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Improved Detection of Botulinum Neurotoxin Serotype A by Endopep-MS through Peptide Substrate Modification

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Abstract

Botulinum neurotoxins (BoNTs) are a family of seven toxin serotypes that are the most toxic substances known to man. Intoxication with BoNT causes flaccid paralysis and can lead to death if untreated with serotype specific antibodies. Supportive care, including ventilation, may be necessary. Rapid and sensitive detection of BoNT is necessary for timely clinical confirmation of clinical botulism. Previously, our laboratory developed a fast and sensitive mass spectrometry (MS) method termed the Endopep-MS assay. The BoNT serotypes are rapidly detected and differentiated by extracting the toxin with serotype specific antibodies and detecting the unique and serotype specific cleavage products of peptide substrates that mimic the sequence of the BoNT native targets. To further improve the sensitivity of the Endopep-MS assay, we report here the optimization of the substrate peptide for the detection of BoNT/A. Modifications on the terminal groups of the original peptide substrate with acetylation and amidation significantly improved the detection of BoNT/A cleavage products. The replacement of some internal amino acid residues with single or multiple substitutions led to further improvement. An optimized peptide increased assay sensitivity five fold with toxin spiked into buffer solution or different biological matrices.

Keywords

Botulinum neurotoxin; Detection; Mass spectrometry; Peptide substrate

Introduction

Botulinum neurotoxins (BoNTs) are proteins produced by some species of anaerobic *Clostridium botulinum*, *C. butyricum*, *C. baratii*, and C. argentinense(Schiavo, Matteoli et al. 2000; Turton, Chaddock et al. 2002). Ingestion of BoNTs results in a severe disease known as botulism in humans and animals, and is usually contracted through the intake of contaminated food, bacterial colonization in the gastrointestinal tract of infants, or contact of

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the bacterium with a wound. The extreme toxicity and the ease of preparation make this toxin a likely agent for bioterrorism(Arnon, Schechter et al. 2001).

BoNTs are synthesized as a single protein of 150 kDa which consists of two polypeptide chains linked through a disulfide bond(DasGupta and Dekleva 1990). The heavy chain of approximately 100 kDa is responsible for binding and entering targeted cells. The 50 kDa light chain functions as a metalloprotease domain which cleaves one of three SNARE proteins in peripheral neurons, subsequently inhibiting neurotransmitter release at the neuromuscular junction. Based on their antigenic properties, BoNTs are classified into seven distinct serotypes designated A to G. Human botulism is usually associated with BoNTs of the serotypes A, B, E, and F(Werner, Passaro et al. 2000), with each BoNT serotype cleaving at a specific site of a member protein forming the SNARE complex. BoNT/A, /C, /E cleave SNAP-25 (synaptosomal-associated protein of 25 kDa) while BoNT/B, /D, /F, and /G cleave synaptobrevin 2 (also known as VAMP2). In addition to SNAP-25, BoNT/C also targets another SNARE protein, syntaxin(Wictome and Shone 1998; Schiavo, Matteoli et al. 2000). BoNT/A causes the most severe and longest lasting effects in humans followed by BoNT/B, /F, then /E(Foran, Mohammed et al. 2003).

Rapid and sensitive detection methods are essential in the case of a public health emergency or the use of these toxins in a bioterrorism domain. The major challenge in detecting BoNT and confirming the diagnosis of botulism is that the analytical method must be selective and the required detection limits are in the attomole/mL range. Of the available detection methodologies, the mouse bioassay is the oldest method and is still used for BoNT detection(Kautter and Solomon 1977). Although the method is sensitive (1 mouse LD_{50} is estimated to be 66 attomoles/mL for BoNT /A) and robust, it is labor intensive, time consuming, slow (requiring up to 4 days to report a result), costly, and necessitates the use of a large number of laboratory animals especially to differentiate the serotypes. To overcome these limitations and reduce the use of live animals, a number of *in vitro* methods have been reported as alternative methods to detect BoNTs.(Cai, Singh et al. 2007) The *in vitro* methods fall into a few major categories including immune-based assays, endoprotease activity assays and a combination immunocapture/endoprotease activity assay. Traditional enzyme-linked immunosorbent assay (ELISA) has been adapted for the detection of botulinum neurotoxins, but the method cannot ascertain whether the toxin detected is still functionally active(Ferreira, Maslanka et al. 2003; Ferreira, Eliasberg et al. 2004; Sharma, Ferreira et al. 2006), lack the necessary sensitivity to detect toxins in many clinical samples, and have been shown to suffer from cross reactivity(Ferreira, Maslanka et al. 2003). BoNT's intrinsic enzymatic function provides a basis for the development of an *in vitro* endopeptidase activity assay. Several laboratories have developed activity methods, by measuring the BoNTs' cleavage products of synthetic peptide substrates, using various detection platforms. One of these approaches uses high-performance liquid chromatography to separate and quantify the cleavage products after toxin hydrolysis of the peptide substrates(Rowe, Schmidt et al. 2010). In fluorescence-based methods, a pair of fluorescence donor and acceptor molecules is incorporated into the peptide substrate. The presence and activity of botulinum neurotoxin is detected by monitoring the fluorescence of the toxin cleavage products using fluorescence resonance energy transfer technology

(FRET) (Schmidt and Stafford 2003; Dong, Tepp et al. 2004; Rasooly and Do 2008; Gilmore, Williams et al. 2011; Piazza, Blehert et al. 2011). An *in vitro* assay (ALISSA) using a large immune-sorbent surface area for toxin capture and enrichment and a fluorogenic peptide substrate for activity measurement has been reported recently and high sensitive detection of BoNTs in complex biological matrices can be achieved by this method (Bagramyan, Barash et al. 2008; Bagramyan and Kalkum 2011).

