Detection, differentiation, and subtyping of botulinum toxins A, B, E, and F by mass spectrometry

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Abstract: Botulinum neurotoxin (BoNT) causes the disease known as botulism, which can be lethal. Rapid determination of exposure to BoNT is an important public health goal. Our laboratory has developed Endopep-MS, a mass spectrometry-based endopeptidase method for detecting and differentiating BoNT. Here, we demonstrate that this method is very sensitive, detecting as little as 0.5 mouse LD_{50} of BoNT/A and as little as 0.05 mouse LD_{50} of BoNT/B, /E, and /F spiked into human serum samples. Additionally, the ability to further differentiate BoNT as the subtype of BoNT/A spiked into milk using toxin proteomics and mass spectrometry has been demonstrated. This method does not require DNA and can be performed on the same sample as that used for Endopep-MS analysis. The combination of these techniques, all performed on the same sample, provides a sensitive and selective analysis of BoNT isolated from a food or clinical sample and measures the toxin's activity.

Keywords: botulinum neurotoxin; BoNT; botulism; mass spectrometry.

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James L. Pirkle is the Director of the Division of Laboratory Sciences, National Center for Environmental Health, CDC and has worked at CDC for 30+ years. He is boarded in clinical pathology with a PhD in Physical Chemistry and has published 117 peer-reviewed articles. His areas of emphasis have been developing the National Biomonitoring Program at CDC to assess US population exposure to environmental chemicals; the CDC laboratory response to chemical and radiologic terrorism; development of advanced methods to measure chemicals and their metabolites in people; and novel mass spectrometry methods to improve diagnosis and prevention of botulism, anthrax and influenza.

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1 Introduction

Botulinum neurotoxin (BoNT) is produced by some species of the genus *Clostridium*, particularly *Clostridium botulinum*, *C. butyricum*, *C. baratii*, and *C. argentinese*. BoNTs cause the disease known as botulism, which can be lethal if untreated. Rapid determination of exposure to BoNT is an important public health goal. Ounce for ounce, BoNT is one of the most lethal substances known with an estimated LD_{50} in humans of approximately 70 μg for the average weight human through oral consumption (Herrero et al., 1967). This extreme toxicity has led, in part, to its current CDC designation as a category A agent for bioterrorism, making it one of the most likely agents for bioterrorism (Arnon et al., 2001).

BoNTs are currently classified into seven serotypes, labelled A-G, and serotypes /A, /B, /E, and /F are known to affect humans. BoNTs are highly specific proteases which target neuronal proteins. BoNT/A, /C, and /E cleave SNAP (synaptosomal-associated protein)-25 (Foran et al., 1996; Binz et al., 1994; Blasi et al., 1993; Schiavo et al., 1993a, 1993b; Williamson et al., 1996) whereas BoNT/B, /D, /F, and /G cleave synaptobrevin-2 (also known as VAMP-2) (Schiavo et al., 1992, 1993c, 1994; Yamasaki et al., 1994a, 1994b; Kalb et al., 2012a). Only BoNT/C is known to cleave more than one protein as it also cleaves syntaxin (Foran et al., 1996; Blasi et al., 1993; Schiavo et al., 1995). Cleavage of any of these proteins which form the soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) complex results in an inability to form this complex and results in the halting of the nerve impulse with flaccid paralysis.

Our laboratory at the CDC has developed an assay for BoNT termed the Endopep-MS method (Barr et al., 2005; Boyer et al., 2005; Kalb et al., 2005, 2006, 2008, 2009; Gaunt et al., 2007). This method involves incubation of BoNT with a peptide substrate that mimics the toxin's natural target. Each BoNT cleaves the peptide substrate in a specific, toxin-dependent location. The reaction mixture then is introduced into a mass

spectrometer, which detects any peptides within the mixture and accurately reports the mass of each. Detection of the peptide cleavage products corresponding to their specific location indicates the presence of a particular BoNT serotype. If the peptide substrate either remains intact or is cleaved in a location other than the toxin-specific site then that BoNT serotype is not present. We have demonstrated that this method can detect BoNT at levels comparable with or lower than levels detected with the mouse bioassay, which historically has been the most common method used to detect the toxin (Kautter and Solomon, 1977).

Endopep-MS is made more selective through the addition of an antibody-extraction step prior to the enzymatic reaction. Magnetic protein G beads coated with antibodies to a specific serotype of BoNT are incubated with the sample matrix which may or may not contain the toxin. If the toxin of that particular serotype is present, it binds to the antibody-coated beads. Other proteins in the sample matrix are washed away, and the beads containing toxin are then incubated with the peptide substrate. This technique allows for purification as well as concentration of the toxin from complex matrices. The use of antibody affinity also adds another level of specificity to the assay.

Mass spectrometry allows for very sensitive and selective measurements. Protein identification via mass spectrometric analysis of a tryptic digest of a protein has become commonplace. The identification of tryptic fragments of BoNT was first reported by van Baar et al. (2002), who demonstrated that BoNT could be specifically identified through mass spectrometric analysis of the tryptic fragments resulting from a digest of the toxin. Although van Baar et al.'s (2002) method as reported is extremely specific and selective for the identification of BoNT, it was not applied to BoNT in complex matrices.

