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High-level expression, purification, crystallization and preliminary X-ray crystallographic studies of the receptor-binding domain of botulinum neurotoxin serotype D

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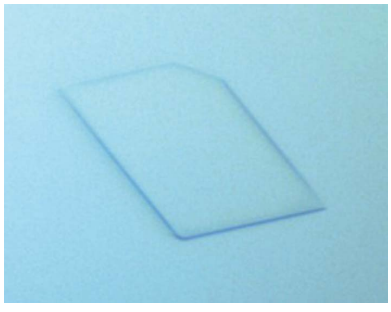
Botulinum neurotoxins (BoNTs) are highly toxic proteins for humans and animals that are responsible for the deadly neuroparalytic disease botulism. Here, details of the expression and purification of the receptor-binding domain (HCR) of BoNT/D in *Escherichia coli* are presented. Using a codon-optimized cDNA, BoNT/D_HCR was expressed at a high level (150–200 mg per litre of culture) in the soluble fraction. Following a three-step purification protocol, very pure (>98%) BoNT/D_HCR was obtained. The recombinant BoNT/D_HCR was crystallized and the crystals diffracted to 1.65 Å resolution. The crystals belonged to space group $P2_12_12_1$, with unit-cell parameters a = 60.8, b = 89.7, c = 93.9 Å. Preliminary crystallographic data analysis revealed the presence of one molecule in the asymmetric unit.

1. Introduction

Botulinum neurotoxins (BoNTs) produced by the bacterium *Clostridium botulinum* are highly toxic proteins with an extremely low LD_{50} of 1 ng kg^{-1} (Willis *et al.*, 2008; Lamanna, 1959; Middlebrooks *et al.*, 1997). There are seven serotypes of BoNTs (A–G), all of which can cause the neuroparalytic disease botulism (Simpson, 1986). These neurotoxins affect the nervous system by inhibiting the release of a neurotransmitter, acetylcholine, resulting in flaccid paralysis (Simpson, 1986; Schiavo *et al.*, 1992; Habermann & Dreyer, 1986). The BoNTs contain two parts, an N-terminal 50 kDa light chain and a C-terminal 100 kDa heavy chain, which are linked by a disulfide bond after cleavage of the originally translated protein by a protease (DasGupta & Sugiyama, 1972). The light chain possesses an endoprotease activity, cleaving proteins that are essential for neurotransmitter release such as SNAP25 (synaptosome-associated protein) and synaptobrevin (also known as VAMP; vesicle-associated membrane protein). Different serotypes recognize different substrates. BoNT/A, BoNT/C and BoNT/E cleave SNAP25 with distinct substrate specificities (Breidenbach & Brunger, 2005; Tonello *et al.*, 1996). BoNT/B, BoNT/D, BoNT/F and BoNT/G cleave synaptobrevin (Breidenbach & Brunger, 2005; Montecucco *et al.*, 2005; Schiavo *et al.*, 2000). The heavy chain consists of an N-terminal translocation domain and a C-terminal receptor-binding domain (HCR). The translocation domain is involved in the transportation of BoNTs from the endosome into the cytosol, while the HCR domain is responsible for interactions with neuronal membranes.

Among the seven serotypes, BoNT/C and BoNT/D predominately cause avian and animal botulism (Lindstrom *et al.*, 2004; Collins & East, 1998; DasGupta & Sugiyama, 1972), with the other serotypes being responsible for human botulism. BoNT/D is the only serotype that does not bind gangliosides, but instead appears to interact with lipids such as phosphatidylethanolamine (PE; Tsukamoto *et al.*, 2005). Consequently, structural analysis of the HCR domain of BoNT/D would help to elucidate how differently this neurotoxin interacts with membranes compared with the other serotypes, giving insights into the molecular mechanisms of BoNT/D toxicity. Towards achieving this goal, we have highly overexpressed BoNT/D_HCR with high solubility in *Escherichia coli* using a codon-optimized cDNA and optimized expression conditions. Approximately 150–



200 mg protein was purified from 1 l cell culture. The recombinant BoNT/D_HCR was crystallized and the crystals diffracted to 1.65 Å resolution.