Similarly, a mass spectrometry (MS) based *in vitro* activity assay (Endopep-MS method) has been developed in our laboratory and has been the first *in vitro* method to prove effective in clinical samples such as serum and stool (scheme 1) (Barr, Moura et al. 2005; Kalb, Garcia Rodriguez et al. 2010; Boyer, Gallegos Candela et al. 2011). In this method, affinity enriched BoNT was incubated with a peptide substrate derived from or mimicking the active-site containing region of a BoNT's natural target. The detection of specific cleavage products by MS can be used to determine the presence of BoNT in clinical and food samples and determine the serotype. All seven serotypes of BoNT toxin can be rapidly and sensitively detected and differentiated by this method, using different peptide substrates combined with matrix-assisted laser desorption/ionization (MALDI) or electrospray ionization (ESI) mass spectrometry techniques. In addition, the Endopep-MS method can detect BoNT in various sample matrices at detection limits below the mouse bioassay. Recently, we have reported an improvement for the detection of BoNT present in stool matrix, a problematic biological complex for *in vitro* assays (Wang, Baudys et al. 2011). Non-specific binding of endogenous proteases in stool samples to the toxin-antibody-bead complex interferes with the assay performance by cleaving peptide substrates, thereby reducing assay sensitivity. We have demonstrated that stool proteases can be reduced dramatically by applying a wash step with extremely high salt concentrations (2 molar). The detection limits in serum for BoNT/A were 1.0 mouse i.p. LD_{50}/mL for BoNT/A, 0.1 mLD_{50}/mL for BoNT/B, 0.2 mLD_{50}/mL for BoNT/E, and 0.1 mLD_{50}/mL for BoNT/F (Kalb, Garcia Rodriguez et al. 2010) and in stool 1.0 mLD_{50}/mL for BoNT /A(Wang, Baudys et al. 2011), and 1.0, 0.2, 1.0 mLD_{50}/mL for BoNT/B, /E, /F, respectively(Kalb, Moura et al. 2006).

We report here a further improvement on the sensitivity of the Endopep-MS assay by optimizing the peptide substrate currently used for the detection of BoNT/A. Through a modification of the peptide termini as well as modification of the internal amino acid residues of respective synthesized peptides, a significant sensitivity increase in the mass spectrometric detection of BoNT/A cleavage products can be achieved.

Materials and Methods

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). The complex forms of the botulinum neurotoxin serotypes A, B, C, D, E, F, and G were obtained from Metabiologics (Madison, WI). Monoclonal antibodies were provided by Dr. James Marks at the University of California, San Francisco. Protein G coupled Dynabeads were purchased

from Invitrogen (Lake Success, NY). Peptide substrate, Pep-1, was synthesized by Los Alamos National Laboratory (Los Alamos, NM). Milk (2%) was purchased from a local grocery store. Cell culture supernatant was provided by the National Botulism Surveillance and Reference Laboratory at the CDC. Serum and stool extracts were collected from anonymous donors, and no demographic information was obtained. Because samples were collected without any identifiers or demographic information, these collections were exempt from human subjects review.

Peptide synthesis

All peptides except Pep-1 were prepared in house by a solid phase peptide synthesis method using Fmoc chemistry on a Tribute peptide synthesizer (Protein Technologies, Tucson, AZ, USA). Peptides were cleaved and deblocked using a reagent mixture of 95% trifluroacetic acid (TFA):2% water: 2% anisole:1% ethanedithiol and purified by reversed-phase HPLC using a water:acetonitrile:0.1% TFA 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.

Endopep-MS assay

In-solution or on-bead Endopep-MS assays were carried out as previously described (Wang, Baudys et al. 2011). 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 37°C for 1 or 4 hrs. For the in-solution assays without antibody-coated beads, various concentrations of BoNT as indicated in the text were directly added into the reaction mixture. For samples including complex matrices, the toxin spiked in matrix was first purified by antibodies immobilized on Protein-G beads followed by an activity assay as described previously (Wang, Baudys et al. 2011).

After reaction, 2 μL of the supernatant was mixed with 20 μL of α-cyano-4-hydroxy cinnamic acid (CHCA) at 5 mg/mL in 50% acetonitrile/0.1% TFA/1 mM ammonium citrate; 2 μL of a 1 μM internal standard peptide (IS, 1480.7Da) 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 N-terminal product (A_{NT}) or C-terminal product (A_{CT}) versus an internal standard (A_{IS}).

MS detection

Each sample was spotted in triplicate on a MALDI plate and analyzed on a 4800 or 4800plus MALDI-TOF instrument (Applied Biosystems, Framingham, MA). Mass spectra of each spot were obtained by scanning from 800 to 3000 *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.

Results and discussion

Terminal modifications

The peptide substrate (Pep-1) most-recently used for BoNT/A detection in Endopep-MS assay was derived from a special region of the toxin's native substrate, SNAP-25, that includes a BoNT/A active site (Scheme 2). Several internal residues and the terminal structure of this peptide were modified from the corresponding sequence of the $SNAP\text{-}25_{185\text{-}260}$ based on previous reported study (Schmidt and Bostian 1997) and experience gained during the initial development of the assay(Barr, Moura et al. 2005; Boyer, Moura et al. 2005). To evaluate whether higher sensitivities of the assay could be accomplished by further optimization of this substrate, we re-examined its function and primary structure.

Two strategies are usually applied for altering a peptide's chemical and physical properties: terminal modification and internal residue substitution. We initially explored the modifications on Pep-1's C-terminal and N-terminal structures. In comparison with the sequence of $SNAP\text{-}25_{185\text{-}260}$, biotin groups were placed on both ends of Pep-1 for the purposes of facilitating peptide purification or cleavage product extraction. To evaluate the function of those biotin groups toward substrate efficiency and cleavage product detection, a new peptide (Pep-2) terminated with non-biotin, unprotected lysine residues was designed and tested in the activity assay. Figure 1 shows that removal of the biotin groups from both N- and C-terminal lysine residues resulted in an approximately 2 fold increase in signal detection of both C-terminal product (CT-product) and N-terminal product (NT-product) after a proteolytic reaction, indicating that omitting biotinylation on the terminal lysines improves assay sensitivity. Given the fact that the non-biotinylated Pep-2 could be purified as desired by reverse phase HPLC without significant difficulty, biotin was no longer considered as a necessary tag to the peptides prepared during the substrate optimization.

A chemically synthesized peptide usually carries free amino and carboxy termini. N-terminal acetylation and C-terminal amidation are two common modifications on synthetic peptides for the development of peptide substrates or inhibitors (Shalev, Mor et al. 2002; Nguyen, Chau et al. 2010). In contrast to unprotected amino and carboxy termini, acetylated N-terminus and amidated C-terminus avoids unnatural charges at the ends of a peptide and in some cases generates a close mimic to native proteins, which can increase the biological activity of a peptide and protect the peptide from nonspecific cleavages by exopeptidases. Two new peptides were designed to test the effect of these terminal modifications, one with a C-terminal amidation (Pep-3) and another with modifications on both termini (Pep-4). While slightly increased cleavage products were detected from the amidated Pep-3, more than 50% improvement was achieved by Pep-4 with both acetylation and amidation in comparison to the unprotected Pep-2 (Fig. 1). These results demonstrate that modifications of the N-terminal acetylation and C-terminal amidation on the peptide substrate enhance the detection of BoNT/A cleavage products.

