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Crystallization and preliminary X-ray diffraction study of a cell-wall invertase from *Arabidopsis thaliana*

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Cell-wall invertase 1 (AtcwINV1), a plant protein from *Arabidopsis thaliana* which is involved in the breakdown of sucrose, has been crystallized in two different crystal forms. Crystal form I grows in space group $P3_1$ or $P3_2$, whereas crystal form II grows in space group $C222_1$. Data sets were collected for crystal forms I and II to resolution limits of 2.40 and 2.15 Å, respectively.

1. Introduction

Sucrose (α-D-glucopyranosyl β-D-fructofuranoside) is a key metabolic compound in higher plants (Salerno & Curatti, 2003). Most higher plants use sucrose as their major transport compound to bring energy and carbon skeletons from source to sink tissues. Sessile life forms such as plants use complex regulatory mechanisms to respond in a flexible way to different abiotic and biotic signals and important changes in source–sink relations often need to occur (Sturm, 1999). It is now becoming increasingly evident that sucrose and hexoses play major roles as metabolic signals, regulating plant development by affecting different classes of genes (Smeekens, 2000; Gibson, 2005). Carbohydrate partitioning and sugar sensing are intimately connected to sucrose metabolism and these processes are vital throughout plant development (Koch, 2004).

The channelling of sucrose into sink metabolism often requires the cleavage of the $\alpha1$–$\beta2$ glycosidic bond. In plants, this can be catalyzed by sucrose synthases (glycosyl transferases; EC 2.4.1.13) and/or invertases (hydrolases splitting sucrose into glucose and fructose; EC 3.2.1.26). Different classes of plant invertases can be discerned: (i) soluble cytoplasmic invertases with a neutral to alkaline pH optimum, (ii) soluble vacuolar invertases, (iii) soluble apoplastic invertases and (iv) insoluble cell-wall-bound invertases. The latter three have acidic pH optima (Tymowska-Lalanne & Kreis, 1998; Kim *et al.*, 2000). Two large invertase gene families exist in *Arabidopsis thaliana*. The first family has eight members encoding six putative cell-wall invertases and two vacuolar invertases (Ji *et al.*, 2005). However, it was recently found that at least two of the so-called cell-wall invertase genes do not encode functional invertases but fructan exohydrolases (FEH; De Coninck *et al.*, 2005). All these enzymes are classified in family 32 of the glycosyl hydrolases (Henrissat & Davies, 1997). The second family contains nine genes encoding cytosolic neutral/alkaline invertases.

Most work has been focused on acid invertases, especially on cell-wall invertases. These enzymes play a crucial role in apoplastic phloem unloading and in doing so intervene in carbohydrate partitioning and the long-distance transport of sucrose. In addition, cell-wall invertases have been implicated in defence responses (Roitsch *et al.*, 2003) and the regulation of seed (Miller & Chourey, 1992) and pollen development (Goetz *et al.*, 2001). Because sucrose and hexoses also regulate gene expression, it is evident that invertase enzymes play a fundamental role in controlling gene expression, cell differentiation and development (Roitsch & González, 2004).

A. thaliana cell-wall invertase 1 (AtcwINV1) shows the highest expression level of the six cell-wall-type hydrolases (Sherson *et al.*, 2003) and its expression level can be further induced after infection (Benhamou *et al.*, 1991; Fotopoulos *et al.*, 2003). Heterologous expression of AtcwINV1 in *Pichia pastoris* demonstrated that it is a typical invertase and not an FEH (De Coninck *et al.*, 2005).

Fructosyl transferases, FEHs, cell-wall invertases and vacuolar invertases from plants are grouped together with microbial β-fructosidases and FEHs within family 32 of the glycosyl hydrolases. The related family 68 harbouring invertases, bacterial levansucrases and inulosucrases can be combined with family 32 in clan GH-J (the β-fructosidase superfamily; Naumoff, 2001). Within this clan, four three-dimensional structures of microbial enzymes have been unravelled to date: levansucrases (GH family 68) from *Bacillus subtilis* (Meng & Fütterer, 2003) and *Gluconacetobacter diazotrophicus* (Martinez-Fleites *et al.*, 2005), a β-fructosidase (GH family 32) from *Thermotoga maritima* (Alberto *et al.*, 2004) and an inulinase (β-fructosidase, GH family 32) from *Aspergillus awamori* (Nagem *et al.*, 2004). The first plant GH family 32 enzyme structure, a 1-FEH from *Cichorium intybus*, has recently been obtained (Verhaest *et al.*, 2005). This is a unifunctional enzyme that only catalyzes the breakdown of inulin-type fructan. A new EC number was recently assigned to this enzyme (EC 3.2.1.153) because it shows zero invertase activity, in contrast to the classic microbial inulinases or β-fructosidases (EC 3.2.1.80) which also break down sucrose.

