Sensitive and Specific Assay to Measure the Endoprotease Activity of *Botulinum Toxin Type A Holotoxin in Milk*

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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 a synthetic peptide substrate. Measurement of the proteolytic activity of these botulinum toxins provides a potentially sensitive and direct means for monitoring the presence of toxin. Substantial signal amplification can be expected as a result of catalytic turnover of the substrate. Also, while a genetically engineered toxin might evade other means of detection based on immunoassay or PCR, for example, changes in the enzyme activity which is a fundamental attribute of *C. botulinum* intoxication are less likely. Therefore, the endoprotease activity would always be available to use for detection. Also, unlike immunodetection, functional assays can distinguish active from inactive toxins.

The assay described here analyzes the cleavage of a biotinylated derivative of SNAPtide™ (US patent #6504006) a FRET substrate for botulinum neurotoxin, type A (BTA) which was designed at List Laboratories. The FRET pair has been replaced by a single dinitrophenyl group (Dnp) for detection of the cleaved substrate. A rapid and sensitive HPLC protocol has been developed for specific detection of the substrate cleaved by BTA. In undiluted whole milk the lowest concentration of BTA holotoxin detectable is less than 1.0 ng/ml after 5 hours of digestion. The sample preparation of milk is simple and efficient, resulting in HPLC chromatograms free of interfering components. The peak for the cleaved product is well-resolved with a baseline allowing confident assignment of low intensity peaks. Still lower levels of detection could be achieved in a shorter time frame by including a BTA concentration step during the sample preparation and by using a fluorophore instead of the Dnp.

The use of a peptidic substrate in serum, on the other hand, is problematic due to the presence of other proteases and the likelihood of nonspecific cleavage of the substrate. Preliminary studies with bovine serum spiked with BTA indicated nonspecific hydrolysis of the biotinylated substrate. The results of the HPLC experiments will be described.

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 peptides designed for the BTA enzyme (**Figure 1**) are biotinylated at the N-terminal, have 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.

Under conditions where >40% cleavage by BTA occurs in buffer, no digestion is observed in BTA spiked whole milk. The detection of the BTA substrate from the milk sample is < 50% of that observed in the buffer sample indicating that milk contains some component that binds the peptide. A simple 4 step procedure was developed to improve detection of BTA in milk .(**Figure 3**).

When BTA-spiked serum samples were tested, similar non-specific binding and inhibition of activity occurred. An additional problem unfolded in the HPLC analysis of substrate. Several cleaved peptide fragments were detected, beyond those expected as a result of BTA cleavage. A number of schemes were investigated to improve detection. The best results were obtained by dilution followed by desalting.

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 (Prod #130A) and the recombinant light chain of the Type A neurotoxin (Prod #610A) are products of List Biological Laboratories.

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 to100% 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 of LcA were performed in 50 mM HEPES, pH 7.4 containing 0.05%TWEEN-20. When working with the holotoxin, reduction with dithiothreitol (DTT), was used to activate the enzyme. In order to identify the optimum buffer conditions for the BTA, cleavage was monitored using 100 mM HEPES buffer and varying the pH and the concentration of zinc, DTT, and TWEEN-20. The optimum buffer was 100 mM HEPES, containing 1.0 mM $ZnCl_2$, 5 mM DTT and 0.2% TWEEN-20, pH 7.4. This buffer is referred to as ASSAY BUFFER. The optimum temperature conditions were also investigated. Maximum cleavage was observed using a 30 min incubation of BTA and substrate at 37°C followed by incubation at room temperature.

Results

I. Digestion of BTA Substrate peptide by LcA

Figure 1: Peptide Substrate and Control for Botulinum Toxin, Type A

BTA Substrate:

Biotin-(ε amino caproic acid)$_2$-S-N-K-SNAPtide™ with Lys-Dnp

BTA Control:

Biotin-(ε amino caproic acid)-S-N-K-(Q? E)-SNAPtide™ with Lys-Dnp

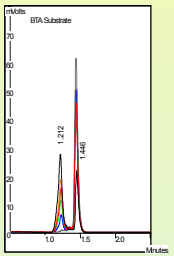

Figure 2A: Cleavage of 20 µM BTA substrate by 50 ng/ml LcA at room temperature. Chromatograms were obtained at 363 nm before the addition of LcA (gray) and after 10 min (blue), 20 min (green), 30 min (red), 40 min (orange) and 110 min (black).

