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Crystallization and preliminary X-ray studies of xylanase 10B from *Thermotoga maritima*

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Xylanases catalyze the hydrolysis of the β-1,4-glycosidic bonds of xylan, which is the second most abundant component of plant cell walls after cellulose. The recombinant xylanase 10B from *Thermotoga maritima* MSB8 was prepared and crystallized by the sitting-drop vapour-diffusion method using 40 m*M* zinc acetate, 20 m*M* MES buffer pH 6.0 and 3% ethanol. Intensity data were collected to 2.5 Å resolution at beamline BL26B2 of SPring-8. Preliminary X-ray analysis showed that the crystal belongs to space group $P2_12_12$, with unit-cell parameters a = 77.3, b = 80.6, c = 58.2 Å and one molecule per asymmetric unit.

1. Introduction

Xylanases (1,4-β-D-xylan xylanohydrolases; EC 3.2.1.8) have been isolated from a wide range of microorganisms and studied extensively with respect to their biochemical and structural properties. These enzymes catalyze the hydrolysis of β-1,4-glycosidic linkages of xylan, the second most abundant polysaccharide on earth after cellulose, and produce short-chain xylo-oligosaccharides of various lengths (Kulkarni *et al.*, 1999).

Xylanases are utilized commercially in the pulp and paper, food and animal feed industries. Great interest has focused on the application of cellulase-free xylanases in the paper industry in order to protect the environment. Although xylanases reduce the consumption of chlorine chemicals in the pulp-bleaching process significantly (Li *et al.*, 2000), most of them are unable to withstand the high temperatures and the alkaline pHs commonly encountered in commercial paper production. Therefore, hyperthermostable xylanases are highly desirable as industrial catalysts.

On the basis of amino-acid sequence similarities in their catalytic domains, xylanases are mainly grouped into two families, 10 and 11 (Henrissat & Davies, 1997). *T. maritima* MSB8 produces two family 10 xylanases (xylanase 10A and xylanase 10B), which are expressed by two distinct genes, *xyn*A and *xyn*B, respectively (Nelson *et al.*, 1999). Xylanase 10A (XynA) is a 120 kDa modular xylanase. It consists of N-terminal domains A1 and A2, which have been shown to be family 22 carbohydrate-binding modules (CBMs), a catalytic domain and the C-terminal non-catalytic domains, referred as C1 and C2, which are family 9 CBMs (Goldstein *et al.*, 1993; Winterhalter *et al.*, 1995). Xylanase 10B (XynB) is a 40 kDa non-modular xylanase consisting of a family 10 glycoside hydrolase catalytic domain. The two enzymes share 34% sequence identity at the amino-acid level (Thompson *et al.*, 1994). One of the most outstanding properties of both xylanases is their thermostability. Xyn10A has an optimal temperature of 365 K at pH 6.2, but XynB is still stable at 378 K at pH 5.4 (Winterhalter & Liebl, 1995).

The catalytic domain structures of family 10 xylanases from several microorganisms have been solved by X-ray crystallography. All the enzymes fold into an eight-stranded α/β barrel (TIM-barrel) with a deep active site (Harris *et al.*, 1994; Derewenda *et al.*, 1994; White *et al.*, 1996; Dominguez *et al.*, 1995; Schmidt *et al.*, 1998; Natesh *et al.*, 1999; Fujimoto *et al.*, 2000). There are two acidic amino acids in the active site that are conserved among this family. One plays a role as a nucleophile and the other acts as a proton donor, confirming enzymatic hydrolysis *via* the double-displacement mechanism (Harris *et al.*, 1994; White *et al.*, 1996; Notenboom *et al.*, 1998). To date, however, the catalytic domain structure and the reaction mechanisms of *T. maritima* xylanases have not been studied.

Despite both the structure and the catalytic mechanism of family 10 xylanases being well known, little information exists regarding *T. maritima* xylanases. Therefore, structure determination of hyperthermostable *T. maritima* XynB will shed light on the structure–thermostability relationship. Moreover, the knowledge derived from these structures may be of use in designing and constructing new mutants which are stable both at elevated temperatures and under basic conditions for

use as industrial biocatalysts. In this paper, we report the crystallization and the results of preliminary X-ray studies of *T. maritima* XynB.

