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13. ABSTRACT *(Maximum 200 words)*
Botulinum neurotoxin, type E (Bot E) was purified to evaluate the affects of purity on crystallization. It was found that purification of the product from PHLS/CAMR Proton, Salisbury, VK did not improve crystal quality significantly. Accordingly, the product was used as supplied in further crystallization experiments. Diffraction quality crystals were grown by VAMC personnel at Fort Detrick, but the crystals which were transported in sealed capillary tubes deteriorated in transportation. To overcome this problem, a safe and secure laboratory was set up in the Pittsburgh VAMC (UD) in compliance with Federal regulations to handle Bot E neurotoxin. Crystals were grown which diffracted to 3.A, and a set of intensity data was collected from it. Work is progressing with the aim of getting more mature and derivative data.

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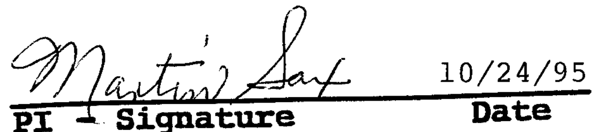

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BOTULINUM NEUROTOXINS - MIDTERM REPORT

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 microlitres) of 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 parameters $a = 81.6$, $b = 172.87$, $c = 139.2$ Å and $\beta = 98.7^\circ$ with two molecules per asymmetric unit. X-ray diffraction data extending to 3.1 Å 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. Further work is in progress.

Botulinum neurotoxins and tetanus neurotoxins have considerable sequence homology and may have structural similarity. The C- terminal half of the heavy chain of tetanus toxin is commercially available and if the three dimensional structure of this fragment could be determined it will serve two purposes. The structure of this fragment by itself could shed light on the binding of this fragment to specific receptors in presynaptic membranes. It could also be used as partial structural information in solving the structure of botulinum neurotoxin. In view of this, the 50 kDa C-fragment (rTTC) of the heavy chain of tetanus toxin was purchased from the Boehringer Mannheim Corporation. rTTC has been crystallized using the vapor diffusion method. Crystals are obtained with a precipitant of 14 to 18% (w/v) PEG 4000, 0.1 M imidazole, pH 6.5. Addition of salts, like $MgCl_2$, NaCl and $NaCH_3CO_2$ also encourage crystallization. Two different crystal forms grow under similar conditions depending on the initial concentration of protein and the ratio of the volume of protein to the volume of reservoir. The morphology of these two crystal forms are different and could be recognized under a microscope Plate like crystals are in space group $P2_12_12_1$ with unit cell parameters a = 79.7, b = 94.0 and c = 71.3 Å. Chunkier, rod shaped crystals are also in space group $P2_12_12_1$ with slightly smaller unit cell volume. The cell parameters are a = 67.4, b = 79.7 and c = 91.1 Å. Chunkier crystals diffract to better resolution and are more stable under irradiation by x-rays and are thus being used for structure determination.

X-ray diffraction data have been collected from native crystals to 2.6 Å resolution. Extensive heavy atom derivative search is being conducted and two suitable heavy atom derivative crystals have already been identified and data collected on them to 3.0 Å resolution. These data were used to calculate MIRAS maps. The phases were further improved by solvent flattening and the resulting map is being analyzed.

CONCLUSION:

1. More crystallization experiments are needed to get more native and derivative diffraction intensity data.

1. Though the electron density map for rTTc reveals the general structure, it is not good enough to trace the entire polypeptide chain. Currently, heavy atom derivative search is being continued to identify more derivatives to improve protein phases. With more derivatives a better map will be obtained and the chain traced.