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Crystallization and preliminary crystallographic analysis of a family 43 β-D-xylosidase from *Geobacillus stearothermophilus* T-6

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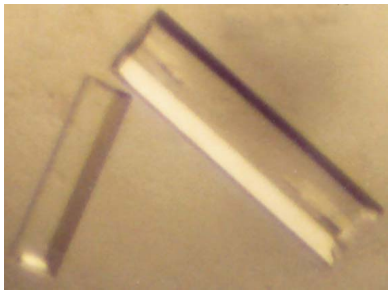

β-D-Xylosidases (EC 3.2.1.37) are hemicellulases that cleave single xylose units from the nonreducing end of xylooligomers. In this study, the crystallization and preliminary X-ray analysis of a β-D-xylosidase from *Geobacillus stearothermophilus* T-6 (XynB3), a family 43 glycoside hydrolase, is described. XynB3 is a 535-amino-acid protein with a calculated molecular weight of 61 891 Da. Purified recombinant native and catalytic inactive mutant proteins were crystallized and cocrystallized with xylobiose in two different space groups, $P2_12_12$ (unit-cell parameters a = 98.32, b = 99.36, c = 258.64 Å) and $P4_12_12$ (or the enantiomorphic space group $P4_32_12$; unit-cell parameters $a = b$ = 140.15, c = 233.11 Å), depending on the detergent. Transferring crystals to cryoconditions required a very careful protocol. Orthorhombic crystals diffract to 2.5 Å and tetragonal crystals to 2.2 Å.

1. Introduction

Xylan is the most abundant hemicellulosic polysaccharide in the plant cell wall, representing up to 30–35% of its total dry mass. This heteropolysaccharide is composed of a β-1,4-linked xylopyranosyl backbone substituted with different groups such as α-L-arabinofuranosyl, D-glucuronopyranosyl, 4-*O*-methyl-D-glucuronopyranosyl and acetyl groups. The complete degradation of xylan is a key step in the carbon cycle in nature and owing to its structural complexity, it requires the synergistic action of several hemicellulases (Shallom & Shoham, 2003; Beg *et al.*, 2001; Collins *et al.*, 2005). Among these enzymes, β-D-xylosidases (EC 3.2.1.37) are responsible for the final release of xylose units from xylooligomers generated by endo-1,4-β-xylanases (EC 3.2.1.8), which hydrolyze the xylan backbone.

Hemicellulases are mainly glycoside hydrolases (EC 3.2.1–3.2.3), a widespread group of enzymes hydrolyzing the glycosidic bond between two or more carbohydrates or between a carbohydrate and a noncarbohydrate moiety (Henrissat *et al.*, 1995). Hydrolysis of the glycosidic bond can be carried out by one of two mechanisms, leading to retention or inversion of the anomeric configuration of the substrate. Both mechanisms require two carboxylic acids. Retaining glycosidases use a double-displacement mechanism, where one catalytic residue functions as a nucleophile and the other as a general acid–base. Inverting glycosidases use a single-displacement mechanism, in which one carboxylic acid acts as a general acid and one as a general base (Sinnott, 1990). Currently, more than 17 000 glycosidase sequences are known and the sequence-based classification of their catalytic domains into glycoside hydrolase (GH) families and clans is available on the continuously updated Carbohydrate-Active Enzymes (CAZY) server (http://afmb.cnrs-mrs.fr/CAZY/). β-Xylosidases are found in the retaining GH families 3, 39, 51, 52 and 54 and in the inverting GH family 43.

Geobacillus stearothermophilus T-6 is a thermophilic bacterium that was originally isolated owing to its ability to produce alkaline-tolerant thermostable xylanases for the biobleaching of wood pulp (Gat *et al.*, 1994; Khasin *et al.*, 1993; Lapidot *et al.*, 1996; Shulami *et al.*, 1999). Strain T-6 possesses an extensive hemicellulolytic system clustered on a 39 kbp chromosomal segment. This system includes an α-glucuronidase (GH68), two arabinofuranosidases (GH51), two

