Crystallization and preliminary X-ray analysis of a novel haemagglutinin component of the toxin complex of serotype C *Clostridium botulinum*

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The botulinum toxin complex, the causative agent of botulism, passes through the intestinal wall *via* sugar-chain-dependent cell binding of a haemagglutinin of 33 kDa molecular weight (HA-33). The amino-acid sequence of the C-terminal half of HA-33 of the serotype C strain Yoichi (C-Yoichi) shares only 46% identity with those of the major serotype C strains. Additionally, C-Yoichi HA-33 exhibits a unique sugar-binding specificity. In the present work, C-Yoichi HA-33 was expressed in *Escherichia coli* and crystallized. Diffraction data were collected at a resolution of 2.2 Å. The crystals belonged to space group R3. The complete detailed protein structure will yield insight into how the unique HA-33 protein recognizes sugar moieties.

1. Introduction

The botulinum toxin complex (TC) produced by the anaerobic Gram-positive bacterium *Clostridium botulinum* causes food-borne botulism. The TC is composed of the botulinum neurotoxin (BoNT; 150 kDa) and nontoxic proteins including nontoxic nonhaemagglutinin (NTNHA; 130 kDa) and three types of haemagglutinin (HA) (HA-70, HA-33 and HA-17; 70, 33 and 17 kDa, respectively). BoNT is serologically classified into seven serotypes, A–G, and these are also used as descriptors of bacterial strains. Human botulism is caused predominantly by serotypes A, B, E and F, and animal and avian botulism by serotypes C and D.

After ingestion of food contaminated with the TC, the complex is exposed to acidic (pH 2) gastric juices, containing pepsin, in the stomach, and to several other proteases in the intestine. Despite such challenges, BoNT and other nontoxic components can nevertheless be detected in the blood, wherein BoNT becomes detached from the other peptides and reaches neuromuscular junctions. BoNT is next internalized by nerve endings and cleaves specific sites in target proteins, inhibiting neurotransmitter release (Montecucco & Schiavo, 1993). BoNT alone is easily degraded into short peptides when exposed to digestive enzymes of the stomach and intestine. Thus, the NTNHAs and HAs attached to BoNT are thought to function as delivery vehicles for BoNT (Miyata *et al.*, 2009).

The first event in food-borne botulism is toxin absorption by the upper small intestine. It has been reported that L-TC (large-sized botulinum toxin complex) binds and passes through intestinal epithelial monolayers much more efficiently than does BoNT, because HA-33 binds to cells with high affinity (Niwa *et al.*, 2007; Ito *et al.*, 2011). Thus, HA-33 is thought to play an important role in binding of the TC to intestinal epithelial cells.

Fujinaga *et al.* (2000) showed that serotype A HA-33 bound to intestinal microvilli of guinea pig upper small intestinal sections and that binding was inhibited by the addition of lactose or galactose, but not by the removal of sialic acid moieties by neuraminidase. Kojima *et al.* (2005) reported similar results. Thus, the binding of serotype A L-TC to the human intestinal cell line Intestine-407 was inhibited by *N*-acetylglucosamine, lactose and galactose, but not by *N*-acetylneuraminic acid. These results suggested that serotype A L-TC and HA-33 preferentially bound to galactose. On the other hand, it has been reported that the binding of serotype D L-TC and HA-33 to the mouse intestinal cell line IEC-6 was inhibited by *N*-acetylneuraminic acid, but not by either galactose or lactose (Niwa *et al.*, 2010).

Inhibition of serotype C L-TC and HA-33 binding by *N*-acetylneuraminic acid has also been reported. Thus, it appears that serotypes C and D HA-33 bind preferentially to sialic acid (Inui *et al.*, 2010). The specificities of binding of HA-33 to sugars thus differ depending on the toxin serotype. Serotype A HA-33 predominantly recognizes galactose, whereas serotypes C and D HA-33 predominantly recognize sialic acid.

Recently, we showed that HA-33 produced by strain Yoichi of serotype C (C-Yoichi) exhibits a unique property in terms of sugar recognition (Matsuo *et al.*, 2011). Binding of C-Yoichi L-TC to erythrocytes and IEC-6 cells was only about 10% of that of the L-TC of the Stockholm serotype C strain (C-St). However, when the cells were treated with neuraminidase, the binding of C-St L-TC decreased, whereas that of C-Yoichi L-TC increased. These changes were sensitive to the neuraminidase concentration. Furthermore, binding of the HA-33/HA-17 complexes to neuraminidase-treated IEC-6 cells decreased upon the addition of galactose and lactose, suggesting that C-Yoichi HA-33 preferentially recognizes galactose, in contrast to the HA-33 of C-St or D-4947.

