Chromatographic Purification of the Toxin of Type E *Clostridium botulinum*[1]

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Summary

Clostridium botulinum Type E extracellular toxin was purified on DEAE cellulose columns following ammonium sulfate precipitation and dialysis. Two fractions, one toxic and one non-toxic, were obtained. Disc electrophoresis of the toxic fraction showed three bands, two of which were not toxic. Passage of the toxic fraction through a Sephadex G-200 column separated it into two fractions with equal toxicity. The estimated molecular weights of the two toxic fractions were about 9000 and 5000, respectively.

Purification of the toxin formed by Type E *Clostridium botulinum* has been carried out by several techniques. Gordon *et al.* (1957) precipitated the toxin with ethanol and used ionized cellulose columns for purification. Gerwing *et al.* (1964) used ammonium sulfate to precipitate the toxin, further purified the concentrated dialysate on DEAE cellulose columns, and calculated a molecular weight of 18,600 (S_{20w} = 1.70S). Sakaguchi and Sakaguchi (1967) digested the "precursor" toxin first with ribonuclease and then used CM-Sephadex and Sephadex G-200 columns for purification and molecular weight estimation. Their results in contrast to those of Gerwing *et al.* indicated a uniform molecular weight toxin (200,000 or more). In our study we attempted to clarify some of the discrepancies relative to the homogeneity, aggregation, and the molecular weight of the Type E toxins.

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Experimental and Discussion of Results

C. botulinum Type E VH strain was used for toxin production. The toxin was produced by using a modified version of the sac method of Vinet and Fredette (1951). The culture medium was trypticase-peptone-sucrose-yeast extract (TPSY) pH 7.0 (Emodi and Lechowich, 1969).

The cells were separated from the toxin by centrifugation at 5000 x g for 20 min, and the toxin was precipitated overnight at 4 C with crystalline $(NH_4)_2SO_4$ at 60% saturation. The precipitate was then collected by centrifugation 10,000 x g at 4 C, dissolved in 0.05 M sodium phosphate

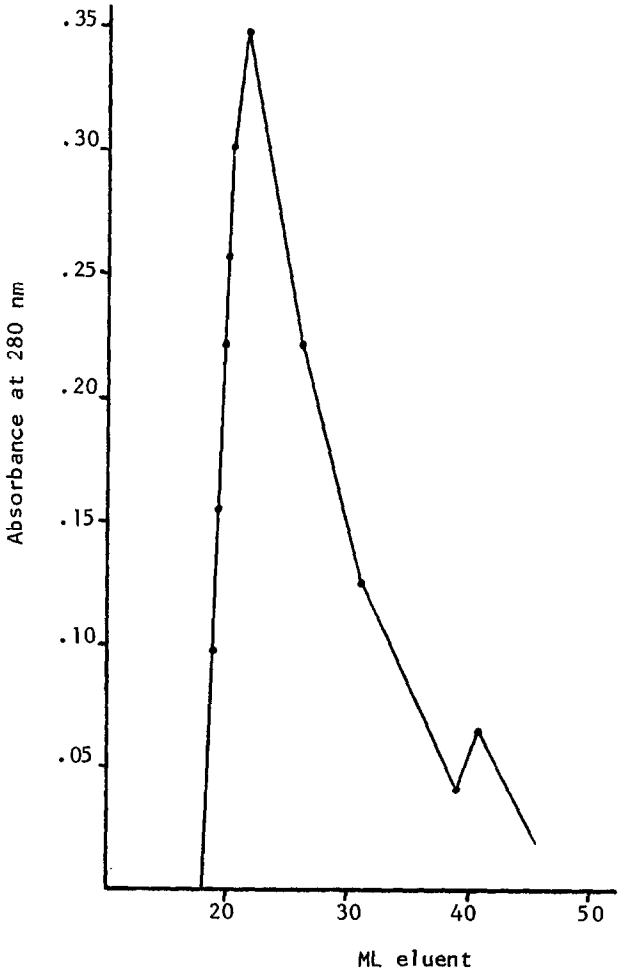

Figure 1. Elution patterns of Type E VH toxin on DEAE cellulose with .05 M sodium phosphate buffer, pH 6.0. Major peak contains the toxin.

buffer, pH 6.0, and dialyzed overnight at 4 C. This partially purified toxin preparation, approximately 1/10 of the original volume, containing 5.7×10^5 MLD toxicity per mg protein was then purified on DEAE cellulose columns (1 x 28 cm). Columns were equilibrated and toxin was eluted with the phosphate buffer used to dissolve the precipitated toxin. Quantities of 4.0 ml of the partially purified preparations were placed on the column. The elution curve of the toxin showed one major peak and one minor peak (Fig. 1). The major peak was highly toxic, 1.04×10^6 MLD per mg protein, while the small peak was only slightly toxic, and contained 1×10^3 MLD per mg protein. Application of NaCl gradient on the columns up to 0.5 M did not elute more toxin.

