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Optimization of peptide substrates for botulinum neurotoxin E improves detection sensitivity in the Endopep-MS assay

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Abstract

Botulinum neurotoxins (BoNTs) produced by *Clostridium botulinum* are the most poisonous substances known to mankind. It is essential to have a simple, quick and sensitive method for the detection and quantification of botulinum toxin in various media, including complex biological matrices. Our laboratory has developed a mass spectrometry-based Endopep-MS assay that is able to rapidly detect and differentiate all types of BoNTs by extracting the toxin with specific antibodies and detecting the unique cleavage products of peptide substrates. Botulinum neurotoxin type E (BoNT/E) is a member of a family of seven distinctive BoNT serotypes (A to G) and is the causative agent of botulism in both humans and animals. To improve the sensitivity of the Endopep-MS assay, we report here the development of novel peptide substrates for the detection of BoNT/E activity through systematic and comprehensive approaches. Our data demonstrate that several optimal peptides could accomplish 500-fold improvement in sensitivity compared to the current substrate for the detection of both not trypsin-activated and trypsin-activated BoNT/E toxin complexes. A limit of detection of 0.1 mouseLD_{50}/mL was achieved using the novel peptide substrate in the assay to detect not trypsin-activated BoNT/E complex spiked in serum, stool and food samples.

Keywords

botulinum neurotoxin; botulism; mass spectrometry; peptide substrate

Introduction

The neurotoxins produced by *Clostridium botulinum* (Botulinum Neurotoxins, BoNT) are the most poisonous substances known to mankind. The life threatening diseases caused by these toxins include food-borne botulism, infant botulism, wound botulism, and adult intestinal colonization[1]. BoNTs also constitute a potential biological weapon as they are easy to produce[2]. On the other hand, botulinum toxins have been used for therapeutic or

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aesthetic applications[3]. For all these applications, it is essential to have a simple, quick and sensitive method for the detection and quantification of botulinum toxin in various media, including complex biological matrices.

The botulinum neurotoxins are synthesized as single chain polypeptides of 150 kDa which undergo proteolytic cleavage to generate active holotoxins constituted of two protein sub-units linked by a disulfide bond: a heavy chain (100 kDa) involved in target binding and a light chain (50 kDa) responsible for the toxicity through its peptidase activity[4]. In fact, the BoNTs belong to a family of zinc-dependent metallopeptidases. They cleave neuronal proteins involved in the exocytosis of neurotransmitters, such as SNAP-25, synaptobrevin and syntaxin, at the site specific to each toxin[5; 6]. This cleavage consequently blocks the release of neurotransmitter molecules at the neuromuscular junction ultimately leading to flaccid paralysis of muscle activity.

The neurotoxin type E (BoNT/E) forms part of a family of seven confirmed, related serotypes (botulinum toxins A to G) produced by different strains of *Clostridium botulinum[7]*. BoNT/E is a neurotoxin that causes botulism in both humans and animals. The most common intoxication by toxin type E is associated with eating contaminated fish [8; 9]. BoNT/E is unique because it is released from the bacterium as a single chain and cleaved into an active di-chain form by unidentified host cell proteases or other exogenous proteases such as trypsin [10; 11]. Activation of a single chain BoNT/E by trypsin leads to an approximately two orders of magnitude more potent neurotoxin than the single chain molecule [10; 12].

The mouse bioassay is the historic method for the detection of botulinum toxins[2]. It is very sensitive, detecting as little as approximately 10 picograms of active toxin which is defined as 1 mouse LD_{50} (mLD_{50}), but the assay can be slow in obtaining final results and requires the sacrifice of many animals. Therefore, much effort has been undertaken to develop alternative *in vitro* endopeptidase activity assays based on BoNT's intrinsic enzymatic function. Several laboratories, including ours, have developed activity methods, by measuring the BoNTs' cleavage products using synthetic peptide substrates with various detection platforms[13].

