Rapid, Sensitive, and Specific Assay to Measure the Endoprotease Activity in *Botulinum Toxin Type A*

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ABSTRACT

An assay focused on the enzymatic property of botulinum toxin has been developed. This assay is designed to detect specific cleavage products resulting from activity of the toxin on several synthetic peptides. Measurement of the proteolytic activity of the neurotoxins provides a potentially sensitive and direct means for monitoring the presence of toxin. The assay distinguishes active from inactive botulinum toxin in sample solutions. Also, while a genetically engineered toxin might evade detection based on immunoassay or PCR, the enzyme activity, which is a fundamental attribute of *C. botulinum* intoxication and can not be altered, is measured in this assay.

The assay described here analyzes the cleavage of a series of peptides based on the FRET peptide SNAPtide® (US patent #6504006) designed at List Laboratories as a substrate for botulinum neurotoxin, type A (BTA). Substantial signal amplification can be expected as a result of catalytic turnover of the substrate. The generation of cleaved peptides is monitored using reverse phase HPLC. Several peptide sequences were examined to identify both the substrate which is efficiently cleaved by BTA and contains the minimum number of sites for non-specific hydrolysis and a control peptide which contains all potential non-specific cleavage sites but is not cleaved by BTA. In an initial study, cleavage of 5µM peptide substrate by 1 nM type A light chain (LcA) at room temperature was easily detected by RPLC in 10 min. Optimum conditions including buffer, substrate concentration and temperature were established. The detecting limit for LcA has been determined under various conditions.

This study establishes this assay as a sensitive, specific and rapid means of detecting the BTA enzyme.

INTRODUCTION

The specific target for the proteolytic activity of the botulinum toxin serotype A is SNAP-25. SNAPtide® was designed as a substrate for the type A neurotoxin. It is also cleaved by LcA as expected.

The seven peptides designed for the BTA enzyme are given in **Figure 1**. Each peptide has a short peptide sequence derived from SNAPtide® and a C-terminal 2,4 dinitrophenyl (Dnp) tag used to detect cleaved and uncleaved peptide by absorbance at 363 nm.

Sequence LBL 7951 was designed for increased specificity. A substitution of D-Lys for Lys within the SNAPtide® sequence decreases non-specific cleavage.

In sequence LBL 7950 the substitution of E for Q eliminates hydrolysis by BTA. This sequence was designed as a control peptide since it contains the same potential non-specific cleavage sites as the BTA substrate.

The remaining five sequences were designed as potential BTA substrates. Sequence LBL 7949 tests the potential advantage of adding 2 consecutive ε-amino caproic acid linkers to the N-terminal while LBL 8115 and LBL 8116 contain an additional one or two linkers on the C-terminal.

MATERIALS

The peptides were commercially synthesized and shown to be >95% pure by reverse phase HPLC. The expected molecular weight was obtained for each, using mass spectroscopy.

Botulinum Neurotoxin Type A Light Chain is a product of List Biological Laboratories, Prod #610A.

METHODS

HPLC was performed using a SWIFT, monolithic RP-all reverse phase column, 4.6 X 50 mm (ISCO, Lincoln NE) 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 8 to 100% B in 5 minutes with a flow of 5 ml/min was used. The column effluent was monitored at 363 nm, the absorbance maximum for Dnp. The injection volume was 100 µl. The intensity for the cleaved C-terminal fragment is reported as the per cent of the total peak areas for the cleaved and uncleaved substrate. Details of each experiment are described in the figure legends.

Digestions were performed in 50 mM HEPES, pH 7.4 containing 0.05% TWEEN-20. This buffer is referred to as ASSAY BUFFER.

Studies in 25% skim milk were performed as follows. Twenty micromolar solutions of LBL 7949 or LBL 7950 in 25% skim milk or ASSAY BUFFER were aliquoted into Microcon YM-30 filter devices (Millipore, Billerica, Mass.) with 30,000 MWCO filters. The digestion reaction was initiated by the addition of appropriate concentration of LcA and the hydrolysis was allowed to proceed for 3 hours at room temperature. The reaction mixture was spun at 12,000 for 15 minutes and the filter was washed with ASSAY BUFFER and spun a second time. The filtrate (100 µl) was injected onto the HPLC column for analysis.

