Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Crystallization and preliminary crystallographic analysis of exo-α-1,5-L-arabinofuranosidase from *Streptomyces avermitilis* NBRC14893

Zui Fujimoto,[a] Hitomi Ichinose[b] and Satoshi Kaneko[b]*

[a]Protein Research Unit, National Institute of Agrobiological Sciences, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8602, Japan, and [b]Food Biotechnology Division, National Food Research Institute, 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan

Correspondence e-mail: sakaneko@affrc.go.jp

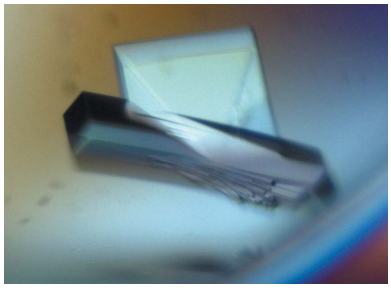
Received 27 May 2008
Accepted 16 September 2008

Exo-α-1,5-L-arabinofuranosidase from *Streptomyces avermitilis* NBRC14893 (SaAraf43A) is composed of a single-chain peptide containing a catalytic domain belonging to glycosyl hydrolase family 43 and a substrate-binding domain belonging to carbohydrate-binding module family 42. The enzyme catalyzes the hydrolysis of an α-linked L-arabinofuranosyl residue from hemicelluloses. SaAraf43A was crystallized at 293 K using the sitting-drop vapour-diffusion method. The crystals belonged to space group $P2_12_12_1$ and diffracted to a resolution of 2.2 Å.

1. Introduction

L-Arabinose is widely distributed as a component of hemicelluloses, arabinoxylan, arabinogalactan or arabinan (Carpita & Gibeaut, 1993). L-Arabinose is poorly absorbed through the wall of the animal intestinal tract (Osaki *et al.*, 2001) and is a functional sugar that inhibits intestinal sucrase and therefore the uptake of sucrose (Seri *et al.*, 1996). α-L-Arabinofuranosidase (EC 3.2.1.55) is an enzyme that catalyzes the hydrolysis of an α-linked L-arabinofuranosyl residue in hemicelluloses. The enzyme is classified into glycosyl hydrolase (GH) families 3, 43, 51, 54 and 62 according to the CAZY database (http://www.cazy.org/; Coutinho & Henrissat, 1999). *Streptomyces chartreusis* produces two types of arabinofuranosidases, which belong to the GH43 and GH51 families. The GH43 enzyme is known to be an exo-α-1,5-L-arabinofuranosidase that only catalyzes the hydrolysis of linear α-1,5-L-arabinan (Matsuo *et al.*, 2000). Although expression of this enzyme in *Escherichia coli* failed, we succeeded in cloning and expressing in *E. coli* a similar gene found in the *S. avermitilis* NBRC14893 genome database (http://avermitilis.ls.kitasato-u.ac.jp). This protein was found to have an amino-acid sequence similarity of 59.3% when compared with the GH43 enzyme from *S. chartreusis*. This recombinant enzyme is a single-chain protein with a molecular weight of 51 kDa. The protein shows a modular architecture that retains the GH43 catalytic domain and the carbohydrate-binding module family 42 (CBM42) domain. The enzyme only cleaves α-1,5-linked arabinofuranosyl linkages and releases arabinose in an exo-acting manner, thereby establishing that the protein is an exo-α-1,5-L-arabinofuranosidase (Ichinose *et al.*, 2008).

In addition to α-L-arabinofuranosidases, many different enzymes, including endo-α-L-arabinanases, exo-α-L-arabinanases, β-xylosidases, β-xylanases and galactan 1,3-β-galactosidases, belong to the GH43 family. The first crystal structure of a GH43-family member was determined for *Cellvibrio japonicus* α-L-arabinanase 43A (Arb43A; Nurizzo *et al.*, 2002). The catalytic domain consists of a five-bladed β-propeller fold. At present, eight independent crystal structures are available of GH43-family proteins. Both exo-type and endo-type enzymes that utilize α-1,5-L-arabinan as a substrate are



found in the GH43 family. The crystal structure of an endo-type enzyme, arabinan endo-1,5-α-L-arabinanase (Arb43A) from *Bacillus subtilis*, has been determined (Proctor *et al.*, 2005). No structural information is currently available for arabinofuranosidases; however, the crystallization of *B. subtilis* arabinoxylan arabinofuranohydrolase has been reported (Vandermarliere *et al.*, 2007). α-L-Arabinofuranosidase is expected to produce only L-arabinose efficiently and is therefore an important enzyme for both the food and the medicinal industries. The structures of these enzymes should provide important information describing the substrate-recognition mechanisms of the endo-arabinanases and exo-arabinosidases. Given this importance, crystallization trials of *S. avermitilis* exo-α-1,5-L-arabinofuranosidase (SaAraf43A) were undertaken.

