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Crystallization and preliminary X-ray diffraction study of fructan 1-exohydrolase IIa from *Cichorium intybus*

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Fructan 1-exohydrolase IIa (1-FEH IIa), a plant enzyme involved in fructan breakdown, has been crystallized using the hanging-drop vapour-diffusion method at 277 K. The crystals are tetragonal, belonging to space group $P4_12_12$ or $P4_32_12$, with unit-cell parameters a = 139.83, b = 139.83, c = 181.94 Å. Calculation of the Matthews coefficient indicates there to be two or three molecules in the asymmetric unit. Synchrotron radiation was used to collect a complete native data set to a resolution of 2.35 Å.

1. Introduction

About 15% of flowering plants use fructans, fructose-based oligosaccharides or polysaccharides, as storage carbohydrates instead of starch or sucrose (Hendry, 1993). Among these plants are several economically important crops (*e.g.* wheat, barley, chicory, onion, leek, garlic *etc.*). In addition to their function as reserve compounds, fructans may be of importance for stress protection (drought and cold) and osmoregulation (Vergauwen *et al.*, 2000; Hincha *et al.*, 2002). Various types of fructans exist. The inulin-type fructans occur mainly in dicotyledonous species (*e.g.* chicory, *Cichorium intybus*) and consist of linear β-(2,1)-linked fructofuranosyl units. Levan-type fructans consist of linear β-(2,6)-linked fructofuranosyl units. Branched fructan types are found in monocots (Vijn & Smeekens, 1999). Fructans also occur in bacteria and are mostly of the levan type. They are much longer than plant fructans and are biosynthesized by levansucrases (Han, 1990).

Inulins are commercially extracted from chicory roots. The food industry is interested in inulins because of their various health-promoting effects (reviewed by Niness, 1999). Inulin functions as dietary fibre and a low-calorie fat substitute and stimulates the growth of beneficial *Lactobacillus* and *Bifidobacteria* species in the intestine. Since inulin is not digested by human intestinal enzymes and as such cannot affect blood sugar levels, it is also suitable for consumption by diabetics. It is important to note that inulin decreases serum triglycerides and blood cholesterol levels. Promotion of calcium absorption serves in the prevention of osteoporosis. Finally, inulin may also play a role in cancer prevention. However, an important drawback to industrial harvesting of inulin from chicory is caused by the endogenous degradation of fructan, which significantly reduces the production efficiency of inulin.

Inulin synthesis involves two distinct enzymes, sucrose:sucrose 1-fructosyl transferase (1-SST; GF + GF $\rightarrow$ GFF + G) and fructan:fructan 1-fructosyl transferase (1-FFT; $GF_n + GF_m \leftrightarrow GF_{n-1} + GF_{m+1}$ with $n > 1$, $m \geq 1$) (Edelman & Jefford, 1968). The breakdown of fructans, catalyzed by fructan exohydrolase (FEH), a glycosyl hydrolase, results in the removal of the last fructose. Both inulin-degrading (inulinases, 1-FEH) and levan-degrading enzymes (levanases, 6-FEH) can be discerned. In chicory, two types of 1-FEH exist: 1-FEH I and 1-FEH II, with the latter having two isoforms, 1-FEH IIa and 1-FEH IIb (Van den Ende *et al.*, 2001). Chicory fructan 1-exohydrolase IIa (1-FEH IIa) is a 61 kDa enzyme (De Roover *et al.*, 1999).

All glycosyl hydrolases are classified into a number of families based on general amino-acid sequence similarities (Henrissat, 1991; Henrissat & Davies, 1997). Chicory 1-FEH IIa belongs to family 32 of the glysosyl hydrolases. At present, no structural information is available for any member of this family, although the crystallization of some members has been reported (Arand *et al.*, 2002; Tsujimoto *et al.*, 2003).

A crystallographic study was therefore undertaken in order to determine the structure of chicory 1-FEH IIa. The elucidation of this structure will not only contribute to a better understanding of the catalytic mechanism of 1-FEH IIa, but may also provide a model for other important enzymes in family 32 (including invertases and fructan biosynthetic enzymes). Here, we describe the crystallization of 1-FEH IIa and its preliminary diffraction analysis.

