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Expression, crystallization and preliminary X-ray diffraction studies of thermostable β-1,3-xylanase from *Thermotoga neapolitana* strain DSM 4359

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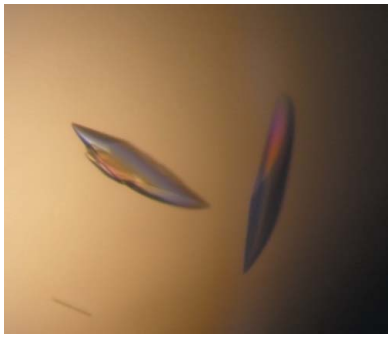

Crystals of β-1,3-xylanase (1,3-β-D-xylan xylanohydrolase; EC 3.2.1.32) from *Thermotoga neapolitana* strain DSM 4359 with maximum dimensions of 0.2 × 0.1 × 0.02 mm were grown using the sitting-drop vapour-diffusion method at 293 K over 24 h. The crystals diffracted to a resolution of 1.82 Å, allowing structure determination. The crystals belonged to space group $P2_1$, with unit-cell parameters a = 39.061, b = 75.828, c = 52.140 Å; each asymmetric unit cell contained a single molecule.

1. Introduction

β-1,3-Xylan is a homopolymer of β-1,3-linked D-xylose units found in the cell walls of red algae (*Porphyra* and *Bangia* spp.) and green algae (*Caulerupa*, *Bryopsis* and *Udotea* spp.) (Iriki *et al.*, 1960). β-1,3-Xylanase (1,3-β-D-xylan xylanohydrolase; EC 3.2.1.32) catalyzes the hydrolysis of the β-1,3-glycosidic linkages of β-1,3-xylan to produce various short-chain β-1,3-xylooligosaccharides. This enzyme is useful for structural analysis of the cell walls of red and green algae (Kiyohara *et al.*, 2006) and the preparation of protoplasts (Araki *et al.*, 1994), as well as for algae biomass conversion.

Until recently, enzymatic studies of β-1,3-xylanase have focused on those in four types of bacteria, *Vibrio* sp. strain AX-4 (Aoki *et al.*, 1988), *Pseudomonas* sp. strain PT-5 (Yamaura *et al.*, 1990), *Alcaligenes* sp. strain XY-234 (Araki *et al.*, 1998) and *Vibrio* sp. strain XY-214 (Araki *et al.*, 1999), and one fungus, *Aspergillus terreus* strain A-07 (Chen *et al.*, 1986). Gene analyses have only been reported for β-1,3-xylanases from *Vibrio* sp. strain XY-214 (Araki *et al.*, 2000), *Alcaligenes* sp. strain XY-234 (Okazaki *et al.*, 2002), *Vibrio* sp. strain AX-4 (Kiyohara *et al.*, 2005) and *Pseudomonas* sp. strain ND137 (Aoki & Kamei, 2006). These enzymes exhibit a modular architecture consisting of a catalytic module and a β-1,3-xylan-binding module (Okazaki *et al.*, 2005; Hashimoto *et al.*, 2005), which are assigned to glycoside hydrolase family 26 (GH26) and carbohydrate-binding module family 31 (CBM31), respectively, in CAZy (Carbohydrate Active Enzymes database; http://www.cazy.org/; Cantarel *et al.*, 2009). Knowledge of the structure of β-1,3-xylanases is limited. To date, only one structure of the catalytic module of β-1,3-xylanase has been determined (Sakaguchi *et al.*, 2004).

Recently, we characterized a highly thermostable β-1,3-xylanase (Xyn26A) from the hyperthermophilic bacterium *Thermotoga neapolitana* strain DSM 4359 (F. Okazaki, C. Ogino & A. Kondo, unpublished work). Xyn26A is composed of 346 amino acids with a molecular mass of 39 816 Da. Our data suggest that Xyn26A is composed of a single module with no CBM. The N-terminal amino-acid sequence exhibited a putative signal peptide in which the predicted cleavage site is located between position 15 (Val) and position 16 (Leu). Therefore, removal of the signal peptide yields a mature protein with 331 amino acids, a molecular mass of 38 033 Da and a pI of 5.0. Optimal conditions for the activity of the enzyme are a pH of 6.5 and a temperature of 358 K.

Here, we report a preliminary structural study of a β-1,3-xylanase from *T. neapolitana* strain DSM 4359. This is the first structural study of a β-1,3-xylanase from a hyperthermophilic bacterium. Comparison of this hyperthermophilic β-1,3-xylanase structure with its mesophilic counterparts (Sakaguchi *et al.*, 2004) should provide insight into

features that stabilize the structure at high temperature, the chemistry of its reaction and the substrate-binding specificity of this enzyme.

