FRET Peptide Substrates for Botulinum Neurotoxins Types A, B, C, and E

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Abstract

The botulinum neurotoxins are among the most toxic substances known to man. Four of the seven immunologically distinct serotypes, A, B, E, and F, cause botulism in humans. A zinc endoprotease on the 50 kDa light chain of the 150 kDa holotoxin, cleaves a single target protein essential for synaptic vesicle membrane fusion. This inhibits neurotransmitter release and leads to muscular paralysis. Measurement of the proteolytic activity provides a potentially sensitive and direct means for detecting these potent toxins. Also, the neurotoxin light chains present an ideal target for the development of potential therapeutic inhibitors. The classic approach for monitoring enzymatic activity utilizes short peptides containing a chromophore or fluorophore moiety at the site of cleavage. However, for all the botulinum serotypes, efficient cleavage requires larger peptide substrates. Also, hydrolysis occurs only for peptides that span both sides of the cleavage site. An alternative approach is to use fluorescence resonance energy transfer (FRET) peptides. These fluorogenic peptides contain a fluorescent group at one end and a suitable chromogenic acceptor group at the other. This allows for the inclusion of amino acids on either side of the enzymatically cleaved bond. The fluorescence is quenched initially by intramolecular energy transfer between the donor/acceptor pair. Upon cleavage, the fluorescence is recovered.

Peptide substrates specific for botulinum toxin types A, B, C, and E have been designed taking advantage of the fact that each serotype of the neurotoxin selectively cleaves one of the SNARE membrane fusion proteins at an unique site. The substrates for type A, SNAPtide®, and type E, SNAP Etide®, are based on the native synaptosomal substrate for these toxins, SNAP-25. The substrates for type B, VAMPtide®, and type C, SYNTAXtide™ are based on the native target SNARE proteins, VAMP and syntaxin, respectively. The peptides contain FRET pairs in varied positions in the sequence. Data demonstrating the efficiency of cleavage of these peptides by their respective neurotoxin light chains as well as studies of the minimum concentration of enzyme detectable by each peptide substrate, is presented.

Introduction

The following table lists the five botulinum toxin serotypes for which a FRET peptide has been designed, its corresponding native substrate and the site where cleavage occurs. The FRET substrates designed for each serotype are included.

Toxin	Substrate Protein	Cleavage Site	FRET Substrate
BoNT/A	SNAP-25	Gln^{197}-Arg^{198}	SNAPtide® 520 (o-Abz/Dnp) SNAPtide® 521 (FITC/DABCYL) SNAPtide® 522 (Mca/Dnp)
BoNT/B	VAMP	Gln^{76}-Phe^{77}	VAMPtide® 540 (o-Abz/Dnp)
BoNT/C	Syntaxin SNAP-25	Lys^{253}-Ala^{254} Arg^{198}-Ala^{199}	SYNTAXtide™ 560(o-Abz/Dnp)
BoNT/E	SNAP-25	Arg^{180}-Ile^{181}	SNAP Etide® 550 (o-Abz/Dnp)

Materials and Methods

SNAP Etide® 550, VAMPtide® 540, SNYTAXtide™ 560, and SNAPtide® substrates 520, 521, and 522, and the Botulinum neurotoxin types A, B, C, and E light chains, recombinant (Product #610A, 620A, 625A, and 635A) are products of List Biological Laboratories, Inc.

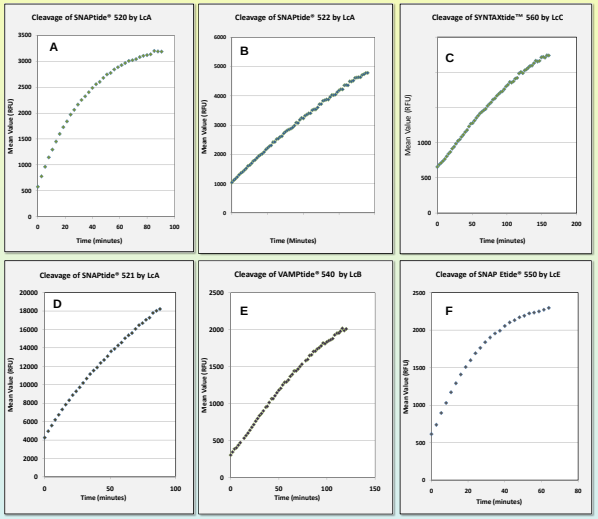
Fluorimetric assays: Continuous assays were performed on a SPECTRAmax GEMINI XS fluorescence microplate reader (Molecular Devices, Sunnyvale, CA) using black flat-bottomed non binding plates (E&K Scientific, Campbell, CA). Assays were performed starting with plates containing FRET substrates and incubated at 37ºC for 15 minutes to ensure equilibrium prior to addition of light chain. The reaction was initiated by the addition of light chain. For the sensitivity assays, plates containing serial dilutions of light chain were equilibrated for 15 min at 37ºC prior to addition of 10 µM substrate. For both assays, the time-dependent increase in fluorescence intensity was monitored at 37ºC unless otherwise stated. The excitation and emission wavelengths were set to 321 nm and 418 nm, respectively, for the o-Abz-substrates, 325 nm and 398 nm for the Mca-substrate and 490 nm and 523 nm using a cutoff filter of 495 nm for the FITC-substrate .