BoNT/E cleaves specifically one of the SNARE complex proteins, SNAP-25, at the Arg^{180}-Ile^{181} bond [14]. Montecucco and coworkers revealed that the minimal length for proteolysis of SNAP-25 by BoNT/E includes a SNARE motif starting from Ala^{141} [15]. Binz and coworkers defined the minimal essential domain of SNAP-25 required for cleavage by BoNT/E as Ile^{156}-Asp^{186} [16]. Through saturation mutagenesis and deletion mapping, Barbieri and Chen defined a short optimal cleavage domain of Met^{167}-Asp^{186}, where the subsite of Met^{167}-Thr^{173} was considered as a binding domain contributing to substrate affinity [17; 18]. These findings led to the development of peptide substrates used in various *in vitro* activity assay platforms for the detection of the BoNT/E toxin. For instance, a fluorescence based assay uses a recombinant substrate consisting of the SNAP-25 sequence Ile^{134}-Gly^{206} flanked by a green fluorescent protein (GFP) and a blue fluorescent protein (BFP)[19]. A 70-mer peptide of Val^{137}-Gly^{206} as a substrate is included in an immuno-assay where the cleavage product was detected by a specific antibody [20]. The sequence of

Ala^{141}-Gly^{206} with a fluorescent tag on either terminus of the peptide formed a substrate included in the BoTest^{TM} kit that uses Förster resonance energy transfer (FRET) technology to detect BoNT/E activity [21]. A 61-mer peptide consisting the sequence of Met^{146}-Gly^{206} is reported in a capillary electrophoresis method [22]. The peptides of Ile^{156}-Asp^{186} and Ile^{156}-Thr^{190} are used in a mass spectrometry-based Endopep-MS assay developed in our laboratory[23; 24]. During the preparation of this manuscript, a new paper published claimed the peptide of Met^{167}-Asp^{186} and its derivative with two Met replaced by Nle residues were effective substrates for the Endopep-MS platform[25]. This report described the development of a novel peptide substrate to improve the sensitivity of the Endopep-MS assay for the determination of BoNT/E catalytic activity. Through comprehensive optimization using approaches of truncation, deletion, single and multiple substitution and other modifications, we have developed several highly efficient peptides that showed more than a 500-fold improvement than the substrate currently used in the Endopep-MS assay.

Materials and method

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). The complex forms of the botulinum neurotoxin without pre-activation and the trypsin activated BoNT/E toxin were obtained from Metabiologics (Madison, WI). Botulinum neurotoxin is highly toxic and appropriate safety measure is required. All BoNT neurotoxins were handled in a class 2 biosafety cabinet equipped with HEPA filters. Monoclonal antibodies were provided by Dr. James Marks at the University of California, San Francisco. Streptavidin coated Dynabeads were purchased from Invitrogen (Lake Success, NY). Serum and stool extracts were purchased from commercial source or collected from anonymous donors, and no demographic information was obtained (CDC IRB 4307).

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 a Tribute peptide synthesizer (Protein Technologies, Tucson, AZ, USA). Peptides were cleaved and deblocked using a reagent mixture of 95% trifluroacetic acid :2% water: 2% anisole:1% ethanedithiol and purified by reversed-phase HPLC using a water:acetonitrile: 0.1% TFA gradient (90-95% purity). 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 [26]. 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/E, 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 streptavidin beads followed by an activity assay as described [26].

After reaction, 2 μL of the supernatant was mixed with 20 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 1 μM internal standard peptide (isotope labeled peptides resembling the sequence of either the C- or N-terminal cleavage product) was added to the solution. The formation of cleavage products was measured as the ratio of the isotope cluster areas of the cleavage product versus an internal standard.

MS detection

Each sample was spotted in triplicate on a MALDI plate and analyzed on a 5800 MALDI-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.

Results and discussion

Optimal length of the peptide substrate of BoNT/E determined by truncation, deletion and mutation

Endopep-MS assay is a method using mass spectrometry, matrix assisted laser desorption ionization (MALDI) or electrospray ionization (ESI), to detect either one or two cleavage products hydrolyzed from a peptide substrate by an affinity enriched toxin. Therefore, assay sensitivity will not only depend on the hydrolysis efficiency (substrate binding and catalysis), but also rely on the ionization of cleaved peptide fragments, which is associated with their amino acid sequence. While different lengths of peptides including the essential elements for substrate binding and cleavage are applied in various in vitro BoNT activity assays as described above, a study on the optimal length of a peptide specifically suitable for the detection of BoNT/E by Endopep-MS was lacking. The peptide substrate (Pep-8 in Table 1) currently used in the Endopep-MS assay was derived from the partial sequence of SNAP-25, ranging from the amino acid residues Ile^{156} to Thr^{190} [27]. To examine whether further improvement can be achieved by optimizing peptide length for our Endopep-MS assay, we systematically prepared a series of peptides with extended or shortened sequences from either the N-terminal or C-terminal direction of the Pep-8, where the opposite end of the substrate remained the same (Table 1). In order to avoid bias caused by sequence-dependent ionization of cleavage fragments, the hydrolysis of the peptides with variable N-termini (Pep-1 to Pep-6) was compared by measuring the formation of C-terminal cleavage products by MALDI-TOF-MS, while that of the C-terminal varied peptides (Pep-7 to Pep-14) was compared with the production of N-terminal cleavage products. For those N-terminal truncated peptides, the longest peptide (Pep-1) with the SNAP-25 sequences of Ala^{141}-Gly^{206} yielded the highest production of the cleavage product. When 10 or 15 N-terminal residues were removed (Pep-2 and Pep-3), the cleavage efficiency of these two

