NEW SV2c CONSTRUCT FOR USE IN BINDING AND DETECTING BOUTULINUM NEUROTOXIN TYPE A

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ABSTRACT

Purpose of Study: The purpose of this study was to express and purify the luminal domain loop of human SV2c containing dual His_6 tags, one each on the C- and N-terminals. This region, residues 454-579, has been identified as the protein receptor specific for botulinum neurotoxin type A (BoNT/A) binding. This construct, when attached to, for example, nickel coated beads, has the potential to form a loop similar to the native structure and increase the binding affinity of BoNT/A for *in vitro* assays.

Methods Used: The His_6- SV2c- His_6 was expressed as a GST fusion protein to increase solubility during isolation and purification. Purified His_6- SV2c- His_6 was attached to magnetic nickel coated beads using various ratios of beads to protein. Each set of coated beads was exposed to varying amounts of BoNT/A in buffer, washed, and incubated with our FRET peptide SNAPtide®, Prod #520. Generation of cleaved peptide was monitored using reverse phase HPLC with fluorescence detection.

Summary of Results: The His_6- SV2c- His_6 was purified in a soluble form and linked to magnetic nickel coated beads. Various buffer conditions and binding conditions were tested to optimize the binding and limit the non specific binding of toxin to the beads. We are able to detect cleavage of SNAPtide® after exposure to as little as 5pg of BoNT/A.

Conclusions: Our study shows the ability to purify a soluble form of the SV2c BoNT/A binding domain and its utility in the capture and detection of BoNT/A. This is a key step in establishing an *in vitro* potency assay. This assay can also be used to screen for inhibitors that interfere in the binding of BoNT/A to the SV2c protein receptor.

INTRODUCTION

Multiple assays have been developed to detect botulinum toxin based solely on antigenic properties and/or enzymatic activity. Now with the identification of SV2c as the protein receptor for BoNT/A[1,2,3] new functional assay methods can be attempted that combine multiple steps of the disease process of botulinum toxin. We can utilize the SV2c receptor domain to obtain specific binding of BoNT/A followed by exposure to a FRET peptide to detect this binding through specific endopeptidase activity.

The specific binding domain of SV2c for BoNT/A has been shown to be the luminal domain loop between transmembrane domains 7 and 8.[1,2] We have purified the luminal binding domain as a GST fusion with His_6 tags on both the carboxy and amino termini of the domain and utilized it in capture assays. See Figure 1 for an illustration of the SV2c construct and the overall assay method.

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

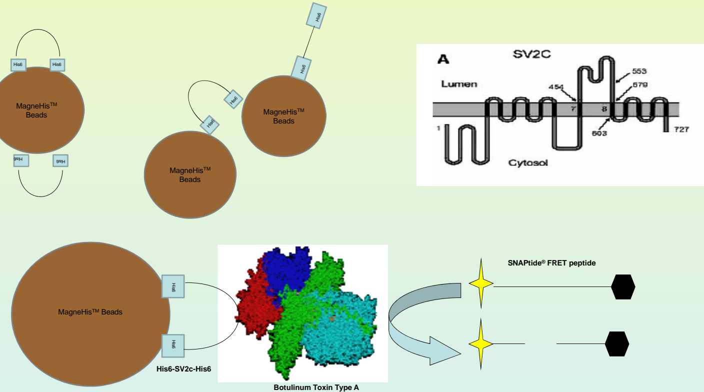


Figure 1: Design of the capture assay using His_6-SV2-His_6.

MATERIALS/METHODS

Reagents: SNAPtide® substrate (Product # 520) and Botulinum Neurotoxin Type A (Product #130A) are products of List Biological Laboratories. MagneHis™ Ni-Particles #V8560 are supplied by Promega. The 2xHis_6-SV2c protein is expressed as a GST fusion using plasmid pGEX-6P-1 (GE Healthcare). The GST tag is removed using PreScission Protease (GE Healthcare).

Protein Purification: GST::2xHis_6-SV2c is expressed in *E. coli* and purified under native conditions. The protein is purified using a GSTrap column (GE Healthcare) attached to an AKTA Explorer FPLC (GE Healthcare). After cleavage of the GST tag a HisTrap Column (GE Healthcare) followed by an additional GSTrap (GE Healthcare) column is run to purifiy the 2xHis_6-SV2c. See Figure 2 for protein purification.

Sample preparation: Five micrograms of 2xHis_6-SV2c is mixed with 10 µl of MagneHis™ Ni-Particles in 1 ml of 100mM HEPES, pH 7.4 + 10 mM imidazole. The binding is done at room temperature for 30 minutes according to the manufacturers' recommendation. The beads are placed on a magnetic stand and washed. The BoNT/A is prepared as a 100 ng/µl solution in 20 mM HEPES, pH 8.0 containing 0.2% Tween-20. Appropriate dilutions are made in 20 mM HEPES, pH 8.0. Dilutions of BoNT/A are added to SV2c coated beads and incubated at room temperature for 1 hour with rotation or shaking. After toxin binding, the beads are placed on a magnetic stand and washed. Reaction buffer is added to beads and incubated at room temperature overnight. Stock solutions (5 mM) of the SNAPtide® (Product #520) FRET peptide are made in dimethyl sulfoxide (DMSO). The SNAPtide® is diluted to 20 µM in 20 mM HEPES, pH 8.0 containing 1.0 mM $ZnCl_2$, 5 mM dithiothreitol (DTT), and 0.1 % Tween-20 (reaction buffer).

