



# SNAP Etide™, a FRET Substrate for Botulinum Toxin Type E

Yuliya Primak, Todd Christian, Nancy Shine  
List Biological Laboratories, 540 Division St., Campbell, CA 95008

Poster presented at the 43rd Interagency Botulism Research Coordinating Committee (IBRCC) Meeting, Silver Springs, MD, November 14-17, 2006.

## Abstract

Botulinum neurotoxins have seven distinct serotypes, four of which (types A, B, E and F) cause botulism in humans. The neurotoxins are composed of a 100 kD heavy chain, and an enzymatically active zinc-dependent light chain (50 kD). The metalloprotease presents an ideal target for the development of potential therapeutic inhibitors of botulism. One of the most efficient methods for rapid high throughput screening of potential inhibitory compounds is based on the use of intramolecularly quenched fluorescent substrates. As previously reported, the fluorescence resonance energy transfer (FRET) peptides, SNAP Etide™ and VAMP Etide™, were devised as substrates for botulinum toxin type A and B, respectively.

Recently, a FRET substrate for botulinum neurotoxin type E, SNAP Etide™, has been designed and evaluated. This peptide, based on the eukaryotic target SNAP-25, contains an *o*-aminobenzoic acid fluorophore (*o*-Abz) and a 2,4-dinitrophenol (Dnp) acceptor chromophore. It is readily cleaved by botulinum neurotoxin type E, light chain (LcE). Tests of the activity of LcE with SNAP Etide™ as a function of pH, ZnCl<sub>2</sub>, and Tween-20 concentrations indicate that the optimum buffer for the cleavage of the substrate is 50 mM HEPES, pH 7.8, 0.1% Tween-20. As observed for both type A and type B light chains, the addition of ZnCl<sub>2</sub> is inhibitory to the FRET substrate cleavage reaction. The hydrolysis of SNAP Etide™ by LcE shows a linear response to the enzyme concentration. A total enzyme digest of increasing concentrations of SNAP Etide™ using trypsin, indicates a linear increase in fluorescence up to 40 μM substrate. At higher concentrations a significant inner filter effect is observed. A value for K<sub>m</sub> was estimated using *o*-Abz-Lys to correct for the inner filter effect. Substrate inhibition is observed for concentrations greater than 100 μM SNAP Etide™.

## Introduction

Botulinum neurotoxins are the most deadly bacterial toxins known in nature. Each neurotoxin is composed of a 50 kD enzymatically active zinc-dependent light chain. This metalloprotease cleaves specific SNARE proteins blocking the release of acetylcholine from the synaptic vesicles and causing potentially lethal paralysis. As such, the light chain presents an ideal target for direct inhibition of the toxin. Identification of potential inhibitors is dependent on a rapid and highly sensitive method for screening large chemical compound libraries. An efficient method for such "high throughput screening" is based on the use of fluorescence resonance energy transfer (FRET) substrates. FRET substrates are substrates containing a fluorescence donor and a quenching acceptor that are separated by an enzymatic cleavage bond. Intramolecular resonance works to suppress the intrinsic fluorescence of the donor by transferring the energy to a chromophore quenching group while the bond is intact. However, once the enzymatic cleavage bond is broken the full fluorescence of the fluorophore is observed. The increase in fluorescence is directly proportional to the amount of substrate cleaved and the enzymatic activity can be monitored continuously.

A new FRET substrate, SNAP Etide™ (Product #550), has been designed based on the synaptosomal SNAP 25 protein. SNAP Etide™ contains an *o*-aminobenzoic acid (*o*-Abz) fluorophore and 2,4-dinitrophenol (Dnp) quencher chromophore. Studies illustrate that the SNAP Etide™ FRET substrate is readily cleaved by botulinum neurotoxin light chain type E (LcE) *in vitro* and therefore is suitable for high throughput screening assays.