RESULTS

I. Optimization

A. Identification of the optimal peptide sequence.

Each of the 7 peptide sequences listed in **Figure 1** were incubated at 20 µM with 50 ng/ml (1 nM) LcA in ASSAY BUFFER at room temperature. At timed intervals, they were analyzed by HPLC. Chromatograms obtained using LBL 7949 before addition of the LcA and at a series of time points after incubation with LcA are shown in **Figure 2A**. Before incubation with LcA, a single peak at 1.446 minutes was observed. After digestion, the Dnp-containing C-terminal fragment was visible at 1.212 min. This peak was easily detected after 10 min of digestion. For LBL 7950, a single peak was observed before and after 180 minutes of incubation with LcA confirming that this peptide is not cleaved by LcA (**Figure 2B**).

Figure 1: Peptide Substrates and Controls for Botulinum Toxin, Type A (BTA)

LBL 7949: Biotin-(ε-amino caproic acid)-SNAPtide® with Lys-Dnp

LBL 7592: Biotin-(ε-amino caproic acid)-S-N-K-SNAPtide® with Lys-Dnp

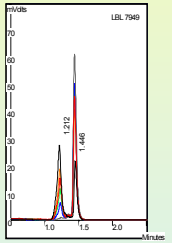
LBL 7951: Biotin-(ε-amino caproic acid)-S-N-K-(D-Lys)-SNAPtide® with Lys-Dnp

LBL 7950: Biotin-(ε-amino caproic acid)-S-N-K-(Q? E)-SNAPtide® with Lys-Dnp

LBL 7948: Biotin-(ε-amino caproic acid)$_2$-S-N-K-SNAPtide® with Lys-Dnp

LBL 8115: Biotin-(ε-amino caproic acid)$_2$-S-N-K-(D-Lys)-SNAPtide®-(ε-amino caproic acid)-Lys-Dnp

LBL 8116: Biotin-(ε-amino caproic acid)$_2$-S-N-K-(D-Lys)-SNAPtide®-(ε-amino caproic acid)$_2$-Lys-Dnp



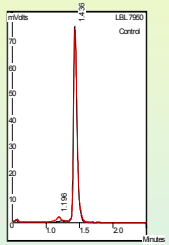


Figure 2A: Cleavage of LBL 7949 by 50 ng/ml LcA at RT. Chromatograms were obtained before the addition of LcA (gray) and after 10 min (blue), 20 min (green), 30 min (red), 40 min (orange) and 110 min (black).

Figure 2B: Cleavage of LBL 7950 by 50 ng/ml LcA at RT. Chromatograms were obtained before the addition of LcA (black) and after 180 min (red).

The time course of the reaction is shown for the 7 peptides in **Figure 3**. The data expressed as the % area represent the appearance of the C-terminal Dnp containing fragment.

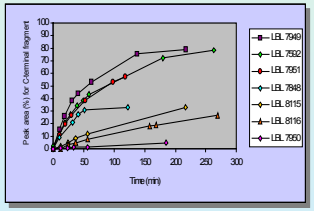


Figure 3: Cleavage of 20 µM of each of the seven peptides listed on the right by 50 ng/ml LcA.

These data indicate:

1). Sequence LBL 7592 is efficiently cleaved by LcA, while elimination of three amino acids on the N-terminal, represented by LBL 7948, significantly decreases hydrolysis by LcA.

2). Sequence LBL 7951 is cleaved as efficiently as LBL 7592 and so the D-Lys SNAPtide® does not affect cleavage and this substitution can be incorporated to obtain a more specific substrate.

3). Sequence LBL 7950 is not digested by LcA in this assay; however, since it contains all of the sites for non-specific cleavage found in Sequence LBL 7949, it is an ideal control peptide.

4). Sequence LBL 7949 contains an additional ε-amino caproic acid linker at the N-terminal. It is the best BTA enzyme substrate.

5). The addition of ε-amino caproic acid linker(s) on the C-terminal end of the peptide in LBL 8115 and LBL 8116 significantly decreases digestion by LcA.