To the best of our knowledge, no three-dimensional structure has been obtained for a typical unifunctional invertase from a plant or from any other organism. Here, we describe the crystallization of AtcwINV1, a key metabolic enzyme involved in the regulation of overall plant growth and development.

2. Materials and methods

2.1. Purification

Cell-wall invertase 1 (AtcwINV1, gene accession code At3g13790) from *A. thaliana* was cloned into pPICZ α vector and heterologously expressed in *P. pastoris* as described by De Coninck *et al.* (2005). The yeast culture was centrifuged (1100*g*, 10 min at 277 K) and the pH of the supernatant was adjusted to pH 5.0 using acetic acid. The supernatant was applied onto an S Sepharose column (25 × 100 mm) equilibrated with 20 m*M* sodium acetate buffer pH 5.0. After washing the column with 20 m*M* Tris–HCl pH 7.5, the enzyme was eluted with 1 *M* NaCl in 20 m*M* Tris–HCl pH 7.5. Active fractions were subsequently loaded onto a Superdex TM 75 (16/60) column (Pharmacia) equilibrated with 20 m*M* sodium acetate buffer pH 5.0 and 50 m*M* NaCl. Finally, the enzyme was loaded onto a Mono S column equilibrated with 50 m*M* sodium acetate buffer pH 5.0. After washing the column with 20 m*M* Tris–HCl pH 7.5, the enzyme was eluted with a gradient of 0–0.3 *M* NaCl in 20 m*M* Tris–HCl pH 7.5 and only the two most active fractions (purity was checked with SDS–PAGE) were used for crystallization. Routine invertase activity measurements were performed as described in De Coninck *et al.* (2005).

2.2. Crystallization

The protein solution was concentrated to approximately 10 mg ml^{-1} by ultrafiltration using a Microcon concentrator (Amicon) with a 3 kDa cutoff. Hampton Research Crystal Screen I (Jancarik & Kim, 1991) was used to determine the initial crystallization conditions. Crystallization was carried out using the hanging-drop vapour-diffusion method in Linbro multiwell tissue plates. Each reservoir was filled with 700 µl reservoir solution. Drops of 1.5 µl protein solution mixed with 1.5 µl reservoir solution were placed on cover slips and equilibrated against the reservoir. Crystals grew under two conditions at a temperature of 277 K. The first crystallization conditions contained 0.2 *M* zinc acetate, 0.1 *M* sodium cacodylate pH 6.5 and 18% PEG 8000 and gave unusual bar-shaped crystals (type I; Fig. 1*a*). The second crystallization condition consisted of 2.0 *M* ammonium sulfate and gave needle-shaped crystals (type II; Fig. 1*b*). Type I crystals appeared after 4 d and reached their maximum size about two weeks later. In contrast, type II crystals only appeared after a year.

Table 1
Data-collection and reduction statistics for *A. thaliana* AtcwINV1.

Values in parentheses are for the highest resolution shell.

	Type I crystals	Type II crystals
Space group	$P3_1$ or $P3_2$	$C222_1$
Unit-cell parameters (Å)		
a	105.1	112.9
b	105.1	162.9
c	50.9	74.1
Wavelength used (Å)	0.984	0.931
Resolution limit (Å)	25.0–2.40 (2.49–2.40)	20.0–2.15 (2.19–2.15)
Total observations	72547	149921
Unique observations	23906 (1953)	38119 (1880)
Redundancy	3.0	3.9
Completeness (%)	97.4 (79.5)	99.9 (99.9)
Mean $I/\sigma(I)$	14.09 (1.9)	12.22 (2.48)
R_{sym} (%)	7.6 (33.2)	9.2 (50.3)

