Enhanced detection of type C botulinum neurotoxin by the Endopep-MS assay through optimization of peptide substrates

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Abstract

It is essential to have a simple, quick and sensitive method for the detection and quantification of botulinum neurotoxins, the most toxic substances and the causative agents of botulism. Type C botulinum neurotoxin (BoNT/C) represents one of the seven members of distinctive BoNT serotypes (A to G) that cause botulism in animals and avians. Here we report the development of optimized peptide substrates for improving the detection of BoNT/C and /CD mosaic toxins using an Endopep-MS assay, a mass spectrometry-based method that is able to rapidly and sensitively detect and differentiate all types of BoNTs by extracting the toxin with specific antibodies and detecting the unique cleavage products of peptide substrates. Based on the sequence of a short SNAP-25 peptide, we conducted optimization through a comprehensive process including length determination, terminal modification, single and multiple amino acid residue substitution, and incorporation of unnatural amino acid residues. Our data demonstrate that an optimal peptide provides a more than 200-fold improvement over the substrate currently used in the Endopep-MS assay for the detection of BoNT/C1 and /CD mosaic. Using the new substrate in a four-hour cleavage reaction, the limit of detection for the BoNT/C1 complex spiked in buffer, serum and milk samples was determined to be 0.5, 0.5 and 1 mouseLD$_{50}$/mL, respectively, representing a similar or higher sensitivity than that obtained by traditional mouse bioassay.

1. Introduction

Botulinum neurotoxins (BoNTs) produced by some species of the genus *Clostridium* can cause a life-threatening disease, botulism, in humans and animals through blocking the transmission of acetylcholine at the neuromuscular junction leading to flaccid paralysis[1]. There are seven structurally related serotypes of BoNTs; type A, B, E, and F cause human botulism, type C, D, and E are responsible for animal and avian botulism, and type G was isolated from soil but has not been known to cause a natural case of botulism [2]. The BoNTs belong to a family of zinc-dependent metalloproteases which consists of two polypeptide chains linked through a disulfide bond. The heavy chain of approximately 100 kDa is responsible for binding and entering targeted cells. The 50 kDa light chain functions as an endoprotease domain which cleave neuronal proteins involved in exocytosis of

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neurotransmitters, such as SNAP-25, synaptobrevin (VAMP2) and syntaxin, and each BoNT has a distinct cleavage site on these SNARE substrates[3].

BoNT/C1 is a common term for the type C botulinum neurotoxin which will be referred as BoNT/C throughout this manuscript. BoNT/C is different from the botulinum C2 toxin, which is not a neurotoxin but is a toxic protein that catalyzes the ADP-ribosylation of G-actin[4]. Some *Clostridium botulinum* strains produce neurotoxins as mosaic isoforms of BoNT /C and /D known as C/D or D/C mosaics. The BoNT C/D and D/C mosaics are composed of a chimeric structure of type C and D neurotoxins where the first letter designates the light chain activity and the second the heavy chain specificity [5]. It has been reported that BoNT/CD and /DC mosaics are associated with avian and bovine botulism [6–9]. Unlike other BoNTs that only target one native substrate, either SNAP-25 or VAMP2, BoNT/C and BoNT/CD are able to hydrolyze two substrates: SNAP-25 [10,11] and syntaxin[12, 13]. The BoNT/C cleavage site on SNAP-25 is Arg198-Ala199, just one residue towards the C-terminus from the scission bond for BoNT/A on this substrate. The hydrolysis of syntaxin by the light chain of BoNT/C and /CD occurs at Lys253-Ala254 near the carboxyl-terminal region of the substrate. Although BoNT/C was reported to be almost as active as BoNT/A in an *in vivo* assay, the *in vitro* cleavage efficiency of SNAP-25 by BoNT/C, was demonstrated to be much lower than that by BoNT/A[14].

For rapid and sensitive detection of botulinum neurotoxins, many *in vitro* endopeptidase activity assays based on BoNT's intrinsic enzymatic function have been developed as alternatives to the mouse bioassay. The mouse bioassay is the historic standard method. It requires the use of many laboratory animals and is relatively slow [15]. Peptides derived from the native substrates have been used as substrates for endopeptidase methods and the presence of BoNT is determined through the detection of cleavage products using various detection platforms. A previous report has shown that a long peptide, SNAP-25(93–206), can be cleaved by BoNT/C as efficiently as full-length SNAP-25[14]. Short versions of a SNAP-25 peptide have also been used as substrates to determine the proteolytic activity of BoNT/C in endopeptidase immunoassays and HPLC assays[16, 17]. In addition to SNAP-25, peptides derived from syntaxin (232–266) have also been utilized as BoNT/C substrate in an endopeptidase assay[17].

A mass spectrometry-based Endopep-MS assay which detected all seven BoNT serotypes was developed in our laboratory utilizing modified SNAP-25 or syntaxin peptide substrates for the determination of BoNT/C and BoNT/CD cleavage activities [8, 18, 19]. BoNT/C detection by this strategy was not very sensitive in relation to the mouse bioassay and needed improvements. In this report, we describe the development of novel peptide substrates for improving sensitivity of the Endopep-MS assay for the sensitive detection of BoNT/C and /CD toxins.

