FEBS Letters 586 (2012) 109–115

journal homepage: www.FEBSLetters.org

Discovery of a novel enzymatic cleavage site for botulinum neurotoxin F5

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ARTICLE INFO

Article history:
Received 20 October 2011
Revised 23 November 2011
Accepted 28 November 2011
Available online 9 December 2011

Edited by Maurice Montal

Keywords:
Botulinum neurotoxin
BoNT/F
Synaptobrevin-2
Mass spectrometry

ABSTRACT

Botulinum neurotoxins (BoNTs) cause botulism by cleaving proteins necessary for nerve transmission. There are seven serotypes of BoNT, A–G, characterized by their response to antisera. Many serotypes are further distinguished into differing subtypes based on amino acid sequence, some of which result in functional differences. Our laboratory previously reported that all tested subtypes within each serotype have the same site of enzymatic activity. Recently, three new subtypes of BoNT/F; /F3, /F4, and /F5, were reported. Here, we report that BoNT/F5 cleaves substrate synaptobrevin-2 in a different location than the other BoNT/F subtypes, between ^{54}L and ^{55}E. This is the first report of cleavage of synaptobrevin-2 in this location.

Structured summary of protein interactions:
BoNT/F5 cleaves **Synaptobrevin-2** by protease assay (View interaction: 1, 2)
BoNT/F1 cleaves **Synaptobrevin-2** by protease assay (View interaction: 1, 2)

1. Introduction

Botulism is a disease which can be fatal if untreated and is caused by exposure to any one of the highly toxic proteins known as botulinum neurotoxins (BoNTs). BoNT are composed of a heavy chain, which binds to receptors on the neuron, and a light chain that is a protease. *In vivo*, the BoNT light chain cleaves proteins necessary for nerve signal transmission. This enzymatic cleavage leads to flaccid paralysis. Botulinum neurotoxins are currently classified into seven serotypes, labeled A–G. BoNT/A, /C, and /E cleave SNAP-25 (synaptosomal-associated protein) [1–6]whereas BoNT/B, /D, /F, and /G cleave synaptobrevin-2 (also known as VAMP-2) [7–11].

Our laboratory previously reported on BoNT's ability to cleave a peptide substrate that mimics its natural target, with peptide cleavage detected by mass spectrometry [12,13]. BoNT/A through /F are known to exhibit genetic and amino acid variance within each serotype, and this has led to the designation of subtypes, some with identified antigenic differences [14–16]. However, it was uncertain if the amino acid variance would also result in differential enzymatic activity. Therefore, previous work in our laboratory focused on determining the cleavage location of all available subtypes of BoNT/A, /B, /E, and /F, the serotypes known to cause botulism in humans. Previously, 15 such subtypes were available in sufficient quantities for testing, and all BoNT subtypes within the same serotype cleaved their respective substrate in the same location [17]. At the time that this work was reported, only four subtypes of BoNT/F were known.

In 2010, three new subtypes of BoNT/F; /F3, /F4, and /F5, were reported based on phylogenetic analysis of botulinum neurotoxin type F genes [18]. All three subtypes were defined as BoNT/F based on their ability to cause signs of botulism in mice which could be prevented by preincubation of toxin with F antitoxins, although it was reported that the BoNT/F5 toxin required 20 times more type F antiserum to neutralize an equivalent amount of BoNT/F1 toxin [19]. Additionally, it was noted that BoNT/F5 was highly different in its gene and amino acid sequences compared to the other BoNT/F subtypes [18]. For instance, Table 1 shows that the whole toxin (holotoxin) of BoNT/F1 and BoNT/F2 have similar (83.5% identical) amino acid sequences. However, BoNT/F1 and /F5

Abbreviations: BoNT, botulinum neurotoxin; SNAP-25, synaptosomal-associated protein; PBS, phosphate buffered saline; TPGY, trypticase-peptone-glucose-yeast extract; CHCA, alpha-cyano-4-hydroxy cinnamic acid; TFA, trifluoroacetic acid; MALDI, matrix-assisted laser desorption/ionization; FA, formic acid

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0014-5793/$36.00 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2011.11.033

Table 1
Percent amino acid identity between BoNT/F [18].

