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Crystallization and preliminary X-ray analysis of the HA3 component of *Clostridium botulinum* type C progenitor toxin

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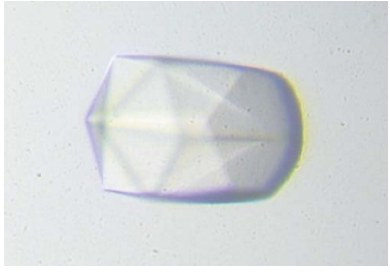

HA3, a 70 kDa haemagglutinating protein, is a precursor form of HA3a and HA3b, the subcomponents of *Clostridium botulinum* type C 16S progenitor toxin. In this report, recombinant HA3 protein was overexpressed in *Escherichia coli*, purified and crystallized. Diffraction data were collected to 2.6 Å resolution and the crystal belonged to the hexagonal space group $P6_3$. Matthews coefficient and self-rotation function calculations indicate that there is probably one molecule of HA3 in the asymmetric unit. A search for heavy-atom derivatives has been undertaken.

1. Introduction

Clostridium botulinum, a spore-forming bacterium, has been classified into seven types (types A–G) based on antigenic differences in its neurotoxin (Sugiyama, 1980). Most of these neurotoxins (NTs) exist as a large stable forms in association with nontoxic proteins [a nontoxic nonhaemagglutinin (NTNH) component and several haemagglutinin (HA) components] in complexes that are termed progenitor toxins. In type C *C. botulinum*, the progenitor toxins are found in two forms with molecular weights of 500 kDa (C16S) and 300 kDa (C12S) (Sakaguchi *et al.*, 1984). The 16S toxin consists of one molecule of NT, one molecule of NTNH and several HA molecules, while the 12S toxin lacks HA components. HAs also consist of four subcomponents, termed HA1 (33 kDa), HA2 (17 kDa), HA3a (21–26 kDa) and HA3b (55 kDa) (Inoue *et al.*, 1996; Fujinaga *et al.*, 1994). HA3 is the precursor component of HA3a and HA3b and is split into the two proteins by proteolytic cleavage.

After oral ingestion of the toxin, NT passes through the gastrointestinal tract and finally reaches the neuromuscular junctions. NT binds to the presynaptic membrane and is internalized by receptor-mediated endocytosis into the nerve cell, where it cleaves syntaxin and then blocks the docking of synaptic vesicles. These events lead to inhibition of neurotransmitter release (Montecucco & Schiavo, 1993; Li & Singh, 1999). It has been reported that the progenitor toxin shows greater toxicity than NT alone, since it is more resistant to low pH and proteolytic cleavage in the digestive tract (Sakaguchi *et al.*, 1984). The nontoxic components are thus presumed to play a role in protecting NT against acidity and proteases in the digestive tract and it has been reported that HA functions as an adhesin in the attachment of the C16S toxin to the microvilli of the upper small intestine (Fujinaga *et al.*, 2004). In our previous study, we proposed that the C16S toxin recognizes sialic acid and is taken up into cells with O-linked oligosaccharides derived from high-molecular-weight glycoproteins, such as mucin, located on the cell surface (Nishikawa *et al.*, 2004). Furthermore, we also found that the C16S toxin can bind to bovine submaxillary mucin and porcine gastric mucin *via* sialo- and asialo-oligosaccharides (Nakamura *et al.*, 2007). Meanwhile, other researchers have concluded that the HA component of type C progenitor toxin contains two distinct carbohydrate-binding subcomponents, HA1 and HA3b, which recognize carbohydrates with different specificities (Fujinaga *et al.*, 2000).

The three-dimensional structure of type D HA2 has been determined (Hasegawa *et al.*, 2007). The HA1 and neurotoxins from

several serotypes have also been extensively studied (Inoue *et al.*, 2003; Arndt *et al.*, 2005; Lacy *et al.*, 1998; Hanson & Stevens, 2000). However, the structure of HA3 has not yet been determined. The elucidation of the structure of HA3 is very important for understanding the formation of the whole toxin complex and for clarification of the internalization mechanism of the C16S toxin. Here, we describe the crystallization and preliminary X-ray diffraction studies of HA3.

