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Crystallization and preliminary X-ray crystallographic studies of rice α-galactosidase

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α-Galactosidases catalyze the hydrolysis of galactooligosaccharides and galactopolysaccharides to α-galactose residues and are widely distributed in microorganisms, plants and animals. α-Galactosidase from rice (*Oryza sativa* L. ssp. *japonica*) was crystallized by the hanging-drop vapour-diffusion method. The crystals belong to space group $P2_12_12_1$, with unit-cell parameters $a = 63.1$, $b = 71.3$, $c = 85.6$ Å, and diffract beyond 1.9 Å resolution.

1. Introduction

α-Galactosidase (α-Gal; EC 3.2.1.22) is one of the exoglycosidases, capable of hydrolysing α-1,6-linked α-galactose residues, and is widely distributed in microorganisms, plants and animals. In humans, α-Gal is a lysosomal exoglycosidase that cleaves glycolipids and glycoproteins at the terminal α-galactose residues; mutations in α-Gal genes cause incomplete degradation of carbohydrate moieties and result in Fabry disease (Brady *et al.*, 1967; Ioannou *et al.*, 2001). In higher plants, galactomannan is one of the major storage polysaccharides in seeds and has commercial importance (Reid, 1995). α-Gals hydrolyse galacto(gluco)mannan and oligosaccharides such as raffinose, melibiose and stachyose at the terminal galactose residues. Raffinose and stachyose in beans are known to cause flatulence and α-Gal has the potential to alleviate these symptoms (Dey *et al.*, 1993). In the degradation of cell-wall galactomannan during germination, α-Gal is one of the key enzymes (Reid *et al.*, 1992; Reid, 1995).

We have purified and sequenced α-Gals from *Mortierella vinacea*, *Penicillium purpurogenum*, *Thermus* sp. T2 and rice and elucidated the substrate specificities of these enzymes in detail (Shibuya, Kobayashi, Kasamo *et al.*, 1995; Shibuya, Kobayashi, Park *et al.*, 1995; Shibuya *et al.*, 1997, 1998, 1999; Ishiguro *et al.*, 2001). The primary structures of more than 50 α-Gals have been deduced from gene or cDNA sequences. Primary structure and hydrophobic cluster analyses have shown that α-Gals can be classified into two glycoside hydrolase families, 27 and 36 (Henrissat & Davies, 1997, 2000). α-Gals from eukaryotes share a high amino-acid sequence similarity and are classified into family 27, whereas prokaryotic α-Gals are grouped into family 36. Family 27 is classified into the glycoside hydrolase clan D together with family 36; the amino-acid sequences of the two families have a low level of amino-acid sequence similarity between them, indicating that the folds of both families 27 and 36 α-Gals may be similar. The hydrolysis mechanism of the glycoside hydrolase clan D is known to be a retention of double-displacement mechanism and the experimentally determined nucleophile of the catalytic residue is an aspartic acid (Hart *et al.*, 2000; Ly *et al.*, 2000). Recently, the crystal structures of the chicken α-*N*-acetylgalactosaminidase in its free form and its complexed form with α-*N*-acetylgalactosamine have been solved; this enzyme catalyzes the hydrolysis of the glycosylated substrate at the terminal α-*N*-acetylgalactosamine and belongs to family 27 of glycoside hydrolases (Garman *et al.*, 2001). This enzyme consists of two domains: the N-terminal domain comprises a $(\beta/\alpha)_8$ barrel as a catalytic domain and the C-terminal domain has eight β-strands containing a Greek-key motif. On the other hand, only a few preliminary crystallization papers have been published for α-Gals (Golubev & Neustroev, 1993; Murali *et al.*, 1994) and there has been no report of a detailed three-dimensional structure of an α-Gals. Recently, we have succeeded in cloning the α-Gal cDNA from rice (*O. sativa* L. ssp. *japonica*; DDBJ, EMBL, GenBank database accession No. AB039671). Rice α-Gal consists of 362 amino-acid residues and its molecular weight is approximately 40 kDa. We report here preliminary X-ray crystallographic results of the family 27 α-Gal from rice. The three-dimensional structure of α-Gal will be helpful in clarifying its catalytic mechanism as well as in making a structural comparison with