Three factors should be considered to affect the detection of the BoNT cleavage products from a peptide substrate measured by mass spectrometry: substrate binding affinity, cleavage efficiency, and ionization efficiency. We define here the substrate efficiency as the

combined effect of the binding affinity and cleavage efficiency. In the MS analysis of two sequence-different peptides at identical concentration, a higher mass signal will be yielded by the peptide with higher ionization efficiency. In comparing Pep-3 and Pep-4, increase in the detection of the CT-products of the Pep-4 is likely caused by enhanced substrate efficiency of the latter one because both peptides should produce an identical CT-product. Similar results were observed in comparing the pair of Pep-2 and Pep-3. This implies that the N-terminal acetylation and C-terminal amidation improved the substrate efficiency and thus enhance the sensitivity of the activity assay.

The next step was to evaluate the role of the two lysine residues at the termini of a peptide substrate. Basic amino acid residues such as lysine, arginine and histidine in a peptide can promote its ionization efficiency in positive ion mode mass spectrometric analysis due to the high proton affinity of these amino acids. Pep-1 contains lysine residues at both C- and N-termini where the C-terminal lysine is not present in the original sequence of $SNAP\text{-}25_{185\text{-}206}$ and was introduced to allow the incorporation of a biotin group. Pep-5 was prepared by deleting the two terminal lysine residues of the Pep-4. This modification, however, significantly reduced the detection of both CT- and NT-products to the amounts similar to Pep-1. This is not surprising because removal of the lysines results in decreased ionization efficiency. The benefit gained from removing biotin groups, N-terminal acetylation, and C-terminal amidation were nearly lost after removing two basic residues. In contrast, maintaining an N-terminal lysine residue in Pep-6 elevated the detection of the NT-product while the CT-product remained unchanged. Since an identical CT-product can be produced from both Pep-5 and Pep-6, this data suggests that basic residues at the termini of the latter plays a positive role by promoting the ionization efficiency of the cleavage products and thus increasing the MS signals.

Arginine is another naturally occurring basic amino acid. It has been previously reported that an arginine-terminated tryptic peptide often produce a more intense signal than its lysine terminated counterpart in MALDI mass spectra, presumably due to the higher basicity of its side chain (Krause, Wenschuh et al. 1999). To examine the effect of arginine terminated substrate on the detection of BoNT cleavage products, two peptides were designed and tested incorporating arginine at the termini. Pep-7 has an N-terminal lysine and C-terminal arginine while Pep-8 contains arginine residues at either end. It was observed, however, that the two peptides did not produce a significant change in the mass signals of CT- and NT-products compared with lysine terminated Pep-4, suggesting that a terminal lysine and arginine residue have a similar effect on the detection of cleavage products by MALDI mass spectrometry.

Internal residue substitutions

We then explored the substitution of internal amino acid residues using a positional scanning synthetic combinatorial library approach in an attempt to further optimize the peptide substrate. For this purpose, Pep-8, Ac-$R_1G_2S_3N_4R_5T_6R_7I_8D_9Q_{10}G_{11}N_{12}Q_{13}R_{14}A_{15}T_{16}R_{17}X_{18}L_{19}G_{20}G_{21}R_{22}\text{-}NH_2$ (X = norleucine), was used as a template for the construction of peptide libraries. As previously indicated, BoNT/A cleaves the peptide bond between Q_{13} and R_{14}. We initially selected four

internal arginines and their flanking residues for library construction because those positions were found to be the most susceptible to hydrolysis by non-specific proteases during the analysis of stool extracts (data not shown). Hundreds of singly substituted peptides were prepared where the R_5, T_6, R_7, I_8, Q_{13}, R_{14}, A_{15}, R_{17}, or X_{18} in the Pep-8 was replaced with natural occurring amino acids, either all or selected ones, respectively. Table 1 shows the results of some selected peptides that yielded relative responses to BoNT/A cleavage within or above 90% production of either C- or N-terminal cleavage fragments from the template peptide (Pep-8). Substitutions of the basic residues, R_5 or R_7, with another basic residue, lysine, in the Pep-9 or Pep-11, respectively, produced similar or a slightly higher detection of the cleavage products. In contrast, replacement of R_7 with a nonpolar valine residue in Pep-12 reduced NT-product detection by 60% while the detection of CT-product remained almost unchanged compared to the cleavage of the Pep-11. Similar detection of the CT-product but different detection for the NT-product from Pep-11 and Pep-12 implied that the two peptides possess similar substrate efficiency but a lysine to valine replacement lowers the ionization efficiency of Pep-12's NT-product. These data implied that the basic or positive charge residue, arginine or lysine, at the positions 5 and 7 plays a role in promoting high ionization efficiency of the NT-product in mass spectrometric analysis. In addition, the hydrolysis of Pep-10, -13, -14, and -15 revealed that the polar residues of T_6 and Q_{10} could be replaced with nonpolar residues of alanine, proline or norleucine. Substituting T_6 with nonpolar proline residue in the Pep-10 resulted in a 30% increase in the measurement of CT-product whereas replacing glutamine at the position 10 with alanine or proline led to significant increase in CT-product detection as well. Furthermore, the substitutions of the amino acid residues R_{14}, A_{15}, R_{17}, or X_{18} did not yield peptide substrates with the detection of cleavage products (data not shown), implying that the region C-terminal to the cleavage site had a lower tolerance for substitution than the N-terminal region. The inherent mechanism of this phenomenon needs further study.

It is interesting to observe that the asparagine at the position 13 (Q_{13}), a residue that forms a scission bond with R_{14}, could be substituted by the smallest amino acid residue, glycine. This substitution only caused a 10% and 30% reduction in the detection of the CT-product and NT-product, respectively, suggesting that the side chain of the asparagine at this position of a substrate might only play a limited role or might not be directly involved in the process of BoNT proteolysis. Similar results have been reported previously in mutagenesis analyses, where the single point mutation of scissile bond-forming Gln to Met, Ser, Thr, Glu, Lys or Ala in a recombinant full length or fragment of SNAP-25 caused no or slight reduction of BoNT/A cleavage (Vaidyanathan, Yoshino et al. 1999; Chen and Barbieri 2006).