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.

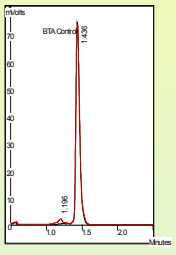

Figure 2B: Cleavage of BTA Control peptide (20 µM) by 50 ng/ml LcA at room temperature. Chromatograms were obtained before the addition of LcA (black) and after 180 min (red).

For the BTA Control, a single peak was observed before and after 180 minutes of incubation with LcA confirming that this peptide is not cleaved by LcA.

II. Studies of BTA-spiked milk

A. Sample preparation and digestion:

Step 1: Milk proteins are precipitated. The majority of the BTA remains in the supernatant.

Step 2: The samples are desalted using a 30,000 MWCO filter and the buffer exchanged to the reduction/reaction buffer (ASSAY BUFFER).

Step 3: The BTA substrate (20 µM) is added to the sample and the reduction/digestion is initiated by incubation for 30 min. at 37°C. Hydrolysis is allowed to proceed at room temperature.

Step 4: The digested samples are filtered again through a 30,000 MWCO filter. Cleaved and uncleaved BTA substrate is collected for HPLC analysis.

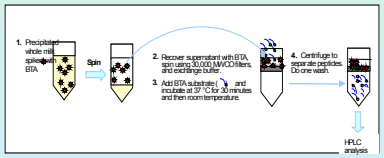

Figure 3: Sample preparation for BTA detection in milk

B. HPLC Analysis:

The hydrolysis of the BTA substrate by increasing concentrations of BTA in milk is demonstrated in the 363 nm HPLC chromatograms shown in **Figure 4**.

1. The C-terminal Dnp-containing fragment produced in BTA cleavage elutes with a retention time at 1.169 minutes.
2. In this 5-hour digest, 1.88 ng/ml BTA (orange) has significant intensity above that recorded for the BTA substrate incubated without BTA (gray). The minimum amount of BTA observable is 0.47 ng/ml (green).

This cleavage of the substrate by BTA in milk shows a linear response as indicated in **Figure 5**.

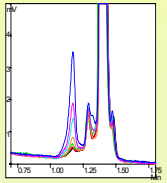

Figure 4: HPLC chromatograms obtained for 100 µl sample from BTA digestion of BTA substrate by 15 ng/ml (blue), 7.5 ng/ml (pink), 3.75 ng/ml (aqua), 1.88 ng/ml (orange), 0.47 ng/ml (green) and 0.19 ng/ml (red) BTA. Chromatogram for substrate sample without BTA is black.

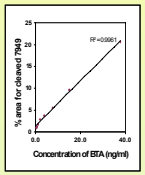

Figure 5: Cleavage of 20 µM peptide substrate as a function of BTA concentration in milk. Incubations were carried out for 30 min. at 37°C and 4.5 hrs at room temperature. Data indicate a linear response.

III. Studies of BTA-spiked Serum

A. Sample preparation and digestion:

Serum samples (50%) were spiked with 5nM BTA and desalted using 30,000 MWCO filters. The buffer was exchanged to the ASSAY BUFFER and 20 µM of peptide was added. Samples were placed at 37°C for 30 min to start the toxin reduction and digestion, and then at room temperature for an additional 1.5 hrs.

B. HPLC Analysis:

The hydrolysis of the substrate by BTA is demonstrated in the 363 nm HPLC chromatograms shown in **Figure 6**. An equivalent digestion of the control was conducted in parallel for comparison (**Figure 7**).

