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Crystallization and preliminary X-ray analysis of α-D-glucuronidase from *Bacillus stearothermophilus* T-6

Anna Teplitsky,[a] Smadar Shulami,[b] Sara Moryles,[b] Galia Zaide,[b] Yuval Shoham[b] and Gil Shoham[a]*

[a]Department of Inorganic Chemistry and the Laboratory for Structural Chemistry and Biology, The Hebrew University of Jerusalem, Jerusalem 91904, Israel, and [b]Department of Food Engineering and Biotechnology, Technion – Israel Institute of Technology, Haifa 32000, Israel

Correspondence e-mail: gil@vms.huji.ac.il

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α-D-Glucuronidases cleave the α-1,2-glycosidic bond of the 4-*O*-methyl-α-D-glucuronic acid side chain in xylan. Of the xylan-debranching hydrolases, these enzymes are the least studied and characterized. The α-glucuronidase gene (*aguA*) from *Bacillus stearothermophilus* T-6 has been cloned, sequenced and over-produced in *Escherichia coli*. The gene encodes for a protein of 679 amino acids with a calculated molecular weight of 78480 and a p*I* of 5.42. α-Glucuronidase T-6 shows high homology to the α-glucuronidases of *Thermotoga maritima* (60% identity) and of *Trichoderma reesei* (44% identity). Based on the amino-acid sequence similarity, it is likely that these enzymes represent a new class of glycosyl hydrolases. Crystallographic studies of α-glucuronidase T-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. In this report, the crystallization and preliminary crystallographic characterization of the native α-glucuronidase T-6 enzyme is described. Two crystal forms were found suitable for detailed crystal structure analysis. The T1 form was obtained by the vapour-diffusion method using PEG 4000 as a precipitant and 2-propanol as an organic additive. The crystals belong to a primitive tetragonal crystal system (space group $P4_12_12$ or $P4_32_12$) with unit-cell dimensions $a = b = 76.1$ and $c = 331.2$ Å. These crystals are mechanically strong, are stable in the X-ray beam and diffract X-rays to better than 2.4 Å resolution. A full 3.0 Å resolution diffraction data set (97.3% completeness, $R_{\rm merge}$ 9.8%) has recently been collected on one crystal at room temperature using a rotating-anode X-ray source and an R-AXIS IIc imaging-plate detector. The M1 form was obtained and characterized by similar techniques. The best crystallization occurred at a slightly lower pH and a lower concentration of 2-propanol. The crystals belong to a primitive monoclinic crystal system (space group $P2_1$) with unit-cell dimensions $a = 65.8$, $b = 127.4$, $c = 96.6$ Å and $\beta = 97.9^\circ$. These crystals are also quite strong and stable, and diffract to better than 2.8 Å resolution. A full 2.8 Å resolution diffraction data set (96.2% completeness, $R_{\rm merge}$ 7.6%) has recently been collected on one crystal at room temperature using the same R-AXIS IIc setup. Both forms are currently being used to obtain crystallographic phasing *via* isomorphous heavy-atom derivatives and selenomethionine MAD experiments.

1. Introduction

After cellulose, xylan is the most abundant polysaccharide in nature. Xylans are natural heteropolysaccharides composed of a backbone of D-xylopyranosyl units linked by β(1–4) glycosidic bonds. Unlike cellulose, the structure of xylan is not uniform and, depending on the source, xylan structures vary from linear to highly branched heteropolysaccharides. In hardwood, the xylan backbone contains various side chains, including α-1,3-linked arabinofuranosyl, α-1,2-4-*O*-methylglucuronic acid linked to the xylose units *via* α-1,2-glycosidic linkages and acetic acid that esterify the xylose units at position O3 or O2. In non-acetylated softwood xylans, the substituents are 4-*O*-methyl-D-glucuronosyl and L-arabinofuranosyl residues attached to the main chain by α-1,3-glycosidic linkages (Puls & Schuseil, 1993). In the plant cell wall, xylan is closely associated with other wall components. The 4-*O*-methyl-α-D-glucuronic acid residues can form ester linkages to the hydroxyl groups of lignin, providing cross-links between the cell walls and lignin (Das *et al.*, 1984).