2. Material and methods

2.1 Materials

All chemicals were obtained from Sigma–Aldrich (St. Louis, MO) except where indicated otherwise. Fmoc-amino acid derivatives and peptide synthesis reagents were purchased from

EMD Chemicals, Inc. (Gibbstown, NJ) or Protein Technologies (Tucson, AZ). Isotopically labeled Fmoc-amino acid derivatives were purchased from Cambridge Isotope Laboratories (Tewksbury, MA). Botulinum type C toxin complex was obtained from Metabiologics (Madison, WI). The specific activity determined by mouse bioassay and protein concentration of the toxin was provided by the manufacturer. The culture supernatant of BoNT/CD mosaic was provided by Theresa Smith of the United States Army Medical Research Institute of Infectious Diseases (USAMRIID). Monoclonal antibody 8DC1.2 was provided by Dr. James Marks at the University of California, San Francisco. Streptavidin coated Dyna-beads were purchased from Invitrogen (Lake Success, NY). Botulinum neurotoxin is a highly toxic substance and therefore requires appropriate safety measures. All neurotoxins were handled by trained personnel in a level 2 biosafety cabinet equipped with HEPA filters and all standard safety handling and decontamination procedures were followed. Serum was collected from anonymous donors and was purchased from the Interstate Bloodbank. No demographic information was obtained.

2.2 Peptide synthesis

All peptides were prepared in house by a solid phase peptide synthesis method using Fmoc chemistry on a Liberty microwave peptide synthesizer (CEM, Matthews, NC, USA) or Symphony X peptide synthesizer (Protein Technologies, Tucson, AZ, USA). Peptides were cleaved and deblocked using a reagent mixture of 94% trifluroacetic acid: 2.5% water: 2.5% 1,2-ethanedithiol:1% triisopropyl silane and purified by reversed-phase HPLC using a 0.1% TFA water:acetonitrile gradient. Correct peptide structures were confirmed by MALDI mass spectrometry. All peptides were dissolved in deionized water as a 1 mM stock solution and were stored at −70°C until further use.

2.3 Endopep-MS assay

In-solution or on-bead Endopep-MS assays were carried out as previously described [20]. In brief, the reaction was conducted in a 20 μL reaction volume containing 0.1 mM peptide substrate, 10 μM $ZnCl_2$, 1 mg/mL BSA, 10 mM dithiothreitol, and 200 mM HEPES buffer (pH 7.4) at 42°C for 4 hrs or as indicated in the text. For the in-solution assays without antibody-coated beads, various concentrations of BoNT/C (as indicated in the results and discussion section) were directly added into the reaction mixture. For samples including complex matrices and BoNT/CD mosaic supernatant, the toxin spiked in matrix was first purified by a BoNT/D specific antibody and immobilized on streptavidin beads, followed by an activity assay as described [20].

After completion of the timed reaction, 2 μL of the supernatant was mixed with 16 μL of α-cyano-4-hydroxy cinnamic acid at 5 mg/mL in 50% acetonitrile/0.1% TFA/1 mM ammonium citrate; 2 μL of a 0.5 or 1 μM internal standard peptide (isotope labeled peptides resembling the sequence of either the C- or N-terminal cleavage product of some peptide substrates during optimization process) was added to the solution if the peptide was used. The formation of cleavage products was measured as the ratio of the isotope cluster areas of the MS peak of the cleavage product versus an internal standard. The data were usually an average of three experiments. CV values for the samples were typically below 20%.

2.4 MS detection

Each sample was spotted in triplicate on a MALDI plate and analyzed on a 5800 MALDI-TOF-TOF MS instrument (Applied Biosystems, Framingham, MA). Mass spectra of each spot were obtained by scanning from 800 to 4000 *m/z* in MS-positive ion reflector mode. The instrument uses a Nd-YAG laser at 355 nm, and each spectrum is an average of 2400 laser shots.

3. Results and discussion

3.1 Selection of an appropriate template as a BoNT/C substrate

The principle of the mass spectrometry-based Endopep-MS assay includes the detection of cleavage products hydrolyzed from a peptide substrate by BoNT by matrix-assisted laser desorption/ionization (MALDI) or electrospray ionization (ESI) mass spectrometry. The focus of this work will be on the detection by MALDI TOF MS. Improvements in assay sensitivity directly rely on elevated peak intensities of the cleavage peptide fragments arising from either increased hydrolysis efficiency or amplified ionization efficiency of the products or both effects combined. Therefore, the only parameter we focused on in this study was the change in the intensity of the cleavage product peak in a mass spectrum resulting from optimization of the structure of a BoNT/C peptide substrate, regardless if the change was caused by creation of a better substrate (related to hydrolysis), or a better cleavage product (related to ionization by MALDI) or a combination of both effects.

