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Crystallization and preliminary X-ray analysis of a family 51 glycoside hydrolase, the α-L-arabinofuranosidase from *Geobacillus stearothermophilus* T-6

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α-L-Arabinofuranosidases (EC 3.2.1.55) are hemicellulases that cleave the glycosidic bond between L-arabinofuranoside side chains and various oligosaccharides. In this study, the first crystallization and preliminary X-ray analysis of the α-L-arabinofuranosidase from *Geobacillus stearothermophilus* T-6 (AbfA T-6), a family 51 glycoside hydrolase, is described. AbfA T-6 is a hexameric protein consisting of six identical subunits of 502 amino acids and with a calculated molecular mass of 57 218 Da. Purified recombinant native and selenomethionine-containing AbfA T-6 were crystallized by the sitting-drop method in two different space groups, $P2_1$ (unit-cell parameters $a = 100.8$, $b = 178.1$, $c = 196.2$ Å, $\beta = 96.1^\circ$) and $R3$ (unit-cell parameters $a = b = 179.3$, $c = 100.4$ Å). The $R3$ crystals diffracted X-rays to a resolution of 1.8 Å.

1. Introduction

α-L-Arabinofuranosidases (EC 3.2.1.55) are hemicellulases that cleave the glycosidic bond between L-arabinofuranoside side chains and various oligosaccharides. These enzymes are part of an array of glycoside hydrolases responsible for the degradation of hemicelluloses such as arabinoxylan, arabinogalactan and L-arabinan (Biely, 1985; Hazlewood & Gilbert, 1998; Sunna & Antranikian, 1997). Hemicellulases, together with cellulases, play a key role in the carbon cycle in nature, since they are responsible for the complete degradation of the plant biomass to soluble saccharides. These in turn are used as carbon or energy sources for microorganisms and higher animals. Hemicellulases have attracted much attention in recent years because of their potential industrial uses in biobleaching of paper pulp, bioconversion of lignocellulose material to fermentative products, improvement of animal feedstock digestibility and organic synthesis (Mackenzie *et al.*, 1998; Saha, 2000; Suurnakki *et al.*, 1997; Wong & Sanddler, 1993). In many cases, microorganisms that utilize hemicelluloses possess α-L-arabinofuranosidases with varying substrate specificities and biochemical properties (Saha, 2000). To date, more than 130 α-L-arabinofuranosidase sequences are known. According to the sequence-based classification of glycoside hydrolases (available at the Carbohydrate Active Enzymes server at http://afmb.cnrs-mrs.fr/CAZY), α-L-arabinofuranosidases belong to four families: 43, 51, 54 and 62 (Henrissat & Bairoch, 1996; Henrissat & Davies, 1997).

The glycosidic bond is one of the most stable bonds in nature, with a half-life of over 5×10^6 y (Wolfenden *et al.*, 1998). Glycoside hydrolases can accelerate the hydrolysis of the glycosidic bond by more than 10^{17}-fold, making them one of the most efficient catalysts known. The hydrolysis of the glycosidic bonds occurs *via* two major mechanisms, giving rise to either overall retention or inversion of the anomeric configuration (Davies *et al.*, 1998; Sinnott, 1990). Both catalytic mechanisms are found in the various α-L-arabinofuranosidases families: enzymes from glycoside hydrolase families 51 and 54 are retaining (Pitson *et al.*, 1996), while the glycoside hydrolase family 43 enzymes are inverting (Pitson *et al.*, 1996); the stereochemistry of family 62 is not yet characterized. Recently, the crystal structure of the family 43 α-L-arabinanase from *Cellvibrio japonicus* (formerly known as *Pseudomonas callulosa*) was determined (Nurizzo *et al.*, 2002). This enzyme displays a five-bladed β-propeller fold and is the first known enzyme to display this topology.

In this study, we describe the crystallization and preliminary X-ray analysis of the α-L-arabinofuranosidase from *G. stearothermophilus* T-6, a family 51 glycoside hydrolase. This enzyme exhibits a wide substrate specificity: in addition to hydrolyzing aryl-α-L-arabinofuranosides, 1,5-α-L-arabinofuranooligomers and 1,2- or 1,3-α-L-arabinofuranosyl xylooligomers (Shallom & Shoham, unpublished results), it can also hydrolyze various aryl-β-D-xylopyranosides (Shallom, Belakhov, Solomon, Gilead-Gropper *et al.*, 2002; Shallom, Belakhov, Solomon, Shoham *et al.*, 2002). The *abfA T-6* gene encodes a 502-amino-acid protein

with a calculated molecular mass of 57 218 Da. Based on gel-filtration experiments, the enzyme was initially proposed to be a homotetramer in solution (Gilead & Shoham, 1995). However, recent measurements indicate that the molecular weight of the protein in solution is above 310 kDa, which may correspond better to a hexamer.

