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Crystallization and preliminary X-ray analysis of the thermostable alkaline-tolerant xylanase from *Bacillus stearothermophilus* T-6

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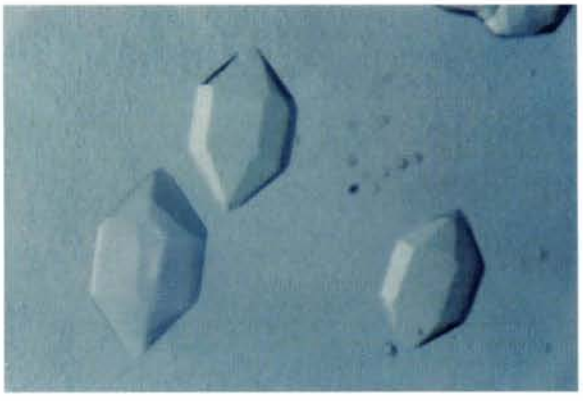
Abstract

The extracellular thermostable xylanase (XT-6) produced by the thermophilic bacterium *Bacillus stearothermophilus* T-6 was shown to bleach pulp optimally at pH 9 and 338 K, and was successfully used in a large-scale biobleaching mill trial. The xylanase gene was cloned and sequenced. The mature enzyme consists of 379 amino acids with a calculated molecular weight of 43 808 and p*I* of 9.0. Crystallographic studies of XT-6 were initiated to study the mechanism of catalysis as well as to provide a structural basis for rational introduction of enhanced thermostability by site-specific mutagenesis. This report describes the crystallization and preliminary crystallographic characterization of the native XT-6 enzyme. The most suitable crystals were obtained by the vapor-diffusion method using ammonium sulfate and 2-methyl-2,4-pentanediol as an organic additive. The crystals belong to a primitive trigonal crystal system (space group $P3_1$ or $P3_2$) with room-temperature cell dimensions of $a = b = 114.9$ and $c = 122.6$ Å. At 103 K the volume of the unit cell decreased significantly with observed dimensions of $a = b = 112.2$ and $c = 122.9$ Å. These crystals are mechanically strong and diffract X-rays to better than 2.2 Å resolution. The crystals exhibit considerable radiation damage at room temperature even at relatively short exposures to X-rays. A full 2.3 Å resolution diffraction data set (99.8% completeness) has recently been collected on flash-frozen crystals at 103 K using synchrotron radiation. Two derivatives of XT-6 were recently prepared. In the first derivative, a unique Cys residue replaced Glu265, the putative nucleophile in the active site. The second derivative was selenomethionyl xylanase which was produced biosynthetically. These derivatives have been crystallized and the resulting crystals were shown to be isomorphous to the native crystals and diffract X-rays to comparable resolutions.

1. Introduction

Xylanases (1,4-β-D-xylan xylanhydrolase; E.C. 3.2.1.8) are hemicellulases that hydrolyze internal β-1,4-glycoside bonds of xylan, which is a major constituent of the hemicellulose complex. A major potential application for hemicellulases in general, and xylanases in particular, is in the paper and pulp industry for biobleaching and biopulping (Grant, 1991; Koponen, 1991; Viikari, Kantelinen, Sundquist & Linko, 1994). High-quality white paper is produced today mainly using chlorine-based compounds (Singh, 1979). The chlorine-based bleaching steps are highly effective, nevertheless they produce large amounts of organochlorine compounds such as chlorinated dioxins (Amato, 1993). Therefore, the paper industry is seeking new technologies for bleaching pulp without chlorine. It was first shown by Viikari *et al.* (Viikari, Ranuae, Kantelinen, Linko & Sundquist, 1986, 1987) that hemicellulases can be used to enhance delignification and bleaching of unbleached pulp, and indeed several full-scale mill trials of enzyme prebleaching have been performed (Grant, 1991; Koponen, 1991; Lundgren *et al.*, 1994; Viikari *et al.*, 1994). Hemicellulases that are active at high temperature and pH are of great interest



(*a*)

(*b*)

Fig. 1. Single crystals of native xylanase T-6 (T1 crystal form). (*a*) Representative crystals used for native diffraction data measurement (magnification ×110). (*b*) Extinction of typical crystals under a polarized light (magnification ×70).

since they can be introduced in the bleaching lines of plants using alkaline pulping (the Kraft process) without the need of physical changes of the lines, thus, reducing the cost for changing temperature and pH and building new facilities. Most xylanases characterized to date are active at acidic pH and moderate temperatures.