Here, we describe a method that relies upon multiple levels of specificity to detect BoNT/A, /B, /E and /F in food or clinical matrices. BoNT is isolated from abundant proteins in a food or clinical sample through immunoaffinity capture on antibody-coated beads. The activity of BoNT is examined through interaction of the toxin with a peptide substrate that mimics the toxin's *in vivo* target. The peptide substrate is analysed through matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry, allowing for sensitive and selective measurements of the cleavage of the peptide substrate and an identification of the serotype of toxin. Finally, the BoNT is subjected to tryptic digestion, and the resulting tryptic fragments are analysed by mass spectrometry, allowing for direct examination of the composition of the toxin protein. This allows for an identification of the subtype of the toxin which is useful in epidemiological investigations. The combination of these techniques, all performed on the same sample, provides a sensitive and selective analysis of BoNT isolated from a food or clinical sample and measures the toxin's activity.

2 Materials and methods

2.1 Materials

Commercially purified BoNT complexes were purchased from Metabiologics (Madison, WI). Polyclonal rabbit-specific IgGs, antitype E and F, were also purchased from Metabiologics. Monoclonal antibodies RAZ1, CR2, B12.1, and 1B18 were obtained from Dr. James Marks at the University of California at San Francisco. Dynabeads® Protein G were purchased from Invitrogen (Carlsbad, CA). All chemicals were from Sigma-Aldrich

(St. Louis, MO) except where indicated. Peptide substrates were synthesised by Los Alamos National Laboratory (Los Alamos, NM, Table 1). Control human serum was purchased from Interstate Blood Bank (Memphis, TN). Milk with 2% fat was obtained from retail sources and used as purchased.

Table 1 Peptide sequences for the Endopep-MS method are listed along with the observed (M+H) + of substrate and cleavage products for each serotype

Peptide	*Sequence*	*m/z observed*
BoNT A substrate	Biotin-KGSNRTRIDQGN**QR**ATRXLGGK-Biotin	2,878.7
BoNT A NT product	Biotin-KGSNRTRIDQGNQ	1,699.9
BoNT A CT product	RATRXLGGK-Biotin	1,197.8
BoNT B substrate	LSELDDRADALQAGAS**QF**ETSAAKLKRKYWWKNLK	4,026.8
BoNT B NT product	LSELDDRADALQAGASQ	1,759.9
BoNT B CT product	FETSAAKLKRKYWWKNLK	2,283.4
BoNT E substrate	IIGNLRHMALDMGNEIDTQNRQID**RI**MEKADSNKT	4,042.6
BoNT E NT product	IIGNLRHMALDMGNEIDTQNRQIDR	2,922.8
BoNT E CT product	IMEKADSNKT	1,136.6
BoNT F substrate	LQQTQAQVDEVVDIMRVNVDKVLERD**QK**LSELDDRADAL	4,497.1
BoNT F NT product	LQQTQAQVDEVVDIMRVNVDKVLERDQ	3,168.9
BoNT F CT product	KLSELDDRADAL	1,345.8

Note: The cleavage site of each substrate is depicted in bold and underlined.

2.2 BoNT extraction

Monoclonal antibodies RAZ1, CR2, 1B18, and B12.1 were immobilised and cross-linked to the Dynabeads® Protein G as described in the manufacturer's protocol using 30 µg of antibody diluted into 500 µL of phosphate buffered saline (PBS) for every 100 µL of Dynabeads® Protein G. Cross-linked IgG-coated Dynabeads® were stored in PBST (PBS with 0.05% Tween®-20) at 4ºC for up to 12 weeks. An aliquot of 20 µL of antibody-coated beads was mixed for 1 hr with a solution of 500 µL of human serum or milk mixed with 50 µL of 10× PBST and spiked with BoNT at levels listed in the results section. After mixing for 1 hr with constant agitation at room temperature, the beads were washed twice in 1 mL each of PBST and then washed once in 100 µL of water. Negative controls consisted of human serum or milk with no spiked BoNT. The remainder of the extraction protocol was as above.

2.3 Endopep-MS reaction

The reaction was performed as previously described (Barr et al., 2005; Boyer et al., 2005; Kalb et al., 2005, 2006, 2008, 2009; Gaunt et al., 2007). In all cases, the final reaction volume was 20 µL; the final concentration of the reaction buffer was 0.05 M Hepes (pH 7.3), 25 mM dithiothreitol, 20 µM $ZnCl_2$, and 1 mg/mL bovine serum albumin; and the final concentration of the peptide substrate was 50 pmol/µL. All samples then were incubated at 37ºC for 4 hrs.