HPLC: For BoNT/A detection studies, the HPLC was performed using a Zorbax Eclipse Plus C18 reverse phase column, 4.6 x 150 mm (Agilent Technologies, Santa Clara, CA) attached to a Varian ProStar HPLC system (Agilent Technologies, Santa Clara, CA). Solvent A was 0.1% TFA and solvent B was 100% acetonitrile containing 0.1% TFA. The column gradient was as follows: 12% B for 5 min, 12-20% B in 5 min, 20-100% B in 5 min, 100% B for 5 min, and 9 min equilibration with 12% B. The column effluent was monitored using a Hitachi fluorescence detector with excitation set to 320 nm and emission at 418 nm to detect the o-Abz fluorophore on the N-terminal cleaved fragment of SNAPtide® 520. The injection volume was 50 µl.

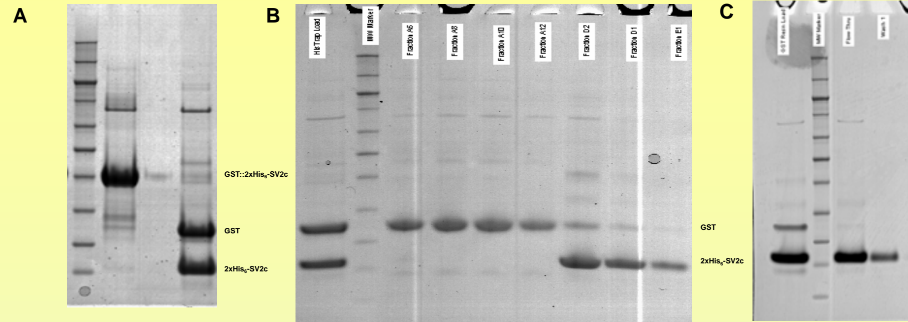
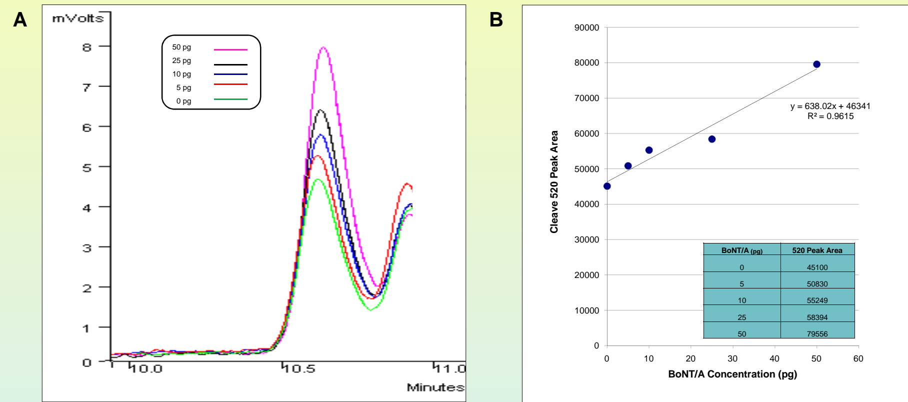


Figure 2. 2xHis_6-SV2c Purification: Panel A shows the initial purification GST::2xHis_6-SV2c on a GSTrap column and subsequent digestion of the GST tag. Panel B shows the separation of the GST tag and 2xHis_6-SV2c using a HisTrap column. Panel C shows a repeat of the GSTrap column to remove contaminating GST protein and yield highly pure 2xHis_6-SV2c protein. Invitrogen NuPAGE 4-12% or 12% Bis-Tris gels with MES running buffer were used.



BoNT/A (pg)	520 Peak Area
0	45100
5	50830
10	55249
25	58394
50	79556

Figure 3. SV2c:BoNT/A bead assay data: The figures above show a representative data set from the SV2c:BoNT/A capture assay. A series of BoNT/A concentrations were captured using 2xHis_6-SV2c coated magnetic beads in buffer and incubated with 20 µM SNAPtide® substrate at room temperature. These assays were run with one hour toxin binding at room temperature and overnight room temperature cleavage reaction with SNAPtide®. The assay samples were stopped with 0.1% TFA and placed in glass vials. The samples were analyzed on the HPLC using a fluorimeter with excitation and emission wavelengths set to 320nm / 418nm. **Figure 3A** shows the overlay of the six chromatograms of the different toxin concentrations tested. The amount of cleaved SNAPtide® substrate, as reflected in the area of the HPLC peak at 10.6 min, is shown as a function of BoNT/A concentration (pg) after overnight digestion in **Figure 3B**. The lowest amount of BoNT/A that can be detected is 5 pg in buffer.

FUTURE DIRECTIONS

A. Studies will be performed to improve the assay by lowering the non-specific binding of toxin and background. Preliminary data suggests a toxin binding buffer of 20 mM Tris, 150mM NaCl, 0.5% Triton x-100, pH 7.4 lowers the non-specific binding significantly. We will continue to explore whether this buffer system will allow the appropriate sensitivity that is needed for this assay.

B. Other potentially useful beads, cobalt or copper coated, from other vendors will be tested. Anti-His_6 antibody coated beads will be examined to see if they would work in the assay.

C. Other FRET peptides including SNAPtide® fIP6 (DABCYL/5-IAF) will be tested.

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.
3. European Patent (EP1940874) Botulinum Neurotoxin A Protein Receptor and Uses Thereof.