B. Substrate concentration dependence of the digestion.

Three concentrations of LBL 7949, 5, 20, and 50 µM, were incubated with 50 ng/ml (1.0 nM) LcA in ASSAY BUFFER at room temperature. At timed intervals, 100 µl of the reaction solution was injected. The time course of the reaction is shown in **Figure 4**. The rate of hydrolysis is similar for both 5 and 20 µM LBL 7949. There appears to be some substrate inhibition using 50 µM LBL 7949. All further studies were conducted using 20 µM of LBL 7949.

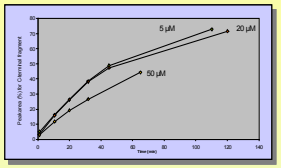


Figure 4: Cleavage of 5 µM (green), 20 µM (orange), and 50 µM (yellow) LBL 7949 with 50 ng/ml LcA.

C. Temperature dependence of the digestion.

LBL 7949 (20 µM) was incubated with 5 ng/ml (0.1 nM) LcA in ASSAY BUFFER at room temperature and 37 °C. At timed intervals, 100 µl of the reaction solution was injected. The time course of the reaction is shown in **Figure 5**. While the initial rate of digestion is similar for both temperatures, the amount of peptide hydrolyzed at 37°C levels off at longer time intervals. The digestion continues in the room temperature sample increasing the potential sensitivity at this temperature. All further studies were conducted at room temperature.

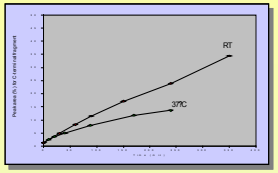


Figure 5: Cleavage of 20 µM LBL 7949 by 5 ng/ml LcA at room temperature (red) and 37°C (green).

II. Sensitivity

The sensitivity of LBL 7949 to LcA is demonstrated in **Figures 6 and 7**. A series of LcA concentrations were incubated with 20 µM substrate at room temperature in Assay Buffer. Every 60 min, 100 µl of each sample was injected on the HPLC column. The % of cleaved product is plotted versus time for 0.25, 0.50, 2.5, 5.0, 12.5, and 25 ng/ml LcA in **Figure. 6A**. In one hour of digestion, 2.5 ng/ml LcA is easily detected. Lower amounts of 0.5 and 0.25 ng/ml LcA can be detected under these conditions at times = 5 hours. The % of cleaved product as a function of LcA concentration is plotted for 1 and 2 hours of digestion in **Figure 6B**. The digestion is linear up to 12.5 ng/ml at these time points.

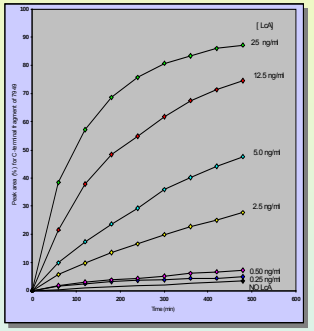


Figure 6A: Cleavage of LBL 7949 by 25 ng/ml (green), 12.5 ng/ml (red), 5.0 ng/ml (aqua), 2.5 ng/ml (yellow), 0.5 ng/ml (pink) and 0.25 ng/ml (blue) LcA.

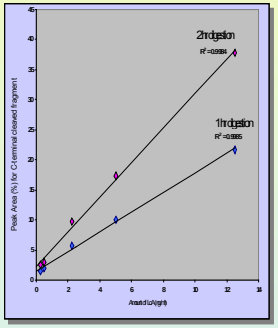


Figure 6B: Cleavage of LBL 7949 as a function of LcA concentration for 1 hour digestion at RT (blue) and 2 hours of digestion (pink) at RT.

The chromatograms for each concentration of LcA at one hour and eight hours of digestion are shown in **Figure 7A and 7B**, respectively.

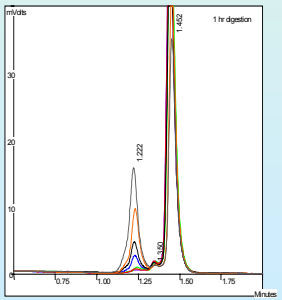


Figure 7A: One hour digestion of LBL 7949 by 0.25 ng/ml (red), 0.5 ng/ml (green), 2.5 ng/ml (blue), 5.0 ng/ml (black), 12.5 ng/ml (orange), and 25 ng/ml (gray) LcA. Chromatogram in the absence of LcA is purple and obscured by the red and green traces.