2.4 MS detection of Endopep-MS reaction

2 µL of each reaction supernatant was mixed with 18 µL of matrix solution consisting of alpha-cyano-4-hydroxy cinnamic acid (CHCA) at 5 mg/mL in 50% acetonitrile, 0.1% trifluoroacetic acid (TFA), and 1 mM ammonium citrate. We pipeted 0.5 µL of this mixture onto each spot of a 192-spot matrix-assisted laser desorption/ionisation (MALDI) plate (Applied Biosystems, Framingham, MA). Mass spectra of each spot were obtained by scanning from 1,000 to 5,000 *m*/*z* in MS-positive ion reflector mode on an Applied Biosystems 4800 Proteomics Analyser (Framingham, MA). The instrument uses a ND-YAG laser at 355 nm, and each spectrum is an average of 2400 laser shots.

2.5 Tryptic digests of BoNT/A1 and /A2 subtypes

A 20 µg aliquot of BoNT/A1 or /A2 at 1 mg/mL was added to 20 µL of acetonitrile and incubated at room temperature for 30 min. Then, a 140 µL solution of 50 mM ammonium bicarbonate (tryptic digest buffer) and 20 µL of trypsin, diluted to 10 µg/mL in tryptic digest buffer, were added to produce a final solution of 100 µg/mL of BoNT/A. The solutions were incubated overnight at 37ºC.

2.6 Tryptic digestion of BoNT/A isolated from spiked milk

Following the Endopep-MS reaction, all reaction supernatant was removed from the toxin-coated beads and replaced with a solution of 10 µL of acetonitrile with 1%TFA. This mixture was incubated at room temperature for 30 min to elute BoNT/A from the antibody-coated beads. The supernatant was removed from the beads, evaporated, and reconstituted in 15 µL of tryptic digest buffer and 5 µL of trypsin. The solutions were incubated overnight at 37°C.

2.7 MALDI analysis of tryptic digests

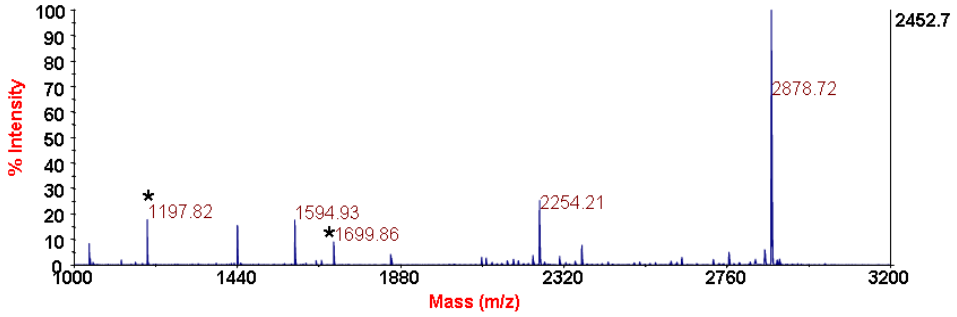
A 10 µL portion of each digestion was purified in a C_{18} minicolumn (C_{18} Zip Tips, Millipore, Bedford, MA) using the standard protocol obtained from Millipore. The peptides were eluted in 2 µL of 80% acetonitrile/0.1% TFA and mixed with 8 µL of matrix solution as described above, with preparation for mass spectrometry as described above. Mass spectra of each sample well were obtained from 650 to 4,500 *m*/*z* in MS positive-ion reflector mode on the Applied Biosystems 4800 Proteomics Analyser. Peptides believed to originate from BoNT/A were subjected to MS/MS analysis to confirm identify through sequence determination.

3 Results

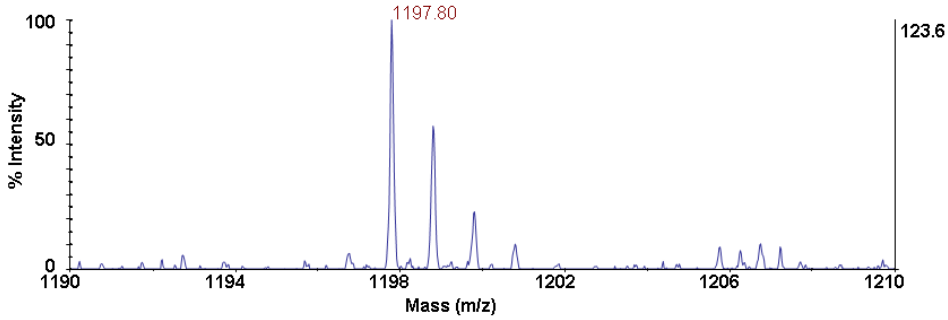
3.1 Detection of BoNT/A in serum

The Endopep-MS method is a rapid assay to detect and differentiate active BoNT serotypes. Because the mouse bioassay has historically been the standard method for detection and quantification of BoNT, the toxin is usually quantified via activity in terms of mouse LD_{50}. The Endopep-MS method also measures the activity of the toxin; therefore, we report here the activity of the toxin in mouse LD_{50}. Figure 1(a) depicts the mass spectrum of 10 mouse LD_{50} of BoNT/A concentrated from 500 µL of human serum using magnetic beads coated with antibodies to monoclonal antibodies, RAZ1 (Razai et al., 2005) and CR2 (Garcia-Rodriguez et al., 2007), followed by a reaction in buffer with peptide substrate. The N-terminal cleavage product is present at 1,699.9 *m/z*, and the C-terminal cleavage product is present at 1,197.8 *m/z*, and these peaks demonstrate the presence of BoNT/A in this sample. A negative control of serum without BoNT/A did not yield peaks at these *m/z*. Various toxin levels were spiked into serum, with a level as low as 0.5 mouse LD_{50} of BoNT/A spiked into 500 µL of serum detectable by Endopep-MS [Figure 1(b)].