In order to determine an appropriate sequence template as a starting point for the optimization of BoNT/C substrate, three synthetic peptides were initially examined (Table 1). Pep-1 was derived from SNAP-25 with some terminal and internal modifications. This is a dual substrate of BoNT/A and BoNT/C used in the Endopep-MS activity assay [8, 18, 19]. Pep-2 is among some syntaxin peptides prepared in our laboratory in a previous investigation of potential BoNT/C substrates (unpublished data). Pep-3 is a 15-mer peptide comprising SNAP-25 (191–204) sequence with the addition of Arg to its C-terminus and modifications at both C- and N-termini. This peptide was found to be one of the promising candidates as a BoNT/A substrate in a previous study, but was not examined for BoNT/C hydrolysis (unpublished data). Assessment of these peptides for BoNT/C activity showed the absence of any fragment peaks in control samples (Fig. 1A and 1C) but the presence of pairs of new peaks in reaction samples (Fig. 1B and 1D), showing that Pep-2 and Pep-3, similar to Pep-1, are effective substrates of BoNT/C in the Endopep-MS activity assay. A comparison of the peak area ratios of the cleavage products versus the internal standard (Table 1) indicates that Pep-3 yielded more C-terminal cleavage product (CT-product) and N-terminal product (NT-product) than the currently used substrate, Pep-1, and thus it was selected as a SNAP-25 peptide template for further improvement. In parallel, we also attempted to develop a better syntaxin sequence-based substrate using Pep-2 as a template. However, the development procedure of this type substrate is not included in the present discussion, since the final optimal peptide from the template showed less ideal results than the optimized SNAP-25 sequence-based peptides.

The ionization efficiency of a peptide can sometimes be promoted in positive ion mode mass spectrometry, if it contains basic amino acid residues such as lysine and arginine in its sequence, due to the high proton affinity of these amino acid residues[21]. For this reason, we incorporated two more arginine residues into the Pep-3 sequence by replacing existing C-terminal leucine and glycine to form Pep-4 as the first optimization step, in order to achieve better detection of the CT-product. An additional substitution of methionine with norleucine (X) was made in Pep-5 in an attempt to avoid unwanted oxidation of this residue during the peptide preparation and storage. Unlike Pep-4, two arginine residues were directly added in Pep-6 to the C-terminus of the template without making any other changes to the template sequence. Evaluation of these three new peptides demonstrated that the replacement of LG with RR in Pep-4 led to a significant decrease in the detection of both CT- and NT-products (Table 1). Because Pep-4 and Pep-3 share the same sequence in their N-terminal cleavage parts, the 10-fold reduction in the NT-products suggested that reduced detection of the cleavage products from Pep-4 were caused by decreased substrate efficiency of the modified peptide. By comparing Pep-5 with Pep-4, the substitution of methionine by norleucine, a general alternate used in peptide mutation, completely diminished the capability of the Pep-5 as a BoNT/C substrate under the experimental conditions used, indicating that residues at the position close to the cleavage site (such as Leu, Gly, and Met here) are susceptible to change in their structures. By contrast, addition of two extra arginine residues (Pep-6) did not impact its substrate cleavage efficiency but led to a slight improvement in the detection of both cleavage products as compared to Pep-3.

We next examined if extending the length of Pep-6 enhanced assay sensitivity. Pep-7 through Pep-10 was constructed by adding a single amino acid residue each time to the template, based on the SNAP-25 sequence, to the N-terminus of Pep-6 (Table 1). While a similar level of CT-product was generated from Pep-7 (Peak area ratio: 2.6) and Pep-10 (2.6) compared to that for Pep-6 (2.6), significant elevation of the CT-product was observed from the cleavage of Pep-8 (3.1) and Pep-9 (4.5). An almost two-fold increase in both CT-product and NT-product from the cleavage of Pep-9 revealed that the string of NKT in the N-terminus of Pep-9 improved its hydrolysis efficiency as a BoNT/C substrate and the ionization efficiency of its NT-product.

3.2 Optimization by single and multiple amino acid residue substitution

Using Pep-9 as a new template, the effect of substitutions of some residues located around the cleavage site and the peptide termini were investigated. The N-terminal asparagine (N_1) was replaced with the residues of various properties (Fig. 2A). By comparing the formation of the CT-product of these new peptides, it was observed that the mutants of N1Q and N1D performed similarly or relatively better than the wild type (Pep-9) did. By contrast, no response was detected on a N2R mutant, indicating that the introduction of a positive charge side chain at this position diminished the substrate capability of binding. Interestingly, replacement of the N_1 residue with three nonpolar residues G, A, or V, generated a more effective substrate, where the CT-products were more than two-fold higher than that of the original Pep-9, presumably due to improved substrate-enzyme interaction. On the other hand, reduced CT-product formation from the N1F mutant suggests that a bulky aromatic ring of the phenylalanine side chain, even though nonpolar, might disfavor the substrate-

enzyme interaction. Consequently the N1V mutant (Pep-11) was subjected to further improvement; where the residue Q_{10}, immediately adjacent to the scission bond residue R_{11} was exchanged to A, K, D and N. As shown in Figure 2B, both positive and negative charges at this position completely destroyed the substrate activity of the modified peptides, while the nonpolar and small sized alanine was tolerated and did not enhance or diminish the peptide cleavage. By contrast, a structurally minor substitution (Pep-12), Q10N with one methyl group removed in this side chain, surprisingly resulted in a dramatic improvement where the production of the cleavage product increased five-fold. These results underline the importance of the amino acid residues at or around the cleavage site, where a subtle change in structure can make an enormous difference in peptide activity. Figure 2C displays the result from the peptides derived from substitutions of N_9 in Pep-12 by the amino acids Q, A, D, E, or K. Unlike previous modifications at position 10, all peptides with substitution at position 9 underwent only moderate changes in cleavage efficiency. The Pep-13 bearing the N9Q mutation was then selected as a new template, due to its slight improvement on assay sensitivity.

