EVALUATION OF THREE VAMPTIDE® SUBSTRATES FOR BOTULINUM NEUROTOXIN TYPE B

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ABSTRACT

Background: Peptide substrates which are specific for Botulinum neurotoxin type B (BoNT/B) have been designed by taking advantage of the fact that each serotype of the neurotoxin selectively cleaves one of the SNARE membrane fusion proteins at a unique site. Substrates for BoNT/B, VAMPtide®, are based on the native target SNARE protein, synaptobrevin-2 or VAMP2. Three VAMPtide® substrates with similar peptide sequences but different fluorophores quenched by an appropriate chromophore are compared. Data demonstrating the efficiency of cleavage of these peptides by BoNT/B holotoxin and light chain as well as studies of the minimum concentration of enzyme detectable by each peptide substrate, is presented.

Methods: VAMPtide® (o-Abz/Dnp), VAMPtide® (FITC/DABCYL), and VAMPtide® (PL 150, Pya/Nop) are product numbers 540, 541, and 542, respectively, from List Biological Laboratories, Inc. VAMPtide®(PL 150, Pya/Nop) is the original product of and is also marketed by Pharmaleads, Paris, France. For each BoNT/B substrate, the time-dependent increase in fluorescence intensity was monitored as a function of BoNT/B holotoxin or light chain concentration using the optimum buffer and temperature conditions established for each in prior experiments. The excitation and emission wavelengths were set to 321 nm and 418 nm, respectively, for the o-Abz-substrate, 490 nm and 523 nm using a cutoff filter of 495 nm for the FITC-substrate, and 340 nm and 400 nm for the PL 150, Pya/Nop substrate.

Results: Evaluation of all three peptides indicated that VAMPtide®(PL 150, Pya/Nop) was most efficiently cleaved by BoNT/B. This substrate was cleaved at least 20 times faster than the other two VAMPtide® substrates using approximately 10 times less enzyme.

Conclusions: Of the three BoNT/B substrates compared in this evaluation, VAMPtide® (PL 150, Pya/Nop), Prod #542, is the preferred peptide substrate for use in both detection and high throughput studies of BoNT/B.

INTRODUCTION

The botulinum neurotoxins are among the most potent toxins in nature. They are synthesized as single 150 kDa polypeptide chains which are subsequently cleaved to produce a 100 kDa heavy chain and a 50 kDa light chain linked by a disulfide bond. Four of the seven immunologically distinct serotypes, A, B, E, and F, cause botulism in humans. The 50 kDa light chain of each serotype is a zinc endoprotease that cleaves a single target protein which is essential for synaptic vesicle membrane fusion. This inhibits neurotransmitter release which leads to muscular paralysis.

These secreted toxins are regarded as major biological warfare threats. Due to their extreme potency and lethality, detection of these toxins requires a highly sensitive and reliable assay. Measurement of proteolytic activity provides for, 1) a potentially sensitive and direct means for detecting these potent toxins, and 2) a method for identifying potential toxin inhibitors using high throughput screening. Substantial signal amplification can be expected as a result of catalytic turnover of the substrate.

Botulinum neurotoxin,Type B cleaves the vesicle-associated membrane protein, synaptobrevin-II, also known as VAMP-2, exclusively between residues Gln^{76} and Phe^{77}. The minimum effective BoNT/B substrate is 35 amino acids in length consisting of residues 60-94 of VAMP II (Shone CC and Roberts AK, Eur. J. Biochem. 1994, **225**:263-270) as shown below. The blue letters indicate the BoNT/B cleavage site.

---L^{60}-S-E-L-D-D^{65}-R-A-D-A-L^{70}-Q-A-G-A-S^{75}-**Q↓F**-E-T-S^{80}-A-A-K-L-K^{85}-R-K-Y-W-W^{90}-K-N-L-K---

The three peptide substrates for botulinum toxin type B (BoNT/B), VAMPtide®, have been designed based on this native sequence. Each peptide is an intramolecularly quenched fluorogenic substrate and cleavage of the substrate by BoNT/B light chain or holotoxin releases the fluorophore from the quencher moiety and full fluorescence is restored. The increase in fluorescence intensity is directly proportional to the amount of enzyme present.

These three substrates each contain a fluorophore and corresponding quenching group on either side of the cleavage site.

1. **VAMPtide®, Product #540**, which contains ortho-amino benzoic acid (o-Abz)-lysine as the fluorophore and 2,4 dinitrophenyl (Dnp)-lysine as the quenching group at positions 79 and 75, respectively.

2. **VAMPtide®, Product #541**, which contains fluorescein-thiocarbamoyl (FITC), as the fluorophore and 4[[4'(dimethyl-amino) phenyl]azo]-benzoic acid (DABCYL) as the quenching group at positions 81 and 70, respectively.

