Sensitive, *In Vitro,* Bifunctional, Potency Assay For Botulinum Neurotoxin Type A

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Abstract

Botulinum toxin progresses through a three step process leading to the interruption of synaptic transmission. The first step is binding of toxin to neuronal cells and incorporation of the toxin into an endosome; the second step is translocation of the enzymatic light chain out of the endosome and into the cytosol of the neuron; and the final step is cleavage of synaptosomal proteins to prevent vesicle docking and neurotransmitter release.

In this assay, the luminal domain loop of SV2c has been attached to magnetic beads and used as a capture reagent for botulinum neurotoxin type A. After capture, the activity of the toxin is assessed using one of our FRET substrates, SNAPtide®. Analysis of the cleaved peptide is via HPLC using a fluorescence detector. Optimization of reaction conditions and detection limits will be described.

This assay monitors two of the three steps of toxin activity, binding and cleavage. As such, this assay has the potential to be part of an alternative method to replace the mouse bioassay.

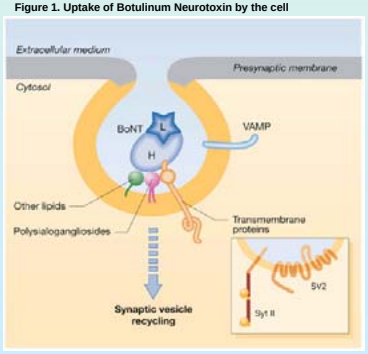
Introduction

A multitude of assays have been developed to detect botulinum toxin based solely on antigenic properties and/or enzymatic activity. As shown in **Figure 1**, toxin uptake into the cell is mediated by both protein and non protein receptors. The identification of the protein receptor for BTA has been shown to be SV2c[1,2]. With this information, new detection methods can now be attempted that combine multiple steps of the disease process of botulinum toxin. Capture of BTA with the SV2c receptor binding domain demonstrates the specific binding which is then detected using a FRET peptide. Cleavage of this specific BTA substrate indicates the specific endopeptidase activity of BTA. By combining these activities this assay mimics two of the three *in vivo* functions of the toxin.

The specific binding domain of SV2c for BTA has been shown to be the luminal domain loop between transmembrane domains 7 and 8.[1,2] A diagram of the structure of SV2c is shown in **Figure 2**. We have purified the SV2c luminal binding domain as a GST fusion and utilized it in our capture assays. We have immobilized the GST-SV2c to magnetic beads coated with glutathione (MagneGST). See **Figure 4** for an illustration of this strategy.

Our goal for this research is to establish an assay which demonstrates binding and catalytic activity and that can compete with the mouse bioassay.

Figure 1. Uptake of Botulinum Neurotoxin by the cell



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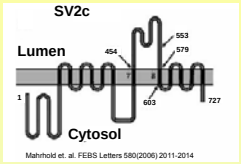

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Figure 2: SV2c structure
The binding domain for Botulinum Neurotoxin Type A has been shown to be in the luminal loop, amino acids 454-579.

Materials

SNAPtide® substrate # 520 contains the FRET pair o-aminobenzoic acid/2,4 dinitrophenyl (o-Abz/Dnp) and SNAPtide® substrate Mca/Dnp contains (7-methoxy-coumarin-4-yl)acetyl/2,4 dinitrophenyl as its FRET pair. Botulinum Neurotoxin Type A (Product #130A) and the SNAPtide® substrates are products of List Biological Laboratories. Plasmid encoding the binding domain of human SV2c was provided by Dr. Andreas Rummel from the Institut für Toxikologie, Hanover, Germany. MagneGST beads (V8611) were obtained from Promega Corporation, Madison, WI. The HPLC is a Varian ProStar with an attached Hitachi fluorescence detector. The column is a MicroSorb-MV C-4, 250 x 4.6.

Methods

MagneGST beads (200µl) were mixed with 125µg of GST-SV2c and incubated for 1 hour with mixing in the presence of 1% BSA. The SV2c coated MagneGST beads were then washed and reconstituted in 1.0 ml of 50mM HEPES, pH 8 buffer. Fifty microliters of the beads were used in each reaction. Various concentrations of Botulinum Neurotoxin Type A were added to each tube. Toxin binding was 1-4 hours at room temperature with mixing. Unbound toxin was washed away and 400µl of SNAPtide® reaction buffer (50 mM HEPES, pH 8, 5 mM DTT, 1 mM $ZnCl_2$, 0.1% BSA) and 20 µM SNAPtide® (o-Abz/Dnp) #520 or SNAPtide ® (Mca/Dnp) was added to each tube. The reaction tubes were mixed at room temperature, unless otherwise noted. They were then placed on a magnetic stand and the reaction mixture was removed from the tubes. To quench the reaction and prepare for HPLC analysis 50 µl of 0.1% TFA (Trifluoroacetic acid) was added to each reaction sample. The HPLC method run is a linear gradient from 0 – 100% Acetonitrile containing 0.1% TFA in 10 minutes at 1.5 ml/min. The excitation and emission wavelengths were set to 321 nm and 418 nm for the o-Abz/Dnp substrate and 325nm/398nm for the Mca/Dnp substrate.