To examine the contribution and potential of other amino acid residues in Pep-13 for the detection of the cleavage products, an Ala-scanning peptide library was prepared where each non-alanine residue (not modified previously) was sequentially substituted by alanine. In addition, glycine was used to substitute each of the two alanine residues to form two more peptides. Also, the cleavage-site forming arginine was replaced by lysine rather than alanine to retain the positive charge property at this position, considering that other non-trivial changes in this position might negatively alter the function of a peptide substrate. Moreover, two more peptides were prepared by either removing the acetyl group on the N-terminus or by omitting C-terminal amidation in Pep-13. Only the CT-products were compared since the CT-product peaks cleaved from all peptides tested were higher than that of its N-terminal counterpart. Figure 3 displayed the response of these single-mutated peptides under the cleavage of BoNT/C and the results were grouped into five categories in terms of relative CT-product measurement. The level of the cleavage products declined more than 80% when either of the only two acidic residues, D_6 and E_7, were substituted by alanine, implying that negative charges at these positions are necessary to maintain the high hydrolysis efficiency of the substrate (Pep-13). Similar impact was detected when the original alanine residues, A_8 and A_{12}, were switched to the smaller glycine residues, suggesting the importance of retaining at least the side chain of alanine or maintaining a chiral configuration at these positions. Reduced hydrolysis (30 – 50%) was also observed on peptides where either of the terminal protection groups were removed, indicating that terminal acetylation and amidation, which was originally designed to make the peptide stable in a biological matrix, somehow promoted enzymatic hydrolysis of Pep-13 by the BoNT/C.

While some mutations or modifications described above led to a reduced cleavage response, others either improved or did not alter the cleavage of the modified peptides. The alanine substitution of the isoleucine residue at position 5 and the glycine residue at position 11 led to a 70% and 30% improvement, respectively. This demonstrated that just small changes in the size, but not the polarity, of the side chains of these two residues improve the BoNT/C hydrolysis of these substituted peptides. The most striking improvement occurred when the cleavage-site residue arginine was changed to lysine, a similar residue that possesses a

positive charge. The R11K substituted peptide, Pep-14, resulted in a 2.6-fold enhancement in the formation of the CT-product, therefore it was selected as a new template for further optimization.

The next refinement step was to manipulate the sequence of Pep-14 with multiple substitutions in an attempt to gain additional improvements. Although several single-substitutions displayed improved results, simply placing together these substitutions into a single sequence may not guarantee an augmented effect. Any single substitution on the peptide may or may not alter its conformation and affect its intra- and/or intermolecular interactions. Therefore, the introduction of a specific additional substitution(s) to a singly-substituted peptide (with positive results) may not provide the same positive benefit on a new doubly or multiply-modified one; as it did on the original, non-substituted peptide. For this reason, we applied a strategy that screens selected residues from C- to N-terminus of Pep-14 with various substitutions, based on the information obtained from the single-substitution study described above. Once a better residue is determined at a specific position, the residue becomes a fixed substitution incorporated into the sequence for the next screening. Table 2 lists multiple-substituted peptides obtained following this approach, where red letters represent the newly incorporated residues on the sequence of Pep-14. Starting from the glycine residue at position 17, the effect of G17A in Pep-15 gave rise to a similar increase (from 1.0 to 1.3) on the CT-product level as the same mutation on Pep-13. Extension of the nonpolar side chain of isoleucine at position 17 in Pep-16 resulted in more CT-product detected; presumably due to enhanced hydrophobic-hydrophobic interaction between enzyme and substrate or increased ionization efficiency of the cleavage product. This effect, however, was diminished by an even larger phenylalanine group in Pep-17. As a result, the Ile residue was fixed in this position for further optimization. Ala-scanning data indicated that the original residues in the region of $T_{13} - L_{16}$ were important, hence only conservative substitutions were conducted on these positions. L16G caused a slight improvement (2.3 in Pep-16 vs 2.7 in Pep-18), leading to a permanent placement of glycine at this position. Added advantages of this replacement include potentially reducing hydrophobicity of a peptide, which may decrease the difficulty of peptide synthesis, and improve peptide solubility in enzymatic reaction solutions. By contrast to L16G, replacement of L_{16} with a positively charged K or a negatively charged D yielded inferior substrates (Pep-19 and Pep-20), underscoring the significance that a conservative substitution is required in this position. For the residue at the position 14, however, even a substitution with similar properties, K14R, gave rise to a significant reduction in the cleavage product (Pep-21 vs Pep-18). Meanwhile, the amount of the product (5.2) doubled once the T_{13} was substituted with serine (Pep-22 vs Pep-18). Interestingly, this significant improvement could be negated by combining the T13S and K14R substitutions (Pep-21). This effect was not unexpected, since the residues at these positions are very close to the cleavage site of the BoNT/C substrate, and may directly participate in the hydrolysis process and are therefore more susceptible to any changes.