3. **VAMPtide®, Product #542**, which contains (L)-pyrenylalanine (Pya), as the fluorophore and p-nitrophenylalanine (Nop) as the quenching group at positions 74 and 77, respectively. VAMPtide®, Product #542 is supplied by and is also marketed as PL 150 by Pharmaleads, Paris, France. It is described in the publication by Anne, C, *et al.*, Anal. Biochem. 2001, **291**:253-261.

MATERIALS and METHODS

VAMPtide® substrates (Products #540, #541 and #542), BoNT/B light chain (LC) (Product #620A), and BoNT/B holotoxin (Prod #136A) are products of List Biological Laboratories, Inc.

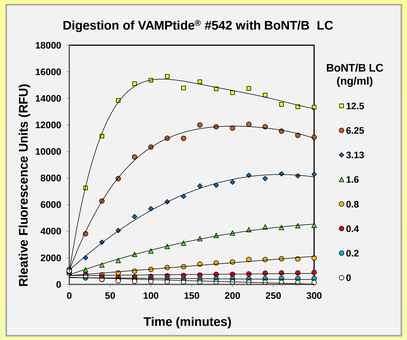
Fluorimetric Assay: Continuous assays were performed on a SPECTRAmax GEMINI XS fluorescence microplate reader (Molecular Devices, Sunnyvale, CA) using Greiner FLUO-TRAC black flat-bottomed half well plates (E&K Scientific, Campbell, CA). Stock solutions of VAMPtide®, Product #540 and #541 were made at 2.5 mM in dimethyl sulfoxide (DMSO). VAMPtide®, Product #542 was reconstituted in 20% dimethylformamide (DMF) to 1.0 mM. Final dilutions were made in the appropriate reaction buffer described in the figure legends. Plates were equilibrated at 37°C for 5 min prior to initiation of the reaction by addition of 10 µM substrate. For all experiments the time-dependent increase in fluorescence intensity was monitored at 37°C for 5 hrs. Plates were moved to ambient room temperature and read again after 24 hrs. The excitation/emission wavelengths were set to 320/418, 490/523 with a cutoff at 495 nm, and 343/397 nm for Product #540, 541 and 542, respectively.

Sensitivity and LOD: Serial dilutions of BoNT/B holotoxin concentrations were prepared in reaction buffer. Seven replicates of each concentration were performed. Following equilibration, the cleavage reaction was initiated with addition of 10 µM VAMPtide®. Relative fluorescence units (RFU) obtained after 5 hours at 37°C and an additional 19 hours at ambient room temperature are plotted against BoNT/B concentration **(Figure 3)**.

The limit of detection (LOD) was measured for VAMPtide® 542 digestion with BoNT/B holotoxin. LOD is the minimum concentration of BoNT/B holotoxin that can be measured with 99% confidence that the concentration present is greater than zero under the conditions using this fluorogenic assay. A calibration curve was generated by measuring the increase in RFU as a function of enzyme concentration after 5 hours at 37°C and an additional 19 hours at ambient room temperature. Seven replicates of a concentration estimated to be around the expected detection limit were performed. Each analysis was calculated as ng/ml using the calibration curve. The detection limit was calculated as 3.143 (Student's t-Distribution with 99% confidence and 6 degrees of freedom) times the standard deviation of the 7 replicates. The results are given in **Table 1**.

RESULTS

A. Progress curves



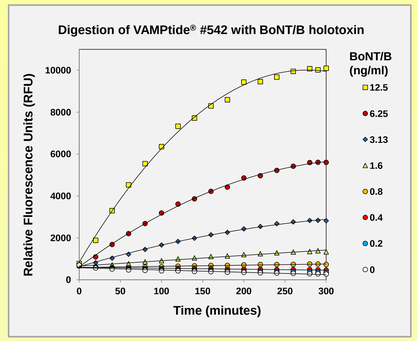
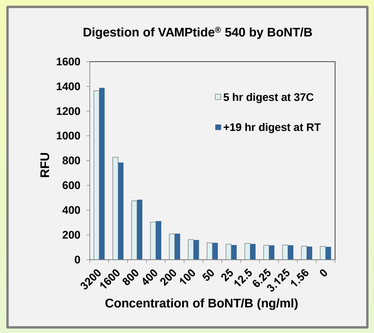


Figure 1: Representative plots of relative fluorescence intensity (RFU) versus time (minutes) observed for cleavage of 10 µM VAMPtide® #542 with a series of BoNT/B LC (left) and BoNT/B holotoxin (right) concentrations at 37° C in 20 mM HEPES, pH 7.4 with 1.5 mM Tris(2-carboxyethyl)-phosphine (TCEP) and 2 µM $ZnSO_4$. As indicated in the graph, the initial rate of VAMPtide® #542 digestion by 12.5 ng/ml BoNT/B LC is higher than that observed for the same amount of holotoxin. However, based on the 3:1 molar ratio of light chain to holotoxin, VAMPtide® #542 is actually cleaved more efficiently by the holotoxin.

