Comparison of Activity of Botulinum Neurotoxin Type A Holotoxin and Light Chain Using SNAPtide® FRET Substrates

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Abstract

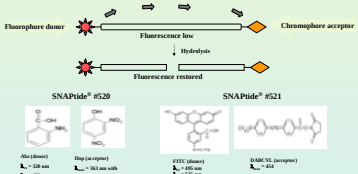
The botulinum neurotoxins are composed of two subunits, a 100 kD heavy chain and a 50 kD light chain, which are linked by a single disulfide bond. The heavy chain is responsible for binding and translocation while the light chain domain contains the enzymatic activity. The light chain is a zinc dependent metalloprotease. The eukaryotic substrate for botulinum neurotoxin type A is the synaptosomal protein SNAP-25. As previously reported, we have developed fluorescence resonance energy transfer (FRET) substrates based on SNAP-25 that are readily recognized and cleaved by the type A holotoxin and light chain. One of the substrates contains an oAbz/DNP FRET pair and the other a FITC/DABCYL FRET pair (US patent #6504006).

In this study, the specific activity of the holotoxin and recombinant light chain were determined using both FRET substrates. Specific activity is expressed in terms of µmoles of SNAPtide® cleaved per minute per milligram of enzyme. For comparison, the specific activity of holotoxin and light chain were ascertained under optimal condition for the holotoxin. The specific activity was also calculated for the light chain under its optimal conditions. A series of $ZnCl_2$ concentrations were tested in several buffer systems to determine whether addition of $ZnCl_2$ would improve the enzymatic activity of the light chain. In order to determine the ultimate sensitivity of this method for detecting the presence of the protease, the limit of detection (LOD) of the SNAPtide® assay was established using both substrates with the holotoxin and the light chain.

Introduction

The botulinum neurotoxins are one of the most potent toxins in nature. Their potency in humans is due to the cleavage and inactivation of specific neuronal proteins. The toxin has two components. The large, heavy chain, fragment is approximately 100,000 daltons. It is responsible for the binding and translocation of the toxin into specific neuronal cells. The smaller, light chain, is approximately 50,000 daltons. The light chain is the enzymatic portion of the toxin. It is a zinc dependent metalloprotease which cleaves at a specific site in one of the synaptosomal proteins. There are seven serotypes that differ in their target specificity. As reported previously, we have developed FRET (fluorescence resonance energy transfer) substrates for botulinum neurotoxin type A. These substrates are known as SNAPtide®. The substrate is named from the eukaryotic target of type A neurotoxin, SNAP-25. SNAPtide® is an internally quenched fluorescent substrate. When it is cleaved by botulinum neurotoxin full fluorescence is restored. Enzymatic activity can be monitored continuously by recording the increase in fluorescence intensity with time. We have recently designed a FRET substrate for botulinum neurotoxin type B as well. VAMPtide® is a peptide substrate based on the sequence of the eukaryotic protein VAMP-2 (synaptobrevin-II). Like the SNAPtide® substrate #520, it contains o-aminobenzoic acid/2,4 dinitrophenyl as the FRET pair.

Several of the light chains of the botulinum neurotoxins have been expressed and purified as fusion proteins in *E. coli* at List Labs. The light chains retain the enzymatic activity of the full neurotoxin but are unable to penetrate cells and cause toxicity. They represent an alternative means of studying botulinum neurotoxins without worrying about the high toxicity and BL3 conditions required when working with the holotoxin. Here we compare the enzymatic activity of the full botulinum neurotoxin type A (BTA) and the botulinum neurotoxin type A light chain, recombinant (LcA). The specific activity and limit of detection of both forms of the toxin are measured using the SNAPtide® fluorogenic assay. Studies illustrating hydrolysis of VAMPtide® with botulinum neurotoxin type B light chain, recombinant (LcB) are also presented.



