Preliminary X-ray diffraction analysis of thermostable β -1,4-mannanase from *Aspergillus niger* BK01

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Received 5 July 2013
Accepted 19 August 2013

β -1,4-Mannanase (β -mannanase) is a key enzyme in decomposing mannans, which are abundant components of hemicelluloses in the plant cell wall. Therefore, mannan hydrolysis is highly valuable in a wide array of industrial applications. β -Mannanase isolated from *Aspergillus niger* BK01 (ManBK) was classified into glycoside hydrolase family GH5. ManBK holds great potential in biotechnological applications owing to its high thermostability. Here, ManBK was expressed and purified in *Pichia pastoris* and the recombinant protein was crystallized. Crystals belonging to the orthorhombic space group $C222_1$, with unit-cell parameters $a = 93.58$, $b = 97.05$, $c = 147.84$ Å, were obtained by the sitting-drop vapour-diffusion method and diffracted to 1.57 Å resolution. Structure determination using molecular-replacement methods is in progress.

1. Introduction

Mannans are part of the most abundant hemicellulose in the plant cell wall of softwood, plant seeds and beans (Dhawan & Kaur, 2007). Recently, mannan decomposition has been widely applied in various industrial processes, including oil extraction from coconuts, bio-bleaching of softwood pulps in the paper and pulp industries, reducing the viscosity of coffee extracts and as feedstock and food additives (Dhawan & Kaur, 2007). Four types of oligosaccharides have been identified in mannans: linear mannan, galactomannan, glucomannan and galactoglucomannan (Singh *et al.*, 2003). These polysaccharides are linked via β -1,4-glycosidic bonds and the backbone contains mannose or a combination of glucose and mannose residues. β -1,4-Mannanases (β -mannanases; EC 3.2.1.78) can catalyze the random hydrolysis of β -1,4-mannopyranoside bonds and thus are key enzymes for mannan degradation. In addition, the major products of β -mannanase, the manno oligosaccharides (MOS, including mannotriose and mannobiose), have also been found to have prebiotic activity (Funaguchi *et al.*, 2007). Collectively, β -mannanases are of great interest for industrial and nutraceutical applications.

Enzyme thermostability is a valuable property because industrial processes usually involve high-temperature treatments (343–363 K). Therefore, searching for thermophilic enzymes is of great interest for academic research and commercial applications. Recently, an acidic thermophilic (optimal pH and temperature pH 4.5 and 353 K) GH5 β -mannanase was cloned and identified from *Aspergillus niger* BK01 (ManBK; Do *et al.*, 2009). The enzyme is highly thermostable and has a half-life as long as 56 h at 343 K. ManBK is active towards structurally distinct mannans and also exhibits low activity towards birchwood xylan (Do *et al.*, 2009). More importantly, ManBK can be heterogeneously expressed in the industrial strain *Pichia pastoris*. Accordingly, ManBK holds great potential for further applications in various industries. Therefore, solving the crystal structure and complex structures of ManBK is a prerequisite for understanding the enzyme's overall structure and to explore the catalytic machinery of its wide substrate specificity.

Table 1

Data collection statistics for the ManBK crystal.

Values in parentheses are for the outermost resolution shell.

Beamline	BL13C1, NSRRC
Wavelength (Å)	0.97622
Resolution (Å)	25–1.57 (1.63–1.57)
Space group	C222 ₁
Unit-cell parameters	
<i>a</i> (Å)	93.58
<i>b</i> (Å)	97.05
<i>c</i> (Å)	147.84
No. of measured reflections	514024 (48924)
No. of unique reflections	94099 (9231)
Completeness (%)	99.5 (98.8)
<i>R</i> _{merge} † (%)	4.0 (36.7)
Mean <i>I</i> /σ(<i>I</i>)	39.8 (5.2)
Multiplicity	5.5 (5.3)
Mosaicity (°)	0.31–0.63
Wilson <i>B</i> factor (Å ²)	18.6
Detector	MX300HE

† $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, in which $\sum_i$ is over all of the *i* measured reflections with equivalent Miller indices *hkl*, $\langle I(hkl) \rangle$ is the averaged intensity of these *i* reflections and $\sum_{hkl}$ is over all measured reflections in the data set.

