Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Expression, purification, crystallization and preliminary X-ray analysis of α-L-arabinofuranosidase B from *Aspergillus kawachii*

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Received 13 February 2004
Accepted 26 April 2004

α-L-Arabinofuranosidase (EC 3.2.1.55) is one of the hemicellulases that cleave the glycosidic bonds between L-arabinofuranoside side chains and various oligosaccharides. In this study, the first crystallization and preliminary X-ray analysis of α-L-arabinofuranosidase B from *Aspergillus kawachii* IFO4308 (AkAbfB), a family 54 glycoside hydrolase, is described. Recombinant AkAbfB was expressed in *Escherichia coli* and *Pichia pastoris*. The native crystals of recombinant AkAbfB produced by *P. pastoris* belong to the orthorhombic space group $P2_12_12_1$ (unit-cell parameters a = 39.5, b = 98.2, c = 144.0 Å) and diffracted X-rays to a resolution of 1.82 Å.

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1. Introduction

The plant cell wall is a composite structure consisting mainly of celluloses, hemicelluloses and lignins. Arabinoxylan, a major component of hemicellulose, consists of a 1,4-β-linked xylose backbone and generally contains heterogenous substituents such as L-arabinose, *O*-acetyl ferulic acid, *p*-coumaric acid and 4-*O*-methylglucuronic acid (Puls & Schuseil, 1993). The arabinoxylan-degrading enzyme system involves endo-β-1,4-xylanases (EC 3.2.1.8), β-xylosidases (EC 3.2.1.37) and side-chain-debranching enzymes such as α-L-arabinofuranosidases (EC 3.2.1.55), α-glucuronidases (EC 3.2.1.139), acetyl xylan esterases (EC 3.1.1.72) and feruloyl esterases (EC 3.1.1.73). α-L-Arabinofuranosidases catalyze the hydrolysis of the α-1,2- and α-1,3-L-arabinofuranosidic bonds in L-arabinose-containing hemicelluloses such as arabinoxylan and L-arabinan. The α-L-arabinofuranosidases belong to five distinct glycoside hydrolase families, 3, 43, 51, 54 and 62, based on sequence similarity (Carbohydrate-Active Enzymes server; http://afmb.cnrs-mrs.fr/CAZY; Coutinho & Henrissat, 1999). The crystal structures of the family 3 β-D-glucan glucohydrolase from *Hordeum vulgare* (Varghese *et al.*, 1999), the family 43 α-L-arabinanase from *Cellvibrio japonicus* (Nurizzo *et al.*, 2002) and the family 51 α-L-arabinofuranosidase from *Geobacillus stearothermophilus* T-6 (Hovel *et al.*, 2003) have been reported. *Aspergillus kawachii* IFO4308 produces two different α-L-arabino furanosidases, arabinofuranosidase A and arabinofuranosidase B (AkAbfB), which are assigned to glycoside hydrolase families 51 and 54, respectively (Koseki *et al.*, 2003). In this study, we describe the expression, purification, crystallization, and preliminary X-ray analysis of AkAbfB. This is the first report of the crystallization of a family 54 glycoside hydrolase.

2. Materials and methods

2.1. Expression and purification of recombinant AkAbfB

The AkAbfB gene was cloned into the pGEM-T Easy vector as described previously (Koseki *et al.*, 2003). Recombinant AkAbfB protein without the native signal sequence region (Gly19–Ser499) was expressed in *Escherichia coli* BL21 (DE3) CodonPlus RIL (Stratagene, La Jolla, CA, USA) using pET17b vector (Novagen, Madison, WI., USA) according to the manufacturer's instruction manuals. The crude extract was heated to 328 K for 30 min to remove *E. coli* proteins and then purified using a DEAE-Sepharose (Amersham Biosciences, Piscataway, NJ, USA) column.

