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

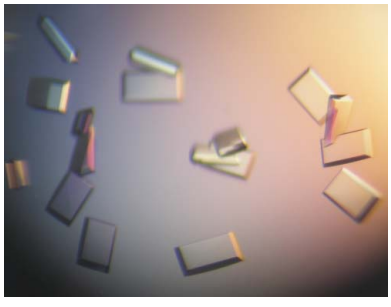
Crystallization and preliminary X-ray study of native and selenomethionyl β-1,4-mannanase AaManA from *Alicyclobacillus acidocaldarius* Tc-12-31

Yueling Zhang,[a,b] Feng Gao,[a] Yanfen Xue,[a] Yan Zeng,[a] Hao Peng,[a] Jianxun Qi[b,c] and Yanhe Ma[a]*

[a]State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, People's Republic of China, [b]Graduate School, Chinese Academy of Sciences, Beijing 100049, People's Republic of China, and [c]Institute of Physics, Chinese Academy of Sciences, Beijing 100080, People's Republic of China

Correspondence e-mail: mayanhe@im.ac.cn

Received 29 November 2007
Accepted 25 January 2008



AaManA, a β-1,4-mannanase from the thermoacidophile *Alicyclobacillus acidocaldarius* Tc-12-31, was expressed in *Escherichia coli* and purified in a form suitable for X-ray crystallographic analysis. Crystals were obtained using the hanging-drop vapour-diffusion method at 291 K using ammonium dihydrogen phosphate as a precipitant. Data were collected from native mannanase and from a selenomethionyl derivative to 1.90 and 1.99 Å, respectively, at 100 K. The native crystal belongs to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 44.34, b = 75.55, c = 88.02 Å. The derivative crystal belonged to the same space group as native AaManA, with unit-cell parameters a = 44.55, b = 75.70, c = 92.66 Å.

1. Introduction

Endo-β-1,4-mannanases (EC 3.2.1.78), which specifically cleave the β-1,4-mannosidic linkage in the backbone of mannans, galactomannans and glucomannans, have drawn much interest because of their important roles in hemicellulose hydrolysis and their potential application in the feed/food and pulp/paper industries (Dekker & Richards, 1976; Kansoh & Nagieb, 2004; Kurakake *et al.*, 2006). According to the continuously updated glycoside hydrolase family webserver at http://afmb.cnrs-mrs.fr/CAZY/fam/acc_GH.html, more than 80 sequences of mannanase catalytic domains have been obtained, mainly from bacteria. These mannanases are classified into GH families 5 and 26 based on sequence similarity (Henrissat, 1991). Several mannanases from mesophilic, thermophilic and alkaliphilic bacteria have been purified and characterized (Stoll *et al.*, 1999; Parker *et al.*, 2001; Ma *et al.*, 2004). Recently, a mannanase gene (*AamanA*) from the thermoacidophilic bacterium *Alicyclobacillus acidocaldarius* has been cloned and characterized (Genbank accession No. DQ680160; Zhang *et al.*, manuscript in preparation).

The *AamanA* gene from *A. acidocaldarius* consists of a 960 bp open reading frame (ORF) which encodes a protein of 320 amino acids. It is a particularly interesting mannanase in that it shows no sequence similarity to any known mannanases on searching the NCBI protein database using the *PSI-BLAST* algorithm (Altschul *et al.*, 1997). To date, seven mannanases have been structurally characterized: five from three subfamilies of family 5, namely subfamilies 7 (Hilge *et al.*, 1998; Akita *et al.*, 2004), 8 (Bourgault *et al.*, 2005; Sabini *et al.*, 2000) and 10 (Larsson *et al.*, 2006), and two from family 26 (Le Nours *et al.*, 2005; Hogg *et al.*, 2001). These enzymes share a common $(\alpha/\beta)_8$-barrel fold, two conserved active-site residues (two glutamate residues) and a retaining cleavage mechanism; they are therefore suggested to have evolved from the same ancestor (Henrissat *et al.*, 1995). However, owing to the lack of sequence similarity, these studies shed little light on the structure and characteristics of *A. acidocaldarius* mannanase.