2.3. Data collection

Data collection was performed at 100 K (Oxford Cryosystems Cryostream) using an N_2-gas stream. Type I crystals were soaked for 3 min in a cryosolution containing 0.2 *M* zinc acetate, 0.1 *M* sodium cacodylate pH 6.5, 18% PEG 8000 and 20% glycerol. Crystals were then mounted in cryoloops and flash-cooled in liquid nitrogen. Diffraction data were collected at the BM14 beamline at the ESRF synchrotron (Grenoble, France) using a MAR Mosaic 225 CCD

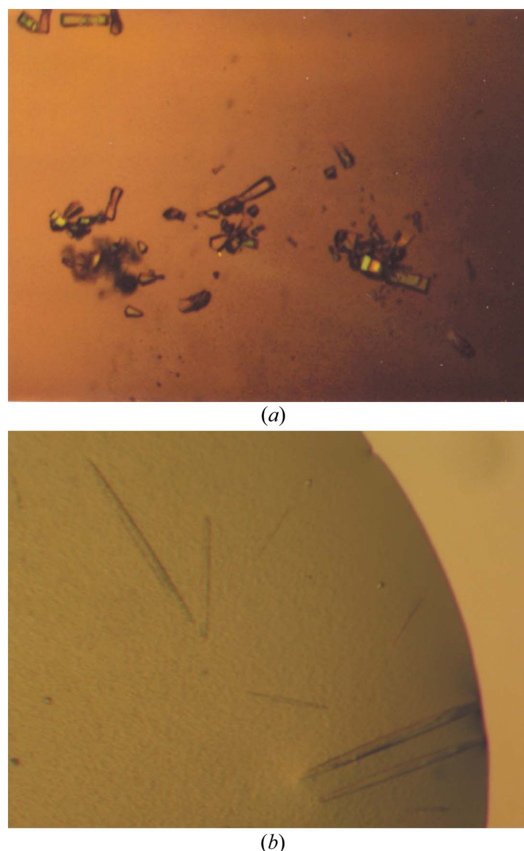

Figure 1
(*a*) Type I crystals and (*b*) type II crystals. The dimensions of the largest crystals were 0.6 × 0.075 × 0.075 mm (*a*) and 0.6 × 0.04 × 0.04 mm (*b*).

detector. Type II crystals were soaked for 10 min in a cryosolution containing 2.0 M ammonium sulfate and 25% glycerol. Data collection for these crystals was performed at the ID14-3 beamline at the ESRF synchrotron.

3. Results and discussion

Type I crystals diffracted to a resolution of 2.4 Å, while crystals of type II diffracted to 2.15 Å resolution. The data sets were processed using *DENZO* and *SCALEPACK* v.0.97.647d (type I crystals) or v.1.97.2 (type II crystals) (Otwinowski & Minor, 1997). Data-collection statistics are summarized in Table 1.

The unit-cell parameters of the type I crystals were a = 105.1, b = 105.1, c = 50.9 Å. As the a axis equals the b axis and the unit-cell angles α and β are 90° and γ is 120°, together with the intensity observations and the systematic absences of $00l$ reflections with $l \neq 3n$, we concluded that our protein crystallized in the trigonal space group $P3_1$ or $P3_2$. Crystal type II belongs to the C-centred orthorhombic space group $C222_1$. This could be concluded from the unit-cell parameters, which showed three unequal axes (a = 112.9, b = 162.9, c = 74.1 Å) and three unit-cell angles of 90°, together with the intensity observations and systematic absences of the $00l$ reflections if $l \neq 2n$.

The Matthews coefficient (Matthews, 1974) for invertase was calculated using a molecular weight of 61 400 Da, a unit-cell volume of 5.624×10^5 Å^3 and three molecules in the unit cell for type I crystals. According to these Matthews coefficient calculations, the asymmetric unit should contain one molecule, with a corresponding $V_{\rm M}$ value of 3.05 Å^3 Da^{-1}. This corresponds to a solvent content of 60%. The type II crystals have a unit-cell volume of 1.363×10^6 Å^3. Matthews coefficient calculations (2.78 Å^3 Da^{-1}) are consistent with one molecule per asymmetric unit and a solvent content of 56%.

As AtcwINV1 shares 52% sequence identity with 1-FEH IIa (PDB code 1st8; Verhaest *et al.*, 2005), the latter protein structure will be used to solve the phase problem of invertase *via* the molecular-replacement technique (Hoppe, 1957; Rossmann & Blow, 1962).

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