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Crystallization and preliminary X-ray analysis of xylanase B from *Clostridium stercorarium*

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Recombinant mature xylanase B from *Clostridium stercorarium* has been prepared and crystallized by the sitting-drop vapour-diffusion method using 4 mg ml^{-1} purified enzyme, 10.3%(*w*/*v*) polyethylene glycol 1500, 8.6%(*v*/*v*) glycerol and 0.34 *M* non-detergent sulfobetaine 195. A suitable crystal grew after incubation for ten weeks at 293 K. The crystal belonged to space group $P2_12_12_1$, with unit-cell parameters a = 64.76, b = 96.60, c = 138.44 Å. X-ray diffraction data were collected to 1.80 Å resolution.

1. Introduction

Xylanases (EC 3.2.1.8) catalyze the hydrolysis of the main polysaccharide chain (β-1,4 xylosidic bond) in xylan, the most abundant component of plant cell walls after cellulose. Xylanases have found applications in the food industry, where they are used to produce xylo-oligosaccharide and xylose from xylan, and as additives in the animal feed industry. However, it is in the paper industry where xylanases have found arguably their most important niche, replacing the use of toxic chlorinated species to remove lignin from kraft pulp (Bissoon *et al.*, 2002; Blanco *et al.*, 1995; Kulkarni & Rao, 1996; La Grange *et al.*, 2001).

Glycosyl-hydrolyzing (GH) enzymes have been classified into 85 families based on sequence similarity (Bourne & Henrissat, 2001) and the xylanase B from *Clostridium stercorarium* reported here is a member of family 10 (GH10). Two catalytic residues in GH10 xylanase, identified as glutamic acid residues, have been identified from analysis of mutated enzymes (MacLeod *et al.*, 1994; Notenboom *et al.*, 2000). The three-dimensional structures of several GH10 xylanases have been determined (Derewenda *et al.*, 1994; Fujimoto *et al.*, 2002; Harris *et al.*, 1994; Lo Leggio *et al.*, 2001; Natesh *et al.*, 1999; White *et al.*, 1994) and all contain the same $(\alpha/\beta)_8$ or TIM-barrel fold. While these GH10 xylanases exhibit identical barrel folds, their thermal and pH stabilities, molecular activities and optimum pH values show marked differences. We have previously reported these difference for the GH10 xylanases xylanase B from the thermophile *C. stercorarium* (XynB; Nishimoto, Kitaoka *et al.*, 2002), xylanase A from the alkaliphile *Bacillus halodurans* C-125 (Nishimoto, Honda *et al.*, 2002) and xylanase B from the hyperthermophile *Thermotoga maritima* MSB8 (Jiang *et al.*, 2001) using *p*-nitrophenyl xylobioside as a substrate. Here, thermal stability (the temperature at which the residual activity is higher than 90% after incubation for 20 min at pH 6.6) has been observed up to 338, 323 and 363 K and the pH optima (the pH range over which the V_{max} values are higher than 90% of the maximum V_{max} at 313 K) are 5.3–6.6, 5.8–8.8 and 5.8–6.7 for XynB from *C. stercorarium*, xylanase A from *B. halodurans* and xylanase B from *T. maritima*, respectively. However, the structures of these have yet to be determined, making it difficult to ascertain whether the differences in the thermal stabilities and the pH optima are the result of structural anomalies (crystallization and preliminary X-ray studies of xylanase B from *T. maritima* MSB8 has recently been reported; Ihsanawati *et al.*, 2003). In this report, we describe the crystallization and preliminary X-ray analysis of XynB from *C. stercorarium*.

2. Materials and methods

2.1. Preparation of the expression plasmid and the recombinant enzyme

An expression plasmid for residues Ala41–Glu388 was constructed using the XynB expression plasmid template described previously (Nishimoto, Kitaoka *et al.*, 2002). The *XynB* gene was amplified by the polymerase chain reaction method using KOD-Plus polymerase (Toyobo, Osaka, Japan) and the two oligonucleotide primers GATCAAA**C-CATGG**CTGAAGACATCCCATCGCTTGC and ATC**CTCGAG**TTCCCGCAACCGTG-AA, where the sequences in bold represent the restriction endonuclease sites for cloning. The amplified fragment was inserted into the pET28 vector (Novagen, Darmstadt, Germany) at the *Nco*I/*Xho*I site using a DNA ligation kit (Ligation High; Toyobo). The expression plasmid was transformed into *Escherichia coli* BL21 (DE3) cells and cultivated at 303 K by shaking in 1 l of Luria–Bertani media (containing 50 μg ml^{-1}

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kanamycin) until the absorbance at 600 nm reached 0.5. Protein production was then induced by the addition of isopropyl-1-thio-β-D-galactoside to the culture to a final concentration of 0.5 mM, followed by further incubation for 20 h (303 K) with shaking. The cells were harvested by centrifugation at 15 000g for 10 min and then resuspended in 20 mM sodium phosphate buffer pH 7.0. The resuspended cells were then sonicated (Branson Ultrasonic Corporation, Danbury, CT, USA) and the cell debris was removed by centrifugation at 17 000g for 30 min. The crude enzyme was purified by column chromatography using Ni–NTA agarose (Qiagen, Hilden, Germany) and Mono Q (Tosoh, Tokyo, Japan) columns as described previously (Nishimoto, Kitaoka *et al.* 2002). The purified enzyme was concentrated to 9.3 mg ml^{-1} using an Amicon Ultra centrifugal filter device (Millipore, Billerica, MA, USA) after dialysis against distilled water. The homogeneity of the purified enzyme was established by SDS–PAGE analysis (Laemmli, 1970), while the protein concentration was determined by the Bradford method (Bradford, 1976).