2. Materials and methods

2.1. DNA synthesis and protein expression

The DNA sequence encoding BoNT/D_HCR (Ser863–Glu1276) was codon-optimized for bacterial expression and synthesized with a C-terminal 6×His tag and with *Nde*I and *Xho*I restriction-enzyme sites at the N- and C-termini, respectively (DNA 2.0; Welch *et al.*, 2009). The optimized cDNA (see Supplementary Material[1] for the sequence) was then inserted into the expression vector pJexpress411 using standard restriction-endonuclease digestion with designed overhangs followed by standard ligation. The plasmid containing BoNT/D_HCR was then transformed into the expression host, *E. coli* BL21 (DE3) competent cells (Invitrogen). A single colony was picked, inoculated into 20 ml LB medium containing 50 µg ml^{-1} kanamycin and incubated overnight at 310 K with shaking at 200 rev min^{-1}. This culture was then transferred into 1 l LB medium and further incubated at 310 K until the OD_{600} absorbance reached 0.8–1.0. The cells were then chilled on ice for 10 min and BoNT/D_HCR expression was induced by the addition of 0.02 m*M* IPTG. After growth at 285 K overnight, cells were harvested by centrifugation and stored at 193 K.

2.2. Protein purification

For BoNT/D_HCR protein purification, cell pellets were resuspended in buffer *A* (50 m*M* sodium phosphate, 300 m*M* NaCl, 10% glycerol, 10 m*M* *β*-mercaptoethanol pH 8.0) with protease-inhibitor cocktail (Fisher Scientific). After sonication, the crude cell extract was centrifuged for 20 min at 12 000*g* (277 K). The supernatant was loaded onto a 20 ml Ni–NTA agarose column (Qiagen) pre-equilibrated with buffer *A*. The column was washed with 200 ml buffer *A* containing 20 m*M* imidazole followed by 50 ml buffer *A* containing 200 m*M* imidazole. The protein eluates under the latter condition were pooled and concentrated to ∼1 ml (Amicon Centriprep-30, Millipore).

After Ni–NTA purification and Amicon concentration, BoNT/D_HCR was loaded onto a 1 ml HiTrap Q ion-exchange column (GE Healthcare) pre-equilibrated with buffer *B* (20 m*M* Tris pH 8.5). The protein was eluted from the column with a linear concentration gradient of NaCl (0–1 *M*) at a flow rate of 1 ml min^{-1}. Peak fractions containing highly purified protein were combined and concentrated as described above.

The oligomeric state of BoNT/D_HCR was determined by size-exclusion chromatography using a Superdex 200 10/30 GL column (GE Healthcare) equilibrated with PBS buffer containing 5% glycerol at a flow rate of 0.5 ml min^{-1}. Protein concentration was measured using the Bradford assay with bovine serum albumin as the standard. SDS–PAGE was performed using precast NuPAGE 4–12% bis-tris polyacrylamide gels (Invitrogen) followed by Coomassie Brilliant Blue staining (Sigma).

2.3. Western blot

Western blot analysis was performed using either a biotinylated anti-6×His antibody (USBiological) or an anti-*C. botulinum* D toxoid antibody (HyTest) as the primary antibody. Following SDS–PAGE, proteins were transferred onto a PVDF membrane (Invitrogen) and blocked for >6 h at room temperature with TBS (20 m*M* Tris, 150 m*M* NaCl pH 8.0) containing 5% milk. The membrane was then incubated overnight at 277 K in TBST buffer (TBS plus 0.1% Tween-20) containing the primary antibody. Next, the membrane was washed three times with TBST alone and incubated with streptavidin-HRP (for biotinylated anti-6×His antibody) or rabbit anti-goat IgG-HRP (for anti-*C. botulinum* D toxoid antibody) at 298 K for 1 h. The signal was developed with SuperSignal West Dura solution (Thermo Scientific) and detected using a Lumi-imager (Roche).

2.4. MALDI–TOF mass spectrometry

A 600 mm AnchorChip target plate (Bruker Daltonics GmbH) was used to mix 1 µl protein solution (3 mg ml^{-1}) with 1 µl matrix solution (10 mg ml^{-1} 2,5-dihydroxybenzoic acid). Using the *FlexControl* software (Bruker Daltonics GmbH), MALDI–TOF mass spectra were obtained in the linear mode using a Bruker ultrafleXtreme mass spectrometer (Bruker Daltonics GmbH) equipped with 1 kHz smartbeam-II laser technology. The system used a 1000 Hz pulsed nitrogen laser emitting at 337 nm. Spectra from various locations over the surface of the matrix spot were analyzed.

