Purification, crystallization and preliminary X-ray analysis of a hexameric β-glucosidase from wheat

Masayuki Sue,[a] Kana Yamazaki,[a] Jun-ichi Kouyama,[b]‡ Yasuyuki Sasaki,[b] Kanju Ohsawa,[b] Toru Miyamoto,[a] Hajime Iwamura[c] and Shunsuke Yajima[b]*

[a]Department of Applied Biology and Chemisry, Tokyo University of Agriculture, Setagaya-ku, Tokyo 156-8502, Japan, [b]Department of Bioscience, Tokyo University of Agriculture, Setagaya-ku, Tokyo 156-8502, Japan, and [c]Department of Biotechnology, School of Biology Oriented Science and Technology, Kinki University, Naga-gun, Wakayama 649-6493, Japan

‡ Present address: Structural Biology Research Center, Photon Factory, High Energy Accelerator Research Organization, Tsukuba, Ibaraki 305-0801, Japan.

Correspondence e-mail: yshun@nodai.ac.jp

The wheat β-glucosidase TaGlu1b, which is only active in a hexameric form, was tagged with 6×His at the N-terminus, overexpressed in *Escherichia coli* and purified in two steps. The protein complexed with a substrate aglycone was crystallized at 293 K from a solution containing 10 mM HEPES pH 7.2, 1 M LiSO$_4$ and 150 mM NaCl using the hanging-drop vapour-diffusion method. Diffraction data were collected to 1.7 Å at the Photon Factory. The crystal belongs to space group $P4_132$, with unit-cell parameters $a = b = c = 194.65$ Å, $\alpha = \beta = \gamma = 90°$. The asymmetric unit was confirmed by molecular-replacement solution to contain one monomer, giving a solvent content of 72.1%.

1. Introduction

β-Glucosidases (EC 3.2.1.21) are one of the largest groups of enzymes in the glycoside hydrolase families and belong to families 1 or 3 (Henrissat, 1991). These enzymes are responsible for cleavage of the β-glucosidic linkage between two (or more) sugars or between a sugar and an aglycone. In plants, β-glucosidases are involved in lignification (Dharmawardhana *et al.*, 1995), regulation of the physiological activity of cytokinin (Falk & Rask, 1995; Haberer & Kieber, 2002), regulation of the biosynthesis of IAA (Ljung *et al.*, 2001; Persans *et al.*, 2001) and chemical defence against pathogens and herbivores (Niemeyer, 1988; Zagrobelny *et al.*, 2004). Many secondary products in plants exist in the form of a glucoconjugate with one or two glucose units attached to their hydroxy or thiol groups. Since glucosylation changes physiological activity, chemical stability and solubility in water, β-glucosidases play important roles by cleaving glucosidic bonds to release monosaccharides and active aglycones such as cyanogenic compounds, flavonoids and hydroxamic acids. The glucosidases involved in the hydrolysis of plant secondary metabolites are members of the family 1 glycohydrolases.

In recent years, we have purified and characterized β-glucosidases from young wheat and rye (Sue *et al.*, 2000*a,b*). Both plants accumulate hydroxamic acids (Hxs; 2,4-dihydroxy-1,4-benzoxazin-3-one, DIBOA, and its 7-methoxy derivative, DIMBOA) as defensive compounds against pathogens and herbivores (Niemeyer, 1988). These compounds are glucosylated in intact plants and are stored in different subcellular compartments from the glucosidase. Although the wheat and rye glucosidases hydrolyze DIBOA-Glc and DIMBOA-Glc efficiently, the preferred natural substrate for each glucosidase is consistent with the predominant Hx species in each plant, namely DIMBOA-Glc in wheat and DIBOA-Glc in rye. These observations raise the issue of how plants have developed these defence chemicals and the enzymes involved in their metabolism.