Recently, we have characterized an extracellular thermostable xylanase produced by the bacterium *Bacillus stearothermophilus* T-6 (Khasin, Alchanati & Shoham, 1993). Xylanase T-6 was shown to bleach pulp optimally at pH 9 and 338 K, and was successfully used in a large-scale biobleaching mill trial (Hogman, Joves, Rosenberg & Shoham, 1992; Shoham *et al.*, 1993; Lundgren *et al.*, 1994). The xylanase gene was cloned, sequenced and overproduced in *E. coli* (Gat, Lapidot, Alchanati, Regueros & Shoham, 1994; Fishman, Berk & Shoham, 1995; Lapidot, Mechaly & Shoham, 1996). The mature enzyme consists of 379 amino acids with a calculated molecular weight of 43 808 and the p*I* of the native protein is 9.0. Amino-acid analysis indicates that the enzyme shares high homology to β-glycanases which belong to group F, according to Gilkes (Gilkes, Henrissat, Kilburn, Miller & Warren, 1991). To date, the three-dimensional structure of only the catalytic domains of three xylanases from group F are known (Derewenda *et al.*, 1994; Harris *et al.*, 1994; White, Withers, Gilkes & Rose, 1994), all of which are eightfold α/β barrels. These enzymes share about 30% homology with xylanase T-6 (Gat *et al.*, 1994). The three-dimensional structure of xylanase T-6 is of great interest because of its unusual natural thermostability, its favorable starting point for a rational improvement of its already high thermal and pH stability, and its potential use in the pulp industry (Rosenberg & Shoham, 1995). In this communication, we report the crystallization and preliminary X-ray analysis of a complete native thermostable xylanase of *B. stearothermophilus* T-6 (XT-6), constituting the first step towards a detailed three-dimensional structure analysis of this enzyme at high resolution.

2. Experimental

2.1. *Purification of XT-6 from B. stearothermophilus T-6*

Native non-recombinant xylanase T-6 was produced and purified as described previously (Fishman *et al.*, 1995; Khasin *et al.*, 1993) where the extracellular enzyme was first adsorbed to a cation exchanger (SE-52, Whatman) directly from the supernatant broth of *B. stearothermophilus* T-6 cultures. The enzyme was then eluted with 1 *M* KCl and further purified by gel filtration (FPLC Superose 12 HR 10/30, Pharmacia).

2.2. *Expression and purification from E. coli*

The mature xylanase gene, without its leader sequence, was cloned into the T7 polymerase expression vector pET9d (Novagen) (Studier, Rosenberg, Dunn & Dubendorff, 1990) and overexpressed in *E. coli* strain BL21DE3 (Novagen) (Lapidot *et al.*, 1996). The recombinant xylanase contained an additional methionine residue in its N terminus and as a result, the M_r of the recombinant xylanase (consisting of 380 amino acids) is 43 939. Following 4–12 h induction with isopropyl-β-thiogalactopyranoside (IPTG), the cells were harvested and disrupted either by sonication or French press. The cell extract was centrifuged (30 000*g*, 30 min at 277 K) and the soluble portion was heated at 333 K for 30 min and then centrifuged again (30 000*g*, 30 min at 277 K). Following the heat treatment the protein was over 98% pure as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and specific activity. The enzyme was further purified in a single adsorption step to the cellulosic cation exchanger SE-52 (Whatman). In this expression system XT-6 comprised over 70% of the soluble protein, and no inclusion bodies were detected. We routinely obtained over 3 g of purified enzyme per 1 l of *E. coli* cultures grown overnight on Terrific Broth (Sambrook, Fritsch & Maniatis, 1989).