substrates underwent a slight decrease. On the other hand, further deleting N-terminal resides (Pep-4 to Pep-6) led to a drastic reduction or non-detection of the cleavage products. For C-terminal truncated peptides, extending amino acid residues all the way to the last SNAP-25 residue at the position 206 (Pep-7) did not provide any benefit, compared to the activity of Pep-8 substrate which ends at position 190. Further C-terminal truncation of the peptides caused a steady decrease in their substrate hydrolysis (Pep-9 to Pep-14). In summary, Pep-1 performed the best among the tested BoNT/E substrate peptides of various lengths. This likely explained why the peptide itself, or with added fluoresence tags, was used as an efficient substrate in other studies and commercial kits (BoTest™ A/E Botulinum Neurotoxin Detection Kit, Biosentinel, Inc.). The large size of this peptide (7.5kDa), however, raised issues such as difficulty of peptide preparation and low solubility in assay buffers. In contrast, the molecular weight of Pep-8 (4.0kDa) is almost half of that of the Pep-1, but it has retained 90% of the substrate activity. For these reasons, we decided to use this peptide as a template for further optimization.

The next modification to optimize the substrate focused on adding or deleting single amino acids to or from either end of the Pep-8 in order to examine whether smaller changes in peptide size impact the substrate activity. As shown in Table 2, removing one or two Ile residues from the N-terminus of the peptide resulted in a 30 to 40% decrease in cleavage efficiency of the newly formed peptides (Pep-15, Pep-16). In addition, extending the sequence (Pep-17) by adding a glycine (residue 155 of SNAP-25) to the N-terminus led to reduced production of enzyme cleavage products as well. The importance of two N-terminal hydrophobic residues led us to speculate that the side chains of these two Ile residues might have some direct contact with the enzyme through a hydrophobic-hydrophobic interaction. An increase in the cleavage product from Pep-18, after the third Ile residue was incorporated into the N-terminus of Pep-8, provided some supportive evidence for this hypothesis. More studies attempting to address this issue will be described below.

A remarkable improvement was obtained when one or two additional SNAP-25 residues were extended on the C-terminus of the Pep-18. Incorporation of Arg^{191} and Arg^{191}-Ile^{192} into the Pep-19 and Pep-20, respectively, resulted in a 20-fold increase in the detection of the C-terminal cleavage products (CT-product) (Table 2). Since the N-terminal products (NT-product) cleaved from these peptides did not show significant changes, the elevated measurement of the CT-product should not come from altered cleavage efficiency of the new substrates, but must be due to the contribution of elevated ionization efficiency of the CT-products measured in positive ion mode by MALDI-TOF-MS, presumably directly associated with a positively charged Arg residue. In contrast, deletion of two C-terminal residues containing a positively charged Lysine from the Pep-18 led to a peptide (Pep-21) with reduced cleavage efficiency, indicated by a 52% production of the NT-product and decreased ionization of its CT-product (20%) as well.

In an attempt to shorten peptide size to improve synthesis yields and/or peptide solubility in the reaction buffer, internal deletions were applied on the N-terminal portion of the new template peptide, Pep-20. Table 3 shows that three peptides, Pep-22, Pep-23 and Pep-24, deleting 5 consecutive residues in different regions yielded different consequences. While less than 20% of the CT-product was detected from Pep-22 and Pep-23, removal of the area