The residue at position 5 was the next one to be considered, due to increased CT-product from an I5A substitution. Various amino acids of different side chain properties were tested to replace isoleucine at this position (Pep-24 to Pep-29), however, reduced performance or inferior results were observed for all of these mutants. Although the I5A substitution on the

original Pep-13 resulted in an elevated cleavage product, this mutation (Pep-24) derived from the sequence of Pep-22 (which included four newly replaced residues) led to the detection of fewer CT-products (3.9 for Pep-24 vs 5.2 for Pep-22); underscoring the difference between single- and multiple-substitutions as discussed above. When the arginine residue in position 4 was replaced by a different type of amino acid, all peptides (Pep-30 to Pep-34) displayed better performance as BoNT/C substrates compared to Pep-22, demonstrating the flexible requirements of the amino acid residues in this position. With Pep-34 as a new template, conservative substitution of threonine in position 3 with serine (Pep-35) or tyrosine (Pep-36), further improved the yield of the cleavage products (9.6 and 10.1) compared to the template (8.6). By contrast, lower hydrolysis products resulted from another conservative amino acid change in position 2 (K2R) in Pep-37 (7.2) and this reduction was not overcome by a double mutation of K2R and T3S in Pep-38 (7.3).

Given the success of the substitution of the original cleavage site residue, R_{11}, with another positively charged lysine residue in Pep-14; we then explored the effect of replacing two lysine residues (K_{11} and K_{14}) in Pep-36, the best BoNT/C substrate up to now, with ornithine (represented by O). Ornithine is a non-essential amino acid derived from the breakdown of arginine during the citric acid cycle and has a structure similar to lysine but one methyl group shorter than the latter (Fig. 4). When K_{14}, two residues downstream from the cleavage site, was changed to ornithine (Pep-39), a remarkable enhancement (2.6 fold) was obtained (Table 2). In addition, a moderate improvement (10.1 to 12.3) was detected from K11O substitution (Pep-40) and almost two-fold increase in CT-product detection (10.1 to 19.2) was achieved with K11O/K14O combination (Pep-41). Because the chemical properties of lysine and ornithine are similar, these improvements were unlikely to result from the elevated ionization efficiency of the CT-product during mass spectrometric acquisition. A more reasonable explanation could be that the positively charged residue in position 14 is directly involved in an enzyme-substrate interaction(s) or cleavage process, and a short side chain of ornithine further enhances such interaction(s). The elucidation of the mechanism that underlies this phenomenon will require additional biochemical and biophysical studies, such as crystallography analysis.

3.3 Comparison of the optimal peptide with the original substrate and its application in biological sample matrices

The optimal peptide, Pep-39, was then compared to Pep-1, the originally-used substrate described in the Endopep-MS assay for the detection of type C botulinum neurotoxin[18]. The substrates were tested with two major forms of toxins: one is BoNT/C as a complex with neurotoxin-associated proteins, and another one is the C/D mosaic containing an enzymatic light chain very similar to BoNT/C and a heavy chain similar to BoNT/D. As shown in Table 3, the area ratio of CT-product versus the internal standard was measured as 0.3 from Pep-1 compared to the value of 70.6 from Pep-39 cleaved by BoNT/C under identical experimental conditions. The product peak increased from 0.2 for Pep-1 to 41.3 for Pep-39 with the cleavage by C/D mosaic. Both results represent more than 200-fold increase in the detection of the cleavage product, demonstrating a dramatic enhancement in the sensitivity of the Endopep-MS assay. Figure 5 displays the assay results for various concentrations of BoNT/C spiked in reaction buffer and two complex matrices, serum and milk. Good

linearity within the test range of toxin concentrations was obtained and the limit of detection of 0.5, 0.5 and 1.0 mouseLD$_{50}$ (1pg/mL or 5.5 attomole/mL) toxin in buffer, serum and milk was accomplished after a 4 hour cleavage reaction (Fig. 5) in contrast to the LOD of 50 mouseLD$_{50}$ of the toxin in buffer using the old substrate under the same assay conditions (data not shown).

4. Conclusion

Novel peptide substrates were developed for the mass spectrometry-based Endopep-MS assay for the detection of type C botulinum neurotoxin. Based on the sequence of a small, modified, SNAP-25 peptide, we conducted optimization through a comprehensive process including length determination, terminal modification, single and multiple amino acid residue substitution, and incorporation of unnatural amino acid residues. Our data demonstrate that an optimal peptide substrate (Ac-VKYNIDEAQNKASOMGIRRR-NH$_2$, O = ornithine) provided more than 200-fold improvement over the original substrate used for the detection of BoNT/C and /CD mosaic. The sensitivity for the detection of BoNT/C using the Endopep-MS assay was significantly increased. The new peptide substrate allows the accomplishment of a similar or higher sensitivity than that obtained using the traditional mouse bioassay, however the Endopep-MS assay is more rapid, requiring only a four hour reaction time. The limit of detection for the BoNT/C complexes spiked in buffer, serum and milk samples was determined to be 0.5, 0.5 and 1 mouseLD$_{50}$/mL, respectively.

