Applications of Rat Brain Synaptic Vesicle Proteins for Sensitive and Specific Detection of Botulinum Neurotoxins

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Abstract

We propose here the application of synaptic vesicle proteins isolated from rat brain as a sole substrate for the specific endoproteinase activities of all seven serotypes of Botulinum Neurotoxin (BoNT/A to G). In this study, we used these proteins for evaluating endopeptidase and receptor binding activity for detecting BoNT/A by western blot and surface plasmon resonance with 6.25 pM and 0.22 fM limit of detection, respectively. Substrate and receptor present in the synaptic vesicle proteins are very robust and stable for more than 6 months to use in BoNT detection.

Keywords: Botulinum neurotoxin; Endopeptidase; Synaptic vesicle; SNAP-25, SPR

Background

Botulinum Neurotoxins (BoNTs) are causative agents of botulism and because of their extreme potency, lethality, ease of production, transport and the need for prolonged medical care they, are classified as category A (the highest priority) bioterrorism agents/diseases by the Centers for Disease Control and Prevention [1]. Seven distinct serotypes of the BoNTs have been identified and are designated type A through G. Humans are usually exposed to the preformed neurotoxins produced by Clostridium botulinum through food poisoning, although there are rare incidents of wound botulism and colonizing infections in the small intestine of neonates known as infant botulism [2]. Once ingested or inhaled into the lung, BoNTs are taken up by the blood stream, target the peripheral cholinergic nerve endings resulting in flaccid muscle paralysis, which leads to death. In recent years, the development of in vitro assays for the detection of BoNTs has been accelerated with the aim of decreasing the Limit Of Detection (LOD) upto femtogram quantities. However, none of the assays developed to date has been validated to be robust enough to completely replace the mouse bioassay, the FDA-approved and widely accepted gold standard method for BoNT detection. The endopeptidase assays and its variants viz. fluorescence endopeptidase assays, Fluorescence Resonance Energy Transfer (FRET) assays which can detect the biologically active neurotoxin, are reported to be more sensitive than the mouse assay [3] and have the potential to replace the mouse lethality assay. Detection of the cleaved peptides by mass spectrometry further increases the sensitivity [4], but due to the requirement for expensive instrumentation, reagents and specialized skills, these techniques are not easily affordable for most laboratories. In the scenario of a bioterror attack, an economic technology compatible with a hand-held type of device would be preferred solution for field deployment and laboratory detection.

Introduction

BoNTs act on the SNARE [soluble NSF (N-ethylmaleimide sensitive factor) attachment receptor] proteins and hydrolyze the peptide bond in a specific location resulting in inhibition of acetylcholine release. Vesicle-Associated Membrane Protein 2 (VAMP-2) is cleaved by BoNT/B, D, F and G; Synaptosomal-Associated Protein 25 (SNAP-25) is cleaved by BoNT/A, C, and E; and syntaxin is an exclusive substrate for BoNT/C [3]. VAMP-2, SNAP-25 and syntaxin are all synaptic vesicle (SV) proteins that can be isolated from the rat brain. This indicates that synaptic vesicle proteins isolated from rat brain can be used as a substrate for detection of all the serotypes of BoNTs. The cleaved fragments of these proteins can be detected by western blot using specific antibodies indicative of BoNT serotype. The assay can differentiate between active and inactive toxin which is generally difficult for other immunoassays. The assay can also be performed in hrs with mouse bioassay that requires several days for serotype detection. Antibodies against SNAP-25, Syntaxin, and VAMP-2 are available from Sigma-Aldrich, Santa Cruz Biotechnology Inc., CRP Inc., Pierce Biotechnology Inc., USA. Those serotypes that exhibit highest sequence similarity share the same protein receptor, e.g., BoNT/A, E, and F bind to SV2, whereas Serotypes B and G bind to synaptotagmin I and II [5]. We herein report the application of the synaptic vesicle proteins from the rat brain as a substrate for detecting the endoproteinase activity of the botulinum neurotoxin. The binding affinity of BoNT/A heavy chain to its protein receptor present on rat brain synapsosome has also been exploited for the development of a specific and sensitive detection system for botulinum neurotoxin type A.

