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Crystallization and preliminary X-ray analysis of an intracellular xylanase from *Bacillus stearothermophilus* T-6

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Xylanases (1,4-β-D-xylan xylanhydrolases; E.C. 3.2.1.8) hydrolyze the 1,4-β-D-xylopyranosyl linkage of xylans. The structural characterization of xylanase active sites is of great interest, since it can lead to a better understanding of their catalytic mechanism and contribute significant knowledge to the rational design of specific oligosaccharide-binding sites *via* protein engineering. An intracellular xylanase gene (*xynA2*) from *Bacillus stearothermophilus* T-6 has recently been cloned and sequenced. The *xynA2* gene encodes for an intracellular enzyme (IXT6) of 331 amino acids, with a calculated molecular weight of 38 639 Da and a pI of 5.72. Based on sequence homology, the enzyme belongs to family 10 of the glycosyl hydrolases. The *xynA2* gene product (IXT6) was overproduced in *Escherichia coli* and purified to homogeneity. Crystallographic studies of IXT6 were initiated in order to study the specificity and mechanism of catalysis of this unique xylanase, as well as to provide a structural basis for rational introduction of enhanced thermostability by site-specific mutagenesis. The M1 crystal form was found to be the most suitable for detailed crystal structure analysis. These crystals belong to a *C*-centered monoclinic crystal system (space group *C*2) with unit-cell parameters a = 170.6, b = 82.5, c = 80.0 Å, β = 91.43°. They are mechanically strong, are fairly stable in the X-ray beam and diffract X-rays to better than 2.5 Å resolution. A full 2.9 Å resolution diffraction data set (97.9% completeness, $R_{\rm merge}$ = 8.4%) has recently been collected from one crystal at room temperature using X-ray synchrotron radiation (λ = 1.125 Å) and a MAR300 imaging-plate area detector. A comparable 2.5 Å data set was measured at 90 K using a rotating-anode X-ray source and an R-AXIS IIc imaging-plate area detector (97.2% completeness, $R_{\rm merge}$ = 6.9%). Molecular-replacement studies and multiple anomalous dispersion (MAD) experiments are currently in progress in order to determine the detailed three-dimensional structure of IXT6.

1. Introduction

Xylanases (1,4-β-D-xylan xylanhydrolases; E.C. 3.2.1.8) hydrolyze the 1,4-β-D-xylopyranosyl linkage of xylans. Xylan is a major component of the plant cell wall and comprises a backbone of D-xylopyranosyl units linked by β-(1–4) glycosidic bonds. The xylose units in the backbone can be modified to include side chains such as arabinose, methyl glucuronic acid and acetyl moieties. The hydrolysis of xylan by endoxylanases produces short modified xylo-oligosaccharides, usually of a size between two and six sugar units. The complete hydrolysis of these units is carried out by additional enzymes, such as β-xylosidases (E.C. 3.2.1.37), arabinofuranosidases (E.C. 3.2.1.55) and α-glucuronidases (E.C. 3.2.1.139). Although all xylanases hydrolyze the same chemical bond, there are multiple forms of xylanases which vary in their substrate specificity and end products (Biely, 1985; Coughlan & Hazlewood, 1993; Eriksson *et al.*, 1990). In this context, culture filtrates of certain fungi, *e.g. Aspergillus niger, Trichoderma viride* and *Talaromyces emersonii*, have been found to possess over 13 different xylanases. The different enzymes vary in specificity. Some prefer unsubstituted sequences and some act close to a substituted residue. For example, a xylanase from *T. emersonii* requires at least 24 unsubstituted xylose residues for its activity, whereas a xylanase from *Bacillus subtilis* requires the presence of 4-*O*-methyl glucuronic acid near the xylan cleavage site. This phenomenon reflects the highly variable structure of xylan in nature. For a given microorganism, the complete degradation of xylan to monomeric sugars or xylobiose can be performed either extracellularly or intracellularly. Fungi such as *Aspergillus* and *Trichoderma* secrete a massive variety of extracellular

hemicellulases which bring about the complete degradation of the hemicellulose polymer to dimers and monomers outside the cells. At the other extreme are *Bacilli* strains that secrete a single extracellular xylanase which partially degrades the xylan substrate. The resulting oligosaccharides enter the cell through specialized permeases and are then further degraded by intracellular hemicellulases. These two completely different strategies for degrading hemicellulose reflect the different natural habitats the microorganisms occupy.