SaAraf43A is composed of an N-terminal GH43 catalytic domain and a C-terminal CBM42 domain. Of enzymes that contain CBM42 domains, *Aspergillus kawachii* α-L-arabinofuranosidase, which belongs to the GH54 family, has been extensively studied and crystal structures have been determined (Miyanaga *et al.*, 2004, 2006). In this enzyme, the CBM42 domain is the substrate-recognition domain that specifically binds to L-arabinofuranose. Structural analyses of our enzyme will clarify the function of the CBM42 domain in SaAraf43A. The analyses will also elucidate the efficient catalytic mechanism of the multidomain glycosidase towards the recalcitrant substrate.

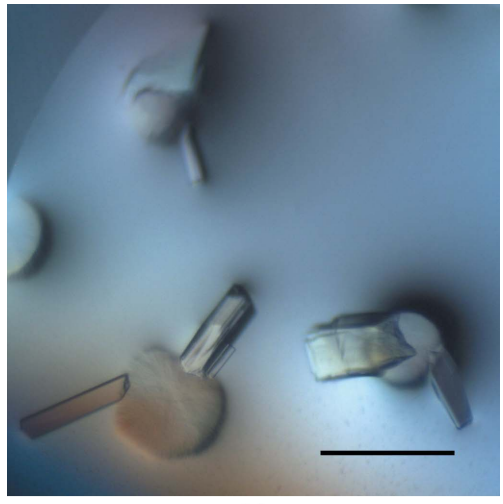
2. Materials and methods

SaAraf43A was expressed in *E. coli* as described elsewhere (Ichinose *et al.*, 2008). The transformants were grown in LB medium at 310 K until an absorbance at 600 nm of 0.2 was reached; protein expression was then induced with 1 m*M* isopropyl β-D-1-thiogalactopyranoside. Growth of the culture was continued for a further 24 h at 298 K. The cells were harvested and resuspended in 50 m*M* phosphate buffer pH 7.2, which was followed by sonication for 5 min. After centrifugation to remove insoluble material, the supernatant was loaded onto an Ni–NTA agarose (Qiagen GmbH, Hilden, Germany) column (5 × 50 mm). The eluted enzyme was identified by SDS–PAGE and the relevant fractions were pooled and dialyzed against deionized water.

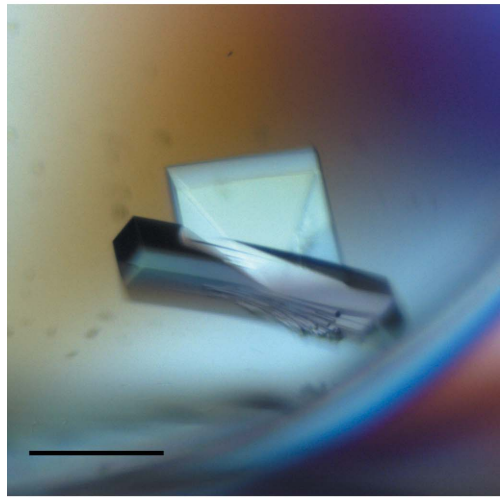
The protein solution was concentrated to 11 mg ml^{-1} (A_{280} = 2.2 U) by ultrafiltration using a YM-10 membrane (Millipore, Massachusetts, USA) and filtered through a 0.1 µm membrane (Millipore, Massachusetts, USA). The pooled solution without buffer reagents was used for crystallization trials. Sparse-matrix crystal screening was performed using several crystallization kits from Emerald Biostructures, Washington, USA (Wizard Screens I and II and Cryo I and II), Hampton Research, California, USA (Crystal Screen HT, Index HT and Salt HT) and Qiagen, Hilden, Germany (JCSG and PACT suites). Sitting-drop vapour-diffusion trials were set up in 96-well IntelliPlates (Art Robbins Instruments, California, USA) at 293 K containing 50 µl reservoir solution; each drop consisted of 0.3 µl protein solution and 0.3 µl reservoir solution. Within a few days, several quasi-crystal spheres were observed in various conditions with PEG 3350 or sodium citrate as the precipitating agent at pH 5–8 and rectangular crystals subsequently appeared from certain conditions (Fig. 1*a*). Crystallization conditions were optimized manually by refinement of the pH and the concentration of the precipitant solution using 96-well CrystalClear Strips sitting-drop plates (Douglas Instruments, Berkshire, England). Crystals with dimensions of 200 × 50 × 20 µm were consistently obtained using 100 µl reservoir solution with a drop consisting of 2–3 µl protein solution and 2 µl reservoir solution (Fig. 1*b*). Crystals of form 1 were obtained using a reservoir solution composed of 0.8 *M* sodium citrate, 0.2 *M* sodium chloride and 0.1 *M* Tris pH 7.0. Crystals of form 2 were obtained using a reservoir solution composed of 18%(*w*/*v*) PEG 3350, 0.1 *M* bis-tris propane pH 7.0 and a salt solution of either 0.1 *M* sodium malonate, 0.2 *M* sodium bromide or 0.2 *M* sodium citrate.