consisting of the sequence of RHMAL in Pep-24 retained 90% substrate activity, revealing that the chain of RHMAL did not play a critical role in peptide binding and/or substrate cleavage. Further deleting several residues sequentially, flanking either end of this 5-residue region, produced seven new peptides, Pep-25 through Pep-31. Among these peptides, Pep-29, with two more residues removed, maintained a relative production of the CT-product (88%) similar to that of the Pep-24. It was also interesting to see how a single residue difference significantly altered the cleavage efficiency of newly formed peptides by BoNT/E, for instance, Pep-28 (2%) versus Pep-29 (88%). In conclusion, this result demonstrated that seven internal residues (RHMALDM) within the BoNT/E peptide substrate seem to not participate in enzyme-substrate interaction and hence can be removed without significant negative impact on the substrate cleavage. To examine the viability of further shortening Pep-29, three new peptides (Pep-32, -33, and -34) were designed, where one to three C-terminal residues (T, KT and NKT) were removed but the terminal Arg residue was maintained. It was observed that the shortest Pep-34 turned out to be a poor BoNT/E substrate whereas the medium length substrate, Pep-33, retained the most substrate capability (Table 3). Pep-32, on the other hand, resulted in significant activity improvement compared to Pep-29. Pep-32 generated over 30% CT- and NT-products more than Pep-29, suggesting that Pep-32 possessed a higher BoNT/E cleavage efficiency, whereas the ionization efficiency of its CT-product remained unchanged. This peptide was then used as a new template for additional optimization discussed below.

Further improvement was accomplished by single or multiple substitutions

Based on the sequence of the best substrate, Pep-32, we carried out a thorough single mutation study where every single residue was substituted with selected amino acids and the peptide mutants were tested as BoNT/E substrates. While about two-third of the mutants tested produced less cleavage products than the wild-type did, another one-third of the single mutated peptides resulted in a higher substrate efficiency, some of those showed three-fold or higher improvement (Fig. 1), demonstrating the power of a mutation approach for substrate optimization.

It was interesting to observe that a substantial improvement was accomplished when each of three N-terminal nonpolar Ile residues were replaced by Phe residues bearing a more hydrophobic side chain. This data emphasized our speculation described above that the N-terminal residues might be involved in direct contact with the catalytic domain of the toxin via hydrophobic-hydrophobic interactions. Substitution with even stronger hydrophobic residues probably enhanced such interaction and therefore increased substrate binding affinity. To explore whether those putative interactions can be further improved, a series of peptides with the modifications of hydrophobic residues in the N-terminal region of the Pep-32 were developed (Fig. 2A). A slight increase in detected CT-product was observed as the six N-terminal residues were replaced by three Phe residues in Pep-35. On the other hand, reduced detection of CT-product resulted when one more Phe was added in Pep-36. This suggested that three hydrophobic residues still retained the special enzyme-substrate interaction, even in a shorter peptide, but the contact might be impacted by a longer hydrophobic chain. When the three N-terminal Ile residues in Pep-32 were replaced by residues with a more hydrophobic structure, such as Phe in Pep-37, Trp in Pep-40, and 1-Nal

in Pep-42, all new peptides acted as better substrates. In addition, the improvement degree, Pep-42 > Pep-40 > Pep-37, seems proportional to the size of the bulky side chain groups (1-Nal > Trp > Phe, Fig. 2B). This data provided additional supportive evidence for the suggestion of a hydrophobic interaction between the BoNT/E enzyme and the peptide substrates. Moreover, the hydrophobicity effect was also demonstrated by the fact that the double Trp substitution (Pep-39) displayed higher cleavage efficiency than the triple Phe substituted peptide (Pep-37) did, and the triple Trp replacement in Pep-40 resulted in better substrate efficiency than the double-Trp one in Pep-39, presumably due to the difference of their combined hydrophobicity. A similar effect was also observed by comparing the cleavage of the double 1-Nal peptide (Pep-41) with the triple Trp and triple 1-Nal ones (Pep-40 and Pep-42, respectively). Furthermore, significantly reduced substrate cleavage by BoNT/E on the peptides with a cluster of five Phe residues (Pep-38) or four 1-Nal residues (Pep-43) suggested that the size of the hydrophobic cluster on the N-terminus of a substrate was not unrestricted. In other words, the space of the putative hydrophobic pocket in the catalytic domain of the toxin was limited and it might not allow the placement of more than three very bulky side chain groups. More study is needed to further confirm or address this proposed hydrophobic interaction between BoNT/E protease and its peptide substrate.