Of the hemicellulases, 1,4-β-xylanases are by far the most characterized and studied enzymes. Over 150 xylanase sequences have been published and are available in the protein sequence data bank. Much of the interest in xylanases stems from their potential biotechnological applications, which includes biobleaching in the pulp and paper industry (Shoham *et al.*, 1993; Viikari *et al.*, 1994), bioconversion of lignocellulose material to fermentative products, partial hydrolysis of animal feedstock to improve digestibility and enzymatic organic synthesis (Fujita & Shoda, 1998; Wong & Whitesides, 1994).

B. stearothermophilus T-6 is a thermophilic strain which was isolated from nature on the basis of its ability to degrade xylan at high temperature and pH. We have recently cloned and sequenced a 23 kb chromosomal segment which contains a gene cluster (presumably an operon) involved in the utilization of glucuronic acid, the monocistronic gene for the extracellular xylanase, xylanase T-6 (XT6, Gat *et al.*, 1994) and part of an operon with an additional xylanase gene (*xynA2*; Shulami *et al.*, 1999). The *xynA2* gene encodes a protein of 331 amino acids with a calculated molecular weight and pI of 38 639 Da and 5.72, respectively. The deduced amino-acid sequence of XynA2 was scanned with the *BLAST*2 program (Altschul *et al.*, 1997) and showed 82% identity to a xylanase from *B. stearothermophilus* 21 (Baba *et al.*, 1994), 59% identity to a xylanase from *Aeromonas caviae* (accession number AB015980) and 41% identity to the extracellular xylanase of strain T-6. Based on sequence homology, the enzyme belongs to family 10 of the glycosyl hydrolases (Henrissat & Bairoch, 1993). Since the *xynA2* gene product lacks a leader peptide, it is likely to be an intracellular xylanase (IXT6). Based on sequence homology and biochemical analysis, it appears that strain T-6 is capable of transporting glucuronyl oligoxylose units and degrading these compounds intracellularly, with α-glucuronidase, IXT6 and β-xylosidase. Thus, strain T-6 possesses an intracellular xylanase, IXT6 (the product of the *xynA2* gene), presumably active on short oligoxylose units (Shulami *et al.*, 1999).

The *xynA2* gene was cloned and overexpressed in *E. coli*. The purified enzyme exhibited different substrate specificity towards synthetic substrates compared with that of the extracellular xylanase (XT6). Since the structure of XT6 is already at a final stage of refinement in our laboratory, we were interested in determining the three-dimensional structure of IXT6 and comparing the two structures at high resolution. This, in turn, may enable us to use the resulting structures as the basis for understanding the structure–function relationships among xylanases with different specificities. Owing to its thermostability and the ease with which it can be overexpressed and purified, IXT6 can also be used for a series of important structurally related mechanistic studies. In this communication, we report the crystallization and preliminary X-ray analysis of IXT6 as a first step in such structural analysis.

2. Experimental

2.1. Purification of IXT6

The *xynA2* gene was cloned into the T7 polymerase expression vector pET9d (Novagen; resulted in pET9d-*xynA2*) and overexpressed in *E. coli* BL21 (DE3) strain (Novagen; Studier *et al.*, 1990). Cells [BL21(DE3)(pET9d-*xynA2*)] were grown overnight (2 × 500 ml in 2 l shake flasks shaken at 200 rev min^{-1}) on Terrific Broth (Sambrook *et al.*, 1989) with kanamycin (25 µg ml^{-1}) at 310 K (without induction), harvested (8000*g*, 5 min), resuspended in 40 ml of 50 m*M* Tris–HCl and 100 m*M* NaCl pH 7 and disrupted by two passages through a French press at room temperature. The cell extract was centrifuged (14 000*g*, 30 min) and the soluble fraction was heat treated (333 K, 30 min) and centrifuged again at room temperature. This step removed most of the *E. coli* proteins and resulted in a soluble fraction (40 ml) which contained the recombinant IXT6 as the main product at a concentration of about 27.5 mg ml^{-1}. The enzyme was further purified by gel filtration at room temperature on a Superdex 200 26/10 column (Pharmacia) running at 2.5 ml min^{-1} with 50 m*M* Tris–HCl buffer pH 7 and 100 m*M* NaCl. The protein appeared as a distinct peak which was then collected and used for crystallization and enzymatic assays. Based on its elution volume, the enzyme is a monomer in solution.

