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A new peptide substrate for enhanced botulinum neurotoxin type B detection by endopeptidase–liquid chromatography–tandem mass spectrometry/multiple reaction monitoring assay

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ABSTRACT

Botulinum neurotoxins (BoNTs) are the most toxic proteins in nature. Rapid and sensitive detection of BoNTs is achieved by the endopeptidase–mass spectrometry (Endopep–MS) assay. In this assay, BoNT cleaves a specific peptide substrate and the cleaved products are analyzed by MS. Here we describe the design of a new peptide substrate for improved detection of BoNT type B (BoNT/B) in the Endopep–MS assay. Our strategy was based on reported BoNT/B–substrate interactions integrated with analysis method efficiency considerations. Incorporation of the new peptide led to a 5-fold increased sensitivity of the assay both in buffer and in a clinically relevant human spiked serum.

Botulinum neurotoxins (BoNTs),[1] the cause of the lethal human disease botulism, are the most toxic proteins known in nature [1]. Of eight serotypes (designated A–H) produced by the species of anaerobic *Clostridium botulinum, Clostridium butyricum, Clostridium baratii,* and *Clostridium argentinense,* the A, B, E, and (rarely) F serotypes are those primarily related to human illness [2–4]. Due to their availability and high toxicity, BoNTs are considered a significant bioterrorism threat mainly as through the poisoning of food [5]. The BoNT is a 150-kDa protein, consisting of a 100-kDa heavy chain (H) linked to a 50-kDa light chain (L) via a disulfide bond. The molecular mechanism of BoNT intoxication includes three consecutive steps: (i) attachment of the receptor binding domain, located on the C terminus of the H chain (the H_C fragment), to its receptors and subsequent internalization by endocytosis; (ii) translocation and release of the L chain into the cytosol, a step considered to be facilitated by the N-terminal fragment of the H chain (H_N); and (iii) specific cleavage of SNAP-25 (synaptosome-associated protein of 25 kDa), VAMP (vesicle-associated membrane protein), or syntaxin (three SNARE [soluble *N*-ethylmaleimide sensitive factor attachment protein receptor] proteins) by BoNT type L chains that possesses endopeptidase activity. This cleavage prevents release of the neurotransmitter acetylcholine from nerve cells to the synapses and leads to muscle paralysis [6–8].

The potent toxicity and bioterrorism potential of BoNT raised the need for rapid and sensitive methods for its detection. Currently, the mouse in vivo bioassay is the only widely accepted and standard method for detecting active BoNT [9]. This method is highly sensitive (1 mouse LD_{50} [$MsLD_{50}$] is estimated to be ~30 fM) but slow (up to 4 days), labor-intensive, time-consuming, and costly and also requires large numbers of laboratory animals for every tested sample. Therefore, several in vitro methods such as enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), and fluorescence assays have been developed [10–12]. One of the most successful in vitro methods is the endopeptidase–mass spectrometry (Endopep–MS) assay introduced by Wictome and coworkers and later by Barr and coworkers [13,14]. Briefly, this assay is based on the unique endopeptidase activity of each neurotoxin serotype [13–16]. Incubation of BoNT of each serotype with a synthetic peptide substrate that mimics its natural target produces specific indicative cleaved products that can be analyzed by various methods, preferably by mass spectrometry.

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[1] *Abbreviations used:* BoNT, botulinum neurotoxin; H chain, heavy chain; L chain, light chain; H_C, C-terminal fragment of H chain; H_N, N-terminal fragment of H chain (H_N); SNAP-25, synaptosome-associated protein of 25 kDa; VAMP, vesicle-associated membrane protein; $MsLD_{50}$, mouse LD_{50}; Endopep–MS, endopeptidase–mass spectrometry; BoNT/E, BoNT type E; LC–MS–MS/MRM, liquid chromatography–tandem mass spectrometry/multiple reaction monitoring; BoNT/B, BoNT type B; aa, amino acids.

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High sensitivity and a broad selectivity range have been reported for different BoNT serotypes tested in different media (buffer, food, serum, and stool) using the Endopep–MS assay [17–20]. Recently, we reported on the design of a new peptide substrate for improved detection of BoNT type E (BoNT/E) [21]. Our strategy was based on previously reported BoNT/E–SNAP-25 interactions integrated with efficiency considerations of the analysis method, that is, liquid chromatography–tandem mass spectrometry/multiple reaction monitoring (LC–MS–MS/MRM). Applying the new modified substrate resulted in significantly increased sensitivity (>1 order of magnitude) over assays using the earlier substrate [21]. Here we describe the design of a new peptide substrate for BoNT type B (BoNT/B) Endopep–MS assay that led to enhanced detection of the toxin.

BoNT/B specifically cleaves VAMPs 1, 2, and 3 [22] between amino acid residues Q^{76} and F^{77} [23]. Each VAMP consists of a highly conserved hydrophilic core flanked by a variable amino terminal head region at its N terminus and a hydrophobic region that anchors the protein to the membrane at its C terminus [23]. The interactions between BoNT/B and its VAMP's substrate have been studied intensively over the past two decades. A peptide derived from the bulk of the VAMP-2 hydrophilic domain (residues 33–94) was shown to be efficiently cleaved by BoNT/B. Shortening the synthetic peptide substrate around the cleavage site to 7 (74–80) or 10 (72–81) amino acid (aa) residues resulted in complete loss of proteolysis in the presence of the toxin [23]. Further reported data revealed that only peptide substrates of more than 30 aa were cleaved by the neurotoxin. More specifically, a peptide corresponding to residues 60 to 94 (^{60}LSELDDRADALQAGASQFETSAAKLKRKYWWKNLK^{94}) was shown to be cleaved by BoNT/B at the same rate as the longer 33- to 94-residue VAMP peptide [24]. Within this 35-aa peptide (aa 60–94), here termed "peptide-1," the C-terminal region is predominantly positively charged, whereas the N-terminal region consists largely of negatively charged residues. Residues flanking the N terminus of the cleavage site were shown to play a key role in the enzymatic substrate recognition. Two aspartate residues (D^{64} and D^{65}) in this region were identified as critical determinants for BoNT/B activity, and neutralization of their negative charge abolished substrate cleavage [25]. Substitution of these residues with asparagine or serine resulted in progressive or complete loss of proteolytic activity of BoNT/B, respectively [26]. Deletion of either A^{69} or A^{72} greatly reduced the cleavage rate by BoNT/B. The addition of an alanine residue at position 72 was also found to completely abolish peptide cleavage [25].