SNAPtide® containing either the o-aminobenzoic acid/2,4 dinitrophenyl (oAbz/DNP) FRET pair or the fluorescein-thiocarbamoyl/DABCYL (FITC/DABCYL) FRET pair have been synthesized. BTA or LcA enzymatic activity can be monitored continuously by recording the increase in fluorescence intensity over time. Fluorescence is a linear function of cleaved SNAPtide® concentration up to 12 µM for the FITC/DABCYL substrate and up to 30 µM for the oAbz/DNP substrate. An 8 fold increase in fluorescence intensity is observed when the oAbz/DNP substrate is fully hydrolyzed with trypsin, while a greater than 30 fold increase in fluorescence intensity is observed for the FITC/DABCYL substrate. The substrate for botulinum neurotoxin type B, VAMPtide®, contains the same FRET pair as SNAPtide® #520 (oAbz/Dnp).

Materials

SNAPtide® substrates (product #520 and #521), SNAPtide® Unquenched Calibration Peptides (product #528 and #529), botulinum neurotoxin type A (product #130A), botulinum neurotoxin type A light chain, recombinant (product #610A), botulinum neurotoxin type B light chain, recombinant (product #620A) and VAMPtide® (product # 540) are all products of List Biological Laboratories, Inc.

Methods

Fluorimetric assays: 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 each FRET substrate were made in dimethyl sulfoxide (DMSO). Final dilutions were made in the assay buffer. Plates were incubated at 37°C for 15 minutes to ensure equilibrium prior to addition of substrate. The reaction was initiated by the addition of SNAPtide® or VAMPtide®. The time-dependent increase in fluorescence intensity was monitored at 37°C. The excitation and emission wavelengths were set to 321 nm and 418 nm, respectively, for the oAbz/DNP-substrate and 490 nm and 523 nm using a cutoff filter of 495 nm for the FITC/DABCYL-substrate.

Zinc titration: LcA is the zinc dependent endopeptidase portion of botulinum neurotoxin type A. During purification of the light chain domain, it is possible that the coordinated zinc molecule may be lost from some of the protein molecules. To test if zinc could be added back to the enzyme and increase its enzymatic activity, zinc chloride was titrated into several reaction buffers. Five nanomolar LcA and 8 µM SNAPtide® #520 were used in these assays. The buffer systems tested were 50 mM HEPES, pH 8.0, 50 mM HEPES, pH 8.0 + 0.05% TWEEN 20, and 50 mM HEPES, pH 8.0 + 1 mg/ml BSA. $ZnCl_2$ was added to reactions starting at 4 mM and titrating down to 0.004 mM in 2 fold dilutions. The reactions were monitored continuously for one hour at 37 degrees Celsius measuring RFU over time (**Figure 4**).

Limit of Detection (LOD): The limit of detection is the minimum concentration of BTA or LcA that can be measured with 99% confidence that the concentration of BTA or LcA present is greater than zero under our conditions using this fluorogenic assay. A calibration curve was generated by measuring the initial rate of proteolysis as a function of enzyme concentration. Seven replicates of a concentration estimated to be slightly greater than the expected detection limit were performed. Each analysis was calculated as pM and ng/ml using the calibration curve. The detection limit was calculated as 2.998 (Student's t-Distribution with 99% confidence and 7 degrees of freedom) times the standard deviation of the 7 replicates. The LOD assays were performed with BTA and LcA using both SNAPtide® substrates at 8 µM. The assay was also done with LcA using excess substrate at a concentration of 20 µM (**Figure 5**). The assays were carried out at 37 degrees Celsius for one and one half hours.

VAMPtide®: VAMPtide® is a new substrate designed for detecting the activity of botulinum neurotoxin type B. To measure the utility of this substrate, it was tested using botulinum neurotoxin type B light chain (LcB). LcB was titrated in the presence of 8 and 20 µM VAMPtide®. The change in fluorescence was monitored over 160 minutes at 37 degrees Celsius (**Figure 6**). Hydrolysis reactions of 8 µM VAMPtide® with 20 nM LcB were done in the presence and absence of 0.05 or 0.1 mM zinc chloride. Several different pH buffers were employed in this assay (**Figure 7**). The pH optimum of the cleavage reaction was also determined by performing the hydrolysis in either 50 mM MES or 50 mM HEPES buffer at several different pH ranges (see **Figure 7** for exact buffer conditions).