Additional effort on further substrate optimization was put on combining single mutations that showed enhanced detection of BoNT/E cleavage products. Since some single mutations may alter the conformation of mutated peptides or intra- and inter molecular interactions, and hence the property of their substrate binding and/or catalysis, it is not realistic to expect a best substrate can be obtained by simply placing together in a single sequence all good single substitutions derived from the studies described above. However, it is reasonable to believe that some combinations of the single mutations may achieve an augmented effect. For this purpose, new peptides were designed where different combinations of the amino acid substitutions, derived from the studies of single mutation and N-terminal modifications described above, were incorporated into their corresponding positions of the template peptide (Pep-32). In addition, some unnatural amino acids, such as homoarginine (hR), and terminal modifications, such as C-terminal amidation, were introduced in some peptides in order to increase peptide stability and reduce their susceptibility to non-specific cleavage by other proteases (e.g. Trypsin) present in biological samples. Table 4 lists some of such modified peptides (Pep-45 to Pep-62) that showed a comparable or better substrate performance than that of the peptide with a single internal substitution (Pep-44). While some multiple substituted peptides (e.g. Pep-45, Pep-46, Pep-48 or Pep-55) exhibited similar or slightly improved detection sensitivity, in terms of the detection of the C-terminal cleavage products, many of the novel peptides achieved a significant improvement with 50% to 300% increase in the CT-product detection, revealing that added benefit on assay sensitivity could be obtained by combining sound single mutations. Among four candidates displaying three-fold improvement over Pep-44, Pep-59 proved to be the best in solubility and the Pep-62 showed highest resistance toward undesired cleavage by nonspecific proteases present in clinical samples (Data not shown). The substitution of the arginine at the cleavage site with an unnatural homoarginine residue seems to contribute to improved resistance toward the cleavage by nonspecific proteases such as trypsin. Therefore, these two optimal peptides

were selected to be used in further experiments and in routine analysis of biological samples for the BoNT/E detection by the Endopep-MS assay.

Evaluation of optimized peptides as BoNT/E substrates in the Endopep-MS assay

To evaluate the outcome of the optimization for the BoNT/E substrates, Pep-59, one of the four best optimized peptides, was compared to Pep-8, the substrate currently used in the Endopep-MS assay. The substrates were hydrolyzed by two forms of BoNT/E toxins: one is the single chain holotoxin without pre-activation as a complex with neurotoxin-associated proteins, and another is the BoNT/E di-chain complex that had been activated by exposing it to trypsin during the manufacturing process. As shown in Table 5, the optimal peptide was able to detect the cleavage products at a 500-fold lower level of BoNT/E compared to the old peptide substrate, for both not activated and trypsin-activated BoNT/E toxin complexes, under the same experimental conditions, demonstrating a dramatic improvement in the assay sensitivity using the new peptide substrate. When testing the sensitivity of the Endopep-MS assay using the newly developed optimal peptide, the limit of detection of 0.1 mLD_{50} (1pg/mL or 5.5 attomole/mL) was accomplished for the detection of not trypsin-activated BoNT/E toxin complex spiked in serum and stool extract, two common biological matrices used for botulism clinical samples, after a 4 hour cleavage reaction (Fig. 3, S/N > 3). This represent an assay sensitivity 10-fold lower than that measured by a traditional mouse bioassay. The specificity of the optimal peptides was examined by exposing them to other serotypes of botulinum neurotoxins including type A, B, and F, and no cleavage product was observed (data not shown). In addition to the specific BoNT/E subtype (E3) used in the experiments described above, the optimized peptides also proved to be effective substrates of other tested BoNT/E subtypes including E1, E2, E4, E7 and the most divergent E9 (Data not shown).

Conclusion

We developed novel peptide substrates for the mass spectrometry-based Endopep-MS assay for the detection of type E botulinum neurotoxin. The systematic and comprehensive optimization process included peptide terminal truncation, internal deletion, single and multiple substitution, terminal residue modification and incorporation of unnatural amino acid residues. Our data demonstrate that one of the four optimal peptides demonstrated a 500-fold improvement in assay sensitivity than the current substrate, used for the detection of both not activated and trypsin-activated BoNT/E toxin complexes. The limit of detection for the toxin complex without pre-activation in serum, stool and food samples using the new substrate is 0.1 mouseLD_{50}/mL. In addition, the troublesome nonspecific cleavage in blank control samples was significantly improved by incorporating an unnatural homoarginine residue in the cleavage site of the optimal peptides. A patent application of these optimized peptides has been filed and the novel peptide substrates continue to be used in our laboratory for routine analysis of clinical samples.

