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Cloning, expression, and purification of C-terminal quarter of the heavy chain of botulinum neurotoxin type A

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Abstract

Botulinum neurotoxins (BoNTs) are highly potent toxins that inhibit neurotransmitter release from peripheral cholinergic synapses. BoNTs consist of a toxifying light chain (LC; 50 kDa) and a binding-translocating heavy chain (HC; 100 kDa) linked through a disulfide bond. The complete sequence of BoNT/A consists of 1296 amino acid residues. The β-trefoil domain for BoNT/A to which gangliosides bind starts at Ser 1092 and this fragment represents the C-half of the C-terminus of the heavy chain (C-quarter HC or HCQ). The recombinant HCQ DNA was successfully cloned into an expression vector (pET15b), which was used to transform *Escherichia coli* strain BL21-Star (DE3) for expression. Expression of HCQ was obtained by an extended post-induction time of 15 h at 30 °C. The recombinant histidine tagged HCQ protein was isolated and purified by nickel affinity gel column chromatography and its molecular weight was verified by gel electrophoresis. The HCQ was positively identified by antibodies raised against BoNT/A employing immunological dot-blot and Western blot assays. HCQ was shown to bind with synaptotagmin (a known BoNT/A receptor) and gangliosides, indicating that the expressed and purified HCQ protein retains a functionally active conformation.

Keywords: Botulinum; Dot-blot; Expression; Gangliosides; Neurotoxin; Purification; Receptor

Botulinum neurotoxins (BoNTs)[2] are a family of seven structurally and pharmacologically similar but antigenically different proteins produced by different strains of *Clostridium botulinum* [1–3]. These proteins are the most toxic substances known and are the cause of flaccid paralysis in botulism. BoNTs are produced as 150 kDa single-chain polypeptides, which are nicked into a 100 kDa heavy chain and a 50 kDa light chain each, linked through a disulfide bond. The heavy chain (HC) is mainly involved in the cell-binding, internalization, and translocation of the BoNT into nerve cells, whereas the light chain (LC) exhibits the intracellular toxic activity [4]. The carboxyl-terminal portion of the HC is responsible for binding with nerve cell receptors [5]. Following binding to the cell surface, the neurotoxin is brought into the endosomal compartment by internalization via receptor mediated endocytosis. The LC gets partially unfolded at low endosomal pH to get across the membrane into cytosol, where it acts as an endopeptidase against one or more of the three SNARE (soluble NSF attachment

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[2] *Abbreviations used:* BoNTs, botulinum neurotoxins; HC, heavy chain; LC, light chain; SNARE, soluble NSF attachment protein receptor; PCR, polymerase chain reaction; SNAP, simple nucleic acid prep; dNTP, deoxy-nucleoside triphosphate; OD, optical density; IPTG , Isopropyl-β-D-thiogalactopyranoside; AEBSF, 4-[2-aminoethyl]benzenesulfonyl fluoride hydrochloride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DPC, dodecylphosphocholine; IEF, isoelectric focusing; PBST, phosphate buffered saline, containing 0.05% Tween 20 ; BCIP 5-bromo-4-chloro-3-indolyl-phosphate; NBT, Nitroblue tetrazolium; ELISA, enzyme-linked immunosorbent assay; pNPP, *para*-nitrophenyl phosphate; MW, molecular weight.

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protein receptor) proteins: SNAP-25 (synaptosomal associated protein of 25 kDa), syntaxin or VAMP/synaptobrevin [6,7]. The cleavage of any of the SNARE proteins prevents the fusion of synaptic vesicles containing acetylcholine, thus blocking the neurotransmitter release [8] that results in the flaccid muscle paralysis.

The carboxyl-terminal domain, referred to as the H_C-fragment, mediates the highly specific binding of clostridial neurotoxins to nerve terminals at the neuromuscular junction through gangliosides and a protein receptor(s) [9]. The N-terminal domain of H_C (H_{CN}) has been speculated to bind with a protein receptor [10–12], whereas the C-terminal domain of H_C (H_{CC}) or HCQ domain has been shown to provide sites for binding with gangliosides [13]. The amino terminal half of HC (H_N-domain) provides the translocation apparatus for the delivery of the LC from the endosome into the cytosol. According to the crystal structure analysis of the BoNT/A, the H_C clearly consists of two distinct domains (H_{CN} and H_{CC}) [14]. The Hcc domain (HCQ) starts at Ser 1092 and theoretically its molecular weight is a quarter of the HC [15,16]. The HCQ fragment in its isolated purified form can enable one to study its interactions with receptors of BoNT/A, and to design binding inhibitors to prevent the neurotoxic action.

