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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_{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.

The neurotoxins produced by *Clostridium botulinum* (botulinum neurotoxins, BoNTs)[1] 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 metallopeptidases. 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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[1] *Abbreviations used:* BoNT, botulinum neurotoxin; SNAP-25, 25-kDa synaptosomal protein; BoNT/E, botulinum neurotoxin type E; mLD_{50}, mouse LD_{50}; 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.

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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_{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 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 Metabiologics (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-μl reaction volume containing 0.1 mM peptide substrate, 10 μM $ZnCl_2$, 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 μl of the supernatant was mixed with 20 μl of α-cyano-4-hydroxycinnamic acid at 5 mg/ml in 50% acetonitrile/0.1% TFA/1 mM ammonium citrate, and then 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 (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