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

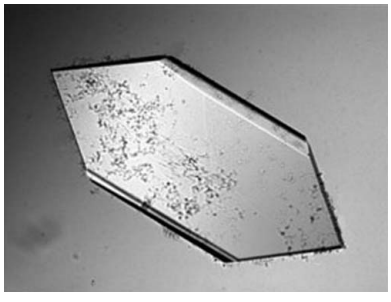
Crystallization and preliminary crystallographic analysis of the family GH78 α-L-rhamnosidase RhaB from *Bacillus* sp. GL1

Zhongli Cui,[a,b] Yukie Maruyama,[a] Bunzo Mikami,[c] Wataru Hashimoto[a] and Kousaku Murata[a]*

[a]Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Uji, Kyoto 611-0011, Japan, [b]Department of Microbiology, College of Life Sciences, Nanjing Agricultural University, 210095 Nanjing, People's Republic of China, and [c]Division of Agronomy and Horticultural Science, Graduate School of Agriculture, Kyoto University, Uji, Kyoto 611-0011, Japan

Correspondence e-mail: kmurata@kais.kyoto-u.ac.jp

Received 1 April 2006
Accepted 23 May 2006



α-L-Rhamnosidases play important roles in the metabolism of plant cell walls, glycosides and bacterial biofilms. This enzyme is also used industrially for debittering citrus fruits by releasing rhamnose from the plant flavonoid naringin. *Bacillus* sp. GL1 α-L-rhamnosidase (RhaB) is a member of glycoside hydrolase (GH) family 78. Native and selenomethionine-derivative enzymes were crystallized at 293 K by hanging-drop vapour diffusion with polyethylene glycol 8000 as a precipitant. This is the first report of the crystallization of a family GH78 enzyme.

1. Introduction

L-Rhamnose is widely distributed in plants and bacteria as a component of the cell wall, glycosides, biofilms and glycolipids, which play physiologically important roles in organisms (Bader *et al.*, 1998; Giavasis *et al.*, 2000). α-L-Rhamnosidases (EC 3.2.1.40) catalyze a hydrolytic reaction, the release of rhamnose from the substrate, and thus are key enzymes in the modification and decomposition of rhamnose-related compounds. A large number of α-L-rhamnosidases have been identified in eukaryotic and prokaryotic organisms (Turecek & Pittner, 1986; Jang & Kim, 1996; Zvelov *et al.*, 2000; Miake *et al.*, 2000; Manzanares *et al.*, 2001; Scaroni *et al.*, 2002). These α-L-rhamnosidases mainly act on substrates from plant or artificial compounds. Those acting on bacterial polysaccharides have, however, been little studied.

Gellan is a bacterial exopolysaccharide with a tetrasaccharide repeating unit (→3)-β-D-Glc*p*-(1→4)-β-D-GlcA*p*-(1→4)-β-D-Glc*p*-(1→4)-α-L-Rha*p*-(1→) produced by *Sphingmonas paucimobilis* (Pollock, 1993). This polysaccharide has broad application in the food industry owing to its high viscosity in the presence of divalent cations. *Bacillus* sp. GL1 was isolated to enzymatically modify the viscous properties of gellan gum (Hashimoto *et al.*, 1999) and has a set of enzymes including an α-L-rhamnosidase responsible for the complete depolymerization of gellan gum. Two α-L-rhamnosidase genes (*rhaA* and *rhaB*) are located in the bacterial genome, but only *rhaB* is expressed in the presence of gellan gum (Hashimoto *et al.*, 2003). RhaB (encoded by *rhaB*; GenBank accession No. AB046706) is a monomeric enzyme of 956 amino-acid residues with a molecular weight of 106 kDa. Although both gene products (RhaA and RhaB) can act on the gellan disaccharide (rhamnosyl-glucose), significant differences exist between RhaA and RhaB in the amino-acid sequence, catalytic characteristics and subunit composition (Hashimoto *et al.*, 2003).

Enzymes involved in the metabolism of carbohydrates are highly important to living organisms and account for 1–3% of the proteins encoded by the genome (Davies *et al.*, 2005). In addition to physiological functions, α-L-rhamnosidases have been used to structurally determine polysaccharides, glycosides and glycolipids and to debitter citrus fruits in the food and beverage industries (Kamiya *et al.*, 1985; Manzanares *et al.*, 2001). Glycoside hydrolases (GHs) constitute one of the categories of the CAZy (Carbohydrate-Active Enzymes) database. These enzymes are divided into more than 100 families based on their primary sequences (http://www.cazy.org/CAZY/fam/acc_GH.html). α-L-Rhamnosidases are categorized into families GH78 or GH106, with most of the enzymes, including *Bacillus* sp.

Table 1
Data-collection statistics for RhaB.

Values in parentheses refer to data in the highest resolution shell.