The separation of the toxin of Type E strain Iwanai into two components using DEAE cellulose was reported by Gerwing et al. (1964). However, there is evidence that some Type E toxins separate into more than two components on DEAE cellulose. The above procedures were applied to the toxin of the Kalamazoo strain and it separated into four components. The details of this experiment will be reported later. Since a second $(NH_4)_2SO_4$ precipitation and DEAE purification did not yield increased purification of the VH toxin as indicated by disc electrophoresis, the toxic samples were pooled and used for further work. All purification procedures were carried out at 4 C.

The purified toxic fraction appeared homogeneous when examined by ultracentrifugation. A very slowly moving peak was observed and a sedimentation coefficient of $S_{obs} = 0.6$ was calculated. However, at the beginning of the centrifugation two small shoulders which later diffused were also observed.

Tests for hemagglutinating activity (Dasgupta and Boroff, 1968) of the eluted sample gave negative results, while unpurified Type A and B toxins used as controls gave positive results.

Disc electrophoresis of the toxic fraction using the method of

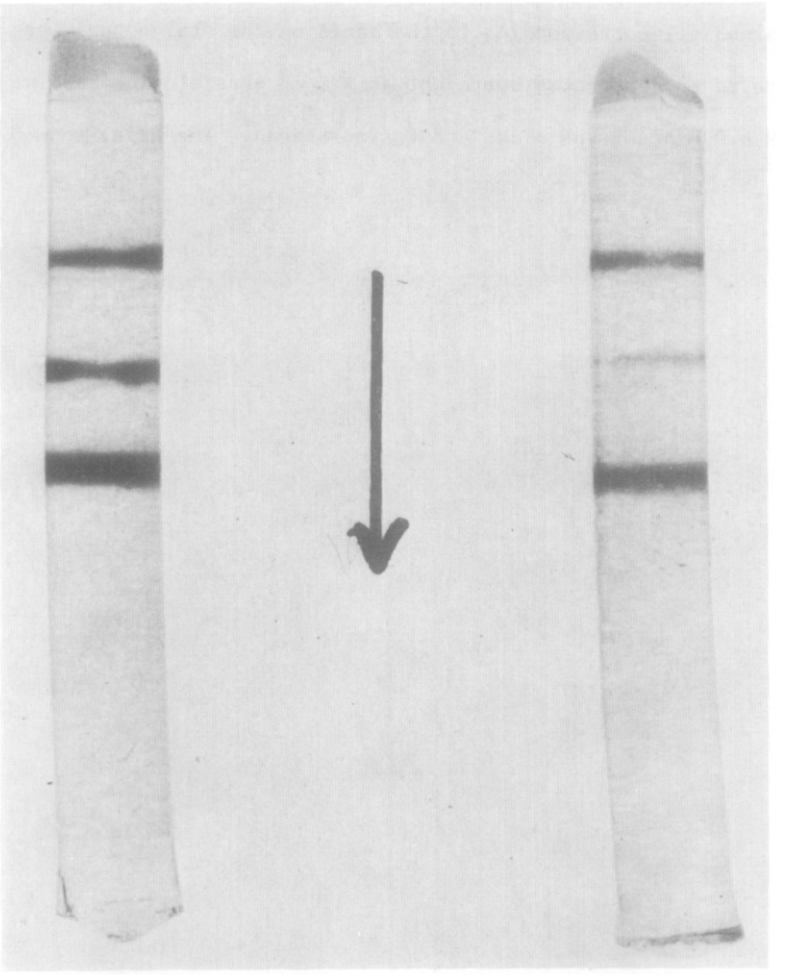

Figure 2. Disc electrophoresis pattern of purified Type E VH toxin on pH 4.3, 7.0% acrylamide gel using pH 5.0 β alanine-acetic acid buffer and a current of 3 mA.