Evaluation of the combination of the effective single substitutions described above was explored for further optimization of the peptide substrate for BoNT/A. For this purpose, a number of new peptides bearing multiple substitutions were synthesized. Two to five substitutions with some specific amino acid residues as described above were incorporated into positions 5, 6, 7, 10, or 13 of the template peptide, Pep-8, in various combinations. Activity measurements revealed that seven of these multi-substituted peptides led to further improvement in the detection of either CT-product, or NT-product, or both products in comparison with a single-residue replacement (Table 1). For example, the NT-product of

Pep-17 (1.6 fold increase in comparing to Pep-8) with a double substitution of K_5 and P_6 was detected at higher levels than the NT-product yielded from Pep-9 with K_5 (1.2) and Pep-10 with P_6 (0.9) alone; Significant increase in the detection of both CT- and NT-products was observed with Pep-19 incorporating a double mutation of P_6 and A_{10} compared to Pep-10 with only the P_6 and the Pep-13 with only the A_{10} substitution.

To find an optimal peptide substrate for the detection of BoNT/A at low concentrations (attomole/mL) by the Endopep-MS assay, we selected five peptides that produced high cleavage products (Pep-14, -17, -18, -19, and -21) for further analysis. The formation of the NT-products measured under low toxin concentration (0 - 0.1 U, 1U = 1 mLD_{50}) is displayed in Figure 2. The data revealed that all of the tested peptides produced detectable cleavage products at the low neurotoxin concentrations; however the linear regression fitting of each data set varied. Pep-21 resulted in the best linearity with an R-squared value of 0.99; this peptide was chosen as the optimal substrate for additional experiments described below.

Specificity of the new substrate

To examine the specificity of optimized peptide substrate, we examined the reaction of the new BoNT/A substrate, Pep-21, with other serotypes (B-G). High dose of the BoNTs (> 1000 U) except BoNT/A (10U) was used in order to detect any cleavage product resulting from low efficiency proteolysis. Figure 3 shows the mass spectra of the reaction solution after incubation of Pep-21 with each of the seven BoNT serotypes. As expected, hydrolysis of the peptide by BoNT/A produced two new mass peaks at 998.6 Da and 1427.6 Da, corresponding to the C- and N-terminal cleavage products, respectively. In contrast, incubation of the peptide with BoNT/B, /C, /D, /E, /F, or /G even at high concentrations for prolonged incubation times (4 hours or overnight at 37°C or 42°C) did not result in any new mass peaks, suggesting that the peptide was not hydrolyzed by any of the BoNT serotypes except type A, neither at the BoNT/A cleavage site nor other peptide bonds within the structure. It should be noted that there were no cleavage products observed by BoNT/C under the conditions used although the peptide bears a putative BoNT/C scissile bond between R_{14} and A_{15}. This was not a surprising observation because previous studies have demonstrated that BoNT/C possess very low *in vitro* cleavage efficiency on SNAP-25, one of the two natural substrates, from which our peptide substrate is derived (Foran, Lawrence et al. 1996). We have also found in previous method development that only the peptide derived from another BoNT/C native substrate, syntaxin 2A, was an effective substrate in the Endopep-MS assay(Moura, Terilli et al. 2011). Together, these data validate that the optimal peptide was a specific substrate of the BoNT serotype A but not the other serotypes.

In addition to the seven distinct serotypes, many BoNT subtypes have been recently identified based on their sequence variations and antigenic differences(Hill, Smith et al. 2007). For the BoNT/A serotype, genetic analysis has identified five subtypes including A1 to A5 (Arndt, Jacobson et al. 2006; Carter, Paul et al. 2009; Jacobson, Lin et al. 2011). All data described above were obtained using the toxin subtype of BoNT/A1. To explore the capability of Pep-21 as a general substrate of BoNT/A, we examined the proteolysis of the Pep-21 by another commercially-available subtype, BoNT/A2. Figure 4 shows the data obtained from the enzymatic hydrolysis of the new peptide substrate by BoNT/A2 and A1 as

well. The detection of the specific CT- and NT-products confirmed the optimal peptide as an effective substrate of BoNT/A2 subtype. Compared to the old substrate (Pep-1), the optimized Pep-21 led to an approximate 5 fold improvement in assay sensitivity for BoNT/A2 detection, similar to the extent of improvement for BoNT/A1. Taken together, the optimal peptide was demonstrated as a specific and effective substrate for the Endopep-MS assay for the detection of the serotype A botulinum neurotoxin.

Complex biological matrix

Botulinum neurotoxins are usually present in complex biological matrices. To evaluate the performance of the new substrate in the assay for detecting BoNT/A present in complex samples, we evaluated the specific cleavage of Pep-21 using BoNT/A toxin spiked into four common sample matrices: serum, stool extract, cell culture supernatant, and milk. The assays were conducted following the general procedure of the Endopep-MS method for clinical samples (Barr, Moura et al. 2005; Kalb, Moura et al. 2006; Wang, Baudys et al. 2011). The toxin was first enriched from a spiked matrix by BoNT/A-specific antibody immobilized on magnetic beads followed by the cleavage reaction in a solution containing toxin bound to beads, Pep-21 and other reaction components. The mass spectra measured from finished reaction solutions are displayed in Figure 5. For the purposes of comparison, the results of BoNT/A cleavage of the template peptide, Pep-1, under identical conditions is shown as well. The observation of two cleavage products at 998.7 Da and 1426.8 Da obtained from all spiking matrices (Fig. 5, A to D) validated the effective cleavage of the Pep-21 by the toxin extracted from a number of biological matrices. Again, the relative intensities of the product peaks generated from the cleavage of the Pep-21 by the toxin in each matrix were about 5-fold higher than those using the old substrate in a corresponding sample matrix (Fig. 5, E to H), further verifying the improvements in assay sensitivity accomplished by the substrate optimization. In addition, the similar extent of signal increase in assay sensitivity revealed that the improvement is independent of the type of sample matrix.

