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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 humankind. 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–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 with 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 mouse LD<sub>50</sub>/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.

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The neurotoxins produced by *Clostridium botulinum* (botulinum neurotoxins, BoNTs)<sup>1</sup> are the most poisonous substances known to humankind. 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 because they are easy to produce [2]. On the other hand, botulinum toxins have been used for therapeutic or aesthetic applications [3]. For all of 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 that undergo proteolytic cleavage to generate active holotoxins composed of two protein subunits 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 metalloproteases. They cleave neuronal proteins involved in the exocytosis of neurotransmitters, such as SNAP-25 (25-kDa synaptosomal protein), 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 botulinum neurotoxin type E (BoNT/E) forms part of a family of seven confirmed related serotypes (botulinum neurotoxins A–G) produced by different strains of *C. 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].

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<sup>1</sup> Abbreviations used: BoNT, botulinum neurotoxin; SNAP-25, 25-kDa synaptosomal protein; BoNT/E, botulinum neurotoxin type E; mL<sub>D50</sub>, mouse LD<sub>50</sub>; SNARE, SNAP receptor; MS, mass spectrometry; TFA, trifluoroacetic acid; MALDI, matrix-assisted laser desorption/ionization; TOF, time-of-flight; CT product, C-terminal product; NT product, N-terminal product; 1-Nal, 1-naphthylalanine.

The mouse bioassay is the historic method for the detection of botulinum toxins [2]. It is very sensitive, detecting as little as approximately 10 pg of active toxin, which is defined as 1 mouse LD<sub>50</sub> (mLD<sub>50</sub>), 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 BoNTs' 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 specifically cleaves one of the SNARE (SNAP receptor) complex proteins, SNAP-25, at the Arg180–Ile181 bond [14]. Montecucco and coworkers revealed that the minimal length for proteolysis of SNAP-25 by BoNT/E includes a SNARE motif starting from Ala141 [15]. Binz and coworkers defined the minimal essential domain of SNAP-25 required for cleavage by BoNT/E as Ile156–Asp186 [16]. Through saturation mutagenesis and deletion mapping, Barbieri and Chen defined a short optimal cleavage domain of Met167–Asp186, where the subsite of Met167–Thr173 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 Ile134–Gly206 flanked by a green fluorescent protein (GFP) and a blue fluorescent protein (BFP) [19]. A 70-mer peptide of Val137–Gly206 as a substrate is included in an immunoassay where the cleavage product was detected by a specific antibody [20]. The sequence of Ala141–Gly206 with a fluorescent tag on either terminus of the peptide formed a substrate included in the BoTest kit that uses Förster resonance energy transfer (FRET) technology to detect BoNT/E activity [21]. A 61-mer peptide comprising the sequence of Met146–Gly206 is reported in a capillary electrophoresis method [22]. The peptides of Ile156–Asp186 and Ile156–Thr190 are used in a mass spectrometry (MS)-based Endopep-MS assay developed in our laboratory [23,24]. During the preparation of this study, a new article published claimed that the peptide of Met167–Asp186 and its derivative with two Met residues 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 a more than 500-fold improvement over the substrate currently used in the Endopep-MS assay.

## Materials and methods

All chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA) except where indicated otherwise. Fmoc–amino acid derivatives and peptide synthesis reagents were purchased from EMD Chemicals (Gibbstown, NJ, USA) or Protein Technologies (Tucson, AZ, USA). Isotopically labeled Fmoc–amino acid derivatives were purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA). The complex forms of the botulinum neurotoxin without preactivation and the trypsin-activated BoNT/E toxin were obtained from Metabio (Madison, WI, USA). Botulinum neurotoxin is highly toxic, and appropriate safety measures are required. All BoNTs were handled in a class 2 biosafety cabinet equipped with HEPA filters. Monoclonal antibodies were provided by James Marks at the University of California, San Francisco. Streptavidin-coated Dynabeads were purchased from Invitrogen (Lake Success, NY, USA). Serum and stool extracts were purchased

from commercial sources or collected from anonymous donors, and no demographic information was obtained (Centers for Disease Control and Prevention institutional review board 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). Peptides were cleaved and deblocked using a reagent mixture of 95% trifluoroacetic acid (TFA)/2% water/2% anisole/1% ethanedithiol and purified by reversed-phase high-performance liquid chromatography (HPLC) using a water/acetonitrile/0.1% TFA gradient (90–95% purity). Correct peptide structures were confirmed by matrix-assisted laser desorption/ionization (MALDI)–MS. 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 described previously [26]. In brief, the reaction was conducted in a 20- $\mu$ l reaction volume containing 0.1 mM peptide substrate, 10  $\mu$ M ZnCl<sub>2</sub>, 1 mg/ml bovine serum albumin (BSA), 10 mM dithiothreitol, and 200 mM Hepes buffer (pH 7.4) at 37 °C for 1 or 4 h. 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 previously [26].