xylanases (GH10) and three β-xylosidases (GH39, GH43 and GH52). The majority of these enzymes have been studied biochemically and structurally (Czjzek *et al.*, 2005, Golan *et al.*, 2004; Bravman, Belakhov *et al.*, 2003; Bravman, Zolotnitsky *et al.*, 2003; Hovel *et al.*, 2003; Shallom, Belakhov, Solomon, Gilead-Gropper *et al.*, 2002; Shallom, Belakhov, Solomon, Shoham *et al.*, 2002; Shallom *et al.*, 2004, 2005; Teplitsky *et al.*, 2004; Zolotnitsky *et al.*, 2004). The family 43 β-xylosidase XynB3 is an intracellular β-xylosidase which was recently cloned, overproduced and its catalytic mechanism characterized (Shallom *et al.*, 2005). XynB3 is a 535-amino-acid protein with a calculated molecular weight of 61 907 Da. The enzyme cleaves single xylose units from the nonreducing end of xylooligomers; the three catalytic residues Asp15, Asp128 and Glu187 were found to be essential for its activity (Shallom *et al.*, 2005). As a member of the GH43 family, hydrolysis is carried out by the inverting mechanism. Interestingly, family GH43 shares a unique five-blade β-propeller architecture with families 32, 62 and 68, which was first revealed for the Arb43 arabinanase from *Cellvibrio japonicus* (Alberto *et al.*, 2004; Nurizzo *et al.*, 2002; Pons *et al.*, 2004). Currently, there are only five members of the GH43 family for which the three-dimensional structures have been determined: three β-xylosidases (PDB codes 1yrz, 1yif and 1yi7) and two arabinanases (PDB codes 1gyd and 1uv4). In this study, we describe the crystallization and preliminary X-ray analysis of the family 43 β-D-xylosidase XynB3 from *G. stearothermophilus*. The three-dimensional structure of this enzyme in complex with various substrates is of great interest since it will provide insight into the binding properties and specificity of family 43 glycoside hydrolases.

2. Experimental

2.1. Expression and purification

The *xynB3* gene (GenBank accession No. AAT98625) from *G. stearothermophilus* T-6 was cloned in the pET9d vector (Novagen), overexpressed in *Escherichia coli* BL21 (DE3) (Novagen) and purified as previously reported (Shallom *et al.*, 2005). In brief, following overnight growth in Terrific Broth medium, the culture was harvested, resuspended and disrupted by two passages through a French press. The cell extract was centrifuged and the soluble fraction was heat-treated (333 K, 30 min) and centrifuged again. The recombinant XynB3 in the soluble fraction was further purified by gel filtration using a Superdex 200 26/60 column.

2.2. Crystallization experiments

Crystallization experiments were performed at 285 K using the sitting-drop setup of the vapor-diffusion method in 24-well plates.

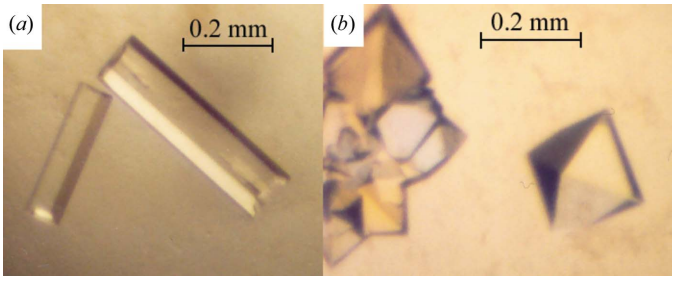

Figure 1
Pictures of crystals of XynB3 (*a*) grown in the primitive orthorhombic crystal form with detergent DDAO and (*b*) grown in the primitive tetragonal crystal form with detergent HEGA-8 under polarized light.

Initial crystallization conditions were screened using the sparse-matrix approach (Jancarik & Kim, 1991) and were further improved using Hampton Research detergent and additive screens. The initial droplets were prepared by mixing 2 µl protein solution with 2 µl reservoir solution and were equilibrated against 300 µl reservoir solution. After improvement of the crystallization conditions, the droplet consisted of 5 µl protein solution at a concentration of 22–30 mg ml^{-1}, 5 µl reservoir solution and 1 µl detergent solution and the volume of the reservoir was increased to 500 µl. In the case of the cocrystallization, 0.4 µl xylobiose solution at 500 m*M* was added to the droplets.

2.3. Cryoprotectant buffer and diffraction experiments

For X-ray diffraction experiments at cryogenic temperature, the crystals were transferred into a solution containing glycerol as cryoprotectant. This step appeared to be very sensitive as the crystals were disrupted if transferred directly to the cryoprotectant buffer. To prevent such disruption, the cryoprotectant concentration was increased gradually. Once the solution contained 17%(*v*/*v*) glycerol the crystals were mounted on a nylon loop and flash-cooled in liquid nitrogen. The raw diffraction data were indexed, integrated and scaled with *DENZO* and *SCALEPACK* from the *HKL* suite (Otwinowski & Minor, 1997).

2.4. Molecular-replacement calculation

Self-rotation calculations were performed with the program *GLRF* (Tong & Rossmann, 1997). For cross-rotation and translation searches, the programs *MOLREP* and *AMoRe* from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994) were used.