Stool is a common clinical specimen collected from botulism patients for disease diagnosis. It was observed in our and other laboratories that stool proteases other than botulinum neurotoxins can cause non-specific cleavages of shortened BoNT substrate peptides; this makes the stool sample one of the most problematic matrices to deal with in the development of an *in vitro* activity assay. We have previously reported an effective method to remove stool proteases nonspecifically bound to the antibody coated magnetic beads used for toxin enrichment (Wang, Baudys et al. 2011). The results from spiked stool samples described above demonstrate that the use of the optimal peptide (Pep-21) further improved assay sensitivity. To determine the limit of detection of the Endopep-MS assay for stool samples using the new peptide substrate, we examined the detection of the toxin at various concentration (0 – 5 U) spiked in 0.5 mL of stool extracts. As shown in Figure 6, the signal intensities of the CT and NT products of the optimized peptide substrate decreased with decreasing toxin concentration. Although some nonspecific cleavage in the blank control sample was detected in the absence of the toxin, the signals of the CT- (S/N = 3.8) and NT-products (S/N = 4.4) at 0.25 U of the toxin exceeded three times the background signal detected in the blank (0U) sample, and therefore are considered as the new LOD of the

Endopep-MS assay for stool matrices. This value (equivalent of 0.5 U/mL) indicates the improvement in the Endopep-MS assay compared to the old peptide substrate, Pep-1 (2.0 U/mL in stool samples under the same experiment condition), and is now more sensitive than the mouse bioassay in stool.

Conclusion

In this study we optimized a peptide substrate used for BoNT/A detection in the Endopep-MS assay using Pep-1 as a template. Our data demonstrates that two terminal biotin groups incorporated in the peptide had an adverse impact on the effective detection of the cleavage products. Improved detection of the BoNT/A cleavage products was achieved by the removal of the biotins from both ends of the template peptide. Further improvements were accomplished by generating a C-terminal amide peptide, and protecting the N-terminus with an acetyl group. In addition to terminal structure modifications, we have applied a positional scanning synthetic combinatorial library approach to examine the effect of substituting internal amino acid residues in the template peptide. Our study demonstrates that an approximately fivefold improvement in the sensitivity of the Endopep-MS assay is achieved after peptide optimization. In addition, the substrate specificity was addressed by examining the incubation of the optimal peptide with other BoNT serotypes and two BoNT/A subtypes. Furthermore, the improvement with the optimal peptide was observed in four different biological sample matrices. The limit of detection of BoNT/A in a stool sample reached to 0.5U/mL using the optimal peptide.

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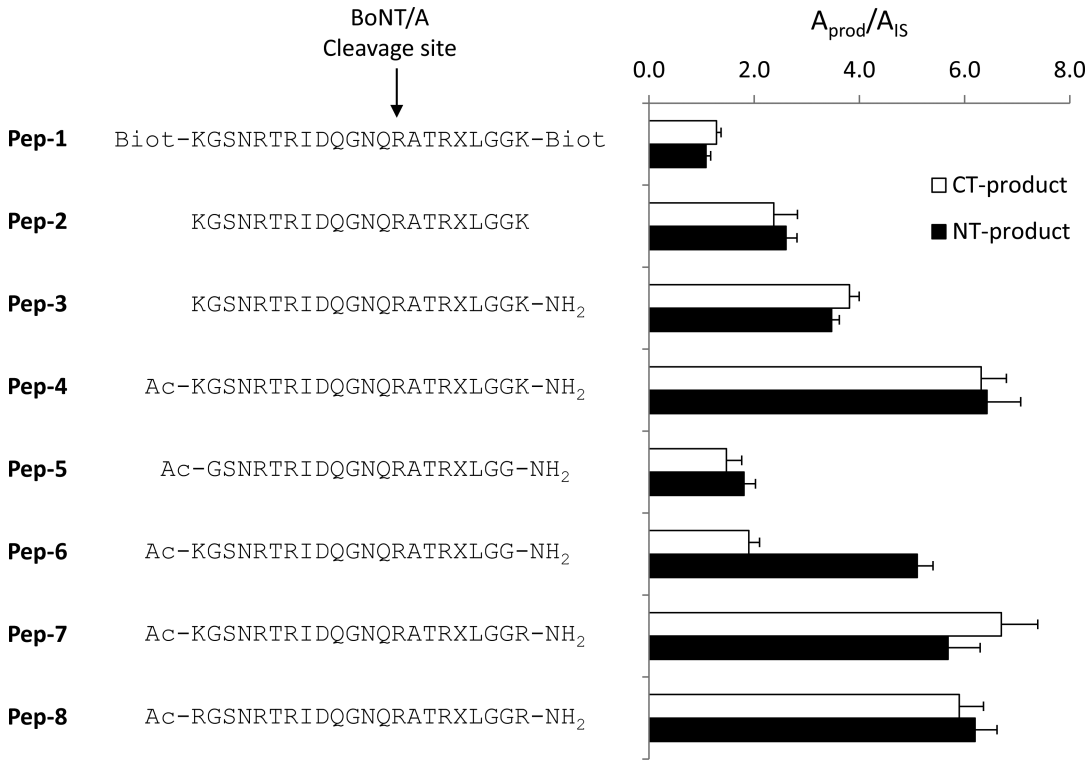


Figure 1.

Effect of modifications of the C-, and N-terminal groups or residues of the peptide substrate currently used in Endopep-MS assay (Pep-1). Conditions: 0.1 mM peptide, 20 U (mLD_{50}) BoNT/A, 37 °C, 4 hours.

