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Purification, crystallization and preliminary X-ray analysis of an HA17–HA70 (HA2–HA3) complex from *Clostridium botulinum* type C progenitor toxin

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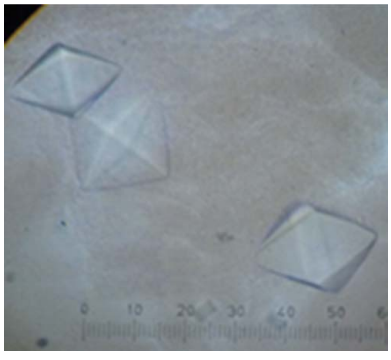

The haemagglutinin (HA) complex of *Clostridium botulinum* type C toxin is composed of three types of subcomponents: HA33, HA17 and HA70 (also known as HA1, HA2 and HA3, respectively). Here, a 260 kDa HA17–HA70 complex was crystallized. His-tagged HA17 and maltose-binding-protein-tagged HA70 were expressed in *Escherichia coli* and their complex was affinity-purified using a combination of amylose resin chromatography and nickel–nitrilotriacetic acid agarose chromatography. Diffraction data were collected to 8.0 Å resolution and the crystal belonged to the tetragonal space group $P4_12_12$. The molecular-replacement solution indicated that one molecule of HA17 was bound to each HA70 monomer.

1. Introduction

Clostridium botulinum, a spore-forming bacterium, has been classified into seven types (types A–G) based on antigenic differences in its neurotoxin (NTX) (Sugiyama, 1980). Most of the NTXs are present as a large stable form in association with nontoxic proteins in complexes that are termed progenitor toxins. The type C strain produces C12S and C16S progenitor toxins. The C12S toxin consists of an NTX and a nontoxic nonhaemagglutinin (NTNHA) component, whereas the C16S toxin is composed of an NTX, an NTNHA and a haemagglutinin (HA) component (Oguma *et al.*, 1997). The HA of type C consists of four subcomponents, which are designated HA33 (also known as HA1), HA17 (HA2) and HA70 (HA3) based on their molecular masses (Fujinaga *et al.*, 1994; Inoue *et al.*, 1996). HA70 is the precursor component of HA22–23 (HA3a) and HA53 (HA3b) and is split into the two proteins by proteolytic cleavage. A hypothetical 14-mer model of the type D progenitor toxin has been proposed based on its crystal structure and electron microscopy (Hasegawa *et al.*, 2007). This model is composed of a 6:3:3:1:1 ratio of HA33, HA17, HA70, NTNHA and NTX.

We have determined the crystal structure of HA70 from type C toxin (denoted HA70/C in this report; Nakamura *et al.*, 2009; Yamashita *et al.*, 2012). In a previous report, a crystal of HA70/C was obtained that belonged to the hexagonal space group $P6_3$ and, although one molecule of HA70/C is present in the asymmetric unit in this structure, HA70/C naturally forms a homotrimer structure which is generated by a crystallographic threefold symmetry axis. As the HA70 protein is split into two subcomponents, HA22–23 and HA53, the entire HA70/C is present as a trimer-of-dimers structure, but in this paper HA70/C is described as a trimer structure in order to facilitate its depiction. HA33/C and HA70/C have lectin activity (Nakamura *et al.*, 2009, 2011) which is critical for the uptake of the toxin into intestinal cells (Uotsu *et al.*, 2006). Elucidation of the structure of the HA complex is important in order to better understand the internalization mechanism of the toxin. Here, we report the purification, crystallization and preliminary X-ray characterization of the 260 kDa HA17–HA70 complex from type C toxin (HA17/C–HA70/C).

