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

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Abstract

Botulinum neurotoxins have seven distinct serotypes, four of which (types A, B, E and F) cause botulism is 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 if botulism. One of the most efficient methods for rapid high throughput screening of potential inhibitory compounds is based on the use of intramolecularly quinched fluorescent substrates. As previously reported, the fluorescence resonance energy transfer (FRET) peptides, SNAPtide® and VAMPtide™, were devised at List 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, $ZnCl_2$ 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 $ZnCl_2$ 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_m was estimated using o-Abz-Lys to correct for the inner filter effect. Substrate inhibition is observed for concentrations greater then 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 thoughput 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 chomophore 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 thoughput 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_m, for FRET peptides is problematic. Analysis of initial rate for SNAP Etide™ cleavage by LcE versus substrate concentration indicates a K_m 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 concentration of this substrate **(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

Fluorimentric 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 was 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 $ZnCl_2$. 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:

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

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 then 100 µM. This is consistent with substrate inhibition. The kinetic data was analyzed using the substrate inhibition equation from KaleidaGraph software:

$$\frac{ax}{b+(x(1+x/c))}, \text{ where } a = V_{max},\ b = K_m, \text{ and } c = K_i \text{ competitive inhibition constant}$$

Results

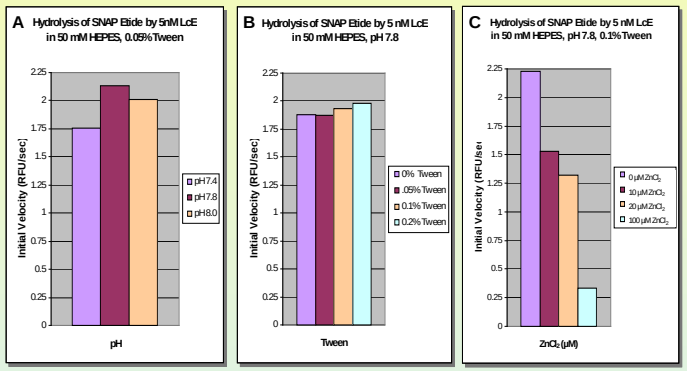


Figure 1: Buffer Optimization. SNAP Etide™ (Product #550) FRET assay conducted in 50 mM HEPES buffers at 37°C with 5 nM LcE (Product #635A) and 10 µM substrate, as a function of pH **(A)**, and Tween **(B)** and $ZnCl_2$ **(C)** concentrations. **Figure 1A** demonstrates that the optimum pH for the cleavage 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 $ZnCl_2$ is clearly shown to be inhibitory to the cleavage reaction of SNAP Etide™ by LcE in **Figure 1C**.

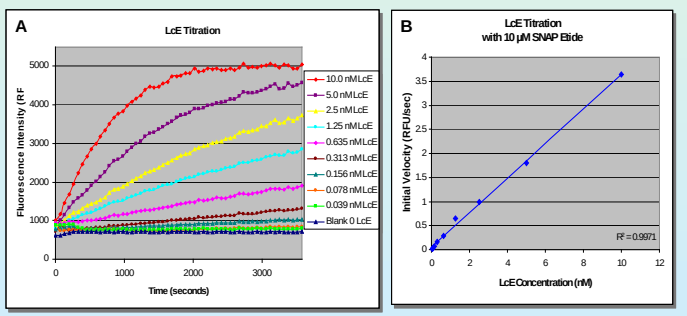


Figure 2: LcE Titration with 10 µM SNAP Etide™. FRET assay with SNAP Etide™ (Product #550) was performed in 50 mM HEPES, pH 7.8, 0.1% Tween-20, at 37°C **(Figure 2A)**. A plot of initial velocities versus LcE concentration demonstrates that the SNAP Etide™ cleavage reaction is linearly proportional to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

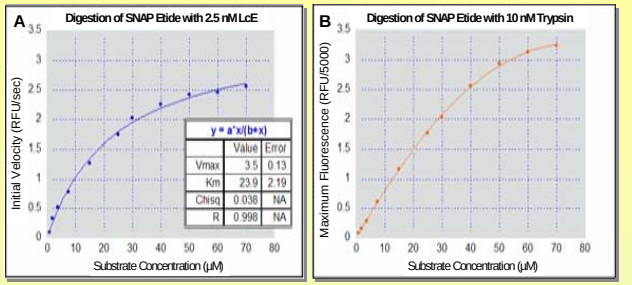
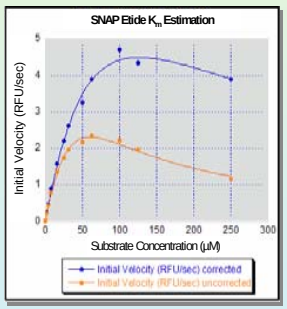


Figure 3: Digestion of SNAP Etide™ with LcE (A) and Trypsin (B). FRET assays were performed in 50 mM HEPES, pH 7.8, 0.1% Tween-20. **Figure 3A** shows SNAP Etide™ digestion with 2.5 nM LcE. The K_m value is estimated to be 23.9 µM using the Michaelis-Menten equation (KaleidaGraph software). Total enzyme digestion with trypsin, **Figure 3B** shows a non-linear response in fluorescence intensity decrease in fluorescence intensity with concentrations greater then 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_m must include correction for the inner filter effect.

SNAP Etide™ (µM)	RFU SNAP Etide™	RFU (o-Abz + SNAPEtide™)	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	559.67	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	3.890

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



y = (a*x)/(b+(x*(1+x/c)))

	Value	Error
Vmax	12.222	2.3751
Km	108.36	28.173
Ki	147.12	48.556
Chisq	0.17042	NA
R	0.99727	NA

y = (a*x)/(b+(x*(1+x/c)))

	Value	Error
Vmax	8.6359	2.0622
Km	82.653	25.107
Ki	44.087	14.033
Chisq	0.046336	NA
R	0.99692	NA

Figure 4: SNAP Etide™ K_m 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_m 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 $ZnCl_2$ 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 then 100 µM.
- K_m value of 108 µM was estimated for SNAP Etide™ using o-Abz-Lys peptide to correct for the inner filter effect.