2.5. Crystallization and X-ray data collection

Prior to crystallization, the protein was exchanged from PBS buffer to buffer *C* (20 m*M* Tris, 150 m*M* NaCl pH 7.4) using two 5 ml spin columns containing Sephadex G-25 Coarse (GE Healthcare). Crystals were screened with the hanging-drop vapor-diffusion method at room temperature (2 µl protein solution at ∼10 mg ml^{-1} and 2 µl reservoir solution equilibrated against 1 ml reservoir solution) using kits from Hampton Research and Emerald BioSystems. The precipitant conditions were optimized after initial crystallization success. The best crystals grew using reservoir solutions consisting of 5%(*w*/*v*) PEG 4000, 0.2 *M* sodium acetate and 0.1 *M* Tris pH 7.5. Crystals were transferred stepwise into cryoprotectant solutions containing increasing concentrations of glycerol for cryoprotection. Crystals were then directly mounted on nylon cryoloops (Hampton Research), flash-frozen and stored under liquid nitrogen.

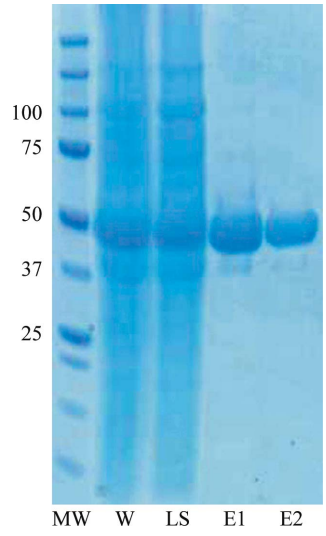


Figure 1
SDS–PAGE of BoNT/D_HCR purification using Ni–NTA and ion-exchange chromatography. Lane MW, molecular-weight standards (kDa); lane W, whole cell extracts; lane LS, supernatant of low-speed centrifugation; lane E1, eluate from Ni–NTA column; lane E2, eluate from ion-exchange chromatography.

[1] Supplementary material has been deposited in the IUCr electronic archive (Reference: XB5024).

X-ray diffraction data were collected on beamline X29A at the National Synchrotron Light Source (NSLS) at Brookhaven National Laboratory. Data were collected with an ADSC Q315 CCD detector, processed using *DENZO* and integrated intensities were scaled using *SCALEPACK* from the *HKL*-2000 program package (Otwinowski & Minor, 1997).

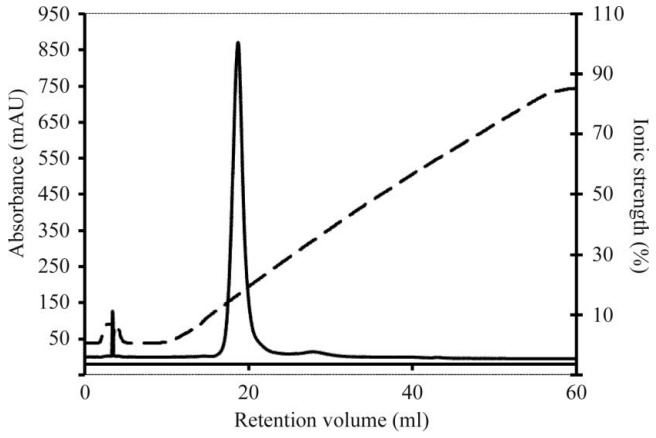


Figure 2
Chromatogram of BoNT/D_HCR purification by ion-exchange chromatography. Solid line, absorbance at 280 nm; dotted line, ionic strength gradient.