After reaction, 2  $\mu$ l of the supernatant was mixed with 20  $\mu$ l of  $\alpha$ -cyano-4-hydroxycinnamic acid at 5 mg/ml in 50% acetonitrile/0.1% TFA/1 mM ammonium citrate, and then 2  $\mu$ l of a 1- $\mu$ M 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 (time-of-flight)–MS instrument (Applied Biosystems, Framingham, MA, USA). Mass spectra of each spot were obtained by scanning from 800 to 4000 *m/z* in MS-positive ion reflector mode. The instrument uses an Nd-YAG laser at 355 nm, and each spectrum is an average of 2400 laser shots.

## Results and discussion

### Optimal length of 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, or electrospray ionization to detect either one or two cleavage products hydrolyzed from a peptide substrate by an affinity-enriched toxin. Therefore, assay sensitivity not only will depend on the hydrolysis efficiency (substrate binding and catalysis) but also will rely on the ionization of cleaved peptide fragments, which is associated with their amino acid sequence. Although 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

**Table 1**  
Hydrolysis of N- or C-terminal truncated peptide substrates by BoNT/E toxin.

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

<sup>a</sup>Relative cleavage rate obtained from the analysis of C-terminal cleavage products (CT prod). Conditions: 37 °C, 4 h.

<sup>b</sup>Relative cleavage rate obtained from the analysis of N-terminal cleavage products (CT prod).

<sup>c</sup>The cleavage site of BoNT/E substrate is depicted in bold.

<sup>d</sup>Pep-8 is the BoNT/E substrate currently used in Endopep-MS assay.

**Table 2**  
Relative production of N- or C-terminal product cleaved from truncated or modified peptide substrates by BoNT/E toxin.

Peptide	Sequence	CT product	NT product
		(%)	(%)
Pep-15	GNLRHMALDMGNEIDTQNRQID <b>RI</b> MEKADSNKT	60	
Pep-16	IIGNLRHMALDMGNEIDTQNRQID <b>RI</b> MEKADSNKT	73	
Pep-8	IIGNLRHMALDMGNEIDTQNRQID <b>RI</b> MEKADSNKT	100	
Pep-17	GIIGNLRHMALDMGNEIDTQNRQID <b>RI</b> MEKADSNKT	81	
Pep-18	IIGNLRHMALDMGNEIDTQNRQID <b>RI</b> MEKADSNKT	123	
Pep-19	IIGNLRHMALDMGNEIDTQNRQID <b>RI</b> MEKADSNKTRI	2000	100
Pep-20	IIGNLRHMALDMGNEIDTQNRQID <b>RI</b> MEKADSNKTR	2177	117
Pep-18	IIGNLRHMALDMGNEIDTQNRQID <b>RI</b> MEKADSNKT	100	130
Pep-21	IIGNLRHMALDMGNEIDTQNRQID <b>RI</b> MEKADSN	20	52

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 residue Ile156 to Thr190 [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- or C-terminal direction of Pep-8, where the opposite end of the substrate remained the same (Table 1). 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, whereas the hydrolysis 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 Ala141–Gly206 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 residues (Pep-4 to Pep-6) led to a drastic reduction or nondetection 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 with 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 fluorescence tags, was used as an efficient substrate in other studies and commercial kits (BoTest A/E Botulinum Neurotoxin Detection Kit, Biosentinel). The large size of this peptide (7.5 kDa), however, raised issues such as difficulty of peptide preparation and low solubility in assay buffers. In contrast, the molecular weight of Pep-8 (4.0 kDa) is nearly half that of 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 Pep-8 in order to examine whether smaller changes in peptide size affect 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 and 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

**Table 3**  
Relative production of the N- or C-terminal product cleaved from internally deleted peptides by BoNT/E toxin.