Holotoxin	F1	F2	F3	F4	F5	F6	F7
F1	–	83.5	83.9	92.3	69.9	87.7	73.7
F2	81.8	–	97.1	83.5	74.2	90.2	68.9
F3	82.9	97.9	–	83.8	74.0	90.1	69.2
F4	96.4	82.7	83.8	–	69.5	87.1	72.2
F5	47.3	46.3	46.3	47.6	–	74.0	63.9
F6	94.3	81.5	82.9	93.6	48.3	–	69.9
F7	63.3	59.5	60.8	64.2	46.9	63.3	–
	F1	F2	F3	F4	F5	F6	F7

Enzymatic domain (light chain).

holotoxins are dissimilar (69.9% identical). The amino acid dissimilarity (47.3% identity) observed in the light chain (the enzymatic domain) between BoNT/F5 and BoNT/F1 is even more pronounced. Until the identification of BoNT/F5, this level of variation in the light chain amino acid sequences within a serotype had not been reported. Indeed, an amino acid identity of only 47% is much less than the light chain identity between differing serotypes which have different sites of enzymatic activity, such as 56% between BoNT/E and /F and 61% between BoNT/B and /G, based on amino acid comparison.

The second-most divergent BoNT/F subtype, F7, was reported to have different peptide substrate recognition requirements [20]. BoNT/F7, produced by *Clostridium baratii*, is 73.7% identical to BoNT/F1 at the holotoxin level and 63.3% identical at the enzymatic domain level. BoNT/F7 was reported to cleave both synaptobrevin-2 and peptides based on synaptobrevin-2 in the same location as the other BoNT/F subtypes, between ^{58}Q and ^{59}K. However, BoNT/F7 did not cleave a peptide substrate cleaved by other available BoNT/F subtypes based on the sequence of synaptobrevin-2, and would only cleave TSNRRLQQTQAQVDEVVDIMRVNVDKVLERDQKLSELDDRADAL, a lengthened peptide substrate [20]. A similar phenomenon was reported with two neurotoxins which also cleave synpatobrevin-2, BoNT/B and tetanus toxin [21]. This was due to differential binding of BoNT/F7 with synaptobrevin-2 as compared to other BoNT/F. Because the amino acid sequence of the BoNT/F5 light chain is even more divergent than BoNT/F7, it was theorized that BoNT/F5 may have a different molecular reaction with its substrate. In this work, we describe the discovery of a novel enzymatic cleavage site on synaptobrevin-2 by BoNT/F5.

2. Materials and methods

2.1. Materials

Synaptobrevin-2, SNAP-25, and synatxin recombinant proteins were purchased from GenWay Biotech, Inc. (San Diego, CA). Monoclonal antibody 6F5 was obtained from Dr. James Marks at the University of California at San Francisco. Dynabeads® (M-280/Streptavidin) were purchased from Invitrogen (Carlsbad, CA.) at 1.3 g/cm^3 in phosphate buffered saline (PBS), pH 7.4, containing 0.1% Tween®-20 and 0.02% sodium azide. All chemicals were from Sigma-Aldrich (St. Louis, MO) except where indicated. Peptide substrates LQQTQAQVDEVVDIMRVNVDKVLERDQKLSELDDRADAL and TSNRRLQQTQAQVDEVVDIMRVNVDKVLERDQKLSELDDRADAL were synthesized by Biopeptide (San Diego, CA). Commercially purified BoNT/F1 (strain Langeland) was purchased from Metabiologics (Madison, WI).