2.2. Crystallization and X-ray data collection

The crystallization procedure was performed using the sitting-drop vapour-diffusion method. Initial conditions were identified with Crystal Screen Cryo and Crystal Screen 2 crystallization kits (Hampton Research, CA, USA) at 293 K. Each drop was prepared by mixing 1.8 µl of purified enzyme solution and the same volume of reservoir solution and was equilibrated against 100 µl of reservoir solution. Crystals of XynB were grown from drops of solutions No. 28 [0.17 M sodium acetate trihydrate, 0.085 M sodium cacodylate pH 6.5, 25.5%(w/v) polyethylene glycol 8000, 15%(w/v) glycerol] and No. 43 [24%(w/v) polyethylene glycol 1500, 20%(w/v) glycerol] from Crystal Screen Cryo and from drops of solutions No. 5 [2 M ammonium sulfate, 5%(v/v) 2-propanol] and No. 26 [0.2 M ammonium sulfate, 0.1 M MES pH 6.5, 30%(w/v) polyethylene glycol monomethyl ether 5000] from Crystal Screen 2. To optimize the crystallization conditions, Additive Screens 1, 2 and 3 (Hampton Research) were used to modify the conditions of solution No. 43 from Crystal Screen Cryo. Each drop was prepared by mixing 1.5 µl of the purified enzyme solution with 2.0 µl of the reservoir solution containing 0.4 µl of each additive and equilibrating it against 100 µl of the reservoir solution.

Table 1
Data-collection statistics.

Values in parentheses refer to the highest resolution shell (1.86–1.80 Å).

Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 64.76, b = 96.60, c = 138.44
Resolution range (Å)	50.14–1.80
$R_{\rm merge}$† (%)	6.2 (27.6)
$\langle I/\sigma(I)\rangle$	8.8 (2.5)
Total No. of reflections ($I > 3\sigma$)	493039
No. of unique reflections	80942
Average redundancy	6.1 (5.7)
Completeness (%)	97.7 (97.7)

† $R_{\rm merge} = \sum_h \sum_i |I(h,i) - \langle I(h)\rangle| / \sum_h \sum_i I(h,i)$, where $I(h,i)$ is the intensity of the ith measurement of reflection h and $\langle I(h)\rangle$ is the mean value of $I(h,i)$ for all i measurements.

Prior to data collection, the crystal was transferred to a reservoir solution containing 20%(w/v) glycerol and flash-cooled in a stream of liquid nitrogen at 100 K. The X-ray diffraction data were collected using a Rigaku R-AXIS IV++ imaging-plate area-detector system mounted on a Rigaku UltraX18 rotating-anode X-ray generator using Cu $K\alpha$ radiation (operating at 50 kV and 100 mA) and processed with *CrystalClear* software. A total of 310 frames were collected with 0.5° oscillations and 6 min exposures.

3. Results and discussion

The signal sequence of the recombinant XynB expressed in *E. coli* BL21 (DE3) was digested at two sites, between Met21 and Ala22 and between Lys24 and Ser25, as described previously (Nishimoto, Kitaoka *et al.*, 2002). In order to obtain enzyme that was homogeneous at the N-terminus, an expression vector coding the mature part of XynB was constructed as described in §2. Initial attempts to prepare crystals of mature XynB from crystallization solutions Nos. 28 and 43 from Crystal Screen Cryo, and Nos. 5 and 26 from Crystal Screen 2 (incubated for 2–4 weeks) failed owing to the formation of small needle-like and thin plate-like crystals that were unsuitable for X-ray analysis. The crystallization conditions were then optimized by the addition of various additives to solution No. 43 from Crystal Screen Cryo, as outlined in §2. A suitable pillar-shaped crystal of mature XynB (approximately 1.0 × 0.2 × 0.1 mm) was eventually grown after ten weeks incubation at 293 K in solution No. 43 containing additive No. 21 (3.0 M non-detergent sulfobetaine 195) from Additive Screen 2. The crystal belongs to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 64.76, b = 96.60, c = 138.44 Å, and diffracted to 1.8 Å resolution with a Rigaku R-AXIS IV++ system. The data statistics are presented in Table 1. Assuming two molecules of XynB per asymmetric unit, the calculated $V_{\rm M}$ value (Matthews, 1968) and solvent content were determined to be 2.8 Å^3 Da^{-1} and 56.0%, respectively. An extended crystallographic analysis using the molecular-replacement method with a GH10 xylanase model (PDB code 1hiz) is currently in progress.

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