Figure 1 Mass spectra of Endopep-MS reactions containing (a) 10 mouse LD_{50} of BoNT/A or (b) 0.5 mouse LD_{50} of BoNT/A spiked into 500 µL of serum



(a)



(b)

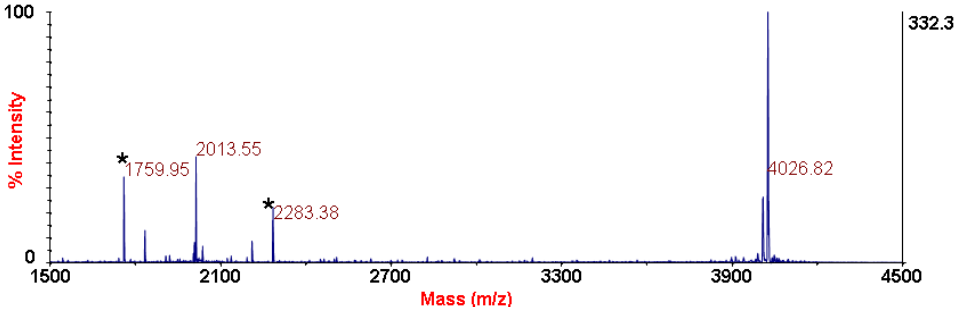
Note: The substrate peptide is present at *m/z* 2,878.7, the N-terminal cleavage product is at *m/z* 1,699.9, and the C-terminal cleavage product is at *m/z* 1,197.8.

BoNT/A can be further differentiated into different subtypes. It is important to be able to detect multiple subtypes within a serotype as any of the subtypes could be used in a terrorist event and have been found in botulism outbreaks. All BoNT/A can currently be classified as BoNT/A1, /A2, /A3, /A4, or /A5. Our laboratory was able to obtain toxins /A1, /A2, /A3, and /A4 and we have tested these toxins to ensure that detection by mass spectrometry is possible (Kalb et al., 2008). These data indicate that these subtypes of BoNT/A all demonstrate the same activity against the peptide substrate.

3.2 Detection of BoNT/B, /E, and /F in serum

We also detected the toxin cleavage products of BoNT/B, /E, and /F in serum samples using Ab-coated beads. In Figure 2(a), the N-terminal and C-terminal toxin cleavage products for cleavage of the peptide substrate with BoNT/B are seen at 1759.9 and 2,283.4 *m/z*, respectively, when 10 mouse LD_{50} of BoNT B extracted with monoclonal antibodies 1B18 (Kalb et al., 2008) and B12.1 (Kalb et al., 2008), from 500 µL of serum is reacted with peptide substrate. In Figure 3(a), cleavage of the peptide substrate by BoNT/E is apparent with the peaks at 1,136.6 and 2,922.8 *m/z* representing the C-terminal and N-terminal cleavage products when 10 mouse LD_{50} of BoNT/E extracted from 500 µL of serum is reacted with peptide substrate. Finally, in Figure 3(b), 10 mouse LD_{50} of BoNT/F extracted from 500 µL of serum is detected through the presence of the C-terminal and N-terminal cleavage products at *m/z* 1,345.8 and 3,168.9. All of the negative controls for BoNT/B, /E, and /F in serum did not yield peaks corresponding to their toxin-cleavage products. Various toxin levels were spiked into serum, with levels as low as 0.05 mouse LD_{50} of BoNT/B, /E, and /F spiked into 500 µL of serum detectable by Endopep-MS [Figures 2(b), 3(c) and 3(d)].

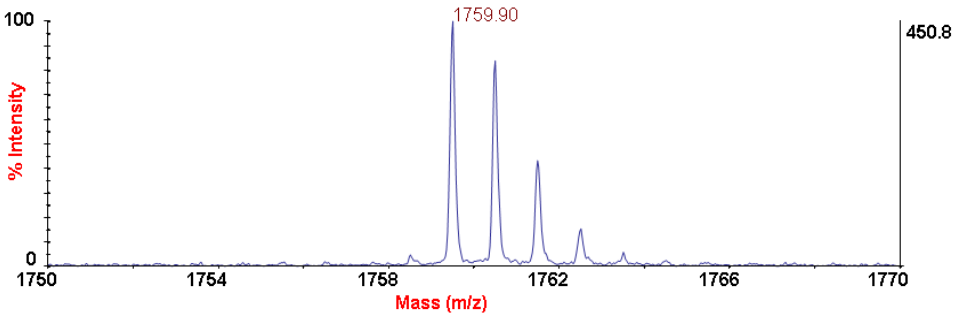
Figure 2 Mass spectra of Endopep-MS reactions containing (a) 10 mouse LD_{50} of BoNT/B or (b) 0.05 mouse LD_{50} of BoNT/B spiked into 500 µL of serum



(a)

Note: The substrate peptide is present at *m/z* 4,026.8, and cleavage products for BoNT/B are present at *m/z* 1,759.9 and 2,283.4.