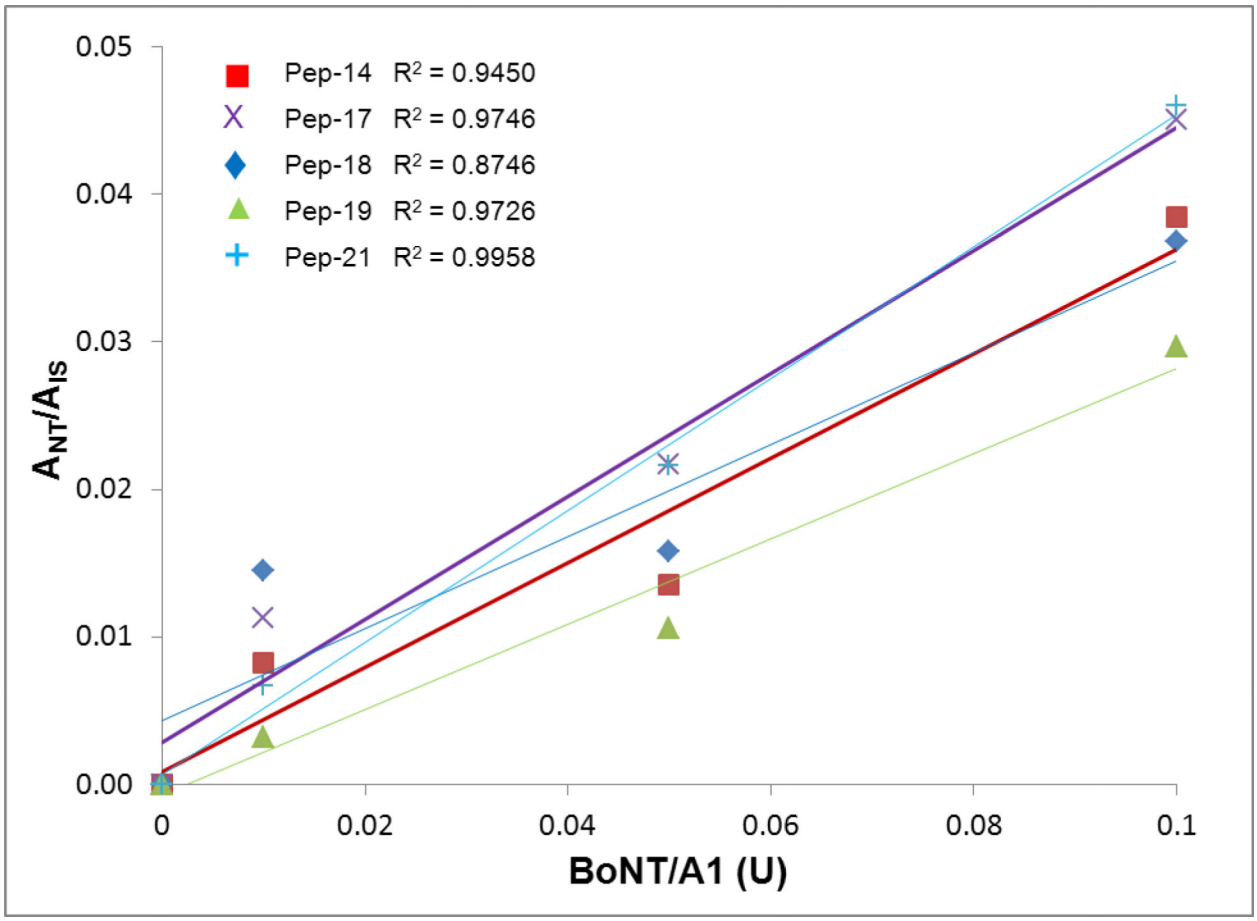


Figure 2.

Formation of the cleavage product of five selected peptides by BoNT/A (0 – 0.1 U). Noise level of the product measured in the absence of the toxin (0 U) was subtracted from all data.

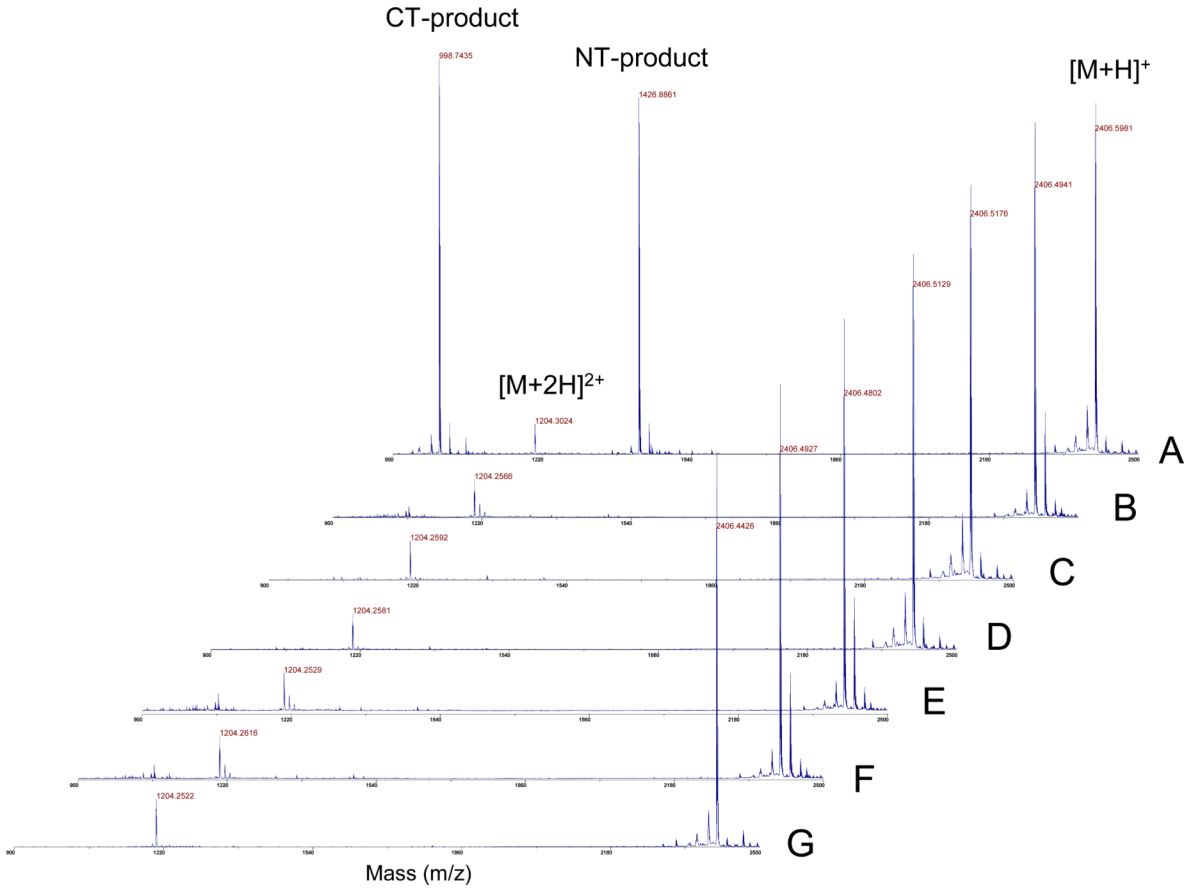


Figure 3.