B. Comparison of VAMPtide® #540 and #542 digestion with BoNT/B



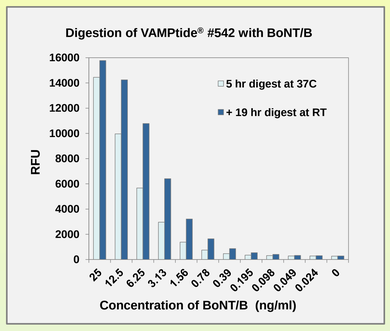
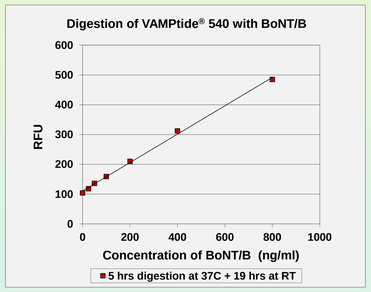


Figure 2: Cleavage of 10 µM VAMPtide® #540 (left) and #542 (right) as observed in a change in relative fluorescence intensity (RFU) as a function of BoNT/B holotoxin concentration (ng/ml). Assays are performed at 37° C for 5 hours followed by an overnight (19 hr) incubation at ambient room temperature. Assays were conducted in 20 mM HEPES, pH 7.4 with 1.5 mM TCEP and 2 µM $ZnSO_4$. Cleavage of BoNT/B by VAMPtide® #540 is not observed for BoNT/B concentrations less than 50 ng/ml while significant cleavage of VAMPtide® #542 is observed. Also while the sensitivity increases for VAMPtide® #542 after the overnight digestion, there is no change for VAMPtide® #540.

C. Sensitivity and LOD



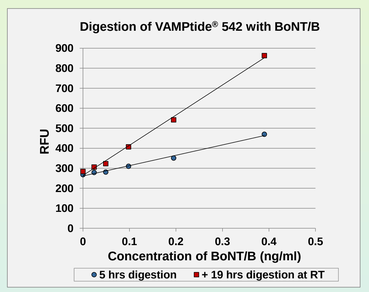


Figure 3: A plot of RFU versus BoNT/B holotoxin concentration for the digestion of 10 µM VAMPtide® #540 (left) and #542 (right). For both substrates the cleavage reaction is linearly proportional to the BoNT/B concentration in the range shown. The LOD for Prod #542 was determined as described in Materials and Methods and is given below.

Table 1. LOD for VAMPtide® Prod #542 digestion with BoNT/B holotoxin.

Digestion time (hr)	5	24*
LOD (pg/ml)	140	83

***5 hours at 37°C + 19 hrs at ambient room temperature.**

D. Rate of cleavage of VAMPtide® #540, 541, 542 with BoNT/B LC

Table 2. Cleavage rates

VAMPtide® #	Concentration of BoNT/B LC	VAMPtide® Concentration (µM)	Initial rate (RFU/sec)
540	10 nM	10	0.653
		5	0.392
		2.5	0.218
541	10 nM	8	0.249
		5	0.196
		2.5	0.141
542	1.25 nM	10	19.5
		5	9.7
		2.5	2.8

The assay buffer used for VAMPtide® #540 and #541 is 50 mM HEPES, pH 6.3, 0.05% TWEEN 20. For VAMPtide® #542 the buffer used is 20 mM HEPES, pH 7.4, 0.1 mM DTT. The digestions were performed at 37°C.

Conclusions

1. VAMPtide® #542, is cleaved more efficiently than VAMPtide® #540 or #541 by both BoNT/B LC and holotoxin. Thus, this substrate allows for a significant increase in the sensitivity. As low as 140 pg/ml of holotoxin can be detected after 5 hrs digest. After an additional incubation for 19 hrs at ambient room temperature, 80 pg/ml are detected. The half well plates contain 100 µl of reaction mixture so the actual amount of toxin detected is 14 and 8 pg after 5 and 24 hrs, respectively.

2. As shown in **Table 2**, VAMPtide® #542 is also ideally suited for high throughput screening for BoNT/B inhibitors since the rate of cleavage is significantly higher than the other VAMPtide® substrates using 8-fold less toxin. However, one disadvantage that may be associated with the VAMPtide® #542 and #540 substrates is that the fluorophores absorb in the range of aromatic compounds that are potential inhibitors, thus eventually complicating the interpretation of the data. VAMPtide® #541 which contains FITC, a fluorophore excited at a longer wavelength, may be preferred for some inhibitor screenings.