Because of its variable structure involving both linear and branched hetero-polysaccharides, the complete degradation of xylan requires the concerted action of several enzymes. These enzymes include endo-1,4-β-xylanase (E.C. 3.2.1.8), β-xylosidase (E.C. 3.2.1.37), α-glucuronidase (E.C. 3.2.1.139), α-L-arabinosidase (E.C. 3.2.1.55) and acetylesterase (E.C. 3.1.1.6) (Sunna & Antranikian, 1997). The key enzyme in xylan degradation is an extracellular endo-1,4-β-xylanase which cleaves the main backbone and releases short xylose units (2–4) with various branched substituents. These branched units can, in many cases, be taken up by the microorganisms *via* specialized permeases and be further degraded with intracellular enzymes such as α-glucuronidase and α-L-arabinosidase, which cleave the side chains and allow the xylose moieties to be hydrolyzed to xylose by intracellular xylanases or xylosidases. Hemicellulases in general, and xylan-degrading enzymes in particular, have attracted much attention owing to their potential industrial use in the biobleaching of paper pulp (Viikari *et al.*, 1994). Hemicellulases are also used for the bioconversion of lignocellulose material to fermentative products and for the improvement of animal feedstock digestibility. Recently, the applications of these hydrolases for organic synthesis has been demonstrated (Wong & Whitesides, 1994).

Of the xylan-debranching enzymes, α-D-glucuronidases are the least studied and characterized (Bronnenmeier *et al.*, 1995; Khandke *et al.*, 1989; Puls, 1992; Smith & Forsberg, 1991). These enzymes catalyze the cleavage of the α-1,2 glycosidic bond of the 4-*O*-methyl-α-D-glucuronic acid side chain. This bond is known to be very stable to acid hydrolysis, and the 4-*O*-methyl-α-D-glucuronic acid residue has a stabilizing effect on the neighbouring xylosidic bonds of the main chain during hydrolysis with 45% formic acid (Roy & Timell, 1968). The hydrolysis catalyzed by α-D-glucuronidases is therefore interesting from theoretical and mechanistic aspects, as well as from practical biotechnological considerations.

In the framework of a wider study of hemicellulases, we have recently cloned, sequenced and overproduced in *Escherichia coli* the α-glucuronidase gene (*aguA*) from *Bacillus stearothermophilus* T-6 (Shoham *et al.*, 1993; Gat *et al.*, 1994). The *aguA* gene appears to be part of a 15.5 kb operon composed of a total of 12 genes which are mostly involved in the transport and utilization of glucuronic acid. The gene encodes for a protein of 679 amino acids with a calculated molecular weight of 78 480 and a p*I* of 5.42. Based on gel filtration, the enzyme is a dimer. The purified gene product was shown to hydrolyze substrates such as 2-*O*-(4-*O*-methyl-α-D-glucupyranosyluronic acid)-D-xylotriose to 4-*O*-methylglucuronic acid and xylotriose. The deduced amino-acid sequence of α-glucuronidase T-6 was scanned with the *TFASTA* program (Genetic Computer Group, Inc.). α-Glucuronidase T-6 has 60% identity (578 amino acids overlap) with the α-glucuronidase of *Thermotoga maritima* (Ruile *et al.*, 1997), 44% identity (581 amino acids overlap) with the extracellular α-glucuronidase of *Trichoderma reesei* (Margolles-Clark *et al.*, 1996) and 45% identity (575 amino acids overlap) with the extracellular α-glucuronidase of *Aspergillus tubingensis* (Vries *et al.*, 1998). Based on the amino-acid sequence similarity, it is likely that these enzymes are related in both structure and mechanism and hence represent a new class of glycosyl hydrolases (Henrissat & Bairoch, 1993).