For expression of the gene in *Pichia pastoris*, the following primer pair was designed to amplify the open reading frame (ORF) without the native signal sequence region (Gly19–Ser499): 5′-CC **ATC GAT** GGG CCCC TGT GAC ATC TAC-3′ (sense; *Cla*I site in bold) and 5′-GC **TCT AGA** GCT ACC GTG AC-3′ (antisense; *Xba*I site in bold). The polymerase chain reaction (PCR) product was cloned into the *Cla*I/*Xba*I-digested pPICZαC vector (Invitrogen, Carlsbad, CA, USA). The AkAbfB-pPICZαC plasmid DNA was linearized with the restriction enzyme *Sac*I to allow integration of the vector DNA into the chromosomal DNA of *P. pastoris* GS115 (Invitrogen). AkAbfB-producing *P. pastoris* cells

were grown in 5 ml of buffered complex medium containing glycerol (BMGH, Invitrogen) at 301 K. Induction was performed by transferring the cells to 1 l buffered complex medium containing methanol (BMMH, Invitrogen) at 299 K. Every 24 h, methanol was added to a final concentration of 0.5%. The expression was allowed to proceed for 72 h. An AkAbfB-containing supernatant was obtained by centrifugation (10 000*g* for 10 min). The supernatant was passed through a 0.22 µm filter and then diluted three times with 20 m*M* sodium acetate buffer pH 5.5. After dilution, this solution was applied onto a DEAE-Sepharose (Amersham Biosciences) column equilibrated with 20 m*M* sodium acetate buffer pH 5.5. After washing with the equilibration buffer to remove unbound contaminants, a linear gradient of 0–500 m*M* sodium chloride was introduced. The active fractions were desalted and concentrated with a Centriprep (Millipore, Billerica, MA, USA).

2.2. Analysis of purified AkAfB enzymes expressed in *E. coli* and *P. pastoris*

AkAbfB activity was measured using 2.5 m*M* *p*-nitrophenyl α-L-arabinofuranoside as the substrate in 100 m*M* sodium acetate buffer pH 4.5 at 310 K. The reaction was stopped by the addition of 0.3 *M* Na_2CO_3 and the liberation of *p*-nitrophenol was measured spectrophotometrically at 405 nm.

2.3. Crystallization, diffraction data collection, processing and initial phasing

The crystallization conditions were initially screened by the sitting-drop vapour-diffusion method using the sparse-matrix kits Crystal Screens I and II (Hampton Research, Laguna Niguel, CA, USA) at 298 K. 1.5 µl of a 15 mg ml^{-1} solution of *P. pastoris*-expressed AkAbfB in 10 m*M* sodium acetate pH 4.5 was mixed with an equal volume of the reservoir solution. Needle-shaped crystals were initially obtained from solution No. 20 of Crystal Screen I. The best crystallization conditions were obtained with a reservoir solution comprising 30% PEG 6000 and 0.1 *M* MES–NaOH buffer pH 6.0. The Xe derivative was prepared by introducing Xe gas under pressure into a native crystal in a Xenon Chamber (Hampton Research) at 0.12 MPa for 60 min (Soltis *et al.*, 1997). Prior to data collection, the crystals were transferred into a cryoprotectant solution comprising 25% PEG 400 in the reservoir solution and then subjected to flash-cooling. Data were collected using a charge-coupled device (CCD) camera on the AR-NW12 station at the Photon Factory, High Energy Accelerator Research Organization (KEK), Tsukuba, Japan at 100 K. Diffraction images were indexed, integrated and scaled using the *HKL*2000 program suite (Otwinowski & Minor, 1997). Isomorphous difference Patterson maps were calculated using the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994). Heavy-atom parameters were calculated using *MLPHARE* (Otwinowski, 1991).

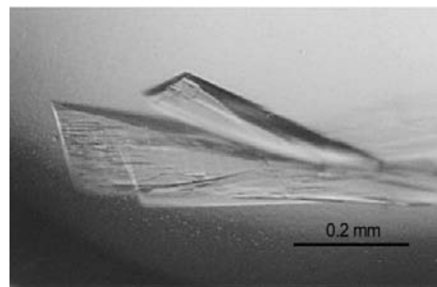

Figure 1
A crystal of AkAbfB.

Table 1
Data-collection statistics.

Data statistics for the highest resolution shells are given in parentheses.