2. Materials and methods

2.1. Construction of the expression vector

The HA3 gene (DDBJ/EMBL/GenBank D38562) was amplified and ligated to the pGEX-5X-3 expression vector as described previously (Fujinaga *et al.*, 2004). To obtain a large amount of HA3, the recombinant plasmid was digested with *Bam*HI and *Sal*I and the DNA fragment encoding HA3 was subcloned into pMAL-cRI vector (New England Biolabs). To apply the purified HA3 protein to cell biological studies, the oligonucleotide 5′-AA TTC GAC TAC AAG GAT CAT GAC ATC GAC TAC AAG GAC GAC GAC GAC AAG TG-3′, which encodes the sequence 2×FLAG (DYKDHDIDYK-DDDDK) and restriction sites for *Eco*RI and *Bam*HI, was also inserted upstream of the HA3 gene. The resulting vector was designated pMAL-FLAG-HA3.

2.2. Production and purification

MBP-2×FLAG-HA3 fusion protein was prepared from *Escherichia coli* JM109 cells harbouring the plasmid pMAL-FLAG-HA3. The cells were grown at 303 K in 4 l Luria–Bertani (LB) medium containing ampicillin (50 mg ml^{-1}) to an A_{600} of 0.5 and were then induced with IPTG at a final concentration of 0.5 m*M* overnight. The harvested cells were suspended in 80 ml PBS and disrupted by sonication. The supernatant was obtained by centrifugation at 18 000*g* for 20 min. The MBP-2×FLAG-HA3 was affinity-purified using an amylose resin. The purified MBP-2×FLAG-HA3 protein was digested with factor Xa, releasing MBP from 2×FLAG-HA3. The sample solution was then dialyzed against 20 m*M* Tris–HCl pH 7.5 and applied onto a Q-Sepharose 16/10 HP anion-exchange column (16 × 100 mm, GE Healthcare) equilibrated with the same buffer. The protein was eluted with a linear gradient of 0–0.5 *M* sodium chloride in the same buffer at a flow rate of 2 ml min^{-1}. The purified protein was detected as a single band on SDS–PAGE.

2.3. Crystallization and data collection

Crystals were grown by the hanging-drop vapour-diffusion method (McPherson, 1982) at 293 K. The protein solution was prepared at a concentration of 20 mg ml^{-1} in distilled water. Initial crystallization screening of 2×FLAG-HA3 was performed using a Crystal Screen kit (HR-2-110, Hampton Research). Each reservoir solution (500 µl) was poured into a VDX plate (HR3-140, Hampton Research) and the wells were individually sealed with cover glasses with crystallization drops. The crystallization drop consisted of 1.0 µl protein solution and an equal volume of crystallization reservoir solution. To perform data collection at cryogenic temperature, the crystal was transferred to a solution consisting of 1.2 *M* ammonium phosphate and 20%(*v*/*v*) ethylene glycol in 100 m*M* sodium citrate pH 4.4 and then immediately flash-cooled in a stream of nitrogen gas at 100 K. Diffraction data were collected at the PF BL-5A beamline (Tsukuba, Japan) and the data set was processed with *HKL*-2000 (Otwinowski & Minor, 1997).

3. Results and discussion

The fusion protein, 2×FLAG-HA3, is composed of 640 amino acids and has a molecular weight of 73.3 kDa. The purity of the protein solution was estimated by SDS–PAGE to be approximately 95%. Hexagonal-shaped crystals with maximum dimensions of 50 × 25 × 25 µm were obtained from condition No. 11 (0.1 *M* trisodium citrate dihydrate pH 5.6, 1.0 *M* ammonium dihydrogen phosphate). The crystallization condition was optimized and larger crystals of similar shape were obtained using a crystallization reservoir solution consisting of 1.2 *M* ammonium phosphate in 100 m*M* sodium citrate pH 4.4. Hexagonal column-shaped crystals of 2×FLAG-HA3 protein reached dimensions of 0.25 × 0.3 × 0.3 mm within several days (Fig. 1). The diffraction data were obtained under cryoconditions and