Peptide	Sequence	CT product (%)	NT product (%)
Pep-20	IIIIGNLRHMALDMGNEIDTQNRQIDRIMEKADSNKTR	100	
Pep-22	IIIIGNLRHMALDMGNE____RQIDRIMEKADSNKTR	6	
Pep-23	IIIIGNLRHMAL____IDTQNRQIDRIMEKADSNKTR	17	
Pep-24	IIIIGNL____DMGNEIDTQNRQIDRIMEKADSNKTR	90	
Pep-25	IIIIGN____DMGNEIDTQNRQIDRIMEKADSNKTR	19	
Pep-26	IIIG____DMGNEIDTQNRQIDRIMEKADSNKTR	43	
Pep-27	III____DMGNEIDTQNRQIDRIMEKADSNKTR	30	
Pep-28	IIIIGNL____NEIDTQNRQIDRIMEKADSNKTR	2	
Pep-29	IIIIGNL____GNEIDTQNRQIDRIMEKADSNKTR	88	
Pep-30	IIIIGNL____MGNEIDTQNRQIDRIMEKADSNKTR	33	
Pep-31	IIIIGNL____LDMGNEIDTQNRQIDRIMEKADSNKTR	53	
Pep-29	IIIIGNL____GNEIDTQNRQIDRIMEKADSNKTR	88	100
Pep-32	IIIIGNL____GNEIDTQNRQIDRIMEKADSNKR	117	136
Pep-33	IIIIGNL____GNEIDTQNRQIDRIMEKADSNR	91	84
Pep-34	IIIIGNL____GNEIDTQNRQIDRIMEKADSR	18	36

Note. Deleted amino acid residues from Pep-20 are depicted by underscore.

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 are described below.

A remarkable improvement was obtained when one or two additional SNAP-25 residues were extended on the C terminus of Pep-18. Incorporation of Arg191 and Arg191–Ile192 into Pep-19 and Pep-20, respectively, resulted in a 20-fold increase in the detection of the C-terminal cleavage products (CT products) (Table 2). Because the N-terminal products (NT products) 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 rather 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 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.

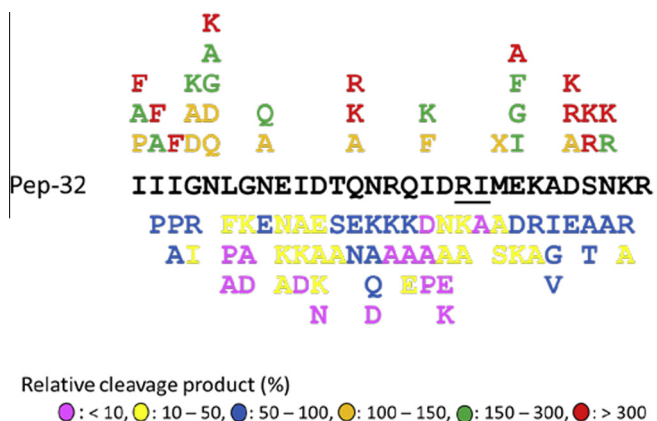
In an attempt to shorten peptide size to improve synthesis yields and/or peptide solubility in the reaction buffer, internal deletions were applied on the N-terminal portion of the new template peptide, Pep-20. Table 3 shows that three peptides (Pep-22, Pep-23, and Pep-24) deleting five consecutive residues in different regions yielded different consequences. Whereas 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 five-residue region, produced seven new peptides, Pep-25 to 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 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, Pep-33,

and Pep-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 with Pep-29. Pep-32 generated at least 30% more CT and NT products 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 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. Whereas approximately two-thirds of the mutants tested produced less cleavage products than the wild type, another one-third of the single mutated peptides resulted in a higher substrate efficiency, with some of those showing 3-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 was replaced by a Phe residue bearing a more hydrophobic side chain. These 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 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 residue 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 affected 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-naphthylalanine (1-Nal) in Pep-42, all new peptides acted as better substrates. In