2. Materials and methods

2.1. Expression of His-tagged HA17/C

The HA17/C gene was amplified and ligated into the pGEX-5X-3 vector as described previously (Fujinaga *et al.*, 2004), but the expression of HA17/C in *Escherichia coli* was low. The recombinant plasmid was digested with *Bam*HI and *Sal*I and the fragment was subcloned into pMAL-cRI vector (New England Biolabs, Ipswich, Massachusetts, USA). The resulting plasmid was digested with *Eco*RI and *Bam*HI and a pair of oligonucleotides, 5′-AA TTC GAC TAC AAG GAT CAT GAC ATC GAC TAC AAG GAC GAC GAC GAC AAG TG-3′ and 5′-GAT CCA CTT GTC GTC GTC GTC CTT GTA GTC GAT GTC ATG ATC CTT GTA GTC G-3′, which encode the sequence 2×FLAG (DYKDHDIDYKDDDDK) and cohesive ends for *Eco*RI and *Bam*HI, was annealed to the digested plasmid, resulting in plasmid pMAL-FLAG-HA17. However, the expression of HA17/C in *E. coli* JM109 cells harbouring pMAL-FLAG-HA17 was still insufficient for crystallization and thus pMAL-FLAG-HA17 was digested with *Eco*RI and *Sal*I and a DNA fragment encoding 2×FLAG-HA17/C was subcloned into pET-28a(+) vector (Merck, Darmstadt, Germany), resulting in plasmid p6×His-T7-2×FLAG-HA17. For protein expression, *E. coli* BL21(DE3) cells harbouring p6×His-T7-2×FLAG-HA17 were precultured in 3 ml Luria–Bertani (LB) medium containing kanamycin (50 mg ml^{-1}) at 37°C overnight. The cells were transferred to 2 l LB medium containing 50 mg ml^{-1} kanamycin and incubated at 37°C until the A_{600} reached 0.6, and were then induced with isopropyl β-D-1-thiogalactopyranoside at a final concentration of 0.1 m*M* at 20°C for 16 h. The harvested cells were suspended in 30 ml of a reconstitution buffer consisting of 5 m*M* sodium phosphate pH 5.8, 350 m*M* KCl, 20 m*M* $MgCl_2$, 6 m*M* β-mercaptoethanol, 0.5 m*M* PMSF (phenylmethylsulfonyl fluoride), 5 µ*M* E-64 [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; Kouguchi *et al.*, 2002] and disrupted by sonication. The crude extract supernatant was obtained by centrifugation at 18 000*g* for 30 min.

2.2. Expression of maltose-binding-protein-tagged HA70/C

The expression of maltose-binding-protein (MBP)-tagged HA70/C has been described previously (Nakamura *et al.*, 2007). In brief, HA70/C was expressed as a fusion with MBP using the plasmid pMAL-FLAG-HA3 (Nakamura *et al.*, 2007) in *E. coli* JM109 cells and disrupted by sonication. The supernatant after centrifugation is referred to as the crude extract.

2.3. Purification of the HA17/C–HA70/C complex

The crude extract of His-tagged HA17/C (30 ml) and an equal volume of the crude extract of MBP-tagged HA70/C were mixed and incubated at 27°C for 16 h. The mixture was applied onto an amylose resin column (5 cm diameter, 30 ml; New England Biolabs) equilibrated with buffer *A* (20 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl) and the protein was eluted with 30 ml buffer *A* containing 10 m*M* maltose. The eluted fraction from the amylose resin column was then applied onto a nickel–nitrilotriacetic acid (Ni–NTA) agarose column (5 cm diameter, 5 ml; Qiagen, Hilden, Germany) equilibrated with buffer *A* and the protein was eluted with buffer *A* containing 30 ml of 50 m*M* imidazole and was further eluted with buffer *A* containing 30 ml of 100 m*M* imidazole. Analysis by SDS–PAGE showed that both fractions contained the complex of His-tagged HA17/C and MBP-tagged HA70/C (data not shown); the two fractions were therefore combined and are referred to as the purified complex of His-tagged HA17/C and MBP-tagged HA70/C.

2.4. Removal of the MBP tag

The purified complex of His-tagged HA17/C and MBP-tagged HA70/C (60 ml) was concentrated to 5 ml by ultrafiltration using an Amicon Ultra 15 ml 30K filter device (Merck) with buffer consisting of 20 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 2 m*M* $CaCl_2$. The protein complex was digested with factor Xa to release the MBP tag at 25°C for 20 h. Factor Xa was then removed using a Benzamidine Sepharose 6B column (1 cm diameter, 1 ml; GE Healthcare, Chalfont St Giles, England) equilibrated with buffer consisting of 20 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 350 m*M* KCl. To remove the MBP tag, the resulting solution was applied onto an amylose resin column equilibrated with buffer *A* (20 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl). The flowthrough was collected to obtain the HA17/C–HA70/C complex and was dialyzed against 5 m*M* Tris–HCl pH 7.4.