2. Materials and methods

2.1. Protein expression and purification

The *xyn*B gene was PCR amplified from the genomic DNA of *T. maritima* MSB8, digested with *Nde*I and *Sal*I and then cloned into the *Nde*I and *Sal*I sites of the pET21b vector system (Novagen) to construct an expression plasmid. The sequences of the primers used were 5′-CCAGTT**CATATG** TCTCAGAATGTATCTCTGAGAGAAC-TCGCAG-3′ and 5′-GCTGAAAATAA-**GTCGAC**ATCGCCCTACTATTTTCTTT-CTTC-3′, where the *Nde*I and *Sal*I sites are shown in bold. An overnight culture of *Escherichia coli* BLR harbouring the expression plasmid was used to inoculate bacteria into fresh medium supplemented with 50 µg ml^{-1} ampicillin. Cells were grown at 310 K to an OD_{600} of 0.6. *xyn*B expression was induced at this point with a final concentration of 1 m*M* isopropyl-*β*-D-thio-galactopyranoside for 4 h at 310 K.

The cells containing the expressed protein were harvested, resuspended in 25 m*M* NaH_2PO_4–NaOH buffer pH 6.0 and then disrupted by sonication. The insoluble components were removed by centrifugation at 15 000*g* for 30 min at 277 K. To denature the *E. coli* proteins, the supernatant was heated to 348 K for 30 min. The clear supernatant was then dialyzed in 10 m*M* MES buffer pH 6.0 at 277 K and was loaded onto a 40 ml DEAE-Toyopearl 650M column (Tosoh Corp.), a weak anion-exchange column, that had been pre-equilibrated with 10 m*M* MES buffer pH 6.0.

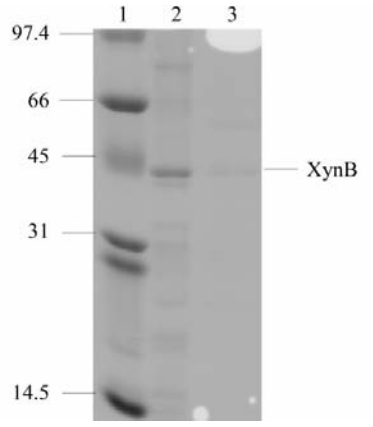

Figure 1
SDS–PAGE analysis of XynB. Lane 1, standard low-molecular-weight markers in kDa; lane 2, the protein before Bioassist-Q; lane 3, after Bioassist-Q (3).

After washing with the same buffer, the proteins were eluted by applying a linear gradient of 0–0.3 *M* NaCl at a flow rate of 1.5 ml min^{-1}. Fractions containing protein were pooled and applied to a Bioassist-Q column (Tosoh Corp.), a strong anion-exchange column. Elution conditions for the Bioassist-Q column were the same as for the DEAE-Toyopearl column. Polyacrylamide-gel electrophoresis analysis of proteins is shown in Fig. 1. All chromatographic processes were carried out at room temperature. Fractions were collected and analysed for xylanase activity. Fractions containing xylanase were pooled and concentrated to 30 mg ml^{-1} in 10 m*M* MES buffer pH 6.0 using a protein concentrator (Millipore) equipped with a 30 kDa cutoff membrane prior to crystallization.

2.2. Enzyme assay

Xylanase activity was determined by both zymogram analysis (Morag *et al.*, 1990) and the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959).

Protein samples were electrophoresed through a 12.5% SDS–polyacrylamide gel (Laemmli, 1970) supplemented with 0.1% larchwood xylan (Sigma). The gel was washed four times for 30 min at room temperature using 25%(*v*/*v*) 2-propanol followed by 100 m*M* MES buffer pH 6.0. The gel was then incubated in 100 m*M* MES buffer pH 6.0 at 310 K for 10 min. For negative staining, the gel was soaked in 0.1% Congo red solution for 15 min and washed with 1 *M* NaCl to remove excess dye.

The enzyme activity was also measured by determining the amount of reduced sugar released from the substrate by the DNS reagent. The reaction mixture, composed of 20 µl of enzyme solution and 80 µl of 0.5% larchwood xylan in 10 m*M* MES buffer pH 6.0, was incubated at 348 K for 10 min. The reaction was stopped by addition of 200 µl of the DNS reagent and boiled at 373 K for 10 min. Absorbance at 545 nm was measured immediately after the addition of 1.2 ml of distilled water.