Optimum conditions for hydrolysis of SNAP Etide™ by LcE have been established. Initial rates of velocity of the SNAP Etide™ cleavage reaction were monitored while varying buffer pH and Tween-20 concentrations (Figures 1A and 1B). Because LcE is a zinc-dependent metalloprotease and some of the zinc may have been lost during the purification process, it is possible that the addition of zinc may increase light chain activity. For this reason the activity of LcE in buffer containing various zinc-chloride concentrations was also evaluated (Figure 1C).

Determination of kinetic parameters, such as K<sub>m</sub>, for FRET peptides is problematic. Analysis of initial rate for SNAP Etide™ cleavage by LcE versus substrate concentration indicates a K<sub>m</sub> value of 24 μM (Figure 3A). However a total trypsin digest of SNAP Etide™ indicates that the fluorescence response is not linear after 40 μM (Figure 3B). This is an indication of the inner filter effect, a phenomenon that occurs when quenching groups on uncleaved substrates or cleaved product molecules absorb some of the fluorescence emitted from the cleaved product. In order to accurately determine the Km the inner filter effect for SNAP Etide™ hydrolyzed by LcE, was corrected using the free fluorophore from SNAP Etide™, *o*-Abz-Lys (Figure 4).

## Materials

SNAP Etide™ substrate (Product #550) and botulinum neurotoxin type E light chain, recombinant (Product #635A), are both products of List Biological Laboratories, Inc. The fluorescently tagged amino acid, *o*-Abz-Lys, was synthesized separately for this project.

## Methods

**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 plates (E&K Scientific, Campbell, CA). Stock solutions of the FRET substrate were made in dimethyl sulfoxide (DMSO). Final dilutions were made in the appropriate buffer. Plates were equilibrated at 37°C for 15 min prior to initiation of the reaction. For all experiments the time-dependent increase in fluorescence intensity was monitored at 37°C. The excitation wavelength was set to 321 nm and emission to 418 nm.

**Buffer Optimization:** FRET assays were performed to test the activity of LcE with SNAP Etide™ as a function of pH, Tween-20 and ZnCl<sub>2</sub>. Three separate experiments were performed (Figure 1). The cleavage reaction was initiated with addition of 5 nM LcE to the wells containing 10 μM SNAP Etide™ in the appropriate buffer. Initial velocities of cleavage in RFU/sec were evaluated and compared for each assay in order to determine the optimum buffer conditions for the reaction.

**LcE Titration:** LcE titration experiment was performed in 50 mM HEPES, pH 7.8, 0.1% Tween-20, using 10 μM SNAP Etide™. LcE was prepared at 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.078, and 0.039 μM concentrations. Following equilibration, the cleavage reaction was initiated with addition of 10 μM SNAP Etide™. Initial velocities of cleavage were plotted against LcE concentration (Figure 2).

**Trypsin Digest:** Dilutions of SNAP Etide™ were prepared in 50 mM HEPES, pH 7.8, 0.1% Tween-20 to achieve 70, 60, 50, 40, 30, 25, 15, 7.5, 3.75, 1.88, and 0.94 μM concentrations. The reaction was initiated with addition of 10 nM trypsin into each well. End point readings were taken after 50 min. A second round of 10 nM trypsin was added to each well in order to achieve total enzyme digestion. The maximum fluorescence reached was graphed as RFU/5000 against SNAP Etide™ concentration (Figure 3A). An identical experiment was run using 2.5 nM LcE for digestion of SNAP Etide™. Initial velocities of cleavage were graphed in RFU/sec against substrate concentration (Figure 3B).

