Preliminary X-ray diffraction analysis of a thermophilic β -1,3–1,4-glucanase from *Clostridium thermocellum*

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β -1,3–1,4-Glucanases catalyze the specific hydrolysis of internal β -1,4-glycosidic bonds adjacent to the 3-*O*-substituted glucose residues in mixed-linked β -glucans. The thermophilic glycoside hydrolase CtGlu16A from *Clostridium thermocellum* exhibits superior thermal profiles, high specific activity and broad pH adaptability. Here, the catalytic domain of CtGlu16A was expressed in *Escherichia coli*, purified and crystallized in the trigonal space group $P3_121$, with unit-cell parameters $a = b = 74.5$, $c = 182.9$ Å, by the sitting-drop vapour-diffusion method and diffracted to 1.95 Å resolution. The crystal contains two protein molecules in an asymmetric unit. Further structural determination and refinement are in progress.

1. Introduction

β -1,3–1,4-Glucan is one of the major noncellulosic constituents of endosperm plant cell walls. Degradation of β -1,3–1,4-glucan has proven to be advantageous in various cereal-based industries such as brewing and animal-feed manufacture (Muralikrishna & Rao, 2007; Planas, 2000). β -1,3–1,4-Glucanase (EC 3.2.1.73; lichenase) catalyzes strict endo-hydrolysis of the β -1,4-glycosidic linkage adjacent to a 3-*O*-substituted glucose residue in the mixed-linked β -glucans via a double-displacement mechanism (Fig. 1). Exogenous supplementation of β -1,3–1,4-glucanases enhances the extraction efficiency and filtration yield in brewing processes, and improves the feed utilizations of poultry diets by reducing digesta viscosity. These biotechnological processes demand enzymes with higher catalytic activities, broader pH adaptabilities, better thermal profiles (stable at up to 70°C) and higher production levels in industrial strains.

A number of β -1,3–1,4-glucanases have been identified from various origins, with those from microorganisms and plants belonging to glycoside hydrolase (GH) families 16 and 17, respectively (CAZy database; <http://www.cazy.org/>). Most of the characterized β -1,3–1,4-glucanases show optimal temperatures lower than 65°C (Planas, 2000). Previously, a GH16 β -1,3–1,4-glucanase was identified and characterized from the cellulolytic bacteria *Clostridium thermocellum* (CtGlu16A; Schimming *et al.*, 1991). Recently, CtGlu16A was demonstrated to increase nutritive values of broiler daily diets when added as a supplement to the barley-based feedstuff (Ribeiro *et al.*, 2012). CtGlu16A exhibits a high specific activity towards barley β -glucan and lichenan, but is not active towards laminarin, curdlan and cellulosic substrates. The enzyme exhibits a pH optimum between pH 8 and 9, and retains more than 50% activity in a broad pH range from 5 to 12 (Schimming *et al.*, 1991). Significantly, CtGlu16A possesses a superior thermal profile to other β -1,3–1,4-glucanases: it has an optimal temperature of 80°C and shows more than 60% activity for at least 7 h at 80°C (Schimming *et al.*, 1991). Although the GH16 family contains a large number of proteins, only five crystal structures of GH16 β -1,3–1,4-glucanase are available, including four from bacteria and one from a thermophilic fungus (CAZy database; <http://www.cazy.org/>; Aÿ *et al.*, 1998; Cheng *et al.*, 2014; Hahn *et al.*, 1995; Tsai *et al.*, 2003). Therefore, the three-dimensional structure of CtGlu16A is of great interest in investigating its exceptional thermostability and catalytic efficiency.

Figure 1
Schematic presentation of the hydrolytic reaction of the GH16 β -1,3-1,4-glucanase.

2. Materials and methods

2.1. Protein preparation

A gene fragment encoding the catalytic domain (residues 31–251) of CtGlu16A (NCBI reference sequence YP_001036645.1) was synthesized and amplified by PCR with forward primer 5'-GGTATTGAGGGTCGCGCTGGTGCTGGTGCTGCAACTGTCGTC AATACCCCA-3' and reverse primer 5'-AGAGGAGAGTTAGAGCCGTTAACCTTTCAAAGGCAAGTTTGGATTG-3' and then cloned into the pET-32 Xa/LIC vector (Invitrogen). The recombinant plasmids were verified by sequencing and transformed into *Escherichia coli* BL21(DE3). Protein expression was induced with 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) at 16°C for 20 h. The cell paste was harvested by centrifugation at 7000g and resuspended in a lysis buffer consisting of 25 mM Tris-HCl pH 7.5, 150 mM NaCl, 20 mM imidazole. Cell lysate was prepared with a French press (GuangZhou JuNeng Biology and Technology Co. Ltd) and then centrifuged at 17 000g to remove cell debris. The supernatant was loaded onto an Ni-NTA column equilibrated with the lysis buffer. The His-tagged CtGlu16A protein was eluted using an imidazole gradient (20–250 mM). The protein solution was then dialyzed against a buffer consisting of 25 mM Tris pH 7.5, 150 mM NaCl and subjected to factor Xa digestion to remove the thioredoxin and His tags. The mixture was passed through an Ni-NTA column again and the untagged CtGlu16A was collected in the flowthrough fraction. The protein was then concentrated to 30 mg ml⁻¹ in the dialysis buffer and its purity was checked by SDS-PAGE analysis

(>95%). About 25 mg purified protein was obtained from 1 l bacteria culture. The recombinant protein contains an extra linker AGAGA at its N-terminus and its molecular weight is calculated as 28.0 kDa.