2. Materials and methods

2.1. Protein expression and purification

The Xyn26A gene (*CTN_0617*; GenBank accession No. CP000916) was amplified from the genomic DNA of *T. neapolitana* strain DSM 4359 by PCR using primers designed to amplify the gene without the putative signal peptide sequences (993 bp encoding amino acids 16–346). The sequences of the primers used were mXyn26A-F, 5′-**AAGGAGATATACATATG**CTGGAAGGGAAAA TACTTCTTGTCA-3′, and mXyn26A-R, 5′-**GGTGGTGGTGCTC-GAG**TTCGCCGTGTTCCCATATGGATTTTTTGATTTCCTTCA-GC-3′. Bold regions represent an additional sequence corresponding to bases upstream and downstream of the destination vector. To express the His-tagged recombinant protein, the PCR products were cloned into the *Nde*I and *Xho*I sites of the pET22b(+) vector (Novagen, San Diego, California, USA) using an In-Fusion PCR Cloning System (Takara Bio Inc., Shiga, Japan) as per the manufacturer's instructions.

Escherichia coli BL21 (DE3) cells harbouring the expression plasmid were incubated in 2 l 2×YT medium supplemented with 100 µg ml^{-1} ampicillin for 16 h at 310 K using Overnight Express Autoinduction System 1 (Novagen) following the manufacturer's instructions. The cells containing the expressed protein were harvested and disrupted using Bug Buster Master Mix (Novagen) according to the manufacturer's instructions. The insoluble components were removed by centrifugation at 20 000*g* for 30 min at 277 K. To denature the *E. coli* proteins, the supernatant was heated to 353 K for 30 min. Protein aggregates were removed by centrifugation at 20 000*g* for 30 min at 277 K and the clear supernatant was then dialyzed in 20 m*M* sodium phosphate, 500 m*M* NaCl and 20 m*M* imidazole at pH 7.4 and 277 K and loaded onto a 5 ml HisTrap HP column (GE Healthcare Biosciences, Uppsala, Sweden) equilibrated with the same buffer. After washing with the same buffer, the proteins were eluted by applying a linear gradient of 20–500 m*M* imidazole at a flow rate of 2 ml min^{-1}. Fractions containing protein were combined, dialyzed in 20 m*M* Bis-Tris buffer pH 6.5 and loaded onto a 5 ml HiTrap Q HP column (GE Healthcare Biosciences) equilibrated with the same buffer. After washing with buffer solution, the enzyme was eluted using a linear gradient of 0–500 m*M* NaCl at a flow rate of 2 ml min^{-1}. Fractions containing β-1,3-xylanase were combined and concentrated to 13 mg ml^{-1} in 10 m*M* Bis-Tris buffer pH 6.5 using a Vivapore 10/20 concentrator (Sartorius Stedim Lab Ltd, Gloucestershire, England) with a 7500 Da cutoff membrane prior to crystallization. All chromatographic separations were performed at 277 K. The homogeneity of the proteins was evaluated by SDS–PAGE analysis (Laemmli, 1970) as shown in Fig. 1.

2.2. Enzyme activity and protein assay

The activity of β-1,3-xylanase was measured by determining the amount of reduced sugar released from β-1,3-xylan using the Somogyi–Nelson method (Somogyi, 1952). A reaction mixture (125 µl) consisting of 0.5% β-1,3-xylan, an appropriate amount of enzyme and 50 m*M* 2-morpholinoethanesulfonic acid–NaOH buffer pH 6.5 was incubated at 358 K for 10 min. After incubation, the amount of reduced sugar generated was measured by the Somogyi–Nelson method and expressed as D-xylose. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 µmol D-xylose per minute under the above conditions.

Protein concentrations were determined using the theoretical molar absorption coefficient (94 310 M^{-1} cm^{-1}) at 280 nm, which was estimated from the numbers of Trp, Tyr and cystine residues in Xyn26A (Pace *et al.*, 1995).