Owing to its thermostability and the ease by which it can be overexpressed and purified, the specific α-glucuronidase from strain T-6 seems to be an excellent representative of this family for a detailed structural analysis. A parallel study of the three-dimensional structure of this enzyme and its catalytic activity is expected to be extremely valuable for the understanding of this as yet uncharacterized family of glycosyl hydrolases. In this communication, we report the isolation, crystallization and preliminary X-ray analysis of the α-glucuronidase from *B. stearothermophilus* T-6 as a first step towards its structural analysis. To the best of our knowledge, this is the first α-glucuronidase for which crystallization has been reported.

2. Experimental

2.1. Purification of α-glucuronidase T-6

The *aguA* gene (GeneBank accession No. AFO98273) was cloned into the T7 polymerase expression vector pET9d (Novagen) and overexpressed in *E. coli* strain BL21DE3 (Novagen) (Studier *et al.*, 1990). Cells [BL21DE3 (pET9d-*aguA*)] were grown overnight (2 × 500 ml in 2 l shake flasks) in Terrific Broth (Sambrook *et al.*, 1989) with kanamycin (25 µg ml^{-1}) at 310 K (without induction), harvested (14000*g*, 10 min), resuspended in 80 ml of 100 m*M* Tris–HCl pH 7 and disrupted by two passages through a French press at room temperature. The cell extract was centrifuged (14000*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 (80 ml) which contained the recombinant α-glucuronidase as the main product at a concentration of about 15 mg ml^{-1}. No loss of activity was detected following the heat treatment. Final purification of the enzyme was performed by gel filtration on a Superdex 200 26/10 column, AKTA explorer (Pharmacia), running with 50 m*M* Tris–HCl buffer pH 7 and 100 m*M* NaCl at 2.5 ml min^{-1} at room temperature. The enzyme appeared as a distinct peak which was then collected and used for crystallization and enzymatic assays.

2.2. Crystallization experiments

Following the cloning and overexpression of the α-glucuronidase T-6 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. Two of these forms were found to be suitable for crystallographic studies, the T1 form and the M1 form. In all of these experiments, the native enzyme was dialyzed overnight against distilled water and was then concentrated to 10 mg ml^{-1}. Drops of 10 µl were generally used for the hanging-drop experiments. These drops were formed by mixing 5 µl of the dialyzed protein solution and an equal volume of the reservoir solution, so that the initial protein concentration in the drop was always 5 mg ml^{-1}. The drops were suspended over a 1 ml reservoir in 4 × 6 Linbro tissue-culture plates.

3. Results and discussion

3.1. Crystallization and characterization of the T1 crystal form

The T1 crystal form of native α-glucuronidase T-6 was obtained by the hanging-drop method described above, using reservoir solution consisting of 0.1 *M* sodium citrate buffer (pH 5.6), 10% PEG 4000 and 15% 2-propanol. The T1 crystals could be initially observed in these conditions after about 3–4 d and grew to their full size in about three weeks. They usually appeared as colorless elongated tetragonal pyramids or tetragonal bipyramids (Fig. 1*a*). In most of the experiments only one or two crystals grew in each drop; they were usually very

well formed with defined faces and sharp edges and displayed a distinct effect under a polarized light (Fig. 1*b*). Typical dimensions of these crystals were 0.5 × 0.2 × 0.1 mm, but they often grew with longer and thinner shapes (up to 2 mm in length and as little as 0.05 mm in width).

Several such crystals were used for crystallographic characterization and measurement of X-ray diffraction data at room temperature. These experiments were performed on a rotating-anode X-ray source. The observed diffraction pattern of these crystals exceeded the 2.4 Å resolution limit and only very slight radiation damage could be detected, even at long exposure times. Nevertheless, even with the best diffracting crystals, only a 3 Å resolution diffraction pattern could be measured and analyzed, owing to the length of one of the cell dimensions and technical restrictions resulting from the size of the detector and its geometrical setup. The diffraction pattern indicated that the crystals belong to a primitive tetragonal crystal system, with unit-cell parameters $a = b = 76.1$ and $c = 331.2$ Å ($\alpha = \beta = \gamma = 90^\circ$). These parameters were determined from 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).