Recently, we cloned three β-glucosidase genes from wheat seedlings and expressed them in *Escherichia coli*. The recombinant glucosidases formed a hexamer, the active form, consisting of 64 kDa monomers (manuscript in preparation). The hexamer was easily dissociated into monomers, resulting in loss of activity. Crystal structures of family 1 β-glucosidases have been isolated from four plant species: white clover (Barrett *et al.*, 1995), white mustard (Burmeister *et al.*, 1997), maize (Czjzek *et al.*, 2001; Zouhar *et al.*, 2001) and sorghum (Verdoucq *et al.*, 2004). The wheat enzyme, however, differs in its active oligomeric structure, since wheat
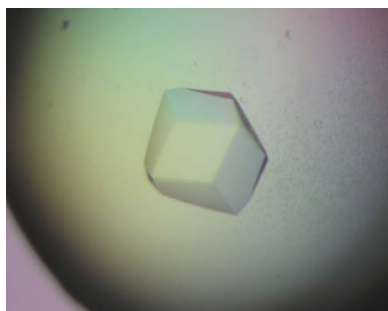
glucosidase must be hexameric to be active as a hydrolase, whereas the other plant glucosidases function as dimers. The crystal structures of family 1 β-glucosidases from several bacteria have been solved as octamers (Sanz-Aparicio *et al.*, 1998; Hakulinen *et al.*, 2000). The wheat enzyme, however, shows lower homologies ($\sim$35%) with bacterial enzymes than with those of plants ($\sim$60%). Thus, it is of interest to compare the monomer-association properties from the quaternary structures. We therefore aimed to crystallize wheat β-glucosidase as a hexamer in order to clarify the relationship between its structure and activity.

2. Materials and methods

2.1. Cloning, expression and purification

Wheat β-glucosidase (TaGlu1b) was expressed with a 6×His tag at the N-terminus in *E. coli*. The DNA fragment corresponding to mature TaGlu1b, with *Nco*I and *Xho*I recognition sites at the 5' and 3' ends, respectively, was amplified by PCR using KOD DNA polymerase (Toyobo). The resulting fragment was introduced into the *Nco*I and *Xho*I sites of pET30a and the product was transformed into BL21 CodonPlus (DE3)-RIL (Stratagene). Luria–Bertani (LB) broth

(3 ml) containing 50 µg ml^{-1} kanamycin and chloramphenicol was inoculated with a single colony of the *E. coli* and was incubated at 310 K and 150 rev min^{-1} for 6 h. A 2 ml aliquot of the culture was then transferred to 150 ml LB broth supplemented with the same antibiotics and was further incubated at 310 K and 150 rev min^{-1} until the OD$_{600}$ of the medium reached 0.6. Expression of TaGlu1b was induced by adding isopropyl β-thiogalactopyranoside to a final concentration of 1 mM, followed by incubation at 293 K and 150 rev min^{-1} for 16 h. The cells were recovered by centrifugation at 15 000g for 15 min and were resuspended in 15 ml ice-cold HEPES pH 7.2 containing 80 µl protease-inhibitor cocktail for purification of the His-tagged protein (Sigma). The cell was disrupted by six bouts of sonication (100 W) for 20 s at 1 min intervals. The lysate was then centrifuged at 15 000g for 20 min at 277 K.

The expressed TaGlu1b was purified using a HiTrap Chelating HP column (Amersham Biosciences) according to the manufacturer's instructions. The 60–300 mM imidazole fraction was collected and concentrated by ultrafiltration. The glucosidase was further purified by gel filtration on Superdex 200 (Amersham Biosciences) equilibrated with 50 mM HEPES pH 7.2 and 150 mM NaCl. The fraction containing glucosidase activity (about 300 kDa) was pooled and concentrated by ultrafiltration. The amount of protein was estimated according to the method of Bradford (1976), using BSA as standard.
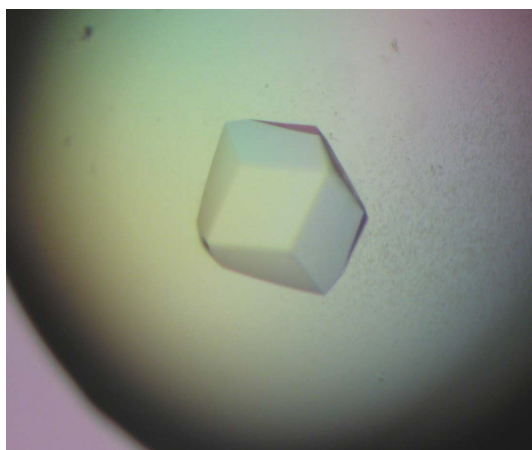
2.2. Crystallization and preliminary X-ray diffraction

The initial crystallization conditions were screened using Hampton Research Crystal Screens I and II (Jancarik & Kim, 1991); the final condition was 10 mM HEPES pH 7.2, 1 M LiSO$_4$ and 150 mM NaCl as the reservoir buffer. All crystallization experiments were performed using the hanging-drop vapour-diffusion method in a 24-well tissue-culture VDX plate at 293 K. Drops consisting of 1 µl protein solution (3.7 mg ml^{-1}) and 1 µl reservoir solution were used for the initial screening and drops consisting of 1.5 µl protein solution and 1.5 µl reservoir solution were used for the final condition.