2. Materials and methods

2.1. Purification

1-FEH IIa from *C. intybus* was cloned into pPICZ alpha vector and heterologously expressed in *Pichia pastoris* as described in

Kawakami & Yoshida (2002). Ammonium sulfate (80% saturation) was added to the yeast supernatant. After centrifugation (40 000*g*, 20 min at 277 K), the pellet was redissolved in 150 ml 50 m*M* sodium acetate buffer pH 5.0 containing 1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$ and 1 m*M* $MnCl_2$. Undissolved material was spun down for 10 min at 40 000*g* and 277 K. The supernatant was applied to a ConA Sepharose column (25 × 100 mm) and eluted as described in Van den Ende *et al.* (1996). Active fractions (adjusted to pH 4.7 with acetic acid) were subsequently loaded onto a Uno S column (Biorad) equilibrated with 50 m*M* sodium acetate buffer pH 4.7. A gradient of 0–0.3 *M* NaCl in 50 m*M* sodium acetate buffer pH 5.0 was used for protein elution. 1-FEH IIa eluted at 150 m*M* NaCl. The two most active fractions (purity was checked with SDS–PAGE) were used for crystallization.

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 Screens I and II (Jancarik & Kim, 1991; Cudney *et al.*, 1994) were used to determine the initial crystallization conditions using the hanging-drop vapour-diffusion method with Linbro multiwell tissue-culture plates (Molecular Dimensions). Several needle-shaped crystals were grown in a condition consisting of 1.6 *M* sodium potassium phosphate and 0.1 *M* HEPES pH 7.5 at a temperature of 277 K.

After optimizing different buffers, pH, drop size, protein and salt concentration, we obtained bar-shaped crystals with a reservoir solution containing 2.1 *M* sodium potassium phosphate and 0.1 *M* potassium phosphate pH 7.0. Drops consisting of 2 µl protein solution mixed with 1 µl reservoir solution were placed on cover slips and equilibrated against a reservoir containing 700 µl reservoir solution. Crystals, as shown in Fig. 1, appeared after approximately 4 d and reached maximum size about two weeks later. The dimensions of the largest crystals were 1.2 × 0.2 × 0.2 mm.

Figure 1
Crystals of 1-FEH IIa.

2.3. Data collection

Data collection was performed at 100 K (Oxford Cryosystems Cryostream) using an N_2 gas stream. Crystals were soaked for 4 min in a cryosolution containing 2.05 *M* sodium potassium phosphate, 0.1 *M* potassium phosphate pH 7.0 and 22.5% glycerol. Crystals were then mounted in cryoloops and flash-cooled in liquid nitrogen. Diffraction data were collected at the X11 beamline of the DESY synchrotron (Hamburg) with a MAR CCD 165 detector. This 2.35 Å resolution data set was collected at a wavelength of 0.81100 Å (Fig. 2).

3. Results and discussion

This data set was processed using *DENZO* and *SCALEPACK* v.1.97.2 (Otwinowski & Minor, 1997). The space group was assigned as $P4_12_12$ or $P4_32_12$. The unit-cell parameters are a = 139.83, b = 139.83, c = 181.94 Å. Data-collection statistics are summarized in Table 1. According to Matthews coefficient calculations (Matthews, 1974), the asymmetric unit should consist of two or three molecules per unit cell, with corresponding V_M values of 3.6 and 2.4 $Å^3$ Da^{-1} and solvent contents of 66 and 49%, respectively. However, only one peak was observed in a self-rotation function, corresponding to the origin peak. This peak has a signal-to-noise ratio of 12.3. All subsequent peaks were at noise level.

We are currently screening heavy-atom derivatives for phase determination, as no similar structure is known.

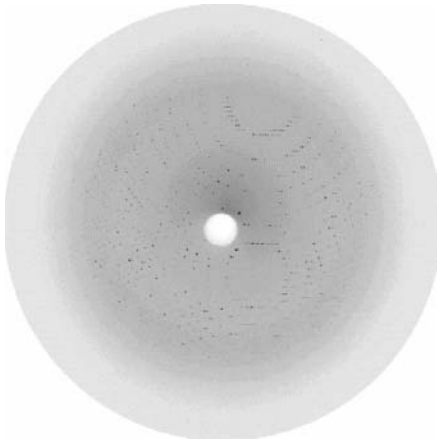

Figure 2
Diffraction pattern from the 1-FEHIIa crystal. The resolution of the outer edge of the image is 2.30 Å.

Table 1
Data-collection and reduction statistics.

Values in parentheses indicate data in the highest resolution shell (2.39–2.35 Å).

Wavelength used (Å)	0.81100
Resolution limit	2.35 (2.39–2.35)
Total observations	362168
Unique observations	75024 (3698)
Redundancy	4.83
Completeness (%)	99.1 (98.7)
Completeness [$I > 2\sigma(I)$] (%)	84 (58.1)
Mean $I/\sigma(I)$	12.62 (2.23)
R_{sym} (%)	7.5 (39.3)

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