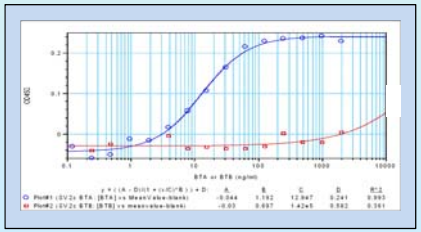

Figure 3: ELISA assay showing specific binding of BTA to SV2c
GST-SV2c (2 µg) was coated overnight at 4ºC in 96 well microtiter plates. Botulinum Neurotoxin Type A and B were applied to the wells and incubated for 1 hr at 37ºC. Toxin was detected using serotype specific antibodies: horse anti-BTA / anti-horse HRP and rabbit anti-BTB / anti-rabbit HRP. The assay was developed using One Step Turbo ELISA-TMB from Pierce.

Figure 4: Design of the capture assay using GST-SV2.

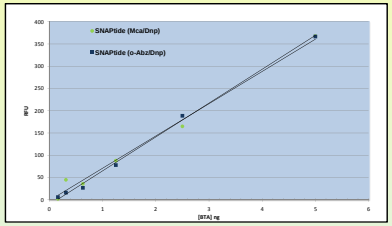

Figure 5: MagneGST-SV2c capture assay
Botulinum toxin was titrated to tubes containing GST-SV2c labeled beads. These assays were run with one hour toxin binding at room temperature and a six hour room temperature cleavage reaction with either SNAPtide® (Mca/Dnp) or SNAPtide® (o-Abz/Dnp). The assay samples were placed in a black 96 well plate and read using a SPECTRAmax GEMINI XS fluorescence microplate reader (Molecular Devices). Both substrates show a linear response to the toxin concentrations. Three hundred picograms of BTA can be detected with either peptide in the plate format.

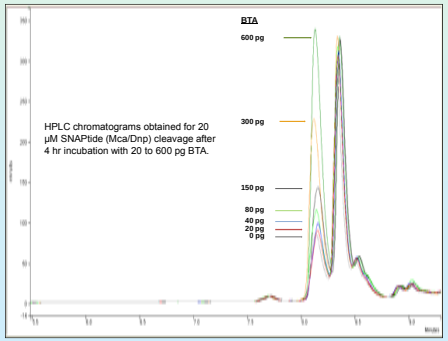

Figure 6: MagneGST-SV2c capture assay with HPLC analysis
Botulinum Toxin was titirated to tubes containing GST-SV2c labeled beads. These assays were run with one hour toxin binding at room temperature and a four hour room temperature cleavage reaction with SNAPtide® (Mca/Dnp). The assay samples were stopped with 0.1% TFA and placed in glass vials. The samples were read on the HPLC using a fluorimeter with excitation and emission wavelengths set to 325nm / 398nm. The substrate shows a linear response to the toxin concentrations. Twenty picograms of BTA can be detected above the background.

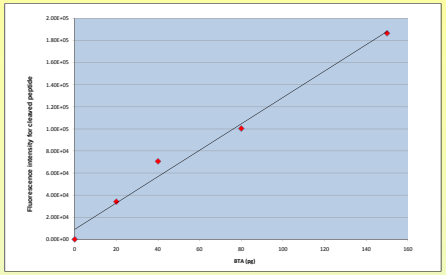

Figure 7: HPLC peak intensity for cleaved SNAPtide® as a function of BTA.
The data shown here is another example of the linear response seen with the SV2c capture assay using HPLC analysis. The background intensity has been subtracted.

Figure 8: Comparison of mouse bioassay to SV2c capture assay.

Characteristic	Mouse Bioassay	Alternative Potency Assay
Sensitivity	~ 5 pg	~ 20 pg
Animal use	yes	no
Cost	expensive	inexpensive
Duration	3 days minimum	2 days maximum
Specificity	Separate assay using serotype specific antibodies, adds to minimum days	Using specific binding receptor, SV2c, and specific substrate, SNAPtide®
Easy to perform	Needs qualified lab and personnel	Simple assay procedure
Availability of reagents	Limited number of labs available to perform	Reagents will be commercially available
Variability	~ 30%	Not measured

Future Directions

Improve sensitivity

1. Reduce background fluorescence
2. Test different fluorophores and higher concentrations of substrates
3. Optimize HPLC methods, columns, and buffers

Improve ease of use

Adapt bead assay to 96-well plate platform

Assay Qualification

Determine specificity, accuracy, precision, linearity, range, robustness, detection limit

Establish correlation with the Mouse Bioassay

References:

1) Mahrold et al. FEBS Letters. 2006 April 3, 580(8):2011-4.
2) Dong et al. Science. 2006 April 28; 312(5773):592-6