3. Results and discussion

3.1. Crystallization and crystal optimization

The first crystals were obtained using the PEG 6000 Grid Screen from Hampton Research. The reservoir solution consisted of 30%(*w*/*v*) PEG 6000 and 0.1 *M* MES buffer pH 6.0. These crystals grew tightly together and could not be improved by seeding techniques. However, use of the Hampton Research detergent screens led, after some optimization (see below), to significantly improved crystals, resulting in two different crystallization conditions. Addition of either *N*,*N*-dimethyldecylamine-β-oxide (DDAO) to a final concentration of 10.4 m*M* or octanoyl-*N*-hydroxyethylglucoamide (HEGA-8) to a final concentration of 10.9 m*M* led to the growth of well defined single crystals (Fig. 1). In both cases, the final optimal reservoir consisted of 19%(*w*/*v*) PEG 6000, 0.1 *M* MES pH 5.4. Although the droplets differed only in the nature of the added detergent (while the reservoir conditions were identical), the resulting crystals were quite different. In the presence of DDAO XynB3 crystals grew as long and thin plates (Fig. 1*a*), whereas with HEGA-8 XynB3 formed more compact diamond-shaped crystals (Fig. 1*b*). The native XynB3 crystallized in both crystal forms, while the three catalytic inactive mutants D15G, D128G and E187G only crystallized in the primitive tetragonal crystal form. The inactive mutants were cocrystallized with the natural substrate xylobiose using HEGA-8 as the detergent additive in the crystallization solution.

3.2. Cryopreservation protocol

The final cryoprotection solution consisted of 17%(*v*/*v*) glycerol, 19%(*w*/*v*) PEG 6000, 0.1 *M* MES pH 5.4 and, as mentioned above, the

Table 1
Selected crystallographic parameters for native XynB3 crystals.

Values in parentheses are for the outer shell.

Crystal form	Orthorhombic (detergent DDAO)	Tetragonal (detergent HEGA-8)
Synchrotron	BL1, PSF, BESSY, Berlin	X13, EMBL Outstation, Hamburg
Wavelength (Å)	0.9797	0.8048
Unit-cell parameters		
a (Å)	98.32	140.15
b (Å)	99.36	140.15
c (Å)	258.64	233.11
Space group	$P2_12_12$	$P4_12_12$ (or $P4_32_12$)
No. monomers in AU	4	4
V_M (Å^3 Da^{-1})	2.5	2.3
Oscillation angle per frame (°)	0.3	0.1
No. of frames	823	772
Data-collection temperature (K)	100	100
Resolution (Å)	2.5 (2.54–2.50)	2.2 (2.23–2.20)
Mosaicity (°)	0.36	0.41
Total No. of reflections	3044731	5966877
No. of rejected reflections	9600	5096
No. of unique reflections	88547	116620
Completeness (%)	99.9 (99.1)	99.5 (97.8)
R_{merge} (%)	6.6 (12.4)	7.1 (19.6)
$\langle I/\sigma(I)\rangle$	22.7 (12.3)	12.0 (6.5)

transfer of the crystals to a cryoprotectant had to be performed in very small steps. If the cryoprotectant concentration increased too rapidly, both types of crystals were disrupted immediately and could not be used for further diffraction experiments. To achieve satisfactory cryoconditions, a modified protocol had to be applied (Garman & Doublié, 2003). Small amounts of solution (2–5 µl) containing glycerol as cryoprotectant were added to the crystal drop and the crystals were then left to equilibrate for at least 2 h before new solution containing a higher glycerol concentration was added. To keep the volume of the droplet constant, the same volume that was added to the droplet was removed shortly before. The modified protocol used for the XynB3 crystals was conducted over 4 d until the final cryoconditions were reached. The substitution of glycerol as the cryoprotectant by PEG, MPD, paraffin oil or glucose did not improve the cryoprotocol.

3.3. Data collection and processing

Diffraction data from crystals grown with DDAO were collected on beamline BL1 of the Protein Structure Factory (PSF) at the Berliner Elektronenspeicherring Gesellschaft für Synchrotronstrahlung (BESSY, Berlin). The best data were measured to a resolution of 2.5 Å (Table 1). The diffraction pattern indicates an orthorhombic unit cell, with unit-cell parameters a = 98.318, b = 99.358, c = 258.642 Å. Previous biochemical characterizations performed using gel filtration indicate that XynB3 is a trimer in solution. However, the most probable V_M value (Matthews coefficient) is 2.5 Å^3 Da^{-1} assuming the presence of four xylosidase monomers in the asymmetric unit (246.5 kDa; 51.4% solvent content; Matthews, 1968). The self-rotation function of this crystal form is consistent with a tetrameric quaternary structure of the enzyme with 222 point symmetry (Fig. 2*a*). In addition to the expected crystallographic peaks, the self-rotation shows eight noncrystallographic peaks in the κ = 180° selection. Two of these peaks belong to a 222 system of three twofold axes perpendicular to each other. The third twofold axis of each 222 system is parallel to the crystallographic c axis. No 120° rotation axes could be observed that would have indicated a trimeric quaternary structure.