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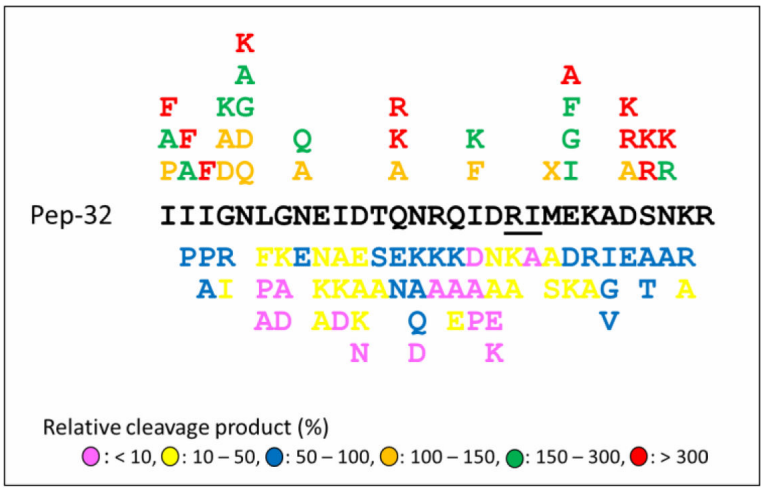

Figure 1.

Effect of single amino acid mutations on the detection of cleavage product of mutated peptide-32 by BoNT/E. The residues at BoNT/E cleavage site are underlined. X represents norleucine.

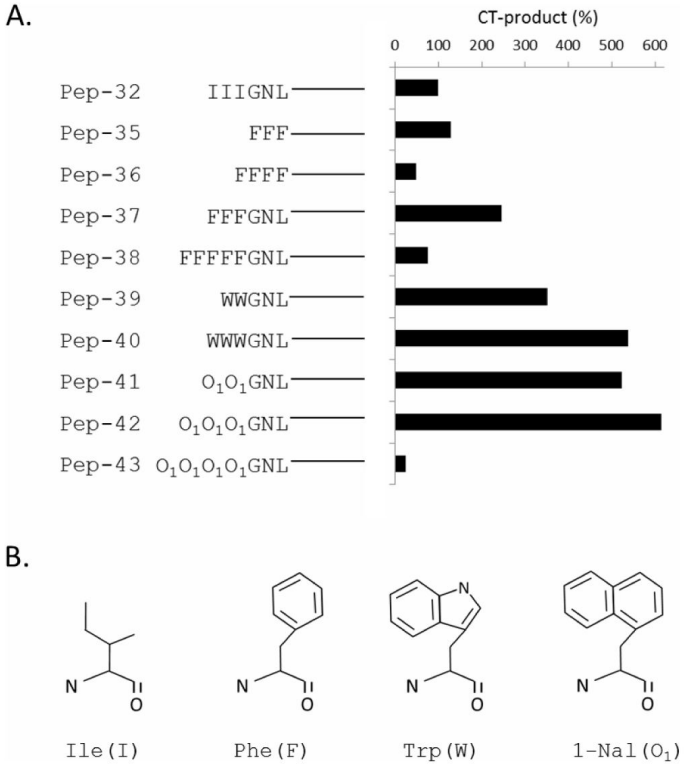

Figure 2.

(A) Cleavage efficiency of the peptides modified with N-terminal hydrophobic residues. (B) the structure of some hydrophobic residues. Several N-terminal residues are represented by letters and other identical regions of the sequences are represented by lines. O_1: 1-1-Naphthylalanine (Nal).

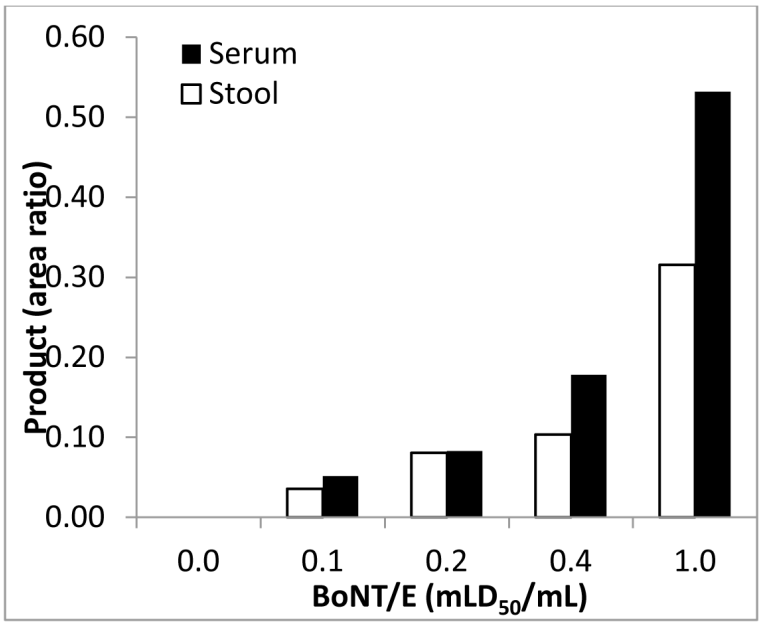

Figure 3.