2. Materials and methods

2.1. Protein preparation

The gene encoding mature ManBK (GenBank access No. FJ268574) from *A. niger* BK01 without signal peptide was amplified by polymerase chain reaction (PCR) with the forward primer 5'-CGCCGGAATTCACCATCATCATCATTCCTTCGCTTC-CACTTCCG-3' and the reverse primer 5'-ATAGTTTACGGCGCCGCTATTAAGCGGAACCGATAG-3', and then cloned into the vector pPICZαA by the *Eco*RI and *Not*I restriction sites. The recombinant plasmid was linearized by *Pme*I and transformed into the *P. pastoris* X33 strain (Invitrogen, California, USA) by electroporation. The transformants were selected on YPD (1% yeast extract, 2% peptone, 2% dextrose, and 2% agar) plates containing 100 µg ml⁻¹ zeocin and incubated at 303 K for 2 d. The protein expression of transformants was tested by the following small-scale expression. The selected colonies were inoculated in 5 ml YPD medium (1% yeast extract, 2% peptone and 2% dextrose) and then amplified in 50 ml BMGY [1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0,

1.34% yeast nitrogen base (YNB) with ammonium sulfate without amino acids, 4 × 10⁻⁵% biotin and 1% glycerol] at 303 K for 24 h. The cells were harvested and then resuspended in 20 ml BMMY (1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34% YNB with ammonium sulfate without amino acids, 4 × 10⁻⁵% biotin and 0.5% methanol) to induce protein expression. Afterwards, the transformants with higher expression levels were chosen for scale-up expression. The cell stock was grown at 303 K in 100 ml YPD medium containing 100 µg ml⁻¹ zeocin for 24 h. Cells were then transferred into 900 ml YPD medium. After a further 24 h, the cells were collected by centrifugation and grown in 1 l BMMY. A total of 0.5% methanol was added every 24 h to induce protein expression for four consecutive days. The supernatant was collected by centrifugation and then purified using an FPLC system with nickel–nitriloacetic acid (Ni–NTA) and diethylaminoethyl (DEAE) columns (GE Healthcare, Uppsala, Sweden). The buffer used for the Ni–NTA column was 25 mM Tris–HCl pH 7.5, 150 mM NaCl and 20 mM imidazole. The target protein was eluted at about 100 mM imidazole when using a 20–250 mM imidazole gradient. The target protein was dialyzed twice against 5 l buffer consisting of 25 mM Tris–HCl pH 7.5 and loaded onto a 20 ml DEAE Sepharose Fast Flow column (GE Healthcare). The buffer and gradient were 25 mM Tris–HCl pH 7.5 and 0–500 mM NaCl, respectively. The target protein was then dialyzed twice against 5 l buffer (25 mM Tris–HCl pH 7.5 and 150 mM NaCl) for storage. ManBK was concentrated to 10 mg ml⁻¹ in 25 mM Tris–HCl pH 7.5 and 150 mM NaCl using an Amicon Ultra-15 Centricon (Millipore, Eschborn, Germany) and the purity was checked by SDS–PAGE analysis (>95%).

2.2. Crystallization and data collection

About 20 mg of recombinant ManBK was treated with 2000 units of endoglycosidase H_f (Endo H_f; New England Biolabs, Hitchin, England) at 310 K for 2 h to remove glycosylation before crystallization. Initial crystallization screening was performed manually using 768 different reservoir conditions from Hampton Research Crystal Screen kits, including Crystal Screen, Crystal Screen 2, Crystal Screen Cryo, Crystal Screen Lite, MembFac, Natrix, Index, SaltRx, SaltRx 2, PEG/Ion, PEG/Ion 2, Quik Screen and Grid Screens (Ammonium Sulfate, MPD, Sodium Chloride, Sodium Malonate, PEG 6000 and PEG/LiCl; Hampton Research, California, USA), and all of the crystallization experiments were conducted at 295.15 K using the sitting-drop vapour-diffusion method.