2.2. Gene construction of BoNT/F1 and F5 LC-GST

An existing BoNT/F1 LC ORF was PCR amplified using primers to add 5' *Bam*HI and 3' *Eco*RI sites. The PCR reaction product was run on a 1% TAE gel and the amplified band at approximately 1400 bp was excised, purified, ligated into the homologous restriction enzymes sites of pGEX6P-1, and transformed into the DH5-α strain of *E. coli*. A single recombinant colony was used to inoculate 5 ml of LB-50 mg/L ampicillin and grown overnight at 37 °C. Plasmid DNA was recovered, completely sequenced to insure accuracy, mobilized into *E. coli* strain BL21 DE3, and spread onto LB plates with 50 mg/L ampicillin. A single recombinant colony was used to inoculate a LB-50 mg/L ampicillin, grown overnight at 37 °C. The overnight culture was used to inoculate a fresh flask of 1L of LB-50 mg/L ampicillin and grown to an OD of 0.7, cooled to 18 °C, IPTG was added to a final concentration of 1 mM, and the induction was allowed to proceed at 18 °C overnight. The same procedure was followed for BoNT/F5, with the exception that the BoNT/F5 light chain ORF (representing nucleotides 1-438 from GU213211.1) was synthesized *de novo* using a generalized *E. coli* K12 codon bias and ligated into the *Bam*HI/*Eco*RI sites of bacterial expression vector pGEX6P-1.

2.3. Purification of fusion proteins

Both proteins bound to glutathione sepharose resin and were eluted in the presence of 10 mM reduced glutathione; however, the protein was not purified at this stage and required further chromatography steps. Both proteins were further separated with cation exchange chromatography after a pH change from ~8.0 to ~5.7. After cation exchange chromatography the purified BoNT/F5 LC-GST was approximately 100 μg/ml, while the BoNT/F1 LC-GST was approximately 40 μg /ml.

2.4. Production and characterization of BoNT/F5 culture supernatant

CDC54075, originally isolated in March 1978 from soil in a cornfield in Mendoza, Argentina at latitude 34° 98' and longitude 67° 59', was inoculated into 10 mls of Trypticase-Peptone-Glucose-Yeast Extract (TPGY) broth (Remel, Inc. Lexana, KS) at 35 °C for 5 days in an anaerobe chamber system (Coy Laboratory Products, Inc. Grass Lake, MI) utilizing 10% Hydrogen, 5% CO_2, and 85% Nitrogen. The culture was tested by PCR for the presence of neurotoxin genes as previously reported [18]. The culture was centrifuged at 4000×*g* for 10 min and filtered using a 0.45 μm syringe filter (Whatman #6876-2504) to remove viable cells. The presence of botulinum toxin in the culture supernatant was confirmed by ELISA and mouse bioassay [22].

2.5. Reaction of BoNT fusion protein with a peptide or protein target

The BoNT fusion proteins were serially diluted in water to achieve three different concentrations of each protein. For F1, concentrations of 40 μg/ml (52.7 pmol/ml), 4 μg/ml (5.27 pmol/ml), and 2 μg/ml (2.64 pmol/ml) were used; whereas concentrations of 100 μg/ml (1.32 nmol/ml), 10 μg/uL (132 pmol/ml), and 5 μg/ml (66 pmol/ml) were used for F5. 2 μl of each concentration of fusion protein was added to 16 μl of reaction buffer consisting of 0.05 M Hepes (pH 7.3), 25 mM dithiothreitol, and 20 μM $ZnCl_2$. Finally, 2 μl of recombinant synaptobrevin-2 or peptide substrate (LQQTQAQVDEVVDIMRVNVDKVLERDQKLSELDDRADAL or TSNRRLQQTQAQVDEVVDIMRVNVDKVLERDQKLSELDDRADAL) was added to achieve a final concentration of 250 ng/μl (18 pmol/μl) for synaptobrevin-2 and 50 pmol/μl for the peptide reactions. All samples then were incubated at 37 °C for 4 hrs with no agitation.

2.6. Reaction of BoNT/F5 holotoxin with a peptide or protein target

Monoclonal antibody 6F5, a clonal relative of mAb E17.1 [23] engineered toward higher affinity for BoNT/F subtypes, was immobilized to streptavidin Dynabeads® after being rinsed three times with