Product response from the cleavage of Pep-59 by not trypsin-activated BoNT/E of various concentrations spiked in serum and stool matrices. Cleavage reaction condition: 37°C, 4 hours.

Table 1

Hydrolysis of N- or C-terminal truncated peptide substrates by BoNT/E toxin.

Peptide	SNAP-25 position	Sequence	CT-prod (%)[a]		NT- prod (%)[b]	
Pep-1	141-206	ARENEMDENLEQVSGIIGNLRHMALDMGNEIDTQNRQID**RI**MEKADSNKTRIDEANQRATKMLGSG[c]	100			
Pep-2	151-206	EQVSGIIGNLRHMALDMGNEIDTQNRQID**RI**MEKADSNKTRIDEANQRATKMLGSG	75			
Pep-3	156-206	IIGNLRHMALDMGNEIDTQNRQID**RI**MEKADSNKTRIDEANQRATKMLGSG	89			
Pep-4	161-206	RHMALDMGNEIDTQNRQID**RI**MEKADSNKTRIDEANQRATKMLGSG	28			
Pep-5	166-206	DMGNEIDTQNRQID**RI**MEKADSNKTRIDEANQRATKMLGSG	19			
Pep-6	171-206	IDTQNRQID**RI**MEKADSNKTRIDEANQRATKMLGSG	0			
Pep-7	156-206	IIGNLRHMALDMGNEIDTQNRQID**RI**MEKADSNKTRIDEANQRATKMLGSG			100	
Pep-8[d]	156-190	IIGNLRHMALDMGNEIDTQNRQID**RI**MEKADSNKT			100	
Pep-9	156-188	IIGNLRHMALDMGNEIDTQNRQID**RI**MEKADSN			81	
Pep-10	156-186	IIGNLRHMALDMGNEIDTQNRQID**RI**MEKAD			68	
Pep-11	156-185	IIGNLRHMALDMGNEIDTQNRQID**RI**MEKA			77	
Pep-12	156-184	IIGNLRHMALDMGNEIDTQNRQID**RI**MEK			38	
Pep-13	156-183	IIGNLRHMALDMGNEIDTQNRQID**RI**ME			15	
Pep-14	156-182	IIGNLRHMALDMGNEIDTQNRQID**RI**M			10	

[a] Relative cleavage rate obtained from the analysis of C-terminal cleavage products (CT-prod). Condition: 37°C, 4 hour.

[b] Relative cleavage rate obtained from the analysis of N-terminal cleavage products (CT-prod).

[c] The cleavage site of BoNT/E substrate is depicted in bold.

[d] Pep-8 is the BoNT/E substrate currently used in Endopep-MS assay.

Table 2

Relative production of the N-or C-terminal product cleaved from truncated or modified peptide substrates by BoNT/E toxin.

Peptide	Sequence	CT-prod (%)		NT- prod (%)	
Pep-15	GNLRHMALDMGNEIDTQNRQID**RI**MEKADSNKT	60			
Pep-16	IGNLRHMALDMGNEIDTQNRQI**RII**MEKADSNKT	73			
Pep-8	IIGNLRHMALDMGNEIDTQNRQID**RI**MEKADSNKT	100			
Pep-17	GIIGNLRHMALDMGNEIDTQNRQID**RI**MEKADSNKT	81			
Pep-18	IIIGNLRHMALDMGNEIDTQNRQID**RI**MEKADSNKT	123			
Pep-19	IIIGNLRHMALDMGNEIDTQNRQID**RI**MEKADSNKTRI	2000		100	
Pep-20	IIIGNLRHMALDMGNEIDTQNRQID**RI**MEKADSNKTR	2177		117	
Pep-18	IIIGNLRHMALDMGNEIDTQNRQID**RI**MEKADSNKT	100		130	
Pep-21	IIIGNLRHMALDMGNEIDTQNRQID**RI**MEKADSN	20		52	

Table 3

Relative production of the N-or C-terminal product cleaved from internally deleted peptides by BoNT/E toxin.