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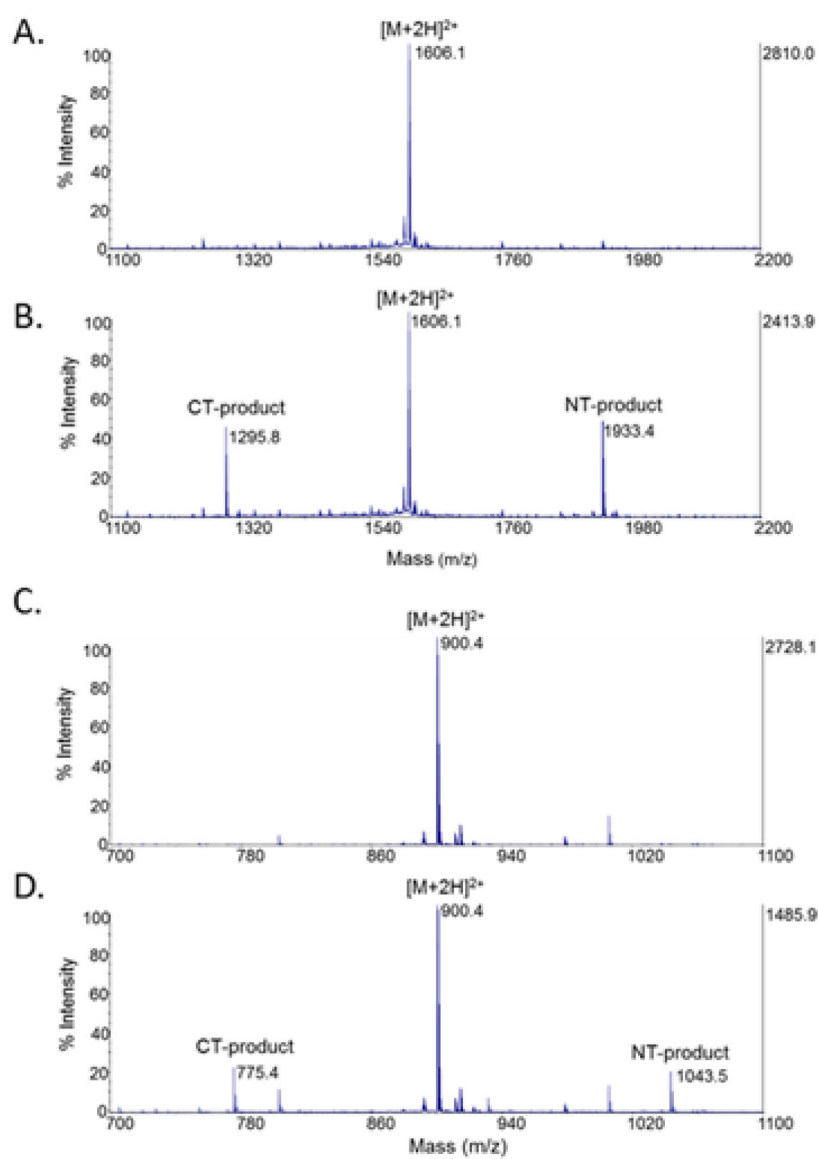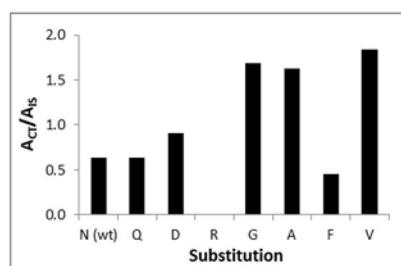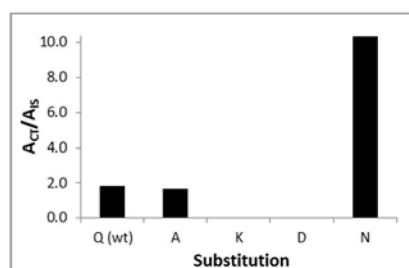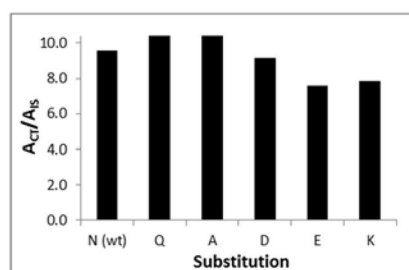
Figure 1.
MALDI mass spectra for the detection of BoNT/C using Pep-2 without (A) and with (B)
1000 mouseLD$_{50}$ toxin added, and using Pep-3 without (C) and with (D) toxin added. The
peaks of doubly charged substrate ions and both N-terminal and C-terminal cleavage
products were labeled.

Figure 2.