A full 3.0 Å resolution data set was collected at room temperature using one T1 crystal of approximate dimensions 0.8 × 0.3 × 0.3 mm. A total of 208038 accepted reflections [$F > 1.0\sigma(F)$] were measured and resulted in 19921 independent reflections with 97.3% completeness to 3.0 Å resolution (90.4% completeness for the highest resolution shell of 3.05–3.00 Å), total $R_{\rm merge}$ of 9.8% and overall mosaicity of 0.3. The systematic extinctions in this diffraction pattern indicated that the space group of the T1 crystal form is either $P4_12_12$ (number 92) or $P4_32_12$ (number 96). Under these conditions, only a small amount of radiation damage was observed during the course of data collection [about 7 d using a rotating-anode source, Cu $K\alpha$ radiation ($\lambda = 1.54$ Å) and an R-AXIS II detector].

A parallel diffraction experiment was performed with a similar T1 crystal using X-ray synchrotron radiation ($\lambda = 1.15$ Å) and a MAR Research imaging-plate detector on the NSLS/X26C station at the Brookhaven National Laboratory. A partial data set was measured (56% completeness to 3.0 Å resolution) and indicated the same potential space groups and similar unit-cell dimensions. Here, the diffraction limit observed (about 2.3 Å) was again significantly higher than the resolution limit that could be practically measured in these conditions.

The volume of the crystallographic unit cell of the T1 form, as determined from the mean value of the unit-cell dimensions at room temperature, is 1.91×10^6 Å^3. Assuming that the unit cell contains eight molecules (the number of symmetry operations in the two potential space groups) of the expressed monomer of native α-glucuronidase T-6 (679 amino acids; M_W = 78 480 Da), the calculated specific ratio of volume per protein molecule in the crystal (V_m) is 3.02 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 eight monomers of α-glucuronidase T-6 (or in fact four dimers) in the unit cell of the T1 crystal form, and hence one monomer (or half dimer) per crystallographic asymmetric unit of the $P4_12_12$ (or $P4_32_12$) space group. Efforts to obtain isomorphous heavy-atom derivatives of this crystal form have already resulted in a potential Pb derivative. This derivative is obtained by soaking the native crystals for 48 h in a solution containing 12 mM (CH_3)PbAc, 0.1 M sodium citrate (pH 7.0), 20% PEG 4000 and 5% 2-propanol. Refinement of these conditions as well as further heavy-atom experiments are in progress. Parallel research is currently being performed to produce and crystallize a fully exchanged selenium-methionine derivative of α-glucuronidase T-6 (each monomer contains 14 methionines) for use in selenium MAD phasing.

3.2. Crystallization and characterization of the M1 crystal form

Attempts to refine the crystallization conditions of the T1 form resulted in another crystal form of native α-glucuronidase T-6, the M1 form. The crystals were obtained by the hanging-drop method described above, using a reservoir solution consisting of 0.1 M sodium citrate buffer (pH 5.2), 10% PEG 4000 and 5% 2-propanol, conditions which are slightly, yet significantly, different from the optimal crystallization conditions for the T1 form. The M1 crystals could initially be observed in these conditions after about 2 d and grew

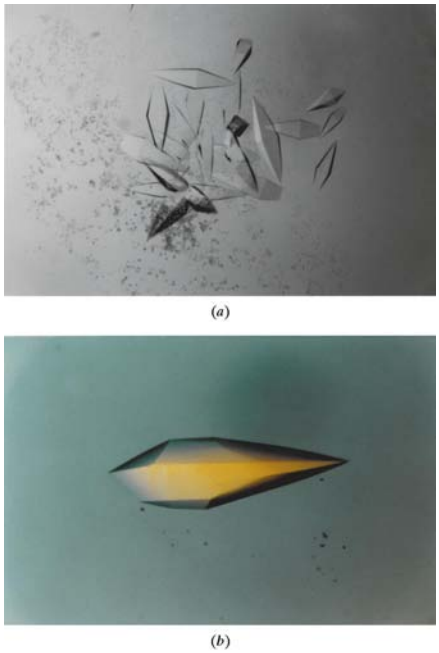

Figure 1
(*a*) Native tetragonal pyramidal and tetragonal bipyramidal α-glucuronidase T-6 crystals (T1 crystal form; representative crystal dimensions are 0.5 × 0.2 × 0.1 mm). (*b*) A typical single crystal of the T1 crystal form of native α-glucuronidase T-6 under polarized light (crystal dimensions are about 1.5 × 0.4 × 0.3 mm).