Peptide	Sequence	CT-prod (%)		NT-prod (%)	
Pep-20	IIIGNLRHMALDMGNEIDTQNRQID**RI**MEKADSNKTR	100			
Pep-22	IIIGNLRHMALDMGNE_____RQID**RI**MEKADSNKTR	6			
Pep-23	IIIGNLRHMAL_____IDTQNRQID**RI**MEKADSNKTR	17			
Pep-24	IIIGNL_____DMGNEIDTQNRQID**RI**MEKADSNKTR	90			
Pep-25	IIIGN_____DMGNEIDTQNRQID**RI**MEKADSNKTR	19			
Pep-26	IIIG_____DMGNEIDTQNRQID**RI**MEKADSNKTR	43			
Pep-27	III_____DMGNEIDTQNRQID**RI**MEKADSNKTR	30			
Pep-28	IIIGNL_____NEIDTQNRQID**RI**MEKADSNKTR	2			
Pep-29	IIIGNL_____GNEIDTQNRQID**RI**MEKADSNKTR	88			
Pep-30	IIIGNL_____MGNEIDTQNRQID**RI**MEKADSNKTR	33			
Pep-31	IIIGNL_____LDMGNEIDTQNRQID**RI**MEKADSNKTR	53			
Pep-29	IIIGNL_____GNEIDTQNRQID**RI**MEKADSNKTR	88		100	
Pep-32	IIIGNL_____GNEIDTQNRQID**RI**MEKADSNKR	117		136	
Pep-33	IIIGNL_____GNEIDTQNRQID**RI**MEKADSNR	91		84	
Pep-34	IIIGNL_____GNEIDTQNRQID**RI**MEKADSR	18		36	

Deleted amino acid residues from the Pep-20 are depicted in underscore.

Table 4

Effect of multiple mutations on the cleavage of modified peptides by BoNT/E toxin*.

Peptide	Sequence	CT-product (%)
Pep-44	IIIGNLGNEIDTQNRQIDRIMEKAKSNKR	100
Pep-45	IIIAKLGNEIDTRNRQIDRIMEKADSNKR	101
Pep-46	IIIGKLGNEIDTRNRQIDRIMEKADSNKR	103
Pep-47	IIIAKLGQEIDTRNRQKDRIMEKADSNKR	152
Pep-48	IIIAKLGNEIDTQNRQKDRIMAKADSNKR	122
Pep-49	IIIAKLGNEIDTRNRQKDRIMAKADSNKR	137
Pep-50	IIIGNLGNEIDTQNRQIDRIXAKAKSKKR	131
Pep-51	IIIGNLGNEIDTQNRQIDRIMAKAKSKKR	192
Pep-52	IIIGNLGNEIDTQNRQIDRIMEKARRKKR	126
Pep-53	IIIGNLGNEIDTQNRQIDRIMEKAKRKKR	132
Pep-54	IIIGNLGNEIDTQNRQIDRIMEKAKKKKR	134
Pep-55	IIIGNLGNEIDTQNRQIDRIMEKAKSKKR	123
Pep-56	IIIGNLGNEIDTQNRQIDhRIMAKAKSKKR	172
Pep-57	IIIGNLGNEIDTQNRQIDhRIMAKAKSKKR-NH_2	165
Pep-58	WWWGNLGNEIDTQNRQIDhRIMAKAKSKKR-NH_2	156
Pep-59	WWWAKLGQEIDTRNRQKDhRIMAKADSNKR-NH_2	314
Pep-60	$O_1O_1O_1$AKLGQEIDTRNRQKDhRIMAKADSNKR-NH_2	302
Pep-61	$O_2O_2O_2$AKLGQEIDTRNRQKDhRIMAKADSNKR-NH_2	288
Pep-62	$O_2O_2O_2$AKLGQEIDTRNRQKDhRIMAhRADSNKR-NH_2	302

* Red letters represent the residues not present in the original SNAP-25 sequence. Bold letters represent the residues at the BoNT/E cleavage site. hR: homoarginine; O_1: 1-Nal; O_2: 2-Nal

Table 5

Comparison of the cleavage of currently used and newly developed peptide substrates by BoNT/E.

Peptide	BoNT/E		Product (Area ratio)	Relative product
	Type	Activity (mLD_{50})*		
Pep-8	not activated	100	0.40	1
Pep-59	not activated	1	2.35	581
Pep-8	activated	0.16	1.07	1
Pep-59	activated	0.0016	5.48	511

* The specific activities of activated and not activated BoNT/E was provided by the manufacturer. Cleavage reactions were conducted at 37°C for 1 hour.