2.2. Crystallization experiments

Following the cloning and overexpression of the IXT6 gene in *E. coli*, we obtained large amounts of highly purified protein which was used for a series of crystallization experiments. The most productive were the factorial and sparse-matrix crystallization screens (Carter & Carter, 1979; Carter *et al.*, 1988; Jancarik & Kim, 1991) combined with the hanging-drop vapour-diffusion method (Hampel *et al.*, 1968), which resulted in several crystal forms. In all of these crystallization experiments, 10 µl drops were formed by mixing 5 µl of protein solution (in 0.05 *M* Tris–HCl buffer at pH 7.0, 0.1 *M* NaCl and 0.02% sodium azide) and an equal volume of the reservoir solution, so that the initial protein concentration in the crystallization drop was always 5 mg ml^{-1}. The drops were suspended over a 1.0 ml reservoir solution in a 4 × 6 Linbro tissue-culture plates. Several forms of IXT6 microcrystals appeared after 3–5 d in some of these crystallization conditions. Refinement of one of these conditions resulted in the M1 crystal form, which was found to be the most suitable for high-resolution structural analysis. The M1 crystals were obtained with reservoir solution consisting of 0.1 *M* sodium cacodylate buffer (pH 6.5), 1.9 *M* sodium acetate and 0.24 *M* citric acid. These crystals grow to their full size in about 10 d. They usually appear as colourless non-symmetrical rhombohedral prisms of slightly varying sizes and shapes (Fig. 1). In most of the experiments, only a small number (5–10) of crystals grew in each drop; they are usually very well formed with defined faces and sharp edges and display a clear extinction effect under polarized light (Fig. 2). Typical dimensions of these crystals are

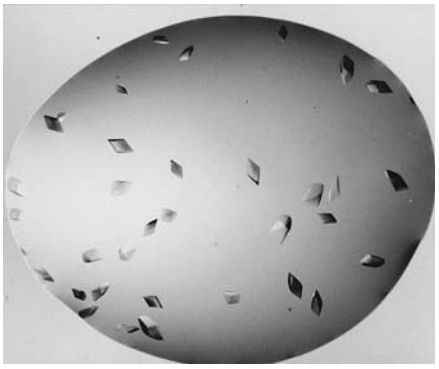

Figure 1
Native IXT6 crystals of the M1 form (magnification ×20; representative crystal dimensions are 0.3 × 0.2 × 0.1 mm).

$0.3 \times 0.2 \times 0.1$ mm, but their sizes range from 0.1 to 0.5 mm in length and from 0.05 to 0.3 mm in width.

3. Results and discussion

3.1. Characterization of the M1 crystal form at room temperature

Several IXT6 crystals of the M1 form were used for crystallographic characterization and measurement of X-ray diffraction data at room temperature. These experiments were performed using X-ray synchrotron radiation (λ = 1.125 Å) and an imaging-plate area detector (MAR300, MAR Research, Germany) at the X26C beamline, National Synchrotron Light Source facility, Brookhaven National Laboratory, NY, USA. The observed diffraction pattern of these crystals exceeded the 2.5 Å resolution limit and relatively little radiation damage could be detected even at long exposure times (about 15% adjusted decay in the diffraction intensity in 6 h). A full oscillation data set to 2.9 Å resolution was measured on a single crystal and indicated that the crystals belong to a *C*-centered monoclinic crystal system (space group *C*2), with crystallographic unit-cell parameters $a = 170.6$, $b = 82.5$, $c = 80.0$ Å, $\beta = 91.43^\circ$.

The raw imaging-plate diffraction data were processed with the *DENZO* and *SCALEPACK* software packages (Otwinowski, 1993). A total of 45 439 accepted reflections [$F > 1.0\sigma(F)$] were measured in the 35.0–2.9 Å resolution range and resulted in 22 644 independent reflections with 97.9% completeness to 2.9 Å resolution (99.8% completeness for the highest resolution shell 2.95–2.90 Å), an overall redundancy of 2.5, an overall mosaicity of 0.7, an average $\langle I/\sigma(I)\rangle$ of 5.9 and a total $R_{\rm merge}$ of 8.4%. These parameters demonstrate the quality of the M1 diffraction data and its suitability for a complete structural analysis.