3. Results and discussion

3.1. Protein expression and purification

In order to highly express BoNT/D_HCR, the native DNA sequence from *C. botulinum* was optimized to eliminate codons that are rarely used by *E. coli*. In the process, the A+T content was reduced from 76 to 56% with frequently used synonymous codons in *E. coli*. The expression results showed that BoNT/D_HCR was produced at a high level (~30% of total cell protein) with excellent solubility (~90% soluble). Initial protein purification using an Ni–NTA column yielded approximately 150–200 mg per litre of cell culture of BoNT/D_HCR at ~90% purity as judged by SDS–PAGE (Fig. 1). Ion-exchange chromatography showed that BoNT/D_HCR eluted as a single peak at an ionic strength between 0.15 and 0.2 *M* NaCl (Fig. 2). Analysis by SDS–PAGE indicated that the protein was >95% pure at this stage (Fig. 1). Size-exclusion chromatography further purified the protein and showed that BoNT/D_HCR eluted with a retention time characteristic of a protein with a molecular weight in the region of 50 kDa, indicating that the protein was a

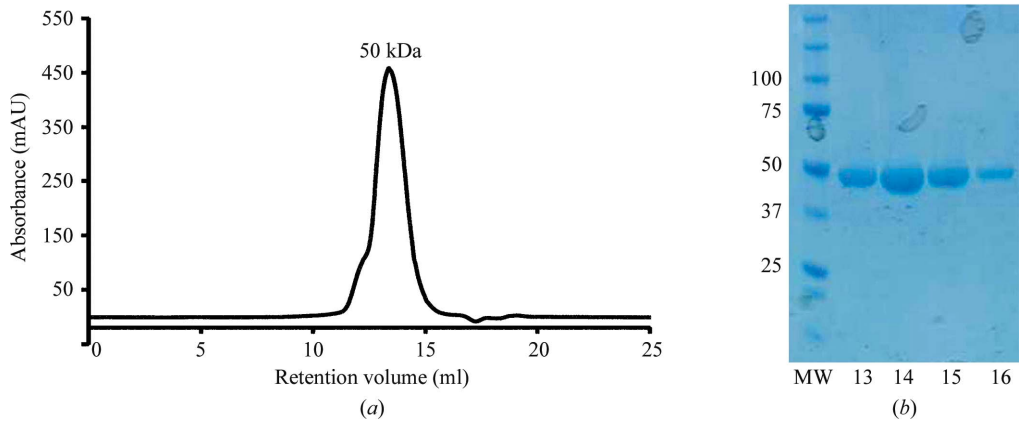


Figure 3
Size-exclusion chromatography of BoNT/D_HCR. (*a*) Chromatogram following size-exclusion chromatography. The major peak corresponds to a molecular weight of ~50 kDa. (*b*) SDS–PAGE of size-exclusion elution fractions sampled across the major peak.

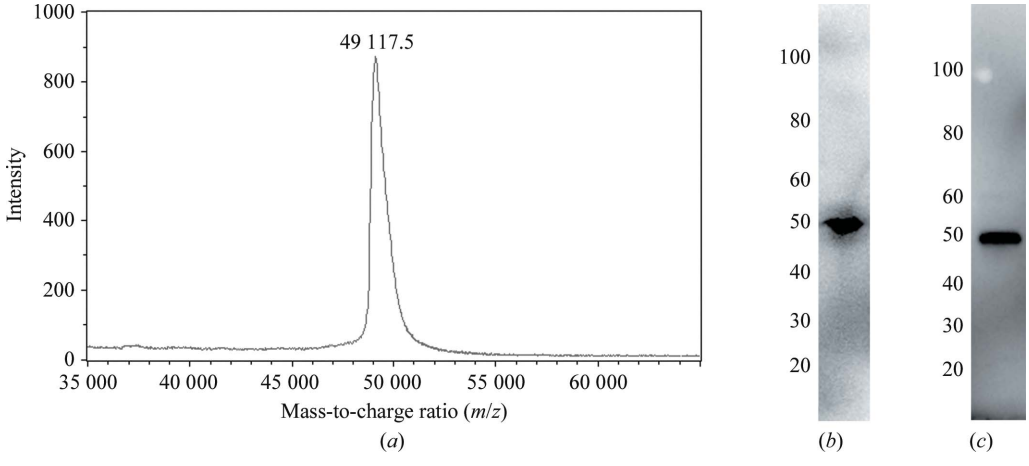


Figure 4
BoNT/D_HCR verification by mass spectrometry and Western blot. (*a*) MALDI mass spectrometry. The protein was detected in the positive-ion mode, therefore 49 117.5 corresponds to the species $[M+H]^+$ and the molecular weight of the protein was 49 116.5 Da. Unmodified BoNT/D_HCR was further confirmed by Western blots using anti-6×His antibody (*b*) or anti-*C. botulinum* D toxoid antibody (*c*).