Here, we report the purification, crystallization and preliminary crystallographic analysis of recombinant native AaManA and of its selenomethionine derivative. The three-dimensional structure, which is being determined using single-wavelength anomalous diffraction (SAD), will provide an important insight into the mechanism of enzymatic reaction of the *A. acidocaldarius* mannanase.

2. Materials and methods

2.1. Cloning, expression and purification

Using the *A. acidocaldarius* genome DNA as a template, the full sequence of *AamanA* was amplified using the primers 5′-GTGCG-CTAGCATGGGACG-3′ and 5′-GCGAAGCTTTCATCGATTTG-3′, which introduced an *Nhe*I restriction site at the 5′ end and a *Hin*dIII restriction site at the 3′ end of the *AamanA* gene. The doubly digested products were cloned into *Nhe*I- and *Hin*dIII-digested pET28a (Novagen Co., USA) to construct pETManA and then transformed into *Escherichia coli* BL21 (DE3) (Novagen Co., USA). The pETManA-containing cells were grown in 1 l LB plus kanamycin (50 µg ml^{-1}) at 310 K to an OD_{600} of 0.6. Expression of recombinant protein was induced by the addition of IPTG (0.8 µ*M*) and growth of the culture continued for 5 h at 310 K. The induced cultures were centrifuged (6000 rev min^{-1}, 10 min) and the cell pellets were resuspended in 20 ml buffer *A* (20 m*M* Tris–HCl pH 7.9, 0.5 *M* NaCl, 10 m*M* imidazole) and disrupted by sonication. Cell debris was removed by centrifugation (15 000 rev min^{-1}, 10 min). The supernatant was loaded onto an Ni-IDA His-Bind Superflow column (Novagen Co., USA), rinsed with 10 ml buffer *B* (20 m*M* Tris–HCl pH 7.9, 0.5 *M* NaCl, 60 m*M* imidazole) and then eluted with 4 ml buffer *C* (20 m*M* Tris–HCl pH 7.9, 0.5 *M* NaCl, 500 m*M* imidazole). Subsequent purification was performed on FPLC by gel filtration (20 m*M* Tris–HCl pH 7.9) on Superdex75 10/300GL columns (GE Healthcare, USA). SDS–PAGE was performed with 10%(*w*/*v*) polyacrylamide gels and stained with Coomassie Brilliant Blue R-250. Selenomethionyl AaManA (SeMet-AaManA) was produced by blocking the methionine-biosynthetic pathway in *E. coli* BL21 (DE3) as described by Van Duyne *et al.* (1993). After 15 min incubation, expression was induced using 0.8 m*M* IPTG and the cells were grown as described for native AaManA. The SeMet-AaManA was purified using the same protocol as for the native protein, except that the purification was carried out in the presence of 10 m*M* DTT. The purified AaManA and SeMet-AaManA solutions were desalted using a HiTrap desalting column (GE Healthcare, USA) against 5 m*M* Tris–HCl pH 7.9 buffer. The protein concentration was measured using a protein assay kit (Bio-Rad, USA) with IgG as a standard.

2.2. Crystallization

Initial crystallization screening was carried out using Crystal Screens I and II from Hampton Research at 291 K with the hanging-drop method. The protein concentration was between 5 and 30 mg ml^{-1}. 5 m*M* Tris–HCl pH 7.9 buffer was used to dissolve and dilute the proteins for crystallization. Drops consisting of 2 µl protein solution mixed with an equal volume of reservoir solution were equilibrated against 200 µl reservoir solution over a period of several days. The crystallization conditions were optimized by varying the pH between 4.6 and 8.5 and the ammonium dihydrogen phosphate (Sigma Chemical Co., St Louis, Missouri, USA) concentration between 0.1 and 1.0 *M*. Crystallization of SeMet-AaManA was performed under the same conditions as used for native AaManA.

