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Crystallization and preliminary X-ray diffraction analysis of an active-site mutant of 'loopless' family GH19 chitinase from *Bryum coronatum* in a complex with chitotetraose

The catalytic mechanism of family GH19 chitinases is not well understood owing to insufficient information regarding the three-dimensional structures of enzyme–substrate complexes. Here, the crystallization and preliminary X-ray diffraction analysis of a selenomethionine-labelled active-site mutant of 'loopless' family GH19 chitinase from the moss *Bryum coronatum* in complex with chitotetraose, (GlcNAc)₄, are reported. The crystals were grown using the vapour-diffusion method. They diffracted to 1.58 Å resolution using synchrotron radiation at the Photon Factory. The crystals belonged to the monoclinic space group *C*2, with unit-cell parameters $a = 74.5$, $b = 58.4$, $c = 48.1$ Å, $\beta = 115.6^\circ$. The asymmetric unit of the crystals is expected to contain one protein molecule, with a Matthews coefficient of $2.08 \text{ Å}^3 \text{ Da}^{-1}$ and a solvent content of 41%.

1. Introduction

Chitin, a β -1,4-linked polysaccharide of *N*-acetylglucosamine (GlcNAc), is hydrolyzed by chitinases (EC 3.2.1.14). The enzymes have been classified into the GH18 and GH19 families according to the CAZy database (<http://www.cazy.org/>; Henrissat & Davies, 1997), with the exception of chitinase C from the thermophilic bacterium *Ralstonia* sp. A-471, which belongs to GH23 (Ueda *et al.*, 2009; Arimori *et al.*, 2013). GH18 chitinases are widely distributed in living organisms from bacteria to humans (Donnelly & Barnes, 2004; Duo-Chuan, 2006; Bhattacharya *et al.*, 2007; Arakane & Muthukrishnan, 2010). In contrast, GH19 enzymes are only found in plants and some bacteria (Arakane *et al.*, 2012). Family GH19 chitinases are further subdivided into two types: 'loopless' and 'loopful'. Hart and co-workers first reported the crystal structure of a 'loopful' family GH19 chitinase from barley seeds (Hart *et al.*, 1993). The barley enzyme is composed of two lobes, both of which are rich in α -helical structures, and several loop structures are located at both ends of the substrate-binding groove lying in between the two lobes. GH19 chitinases lacking these loops at both ends of the binding groove (loopless chitinases) have been isolated from two bacteria, *Streptomyces griseus* HUT6037 (Kazuka *et al.*, 2006) and *S. coelicolor* A3(2) (Hoell *et al.*, 2006), and from the evergreen conifer Norway spruce (Ubhayasekera *et al.*, 2009). In addition, Taira *et al.* (2011) reported the isolation and characterization of a 'loopless' GH19 chitinase from the moss *Bryum coronatum* (BcChi-A).

Very recently, we determined crystal structures of a 'loopful' family GH19 chitinase from rye seeds in a ligand-free form and in a complex with chitotetraose, (GlcNAc)₄ (Ohnuma *et al.*, 2012, 2013). These structures revealed that there are eight substrate-binding subsites in the 'loopful' GH19 enzyme, from -4 to $+4$. Domain opening and closing motions upon substrate binding, which might be essential for chitin hydrolysis, were also observed. In the 'loopless' type GH19 enzymes the number of subsites is still controversial. Based on the kinetics of chitin oligosaccharide hydrolysis and the crystal structure of chitinase G, a 'loopless' GH19 chitinase from *S. coelicolor* A3(2), the enzyme has been shown to have only four subsites (from -2 to $+2$; Hoell *et al.*, 2006). In contrast, we analyzed the binding of chitin oligosaccharides to 'loopless' GH19 chitinase from moss, *Bryum coronatum* (BcChi-A), using isothermal titration calorimetry (ITC) and reported that BcChi-A has at least six subsites in its substrate-binding groove (Ohnuma *et al.*, 2011). The number of subsites in the

Table 1

Data-collection statistics.

Values in parentheses are for the last shell.