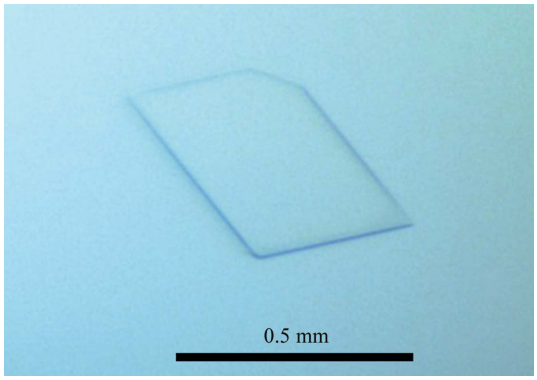


Figure 5
A typical crystal of BoNT/D_HCR. The maximum dimensions of the crystals were 0.5 × 0.4 × 0.05 mm.

Table 1
Data-collection statistics.

Values in parentheses are for the last shell.

Space group	$P2_12_12_1$
Unit-cell parameters	
a (Å)	60.8
b (Å)	89.7
c (Å)	93.9
$\alpha = \beta = \gamma$ (°)	90
No. of molecules per asymmetric unit	1
Matthews coefficient (Å^3 Da^{-1})	2.7
Solvent content (%)	54.5
Data collection	
Detector	ADSC Q315 CCD
Wavelength (Å)	0.9793
Resolution (Å)	50.0–1.65 (1.71–1.65)
Multiplicity	14.1 (13.7)
$\langle I/\sigma(I)\rangle$	26.6 (4.2)
Completeness (%)	100 (99.9)
$R_{\rm merge}$ (%)	10.7 (59.1)

monomer in solution (Fig. 3*a*). After the three-step chromatographic purification, BoNT/D_HCR was purified to near-homogeneity (>98% pure) with high yield (>100 mg per litre of cell culture; Fig. 3*b*).

3.2. Mass-spectrometric analysis and Western blot

Occasionally, recombinant protein expressed in *E. coli* may be truncated owing to unknown causes. Using SDS–PAGE analyses it is difficult to detect if only a few residues are missing since SDS–PAGE is not sufficiently sensitive. Mass-spectrometric methods can measure protein masses accurately. The MALDI-MS-measured molecular weight of purified BoNT/D_HCR was 49 117.5 Da (Fig. 4*a*). Since an extra proton was added to the protein in the positive-ion mode, the experimentally determined molecular weight of purified BoNT/D_HCR was 49 116.5 Da. No other major peaks were observed from the MALDI-MS, confirming the purity of BoNT/D_HCR suggested by SDS–PAGE. These results, in combination with the positive results of the Western blots (Figs. 4*b* and 4*c*), strongly suggest that the purified protein is BoNT/D_HCR without any modifications.

3.3. Crystallization and preliminary crystallographic data analysis

Prior to crystallization, the BoNT/D_HCR was concentrated to ∼10 mg ml^{-1}. Initial crystallization screens using the hanging-drop vapor-diffusion method with commercial kits (Hampton Research and Emerald BioSystems) produced crystals under several conditions. However, most crystals were highly clustered or microcrystalline and the conditions needed further optimization. By varying the precipitating solution composition and concentrations, the best single crystals were obtained using 5%(*w*/*v*) PEG 4000, 0.2 *M* sodium acetate and 0.1 *M* Tris pH 7.5 (Fig. 5) at a protein concentration of 3.5 mg ml^{-1}. The crystals were plate-shaped and reached maximum dimensions of 0.5 × 0.4 × 0.05 mm within 5 d.

The crystals of BoNT/D_HCR diffracted to 1.65 Å resolution with 99.9% completeness and an overall $R_{\rm merge}$ of 10.7%. The space group was $P2_12_12_1$, with unit-cell parameters a = 60.8, b = 89.7, c = 93.9 Å, $\alpha = \beta = \gamma = 90^\circ$. The X-ray diffraction data-collection statistics are summarized in Table 1. Preliminary crystallographic studies revealed one molecule per asymmetric unit with a Matthews coefficient of 2.7 Å^3 Da^{-1} (solvent content 54.5%), which is consistent with the size-exclusion chromatography results. Available structures of other serotypes of botulinum neurotoxin were used as phasing models and structure refinement is in progress.

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