Methods/Discussion

Rat was anesthetized by halothane inhalation, brain was removed immediately and rinsed in ice cold 0.9 % NaCl. Fresh rat brain was homogenized with a teflon homogenizer in ten times volume (w/v) of cold homogenization buffer (25 mM Tris, 100 mM NaCl, 19.2 mM glycine, 100 µg/ml BSA, 0.1 mM DTT, 10 µM ZnCl2, pH 7.5). Homogenized samples were centrifuged at 10,000 xg for 20 min and the supernatant was collected. SV proteins present in the supernatant were then filtered through a 0.22 µ membrane filter and aliquots were stored at -20°C. The synaptic vesicle proteins obtained from a single rat brain were ~20 ml with a concentration of 2.5 mg/ml which would be sufficient for millions of detection and binding experiments and were found to be stable for more than 6 months at -20°C. The animal experiments were approved by the Laboratory Ethical Committee

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To evaluate the functional state of the isolated protein (rBoNT/A), binding of recombinant C-terminal heavy chain fusion protein (rBoNT/A-LC-Hcc) was determined by ELISA as reported in our earlier study [6]. The receptors present in the synaptic vesicle protein preparation were used. In our observation, protein substrate is very robust, stable upon long term storage and can be used to detect endopeptidase activity of botulinum neurotoxin type A (upto 12 months, data not shown). The receptor present in the synaptic vesicle protein preparation was also found to bind with C-terminal binding domain of BoNT/A after long term storage.

**Conclusion**

The work presented here reasonably describes the technical validity of this approach to BoNT detection. The assay is based on synaptic vesicle proteins isolated from rat brain that can be performed in the laboratory with limited infrastructure. To date, the only widely accepted test for the identification of BoNTs in both clinical specimens and food is the mouse bioassay. The advantage of the mouse bioassay is that it is very sensitive and detects biological active toxin. Although mice often exhibit signs of botulism within a few hours after a BoNT sample injection, but it requires 4 days to confirm a negative result, and it can take several days to determine the toxinoype. However, the primary drawback remains as it requires many mice per specimen with a lethal end result. An in vitro alternative to animal use is preferred. None of the in vitro assays developed till date that meet the four requirements which include (1) measuring active toxin, with (2) sensitivity, (3) specificity, and (4) practicality. Several forms of immunoassays for BoNT detection have been developed but they are not as sensitive as the mouse bioassay. Furthermore, they fail to measure biological activity of the toxins that limits their universal application for BoNT analysis. The immunological in vitro methods are unable to discriminate between an active and inactive form of the toxin where as endopeptidase based assay detects biological active neurotoxin. In this sense, endopeptidase assays are closer to the mouse lethality bioassay than immunoassays as detection of the active neurotoxin is arguably more relevant because the in vitro assays developed till date that meet the four requirements which include (1) measuring active toxin, with (2) sensitivity, (3) speed, and (4) specificity. Several forms of immunoassays for BoNT detection have been developed but they are not as sensitive as the mouse bioassay. Furthermore, they fail to measure biological activity of the toxins that limits their universal application for BoNT analysis. The immunological in vitro methods are unable to discriminate between an active and inactive form of the toxin where as endopeptidase based assay detects biological active neurotoxin. In this sense, endopeptidase assays are closer to the mouse lethality bioassay than immunoassays as detection of the active neurotoxin is arguably more relevant because only the active form is associated morbidity and mortality. Using present approach endoproteinasme activities of all seven serotypes of BoNT/A can be detected within hrs. The synaptic vesicle proteins can be used to detect biologically active serotypes of botulinum neurotoxins using the substrate specific commercial antibodies with scope for
miniaturization. The reagents required to conduct experiments are stable for more than 6 months and cost effective as thousands of tests can be performed using synaptic vesicle proteins isolated from a single rat brain. The limit of detection and specificity can also be increased by several folds exploiting receptor binding activity to concentrate/capture these agents.

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References