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Purification, crystallization and preliminary X-ray analysis of a maize cytokinin glucoside specific β-glucosidase

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Zm-p60.1, a cytokinin glucoside specific β-glucosidase from maize, is a key enzyme involved in plant development and growth. It has been overexpressed in soluble form from *Escherichia coli* with a His tag at its N-terminus. The recombinant protein has been purified and crystallized at room temperature using PEG 4000 as the main precipitant. At least three crystal forms have been observed from very similar growth conditions. A flash-annealed monoclinic crystal diffracted to high resolution (beyond 2 Å) with space group $P2_1$ and unit-cell parameters $a = 55.66$, $b = 110.72$, $c = 72.94$ Å, $\beta = 92.10^\circ$. The asymmetric unit is estimated and confirmed by molecular-replacement solution to contain one Zm-p60.1 dimer, giving a crystal volume per protein mass (V_M) of 1.89 Å^3 Da^{-1} and a solvent content of 35%.

1. Introduction

β-Glucosidases (β-glucoside glucohydrolases; E.C. 3.2.1.21) occur widely in prokaryotes and eukaryotes. They catalyze the hydrolysis of aryl- and alkyl-β-D-glucosides as well as glucosides with only a carbohydrate moiety (*e.g.* cellobiose). The variety of biological functions of β-glucosides implicates the engagement of β-glucosidases in numerous essential steps of life. For example, in plants β-glucosidases have been implicated in various aspects of growth, productivity and defense, and reactions such as cyanogenesis related to food and feed toxicity (reviewed in Esen, 1993). In addition, plant β-glucosidases may be involved in the metabolism of plant growth regulators, whose storage forms occur as β-glucosides and are activated by β-glucosidases (Brzobohatý *et al.*, 1994; Sembdner *et al.*, 1994).

The extensive diversity of β-glucosides is likely to be paralleled by a variety of β-glucosidases designed for their selective hydrolysis. This raises the question of the origin of β-glucosidase specificity. An essential step in solving the question is three-dimensional structure determination of individual β-glucosidases. Currently, crystal structures of only a few bacterial β-glucosidases (Aguilar *et al.*, 1997; Hakulinen *et al.*, 2000; Sanz-Aparicio *et al.*, 1998) and only one plant cyanogenic β-glucosidase (Barrett *et al.*, 1995) are available. The data confirmed that the overall fold in these structures is a single domain $(\beta/\alpha)_8$ barrel, as was predicted for family 1 glycosyl hydrolases (Henrissat *et al.*, 1995, Jenkins *et al.*, 1995), to which β-glucosidases belong. A detailed analysis of the individual structures of the active sites has now become a key prerequisite to unravel the molecular determination of substrate specificity in β-glucosidases.

In maize, a β-glucosidase capable of efficiently hydrolyzing cytokinin-O- and N3-glucosides was identified and a corresponding cDNA, *Zm-p60.1*, cloned (Campos *et al.*, 1992; Brzobohatý *et al.*, 1993). Zm-p60.1 had no or very little activity on substrates from several groups of naturally occurring glycosides, including disaccharides involved in plant cell-wall degradation (cellobiose and laminiaribose), phenolic glucosides (salicin) and flavonoid glycosides (rutin). Based on enzyme activity and the highly specific expression pattern, Zm-p60.1 has been suggested to be one of the key enzymes involved in regulation of plant development by releasing biologically active plant growth regulators, cytokinins, from their inactive conjugated storage and transport forms (Brzobohatý *et al.*, 1993; Kristoffersen *et al.*, 2000). The biologically active form of Zm-p60.1 is a homodimer (Rotrekl *et al.*, 1999) or higher oligomers (Esen & Blanchard, 2000) located in plastids/chloroplasts (Kristoffersen *et al.*, 2000).

In order to more deeply understand the biological function and to engineer the catalytic and molecular properties for industrial and field application, a detailed structure–function analysis of Zm-p60.1 is required. Crystal structure determination will thus form a firm base for this structure–function analysis. Here we report (i) a detailed protocol for protein production in quantity and quality

Table 1
Data-collection statistics.