Data set	Native	Xe
Wavelength (Å)	1.1000	1.0500
Unit-cell parameters		
a (Å)	39.5	39.5
b (Å)	98.2	98.8
c (Å)	144.0	144.3
Resolution (Å)	20.0–1.82 (1.89–1.82)	20.0–2.00 (2.07–2.00)
Total reflections	235291	159186
Unique reflections	51344	39359
Completeness (%)	99.3 (99.6)	99.3 (99.1)
R_{merge}† (%)	0.049 (0.123)	0.047 (0.079)
Mean $\langle I/\sigma(I)\rangle$	16.2 (9.2)	16.9 (14.2)
Phasing power, acentric/centric		0.68/0.51
R_{cullis}, acentric/centric		0.93/0.86
Figure of merit		0.21
No. heavy-atom sites		1

† $R_{merge} = \sum_h \sum_i |I(h,i) - \langle I(h)\rangle| / \sum_h \sum_i I(h,i)$, where $I(h,i)$ is the intensity of the *i*th measurement of reflection *h* and $\langle I(h)\rangle$ is the mean value of $I(h,i)$ for all *i* measurements.

3. Results and discussion

E. coli cells did not produce sufficient AkAbfB enzyme for crystallization: the amount of purified protein obtained from 3 l of culture was only 0.3 mg. On the other hand, *P. pastoris* cells produced a sufficient amount of the enzyme: 20 mg from 1 l of culture medium. The specific enzymatic activities of the purified samples produced from *E. coli* and *P. pastoris* were 18.7 and 29.3 µ*M* min^{-1} mg^{-1} and the samples migrated as single bands on SDS–PAGE corresponding to approximately 60 and 64 kDa, respectively. The band of the sample from *P. pastoris* was stained red with the periodic acid–Schiff base (PAS) stain method (Zacharius *et al.*, 1969), which detects protein glycosylation. AkAbfB contains two potential N-glycosylation sites. These results indicate that the recombinant AkAbfB produced by *P. pastoris* contains glycoside chains. The relative molecular size determined by gel-filtration chromatography showed that the recombinant protein eluted as a monomer of 78 kDa. The apparent molecular size may be affected by the shape of AkAbfB protein in the native state. The recombinant AkAbfB produced by *P. pastoris* was used for subsequent crystallization experiments.

The optimum conditions resulted in the formation of thin plate-like crystals of approximately 0.5 × 0.2 × 0.05 mm in size (Fig. 1). The crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters *a* = 39.5, *b* = 98.2, *c* = 144.0 Å. The native crystals diffracted to 1.82 Å resolution with a synchrotron X-ray source (Fig. 2). The statistics for the native and derivative data are presented in Table 1. Assuming one monomer per asymmetric unit, the calculated V_M value (Matthews,

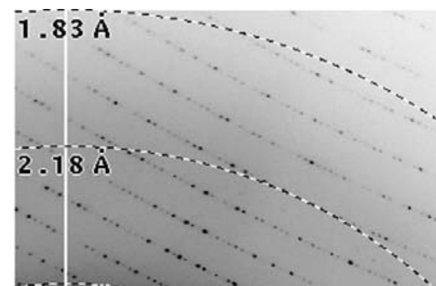

Figure 2
X-ray diffraction pattern of an AkAbfB crystal at high resolution.

1968) and solvent content are 2.74 Å^3 Da^{-1} and 55.1%, respectively. Inspection of a Harker section of the isomorphous difference Patterson synthesis map with the Xe derivative revealed the position of one Xe atom with high occupancy (data not shown). Further derivatives will be prepared in order to solve the three-dimensional structure of AkAbfB.

We wish to thank Drs N. Matsugaki, M. Suzuki, N. Igarashi and S. Wakatsuki for the data collection at the Photon Factory, KEK, Japan, the staff of SPring-8 for preliminary data collection and Dr M. Nishimoto for the helpful suggestion regarding the PAS stain method. This work was supported by the Japan Society for the Promotion of Science (JSPS), a Grant-in-Aid for Scientific Research (15780067) to SF, the National Project on Protein Structural and Functional Analysis and the SBSP (Structural Biology Sakabe Project), FAIS (Foundation for Advancement of International Science).

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