Wavelength (Å)	0.97932
Crystal-to-detector distance (mm)	219.7
Oscillation width (°)	1.0
Total rotation range (°)	720
Exposure time (s)	1.0
Mosaicity (°)	0.324
Space group	C2
Unit-cell parameters (Å, °)	$a = 74.5$, $b = 58.4$, $c = 48.1$, $\alpha = 90$, $\beta = 115.6$, $\gamma = 90$
Resolution (Å)	50–1.58 (1.61–1.58)
Measured reflections	344330 (12560)
Unique reflections	25456 (1256)
Multiplicity	13.5 (10.0)
Completeness (%)	99.7 (96.9)
$\langle I/\sigma(I) \rangle$	43.4 (8.8)
$R_{\text{merge}}^{\dagger}$	0.119 (0.281)
Wilson plot estimated B factor (Å ²)	9.2

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity and $\langle I(hkl) \rangle$ is the average intensity over symmetry-equivalent measurements.

'loopless' family GH19 chitinases estimated from ITC appears to be inconsistent with that estimated by kinetics and from the crystal structure. No crystal structure of 'loopless' family GH19 chitinases in complex with their substrate has been reported to date. This situation veils the catalytic mechanism and substrate-binding mode of 'loopless' family GH19 chitinases. Therefore, it is highly desirable to determine the crystal structure of a loopless GH19 chitinase in complex with a chitin oligosaccharide.

In this study, we produced a selenomethionine-labelled inactive mutant of BcChi-A (SeMetBcChi-A-E61A) using an *Escherichia coli* expression system and purified it to homogeneity. The purified protein was co-crystallized with (GlcNAc)₄ and the crystals were employed for X-ray diffraction experiments.

2. Materials and methods

2.1. Protein preparation

A selenomethionine-labelled inactive mutant of BcChi-A (SeMetBcChi-A-E61A), in which the active-site residue Glu61 was replaced with Ala, was produced in *E. coli* strain B834(DE3) and purified by the methods described previously (Ohnuma *et al.*, 2011). Briefly, for selenomethionine incorporation, *E. coli* B834 (DE3) was transformed with pET-BcChi-A-E61A and grown in M9 minimal medium containing 25 µg ml⁻¹ selenomethionine. At an A₆₀₀ of 0.6, 1 mM isopropyl β-D-1-thiogalactopyranoside was added and the culture was continued for 24 h at 288 K. The cells were harvested by centrifugation, suspended in 20 mM Tris-HCl buffer pH 7.5 and disrupted with a sonicator. After the cell debris had been removed by centrifugation (10 000g, 10 min), the supernatant was dialyzed against 10 mM sodium acetate buffer pH 4.0. After dialysis, the resulting insoluble proteins were eliminated by centrifugation at 10 000g for 15 min. The supernatant was dialyzed against 10 mM sodium acetate buffer pH 5.0. The dialysate was applied onto a Q Sepharose Fast Flow column (1.0 × 6 cm) equilibrated with the dialysis buffer. The column was washed with the same buffer and the adsorbed proteins were eluted with a linear gradient of NaCl from 0 to 0.3 M in the same buffer. The eluted fractions containing SeMetBcChi-A-E61A were further applied onto a Sephacryl S-100 HR column (1.6 × 60 cm) and the eluted fractions giving a single band on SDS-PAGE were collected as the purified recombinant protein. Purified SeMetBcChi-A-E61A was thoroughly dialyzed

against distilled water before crystallization. The protein concentration was determined from the absorbance at 280 nm using an extinction coefficient for BcChi-A (49 390 M⁻¹ cm⁻¹) obtained using the equation of Pace *et al.* (1995).

2.2. Crystallization

A total of 576 crystallization conditions were screened by the sitting-drop vapour-diffusion method at 293 K using the following commercial crystallization screening kits: Crystal Screen, Crystal Screen 2, Crystal Screen Lite, Crystal Screen Cryo, Natrix, PEG/Ion Screen, Index, SaltRx (Hampton Research), Wizard I and Wizard II (Emerald BioSystems). Sitting drops were prepared by mixing 1 µl reservoir solution with 1 µl protein solution (4.75–5.0 mg ml⁻¹ in water) containing 10 mM (GlcNAc)₄ and were equilibrated against 100 µl reservoir solution. The preliminary crystals of the binary complex were obtained with condition No. 51 (100 mM Tris-HCl pH 8.5, 1.5 M diammonium hydrogen phosphate) of SaltRx and with condition No. 47 (100 mM Tris-HCl pH 8.5, 200 mM lithium sulfate, 1.26 M ammonium sulfate) of Wizard I. The preliminary crystals thus obtained were small polycrystals that were unsuitable for X-ray diffraction experiments. We therefore tried to improve the crystallization conditions for these crystals using a pH Buffer kit and an Additive Screen kit (Hampton Research). Since the crystals derived from condition No. 47 of Wizard I were not improved, we focused on condition No. 51 of SaltRx for optimization. Finally, large single crystals that were suitable for X-ray diffraction experiments were obtained within a month using a reservoir consisting of 90 mM Tris-HCl pH 8.5, 1.2–1.4 M diammonium hydrogen phosphate, 1 mM CoCl₂, 10 mM sodium acetate pH 4.6, 100 mM 1,6-hexanediol.