A complete description of the construction and production of Glu265Cys-xylanase and selenomethionyl xylanase (Se-XT-6) will be described elsewhere (Mechaly *et al.*, in preparation). In short, Se-XT-6 was produced biosynthetically in an *E. coli* Met$^-$ strain (B834 λDE3, Novagen) containing the T-7 RNA polymerase gene. The defined growth medium (M-9) contained a cocktail of 19 amino acids, selenomethionine (50 mg l^{-1}), vitamins, and 4% glucose. The cultures were grown overnight at 310 K, and induction (IPTG) was carried at a turbidity of about $\sim$1.5 OD_{600} for 4 h. The cells were centrifuged, broken in a French Press, and the Se-XT-6 was purified *via* heat treatment and a batch adsorption to a cation exchanger (SE-52, Whatman). Using this procedure we obtained from 1 l culture, over 120 mg of purified Se-XT-6.

The Glu265Cys mutation was introduced by site-directed mutagenesis using the unique site elimination procedure of Deng & Nickoloff (1992). Mutagenesis was performed directly on the cloned xylanase gene in the pET9d vector, and the mutant was expressed and purified essentially as described for the native protein.

2.3. *Crystallization*

The crystallization of the non-recombinant native XT-6 (purified enzyme from *B. stearothermophilus* cultures) has proved to be more difficult than initially expected because of protein impurity and inhomogeniety presumably caused by a partial deamination of the native enzyme. The non-recombinant XT-6 crystallization resulted in crystal forms M1 and R1 which are discussed below. Following the cloning and overexpression of the gene in *E. coli* we obtained large amounts of highly purified protein which gave a number of additional crystal forms. Seven of the combined crystal forms were found to be suitable for crystallographic studies. Four of these crystal habits are hexagonal (and labeled accordingly H1, H2, H3, H4), one form is monoclinic (M1), one form is trigonal (T1), and one form is rhombohedral (R1). All seven crystal forms were found to be single crystals and they seem to be rather stable (to mechanical manipulations and air exposure) at room temperature. The diffraction quality of crystal forms H1, H2, H3 and R1 have been found insufficient for high-resolution structural analysis and current attempts to refine their crystallization conditions are in progress. The hexagonal H4 crystal form, the monoclinic M1 crystal form and the trigonal T1 crystal form, however, were found to be suitable for such studies at high resolution and are hence described in more detail below.

The M1 crystal form of non-recombinant XT-6 has also been obtained by the hanging-drop vapor-diffusion method. The drops were prepared by mixing 5 μl of protein solution

(6 mg ml^{-1} xylanase T-6 in distilled water) with an equal volume of 0.1 *M* sodium cacodylate buffer (pH 7.4), 0.2 *M* calcium acetate and 10% polyethylene glycol (PEG) 8 K. These drops were suspended over a 1 ml reservoir of 0.1 *M* sodium cacodylate buffer (pH 7.4), 0.2 *M* calcium acetate and 10% PEG 8 K. The M1 crystals grew to their full size in about 3 months. They were well shaped colorless polygonal pyramids with well formed faces and edges. Their typical dimensions were 0.2 × 0.1 × 0.1 mm and they showed a clear X-ray diffraction pattern to about 3.5 Å resolution. These crystals were found to be rather sensitive to X-ray radiation with an average life time of about 10 h (at room temperature). Partial diffraction data sets indicated a *C*-centered monoclinic unit cell (space group *C*2) with dimensions of $a = 70.6$, $b = 149.4$, $c = 77.8$ Å, $\beta = 90.9°$.

The H4 crystal form of recombinant T-6 has also been obtained by the hanging-drop vapor-diffusion method. The crystallization drops were prepared by mixing 5 µl of protein solution (10–16 mg ml^{-1} XT-6 in distilled water) with an equal volume of 0.2 *M* sodium acetate buffer (pH 5.1), 2.0 *M* ammonium sulfate and 0.02% sodium azide. These drops were suspended over a 1 ml reservoir of 0.2 *M* sodium acetate buffer (pH 5.1) and 2.0 *M* ammonium sulfate. The first H4 crystals appeared after about 4–5 months. Within about another 4–6 weeks these crystals grew to their full size.