Results

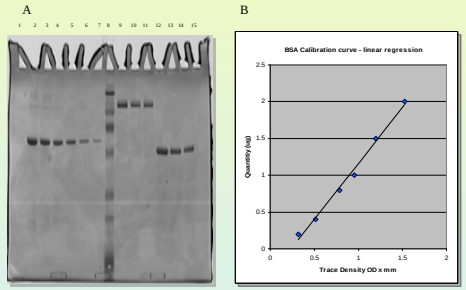


Figure 1: **BTA and LcA quantitation**. A) 12% SDS-PAGE gel with BSA standards equivalent to 2.0, 1.5, 1.0, 0.8, 0.4, and 0.2 µg (lanes 2-7) was run in order to estimate the quantity of BTA and LcA. Three different amounts of BTA (lanes 9-11) and LcA (lanes 12-14) were loaded. After running, the gel was scanned and analyzed using a BioRad G-800 Densitometer. B) Standard curve obtained through plotting the density of the known quantities of BSA. Our 10 µg vial of BTA was determined to contain 12.5 +/- 1.2 µg and our 10 µg vial of LcA was determined to contain 12.3 +/- 0.4 µg.

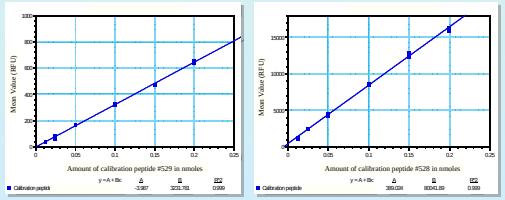


Figure 2: **Calibration peptide curves**. SNAPtide®, Unquenched Calibration Peptides (#528 and #529) were used to generate standard curves for the conversion of RFU to nmoles of cleaved substrate. Calibration peptide #528 corresponds to SNAPtide® substrate #521 and calibration peptide #529 corresponds to SNAPtide® substrate #520. The calibration peptides were diluted to final concentrations of 0.2, 0.15, 0.10, 0.05, 0.025, and 0.0125 nmoles. Dilutions were run in triplicate and conditions for each calibration peptide were identical to those used for the corresponding SNAPtide® substrate. The conversion equation is: nmoles = [(RFU-A)/B] where A = the x -intercept and B = the slope established from the calibration curve.

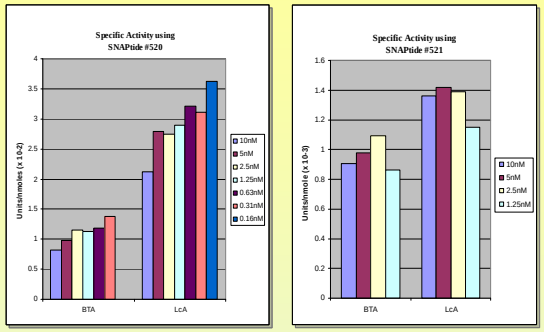


Figure 3: **Specific activity of BTA and LcA using fluorogenic assay**. Endopeptidase reactions with SNAPtide® #520 were conducted in 20 mM HEPES, pH 8.0, 0.3 mM ZnCl2, 1.25 mM DTT, and 0.2% TWEEN 20. Assays with SNAPtide® #521 were conducted in 20 mM HEPES, pH 8.0, 0.3 mM ZnCl2, 1.25 mM DTT, and 0.1% TWEEN 20. These are optimized buffers for hydrolysis using BTA. BTA and LcA were titrated 2 fold starting at 10 nM. Eight micromolar substrate was added to initiate the reactions. The data shown above is plotted in units per nmole of enzyme. The specific activity of LcA is significantly higher than that measured for BTA using substrate #520. The specific activities for both BTA enzymes are closer when testing with substrate #521. In general, both BTA and LcA have a higher specific activity when SNAPtide® #520 is used for measurement than when #521 is used.