Mass spectra after the incubation of the Pep-21 with seven BoNT serotypes, A through G, respectively.

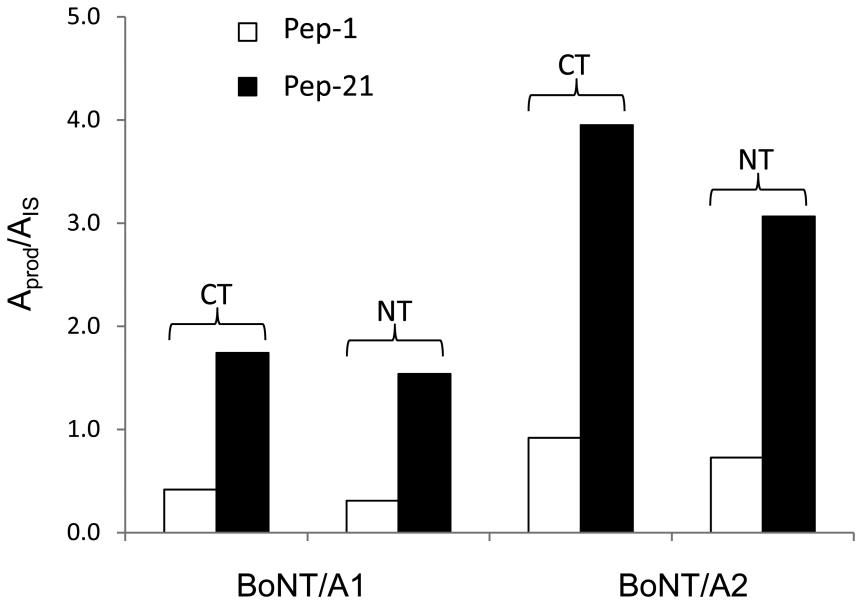


Figure 4.
Comparison of the hydrolysis products of the Pep-21 and Pep-1 cleaved by BoNT/A1 (100 U) and /A2 (400 U).

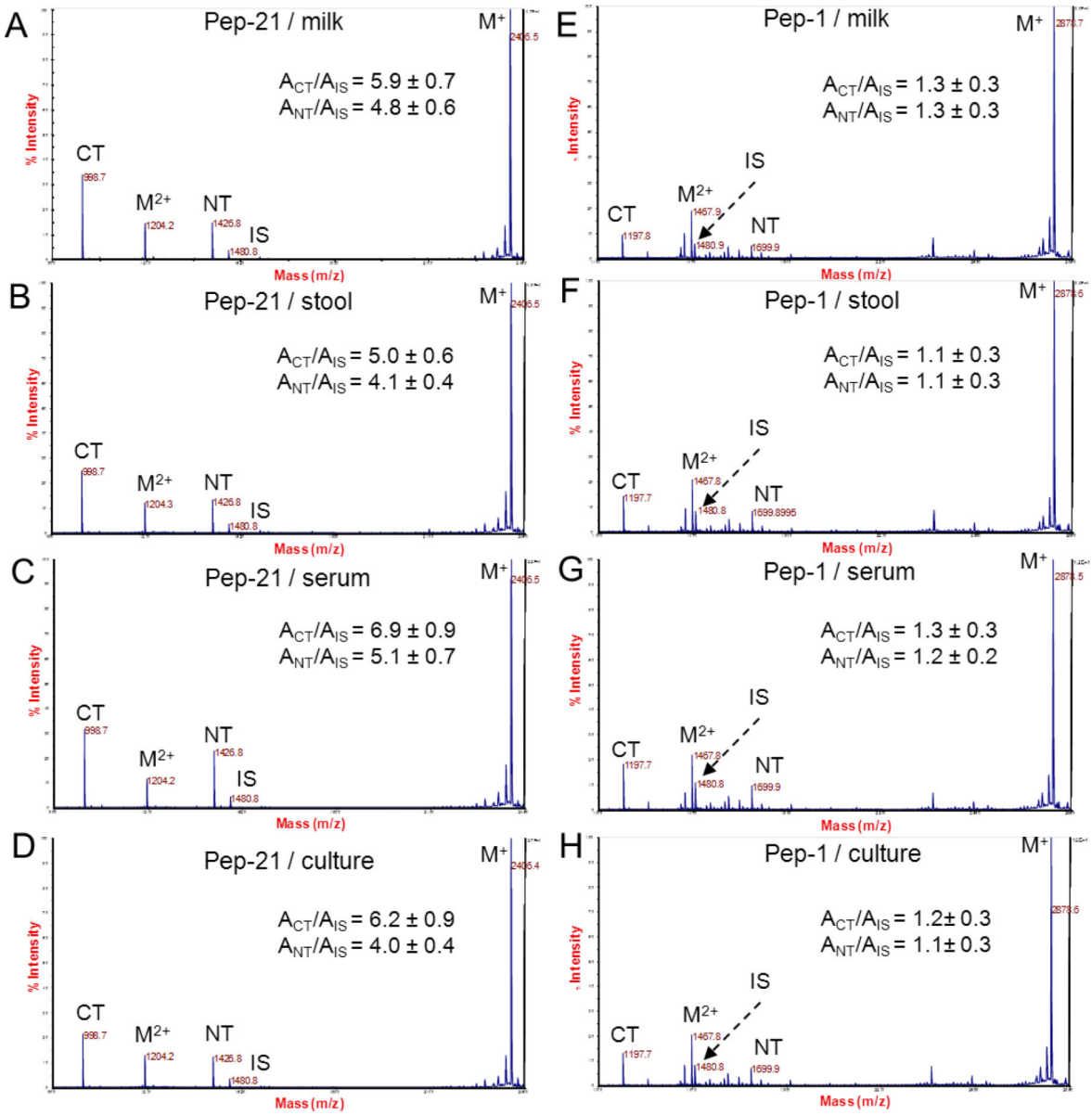


Figure 5.

Cleavage of the Pep-21 (left panel) and Pep-1 (right panel) by 20 U BoNT/A spiked in 0.5 mL of milk (A, E), stool extract (B, F), serum (C, G), or cell culture supernatant (D, H).