Crystals from both conditions could be directly flash-frozen in a nitrogen stream using a 0.3 mm nylon loop (Hampton Research, California, USA) without the addition of cryoprotectant. However, the mosaicity of the data was extremely high. The mosaicity was improved by changing the reservoir solution for crystal form 1 to approximately 5%(*v*/*v*) glycerol before the crystal was flash-frozen.

Native diffraction data for form 1 crystals were collected on beamline BL-6A of the Photon Factory (PF), High Energy Accelerator Research Organization, Tsukuba, Japan. An approximately equal volume of reservoir solution containing 10% glycerol was added to the crystal drop; the crystal was scooped up in a nylon loop after 5 min and flash-frozen in a nitrogen stream at 95 K. Diffraction data were collected with 15 s exposures and 1° oscillations over a total of 200° at a wavelength of 0.978 Å with a Quantum Q4 CCD detector (ADSC, California, USA). Data were integrated and scaled



(*a*)



(*b*)

Figure 1
Typical crystals of SaAraf43A of (*a*) form 2 [reservoir solution 19%(*w*/*v*) PEG 3350, 0.2 *M* sodium bromide and 0.1 *M* bis-tris propane pH 7.3] and (*b*) form 1 (reservoir solution 0.8 *M* sodium citrate, 0.2 *M* sodium chloride and 0.1 *M* Tris pH 7.0). The scale bars represent 200 µm.

Table 1
Data-collection statistics of the SaAraf43A crystal obtained using sodium citrate as the precipitating agent.

Values in parentheses are for the highest resolution shell.

Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 41.0, b = 91.5, c = 135.5
Wavelength (Å)	0.978
Resolution range (Å)	50.0–2.2 (2.28–2.20)
R_{merge}†	0.081 (0.285)
Completeness (%)	97.5 (97.5)
Multiplicity	7.0 (7.2)
Average $I/\sigma(I)$	44.4 (3.1)
Unique reflections	26313 (2601)
Observed reflections	182571

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the weighted average intensity for all observations i of reflection hkl.

using the programs *DENZO* and *SCALEPACK* from the *HKL*-2000 program suite (Otwinowski & Minor, 1997).

3. Results and discussion

Using synchrotron radiation, crystals of form 1 diffracted to a maximum resolution of 2.2 Å and belonged to space group $P2_12_12_1$, with unit-cell parameters a = 41.0, b = 91.5, c = 135.5 Å. The processing statistics of the data collected are summarized in Table 1. Assuming the presence of one molecule in the asymmetric unit, the solvent content of the crystals was 43.7%, corresponding to a Matthews coefficient of 2.19 Å^3 Da^{-1} (Matthews, 1968).

Using the laboratory X-ray diffractometer, crystals of form 2 also diffracted to 2.2 Å resolution and belonged to space group $P2_12_12_1$, with unit-cell parameters a = 40.7, b = 95.0, c = 134.8 Å. These crystals have the same space group and similar unit-cell parameters to crystal form 1. Consequently, the crystal packing of the crystals from the two crystallization conditions may be similar. Crystal form 2 was not used in further structural analysis because of its high mosaicity.

The molecular-replacement method was employed using the structures of the catalytic domains of several GH43-family members (maximum 20% amino-acid sequence identity for xylan β-1,4-xylosidase from *B. halodurans* C-125; PDB code 1yrz) as search models. However, this approach did not give sufficient phases for structure determination. We are currently preparing a selenomethionine-substituted SaAraf43A crystal for phase determination using the MAD technique.

The use of synchrotron radiation for this work was approved by the Photon Factory Advisory Committee (proposal No. 2007G659) and we would like to thank the scientists and staff at the Photon Factory. This work was financially supported in part by a Grant-in-Aid (Development of Biomass Utilization Technologies for Revitalizing Rural Areas) from the Ministry of Agriculture, Forestry and Fisheries of Japan.

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