For the BTA substrate (**Figure 6**):

1. In ASSAY BUFFER there is no hydrolysis (black trace), while addition of 5 nM BTA (blue trace) results in one major cleaved peak (B).
2. In 50% serum with 5 nM BTA (red trace), there are 4 cleaved Dnp-containing fragments (A-D). Peak B is the BTA cleaved fragment. Peaks A, C, and D arise from non-specific cleavage.
3. In 50% serum without BTA two peaks co-eluting with C and D are observed (green trace). These data demonstrate non-specific cleavage of the substrate, with two new peaks C and D in the chromatogram. Also it appears that peak B is hydrolyzed further to peak A in 50% serum.

For the BTA control (**Figure 7**):

1. In ASSAY BUFFER there is no hydrolysis with (blue trace) or without (black trace) 5 nM BTA.
2. In 50% serum with (red trace) or without 5 nM BTA (green trace) peak C appears. The peak coincides with peak C observed in the serum cleavage of the substrate peptide.

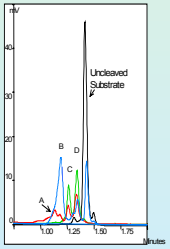

Figure 6: Hydrolysis of BTA substrate in ASSAY BUFFER without (black) and with 5 nM BTA (blue), and in 50% serum without (green) and with 5 nM BTA (red).

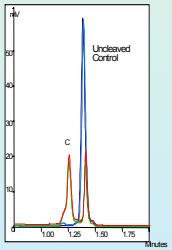

Figure 7: Hydrolysis of BTA control peptide in ASSAY BUFFER without (black) and with 5 nM BTA (blue), and in 50% serum without (green) and with 5 nM BTA (red).

C. Trypsin Digest

HPLC analysis was performed on a trypsin digest of the BTA substrate and control peptides. No peak elutes at the retention time of peak C which demonstrates that peak C observed with serum incubation is not due to trypsin-like cleavage of the substrate.

Conclusions

I. Digestion of BTA Substrate peptide by LcA

The BTA substrate is readily cleaved by LcA while the Control peptide is not hydrolyzed by LcA.

II. Studies of BTA-spiked milk

A. The sample preparation of milk is simple and efficient. In addition, Step 2 allows for BTA concentration that will significantly increase the sensitivity of the assay and decrease digestion time needed to observe the enzyme activity.

B. The HPLC method is totally automated using an autosampler, injecting every 8.5 minutes. Analyses are done at room temperature. Chromatograms are free of interfering components. The peak for the cleaved product is well-resolved with a baseline allowing confident assignment of low intensity peaks.

C. As little as 0.47 ng/ml of BTA can be detected after 5 hours of substrate digestion in whole milk.

D. The rate of cleavage of the BTA substrate is linear with respect to the concentration of BTA.

III. Studies of BTA-spiked serum

A. There is nonspecific cleavage of the BTA substrate as well as the Control peptide in 50% serum. For the BTA Control only one additional Dnp-containing fragment is observed while for the BTA substrate, three extra fragments are created.

B. Fragment under Peak A appears to be further breakdown of the BTA C-terminal cleaved fragment (Peak B). This result suggest that an aminopeptidase may be involved since cleavage with BTA creates an N-terminal amino acid; there are none present in the original sequence.

C. Peak D is not in the Control chromatogram. This Peak D was absent in identical studies using a peptide substrate which contains one ε-amino caproic acid linker, instead of two, suggesting that cleavage is occurring in the linker region.

D. Peak C is not the result of non-specific cleavage by a trypsin -like protease.

Future Directions

Current efforts to optimize this assay include:

A. Modification of the peptide substrate to replace the Dnp chromophore with a fluorophore such as fluorescein. This would greatly enhance the sensitivity of the HPLC assay.

B. Optimization of the HPLC protocol, including method refinement and column evaluation, for maximum sensitivity and peak resolution.

C. Identification of non-specific cleavage sites recognized by serum proteases. Modification of substrate sequence and /or use of specific inhibitors to minimize non-specific hydrolysis.

D. Development of a simple and quick method to remove interfering components in serum, similar to the sample preparation for whole milk.

E. Extension of these studies to include the Type B neurotoxin and the remaining serotypes.

F. Multiplexing the assay to detect all serotypes from one analysis.

G. Adaptation of this functional assay to a lateral flow device.

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