Wavelength (Å)	1.00
Resolution (Å)	50–1.9 (1.97–1.90)
Crystal system	Orthorhombic
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 97.7, b = 119.9, c = 207.4
Total observations	1009571
Independent reflections	188971
Completeness (%)	98.8 (91.0)
$\langle I/\sigma(I)\rangle$	11.2 (2.12)
$R_{\rm merge}$† (%)	5.5 (40.7)

† $R_{\rm merge} = \sum_h \sum_i |I(h)_i - \langle I\rangle| / \sum_h \sum_i I(h)_i$, where $I(h)_i$ is the ith observation of reflection h and $\langle I(h)\rangle$ is the mean intensity of all observations of h.

GL1 RhaA and RhaB, belonging to family GH78. Although crystal structures of representatives from 69 GH families have been determined, no information on the three-dimensional structure of an α-L-rhamnosidase is available.

Structure analysis of family GH78 enzymes will help to clarify their mechanisms of catalysis and substrate specificity and to improve their potential application in a wide variety of industries.

This article focuses on the crystallization and preliminary X-ray crystallographic analysis of RhaB.

2. Methods and results

2.1. Analytical methods

The α-L-rhamnosidase RhaB was assayed in a 0.5 ml reaction system containing 0.4 m*M* *p*NP-α-L-rhamnopyranoside (Sigma) as a substrate and 50 m*M* potassium phosphate buffer (KPB) pH 7.0. The reaction was stopped by adding two volumes of 0.25 *M* sodium carbonate and the resultant *p*-nitrophenol concentration was determined by measuring the absorbance at 400 nm. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of *p*-nitrophenol from the substrate per minute. The protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as a standard.

2.2. Protein expression and purification

RhaB was expressed and purified as described elsewhere (Hashimoto *et al.*, 2003) with slight modification. After supplementation with 1 m*M* PMSF and 0.1 µ*M* pepstatin A, the cell extract prepared from *Escherichia coli* cells containing a plasmid carrying the gene encoding RhaB (pET3a-RhaB) was applied onto a DEAE-Toyopearl 650M (Tosoh) column (2.6 × 30 cm) previously equilibrated with 20 m*M* KPB pH 7.0. The enzyme was eluted with a linear gradient of NaCl (0–0.7 *M*) in 20 m*M* KPB pH 7.0 (500 ml) and 6 ml fractions were collected every 5 min. The active fractions were combined and saturated with ammonium sulfate (25%). The enzyme was applied onto a butyl-Toyopearl 650M (Tosoh) column (2.6 × 15 cm) previously equilibrated with 20 m*M* KPB pH 7.0 saturated with ammonium sulfate (25%). The enzyme was eluted with a linear gradient of ammonium sulfate (25–0% saturation) in 20 m*M* KPB pH 7.0 (400 ml) and 4 ml fractions were collected every 4 min. The active fractions were combined and dialyzed against 20 m*M* Tris–HCl pH 7.5 overnight. The dialysate was applied onto a HiLoad 26/10 Q Sepharose column previously equilibrated with 20 m*M* Tris–HCl pH 7.5 using FPLC (ÄKTA Purifier, Amersham Biosciences) for further purification. The enzyme was eluted with a linear gradient of NaCl (0–0.7 *M*) in 20 m*M* Tris–HCl pH 7.5 (400 ml) and 2 ml fractions were collected every 0.5 min. The enzyme eluted at about 0.4 *M* NaCl was used as purified RhaB. The purified enzyme showed a specific activity of 50 U mg^{-1}.

The purified enzyme was confirmed to be homogeneous using SDS–PAGE (Laemmli, 1970) and then concentrated to 25 mg ml^{-1} by ultrafiltration with a Centriprep (Millipore) for crystallization. The protein solution for crystallization consists of 20 m*M* Tris–HCl pH 7.5 and 0.4 *M* NaCl.

Selenomethionine-derivative RhaB was expressed in *E. coli* B834(DE3) containing pET3a-RhaB in 5 l minimum medium (Doublié & Carter, 1992) implemented with 25 mg selenomethionine per litre. Selenomethionine-derivative RhaB was purified in the same way as the native RhaB described above.

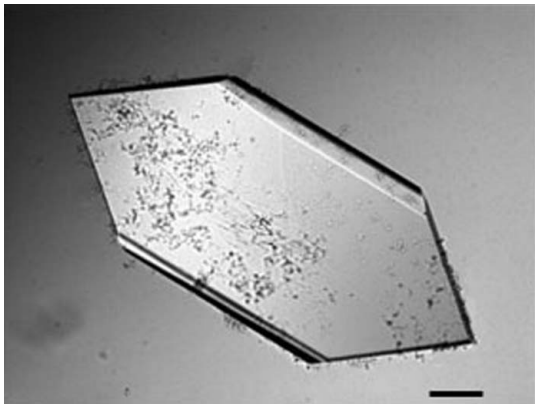


Figure 1
Crystal of α-L-rhamnosidase RhaB from *Bacillus* sp. GL1. The scale bar is 0.1 mm in length.