Formation of CT-product generated from the BoNT/C cleavage of single substituted peptides. (A) In wild type Pep-9 (Ac-$N_1K_2T_3R_4I_5D_6E_7A_8N_9Q_{10}R_{11}A_{12}T_{13}K_{14}M_{15}L_{16}G_{17}R_{18}R_{19}R_{20}$-NH$_2$) template (wt), the N_1 was replaced with Q, D, R, G, A, F, or V, respectively, in seven new peptides. (B) In Pep-11 (Ac-$V_1K_2T_3R_4I_5D_6E_7A_8N_9Q_{10}R_{11}A_{12}T_{13}K_{14}M_{15}L_{16}G_{17}R_{18}R_{19}R_{20}$-NH$_2$), Q_{10} was substituted with A, K, D or N, respectively. (C) In Pep-12 (Ac-$V_1K_2T_3R_4I_5D_6E_7A_8N_9N_{10}R_{11}A_{12}T_{13}K_{14}M_{15}L_{16}G_{17}R_{18}R_{19}R_{20}$-NH$_2$), the N_9 was changed to Q, A, D, E, or K residue, respectively.
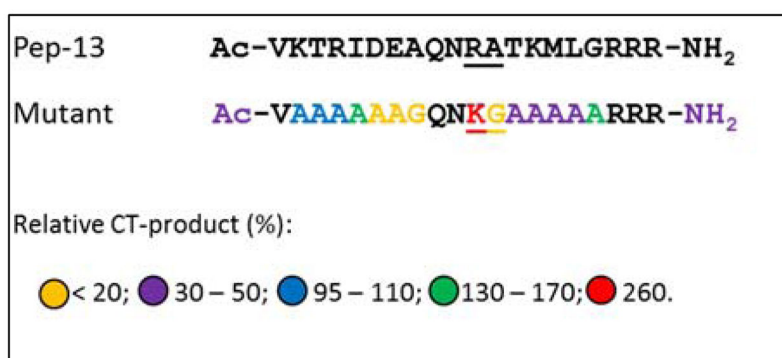
Figure 3.
Cleavage efficiency of single substituted Pep-13 hydrolyzed by 100 mouseLD$_{50}$ BoNT/C. The top row is the primary amino acid sequence of Pep-13. The bottom row represents the point mutated peptide-13. These peptides include two mutants that lack either N-terminal acetylation or C-terminal amidation. Underlined are the residues at the BoNT/C cleavage site.
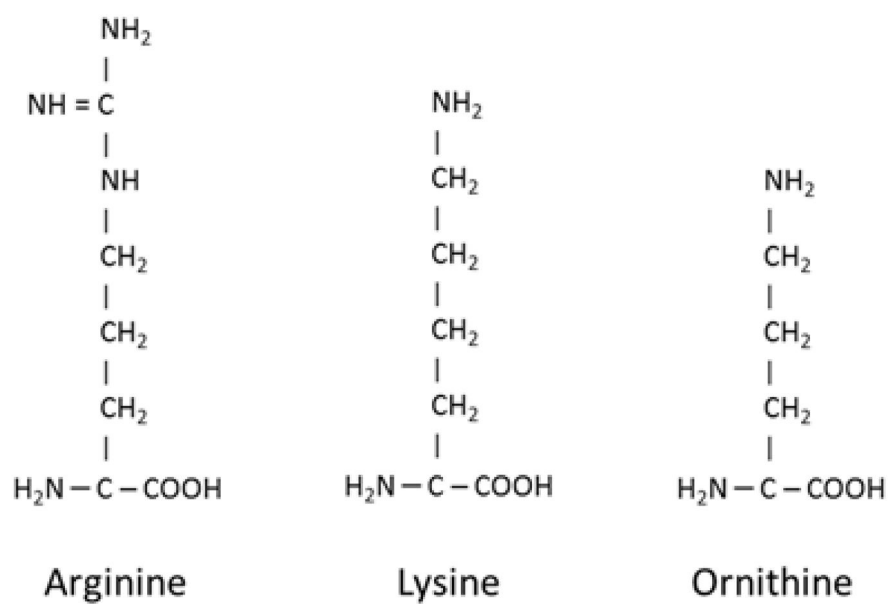
Figure 4.
Structure of the three positively charged amino acids: arginine, lysine and ornithine.
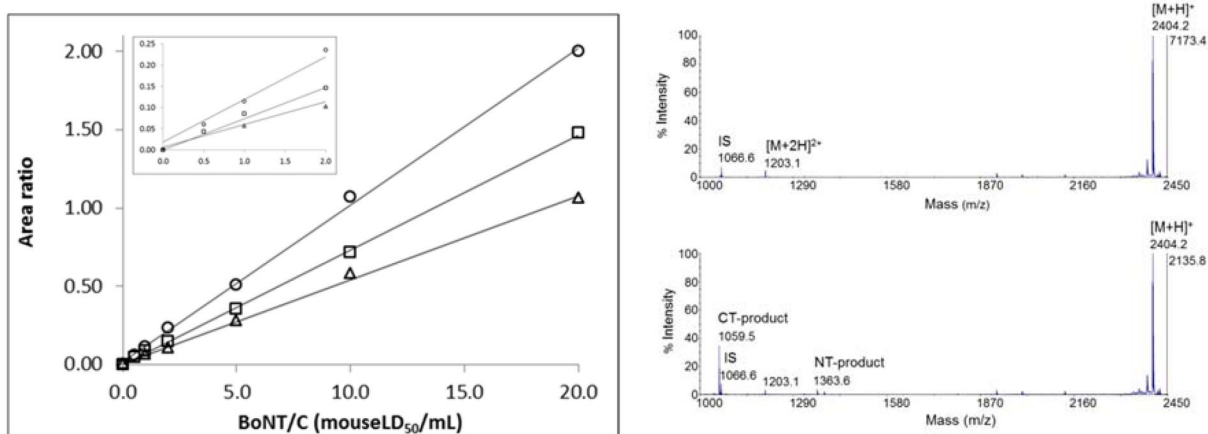
Figure 5.
(left) Product response from the cleavage of Pep-39 by BoNT/C of various concentrations spiked in buffer ($\bigcirc$, R^2=0.999), serum ($\square$, R^2=0.999) and milk ($\triangle$, R^2=0.998). LODs were determined to be 0.5, 0.5 and 1.0 mouseLD$_{50}$/mL for buffer, serum and milk, respectively. Reaction condition: 37°C, 4 hours. (right) Typical mass spectra of the cleavage reactions without (upper) and with (lower) adding toxin.