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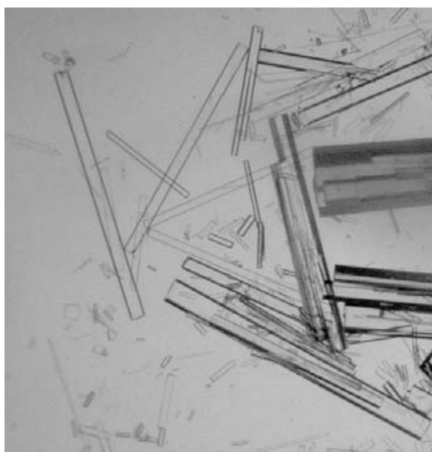

Figure 1
Crystals of rice α-Gal grown by the hanging-drop vapour-diffusion method.

α-*N*-acetylgalactosaminidase and will be useful in further protein engineering and rational design for industrial use.

2. Experimental and results

2.1. Crystallization

α-Gal from rice was purified from the culture supernatant of the rice-cell suspension culture by sequential chromatography on SP-Sepharose FF, ε-aminocaproyl-α-D-galactopyranosylamine Sepharose and Hiprep Sephacryl S-200 (Kim *et al.*, submitted). The purity of the protein homogenity was checked by SDS–PAGE. Crystallization conditions were screened by the hanging-drop vapour-diffusion method using ammonium sulfate and polyethylene glycol 6000 (Hampton Research) as precipitants. A protein solution of concentration corresponding to an OD_{280} of 15 ($\sim$6 mg ml^{-1}) was used for the initial screening. A droplet composed of 5 µl protein solution and 5 µl reservoir solution was made up and equilibrated against 1 ml reservoir solution at 293 K. Clusters of thin needle-shaped crystals grew in less than one month using the following reservoir solution: 5% 2-propanol, 0.1 *M* ammonium sulfate, 0.1 *M* acetate buffer pH 4.5. After refinement of crystallization conditions, crystals of rice α-Gal (0.03 $\times$ 0.05 $\times$ 1.0 mm; Fig. 1) were obtained when 5 µl protein solution at a concentration of 15 mg ml^{-1} was mixed with 5 µl reservoir solution (5% 2-propanol, 0.1 *M* ammonium sulfate, 0.1 *M* acetate buffer pH 4.5 with 5% D-galactose) at 293 K. Addition of D-galactose, which is a product of this enzyme, improved the resolution of the crystal. This might be because the D-galactose binds to the catalytic pocket of the enzyme and provides better crystal packing.

Table 1
Data-collection statistics.

Values in parentheses refer to the highest resolution shell.

Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 63.1, b = 71.3, c = 85.6
Wavelength (Å)	0.977
Resolution (Å)	30.0–1.9 (2.0–1.9)
$R_{\rm merge}$ (%)	8.0 (25.0)
Completeness (%)	99.9 (99.9)
Multiplicity	5.8 (5.9)
Average $I/\sigma(I)$	9.7 (3.3)
Unique reflections	31134 (4481)
Observed reflections	180876 (26337)

2.2. Data collection

Diffraction data from native crystals were obtained at beamline BL6A, Photon Factory, Tsukuba, Japan (λ = 0.977 Å). α-Gal crystals were mounted in nylon loops (Hampton Research) after soaking in a cryoprotectant solution (25% glycerol in the precipitant solution). The crystals were then flash-frozen in a nitrogen stream at 100 K. Diffraction data were collected using the Quantum CCD X-ray detector (ADSC) in 2.0° oscillation steps over a range of 180°. All data sets were processed and scaled using *DPS*/*MOSFLM* (Rossmann & van Beek, 1999).

The crystals of α-Gal belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 63.1, b = 71.3, c = 85.6 Å, and diffract beyond 1.9 Å resolution. The data-collection statistics are shown in Table 1. Assuming one α-Gal molecule in an asymmetric unit, the V_M value is 2.4 Å^3 Da^{-1}, which is within the expected range (Matthews, 1968). This V_M value corresponds to a solvent content of approximately 49.0%.

Initial phases were successfully obtained by the multiple isomorphous replacement (MIR) method using two derivatives with the program *MLPHARE* (Collaborative Computational Project, Number 4, 1994). The MIR phases were significantly improved after density modification with the program *DM* (Collaborative Computational Project, Number 4, 1994). Building and refinement of the structural model are now under way.

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