To date, crystal structures of serotype C HA-33 and the serotype D HA-33–HA-17 complex have been determined (Inoue *et al.*, 2003; Hasegawa *et al.*, 2007); both recognize sialic acid preferentially. Additionally, the crystal structure of serotype A HA-33 (which preferentially recognizes galactose) is available (Arndt *et al.*, 2005). However, the structure of C-Yoichi HA-33, which (uniquely) preferentially recognizes galactose, although belonging to serotype C, has never been determined. Here, we describe the crystallization of, and provide preliminary X-ray diffraction data on, C-Yoichi HA-33.

2. Materials and methods

2.1. Production and purification of C-Yoichi HA-33

The C-Yoichi *ha-33* gene (AB061780) sequence (857 bp) was amplified by PCR using genomic DNA as a template. The primers were: forward, 5'-CACCATGTCTCAAACAAATG-3', and reverse, 5'-TTATAATCTTGTGTTATAATCCATTTTGTGC-3'. The PCR product encoding the *ha-33* gene was cloned into the pET200/D-TOPO plasmid vector and the recombinant plasmid was trans-

formed into *Escherichia coli* TOP 10. Transformants were selected on LB plates with 50 µg ml⁻¹ kanamycin, and plasmid DNA was extracted using a QIAprep Spin Miniprep kit (Qiagen, Dusseldorf, Germany). Sequencing was performed with the aid of vector-specific primers. Extension products were dye-labelled using an ABI PRISM Big Dye Terminator kit (Applied Biosystems, Carlsbad, California, USA) and sequenced on a Model 3130 DNA platform (Applied Biosystems). The pET200/D-TOPO-*ha-33* construct (10 ng) was next transformed into *E. coli* BL21 Star (DE-3) cells for the production of recombinant HA-33 (rHA-33). *E. coli* was inoculated into 10 ml LB broth with 100 µg ml⁻¹ kanamycin and grown overnight (18 h) at 310 K with gentle shaking. This culture was inoculated into 400 ml LB broth and incubated at 310 K until mid-log phase was attained (the OD₆₀₀ was about 0.5). Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM and induction proceeded at 291 K for a further 18 h. Harvested cells were suspended in 40 ml 50 mM phosphate buffer pH 7.4 with 0.3 M NaCl, sonicated and centrifuged at 10 000g for 20 min at 277 K. The supernatant was mixed with 5 ml 50% (w/v) Ni-charged Resin (Bio-Rad, Hercules, California, USA) suspension equilibrated with the same buffer and the mixture was poured into a glass column. After washing, the bound protein was eluted in equilibration buffer with 300 mM imidazole. rHA-33 was precipitated by the addition of ammonium sulfate at 80% saturation. The precipitate was dialyzed against 50 mM phosphate buffer pH 6.0 with 0.15 M NaCl and finally subjected to gel filtration on a Superdex 200 HR 16/60 column (GE Healthcare BioScience, Piscataway, New Jersey, USA) equilibrated with the same buffer.

2.2. Crystallization and X-ray data collection

Crystals were grown at 293 K by the hanging-drop vapour-diffusion method (Adachi *et al.*, 2003). A protein solution was concentrated to minimal volume using Amicon Ultra-0.5 filtration (molecular weight cutoff 10 kDa; Millipore, Billerica, Massachusetts, USA) and diluted to 5.0 mg ml⁻¹ in 50 mM acetate buffer pH 5.0 in the absence of any other salt. This solution was filtered through an Ultrafree-MC (0.22 µm pore diameter; Millipore). Initial crystallization screening was performed using Crystal Screen (Hampton Research, Aliso Viejo, California, USA). Each reservoir solution (500 µl) was transferred to a VDX plate (Hampton Research), crystallization drops were added and the wells individually sealed with cover slips. Crystals were flash-cooled in a stream of gaseous

Figure 1
SDS-PAGE of purified rHA-33. Lane 1, molecular-weight standards (labelled in kDa); lane 2, purified rHA-33.