HPLC: HPLC was performed using a ProSwift, monolithic RP-1S reverse phase column, 4.6 x 50 mm (Dionex, Sunnyvale, CA) attached to a Varian HPLC system. Solvent A was 0.1%TFA and solvent B was 100% acetonitrile containing 0.1% TFA. A linear gradient from 1 to100% B in 12 minutes with a flow of 1 ml/min was used. The column effluent was monitored using an Hitachi fluorescence detector with excitation set to 488 nm and emission at 520 nm to detect the FITC fluorophore on the N-terminal cleaved fragment of SNAPtide® 521. The injection volume was 30 µl. Details of the digestion experiment are described in the figure legend.

Materials and Methods Continued:

Limit of Detection (LOD): The limit of detection is the minimum concentration of light chain that can be measured with 99% confidence that the concentration of light chain present is greater than zero under our conditions using this fluorogenic assay. Twelve replicates of a concentration estimated to be slightly greater than the expected detection limit were performed. The detection limit was calculated as 2.821 (Student's t-Distribution with 99% confidence and 12 degrees of freedom) times the standard deviation of the 12 replicates.

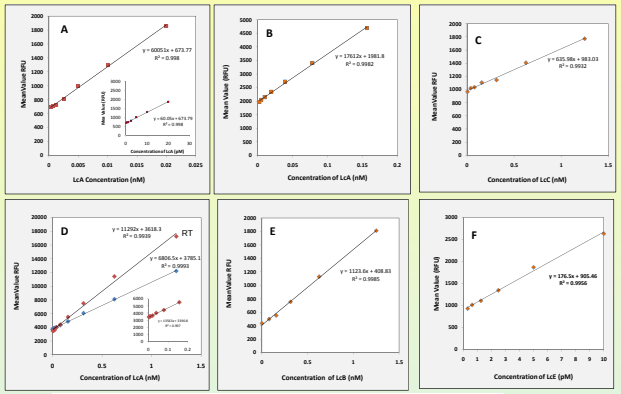
Figure 1: Progress curves for cleavage of FRET peptide substrates by light chains.



Plot	FRET Peptide (µM) (All stock solutions made in DMSO)	Dilution buffer/Hydrolysis buffer	Light Chain (nM)
A	5 µM SNAPtide® 520 (o-Abz/Dnp)	50 mM HEPES, pH 7.4, 0.05% TWEEN-20	2.5 nM LcA
B	5 µM SNAPtide® 522 (Mca/Dnp)	50 mM HEPES, pH 7.4, 0.1% TWEEN-20	10 nM LcA
C	5 µM SYNTAXtide™ 560 (o-Abz/Dnp)	50 mM HEPES, pH 6.8, 0.05% TWEEN-20	10 nM LcC
D	5 µM SNAPtide® 521 (FITC/DABCYL)	20 mM HEPES, pH 8.2, 0.1% TWEEN-20	2.5 nM LcA
E	5 µM VAMPtide® 540 (o-Abz/Dnp)	50 mM HEPES, pH 6.3, 0.0.05% TWEEN-20	10 nM LcB
F	5 µM SNAP Etide® 550 (o-Abz/Dnp)	50 mM HEPES, pH 7.8 , 0.1% TWEEN-20	5 nM LcE

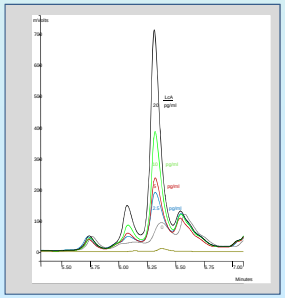
*For all botulinum toxin light chains, even low concentrations of $ZnCl_2$ are inhibitory. Also, DMSO inhibits cleavage, so final concentrations must be less that 2% of the total volume.

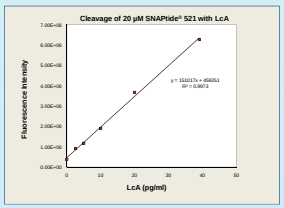
Figure 2: The fluorescence intensity as a function of light chain concentration for six FRET peptide substrates. A linear relationship between the RFU and the concentration of light chain is observed. For each titration 10 µM FRET substrate was used. Endpoint readings were obtained after 24 hours at 37°C. For SNAPtide® 521 an incubation at RT was also performed. These titrations were used to obtain the concentrations used for the LOD determinations.



Plot	FRET substrate	LOD with light chains
A	SNAPtide® 520 (o-Abz/Dnp)	0.5 ng/ml (10 pM LcA)
B	SNAPtide® 522 (Mca/Dnp)	0.625 ng/ml (20 pM LcA)
C	SYNTAXtide™ 560(o-Abz/Dnp)	2 ng/ml (40 pM LcC)
D	SNAPtide® 521 (FITC/DABCYL)	1.25 ng/ml (25 pM LcA)
E	VAMPtide® 540 (o-Abz/Dnp)	4 ng/ml (78 pM LcB)
F	SNAP Etide® 550 (o-Abz/Dnp)	0.008ng/ml (0.16 pM LcE)

Figure 3: HPLC analysis of the digestion of SNAPtide® 521 with LcA. Overnight digestion of 20 µM SNAPtide® 521 by 0 (gray), 2.5 (blue), 5.0 (red), 10 (green) and 20 (black) pg/ml LcA at room temperature.





As little as 2.5 pg/ml of LcA is detected in the HPLC analysis of the generation of the FITC N-terminal fragment from SNAPtide® 521 as a function of LcA concentration.