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J. Martin Sax Aug. 6, 1996  
PI - Signature Date

## Table of Contents

Front Cover	1
SF 298	2
Foreword	3
Table of Contents	4
Introduction	5
Body	5
Conclusion	6
References	7
Figure 1	8

## **BOTULINUM NEUROTOXINS - FINAL REPORT**

### **INTRODUCTION:**

Clostridial toxins are regarded as the most poisonous substances to mankind. There are seven serologically distinct botulinum neurotoxins, labeled A to G, secreted by *Clostridium botulinum*. These are synthesized as single chain proteins of 150kDa molecular weight but some of these serotypes are nicked by endogenous protease at about one third from the N-terminus forming a dichain molecule with one light and one heavy chain. For example, while Bot - type A is a dichain, bot type E remains as a single chain. However, Bot type E could also be nicked in vitro by trypsin. Dichain botulinum neurotoxins are multifold more toxic than single chain neurotoxins. While the precise nature of mechanism of action of these toxins is not known, a three step model has been proposed. In the first step neurotoxin binds to specific receptors on the presynaptic membranes and the binding site is believed to be in the C- terminal half of neurotoxin. The second step is internalization of toxin and the N terminal half of neurotoxin is involved in this. The third and final step is the intracellular lysis which takes place via the light chain. However, to understand the mechanism of action and the nature of binding sites, it is essential to have the three dimensional structure of these neurotoxins. Since the three dimensional structure of none of these toxins is known at present, we have undertaken to determine the three dimensional structure of botulinum neurotoxin, type E.

### **BODY:**

Botulinum neurotoxin, type E (Bot-E) was obtained from PHLS/CAMR Porton Down, Salisbury, UK. The protein as obtained was pure as shown by SDS gels. Initial screening for crystallization was carried out using the hanging drop method in 24 well Linbro culture plates. Equal volumes of (2 to 3 microliters) protein and reservoir solutions were mixed on siliconized cover slips which were then inverted over the reservoir wells and sealed with silicone grease. Crystals were obtained over a range of 6 to 10% PEG 4000 as reservoir solution containing 0.2 M Hepes buffer in the pH range 6.8 to 7.2. These crystals were plate like crystals and preliminary x-ray diffraction experiments showed that they are in monoclinic space group P2 with cell parameter  $a = 81.6, b = 172.87, c = 139.2 \text{ \AA}$  and  $\beta = 98.7^\circ$  with two molecules per asymmetric unit. X-ray diffraction data extending to 3.1 A resolution have been collected.

Crystals were mounted in capillary tubes and then sealed before being transported to Pittsburgh from Fort Detrick. Since it was difficult to transport mounted crystals as was initially done, crystallization experiments were also moved to Pittsburgh. For this purpose a small lab was modified to be P2 biosafety level lab at the VA Medical Center, Pittsburgh. A biosafety cabinet and a refrigerated micro centrifuge were purchased. The lab is now functional and is in operation. This move has increased our ability to try more experiments than in the past. We found that when 8% PEG 6000 and 0.2 M Hepes at pH 6.8 are used we get small but chunky crystals. One data set was collected using these crystals. The unit cell dimensions are  $a = 81.44, b = 172.68, c = 138.31 \text{ \AA}$  and  $\beta = 98.5^\circ$  in the monoclinic space group P2. The Matthews coefficient is  $3.02 \text{ \AA}^3/\text{dalton}$  assuming two molecules per asymmetric unit and the solvent occupies approximately 60% of the cell volume. Recently we have received a batch of BoNT/E from Dr. Johnson's

laboratory and crystallization of this material is in progress.

Three dimensional x-ray diffraction data were collected using a Siemens HiStar area detector system. The area detector was mounted on a Rigaku RU200 rotating anode with Cu target and 2.0x0.2 mm filament. The generator was run at 42KV and 65mA rating. Highly focused monochromatic  $\text{CuK}\alpha$  radiation was obtained using a thin nickel filter and Franks double focusing mirrors. Since at room temperature the crystal life time in the x-ray beam is less than 24 hr, several crystals were used to collect a native data set. The native data set extends to at least 3.1 Å at the present time, with a few reflections in the 3.1 - 3.0 Å shell. Efforts are underway to use flash freezing techniques to prolong the life time of these crystals in x-radiation. The present native data set is 75% complete to 3.1 Å resolution. Low temperature data collection with flash frozen crystals should give better data extending to higher resolution. Data processing was carried out with the Xengen area detector processing package.