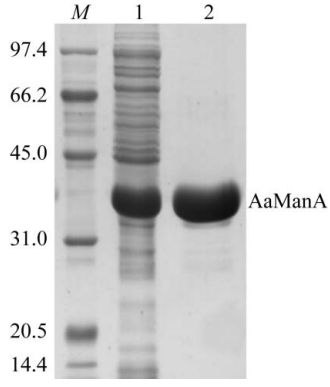


Figure 1
SDS–PAGE of purified AaManA. Lane *M*, low-molecular-weight protein markers (kDa); lane 1, soluble proteins from *E. coli* strain BL21(DE3)/pETMan; lane 2, purified native AaManA.

Table 1
Diffraction data statistics.

Values in parentheses are for the highest resolution shell.

Data set	Native	SeMet
Wavelength (Å)	1.5418	0.9794
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters (Å)	a = 44.34, b = 75.55, c = 88.02	a = 44.55, b = 75.70, c = 92.66
Resolution (Å)	35.07–1.90 (1.97–1.90)	58.82–1.99 (2.07–1.99)
Unique reflections	23386	10598
Redundancy	6.92 (5.80)	6.5 (5.6)
Completeness (%)	97.3 (89.4)	97.1 (96.8)
R_{merge} (%)†	5.5 (14.4)	9.6 (37.9)
$I/\sigma(I)$	20.8 (10.2)	17.4 (3.0)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the ith measurement of reflection hkl and $\langle I(hkl)\rangle$ is the mean intensity of all symmetry-related reflections.

2.3. X-ray data collection and processing

For X-ray diffraction measurements, the AaManA crystals were soaked in a cryoprotectant consisting of the reservoir solution with 15%(*v*/*v*) glycerol for 10 min. The crystals were mounted in a cryo-loop and flash-frozen in a stream of gaseous nitrogen (Parkin & Hope, 1998). Diffraction data from the native AaManA crystal were collected in-house on a Rigaku MicroMax007 rotating copper-anode X-ray generator operated at 40 kV and 20 mA (Cu $K\alpha$; λ = 1.5418 Å), which was equipped with Osmic mirrors and an R-AXIS VII^{++} image-plate detector. Single-wavelength anomalous diffraction (SAD) data were collected for SeMet-AaManA at peak wavelength (0.9794 Å) using synchrotron radiation on BSRF beamline 3W1A equipped with a CCD detector. Native and SeMet-AaManA data were indexed, integrated and scaled using *DENZO* and *SCALEPACK* as implemented in *HKL*-2000 (Otwinowski & Minor, 1997).

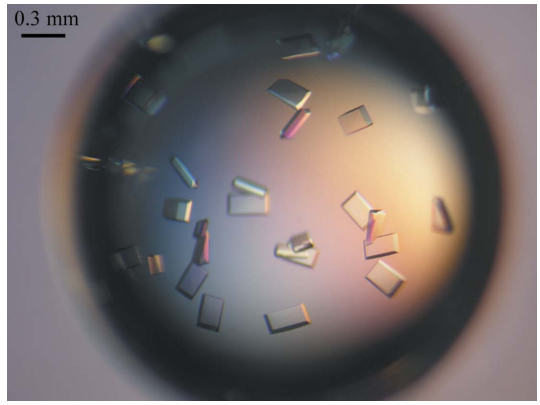


Figure 2
Typical crystals of native AaManA.