2.5. Crystallization

Crystals were grown by the hanging-drop vapour-diffusion method at 20°C. The protein solution was prepared at a concentration of 20 mg ml^{-1} using an Amicon Ultra 15 ml 30K filter device. Initial crystallization screening of the HA17/C–HA70/C complex was performed using Crystal Screen and Crystal Screen 2 (Hampton Research, Aliso Viejo, California, USA). The crystallization drop consisted of 1.0 µl protein solution and an equal volume of crystallization reservoir solution. The crystals were transferred to reservoir solution containing 30% glycerol and then immediately flash-cooled in a stream of nitrogen gas at −173°C. Diffraction data were collected on the PF BL-5A beamline (Photon Factory, Tsukuba, Japan) and the data set was processed with *iMosflm* (Battye *et al.*, 2011) and *SCALA* (Evans, 2006).

3. Results and discussion

The crude extract of a mixture of His-tagged HA17/C and MBP-tagged HA70/C was first applied onto an amylose resin column. The SDS–PAGE profile of each purification step is shown in Fig. 1. Although some impurities were observed, bands for His-tagged HA17/C and MBP-tagged HA/70 were clearly detected (Fig. 1, lane

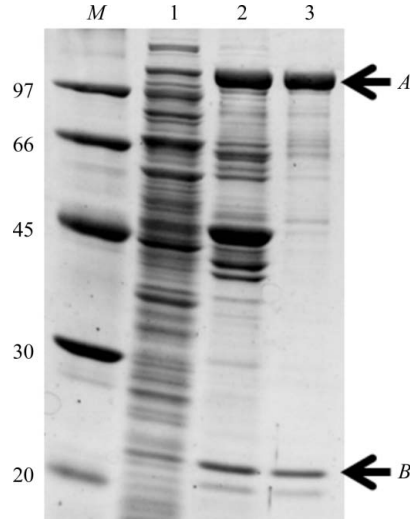

Figure 1
SDS–PAGE of the complex of His-tagged HA17/C and MBP-tagged HA70/C. Lane *M*, molecular-mass marker (labelled in kDa); lane 1, crude extract; lane 2, amylose resin column eluate; lane 3, Ni–NTA agarose column eluate; arrow *A*, MBP-tagged HA70/C; arrow *B*, His-tagged HA17/C.

2). The eluted fraction was further purified using Ni–NTA agarose. The majority of impurities were removed and the result indicated that the complex of His-tagged HA17/C and MBP-tagged HA70/C was purified (Fig. 1, lane 3). The MBP tag was then removed as described in §2.

Crystals of the HA17/C–HA70/C complex with maximum dimensions of 0.2 × 0.2 × 0.15 mm were obtained from condition No. 11 of Crystal Screen 2 [0.01 *M* cobalt(II) chloride hexahydrate, 1.0 *M* 1,6-hexanediol, 0.1 *M* sodium acetate trihydrate pH 4.6] in five days (Fig. 2). The crystallization condition was optimized and larger crystals of a similar shape were obtained using a crystallization reservoir solution consisting of 10 m*M* cobalt(II) chloride hexahydrate, 0.5 *M* 1,6-hexanediol, 0.1 *M* sodium acetate trihydrate pH 4.6. Regular octahedron-shaped crystals were obtained within three weeks.

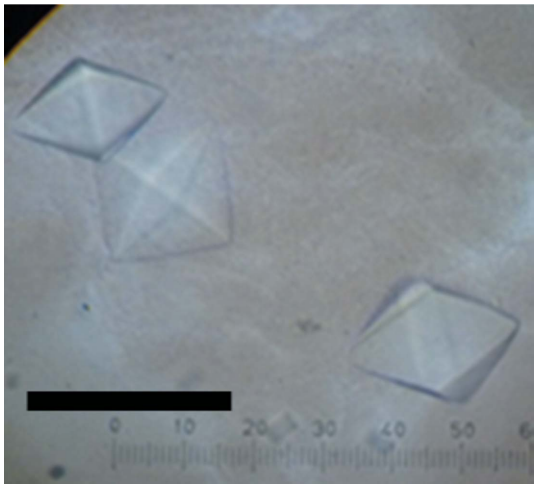

Figure 2
Regular octahedron-shaped crystals of HA17/C–HA70/C. The scale bar represents 0.3 mm.