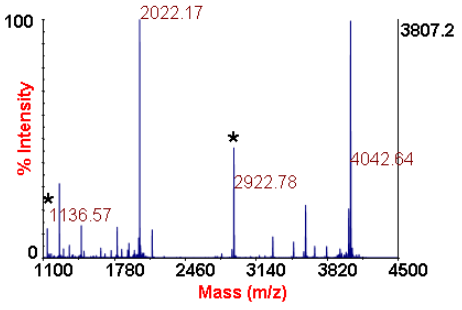
Figure 2 Mass spectra of Endopep-MS reactions containing (a) 10 mouse LD_{50} of BoNT/B or (b) 0.05 mouse LD_{50} of BoNT/B spiked into 500 µL of serum (continued)



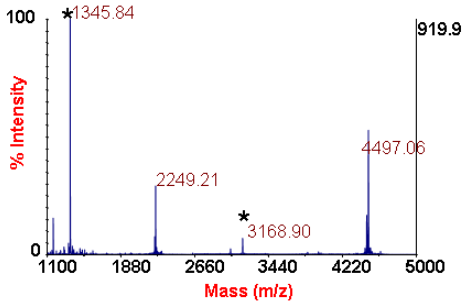
(b)

Note: The substrate peptide is present at *m/z* 4,026.8, and cleavage products for BoNT/B are present at *m/z* 1,759.9 and 2,283.4.

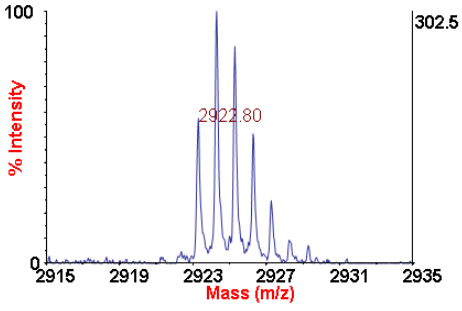
Figure 3 Mass spectra of Endopep-MS reactions containing BoNTs concentrated from 500 µL of serum, (a) 10 mouse LD_{50} of BoNT/E; (b) 10 mouse LD_{50} of BoNT/F; (c) 0.05 mouse LD_{50} of BoNT/E; (d) 0.05 mouse LD_{50} of BoNT/F



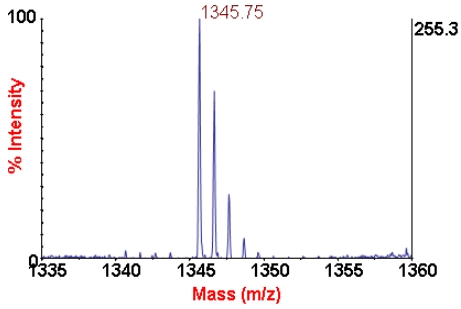
(a)



(b)



(c)



(d)

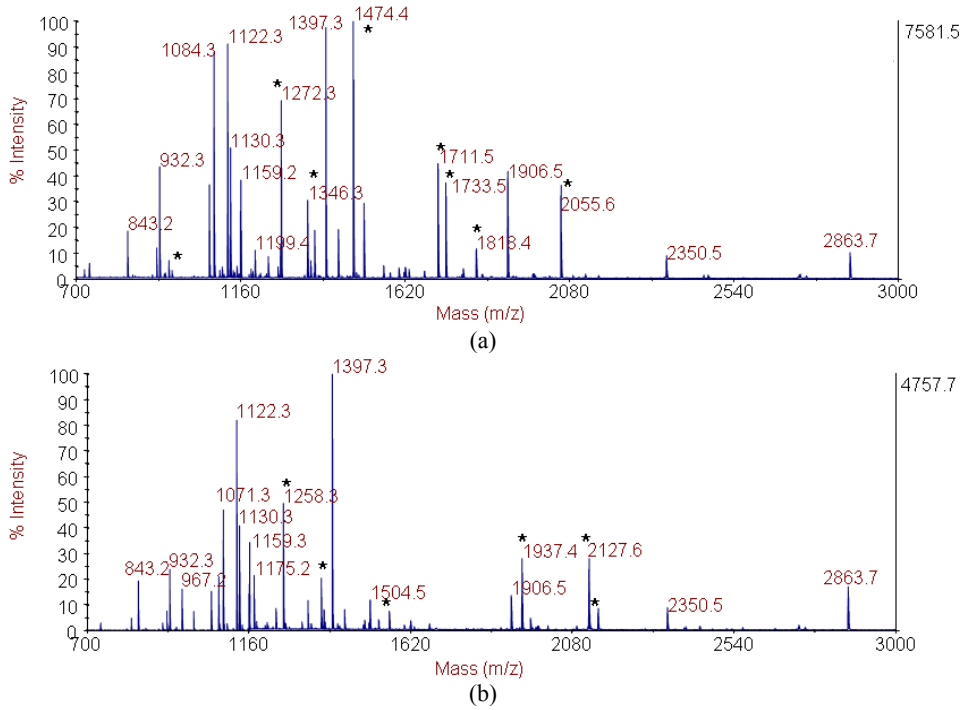
Note: Cleavage products for BoNT/E are present at *m/z* 1,136.6 and 2,922.8; and for BoNT/F at *m/z* 1,345.8 and 3,168.9.