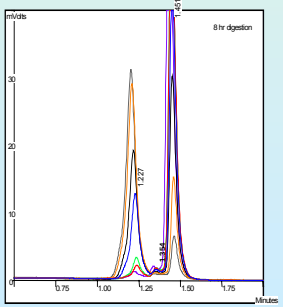


Figure 7B: Eight hour digestion of LBL 7949 by 0.25 ng/ml (red), 0.5 ng/ml (green), 2.5 ng/ml (blue), 5.0 ng/ml (black), 12.5 ng/ml (orange), and 25 ng/ml (gray) LcA. Chromatogram in the absence of LcA is purple.

III. LcA-spiked skim milk

The hydrolysis of 20 µM LBL 7949 by LcA in 25% skim milk is demonstrated in **Figures 8 and 9**. The chromatograms obtained for digestion by 1.25, 2.5, 5.0, and 25 ng/ml LcA in 25% skim milk are shown in **Figure 8**. The peak with retention time at 1.233 minutes represents the C-terminal cleaved fragment. In this time interval, even the lowest concentration of 1.25 ng/ml has significant intensity above that recorded for LBL 7949 incubated without LcA. Identical samples containing 20 µM LBL 7950 were run in parallel. Only the non-cleaved LBL 7950 peptide is observed indicating that there is no detectable non-specific cleavage in 25% skim milk (data not shown).

The % of cleaved LBL 7949 observed for the series of LcA concentrations in 25% skim milk is compared with the cleavage observed for identical samples in ASSAY BUFFER in **Figure 9**. Hydrolysis is greater in ASSAY BUFFER for all concentrations of LcA. These data suggest that substrate cleavage is inhibited by some component in the skim milk.

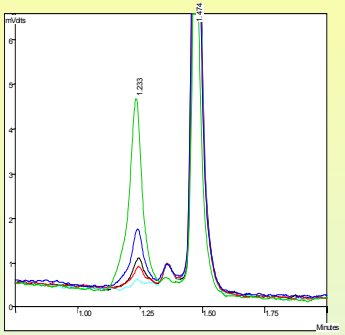


Figure 8: Three hour digestion of LBL 7949 in 25% skim milk by 1.25 ng/ml (red), 2.5 ng/ml (black), 5.0 ng/ml (blue), 25 ng/ml (green) LcA. Chromatogram in the absence of LcA is aqua. Details of this experiment are described in METHODS.

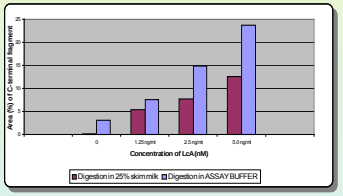


Figure 9: Three hour digestion of LBL 7949 in 25% skim milk (burgundy) and ASSAY BUFFER (blue). Details of this experiment are described in METHODS.

CONCLUSIONS

I. Optimization

A. The preferred peptide substrate was identified as Sequence LBL 7949.

B. Substitution of a D-Lys in Sequence LBL 7951 does not alter cleavage and thus increases the specificity for the BTA enzyme.

C. Sequence LBL 7950 was identified as an ideal control peptide.

D. Hydrolysis of LBL 7949 was linear over a longer time at room temperature versus 37°C.

E. Concentrations up to 20 µM 7949 can be used in the digestion assay.

II. Sensitivity

A. Under the conditions used in this assay 2.5 ng/ml LcA was readily detected at the earliest time point of one hour.

B. As little as 0.25 ng/ml LcA can be detected after 5 or more hours of digestion.

C. The rate of cleavage of LBL 7949 is linear with respect to the concentration of LcA.

III. LcA-spiked skim milk

Under the conditions used in this assay, the lowest concentration of LcA tested, 1.25 ng/ml, was detectable in 25% skim milk after three hours of digestion at room temperature. The sample preparation was simple and efficient resulting in chromatograms free of interfering components. The peak for the cleaved product is well-resolved with a baseline allowing confident assignment of low intensity peaks. These results indicate that 5 ng/ml LcA could be detected in skim milk using this protocol. Lower levels of LcA may be observed at longer incubation times.

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