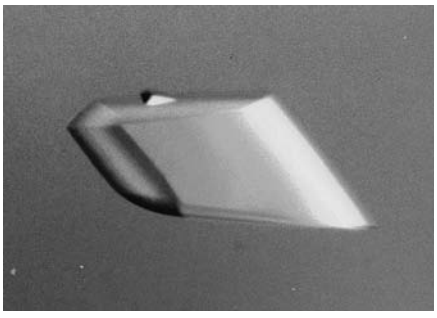

Figure 2
A typical single crystal of native IXT6 of the M1 crystal form under polarized light (crystal dimensions are about $0.4 \times 0.25 \times 0.1$ mm). Note the small satellite crystal grown on the top face of the large crystal. Since their volume difference is more than 1:50, such satellite crystals should not interfere with the diffraction data measurement of the large crystal.

The volume of the M1 crystallographic unit cell as determined from the mean value of the unit-cell dimensions at room temperature is 1.12×10^6 Å^3. Assuming that the specific ratio of volume to protein in the crystal (V_m) is within the normal range of values observed for soluble protein crystals (1.68–3.5 Å^3 Da^{-1}; Matthews, 1968), there should be 8–16 molecules of IXT6 (331 amino acids; M_W = 38 639 Da) in the unit cell. Since there are four symmetry operations in the *C*2 space group, this number should be 8, 12 or 16 (and hence a dimer, a trimer or a tetramer of native IXT6 molecules in the asymmetric unit, respectively), with the highest probability being the middle value (12 molecules or four trimers per unit cell). It is still possible, however, that there is only one monomer per asymmetric unit and that the water content is exceptionally high (~80%) as was surprisingly realised for the related crystal structure of XT6 (Teplitsky, unpublished work).

3.2. Characterization of the M1 crystal form at 90 K

Freezing the IXT6 crystals during the X-ray data measurement proved to be very useful in terms of both the resolution and quality of the resulting diffraction pattern. The best results were obtained when a medium-size crystal ($0.3 \times 0.2 \times 0.1$ mm) of native IXT6 (grown according to the procedure described above) was soaked for 5 min in a cryo-protecting solution containing 1.9 *M* sodium acetate, 0.24 *M* citric acid, 0.1 *M* sodium cacodylate pH 6.5, 30%(*v*/*v*) glycerol and was then submitted to flash-freezing in a cold nitrogen-gas stream (90 K, Oxford Cryosystem). No radiation damage was observed for native IXT6 crystals (M1 form) under these conditions and the diffraction pattern exceeded the 2.5 Å resolution limit even with a standard laboratory X-ray source.

A full such data set (to 2.5 Å resolution) has been recently collected in our laboratory using an imaging-plate area detector (Rigaku R-AXIS IIC imaging plate) mounted on a rotating-anode X-ray generator (Rigaku RU-300 with nickel-filtered Cu $K\alpha$ radiation focused by nickel mirrors; Shibata, 1990; Sato *et al.*, 1992). The diffraction data was processed as described above and indicated that the crystals are completely isomorphous to those analyzed at room-temperature conditions. Nevertheless, as often observed in crystallographic flash-cooling experiments, the unit-cell parameters obtained for frozen IXT6 crystals (a = 169.19, b = 80.64, c = 79.09 Å, $\beta = 91.9^\circ$) differed slightly (less than 1% change) compared with those obtained at room temperature. A total of 127 279 accepted reflections were measured in the resolution range of 30.0–2.5 Å and resulted in 35 997 independent reflections with an overall data redundancy of about 3.5 and an average $\langle I/\sigma(I)\rangle$ of 10.2. The overall mosaicity of this data is 0.32, the completeness is 97.2% (97.0% in the highest resolution shell 2.55–2.50 Å) and the total $R_{\rm merge}$ is 6.9% (28.8% in the highest resolution shell). Preliminary experiments indicated that the quality and resolution of the diffraction data could be further improved by taking measurements from a frozen crystal using well focused synchrotron radiation. Our present efforts are directed towards the measurement of a complete such synchrotron data set at 90 K, as well as the refinement of the most promising crystallization conditions.

In parallel with the improvement of the experimental conditions, efforts are currently made towards phasing the two data sets described above, especially the 2.5 Å resolution data set collected at 90 K. Molecular-replacement phasing calculations are in progress using the recently determined structure of the extracellular xylanase of *B. stearothermophilus* T6 (XT6; Teplitsky *et al.*, 1997; Teplitsky *et al.*, unpublished) as the main reference structure. In addition, we are presently preparing heavy-atom derivatives and a fully exchanged selenomethionyl derivative of IXT6 for alternative (MAD) phasing procedures. The important three-dimensional structure of IXT6, expected to be determined in the near future, will be used for both mechanistic and mutagenesis studies in order to obtain enzyme forms with improved desired properties.

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