As with BoNT/A, BoNT/B, /E, and /F can be further subdivided into various subtypes. Our laboratory has obtained most of those toxin subtypes in sufficient quantities, and we have tested those toxins to ensure that detection by mass spectrometry is possible. Currently, we can detect the presence of BoNT/B1, /B2, /B3, /B4, /B5, /B6, /B7, /E1, /E2, /E3, /E4, /F1, /F2, /F4, /F5, /F6, and /F7 (Kalb et al., 2008; Kalb et al., 2012a, 2012b). With the exception of /F5 (Kalb et al., 2012a), these data indicate that these subtypes of BoNT/B, /E, and /F all demonstrate the same activity against their peptides substrates.

3.3 Differentiation of BoNT/A subtypes

We have tested four of the five currently known subtypes of BoNT/A and found that they are detectable with the Endopep-MS assay. We expect the fifth subtype (A5) will also be capable of detection in the Endopep-MS assay since it is only a small variant of A1 (Carter et al., 2009). The assay yields the same data for each of the subtypes; i.e., the Endopep-MS assay cannot be used to differentiate the subtype of BoNT/A. Differentiation of the subtype can be very useful information for forensic or epidemiologic purposes as it can suggest that samples may originate from a single source or if subtypes are different then must originate from different sources. Therefore, we have devised a method to identify the subtype using the toxin protein itself. This method involves tryptic digestion of the toxin and mass spectrometric analysis of the tryptic fragments.

Figure 4 MALDI mass spectra of a tryptic digest of (a) BoNT/A1 or (b) /A2



Note: Most significant differences between the two spectra are marked with asterisks.

Source: Figure reprinted from Kalb et al. (2005).

Figure 4 are the MALDI-TOF mass spectra acquired following tryptic digestion of BoNT/A1 [4(a)] and /A2 [4(b)]. It is apparent from these spectra that there are many similarities between these two spectra. BoNT/A1 and /A2 are approximately 90% homologous (Willems et al., 1993), so most of the peptides generated from a tryptic digest are homologous. However, there are some peaks which are unique to either BoNT/A1 or /A2, and these can be used to identify a subtype as BoNT/A1 or /A2. These peaks are marked with asterisks in Figure 4. Additionally, Table 2 lists *m/z* values of peptides present in either the BoNT/A1 or /A2 subtype, but not both, as well as their respective peptide sequences determined by MS/MS with amino acid residue substitutions indicated. Peptides listed in this table can therefore be used to distinguish by MALDI analysis between BoNT/A1 and /A2. There are 18 peptides present in both digests, and these can be used to determine the presence of BoNT/A.

Table 2 Peptides unique to either BoNT/A1 or /A2 observed by MALDI listed with their observed *m/z* values

Digest fragment	*m/z obs of BoNT/A1*	*Sequence*
G827-R835	958.3	GTL**IG**QVDR
S166-R176	1,272.3	SFGH**E**VLNLTR
Y882-R892	1,346.3	Y**ESNH**LIDLSR
V381-R392	1,474.4	**V**NYTI**Y**DGFNL**R**
I565-R580	1,711.5	I**A**LTNS**VN**EALL**N**P**SR**
R806-K820	1,733.5	RL**E**DFDAS**LK**D**A**LLK
G1140-R1155	1,818.4	GSV**M**TTNIYLNS**S**LY**R**
F212-R230	2,055.6	FATDPAVTLAHELIHA**G**HR
Digest fragment	*m/z obs of BoNT/A2*	*Sequence*
R1064-K1069	823.3	RYI**M**IK
G827-R835	1,000.3	GTL**VL**QVDR
L807-R815	1,050.3	L**K**DFDAS**VR**
S166-R176	1,258.3	SFGH**D**VNLTR
V720-K730	1,328.3	VNTQIDLIR**E**K
I23-K36	1,529.4	IPN**A**GQMQPVKAFK
G393-R410	1,937.4	**GA**NL**STN**FNGQNTEIN**SR**
F212-R230	2,127.6	FATDPAVTLAHELIHA**E**HR
I897-K914	2,153.6	INIG**DR**V**YY**D**S**IKNQI**K**

