraction (sample B) was treated with RNase, then was centrifuged on a sucrose-density gradient at pH 6.0. The sedimentation pattern of toxicity was the same as that of the nontreated sample (Fig. 2-B); indicating that no RNA was present. The sample (B) was centrifuged to detect the possibility of dissociation at pH 8.0 by sucrose-density-gradient centrifugation with purified proto-Eα as marker. The single band of toxicity sedimented at the position of proto-Eα (Fig. 2-C), at 7.3S, showing dissociation. In an immuno-agar-gel-diffusion test of samples (A) and (B), at least 4 bands were formed; the two bands in these bands fused with proto-Eα and proto-Eβ of pure prototoxin (Fig. 3).

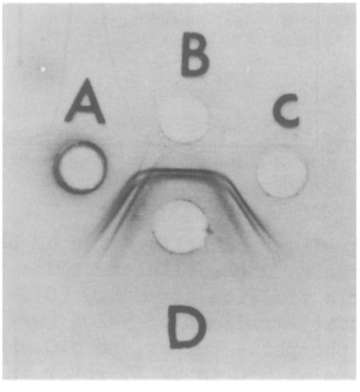

Fig. 3 Agar-gel-diffusion tests performed with prototoxin, DEAE-cellulose toxic fraction and concentrated media. A; concentrated media (sample A), B; prototoxin of type E 35396, C; DEAE-cellulose toxic fraction (sample B), and D; anti-prototoxin with an antitoxin titer of 450 IU/ml. Agar gel, 1% agar in 0.05 M phosphate buffer, pH 8.0; incubation, at 4°C for 3 days.
Two bands can be observed between B and D, and the top band represents proto-Eα and the lower one represents proto-Eβ .

The results indicate that released toxin has a large molecular size of approximately 11-12S and consists of Eα and Eβ . This toxic fraction may also be a mixture of prototoxin and activated 12S toxin because the toxicity ratio after activation to that before activation of sample (A) was approximately 12-fold

by the intraperitoneal injection method (5). The activation ratio of proto-toxin or RNA-prototoxin (5,11) is 200-500 (5,11) and that of proto-Eα is 230 (6). The low activation ratio of the present preparation may indicate an already activated material, possibly by an enzyme system.

Retained toxin was extracted from the cells of the VH strain cultured in 4% proteose peptone (Difco), 0.5% yeast extract (Difco), 1% glucose and 0.03% sodium thioglycolate media at pH 6.4 in which the 35396 strain of C. botulinum type E had been grown with good yield. After incubation at 30° C for 4 days, toxin was extracted from the cells in 0.2 M phosphate buffer pH 6.0 at 37° C for 1 hr. The extract concentrated with Ficoll was centrifuged to estimate molecular size by sucrose-density-gradient centrifugation at pH 6.0. The broad peak of potential toxicity was demonstrated at the position of about 15-19S (Fig. 4). After treatment of the extracted toxin with RNase, the position of the highest potential toxicity changed from 15-19S to 11-12S (Fig. 4). The sharp peak obtained after RNase treatment indicated that the prototoxin was homogeneous, and therefore that the RNA moiety in the RNA-prototoxin consisted of non-uniform RNA fragments.

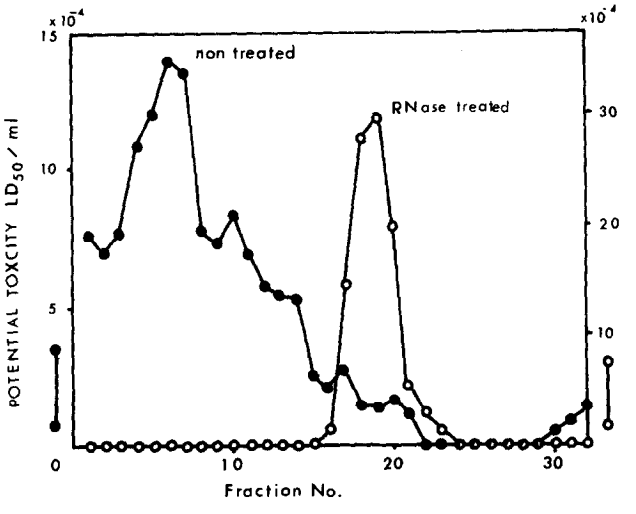

Fig. 4 Sucrose-density-gradient (5-20%) centrifugation at pH 6.0 of cell extraction and RNase treated cell extraction.
Centrifugation procedure and toxin potencies were same as described in Fig. 2. Two tenths ml of cell extraction was treated by RNase (6 mg/ml) at 37°C for 20 min.

The results presented here show that both the released and retained forms of the toxin are of relatively large molecular weight, and identifiable with previously reported toxins of type E (2,5,7). The released toxin was obtained under essentially the same conditions utilized by Emodi et al. (4,9) who reported obtaining two toxic components of M.W. 9,000 and 5,000 after passing the DEAE-cellulose eluted toxic fraction through Sephadex G-200 in a small column (1 x 28 cm) (4). No such small molecular weight components were found in the present investigation. It may be mentioned that small molecular weight toxins could not be confirmed in types A and B by Boroff et al. (12), Beers et al. (13) and Knox et al. (14). It is also suggested here that the sucrose-density-gradient centrifugation gives a more reliable estimate of molecular weight than the methods used by Emodi et al. (4).

The results presented indicate that, within the C. botulinum type E cell, the cell synthesizes proto-Eα of little or no toxicity complexed with RNA and with proto-Eβ . The toxin released to the medium is free of RNA and of high toxicity. These results point to the following plausible mechanism. The synthesized RNA-prototoxin, before or during release from the cells, is dissociated from the RNA and activated by some enzyme system (s). The activation or synthesis of this enzyme system is dependent on certain conditions of culturing the cells (pH or media composition). The RNA may bind proto-Eα to some cell structure, or again may act as a carrier for proto-Eα .

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