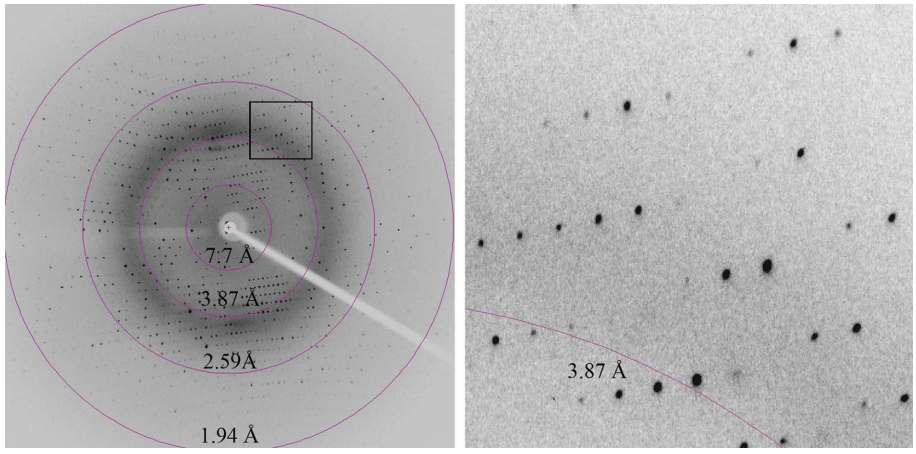


Figure 3
A typical diffraction pattern of a native AaManA crystal. The image on the right is an enlargement of the area framed on the left.

3. Results and discussion

The AaManA gene was expressed in *E. coli* BL21 (DE3) (Fig. 1, lane 1). The recombinant AaManA was purified to apparent homogeneity and its molecular weight was estimated to be 38 kDa on the basis of relative mobility on the SDS–PAGE gel (Fig. 1, lane 2), corresponding to the molecular weight of recombinant AaManA with a 20-amino-acid His tag.

Crystal Screens I and II from Hampton Research were employed for initial crystallization screening using the hanging-drop method. After 24 h, AaManA crystals formed in drops with solutions Nos. 3, 11 and 48 from Crystal Screen I. Interestingly, solutions Nos. 3, 11 and 48 were the only three conditions which contained ammonium dihydrogen phosphate, indicating that ammonium dihydrogen phosphate is particularly useful in the crystallization of AaManA. The best crystals were obtained from solution No. 11, which contained 1.0 *M* ammonium dihydrogen phosphate and 0.1 *M* sodium citrate pH 5.6. Unfortunately, the crystals could not be reproduced using this condition. Good reproducibility was obtained from solutions Nos. 3 and 48, which consisted of 0.4 *M* ammonium dihydrogen phosphate only and of 2.0 *M* ammonium dihydrogen phosphate with 0.1 *M* Tris–HCl pH 8.5, respectively. However, the crystals were of a poor appearance and did not diffract well. By varying the buffer pH and ammonium dihydrogen phosphate concentration, we found that the combination of pH and ammonium dihydrogen phosphate concentration is important for the reproducibility and quality of the crystals. The best crystals were obtained with a reservoir solution containing 0.2 *M* ammonium dihydrogen phosphate and 0.1 *M* sodium citrate pH 4.6 and with a protein solution concentration of 12 mg ml^{-1} (Fig. 2). The brick-shaped crystals grew with good reproducibility using this condition and reached dimensions of 0.1 × 0.15 × 0.3 mm in 24 h. The crystals are stable at 291 K for at least one month. A complete native set was collected to 1.9 Å (Fig. 3). A summary of the data-processing statistics is shown in Table 1. The crystal belongs to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 44.34, b = 75.55, c = 88.02 Å and one molecule per asymmetric unit. The Matthews number (V_M; Matthews, 1968) is about 1.91 Å^3 Da^{-1}, corresponding to a solvent content of 36%.

On comparison with proteins of known tertiary structure using the *Phyre* system (Kelley *et al.*, 2000; http://www.sbg.bio.ic.ac.uk/phyre), AaManA had the highest sequence similarity to human β-glucuronidase (PDB code 1bhg; 13% identity). We attempted to use molecular-replacement methods for phase determination, but failed. Therefore, SeMet-labelled enzyme was produced for phasing. Crystals of SeMet-AaManA of similar size and appearance as those of native AaManA were grown under the same conditions as used for native AaManA. The SeMet-AaManA crystal belonged to the same space group as the native AaManA crystal, with unit-cell parameters a = 44.55, b = 75.70, c = 92.66 Å. The significant c dimension difference indicates a lack of isomorphism between the SeMet-AaManA and native AaManA structures. Therefore, the SAD method was chosen for phase calculation. 12 selenium sites were located using the program *SOLVE* (Terwilliger & Berendzen, 1999). Refinement of the heavy-atom parameters and phase calculation were performed using the program *SOLVE*. At present, about half of the residues have been automatically built using the program *RESOLVE* (Terwilliger, 2003). Manual rebuilding of the other residues is now in progress.