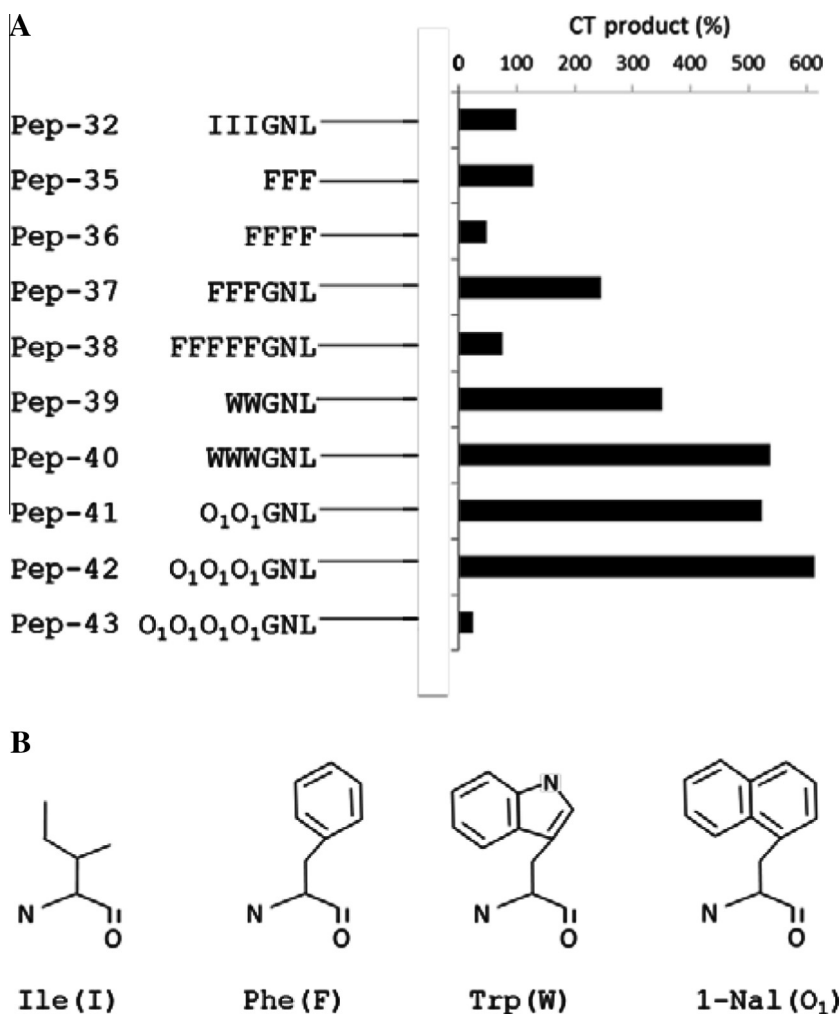


**Fig. 1.** Effect of single amino acid mutations on the detection of cleavage product of mutated Pep-32 by BoNT/E. The residues at the BoNT/E cleavage site are underlined. X represents norleucine.

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; see Fig. 2B). These 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), 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 that of the triple Trp and triple 1-Nal peptides (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 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. Because some single mutations may alter the conformation of mutated peptides or intra- and intermolecular interactions, and hence the property of their substrate binding and/or catalysis, it is not realistic to expect that a best



**Fig. 2.** (A) Cleavage efficiency of the peptides modified with N-terminal hydrophobic residues. (B) 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<sub>1</sub>, 1-naphthylalanine (1-Nal).

