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Crystallization and preliminary X-ray diffraction analysis of a thermostable endo-1,5-α-L-arabinanase from *Bacillus thermodenitrificans* TS-3

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A thermostable endo-1,5-α-L-arabinanase ABN-TS from *Bacillus thermodenitrificans* TS-3 with a molecular weight of 35 kDa was crystallized by the hanging-drop vapour-diffusion method using sodium citrate as a precipitant. The crystals were loop-mounted in a cryoprotectant solution containing 28%(*w*/*v*) sucrose and 1 *M* sodium citrate pH 6.0 and flash-cooled. Sucrose was selected as the most suitable cryoprotectant. The crystal belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 40.3, b = 77.8, c = 89.7 Å. The calculated $V_{\rm M}$ based on one molecule per asymmetric unit was 2.0 $Å^3\,Da^{-1}$. A complete data set from a frozen crystal was collected to 1.9 Å resolution using synchrotron radiation at SPring-8. A molecular-replacement solution was obtained using the structure of α-arabinanase 43A from *Cellvibrio japonicus*.

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1. Introduction

Arabinan is widely distributed in plant cell walls. It consists of a backbone of α-1,5-linked L-arabinofuranosyl residues, some of which are substituted with α-1,2- and/or α-1,3-linked L-arabinose side chains (Tanaka *et al.*, 1981; Bacic *et al.*, 1988). Two enzymes, endo-1,5-α-L-arabinanase (ABN; EC 3.2.1.99) and α-L-arabinofuranosidase (ABF; EC 3.2.1.55), work synergistically in degrading branched arabinan to generate L-arabinose (Voragen *et al.*, 1987; Matsuo *et al.*, 2000). ABN enzymes, which are members of glycoside hydrolase (GH) family 43 (Coutinho & Henrissat, 2004; Henrissat & Bairoch, 1996), hydrolyze α-1,5-L-arabinofuranoside linkages by an endo mechanism (Voragen *et al.*, 1987). ABF enzymes, which are members of the GH families 43, 51, 54 and 62 (Henrissat & Davies, 1997), also degrade arabinan by cleavage of the arabinose side chains.

L-Arabinose selectively inhibits intestinal sucrase in a non-competitive manner and suppresses the increase in the glycaemic response after the ingestion of sucrose in animals (Seri *et al.*, 1996). Therefore, L-arabinose could be used as a physiologically functional sugar that suppresses sucrose digestion. Thermostable arabinan-degrading enzymes are invaluable in the food industry for the efficient production of L-arabinose from plant arabinan at high temperatures.

Recently, a gene that encodes a thermostable ABN (ABN-TS) was isolated from a thermophilic bacterium, *Bacillus thermodenitrificans* strain TS-3 (Takao *et al.*, 2002). ABN-TS showed optimal activity at 343 K and pH 6.0 and its half-life was 4 h at 348 K. Genes encoding mesophilic ABNs have been found in various microorganisms, including *B. subtilis* (Sakamoto *et al.*, 1997), *Pseudomonas fluorescens* (McKie *et al.*, 1997) and *Aspergillus niger* (Flipphi *et al.*, 1993). Of these enzymes, the three-dimensional structure of α-L-arabinanase Arb43A from *Cellvibro japonicus* (formally known as *P. cellulosa*) has been determined by X-ray crystallography (Nurizzo *et al.*, 2002). The enzyme has the unique motif consisting of a five-bladed β-propeller fold. Since ABN-TS showed 46% similarity to Arb43A in its amino-acid sequence, X-ray crystallographic analysis of ABN-TS should provide important information towards clarifying the structural features that contribute to its thermostability. Here, we report the crystallization and preliminary X-ray crystallographic analysis of ABN-TS from *B. thermodenitrificans* TS-3.

2. Materials and methods

2.1. Crystallization

Recombinant ABN-TS was overexpressed in *B. subtilis* MI112 (Takao *et al.*, 2002). A fraction from the culture filtrate precipitated with 20–80% saturated ammonium sulfate was purified by hydrophobic (Phenyl Sepharose, Amersham Biosciences), anion-exchange (UNO Q, Bio-Rad) and gel-filtration (Superdex 200, Amersham Biosciences) chromatography using an FPLC system from Amersham Biosciences at 277 K. The purity of the enzyme was confirmed by SDS–PAGE.

Prior to crystallization trials, the purified protein was dialysed against 20 m*M* Tris–HCl buffer pH 7.5 and concentrated using an

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Ultrafree-MC concentrator with a membrane cutoff of 10 kDa (Millipore) at 277 K. Crystallization trials were performed using the hanging-drop vapour-diffusion method (McPherson, 1999). Conditions for crystallization were initially searched using the commercially available sparse-matrix screening kits Crystal Screen I and II from Hampton Research (Jancarik & Kim, 1991). Each drop was prepared by mixing 1 µl ABN-TS solution with 1 µl reservoir solution and equilibrated against 500 µl of the respective reservoir solution. The best initial conditions were optimized by varying the pH and the sodium citrate and protein concentrations.