## Methods (continue)

**Inner Filter Effect Correction:** Dilutions of SNAP Etide™ were prepared in 50 mM HEPES, pH 7.8, 0.1% Tween-20 to achieve concentrations ranging from 250 μM to 2 μM. Fluorescence end point readings of SNAP Etide™ at each concentration were recorded. In order to determine the inner filter effect at each substrate concentration another set of end point fluorescence (RFU) readings were recorded after addition of 5.0 μM free *o*-Abz-Lys. Fluorescence intensity obtained for SNAP Etide™ was then subtracted from the fluorescence intensity obtained for SNAP Etide™ and *o*-Abz-Lys in order to obtain fluorescence for the free *o*-Abz-Lys peptide. The decrease in fluorescence of the *o*-Abz-Lys in the presence of SNAP Etide™ reflects the inner filter effect (Table 1). A correction factor is obtained for each SNAP Etide™ concentration:

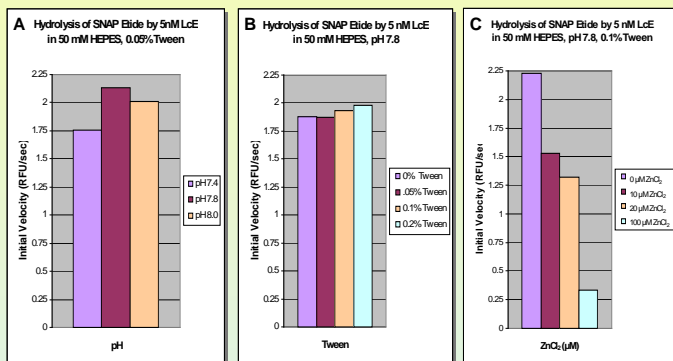
$$\text{correction factor} = \frac{\text{RFU}(\text{o-Abz-Lys}) \text{ at each SNAP Etide}^{\text{TM}} \text{ concentration}}{\text{RFU}(\text{o-Abz-Lys})}$$

Initial reaction rates were obtained for each substrate concentration after addition of 2.5 nM LcE. The rates were corrected as given in Table 1. The plots of initial velocity versus SNAP Etide™ concentration (Figure 4) indicates a decreasing rate of cleavage at concentrations of substrate greater than 100 μM. This is consistent with substrate inhibition. The kinetic data was analyzed using the substrate inhibition equation from Kaleidagraph software:

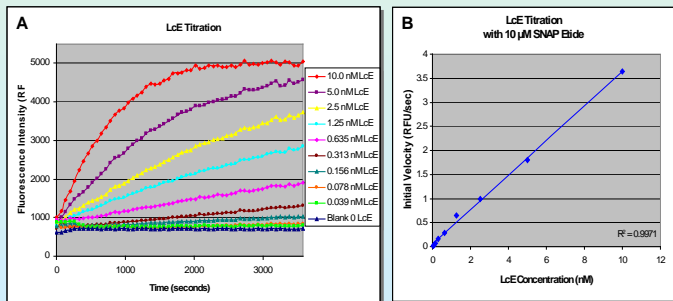
$$\frac{v}{b+(x(1+(x/c)))}$$

where a = V<sub>max</sub>, b = K<sub>m</sub>, and c = K<sub>i</sub> competitive inhibition constant

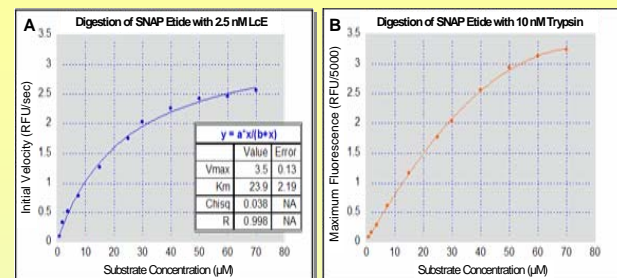
## Results



**Figure 1: Buffer Optimization.** SNAP Etide™ (Product #550) FRET assay conducted in 50 mM HEPES buffers at 37°C with 5 nM LcE (Product #635A) and 10 μM substrate, as a function of pH (A), and Tween (B) and ZnCl<sub>2</sub> (C) concentrations. Figure 1A demonstrates that the optimum pH for the cleavage reaction is 7.8. According to Figure 1B the optimum Tween-20 concentration is 0.2% however since the difference in LcE activity between 0.1% and 0.2% is very slight 0.1% Tween-20 was used in further studies in order to reduce the amount of bubbles obtained during mixing. The use of ZnCl<sub>2</sub> is clearly shown to be inhibitory to the cleavage reaction of SNAP Etide™ by LcE in Figure 1C.