**Table 4**  
Effect of multiple mutations on cleavage of modified peptides by BoNT/E toxin.

Peptide	Sequence	CT-product (%)
Pep-44	IIIGNLGNEIDTQNRQID <b>RI</b> MEKA <b>KS</b> NKR	100
Pep-45	II <b>I</b> AKLGNEIDTRNRQID <b>RI</b> MEKADSNKR	101
Pep-46	IIIGKLGNEIDTRNRQID <b>RI</b> MEKADSNKR	103
Pep-47	II <b>I</b> AKLGQ <b>E</b> IDTRNRQ <b>KD</b> RI <b>ME</b> KADSNKR	152
Pep-48	II <b>I</b> AKLGNEIDTQNRQ <b>KD</b> RI <b>ME</b> KADSNKR	122
Pep-49	II <b>I</b> AKLGNEIDTRNRQ <b>KD</b> RI <b>ME</b> KADSNKR	137
Pep-50	IIIGNLGNEIDTQNRQID <b>RI</b> XAKAK <b>SK</b> KR	131
Pep-51	IIIGNLGNEIDTQNRQID <b>RI</b> MAKAK <b>SK</b> KR	192
Pep-52	IIIGNLGNEIDTQNRQID <b>RI</b> MEKAR <b>RK</b> KR	126
Pep-53	IIIGNLGNEIDTQNRQID <b>RI</b> MEKAK <b>RK</b> KR	132
Pep-54	IIIGNLGNEIDTQNRQID <b>RI</b> MEKAK <b>KK</b> KR	134
Pep-55	IIIGNLGNEIDTQNRQID <b>RI</b> MEKAK <b>SK</b> KR	123
Pep-56	IIIGNLGNEIDTQNRQID <b>hR</b> IMAKAK <b>SK</b> KR	172
Pep-57	IIIGNLGNEIDTQNRQID <b>hR</b> IMAKAK <b>SK</b> KR-NH <sub>2</sub>	165
Pep-58	WWGNLGN <b>E</b> IDTQNRQID <b>hR</b> IMAKAK <b>SK</b> KR-NH <sub>2</sub>	156
Pep-59	WWWAKLGQ <b>E</b> IDTRNRQ <b>KD</b> h <b>R</b> IMAKADSNKR-NH <sub>2</sub>	314
Pep-60	O <sub>1</sub> O <sub>1</sub> O <sub>1</sub> AKLGQ <b>E</b> IDTRNRQ <b>KD</b> h <b>R</b> IMAKADSNKR-NH <sub>2</sub>	302
Pep-61	O <sub>2</sub> O <sub>2</sub> O <sub>2</sub> AKLGQ <b>E</b> IDTRNRQ <b>KD</b> h <b>R</b> IMAKADSNKR-NH <sub>2</sub>	288
Pep-62	O <sub>2</sub> O <sub>2</sub> O <sub>2</sub> AKLGQ <b>E</b> IDTRNRQ <b>KD</b> h <b>R</b> IMAhRADSNKR-NH <sub>2</sub>	302

Note. 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<sub>1</sub>, 1-Nal; O<sub>2</sub>, 2-Nal. (For interpretation of the reference to color in this table note, the reader is referred to the Web version of this article.)

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, 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 these 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). Whereas some multiple substituted peptides (e.g., Pep-45, Pep-46, Pep-48, 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 an added benefit on assay sensitivity could be obtained by combining sound single mutations. Among four candidates displaying 3-fold improvement over Pep-44, Pep-59 proved to be the best in solubility and Pep-62 showed the 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 BoNT/E detection by the Endopep-MS assay.

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

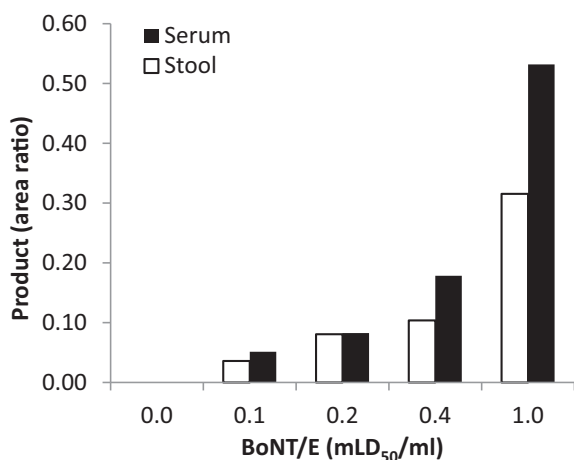
To evaluate the outcome of the optimization for the BoNT/E substrates, Pep-59, one of the four best optimized peptides, was

compared with 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 preactivation as a complex with neurotoxin-associated proteins, and the other 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 with the old peptide substrate, for both not-trypsin-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, a limit of detection of 0.1 mL<sub>D50</sub> (1 pg/ml or 5.5 amol/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-h cleavage reaction (Fig. 3, signal/noise [S/N] > 3). This represents 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 types 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

**Table 5**  
Comparison of cleavage of currently used and newly developed peptide substrates by BoNT/E.

Peptide	BoNT/E		Product (area ratio)	Relative product
	Type	Activity (mLD <sub>50</sub> ) <sup>a</sup>		
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

<sup>a</sup> The specific activities of activated and not-activated BoNT/E was provided by the manufacturer. Cleavage reactions were conducted at 37 °C for 1 h.



**Fig. 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 conditions: 37 °C, 4 h.

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 over the current substrate used for the detection of both not-trypsin-activated and trypsin-activated BoNT/E toxin complexes. The limit of detection for the toxin complex without preactivation in serum, stool, and food samples using the new substrate is 0.1 mouse LD<sub>50</sub>/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 for these optimized peptides has been filed, and the novel peptide substrates continue to be used in our laboratory for routine analysis of clinical samples.

## Disclaimer

The findings and conclusions in this article are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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