2.2. Crystallization and data collection

The CtGlu16A protein containing 10 mM DTT was first crystallized using the PEG/Ion 2 screen kit (Hampton Research, Aliso Viejo, California, USA) at 25°C with the sitting-drop vapour-diffusion method. In general, 2 μ l protein solution (30 mg ml⁻¹ in 25 mM Tris pH 7.5, 150 mM NaCl) was mixed with 2 μ l reservoir solution and equilibrated against 300 μ l reservoir solution in 24-well Cryschem Plates (Hampton Research). Within 3 d, initial crystals were found in a condition consisting of 0.04 M citric acid, 0.06 M bis-tris propane pH 6.4, 20%(w/v) PEG 3350. The crystallization condition was subsequently optimized to 0.04 M citric acid, 0.06 M bis-tris propane pH 6.4, 18%(w/v) PEG 3350. The crystals reached dimensions of about 0.3 $\times$ 0.3 $\times$ 0.4 mm within 7 d. Prior to data collection at -173°C, the crystal was mounted in a cryoloop and flash-cooled in liquid nitrogen with a cryoprotectant consisting of 0.8 M citric acid, 0.12 M bis-tris propane pH 6.4, 20%(w/v) PEG 3350, 15%(w/v) glycerol. An X-ray diffraction data set was collected at beamline BL13C1 of the National Synchrotron Radiation Research Center (NSRRC), Hsinchu, Taiwan and the diffraction images were processed using HKL-2000 (Otwinowski & Minor, 1997). The data-collection statistics are shown in Table 1.

3. Results and discussion

A CtGlu16A crystal obtained in the optimized crystallization solution is shown in Fig. 2. The crystals diffracted to a high resolution of 1.95 Å (Fig. 3) and belonged to the trigonal space group $P3_121$ (or $P3_221$), with unit-cell parameters $a = b = 74.5$, $c = 182.9$ Å. Assuming that there are two molecules in an asymmetric unit, the Matthews coefficient V_M (Matthews, 1968) is $2.93 \text{ Å}^3 \text{ Da}^{-1}$ and the estimated solvent content is 58%.

The structure of CtGlu16A was solved by the molecular-replacement method with *Phaser* (McCoy *et al.*, 2007) from the *CCP4* suite

Figure 2

A crystal of CtGlu16A. The crystal reached approximate dimensions of $0.3 \times 0.3 \times 0.4$ mm in 7 d.

Figure 3

A diffraction pattern of the CtGlu16A crystal. Resolutions of 7.2, 3.5, 2.3 and 1.8 Å are shown as concentric rings from the centre.

Table 1

Data-collection statistics for the CtGlu16A crystal.

Values in parentheses are for the highest resolution shell.

Beamline	BL13C1, NSRRC
Wavelength (Å)	1.0000
Resolution (Å)	25–1.95 (2.02–1.95)
Space group	$P3_121$
Unit-cell parameters (Å)	$a = b = 74.5$, $c = 182.9$
No. of measured reflections	286275 (28830)
No. of unique reflections	43722 (4303)
Completeness (%)	100 (100)
R_{merge} (%)	5.3 (38.7)
$R_{\text{p.i.m.}}$ (%)	2.2 (16.2)
$R_{\text{r.i.m.}}$ (%)	5.7 (41.7)
Mean $I/\sigma(I)$	37.5 (5.4)
Multiplicity	6.5 (6.7)
Horizontal size of X-ray beam (μm)	200
Oscillation range per image (°)	0.5
Time of exposure (s)	25
Crystal-to-detector distance (mm)	250
Detector	ADSC Q315r

(Winn *et al.*, 2011) using the structure of the *Bacillus macerans* β -1,3-1,4-glucanase (51.6% sequence identity to CtGlu16A; PDB entry 1mac; Hahn *et al.*, 1995) as a search model. Preliminary structure refinement using *REFMAC5* (Murshudov *et al.*, 2011) resulted in a model with an R_{work} and R_{free} of 38 and 42%, respectively. The initial electron-density map clearly showed the solvent boundary of two protein molecules in an asymmetric unit. Further structure refinement is in progress. Finally, in an attempt to fully understand the catalytic mechanism and substrate-binding modes, cocrystallization and soaking the CtGlu16A crystals with substrates are also under way.

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