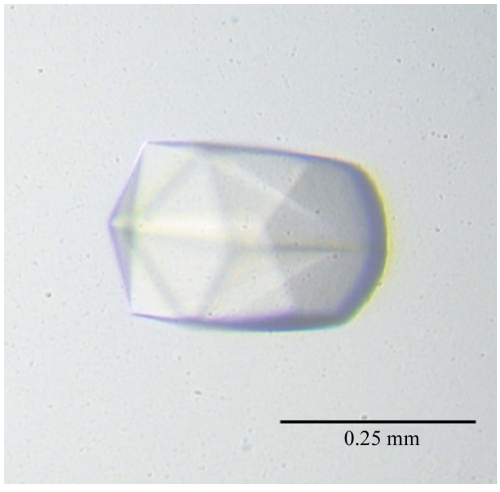

Figure 1
A hexagonal column-shaped crystal of FLAG-HA3.

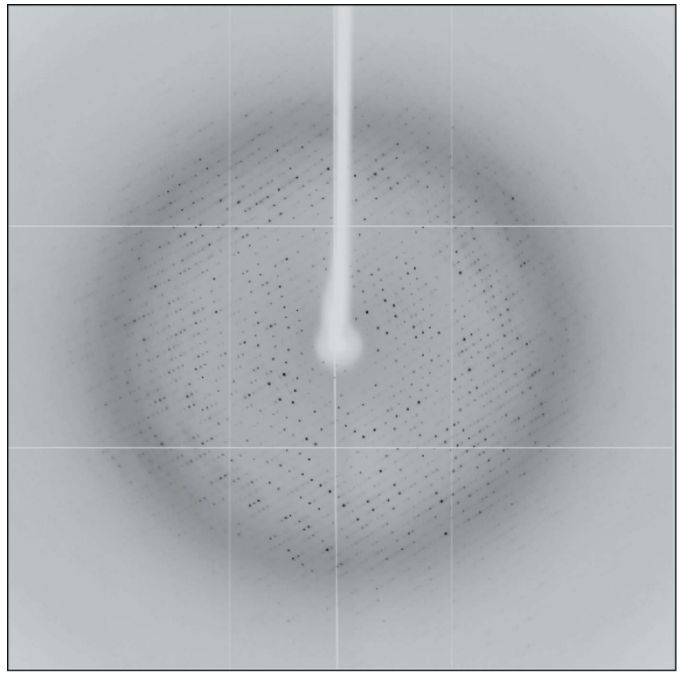

Figure 2
A diffraction image from a FLAG-HA3 crystal.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	1.0
Temperature (K)	100
Resolution (Å)	50–2.6 (2.69–2.60)
No. of observed reflections	472869
No. of unique reflections	44256
Completeness (%)	100 (100)
$R_{\rm merge}$†	0.057 (0.311)
$\langle I/\sigma(I)\rangle$	50.9 (5.9)
Space group	$P6_3$
Unit-cell parameters	
$a = b$ (Å)	176.4
c (Å)	80.9
γ (°)	120

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \overline{I(hkl)}|/\sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith observation of reflection h and $\overline{I(hkl)}$ is the weighted average intensity for all observations i of reflection hkl.

a full set of intensity data was collected to 2.6 Å resolution (Fig. 2). Data-collection statistics and crystal data are summarized in Table 1. A self-rotation function was calculated with the program *MOLREP* from the *CCP*4 software suite (Collaborative Computational Project, Number 4, 1994). Except for the crystallographic peaks, no obvious peaks were observed in the contour map, suggesting that the crystal may contain one HA3 molecule per asymmetric unit. From the results of the program *MATTHEWS_COEFF* from the *CCP*4 software suite, the solvent content was calculated to be 50% ($V_{\rm M}$ = 2.5 Å^3 Da^{-1}) or 75% ($V_{\rm M}$ = 5.0 Å^3 Da^{-1}). This indicates that the crystals would be expected to contain one or two molecules per asymmetric unit. We are now preparing heavy-atom derivatives for MAD (multiple anomalous scattering) phasing.

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