Values in parentheses are for the last resolution shell.

Wavelength (Å)	0.9831
Resolution (Å)	68–2.05 (2.42–2.05)
Completeness (%)	94.7 (85.7)
$R_{\rm merge}$† (%)	5.3 (17.6)
$I/\sigma(I)$	15.5 (4.9)
Space group	$P2_1$
Unit-cell parameters (Å,°)	a = 55.66
	b = 110.72
	c = 72.94
	β = 92.1
No. of possible unique reflections	55455
No. of observed reflections	539430
$V_{\rm M}$ (Å^3 Da^{-1})	1.89
Molecules per asymmetric unit	2

† $R_{\rm merge} = \sum|I_{\rm obs} - I_{\rm avg}|/\sum I_{\rm obs}$, where the summation is over all reflections.

appropriate for crystallization, (ii) successful crystallization procedures and (iii) diffraction data collection from Zm-p60.1 crystals.

2. Experimental

2.1. Protein expression and purification

Zm-p60.1 was expressed and purified as a 518-residue fusion protein with an N-terminal His tag in *E. coli* with modified procedures based on Zouhar *et al.* (1999). Briefly, 2 l of Luria–Bertani (LB) medium supplemented with ampicillin (100 mg l^{-1}) was inoculated with 20 ml of an overnight culture of *E. coli* strain BL21(DE3)pLysS harboring the plasmid pRSET::*Zm-p60.r* (Zouhar *et al.*, 1999) at 310 K. Overexpression of Zm-p60.1 was induced by addition of 0.4 m*M* isopropyl β-thiogalactopyranoside (IPTG) at an OD_{600} of about 0.8. The culture was then cooled and grown at 303 K for another 3 h. Cells were harvested by centrifugation at 7000*g* for 15 min at 296 K (Beckman J2-HS). The pellet obtained from 2 l of the expression culture was resuspended in 5 ml of 50 m*M* phosphate buffer pH 7.0 containing 150 m*M* NaCl and 0.5 m*M* phenylmethylsulfonylfluoride (PMSF). After incubation on ice for 30 min, the cell suspension was sonicated by applying 80 W pulses (Sonics, USA). The lysate was centrifuged on a Beckman J2-HS centrifuge at 17 000*g* for 25 min to remove insoluble cell debris.

Figure 1
Monoclinic crystals of Zm-p60.1 grown in 25%(*w*/*v*) PEG 4000 with 0.1 *M* citrate buffer pH 5.6 and 0.2 *M* ammonium acetate. The approximate dimensions of the larger crystal are 0.4 × 0.3 × 0.1 mm.

The Zm-p60.1 protein was purified by the immobilized metal-affinity chromatography (IMAC) on a cobalt resin TALON (Clontech, USA) column according to the manufacturer's instructions. The fractions containing the protein were then pooled and precipitated by gradually adding ammonium sulfate to 75% saturation. The pellet from the ammonium sulfate precipitation was dissolved in 500 µl of 20 m*M* Tris buffer pH 7.8 with 0.2 *M* NaCl. The protein was further purified by a gel-filtration step on a Superdex 200 column (Amersham Pharmacia Biotech, Sweden) mounted on a BioCAD SPRINT system (PerSeptive Biosystems Inc., USA). Fractions containing the enzyme were pooled and concentrated to 2–3 mg ml^{-1} using a Centricon-30 (Amicon, USA) for crystallization experiments. The protein aliquots were stored either in a refrigerator or in a freezer at 190 K.

2.2. Crystallization

The initial crystallization conditions were screened by the sparse-matrix method (Jancarik & Kim, 1991). All crystallization experiments were performed at 294 K by the hanging-drop vapour-diffusion method in 24-well VDX plates (Hampton Research, USA). In each trial, a hanging drop of 1 µl of protein solution mixed with 1 µl of precipitant solution was equilibrated against a reservoir containing 500 µl of precipitant solution. Macroseeding techniques were also attempted to obtain reproducible large crystals in the drops equilibrated with reservoir solutions in less than 24 h.