We are grateful to Professor Jijie Chai and graduate student Linjie Chen from the National Institute of Biological Sciences (Beijing) for their assistance in the expression of SeMet-AaManA. We thank Professor Peng Liu and Yuhui Dong from the BSRF Laboratory for their help during synchrotron-radiation data collection. This work was supported by grants from the Chinese Academy of Sciences (Knowledge Innovation Program; KSCX2-SW-33), from NSFC (30621005) and from the Ministry of Sciences and Technology of China (973 program; 2004CB719605 and 2007CB707801).

References

Akita, M., Takeda, N., Hirasawa, K., Sakai, H., Kawamoto, M., Yamamoto, M., Grant, W. D., Hatada, Y., Ito, S. & Horikoshi, K. (2004). *Acta Cryst.* D**60**, 1490–1492.
Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). *Nucleic Acids Res.* **25**, 3389–3402.
Bourgault, R., Oakley, A. J., Bewley, J. D. & Wilce, M. C. (2005). *Protein Sci.* **14**, 1233–1241.
Dekker, R. F. & Richards, G. N. (1976). *Adv. Carbohydr. Chem. Biochem.* **32**, 277–352.
Henrissat, B. (1991). *Biochem. J.* **280**, 309–316.
Henrissat, B., Callebaut, I., Fabrega, S., Lehn, P., Mornon, J. P. & Davies, G. (1995). *Proc. Natl Acad. Sci. USA*, **92**, 7090–7094.
Hilge, M., Gloor, S. M., Rypniewski, W., Sauer, O., Heightman, T. D., Zimmermann, W., Winterhalter, K. & Piontek, K. (1998). *Structure*, **6**, 1433–1444.
Hogg, D., Woo, E. J., Bolam, D. N., McKie, V. A., Gilbert, H. J. & Pickersgill, R. W. (2001). *J. Biol. Chem.* **276**, 31186–31192.
Kansoh, A. L. & Nagieb, Z. A. (2004). *Antonie van Leeuwenhoek*, **85**, 103–114.
Kelley, L. A., MacCallum, R. M. & Sternberg, M. J. (2000). *J. Mol. Biol.* **299**, 499–520.

Kurakake, M., Sumida, T., Masuda, D., Oonishi, S. & Komaki, T. (2006). *J. Agric. Food Chem.* **54**, 7885–7889.
Larsson, A. M., Anderson, L., Xu, B., Munoz, I. G., Uson, I., Janson, J.-C., Stalbrand, H. & Stahlberg, J. (2006). *J. Mol. Biol.* **357**, 1500–1510.
Le Nours, J., Anderson, L., Stoll, D., Stalbrand, H. & Lo Leggio, L. (2005). *Biochemistry*, **44**, 12700–12708.
Ma, Y., Xue, Y., Dou, Y., Xu, Z., Tao, W. & Zhou, P. (2004). *Extremophiles*, **8**, 447–454.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Parker, K. N., Chhabra, S. R., Lam, D., Callen, W., Duffaud, G. D., Snead, M. A., Short, J. M., Mathur, E. J. & Kelly, R. M. (2001). *Biotechnol. Bioeng.* **75**, 322–333.
Parkin, S. & Hope, H. (1998). *J. Appl. Cryst.* **31**, 945–953.
Sabini, E., Schubert, H., Murshudov, G., Wilson, K. S., Siika-Aho, M. & Penttilä, M. (2000). *Acta Cryst.* D**56**, 3–13.
Stoll, D., Stalbrand, H. & Warren, R. A. (1999). *Appl. Environ. Microbiol.* **65**, 2598–2605.
Terwilliger, T. C. (2003). *Acta Cryst.* D**59**, 38–44.
Terwilliger, T. C. & Berendzen, J. (1999). *Acta Cryst.* D**55**, 849–861.
Van Duyne, G. D., Standaert, R. F., Karplus, P. A., Schreiber, S. L. & Clardy, J. (1993). *J. Mol. Biol.* **229**, 105–124.