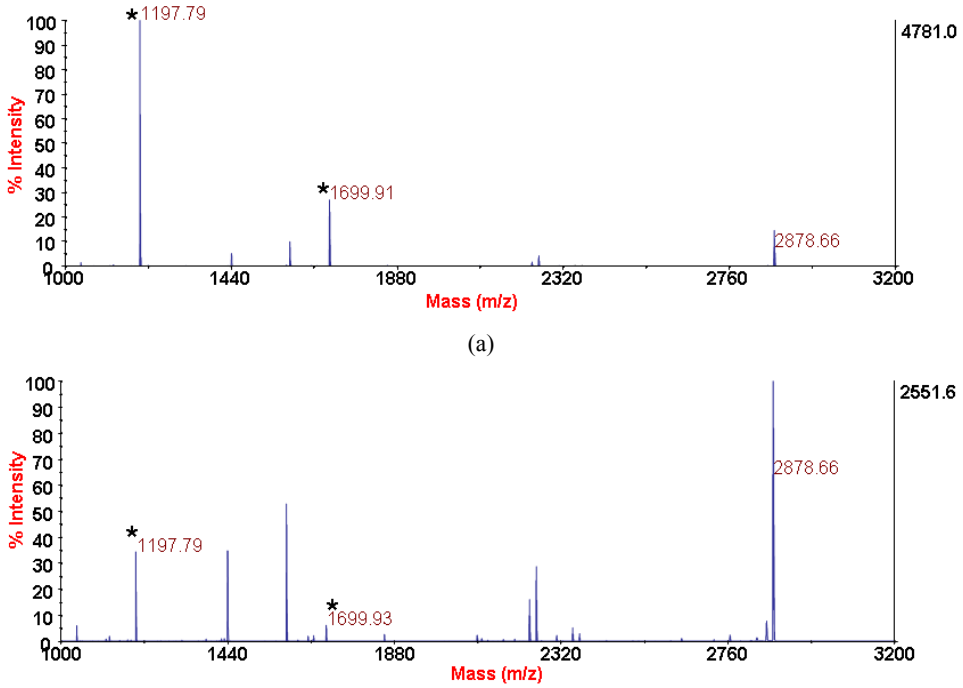
Notes: Residues that differ between subtypes are underlined and listed in boldface type. The amino acid sequences are listed and are predicted from the genomic sequences.

Source: Table reprinted from Kalb et al. (2005).

After determining that mass spectrometric analysis of tryptic digest fragments of BoNT/A yields subtype-specific information, we performed an experiment to examine

whether this process could be used to determine the subtype of BoNT/A present in a spiked milk sample. Antibody-coated beads were combined with a milk sample spiked with either BoNT/A1 or /A2. Following washing, the beads were subjected to the Endopep-MS reaction with mass spectrometric analysis of the reaction supernatant depicted in Figure 5. The peptide substrate is present in both cases at *m/z* 2,878.7. Cleavage of this peptide substrate by BoNT/A results in peptides at *m/z* 1,197.8 and 1,699.9. The presence of these peaks indicates that BoNT is in the milk sample and that the serotype is BoNT/A.

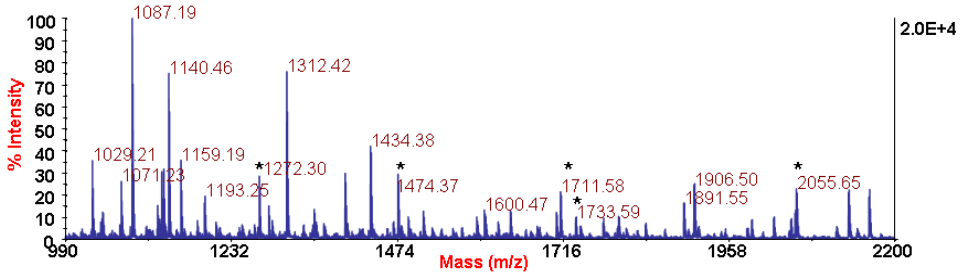
Figure 5 MALDI mass spectrum of Endopep-MS reaction of (a) BoNT/A1 or (b) BoNT/A2 spiked into milk



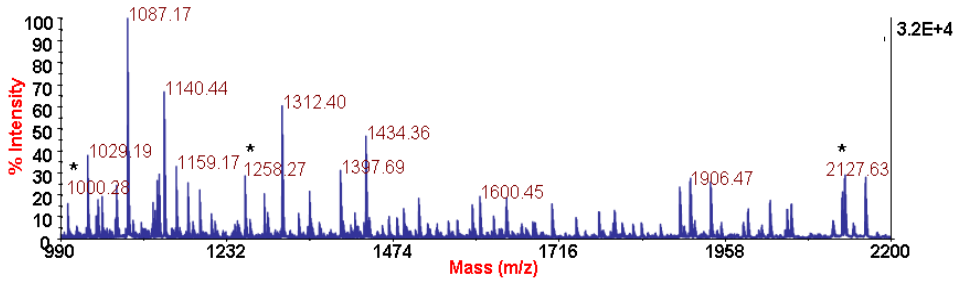
Note: The peptide substrate is present at *m/z* 2,878.7, and the toxin cleavage products at *m/z* 1,197.8 and 1,699.9 are marked with asterisks.