To obtain the complex of TaGlu1b and its substrate aglycone, DIMBOA, crystals were soaked in the crystallization buffer with 0.5 mM DIMBOA and 30% glycerol as a cryoprotectant for 15 min and were then cooled in a nitrogen stream at 100 K. The diffraction data were processed using the *HKL*2000 program (Otwinowski & Minor, 1997).
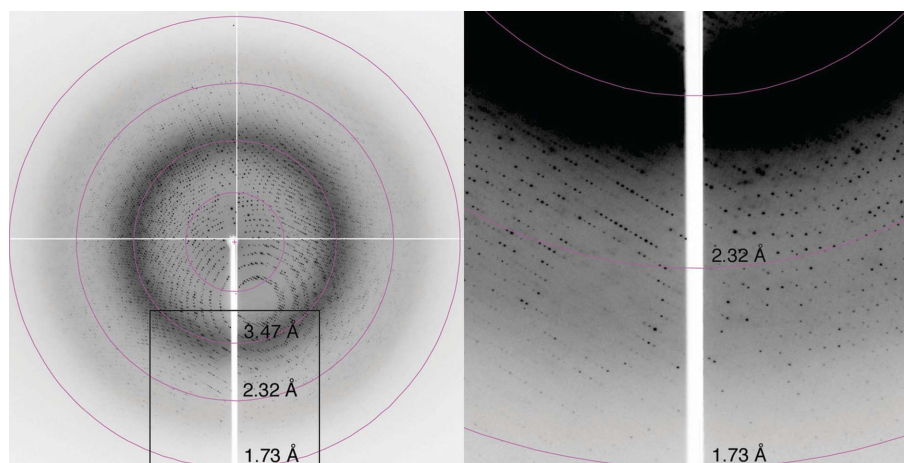

Figure 2
A representative diffraction image of a crystal of TaGlu1b complexed with DIMBOA collected on beamline BL6A at the Photon Factory using an ADSC Quantum 4 detector. The box in the left image indicates the position of the image magnified on the right.

crystallization communications

Table 1
Data-collection and processing statistics.

Values in parentheses correspond to the outer resolution shell.

Wavelength (Å)	1.000
Space group	$P4_132$
Unit-cell parameters	
$\quad a = b = c$ (Å)	194.65
$\quad \alpha = \beta = \gamma$ (°)	90
Matthews coefficient (Å^3 Da^{-1})	4.45
Molecules per AU	One monomer
Solvent content (%)	72.1
Resolution range (Å)	50–1.70 (1.76–1.70)
Total observations	2667849
Unique reflections	137005
Average $I/\sigma(I)$	62.0 (6.6)
R_{merge}	0.079 (0.564)
Completeness (%)	99.8 (98.0)

3. Results and discussion

The 6×His-tagged TaGlu1b was expressed strongly in the soluble fraction in *E. coli* and we were able to obtain 2 mg of the purified protein from 150 ml culture. From the initial screening, crystals were obtained from both 0.1 *M* HEPES pH 7.5, 2 *M* ammonium sulfate, 2% PEG 400 and 10 m*M* HEPES pH 7.2, 1 *M* LiSO$_4$, 150 m*M* NaCl. The first set of conditions produced only tiny crystals, so the latter conditions were subsequently used. The crystals were typically 0.2 mm in their longest dimension, with a maximum of around 0.3 mm (Fig. 1); we used the largest crystals for data analysis. Crystals of TaGlu1b complexed with DIMBOA were obtained by soaking and diffracted to 1.7 Å on beamline BL-6A at the Photon Factory (Fig. 2). However, crystals without the substrate diffracted to less than 2.0 Å. The mosaicity of the crystals was very low (~0.12) and R_{merge} was 0.079 with high redundancy (20). Table 1 shows the data-collection and related processing statistics.

A molecular-replacement solution using the β-glucosidase from sorghum (PDB code 1v03; Verdoucq *et al.*, 2004) as the search model was obtained using the program *MOLREP* (Vagin & Teplyakov, 1997). From this solution, the asymmetric unit was found to contain one monomer and the solvent content was 72.1%, which is relatively high despite the high resolution. Model building and refinement of this structure are under way.

Several structures of O-β-glucosidases from plants, including white clover, maize and sorghum, have been determined. The resolution obtained in the present study is better, however, and promises to provide a better understanding of the relation between the structure and activity of O-β-glucosidases.

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