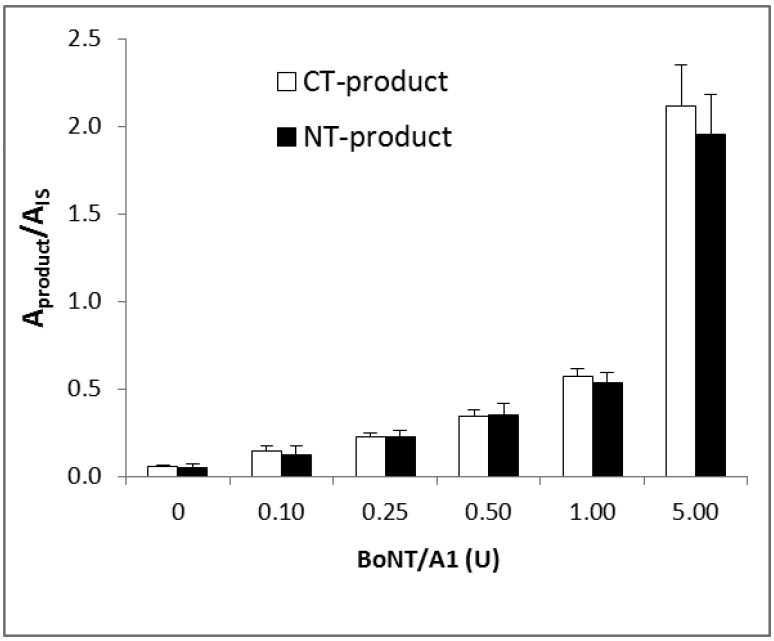
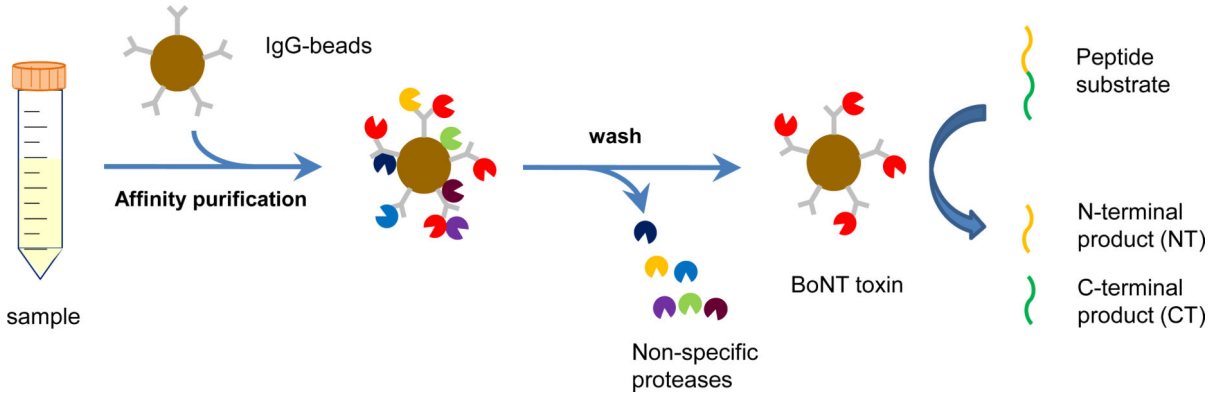
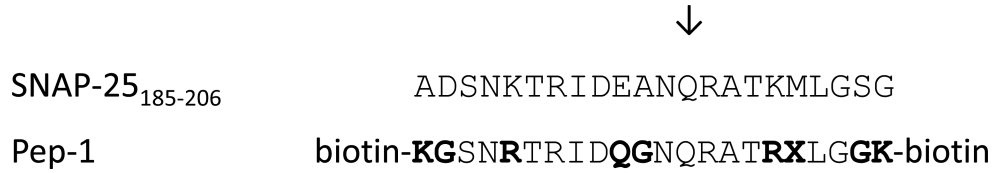


Figure 6.
Cleavage of the Pep-21 by various concentrations of BoNT/A spiked in 0.5 mL of stool extract.



Scheme 1.
Endopep-MS assay for the detection of BoNTs using a peptide substrate.



Scheme 2.

The sequence of the Pep-1 peptide and its corresponding region of the SNAP-25, a native substrate of BoNT/A. X represent norleucine. The arrow indicates the BoNT/A cleavage site. Bold letters in Pep-1 represent the amino acid residues different from the native sequence of SNAP-25.

Table 1

Relative response of selected peptide substrates to BoNT/A cleavage. Condition: 0.1mM peptide and 10-40U toxin in 20μL reaction solution at 37°C for 1, 2, or 4 hours. Bolded letter represents substituted residue. X: neoleucine.

Peptide	Sequence	CT-product	NT-product
Pep-8	Ac-RGSNRTRIDQGNQRATRXLGGR-NH_2	1.0	1.0
Pep-9	Ac-RGSN**K**TRIDQGNQRATRXLGGR-NH_2	0.9	1.2
Pep-10	Ac-RGSNR**P**RIDQGNQRATRXLGGR-NH_2	1.3	0.9
Pep-11	Ac-RGSNRT**K**IDQGNQRATRXLGGR-NH_2	0.9	1.0
Pep-12	Ac-RGSNRT**V**IDQGNQRATRXLGGR-NH_2	0.9	0.4
Pep-13	Ac-RGSNRTRID**A**GNQRATRXLGGR-NH_2	1.2	0.7
Pep-14	Ac-RGSNRTRID**P**GNQRATRXLGGR-NH_2	1.4	1.0
Pep-15	Ac-RGSNRTRID**X**GNQRATRXLGGR-NH_2	1.0	0.7
Pep-16	Ac-RGSNRTRIDQGN**G**RATRXLGGR-NH_2	0.9	0.7
Pep-17	Ac-RGSN**KP**RIDQGNQRATRXLGGR-NH_2	1.3	1.6
Pep-18	Ac-RGSNR**PK**IDQGNQRATRXLGGR-NH_2	1.3	1.3
Pep-19	Ac-RGSNR**P**RID**A**GNQRATRXLGGR-NH_2	1.7	1.2
Pep-20	Ac-RGSN**KPK**IDQGNQRATRXLGGR-NH_2	1.1	1.3
Pep-21	Ac-RGSN**KPK**ID**A**GNQRATRXLGGR-NH_2	1.4	1.4
Pep-22	Ac-RGSN**KPK**ID**P**GNQRATRXLGGR-NH_2	1.4	1.1
Pep-23	Ac-RGSN**KPK**ID**A**GN**G**RATRXLGGR-NH_2	1.3	1.2