2.3. Data collection and processing

Crystals of Zm-p60.1 with dimensions of about 0.3 × 0.2 × 0.1 mm were mounted in a nylon CryoLoop (Hampton Research, USA), immersed in cryoprotectant (20% PEG 4000, 5% glycerol) for a few seconds and then flash-cooled in a cold nitrogen stream (Oxford Cryosystems Cryostream Cooler). Diffraction data were collected at the crystallographic beamline BL711 at the MAX-II synchrotron in Lund (Sweden) at 100 K using a MAR345 image-plate detector and the oscillation method. The so-called *in situ* flash-annealing method (that is, stopping the cold nitrogen stream for several seconds to allow the frozen crystal to thaw completely and then freezing the crystal rapidly again; Yeh & Hol, 1998) was used to impove the diffraction quality of the crystals. The wavelength for the data collection was 0.983 Å and the crystal-to-detector distance was 220 mm, with an oscillation range of 1° per image. A typical exposure time was 60 s per frame. Data were processed using the *DENZO* and *SCALEPACK* packages (Otwinowski & Minor, 1997).

3. Results and discussion

The purity of the Zm-p60.1 protein was estimated to be about 90% by SDS–PAGE (data not shown). About 3 mg of protein can be obtained from 1 l of culture.

The crystals were first observed in drops 9 [0.2 *M* ammonium acetate, 0.1 *M* sodium citrate pH 5.6, 30%(*w*/*v*) PEG 4000] and 15 [0.2 *M* ammonium sulfate, 0.1 *M* sodium cacodylate, 30%(*w*/*v*) PEG 8000]. The crystallization conditions (pH, PEG concentration and ammonium acetate concentration) were then optimized by a finer screen around these conditions. The shapes of the original crystals are often thin plates and long rods.

As shown in Fig. 1, typical plate-like crystals of average dimensions 0.3 × 0.2 × 0.1 mm grew in 20–25%(*w*/*v*) PEG 4000 with 0.1 *M* citrate buffer pH 5.3–5.9 and 0.2 *M* ammonium acetate between a few days and 10 d. However, the spontaneous nucleation and growth of the crystals were not reproducible, particularly for protein stored in a refrigerator for longer than a week. Macroseeding procedures using either a nylon CryoLoop or a piece of hair were tried and could produce good crystals; in fact, it was better to use older protein batches for the macroseeding since fresh protein produced too many small crystals. Although it is difficult to tell from the morphology of the crystals, the diffraction data show that the packing of the protein can be very different. So far, monoclinic, orthorhombic and *C*-centered orthorhombic crystal forms have been observed.

Some Zm-p60.1 crystals diffracted to better than 2.0 Å resolution on beamline BL711 of MAX-lab, Lund. However, most of the crystals showed serious disorder and high mosaicity when frozen directly. The *in situ* flash-annealing method (Yeh & Hol, 1998) improved the mosaicity and diffraction limits dramatically. A flash-annealed monoclinic crystal diffracted well beyond

2.0 Å (in comparison with disordered diffraction to only ∼3.0 Å before flash-annealing) and a complete data set was collected on this crystal; the statistics of this data set are listed in Table 1. The crystal belongs to the space group $P2_1$, with unit-cell parameters a = 55.66, b = 110.72, c = 72.94 Å, β = 92.10°. There are two molecules (a homodimer of Zm-p60.1) per asymmetric unit as estimated by the calculated value of V_M (Matthews, 1968), 1.89 Å^3 Da^{-1}. The solvent content for such a crystal is then approximately 35%.

A molecular-replacement solution using the cyanogenic β-glucosidase from white clover (PDB code 1cbg; Barrett *et al.*, 1995) as the search model has been obtained using the program *AMoRe* (Navaza, 1994); the solution confirmed that there is a dimer in the asymmetric unit. The model building and refinement of this structure are under way.

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