The presence of two molecules in the asymmetric unit suggests that the toxin may exist as a dimer with a non-crystallographic two fold axis present in the unit cell. BoNT/A is known to exist as a dimer in crystals.<sup>1</sup> BoNT/E has been shown to exist as a monomer or a dimer based on the native gel electrophoresis and chemical cross linking experiments.<sup>2</sup> Since non-crystallographic symmetry can be used to advantage in the phasing technique by density averaging, self rotation function studies were carried out to identify the non-crystallographic axis if present. Rotation function calculations were done using Crowther's fast rotation function and the program ROTRAN.<sup>3</sup> Self rotation functions were computed for different resolution cut offs, ranging from 8 to 4 Å and different Patterson search radii. The strongest peaks appeared in the  $\kappa = 180$  of the self rotation function map, calculated with a search radius of 35 Å and with data in the resolution range 40 - 4 Å, is given in Figure 1. An interesting aspect of this self rotation study is the presence of a 222 symmetry indicating a possibility of a tetramer arrangement of BoNT/Es. Three dimensional reconstruction by electron cryomicroscopy and image processing shows channels in vesicle cells arising from interaction with four botulinum neurotoxin molecules.<sup>4</sup> However the presence of a tetrameric arrangement in the crystal is only a conjuncture at this stage of structure analysis.

Search for heavy atom derivatives are underway though no derivative data have been collected since the low temperature set up for data collection is not fully operational yet.

#### CONCLUSION:

1. More crystallization experiments are in progress to get more native and derivative diffraction intensity data.
2. A rotation function analysis of Bot E indicates the presence of local two fold symmetry in the asymmetric unit which consists of two molecules.

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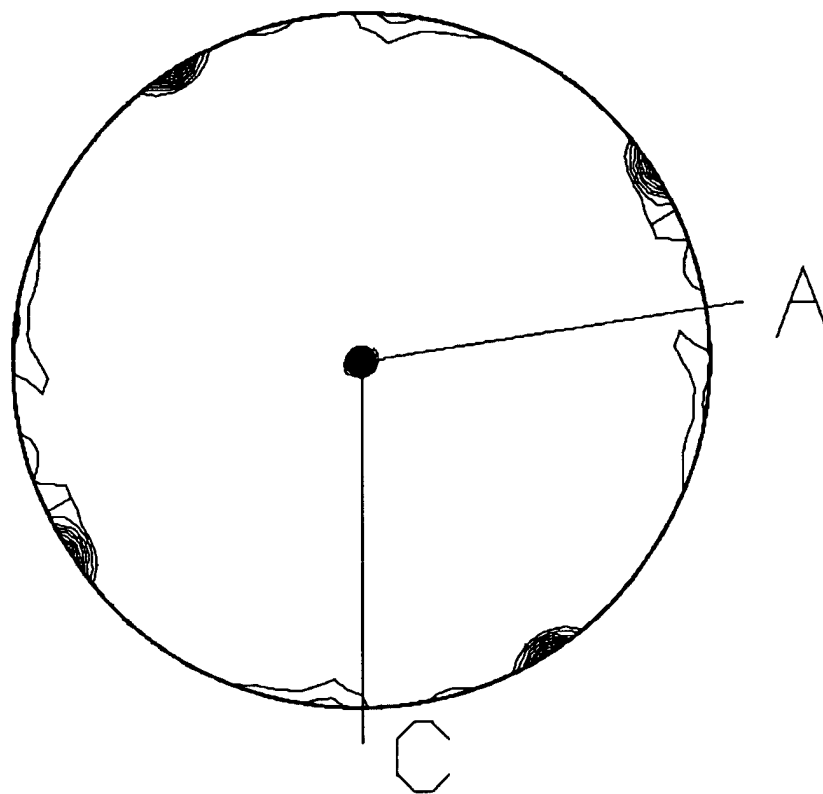


Figure 1: Stereographic projection of rotation function at  $\kappa = 180$  viewed down  $b^*$  axis is shown above for BoNT/E crystals. Non-crystallographic two fold symmetry at  $32^\circ$  to the C axis and orthogonal to the crystallographic two-fold. The peak height of the peak due to non-crystallographic symmetry is 80% of the origin peak. This map was obtained with data extending upto  $4\text{\AA}$  resolution and a  $35\text{\AA}$  Patterson search radius was used.