The T1 crystal form was prepared, similar to H4, from a recombinant XT-6 that was expressed in, and purified from, *E. coli* as described above. Recombinant XT-6 consisted of one extra methionine residue at the amino terminus. Crystals were obtained by the hanging-drop vapor-diffusion method. In comparison to the H4 form, the crystallization hanging drops were set up with lower concentrations of the protein (4–6 mg ml^{-1}) and the precipitant (0.7–1.0 *M* ammonium sulfate), and no buffer or sodium azide were added. These drops were suspended over a 1 ml reservoir of 1.4–2.0 *M* ammonium sulfate solution. In these conditions, first crystals could be observed in about a week and grew to their full size (typically 0.25× 0.25 × 0.15 mm) in two more weeks. They appeared usually as transparent 'diamond-like' crystals with very distinct faces and edges (Fig. 1*a*), and showed very clear and homogenous polarization effects under polarized light (Fig. 1*b*). These crystals typically diffracted to about 3.0 Å resolution but, as with all the other forms, suffered from serious radiation damage in room temperature X-ray exposure.

3. Results and discussion

3.1. *Measurement of native diffraction data*

Refinement of the crystallization conditions of the T1 form resulted in larger crystals (Fig. 1*a*), faster crystal growth and improved stability of the crystals in the X-ray beam. In the best conditions, the initial drop content is 5–6 mg ml^{-1} XT-6 protein, 0.75 *M* ammonium sulfate, and 8%(*v*/*v*) 2-methyl-2,4-pentanediol (MPD), equilibrated against a reservoir solution of 1.5 *M* ammonium sulfate. First crystals could be observed in the drop after about 4–5 d and grow to their full size in an additional 3–4 d. Several such crystals (0.3 × 0.3 × 0.2–0.5 × 0.4 × 0.4 mm) were used recently for native diffraction data measurement at room temperature on a rotating-anode X-ray source. The diffraction pattern exceeded 2.8 Å resolution but significant radiation damage was observed after several hours of X-ray exposure, limiting the practicality of such crystals for a full structural analysis. The diffraction pattern indicated that the crystals belong to a primitive trigonal crystal system, with unit-cell dimensions of $a = 114.9$, $b = 114.9$, $c = 122.6$ Å, $\alpha = 90$, $\beta = 90$, $\gamma = 120°$. These parameters were determined from precession photographs (nickel-filtered Cu $K\alpha$ radiation from a Rigaku RU-300 rotating anode) and a combination of still images and oscillation images on an imaging-plate area detector (Rigaku R-AXIS IIC imaging plate mounted on a Rigaku RU-300 rotating anode with nickel-filtered Cu $K\alpha$ radiation focused by nickel mirrors; Shibata, 1990; Sato *et al.*, 1992). The raw imaging-plate diffraction data were processed with the *DENZO* and *SCALEPACK* software packages (Otwinowski, 1993).

An attempt to freeze the XT-6 crystals and collect a full native data set from one crystal has recently succeeded. A relatively small (0.2 × 0.2 × 0.1 mm) crystal of native XT-6 was submitted to flash freezing in a nitrogen stream (an Oxford Cryosystems Cryostream) without the need of cryo-protectant, and a full data set was measured using X-ray synchrotron radiation ($\lambda = 0.96$ Å) and a MAR Research imaging-plate detector on the NSLS/X12B station at the Brookhaven National Laboratory. No change has been observed for the room-temperature crystal system, and the diffraction data clearly favored the assignment of the primitive trigonal space groups $P3_1$ or $P3_2$. At the cold nitrogen stream (103 K), however, the volume of the unit cell decreased significantly with observed dimensions of $a = b = 112.2$ and $c = 122.9$ Å. The freezing seemed also to improve significantly the resolution and quality of the X-ray diffraction of the crystals, since resolution beyond 2.2 Å could be detected. A total of 536 964 accepted reflections were measured in the range 40.0–2.3 Å resolution, and resulted in 248 268 independent reflections (99.8% completeness to 2.3 Å resolution, $R_{\rm merge} = 7.2\%$, overall mosaicity of 0.43). At these conditions no significant radiation damage was observed during the course of data collection.