Data from the second crystal form grown with the detergent HEGA-8 were collected on beamline X13 at the EMBL Outstation, Hamburg (Table 1). A complete data set was collected to 2.2 Å resolution. Data could be indexed in the primitive tetragonal space group $P4_12_12$ (or $P4_32_12$), with unit-cell parameters $a = b$ = 140.15, c = 233.11 Å. Calculation of the Matthews coefficient suggests that there are four monomers in the asymmetric unit with a V_M of 2.3 Å^3 Da^{-1} and a solvent content of 46.4%. As in the orthorhombic space group, the self-rotation function is consistent with a tetrameric quaternary structure (Fig. 2*b*). In this case, non-crystallographic 422

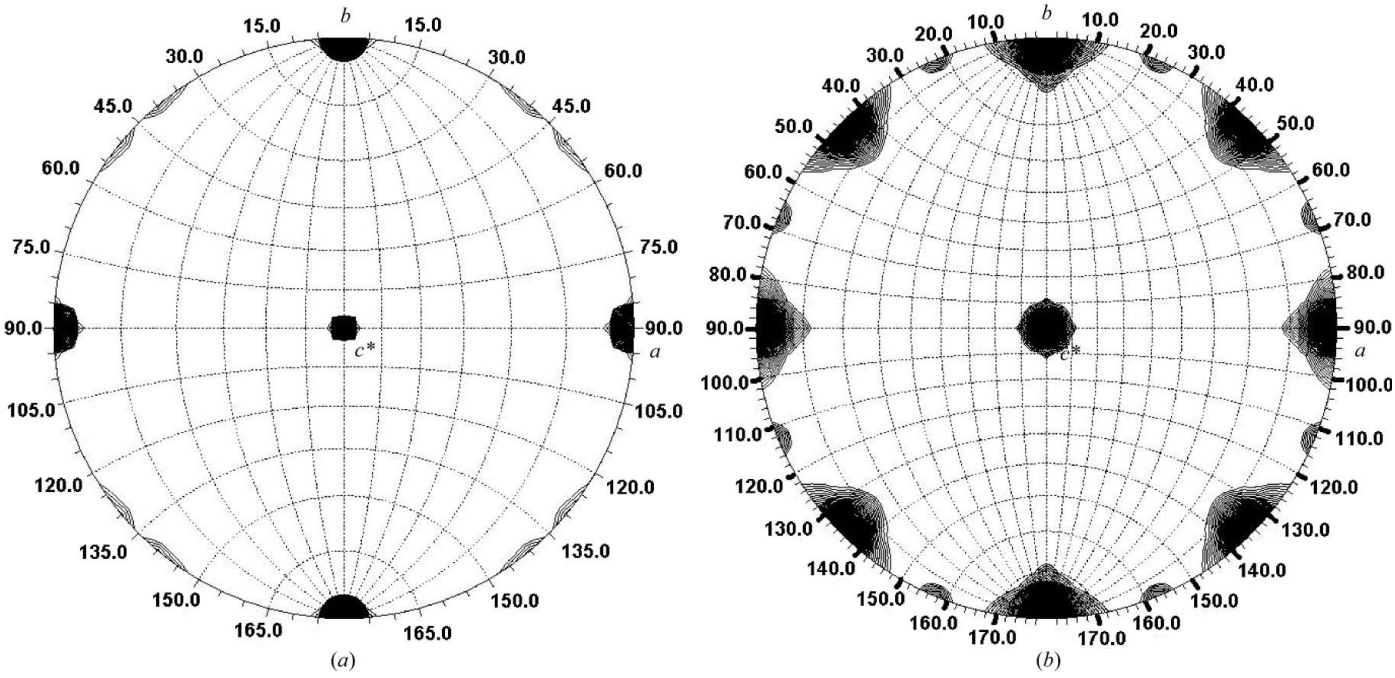

Figure 2
(*a*) Self-rotation function for the orthorhombic space group, κ = 180° section. (*b*) Self-rotation function for the tetragonal space group, κ = 180° section.

systems are also visible with one of the three twofold axes parallel to the crystallographic *c* axis.

3.4. Structure solution

The structure of XynB3 was solved using molecular-replacement techniques, with the structure of family 43 β-D-xylosidase from *Bacillus subtilis* (PDB code 1yif; Patskovsky & Almo, 2005) as a search model. This protein shares a sequence similarity of 65% with XynB3. Using the programs *MOLREP* and *AMoRe* from the *CCP*4 program package (Collaborative Computational Project, Number 4, 1994), significant peaks in the rotation and translation function could be found which led to a plausible crystal packing for each of the two crystal forms. A full structure analysis is now in progress.

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