The three-dimensional structure of this enzyme and especially of its complexes with various substrates is of great interest since it will provide crucial information on the binding mechanism and specificity of family 51 glycoside hydrolases.

2. Experimental

2.1. Purification of native and seleno-methionine AbfA T-6

The *abfA T-6* gene (GenBank accession No. AF159625) from *G. stearothermophilus* T-6 was cloned in the pET9d vector (Novagen) and overexpressed in *Escherichia coli* BL21 (DE3) (Novagen). The purification procedure included heat treatment at 333 K and gel-filtration chromatography as described previously (Shallom, Belakhov, Solomon, Gilead-Gropper *et al.*, 2002) and resulted in gram quantities of >99% pure protein. Production of the selenomethionine derivative of AbfA T-6 was carried out in the methionine-auxotrophic *E. coli* strain B834 (DE3) (Novagen), essentially as described previously (Mechaly *et al.*, 2000), and protein purification proceeded as for the wild-type enzyme. ESI mass spectra of both native and Se-Met AbfA T-6 were in accordance with the calculated molecular masses, indicating complete incorporation of SeMet. The purified enzyme was concentrated to 8 mg ml^{-1} by ultrafiltration. The homogeneity of the protein was checked by standard SDS–PAGE, native–PAGE, dynamic light scattering and mass spectrometry.

2.2. Crystallization experiments

All crystallization experiments were performed at 293 K using the sitting-drop variant of the vapour-diffusion method. Initial crystallization conditions were screened using the sparse-matrix approach (Jancarik & Kim, 1991) and resulted in one promising crystal form. Refinement of the first crystal form led to a second higher symmetrical crystal form. 6 µl droplets were typically used for the crystallization experiments. The drops were prepared by mixing 3 µl of protein solution with an equal volume of the optimized reservoir solution and were equilibrated against 1 ml of reservoir solution.

Table 1
Selected crystallographic parameters for the AbfA T-6 crystal forms.

Data collection	X11, EMBL Outstation, Hamburg	Rigaku rotating-anode X-ray generator
Wavelength (Å)	0.9080	1.54178
Unit-cell parameters		
a (Å)	100.8	179.3
b (Å)	178.1	179.3
c (Å)	196.2	100.4
β (°)	96.1	
Space group	$P2_1$	$R3$
No. monomers in the asymmetric unit	12	2
V_M (Å^3 Da^{-1})	2.56	2.7
Oscillation angle per frame (°)	0.5	1
No. of frames	123	117
Data-collection temperature (K)	100	100
Resolution (Å)	2.6	1.8
Total No. of reflections	483244	429358
No. of rejected reflections	9171	6821
No. of unique reflections	166649	119711
Completeness (%)	84.6	99.7
R_{merge}† (%)	5.4	4.7
R_{merge}† (%) (outer shell)	31.6 (2.65–2.60 Å)	29.2 (1.84–1.80 Å)
$I/\sigma(I)$	19.7	26.5
$I/\sigma(I)$ (outer shell)	3.4 (2.65–2.60 Å)	3.9 (1.84–1.80 Å)

† $R_{merge} = \sum(|I - \langle I\rangle|)/\sum I$.

2.3. Diffraction experiments

For X-ray diffraction experiments at cryogenic temperature, the crystals were transferred stepwise into a cryoprotectant buffer consisting of the reservoir solution containing up to 12.5%(*v*/*v*) glycerol. The crystals were mounted in a nylon loop and flash-cooled in liquid nitrogen. The raw diffraction data were indexed, processed and scaled with *DENZO* and *SCALEPACK* from the *HKL* suite (Otwinowski & Minor, 1997).

3. Results and discussion

3.1. Crystallization and characterization of the $P2_1$ crystal form

The $P2_1$ crystal form of native AbfA T-6 was obtained by the sitting-drop method described above using the PEG/Ion Screen from Hampton Research. The reservoir solution consists of 20% PEG 3350 and 0.2 *M* NH_4F. Small crystals were initially observed under these conditions after about 7 d. For pH screening, 0.1 *M* of Tris–HCl was added to the reservoir and the best crystals were obtained at pH 7.0. Microseeding appeared to be crucial in order to obtain $P2_1$ crystals with defined faces and sharp edges. As the quality and the size of the crystals depended on the quality of the seed crystals, several cycles of seeding were performed. In each cycle, the seeds were transferred directly into the optimized reservoir solution consisting of 18%(*w*/*v*) PEG 3350, 0.2 *M* NH_4F and 0.1 *M* Tris–HCl pH 7.0. Monoclinic crystals usually appeared with typical dimensions of 0.4 × 0.25 × 0.15 mm (Fig. 1).