Receptor-binding domains of clostridial neurotoxins are receiving more attention in recent years for the development of a number of applications including neuronal targeting. Such molecules clearly have a potential application in the treatment of a range of neurologic conditions [17,18]. The binding domain of the HC, which is a nontoxic fragment, can also be used as a potential candidate for the development of therapeutics and diagnostics. In fact, the HCQ fragment of BoNTs seems to be a promising tool in the search for potential vaccines and immunogens [19–22].

In this study, we present results of cloning, expression, and purification of the HCQ fragment to study its binding characteristics that might enable identification of the receptors for BoNT/A. The purified HCQ reacted positively with antibodies raised against BoNT/A and also bound to gangliosides and synaptotagmin.

Materials and methods

Restriction endonucleases and DNA modifying enzymes were purchased from New England Biolabs (Beverly, MA). All oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). Eppendorf–Netheler–Hinz GmbH (Hamburg, Germany) thermal cycler (Model-Mastercycler personal) was used for polymerase chain reaction (PCR) experiments. The recombinant plasmid DNA pET 15b encoding the heavy chain of BoNT/A (pET 15b-HCA) was obtained as described previously by Li and Singh [23]. The reagents used in all the experiments were of analytical grade. Nuclease free water was used from Ambion (Austin, TX).

Plasmid DNA preparation

The plasmid DNA was isolated using SNAP (Simple Nucleic Acid Prep)—a Miniprep Kit supplied by Invitrogen Life Tech (Carlsbad, CA) from *Escherichia coli* strain BL21-Codon Plus (DE3)-RIL (Stratagene, La Jolla, CA). The *E. coli* strain containing two plasmids pACYC and pET15b-HCA (encoding the heavy chain of BoNT/A) was prepared as described previously [23]. The heavy chain fragment (pET15b-HCA) was cleaved using restriction enzymes *Nde*I and *Bam*HI, and then electrophoresed on a 1.0% agarose gel. The DNA of plasmid pET-15b contained the heavy chain insert (*Nde*I–*Bam*HI fragment) which was extracted using QIAquick Gel Extraction Kit (Qiagen, Chatsworth, CA) and used as a template for PCR.

Primer design and polymerase chain reaction

Polymerase chain reaction was performed to generate a DNA fragment encoding the C-half of the C-terminus of the heavy chain of BoNT/A. Both *Nde*I and *Bam*HI restriction sites were incorporated into the 5′ end of the forward sequence and reverse sequence primers, respectively. PCRs were performed in a total volume of 50 μL containing 200 ng of template DNA, 100 ng of each primer, 200 μM of each dNTP (deoxy-nucleoside triphosphate), 1 U Vent polymerase (New England Biolabs, Beverly, MA), and nuclease free water to adjust the total volume. The reaction mixture was denatured for 2 min at 93 °C, then subjected to 30 consecutive cycles consisting of denaturation (1 min at 93 °C), annealing (1 min at 60 °C), and polymerization (1 min at 73 °C).

According to BoNT/A sequence in GenBank data-base under Accession No. M30196 [15], primers for heavy chain binding domain were designed. 5′-GGGCCCC CAT↓ATG TCA AAT TCA GG-3′ was the sense primer for BoNT/A HCQ. The antisense primer sequence used was 5′-GGGCCCC GGA↓TCC TTA CAG TGG CCT TTC T-3′. An expected size (615 bp) band was purified by agarose gel electrophoresis, digested with restriction enzymes *Bam*HI and *Nde*I and ligated with predigested (restriction enzymes *Bam*HI and *Nde*I treated) vector pET15b. An aliquot of the ligation mixture was used to transform *E. coli* strain BL21-Star (DE3). The *E. coli* cells were plated on LB-ampicillin (50 μg/mL) agar plates and the transformants were verified to contain the HCQ by Plasmid DNA isolation and agarose gel electrophoresis.

DNA sequencing

The recombinant plasmid DNA containing the HCQ gene was sequenced with a 3730 × l DNA Analyzer