Figure 2
Two types of rHA-33 crystals grown using the hanging-drop vapour-diffusion method. The average dimensions of the crystals were 0.05 × 0.05 × 0.1 mm (a) and 0.01 × 0.01 × 0.2 mm (b). The latter crystals were used to obtain X-ray diffraction data.

Table 1

Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Crystal-to-detector distance (mm)	212.4
Exposure time (s)	5
Oscillation angle (°)	1
Wavelength (Å)	1.000
Temperature (K)	100
Resolution (Å)	50–2.2 (2.24–2.20)
No. of unique reflections	48719
Multiplicity	2.9 (2.8)
Completeness (%)	100 (99.8)
$R_{\text{merge}}^{\dagger}$	0.085 (0.461)
$\langle I/\sigma(I) \rangle$	15.5 (2.8)
Space group	<i>R</i> 3
Unit-cell parameters (Å, °)	$a = b = 142.52$, $c = 126.79$, $\alpha = \beta = 90$, $\gamma = 120$

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $\langle I(hkl) \rangle$ is the mean intensity of multiple observations of symmetry-related reflections.

nitrogen at 100 K in the presence of a cryoprotectant [30%(w/v) polyethylene glycol 400 in 0.1 M Tris buffer pH 8.5]. Diffraction data were collected on beamline AR-NW12A fitted with an ADSC Quantum 210r CCD detector (Photon Factory, Tsukuba, Japan), and were processed using the *HKL-2000* software (Otwinowski & Minor, 1997).

3. Results and discussion

The serotype C HA-33 protein of C-St has previously been crystallized and the structure has been determined (Inoue *et al.*, 2003). However, the extent of amino-acid sequence identity between the HA-33 proteins of C-Yoichi and C-St is only 73.4%, and decreases to 46.1% in the C-terminal halves of the proteins (Sagane *et al.*, 2001). C-Yoichi HA-33 recognises galactose-terminated sugar chains on the cell surface, whereas most serotype C strains recognise sialic acid

terminated chains. Thus, the C-Yoichi HA-33 protein is atypical. HA-33 of serotype A principally recognises galactose moieties, as does C-Yoichi HA-33. However, the amino-acid sequences of serotypes A and C-Yoichi are only 35.8% identical. Therefore, it may be that the HA-33 of serotype C is novel. We therefore cloned and crystallized the C-Yoichi HA-33 protein and obtained preliminary structural data.

As shown in Fig. 1, highly purified rHA-33 ran as a single band on SDS-PAGE and had a molecular mass of 37 kDa, as expected from addition of the estimated molecular mass of HA-33 and that of the N-terminal His tag. One early report suggested that the HA-33 of C-Yoichi was cleaved in the C-terminal region in an unknown manner, increasing mobility on SDS-PAGE (Sagane *et al.*, 2001). In the present study, we found no evidence of such cleavage, and concluded that recombinant protein produced in *E. coli* was not in fact cleaved. We crystallized the protein.

When incubated in drops comprised of 3.0 µl protein solution and 2.0 µl crystallization reservoir solution consisting of 8%(w/v) polyethylene glycol 8000 and 5%(w/v) polyethylene glycol 400 in 0.1 M Tris buffer pH 8.5, two types of crystals appeared within 3 d of incubation and grew to maximum dimensions of 0.05 × 0.05 × 0.1 mm (Fig. 2a) and 0.01 × 0.01 × 0.2 mm (Fig. 2b). Diffraction data were obtained from the latter crystals under cryoconditions; a full set of intensity data was collected to a resolution of 2.2 Å (Fig. 3). The other type of crystal did not yield diffraction data. Data-collection statistics and crystal data are summarized in Table 1. We needed more exposure time than usual because of beamline problems. Two molecules formed an asymmetric unit, as was also true of crystals of C-St HA-33 (Inoue *et al.*, 2003). Determination of the complete structure of the unique HA-33 from C-Yoichi may help us to understand how L-TC is absorbed from the intestine. We are currently preparing crystals from sugar-containing protein solutions to determine the exact sugar-binding sites in the C-Yoichi HA-33 protein. It will be useful to understand how the novel HA subunit of the TC of serotype C. *botulinum* binds to the cell.

Figure 3

An X-ray diffraction image from an rHA-33 crystal. The edge of the detector corresponds to a resolution of 2.2 Å.

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