2.3. Crystallization and X-ray data collection

Crystallization was performed using the sitting-drop vapour-diffusion method. In initial crystallization attempts, the Crystal Screen Cryo kit (Hampton Research, Laguna Niguel, California, USA) and an MRC MAXI Optimization Plate with 48 wells (Molecular Dimensions Limited, Suffolk, England) were used as the reservoir solution and the crystallization plate. A solution of protein (1.5 µl) was mixed with an equal volume of reservoir solution. Each sitting drop was then vapour-equilibrated against 0.3 ml reservoir solution at 293 K. X-ray diffraction images of the crystal were obtained with a resolution of 1.82 Å using a charge-coupled device detector (Quantum 315, ADSC, Poway, California, USA) and synchrotron radiation from beamline BL38B1 at SPring-8 (Japan

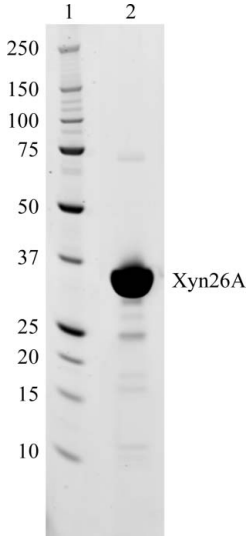

Figure 1
SDS–PAGE analysis of Xyn26A. Lane 1, molecular-weight markers (kDa); lane 2, purified protein.

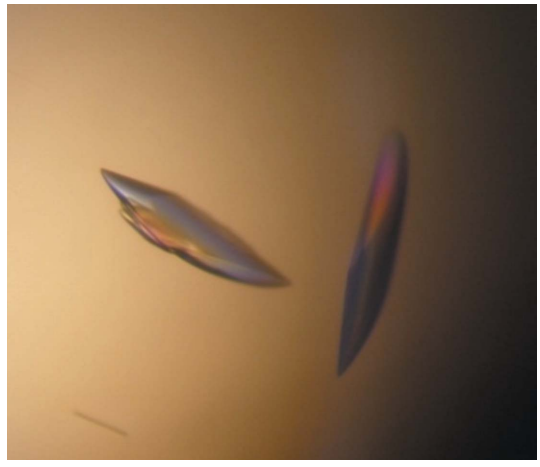

Figure 2
Crystals of Xyn26A. The crystal analyzed was approximately 0.2 × 0.1 × 0.02 mm in size.

Table 1
Statistical data from X-ray analysis of a crystal of Xyn26A.

Values in parentheses are for the highest resolution shell.

X-ray source	SPring-8 BL38B1
Wavelength (Å)	1.000
Temperature (K)	100
Space group	Monoclinic $P2_1$
Unit-cell parameters (Å, °)	a = 39.06, b = 75.83, c = 52.14, β = 109.0
Resolution (Å)	50–1.82 (1.85–1.82)
Total No. of reflections	143543
No. of unique reflections	25557
Completeness (%)	99.7 (100.0)
$R_{\rm merge}$ (%)	8.0 (46.5)
$\langle I/\sigma(I)\rangle$	41.3 (4.5)
Multiplicity	5.7 (5.9)
No. of molecules in each asymmetric unit cell	1

Synchrotron Radiation Research Institute, Hyogo, Japan). During acquisition of the diffraction images the crystal was cooled to 100 K under a stream of nitrogen gas using 30%(*w*/*v*) polyethylene glycol 400 as a cryoprotectant. The images were processed using *DENZO* and *SCALEPACK* in the *HKL*-2000 program package (HKL Research Inc., Charlottesville, Virginia, USA; Otwinowski & Minor, 1997).

3. Results and discussion

Crystals of Xyn26A grew after 1 d in the Crystal Screen Cryo kit under the following conditions: 0.2 *M* magnesium chloride dihydrate, 0.1 *M* sodium HEPES pH 7.5 and 30%(*w*/*v*) polyethylene glycol 400. The crystals grew as plates with dimensions of 0.2 × 0.1 × 0.02 mm (Fig. 2). Diffraction data from the crystal were collected to a resolution of 1.82 Å. The data set consisted of 143 543 observed reflections, which reduced to 25 557 unique reflections with an $R_{\rm merge}$ of 8.0%. The completeness of the data was 99.7%. The space group of the crystal was monoclinic $P2_1$, with unit-cell parameters a = 39.06, b = 75.83, c = 52.14 Å, β = 109.0°. The asymmetric unit contained one molecule and the volume per unit mass, $V_{\rm M}$, was 1.92 Å^3 Da^{-1}. This value is within the range usually found for protein crystals (1.7–3.5 Å^3 Da^{-1}; Matthews, 1968). The calculated solvent content was 36.0%. Data-collection statistics are summarized in Table 1.

This is the first report of the crystallization and preliminary X-ray analysis of a highly thermostable β-1,3-xylanase from a hyperthermophilic bacterium. Structural analysis of this enzyme using the molecular-replacement method is now in progress. Information about the structure of Xyn26A from *T. neapolitana* may contribute towards the understanding of β-1,3-xylanases and GH26. To obtain further information regarding the mechanism of the catalytic reaction and the structural properties that are responsible for catalysis at high temperature in more detail, crystallization and structure determination of a complex of Xyn26A with a substrate (β-1,3-xylooligosaccharide) should be performed.

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