The volume of the crystallographic unit cell of the T1 form as determined from the mean value of the cell dimensions at room temperature is 1.40×10^6 Å^3. Assuming that the unit cell contains 12 molecules of the expressed xylanase T-6 (380 AA; $M_r = 43\,939$) the calculated specific ratio of volume/protein in the crystal (V_m) is 2.65 Å^3 Da^{-1}. This value is well within the normal range of V_m values observed for soluble protein crystals (1.68–3.5 Å^3 Da^{-1}; Matthews, 1968). It is, therefore, concluded that there are probably 12 molecules of xylanase T-6 in the unit cell of the T1 crystal form, and hence four monomers per crystallographic asymmetric unit of the $P3_1$ (or $P3_2$) space group.

3.2. *Additional crystals for phase determination*

All of the traditional attempts to obtain heavy-atom derivatives of the native XT-6 proved to be difficult from a practical point of view. One of the possible reasons for these results is the fact that there are no cysteine residues and only a few histidine residues in native XT-6. Attempts to use the known structures of the partially homologous xylanase catalytic domains in order to phase the native data with molecular replacement showed also only limited success.

In order to allow for another phase determination approach, a mutant of XT-6 was constructed where Glu265 of the active

site has been specifically replaced with a cysteine residue as described above. Such a mutant should provide a high-affinity mercury-specific binding site at a solvent-accessible area of the enzyme. The mutant was expressed, purified and crystallized, and preliminary crystallographic analysis indicated that these crystals bind a single Hg atom at a high relative occupancy. Single crystals of the Glu265Cys-XT-6 mutant were obtained by the hanging-drop method in conditions similar to those of the native XT-6 crystals. Partial data sets were measured on a MAR Research imaging-plate detector ($\lambda = 0.96$ Å, BNL, X12B beamline), and on an off-line Fuji imaging-plate scanner ($\lambda = 0.99$ Å, BNL, X4A beamline). These diffraction studies indicated that the crystals of the Glu265Cys-XT-6 mutant are closely isomorphous to the native crystals in both the space group and unit-cell dimensions. As for the native crystals at room temperature the diffraction pattern exceeded 2.8 Å resolution but significant radiation damage was observed after several diffraction frames.

An alternative approach for the crystallographic phase determination was to biosynthetically produce a derivative of XT-6 where all eight methionine residues of the overexpressed native enzyme were substituted with selenium-methionine. Since selenium gives a usable anomalous-scattering signal, such a derivative could be used for a multi-wavelength anomalous diffraction (MAD) phase determination. Se-XT-6 has recently been expressed and purified, and high-resolution electrospray mass spectrometry confirmed that all eight methionine sites have been fully modified to selenium-methionine. The selenomethionyl derivative has been crystallized in conditions similar to those of the native enzyme and preliminary crystallographic studies using synchrotron radiation ($\lambda = 0.99$ Å, X4A beamline) indicated that the selenium anomalous scattering signal of these crystals will be sufficient (at least in principle) for MAD phasing of the native diffraction data. Several Se-XT-6 crystals were used recently for diffraction studies at room temperature using the X-ray synchrotron facilities mentioned above ($\lambda = 0.96$ Å, X12B beamline; $\lambda = 0.99$ Å, X4A beamline). As for the crystals of the Glu265Cys-XT-6 mutant, these preliminary diffraction studies indicated that the crystals of the Se-XT-6 are isomorphous to the native crystals in both the space group and the unit-cell dimensions, and are also similar to the native crystals in their diffraction properties. These crystals, as expected, are also extremely radiation sensitive at room temperature.

Attempts to flash-freeze the Glu265Cys-XT-6 mutant crystals and the Se-XT-6 derivative crystals in conditions similar to those of the native crystals (*i.e.* with no cryoprotectant) were only partially successful. Studies are currently underway to introduce a suitable cryoprotectant, in order to allow for a full data collection of the mutated enzyme (and its potential heavy-atom derivatives) as well as of its selenomethionyl derivative.

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