2.3. Crystallization

The RhaB purified from *E. coli* cells was crystallized at 293 K using hanging-drop vapour diffusion. Crystallization conditions were initially screened by sparse-matrix screening using commercial crystallization kits from Hampton Research in a 96-well Intelli-plate (Art Robbins Instruments). Crystallization of the enzyme was attempted under a large number of conditions at concentrations of 25 and 12.5 mg ml^{-1}. 500 µl mother liquor was used as reservoir solution and 1 µl RhaB solution was mixed with 1 µl reservoir solution to form the drop. Thin plate-shaped crystals of the enzyme were found in a droplet consisting of 14.4%(*w*/*v*) polyethylene glycol (PEG) 8000, 0.08 *M* sodium cacodylate pH 6.5, 0.16 *M* calcium acetate and 20%(*v*/*v*) glycerol. Crystallization conditions were optimized in 24-well Linbro plates. The crystallization droplet solution was prepared on a siliconized cover slip by mixing 3 µl protein solution with 3 µl precipitant solution. After conditions were optimized, the mother liquor most suitable for crystallization was determined to be a mixture consisting of 13.5%(*w*/*v*) PEG 8000, 0.08 *M* sodium cacodylate pH 6.5, 0.16 *M* calcium acetate and 20%(*v*/*v*) glycerol. Crystals grew in these droplets in two weeks at 293 K to exceed 5 mm with an appropriate thickness (Fig. 1).

2.4. X-ray analysis

A crystal of the enzyme picked up from a droplet with a mounted nylon loop (Hampton Research) was placed directly into a cold nitrogen-gas stream at 100 K. X-ray diffraction images were collected from the crystal at 100 K under a nitrogen-gas stream with a Quantum 4R detector and synchrotron radiation of wavelength 1.0 Å at the BL-38B1 station of SPring-8 (Japan). The distance between the

crystal and the detector was set to 180 mm and 0.5° oscillation images were recorded with an exposure time of 10 s. Diffraction data from the crystal were obtained at 1.9 Å resolution and were processed using *HKL*2000 (*DENZO* and *SCALEPACK*; Otwinowski & Minor, 1997). The preliminary X-ray crystallographic properties of RhaB are summarized in Table 1. V_M (Matthews, 1968), the crystal volume per unit of protein molecular weight, was calculated to be 2.86 $Å^3$ Da^{-1}, assuming the presence of two molecules of the enzyme in an asymmetric unit, and the solvent content was 57.0%. The V_M and solvent content lie within the ranges usually found for protein crystals.

A selenomethionine derivative of RhaB has already been obtained and crystallized. A crystal of selenomethionine-derivative RhaB was obtained under conditions similar to those for native RhaB except that the concentration of PEG 8000 was 10.2%(*w*/*v*). The phase problem will be solved by multiwavelength anomalous dispersion (MAD) experiments as the enzyme shows no homology with proteins of known structure.

This work was supported in part by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN) of Japan. We thank Drs H. Sakai and K. Hasegawa of the Japan Synchrotron Radiation Research Institute (JASRI) for their kind help during data collection. X-ray data at BL38B1 of SPring-8 were collected with the approval of the organizing committee of SPring-8 (proposal No. 2006A1405, WH).

References

Bader, G., Wray, V., Just, U. & Hiller, K. (1998). *Phytochemistry*, **49**, 153–156.
Bradford, M. M. (1976). *Anal. Biochem.* **72**, 248–254.
Davies, G. J., Gloster, T. M. & Henrissat, B. (2005). *Curr. Opin. Struct. Biol.* **15**, 637–645.
Doublié, S. & Carter, C. W. Jr (1992). *Crystallization of Nucleic Acids and Proteins. A Practical Approach*, edited by A. Ducruix & R. Giegé, pp. 311–317. Oxford University Press.
Giavasis, I., Harvey, L. M. & McNeil, B. (2000). *Crit. Rev. Biotechnol.* **20**, 177–211.
Hashimoto, W., Miyake, O., Nankai, H. & Murata, K. (2003). *Arch. Biochem. Biophys.* **415**, 235–244.
Hashimoto, W., Nankai, H., Sato, N., Kawai, S. & Murata, K. (1999). *Arch. Biochem. Biophys.* **368**, 56–60.
Jang, I. S. & Kim, D. H. (1996). *Biol. Pharm. Bull.* **19**, 1546–1549.
Kamiya, S., Esaki, S. & Tanaka, R. (1985). *Agric. Biol. Chem.* **49**, 55–62.
Laemmli, U. K. (1970). *Nature (London)*, **227**, 680–685.
Manzanares, P., van den Broeck, H. C., de Graaff, L. H. & Visser, J. (2001). *Appl. Environ. Microbiol.* **67**, 2230–2234.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Miake, F., Satho, T., Takesue, H., Yanagida, F., Kashige, N. & Watanabe, K. (2000). *Arch. Microbiol.* **173**, 65–70.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Pollock, T. J. (1993). *J. Gen. Microbiol.* **139**, 1939–1945.
Scaroni, E., Cuevas, C., Carrillo, L. & Ellenrieder, G. (2002). *Lett. Appl. Microbiol.* **34**, 461–465.
Turecek, P. & Pittner, F. (1986). *Appl. Biochem. Biotechnol.* **13**, 1–13.
Zvelov, V. V., Hertel, C., Bronnenmeier, K., Hroch, A., Kellermann, J. & Schwarz, W. H. (2000). *Mol. Microbiol.* **35**, 173–179.