In general, 2 µl ManBK-containing solution (25 mM Tris–HCl, 150 mM NaCl pH 7.5; 10 mg ml⁻¹ protein) was mixed with 2 µl reservoir solution in 24-well Cryochem Plates (Hampton Research) and equilibrated against 500 µl reservoir solution. The initial crystals of ManBK were obtained within 7 d using Index condition No. 82 [0.2 M magnesium chloride hexahydrate, 0.1 M Bis-Tris pH 5.5 and 25%(w/v) polyethylene glycol 3350]. The optimized crystallization condition for all crystals mentioned here consisted of 0.4 M magnesium chloride hexahydrate, 0.1 M Bis-Tris pH 5.5 and 29%(w/v) polyethylene glycol 3350. Within 7–8 d, the crystals reached dimensions of about 0.5 × 0.3 × 0.1 mm. Prior to data collection at 100 K, the crystal was mounted in a cryoloop and flash-cooled in liquid nitrogen with a slightly modified cryoprotectant which consisted of 0.4 M magnesium chloride hexahydrate, 0.1 M Bis-Tris pH 5.5 and 34%(w/v) polyethylene glycol 3350, 5%(w/v) glycerol. An X-ray diffraction data set was collected to 1.57 Å resolution on beamline BL13C1 of the National Synchrotron Radiation Research Center (NSRRC, Hsinchu, Taiwan). During data collection, the oscillation range was 0.2°, the exposure time was 6 s and the crystal-to-detector

Figure 1

A crystal of ManBK. The crystal reached approximate dimensions of 0.5 × 0.3 × 0.1 mm in 7–8 d.

Figure 2

A diffraction pattern of the ManBK crystal. The resolution edges (6.8, 3.0, 2.0 and 1.57 Å) are shown by using inner to outer concentric rings.

distance was 200 mm. The diffraction images were processed using *HKL-2000* (Otwinowski & Minor, 1997). Some data-collection statistics are shown in Table 1.

3. Results and discussion

As shown in Fig. 1, the ManBK crystal was obtained in 0.4 M magnesium chloride hexahydrate, 0.1 M Bis-Tris pH 5.5 and 29% (w/v) polyethylene glycol 3350. Based on the diffraction pattern (Fig. 2), these ManBK crystals belonged to the orthorhombic space

group $C222_1$, with unit-cell parameters $a = 93.58$, $b = 97.05$, $c = 147.84$ Å, α , β , $\gamma = 90^\circ$. Assuming there are two molecules per asymmetric unit, the Matthews coefficient V_M (Matthews, 1968) is $2.21 \text{ Å}^3 \text{ Da}^{-1}$ and the estimated solvent content is 44.36%.

The structure of ManBK was solved by the molecular-replacement (MR) method with *Phaser* (McCoy *et al.*, 2007) from the *CCP4* suite (Winn *et al.*, 2011) using the structure of the *Trichoderma reesei* mannanase (57% sequence identity to ManBK) as a search model (PDB code 1qnr; Sabini *et al.*, 2000). Initial structure refinement using *REFMAC5* (Murshudov *et al.*, 2011) resulted in a model with R_{work} and R_{free} of 15.9% and 18.9%, respectively. The initial electron-density map clearly showed that there are two monomer molecules in the asymmetric unit. Further model building and structure refinement are in progress.

The synchrotron data collection was conducted on beamline BL13C1 of the NSRRRC (National Synchrotron Radiation Research Center, Taiwan) supported by the National Science Council (NSC). This work was supported by the National High Technology Research and Development Program of China (2012AA022200) and Tianjin Municipal Science and Technology Commission (12ZCZDSY12500).

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