Table 1

Cleavage of peptide substrates by BoNT/C.[a]

Peptide #	Sequence	Area ratio	
		CT-product	NT-product
Pep-1[b]	Biot-KGSNRTRIDEANQRATRMLGGK-Biot	0.7	0.5
Pep-2	YVERAVSDTKKAVKYQSKARRKKIMII	0.2	0.7
Pep-3	Ac-RIDEANQRATKMLGR-NH$_2$	2.3	1.5
Pep-4	Ac-RIDEANQRATKMRRR-NH$_2$	0.2	0.1
Pep-5	Ac-RIDEANQRATKXRRR-NH$_2$	0.0	0.0
Pep-6	Ac-RIDEANQRATKMLGRRR-NH$_2$	2.6	1.7
Pep-7	Ac-TRIDEANQRATKMLGRRR-NH$_2$	2.5	2.0
Pep-8	Ac-KTRIDEANQRATKMLGRRR-NH$_2$	3.1	1.5
Pep-9	Ac-NKTRIDEANQRATKMLGRRR-NH$_2$	4.5	2.8
Pep-10	Ac-SNKTRIDEANQRATKMLGRRR-NH$_2$	2.6	1.1

[a] cleavage reaction: 20μL reaction solutions including 500 mouseLD$_{50}$ toxin, 100μM peptide and other components. 42°C for 4 hours.

[b] the Pep-1 is the BoNT/C substrate originally used in the Endopep-MS assay. Biot represent biotination modifier. Underline residues represent BoNT/C cleavage site.

Table 2

Effect of single or mutiple substitutions on the cleavage efficiency of modified peptides by BoNT/C.[*]

Peptide #	Sequence	Relative CT-product
Pep-14	Ac-VKTRIDEAQNKATKMLGRRR-NH$_2$	1.0
Pep-15	Ac-VKTRIDEAQNKATKMLARRR-NH$_2$	1.3
Pep-16	Ac-VKTRIDEAQNKATKMLIRRR-NH$_2$	2.3
Pep-17	Ac-VKTRIDEAQNKATKMLFRRR-NH$_2$	1.2
Pep-18	Ac-VKTRIDEAQNKATKMGIRRR-NH$_2$	2.7
Pep-19	Ac-VKTRIDEAQNKATKMKIRRR-NH$_2$	0.0
Pep-20	Ac-VKTRIDEAQNKATKMDIRRR-NH$_2$	0.6
Pep-21	Ac-VKTRIDEAQNKATRMGIRRR-NH$_2$	1.2
Pep-22	Ac-VKTRIDEAQNKASKMGIRRR-NH$_2$	5.2
Pep-23	Ac-VKTRIDEAQNKASRMGIRRR-NH$_2$	1.4
Pep-24	Ac-VKTRADEAQNKASKMGIRRR-NH$_2$	3.9
Pep-25	Ac-VKTRGDEAQNKASKMGIRRR-NH$_2$	0.4
Pep-26	Ac-VKTRLDEAQNKASKMGIRRR-NH$_2$	1.2
Pep-27	Ac-VKTRXDEAQNKASKMGIRRR-NH$_2$	2.2
Pep-28	Ac-VKTRDDEAQNKASKMGIRRR-NH$_2$	0.1
Pep-29	Ac-VKTRKDEAQNKASKMGIRRR-NH$_2$	0.1
Pep-30	Ac-VKTAIDEAQNKASKMGIRRR-NH$_2$	8.4
Pep-31	Ac-VKTKIDEAQNKASKMGIRRR-NH$_2$	8.4
Pep-32	Ac-VKTDIDEAQNKASKMGIRRR-NH$_2$	7.4
Pep-33	Ac-VKTSIDEAQNKASKMGIRRR-NH$_2$	6.3
Pep-34	Ac-VKTNIDEAQNKASKMGIRRR-NH$_2$	8.6
Pep-35	Ac-VKSNIDEAQNKASKMGIRRR-NH$_2$	9.6
Pep-36	Ac-VKYNIDEAQNKASKMGIRRR-NH$_2$	10.1
Pep-37	Ac-VRTNIDEAQNKASKMGIRRR-NH$_2$	7.2
Pep-38	Ac-VRSNIDEAQNKASKMGIRRR-NH$_2$	7.3
Pep-39	Ac-VKYNIDEAQNKASOMGIRRR-NH$_2$	26.2
Pep-40	Ac-VKYNIDEAQNOASKMGIRRR-NH$_2$	12.3
Pep-41	Ac-VKYNIDEAQNOASOMGIRRR-NH$_2$	19.2

[*] Red letters represent substituted residues. X: norleucine; O: ornithine

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Table 3

Cleavage of the current (Pep-1) and newly developed (Pep-39) substrates hydrolyzed by BoNT/C and /CD mosaic. [*]

BoNT	Area ratio (CT-product)	
	Pep-1	**Pep-39**
C1	0.3	70.6
C/D mosaic	0.2	41.3

[*] BoNT/C (100 mouseLD$_{50}$) was directly added into the reaction solution. BoNT/CD mosaic was purified from 2μL culture supernatant spiked PBST buffer via antibody-b eads followed by on-beads reaction. Experiment performed in triplicate.