Thorough analysis of peptide-1 reveals three essential regions that are important for BoNT/B interactions: residues adjacent to the scissile bond cleavage site (cleavage region) and residues located within the N-terminal and C-terminal regions relative to the cleavage region [27]. Residues located within sites N terminal (63–68) and C terminal (83, 85) to the cleavage region influenced binding affinity [27]. Residues within the cleavage region influenced catalysis. In particular, mutation at positions 70, 73, 75, and 77 had the greatest inhibitory effect on BoNT/B activity. Mutations introduced from positions 86 to 94 at the C terminus did not influence the cleavage activity of BoNT/B [22,24,27,28], suggesting that residues downstream from position 86 might not be essential for BoNT/B endopeptidase activity.

To date, the 35-residue peptide-1 (60–94) was reported to be the preferred substrate for BoNT/B Endopep–MS assay [24]. Applying the assay with peptide-1 results in two cleavage products of similar length, 17 and 18 aa, for the N and C termini, respectively. Whereas for enhanced reliable detection of the toxin, a similar contribution of both cleavage products to sensitivity is required, use of the assay in our hands revealed restricted signals for the C-terminus product, which reduced assay sensitivity. This product was found to be sticky, making it rapidly absorb nonspecifically to any reaction/analysis vessel surface, thereby limiting overall reproducibility and sensitivity of the method.

A close examination of the 18-aa C-terminal sequence revealed the existence of a hydrophobic cluster that might underlay this phenomenon. This indication, together with the hypothesis that residues downstream from position 86 of the C terminus might not be essential for BoNT/B endopeptidase activity, encouraged us to design a new synthetic substrate characterized by selective deletion of aa residues at the C terminus. According to that rationale, deletion of the hydrophobic cluster might contribute to reduced nonspecific absorbance of the C-terminal cleavage product and, consequently, improved assay performance. In addition, shortening the peptide sequence might increase its detectability by LC–MS/MS (MRM), as was demonstrated previously [22]. For these reasons, we considered the deletion of aa 86 to 94 at the peptide C terminus. Yet, Arg^{86} and Lys^{87} in this segment are basic and positively charged and, hence, can amplify LC–MS–MS/MRM signal sensitivity due to their high proton affinity and efficient electrospray ionization in positive mode mass spectrometric analysis. Therefore, we deleted residues 88 to 94 and synthesized ^{60}LSELDDRADALQAGASQFETSAAKLKRK^{87}, which we termed "peptide-2." The new peptide is shorter than peptide-1 by 7 C-terminal residues and was expected to be cleaved by BoNT/B into two products of 17 and 11 aa for the N and C termini, respectively.

LC–MS–MS/MRM analysis methods were developed to quantify and identify the N- and C-terminal products expected to result from BoNT/B cleavage of peptide-1 and peptide-2. The analysis methods were first evaluated using synthetic peptides corresponding to these cleavage products. At least two MRM transitions for each product were determined from the multiple charge molecular ions to its fragment ions. A significantly increased signal response, approximately 5-fold higher, was measured for the C-terminal product of peptide-2 (m/z 426.6–120.1) compared with peptide-1 (m/z 574.8–129.1) (Fig. 1A). The enhanced signal obtained can be explained by efficient electrospray ionization being feasible due to formation of fewer multiple charge molecular ions combined with higher intensities of their mass spectrometric fragments obtained in MRM mode.

To test our hypothesis, according to which the selective aa deletion will not influence BoNT/B endopeptidase activity, the assay was applied simultaneously comparing the new peptide-2 with the original peptide-1. Here, 100 $MsLD_{50}$/ml of BoNT/B was incubated for 5 h with either peptide-1 or peptide-2 in equal molar concentration. Because both peptide substrates share common N-terminal cleavage products, their detection levels were expected to be comparable unless there were differences in the catalytic activity of BoNT/B with the two substrates. Fig. 1B shows an LC–MS–MS/MRM chromatogram of the most dominant MRM transition, m/z 880 to 234, of the common N-terminal cleavage product derived from peptide-1 and peptide-2. The results confirm comparable BoNT/B activity with both peptides, indicating that deletion of residues 88 to 94 from the sequence did not influence BoNT/B catalytic activity.

As shown in Fig. 1A, a 5-fold higher LC–MS–MS/MRM signal of the most dominant MRM transition of peptide-2 C-terminal product was observed over the correlated transition of peptide-1 when performing the Endopep–MS assay (Fig. 1C). In addition, and as expected, higher overall method reproducibility was obtained for each cleavage product of the shorter new peptide-2 (calculated precision [coefficient of variation, CV] of <25%). Precision was calculated from seven independent replicates. Signal intensities varied between 1.5×10^5 and 2.9×10^5 for N terminus and between 1.6×10^5 and 3.1×10^5 for C terminus. Furthermore, whereas restricted signal of the C-terminal product of peptide-1 reduced