Davies (1964) was done by placing 50-100 µg protein on pH 4.3, 7.0% acrylamide gel, using pH 5.0 β alanine-acetic acid buffer. Electrophoresis was carried out at 4 C with a current of 3 m. Amp. per tube. Two tubes per sample were used for electrophoresis; one was stained to detect

the bands while the other one was used for toxin assay. Three protein bands (one dense and two faint) were observed (Fig. 2). Portions of the unstained gel corresponding to the bands on the stained gel were cut out, macerated and extracted overnight at 4 C in physiological saline buffered to pH 6.0, then trypsinized and toxin assayed. The assay showed that the

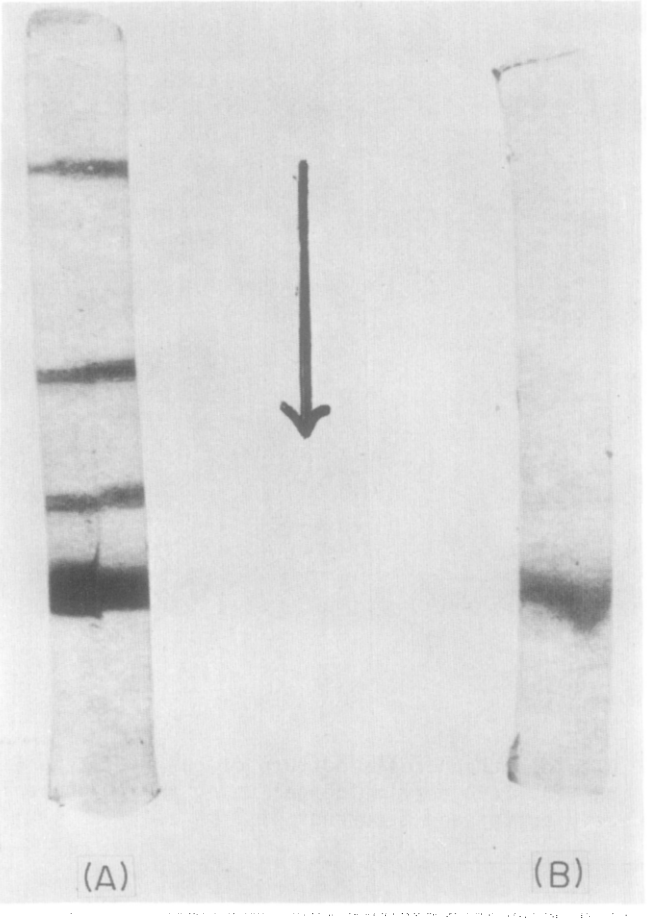

Figure 3. Disc electrophoresis pattern of the toxic and non-toxic fractions eluted from DEAE cellulose after RNase treatment.

A. Toxic Fraction
B. Non-toxic Fraction

band with the lowest electrophoretic mobility contained all the toxic material. Disc electrophoresis of the toxic fraction on pH 8.9, 7.0% acrylamide gel, using pH 8.3 Tris-glycine buffer showed only one very slowly moving band.

Absorbance of the toxic fractions at 280/260 nm yielded ratios of 1.06-1.35 showing that approximately 0.9-3.0% nucleic acid was present in the preparation. Therefore, the preparation was treated with RNase (10 µg/ml) at room temperature. This RNase-digested protein was then rechromatographed on DEAE cellulose columns using the PO_4 buffer system used before. The RNase treated preparation separated into two components; both fractions came through before NaCl gradient application. The gradient produced no more absorption peaks. The first component contained more than 90% of the toxin. Absorbance at 280/260 nm yielded ratios of 1.75-2.0, while the second non-toxic component yielded a ratio of 1.38. Upon disc electrophoreses of the two fractions (Fig. 3), the toxic fraction yielded four bands while the non-toxic fraction produced two bands. Toxin assay showed that the slowest moving band contained essentially all of the toxin.

Rechromatography of the toxic components eluted from DEAE cellulose before and after RNase treatment, on Sephadex G-200 column (1 x 28 cm), produced two peaks of equal toxicity. On a calibrated G-200 column (method of Andrews, 1965), both peaks were eluted after cytochrome C (Fig. 4). Therefore, the estimated molecular weights are believed to be about 9000 and 5000, respectively.