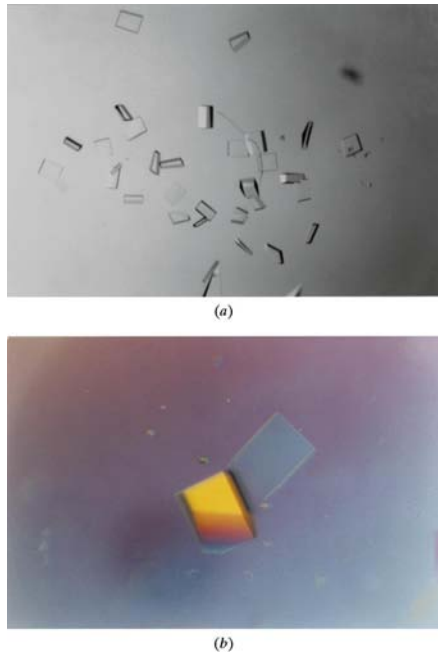

Figure 2
(*a*) Monoclinic rectangular box-shaped α-glucuronidase T-6 crystals (M1 crystal form; representative crystal dimensions 0.1 × 0.1 × 0.05 mm). (*b*) Typical single crystals of the M1 crystal form of native α-glucuronidase T-6 under polarized light (dimensions of the larger crystal are about 0.25 × 0.2 × 0.2 mm).

to their full size in about a week. They were usually colorless and rectangular with well formed faces and edges (Fig. 2*a*) and displayed a distinct polarization effect (Fig. 2*b*). Typical dimensions of these crystals were 0.1 × 0.1 × 0.05 to 0.3 × 0.3 × 0.2 mm.

Several M1 crystals were used for crystallographic characterization and measurement of X-ray diffraction data on a rotating-anode X-ray source at room temperature. The resolution limit of the observed diffraction pattern of these crystals exceeded 2.8 Å and, as for the T1 form, the M1 crystals suffered only slight radiation damage during X-ray exposure. The diffraction pattern indicated that the crystals belong to a primitive monoclinic crystal system, with unit-cell parameters a = 65.8, b = 127.4, c = 96.6 Å and β = 97.9° ($\alpha = \gamma = 90°$). These parameters were determined from a combination of still images and oscillation images on the Rigaku R-AXIS IIC imaging plate described above. The raw imaging-plate diffraction data were processed with the *DENZO* and *SCALEPACK* software packages (Otwinowski, 1993).

A full 2.8 Å resolution data set was recently collected at room temperature using one M1 crystal of about 0.3 × 0.3 × 0.2 mm. A total of 199364 accepted reflections [$F > 1.0\sigma(F)$] were measured and resulted in 41467 independent reflections with 96.2% completeness to 2.8 Å resolution (95.6% completeness for the highest resolution shell of 2.85–2.80 Å), a total $R_{\rm merge}$ of 7.6% and an overall mosaicity of 0.16. The systematic extinctions in this diffraction pattern indicated that the space group of the M1 crystal form is $P2_1$.

The volume of the crystallographic unit cell of the M1 form, as determined from the mean value of the cell dimensions at room temperature, is 7.0×10^5 Å^3. Assuming that the unit cell contains four monomers (or two dimers) of α-glucuronidase, the calculated specific ratio of volume per protein molecule in the crystal (V_m) is 2.22 Å^3 Da^{-1}, a very reasonable V_m value for a soluble protein (Matthews, 1968). This analysis means that in the M1 asymmetric unit there is, most probably, only one dimer of native α-glucuronidase T-6. As with the T1 crystal form, this form is definitely suitable for a full crystallographic structural analysis, and heavy-atom derivative experiments are therefore being conducted with the M1 form as well as with the T1 form.

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