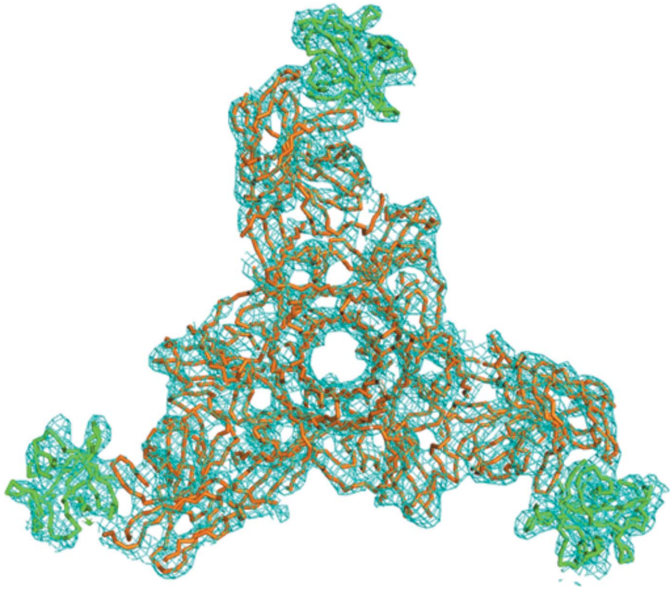

Figure 3
A $2F_o - F_c$ electron-density map of HA17/C–HA70/C contoured at 1.0σ. The backbone structures of HA70/C (orange) and HA17/D (green) are shown.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Beamline	BL5A, Photon Factory
Wavelength (Å)	1.0
Space group	$P4_12_12$
Unit-cell parameters	
$a = b$ (Å)	298.7
c (Å)	276.9
Resolution range (Å)	49.5–8.00 (8.43–8.00)
Measured reflections	133943
Unique reflections	13704
Multiplicity	9.8 (10.3)
Completeness (%)	99.6 (100)
$\langle I/\sigma(I)\rangle$	3.7 (1.3)
R_{meas}	0.160 (0.605)
Unit-cell volume (Å^3)	24698870

The diffraction data were obtained under cryoconditions and a full set of intensity data was collected to 8.0 Å resolution (Table 1). The crystals were of the tetragonal form. To determine the space group, the molecular-replacement program *MOLREP* (Vagin & Teplyakov, 2010) was used with a model of the entire HA70/C trimer structure (PDB entry 4en6; Yamashita *et al.*, 2012) as a search model. When space group $P4_12_12$ was employed for the calculation a reasonable result was obtained and only one HA70/C trimer structure could be placed in the asymmetric unit. The crystal structure of HA17/C has not been reported, but the structure of HA17 from type D toxin (HA17/D) is available (PDB entry 2e4m; Hasegawa *et al.*, 2007) and there are only two amino-acid differences between HA17/C and HA17/D (sequence identity of 98.6%). Therefore, the HA17/D structure was also placed using *MOLREP*. The R_{work} and R_{free} values after rigid-body refinement in *REFMAC* (Murshudov *et al.*, 2011) were 0.409 and 0.418, respectively. Electron density for the HA17/C–HA70/C complex was clearly seen (Fig. 3) and the result indicated that one molecule of HA17/C was bound to each HA70/C monomer. Although the solvent content calculated with *MATTHEWS_COEFF* (Kantardjieff & Rupp, 2003) was high at 90% (V_M = 5.0 Å^3 Da^{-1}; Matthews, 1968), the value was similar to that of the HA70/C crystal (76%; Nakamura *et al.*, 2009). Very recently, crystal structures of a complex of HA17 and the C-terminal part of HA70 from type A toxin (Lee *et al.*, 2013) and a complex of HA33, HA17 and HA70 from type B toxin (Amatsu *et al.*, 2013) have been determined. In both the type A and type B toxins, one molecule of HA17 is bound to each HA70 monomer and their manners of binding appear to be similar to that of the HA17/C–HA70/C complex. Attempts to improve the crystal quality and collect higher resolution diffraction data are currently in progress.

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