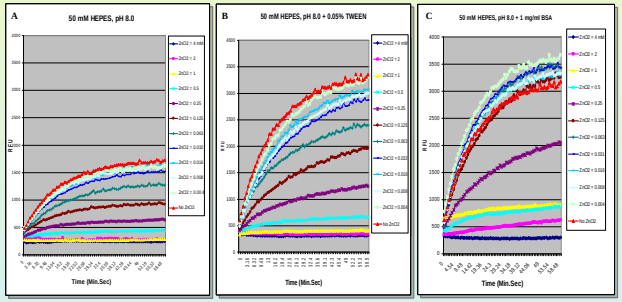
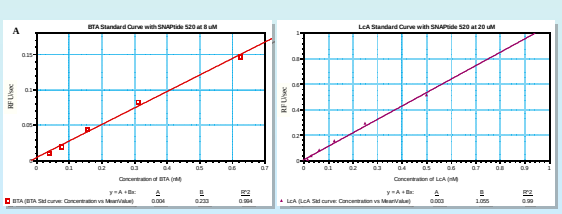


Figure 4: **Titration of $ZnCl_2$ in SNAPtide® assays**. The SNAPtide® assays were run using 5nM LcA and 8 µM SNAPtide® #520. The buffer systems tested were A) 50 mM HEPES, pH 8.0, B) 50 mM HEPES, pH 8.0 + 0.05% TWEEN 20, and C) 50 mM HEPES, pH 8.0 + 1 mg/ml BSA. $ZnCl_2$ was added to reactions starting at 4 mM and titrating down to 0.004 mM in 2 fold dilutions. The reactions were monitored continuously for one hour measuring RFU over time. The addition of TWEEN 20 or BSA significantly improved the RFU achieved in the assay. Under conditions tested, high concentrations of Zinc were inhibitory to the assay under all buffer conditions. Addition of low levels of zinc (< 0.2 mM) to BSA containing buffer did show some positive effect.



B

	LOD for BTA	LOD for LcA
SNAPtide® 520 @ 8 µM	48 pM (7.2 ng/ml)	24 pM (1.20 ng/ml)
SNAPtide® 520 @ 20 µM	No Data	25 pM (1.25 ng/ml)
SNAPtide® 521 @ 8 µM	31 pM (4.65 ng/ml)	25 pM (1.25 ng/ml)
SNAPtide® 521@ 20 µM	No Data	25 pM (1.25 ng/ml)

Figure 5: **Limit of detection of BTA and LcA.** The limit of detection is defined as the minimum concentration of enzyme that can be measured with 99% confidence that the concentration is greater than zero under our testing conditions. A) The top graphs are representative calibration curves generated by measuring the initial rate of proteolysis as a function of protease concentration. Here we show the curves generated when testing BTA with SNAPtide® #520 at 8 µM and when testing LcA with SNAPtide® #520 at 20 µM. B) The limit of detection in this assay for LcA is around 25 pM. This value is true for both fluorogenic substrates and when using excess substrate. When compared to BTA, the LOD of LcA is about 2 fold lower on a molar scale.

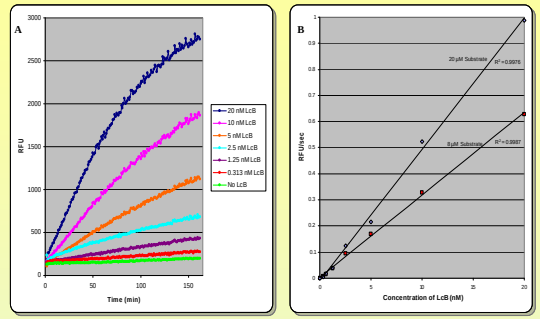


Figure 6: **Hydrolysis of VAMPtide®.** A) 8 µM VAMPtide® was cleaved by a series of concentrations of LcB as indicated on the right. Digestion was performed in 50 mM HEPES, pH 6.2, containing 0.05% TWEEN-20 at 37°C. The lowest concentration, 0.313 nM LcB, is easily detected in 160 minutes. B) Hydrolysis by LcB of either 8 µM substrate (red) or 20 µM substrate (blue) shows a linear response to enzyme concentration.