It appears that the purification procedure used in our studies did not produce a homogeneous compound. The toxic component obtained from DEAE cellulose chromatography separated into small molecular weight species on Sephadex G-200. RNase treatment of the toxin produced one more band upon disc electrophoresis. Further characterization of the toxin and a possible aggregation phenomenon into a larger molecular

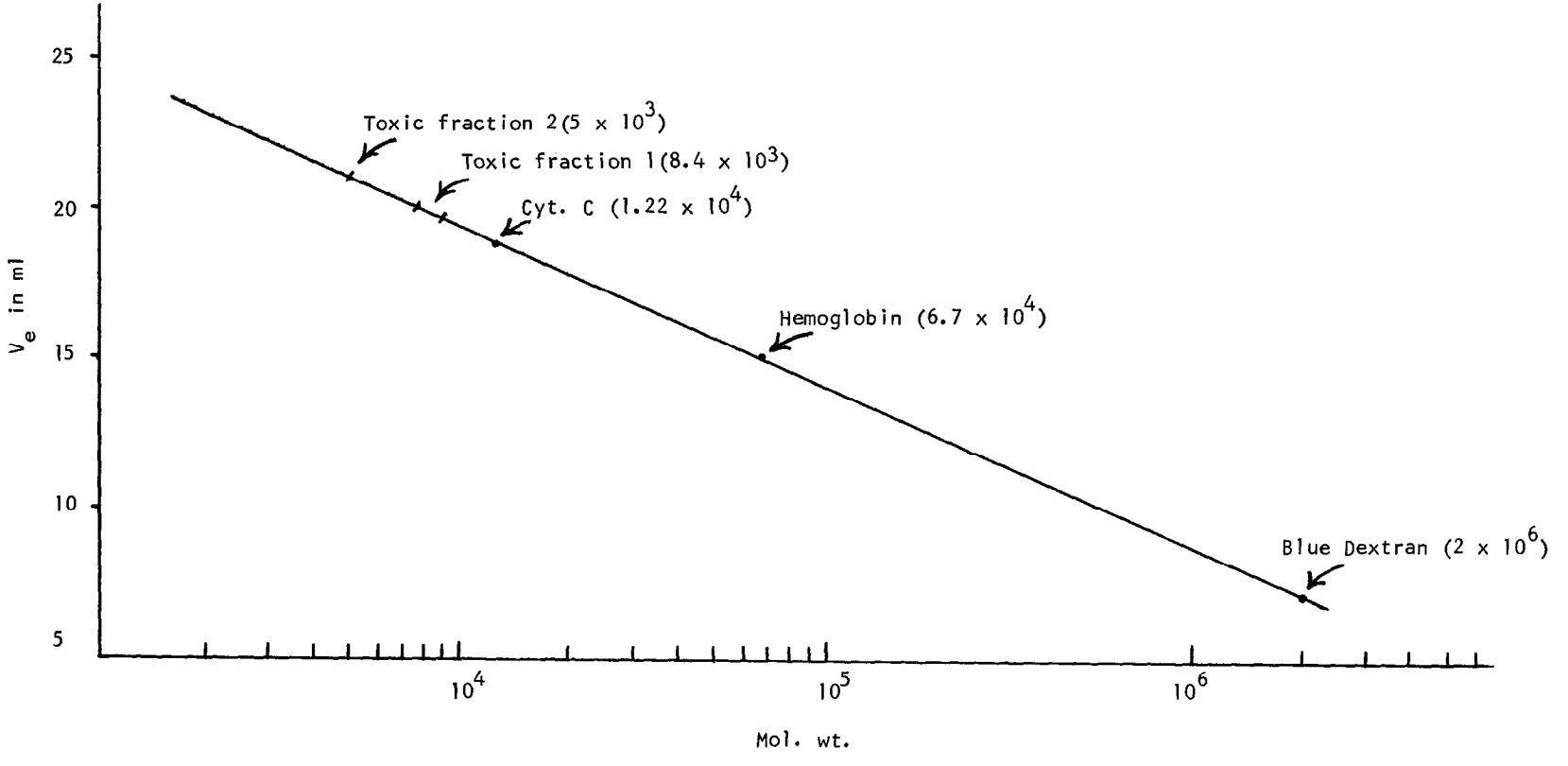

Figure 4. Molecular weights of toxic fractions as determined on Sephadex G-200 column (1 x 28 cm). Proteins were eluted with 0.05 M sodium phosphate buffer, pH 6.0. The molecular weights of proteins are within the parentheses.

weight compound is being investigated.

Acknowledgement

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