Following the Endopep-MS reaction, a tryptic digest was performed on the eluent of BoNT/A1 and /A2 from the antibody-coated beads that were used in the Endopep-MS assay. MALDI mass spectra of those digests are in Figure 6. It is apparent from these spectra that this process can be used to correctly differentiate between BoNT/A1 and /A2. For example, peaks at *m/z* 1,272.3, 1,474.4, 1,711.6, 1,733.6, and 2,055.7 can be used to identify the sample depicted in Figure 6(a) as BoNT/A1, and peaks at *m/z* 1,000.3, 1,258.3, and 2,127.6 identify Figure 6(b) as BoNT/A2. The amino acid sequences of these peaks are listed in Table 2. MS/MS spectra were acquired for several of the peaks present in only the BoNT/A1 or /A2 subtype (data not shown) to confirm the identity of those peaks. This experiment demonstrates the ability to identify the subtype of toxin isolated from a milk sample as either BoNT/A1 or BoNT/A2.

Figure 6 MALDI mass spectrum of a tryptic digest of (a) BoNT/A1 or (b) /A2 recovered from spiked milk



(a)



(b)

Note: Peaks marked with an asterisk are unique for identification of the BoNT/A1 or /A2 subtype.

4 Discussion

A botulism outbreak is a public health emergency, and early diagnosis and treatment of the disease are critical to minimise the morbidity and mortality from the disease. Prompt epidemiology and diagnostic laboratory testing can be important for identifying the source and preventing further cases of botulism. Also, identifying BoNT in a clinical sample or remnants of food consumed by a patient can confirm a diagnosis of botulism. Additionally, BoNTs are now considered potential biological warfare and terrorist agents. Information gained on the toxin may promote an understanding of a botulism outbreak and assist in identification of the source of the outbreak.

We have developed a method called Endopep-MS that can detect and differentiate the BoNT serotype in clinical and food samples using antibody-coated beads for toxin extraction. The effectiveness of this method is based on several levels of specificity. First, antibodies specific for the toxin serotype extract the toxin from serum or milk samples. Next, the toxin cleaves its peptide substrate in a specific, toxin-dependent location. Finally, the cleavage products from the enzymatic reaction are detected by mass spectrometry. This method can detect BoNT/A, /B, /E, and /F spiked into serum at levels

below that of the mouse bioassay. Specifically, we have demonstrated here that this method can currently detect 0.5 mouse LD_{50} of BoNT/A and 0.05 mouse LD_{50} of BoNT/B, /E, and /F spiked into a single 500 μL serum sample. This method produces results within hours of receipt of sample, allowing for rapid diagnosis without the use of live animals.

BoNT/A, /B, /C, /D, /E, and /F are known to exhibit genetic and amino acid variance within each serotype. Because the amino acid variance can result in antigenic differences, it is possible that amino acid variance would also result in enzymatic differences in terms of substrate and cleavage location. Our experiments determined that multiple BoNT/A, /B, /E, and /F subtypes, with the exception of BoNT/F5 (Kalb et al., 2012a), cleave their respective substrates at identical sites, despite having large genetic differences in their neurotoxins. These data imply that the non-commercial subtypes of BoNT/A, /B, /E, and /F have the ability to cleave the natural, *in vivo* substrate, SNAP-25 or VAMP-2, in the same location as the commercial subtypes of BoNT/A, /B, /E, and /F. These data also demonstrate the utility of Endopep-MS for detecting the presence of multiple, diverse BoNT/A, /B, /E, and /F; in some cases, at levels below that of the most commonly used method, the mouse bioassay.

In addition to being able to detect multiple subtypes of BoNT via their activities, it can also be important to differentiate those subtypes. Differentiation of the subtype can be very useful information for forensic or epidemiologic purposes as it can help determine whether multiple samples originate from a single source and might assist in identification of the source of botulism. Typically, subtype identification is determined through DNA analysis via polymerase chain reaction (PCR) (Szabo et al., 1992, 1993; Fach et al., 1993, 1995), or more recently through real-time polymerase chain reaction (RT-PCR) (Lovenklev et al., 2004). However, these methods can only be used if the bacterium that produces the toxin is present, and BoNT can potentially be present in a sample that does not contain the bacterium. In such a situation, subtype identification would not be possible using traditional, DNA-based methods.

Therefore, we have devised a method to identify the subtype using the toxin protein itself rather than bacterial DNA. This method involves tryptic digestion of the toxin and mass spectrometric analysis of the tryptic fragments. This method has been demonstrated on BoNT/A1 and /A2 spiked into milk and has proven effective at first detecting the active toxin and defining the serotype, and then differentiating the subtype of that toxin. It should be noted that the toxin subtype identification requires much higher levels of toxin than serotype identification; however, this level of toxin is comparable to some reports of BoNT/A present in food samples (Kalluri et al., 2003).

It is especially important to note that this technique to differentiate the BoNT subtype is used as an addendum to the Endopep-MS method which identifies the serotype of toxin present in a sample. Once the serotype of toxin is identified by Endopep-MS, the toxin responsible for that activity can be tryptically digested and then analysed for subtype identification. The subtype identification does not require a separate sample, provided that a sufficient level of toxin is present in the original sample. The combination of these selective techniques, all performed on the same sample, provides a sensitive and selective analysis of BoNT isolated from a food or clinical sample and measures the toxin's activity.

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