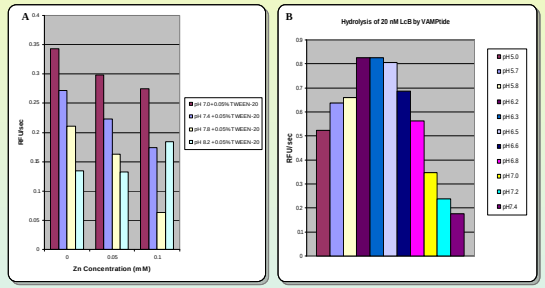


Figure 7: **Addition of zinc to the hydrolysis of VAMPtide® and optimal pH**. A) The cleavage of 8 µM VAMPtide® by 20 nM LcB was performed in 50 mM HEPES at pH 7.0, 7.4, 7.8, and 8.2 containing 0.05% TWEEN-20 at 37 degrees Celsius. For all pH values excpt 8.2, the best catalysis is seen without the addition of zinc. B) The hydrolysis of 8 µM VAMPtide® by 20 nM LcB was tested as a function of pH. The cleavage was performed in 50 mM MES at pH 5.0, 5.7, and 5.8 containing 0.05% TWEEN-20. The cleavage at pH 6.2, 6.3 6.5 6.6, 6.8, 7.0, 7.2, and 7.4 was performed in 50 mM HEPES containing 0.05% TWEEN-20. All reactions were run at 37 degrees Celsius. The optimal pH is between 6.2 and 6.3.

Conclusions

Specific Activity

The botulinum neurotoxin type A light chain (LcA) has higher specific activity based on units per nmole enzyme, using both SNAPtide®substrates when compared to the full length neurotoxin. There is also a large difference in activity when comparing the two FRET substrates. SNAPtide® #520 is a much better substrate than SNAPtide®# 521 when testing with either form of the enzyme. We hypothesize that the large FRET pair on substrate #521 may limit its access to the active site of the enzyme resulting in a decrease in the number of nmoles cleaved.

The specific activity of both LcA and BTA using SNAPtide® #520 appears to increase as enzyme concentration is decreased. We hypothesize that there may be some substrate inhibition occurring during these reactions. A portion of the cleaved substrate may interfere with further cleavage of the substrate.

Addition of Zinc

20 mM HEPES buffer containing 0.05% TWEEN or 1 mg/ml BSA appears to be much more active than 20 mM HEPES buffer alone. We hypothesize that the TWEEN and BSA may help disperse non-specifically bound enzyme or substrate from the walls of the microtiter well. In so doing, more enzyme and substrate are free to interact.

The addition of high levels of zinc chloride to the enzyme reactions are inhibitory. When using 20 mM HEPES with 0.05% TWEEN, no level of zinc was beneficial. When the buffer system included 1 mg/ml BSA, low levels of zinc chloride did improve the ultimate RFU achieved. Working with BSA in the buffer is somewhat problematic for the fact that bubbles are easily introduced into the wells upon mixing and can affect the reading of the fluorescence.

Limit of Detection (LOD)

The limit of detection for botulinum neurotoxin type A light chain is approximately 25 pM using either SNAPtide® substrate. When the substrate concentration was in excess, the LOD remained the same. The limit of detection for the full length botulinum neurotoxin type A is in the 30 – 50 pM range when using 8 µM substrate. The assays were run at 37 degrees Celsius for one and one half hours. If we calculate the LOD in terms of ng/ml the LOD for LcA using either substrate is approximately 1.3 ng/ml. The LOD for BTA is in the 4-7 ng/ml range of detection.

Our FRET substrates are a highly sensitive tool for detecting the activity of the botulinum neurotoxin. The sensitivity make it very useful in the search for small molecule inhibitors of the protease activity of the toxin.

VAMPtide®

VAMPtide® is a new FRET substrate designed for testing activity of the B serotype of botulinum neurotoxin. It has been shown to be a good substrate for botulinum neurotoxin type B light chain. The optimal pH is between 6.2 and 6.3 for the assay. The addition of zinc chloride to the assay was inhibitory, as was seen with LcA. The rate of cleavage of VAMPtide® is linear with respect to the concentration of LcB.