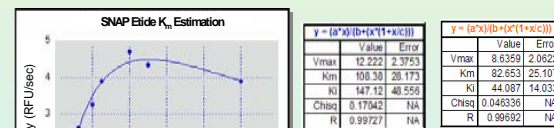
**Figure 2: LcE Titration with 10 μM SNAP Etide™.** FRET assay with SNAP Etide™ (Product #550) was performed in 50 mM HEPES, pH 7.8, 0.1% Tween-20, at 37°C (Figure 2A). A plot of initial velocities versus LcE concentration demonstrates that the SNAP Etide™ cleavage reaction is linearly proportional to the LcE concentration (Figure 2B).



**Figure 3: Digestion of SNAP Etide™ with LcE (A) and Trypsin (B).** FRET assays were performed in 50 mM HEPES, pH 7.8, 0.1% Tween-20. Figure 3A shows SNAP Etide™ digestion with 2.5 nM LcE. The K<sub>m</sub> value is estimated to be 23.9 μM using the Michaelis-Menten equation (Kaleidagraph software). Total enzyme digestion with trypsin, Figure 3B shows a non-linear response in fluorescence intensity decrease in fluorescence intensity with concentrations greater than 40 μM SNAP Etide™ due to inner filter effect. The decrease in initial velocity seen in Figure 3A contains some contribution from this effect. Estimation of K<sub>m</sub> must include correction for the inner filter effect.

SNAP Etide™ (μM)	RFU (SNAP Etide™)	RFU (o-Abz + SNAP Etide™)	RFU (o-Abz)	Correction Factor	RFU/sec	Corrected RFU/sec
0.0	146.54	3211.83	3065.29	1.000	0.018	0.018
2.0	274.46	3193.58	2919.12	0.952	0.234	0.246
3.9	361.42	3257.30	2895.88	0.945	0.435	0.460
7.8	599.67	3250.10	2690.43	0.878	0.796	0.907
15.6	935.41	3574.28	2638.87	0.861	1.364	1.584
25.0	1274.31	3683.18	2408.87	0.786	1.739	2.212
31.3	1474.83	3768.05	2293.22	0.748	1.967	2.630
50.0	1936.44	3982.41	2045.97	0.667	2.170	3.253
62.5	2186.54	4024.53	1837.99	0.600	2.336	3.893
100.0	2752.00	4199.58	1447.58	0.472	2.219	4.701
125.0	3008.91	4403.25	1394.34	0.455	1.977	4.345
250.0	3698.19	4618.11	919.92	0.300	1.167	3.880

**Table 1: Inner Filter Effect Correction at Different SNAP Etide™ Concentrations** (see discussion in the Methods section)



**Figure 4: SNAP Etide™ K<sub>m</sub> Estimation.** SNAP Etide™ (Product #550) hydrolysis rates at different substrate concentrations. Rates corrected for the inner filter effect as given in Table 1 are graphed in blue, uncorrected rates are graphed in orange. Estimated K<sub>m</sub> values are 108 and 82 μM for corrected versus uncorrected data.

## Conclusions

- SNAP Etide™ (Product 550) is readily cleaved by botulinum neurotoxin type E light chain (Product 635A).
- Optimum buffer for the digestion of SNAP Etide™ by LcE is 50 mM HEPES, pH 7.8, 0.1% Tween-20.
- Addition of ZnCl<sub>2</sub> is inhibitory to the cleavage reaction of SNAP Etide™ by LcE.
- Hydrolysis of SNAP Etide™ by LcE shows linear response to the LcE concentration.
- Total enzyme digest of SNAP Etide™ using trypsin shows a linear increase in fluorescence up to 40 μM substrate.
- Substrate inhibition kinetics are observed for SNAP Etide™ digestion with LcE for concentrations greater than 100 μM.
- K<sub>m</sub> value of 108 μM was estimated for SNAP Etide™ using *o*-Abz-Lys peptide to correct for the inner filter effect.