2.3. Crystallization and X-ray data collection

Crystallization was performed using the sitting-drop vapour-diffusion method. Conditions for crystallization were initially investigated using Emerald Biostructures Wizard I and II crystallization screening kits (deCODE Genetics). Each drop was prepared by mixing 1 µl of protein solution with 1 µl of reservoir solution and was equilibrated against 100 µl of reservoir solution. Crystals grew in the drops from solutions No. 17 [0.2 *M* Li_2SO_4, 0.1 *M* acetate buffer pH 4.5 and 30%(*w*/*v*) PEG 8000] and No. 39 [0.2 *M* Li_2SO_4, 0.1 *M* phosphate–citrate buffer pH 4.2 and 20%(*w*/*v*) PEG 1000] of Wizard I and No. 20 [0.2 *M* zinc acetate, 0.1 *M* MES buffer pH 6.0 and 15%(*v*/*v*) ethanol] of Wizard II at 298 K. Subsequently, solutions No. 17 and No. 20 were prepared as starting points for the optimization of crystallization conditions.

For X-ray data collection, crystals were transferred into reservoir solution containing 30%(*w*/*v*) glycerol and flash-frozen in a liquid-nitrogen gas stream at 100 K. Diffraction data were collected at beamline BL26B2 (λ = 1.00 Å) of SPring-8, Japan using a Rigaku/MSC Jupiter 210 CCD detector. A total of 180 images were recorded with an exposure time of 15 s per image and an oscillation angle of 1.0°. Raw data images were indexed, integrated and scaled using *CrystalClear*1.3 (Rigaku/MSC).

3. Results and discussion

In the initial crystallization trials, crystals grew in different shapes. Stacking plate-shaped crystals appeared after 1 d of

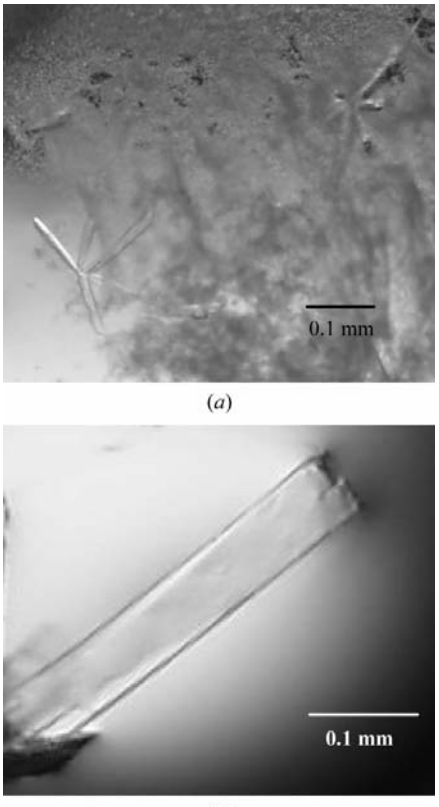

Figure 2
Crystals of xylanase 10B (*a*) from the initial conditions and (*b*) after optimization.

Table 1
Statistical data for the X-ray analysis.

Values in parentheses are for the highest resolution shell (2.59–2.50 Å).

Resolution (Å)	47.18–2.50
Space group	$P2_12_12$
Unit-cell parameters (Å)	a = 77.31, b = 80.58, c = 58.19
R_{merge} (%)	10.0 (25.0)
$\langle I/\sigma(I)\rangle$	14.4 (6.5)
Total No. of reflections	73302
No. of unique reflections	13120
Average redundancy	5.59 (5.75)
Completeness (%)	100 (100)

incubation at 298 K from solution No. 20, while small needle-like crystals appeared after 4–7 d from solutions Nos. 17 and 39, as shown in Fig. 2(*a*). Based on the results of the initial screening, various concentrations of protein, salt and precipitant solutions for both solutions Nos. 20 and 17 were tested with various ranges of buffer pH solutions. Thin plate-shaped crystals from a reservoir solution containing 40 m*M* zinc acetate, 3%(*v*/*v*) ethanol and 20 m*M* MES buffer pH 6.0 were suitable for X-ray analysis (Fig. 2*b*). The crystal diffracted to 2.5 Å resolution and yielded good data processing (Table 1).

The processing data revealed that crystals belong to space group $P2_12_12$, with unit-cell parameters a = 77.31, b = 80.58, c = 58.19 Å. Assuming one molecule with a mass of 38 kDa per asymmetric unit, the calculated Matthews coefficient (V_M) is 2.4 Å^3 Da^{-1} and 47.7% of the crystal volume is occupied by solvent. This is within the range of values tabulated by Matthews (1968). The structure may be solved using the molecular-replacement method with a similar family 10 xylanase as a search model.

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