2.3. X-ray data collection

For data collection, the SeMetBcChi-A-E61A-(GlcNAc)₄ crystals were cryoprotected in a solution consisting of 100 mM Tris-HCl pH 8.5, 1.5 M diammonium hydrogen phosphate, 20% ethylene glycol. The crystals were mounted in a nylon loop and then flash-cooled in a nitrogen stream at 95 K. X-ray diffraction data were collected on beamline BL-17A of KEK (Ibaraki, Japan) using an ADSC Q315r CCD detector. Diffraction data were integrated and scaled with HKL-2000 (Otwinowski & Minor, 1997). The processing statistics are summarized in Table 1.

Figure 1
Crystals of the SeMetBcChiA-E61A-(GlcNAc)₄ complex belonging to space group C2.

Figure 2
Diffraction pattern of a crystal of the SeMetBcChiA-E61A-(GlcNAc)₄ complex. The edge of the detector corresponds to a resolution of 1.58 Å.

3. Results and discussion

The structures of chitinase C from *S. griseus* (PDB entry 1wvu; Kezuka *et al.*, 2006), chitinase G from *S. coelicolor* A3(2) (PDB entry 2cjl; Hoell *et al.*, 2006) and chitinase from Norway spruce (PDB entry 3hbd; Ubhayasekera *et al.*, 2009) have been determined to date by crystallography. Since these proteins share only 49–55% sequence identity with BcChi-A, we supposed that it would be difficult to determine the crystal structure of BcChi-A by the molecular-replacement method using these coordinates as a search model. Therefore, we tried to determine the BcChi-A structure by the single-wavelength anomalous dispersion (SAD) method using selenium as the anomalous scattering atom. The SeMetBcChi-A-E61A protein was over-expressed in *E. coli* cells using the pET-BcChi-A-E61A expression plasmid and was purified to homogeneity through two purification steps: ion-exchange chromatography on Q Sepharose FF (GE Healthcare, Tokyo) and gel filtration on Sephacryl S-100 (GE Healthcare). The amount of recombinant SeMetBcChi-A-E61A obtained by this procedure was 50 mg per litre of induced culture. The SeMetBcChiA-E61A-(GlcNAc)₄ crystals were obtained using reservoir conditions consisting of 90 mM Tris-HCl pH 8.5, 1.2–1.4 M diammonium hydrogen phosphate, 1 mM CoCl₂, 10 mM sodium acetate pH 4.6, 100 mM 1,6-hexanediol. Rod-like crystals grew within a month to dimensions of up to 200 × 20 × 20 µm (Fig. 1). The crystals diffracted to a resolution of 1.58 Å (Fig. 2) and belonged to the monoclinic space group C2, with unit-cell parameters $a = 74.5$, $b = 58.4$, $c = 48.1$ Å, $\beta = 115.6^\circ$. On the basis of the molecular mass of BcChi-A (22.7 kDa), the crystals are expected to contain one protein

molecule per asymmetric unit, which corresponds to a solvent content of 41% and a Matthews coefficient of $2.08 \text{ Å}^3 \text{ Da}^{-1}$ (Matthews, 1968). Recently, we have succeeded in determining the structure of the SeMetBcChiA-E61A-(GlcNAc)₄ complex by the SAD method using the diffraction data from the SeMet-labelled crystals. A total of three selenium sites were identified with SnB (Weeks & Miller, 1999) and were used for phase calculation at 1.58 Å resolution with *SHARP* (de La Fortelle & Bricogne, 1997). The initial phase was improved by solvent flattening with *SOLOMON* (Abrahams & Leslie, 1996). The SAD experimental electron-density map thus obtained had sufficient quality for tracing BcChi-A-E61A and revealed clear density for (GlcNAc)₄ in the active site. The analysis of the structure of the BcChiA-E61A-(GlcNAc)₄ complex will be discussed in a future paper.

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