A 2.6 Å resolution data set was collected using X-ray synchrotron radiation and a MAR CCD 165 mm detector at the EMBL Outstation, Hamburg (Table 1). The diffraction pattern indicated a monoclinic unit cell, with unit-cell parameters a = 100.8, b = 178.1, c = 196.2 Å, β = 96.1°. Assuming

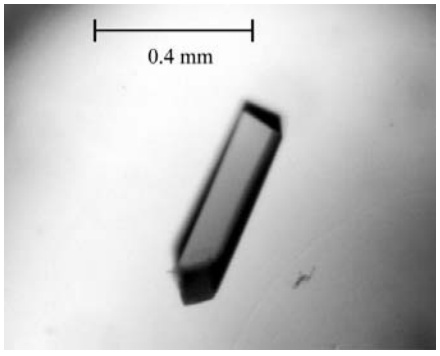

Figure 1
Primitive monoclinic crystal of native AbfA T-6 under polarized light.

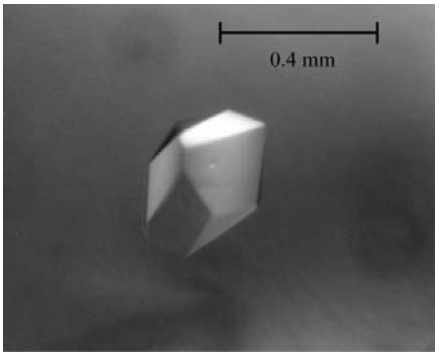

Figure 2
*R*3 crystal of native AbfA T-6 under polarized light.

the specific ratio of volume to protein in the crystal (V_M) to be within the normal range of values observed for soluble protein crystals (Matthews, 1968), there are 12 molecules of AbfA T-6 in the asymmetric unit (684 kDa; V_M value of 2.56 Å^3 Da^{-1}). At this point, owing to uncertainties concerning the quaternary structure, it was possible to assign either three tetramers or two hexamers to the asymmetric unit, as the complex self-rotation functions and native Patterson functions could not clearly be interpreted.

3.2. Crystallization and characterization of the *R*3 crystal form

A second crystal form was observed during the refinement of the $P2_1$ crystals by using the same crystallization solution but increasing the pH to 8.0. Although these crystals could only be obtained by cross seeding with the crushed and diluted $P2_1$ crystals, the new crystals had a rhombohedral shape and displayed a more distinct effect under polarized light. Addition of 2-propanol improved the diffraction quality of the crystals and they grew to typical dimensions of 0.3 × 0.2 × 0.2 mm within one week (Fig. 2). The optimized reservoir consisted of 18%(*w*/*v*) PEG 3350, 0.2 *M* NH_4F, 5%(*v*/*v*) 2-propanol and 0.1 *M* Tris–HCl pH 8.0.

For the *R*3 crystals, a complete data set was measured to 1.8 Å resolution on a Rigaku rotating-anode X-ray generator and a MAR 345 imaging-plate area detector (MAR Research, Germany) (Table 1). Data could be indexed in the primitive rhombohedral space group *R*3, with unit-cell parameters $a = b = 179.3$, $c = 100.4$ Å. The calculated V_M value for two monomers in the asymmetric unit is 2.7 Å^3 Da^{-1}; these results could not be explained by a tetramer and indicated hexameric AbfA T-6.

3.3. Experimental phasing

As no homologous structure is known to date for family 51 glycoside hydrolases, experimental phasing techniques had to be applied. For both crystal forms, the selenomethionine derivative of AbfA T-6 could be crystallized under similar conditions. For the $P2_1$ crystals, the presence of 12 AbfA T-6 molecules in the asymmetric unit requires the identification of 144 Se sites in the course of the MAD experiment. Therefore, these crystals appear to be less suitable for subsequent selenium MAD phasing. In contrast, the *R*3 crystals are highly suitable for subsequent phasing by MAD: their symmetry is higher, the size of the asymmetric unit is smaller (104 kDa), their diffraction power is better and the Se substructure is smaller, with only 24 sites in the asymmetric unit. For the *R*3 crystals, a SeMAD data set was measured on BW7A at the EMBL Outstation, Hamburg (data statistics not shown). All 24 SeMet sites were located using the automated protocol of *SOLVE* (Terwilliger & Berendzen, 1999). Initial phasing with *SOLVE* resulted in an interpretable electron-density map. At the present stage of structure refinement, we are able to identify hexameric AbfA T-6 in both crystal forms.

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