2.2. X-ray diffraction analysis

For data collection at cryogenic temperature, the ABN-TS crystals were briefly immersed in a cryoprotectant solution. Screening for a suitable cryoprotectant took place using the commercially available cryoprotectant kit CryoPro from Hampton Research. The crystals were mounted in loops (Hampton Research) and flash-cooled in the gas stream from a liquid-nitrogen cryostat (Rigaku).

Preliminary X-ray diffraction studies were carried out at 100 K using an R-AXIS IIc image-plate area detector mounted on a Rigaku RU-300 rotating-anode source operating at 40 kV and 100 mA with Cu $K\alpha$ radiation. A complete data set was collected at 100 K on a DIP6040 image-plate detector using synchrotron radiation of wavelength 0.9 Å at the BL44XU station of SPring-8. The crystal-to-detector distance was 270 mm and 100 images were recorded at 1° intervals with an exposure time of 5 s per image. The intensity data were processed with the program *DENZO* and merged with the program *SCALEPACK* (Otwinowski & Minor, 1997).

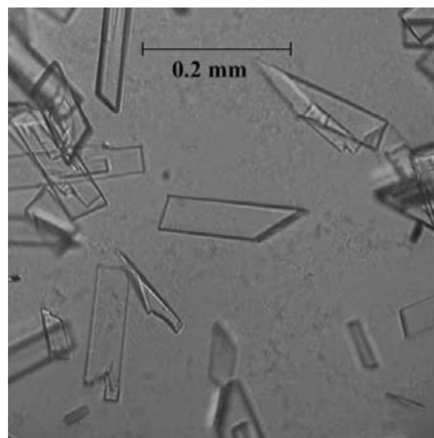

Figure 1
Typical crystals of endo-1,5-α-L-arabinanase ABN-TS from *B. thermodenitrificans* TS-3. The crystal dimensions were approximately 0.2 × 0.05 × 0.01 mm.

3. Results and discussion

The purification of the enzyme yielded 3 mg of protein from a 3 l culture and the purity was evaluated to be over 95% on Coomassie Brilliant-blue stained gels. In the initial crystallization trials at 277 K using 10 mg ml^{-1} protein solution, only amorphous precipitation or phase separation was observed. The trials were continued at 293 K using twofold-diluted protein solution. As a result, small crystals were successfully obtained from formulation No. 28 of Hampton Research Crystal Screen II containing sodium citrate as a precipitant. The final crystallization conditions involved mixing 1 µl ABN-TS (5 mg ml^{-1}) in 20 m*M* Tris–HCl buffer pH 7.5 with the same volume of reservoir solution containing 1 *M* sodium citrate pH 6.0. The crystals grew to maximum dimensions of approximately 0.2 × 0.05 × 0.01 mm in four weeks (Fig. 1).

Crystals mounted in thin-walled glass capillaries did not diffract to beyond 3.5 Å resolution and were easily damaged when exposed to Cu $K\alpha$ radiation at room temperature. We tested several reagents, including those listed in Table 1, as cryoprotectants at various concentrations. X-ray diffraction studies were performed with three to five crystals under each cryoprotectant condition. Of these, sucrose gave good results and the best diffraction was obtained at 28%(*w*/*v*) concentration. Glycerol was also functional, but only with cryogenic cooling at high concentrations. The effects of the glycerol, however, were not reproducible. With other reagents such as polyethylene glycols and other sugars, hardly any diffraction from the crystals was observed.

The crystals were loop-mounted in the established cryoprotectant solution and flash-cooled. A complete data set was collected to a resolution of 1.9 Å using synchrotron radiation. Detailed crystal parameters and data-collection statistics are shown in Table 2. Assuming one molecule of ABN-TS (35 kDa) in the asymmetric unit, the V_M value was calculated to be 2.0 Å^3 Da^{-1}, corresponding to a solvent content of 38.5%. This is within the values tabulated by Matthews (1968).

Molecular-replacement calculations were carried out with the program *AMoRe* (Navaza, 1994) implemented in the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994) using the structure of α-L-

Table 1
Effects of sucrose and glycerol as cryoprotectants.

Cryoprotectant (*w*/*v*)	Resolution (Å)	Spot shape
15% sucrose	—	None
28% sucrose	2.0	Good
38% sucrose	2.6	Long
10% glycerol	—	None
20% glycerol	—	None
30% glycerol	3.0	Long
40% glycerol	2.9	Good

Table 2
Summary of crystal parameters and data-collection statistics.

Values in parentheses are for the highest resolution shell (1.97–1.90 Å).

Resolution (Å)	50–1.9
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 40.3, b = 77.8, c = 89.7
R_{merge} (%)	8.6 (27.6)
$I/\sigma(I)$	10.5 (2.4)
No. reflections	170121
Unique reflections	22936
Redundancy	4.1
Completeness (%)	99.2 (96.5)
Mosaicity (°)	0.7

arabinanase 43A from *C. japonicus* (PDB code 1gyd) as a starting model. A clear peak was found with a correlation coefficient of 35.2 and an *R* factor of 47.3% within the resolution range 10–4 Å after translation-function calculations in space group $P2_12_12_1$. No unfavourable molecular contacts were observed in the crystal packing. Model building by manual fitting to the electron-density map using the program *O* (Jones *et al.*, 1991) is now under way.

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