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Crystallization and preliminary X-ray analysis of β-glucan exohydrolase isoenzyme ExoI from barley (*Hordeum vulgare*)

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Abstract

Crystals of a β-glucan exohydrolase purified from extracts of young barley seedlings have been obtained by vapour diffusion in the presence of ammonium sulfate and polyethylene glycol. The enzyme exhibits broad substrate specificity against (1,3)-, (1,3;1,4)- and (1,3;1,6)-β-glucans, and related oligosaccharides. Crystal dimensions of up to 0.8 × 0.4 × 0.6 mm have been observed. The crystals belong to the tetragonal space group $P4_12_12$ or $P4_32_12$. Cell parameters are $a = b = 102.1$ and $c = 184.5$ Å, and there appear to be eight molecules in the asymmetric unit. The crystals diffract to at least 2.2 Å resolution using X-rays from a rotating-anode generator.

1. Introduction

Two β-glucan exohydrolases have been purified from extracts of young barley seedlings 8 d after the initiation of germination (Hrmova *et al.*, 1996). The enzymes, which are designated isoenzymes ExoI and ExoII, have apparent molecular masses of 69 and 71 kDa, and isoelectric points of 7.8 and 8.0, respectively (Hrmova *et al.*, 1996). The complete amino-acid sequences of both isoforms have now been deduced from corresponding cDNAs (Hrmova *et al.*, 1996; A. J. Harvey and G. B. Fincher, unpublished work).

The barley β-glucan exohydrolases release glucose from the non-reducing ends of substrates, with retention of anomeric configuration (Hrmova *et al.*, 1996), but their classification into a single Enzyme Commission group has proved difficult. They exhibit similarities in substrate specificity and action pattern to plant (1,3)-β-glucan exohydrolases that have been classified in the E.C. 3.2.1.58 group (Cline & Albersheim, 1981; Lienart *et al.*, 1986; Labrador & Nevins, 1989; Kotake *et al.*, 1997) but, in addition to their ability to hydrolyse (1,3)-β-glucans, they are able to hydrolyze (1,3;1,4)-β-glucans, (1,3;1,6)-β-glucans, (1,4)-β-oligoglucosides and 4-nitrophenyl-β-glucoside (Hrmova *et al.*, 1996; Hrmova & Fincher, 1997).

Glycosyl hydrolases have also been classified into a number of families on the basis of amino-acid sequence alignments and hydrophobic cluster analyses (Henrissat & Bairoch, 1993). As more crystal structures of glycosyl hydrolases have been solved, it has become apparent that members of each family have similar three-dimensional conformations. Thus, barley (1,3)-β-glucan endohydrolases and (1,3;1,4)-β-glucan endohydrolases are both classified in family 17 and their $(\beta/\alpha)_8$ folds are very similar; the r.m.s. deviation in C^α positions is 0.65 Å over 278 amino-acid residues (Varghese *et al.*, 1994). Furthermore, if the X-ray structure of one member of a particular family is available, this facilitates the determination of structures of other members of the family, and new computer software programs are allowing the development of reliable three-dimensional models of sibling polypeptides from their primary amino-acid sequences (Rossman, 1972; Varghese *et al.*, 1994).

Sequence alignments and hydrophobic cluster analyses show that the barley β-glucan exohydrolases are members of the family 3 group of glycosyl hydrolases, for which there are currently no crystal structures available. Here, we describe methods for the crystallization of barley β-glucan exohydrolase isoenzyme ExoI, together with crystallographic data.

2. Materials and methods

2.1. *Protein purification*

The barley β-glucan exohydrolase isoenzyme ExoI was purified from extracts of 8 d germinated barley by fractional precipitation with ammonium sulfate, ion-exchange chromotagraphy and gel-filtration chromatography (Hrmova *et al.*, 1996). The purity of the enzyme preparation was confirmed by the presence of a single polypeptide band of apparent M_r 69 kDa on overloaded sodium dodecyl sulfate (SDS)–polyacrylamide gels, and by NH_2-terminal amino-acid sequence analysis, where yields were close to expected values and no secondary sequences were detectable (data not shown).

2.2. *Enzyme assays*

The activities of the purified enzyme and re-dissolved crystals were measured against laminarin from *Laminaria digitata*, barley (1,3;1,4)-β-glucan and 4-nitrophenyl-β-glucoside as described by Hrmova *et al.* (1996). The laminarin and 4-nitrophenyl-β-glucoside were obtained from Sigma Chemical Company (St Louis, MO, USA) and the barley (1,3;1,4)-β-glucan was from Megazyme (Boronia, Victoria, Australia).

2.3. *Crystallization*

The approximate precipitation point of the enzyme was determined at 277 K in a liquid dialysis button (Hampton Research, CA, USA). The enzyme solution was prepared at a concentration of 9 mg ml^{-1} in 10 m*M* sodium acetate buffer, pH 5.25 and 25 µl aliquots were tested against 1 ml 0.8–2.1 *M* ammonium sulfate solutions buffered with 100 m*M* citric acid–NaOH at pH 5.0 (Grid Screen Solutions, Hampton Research). Amorphous precipitates were formed at ammonium sulfate concentrations in the range 1.8–1.9 *M*. Crystals were subsequently grown by vapour diffusion, using the conventional hanging-drop method over 1 ml reservoirs in 24-well Linbro plates (Hampton Research) at 277–279 K. Enzyme drops [8 µl of a 6.8 mg ml^{-1} solution in 75 m*M* Hepes–NaOH buffer, pH

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7.0 containing 7.5 m*M* sodium acetate, 1.2%(*w/v*) polyethylene glycol 400 and 0.8 *M* ammonium sulfate] were placed on siliconized, 22 mm, circular glass cover slips (Hampton Research) over 1 ml 1.7 *M* ammonium sulfate in 50 m*M* Hepes–NaOH buffer, pH 7.0.

To remove crystals for enzyme activity determinations or for seeding other hanging drops, a rabbit whisker was drawn across the surface of the drop and adhering microcrystals were transferred to other drops or to assay solutions as required. For X-ray analysis, crystals were mounted in thin-walled capillaries with a drop of mother liquor.

2.4. *X-ray diffraction*

Data extending to 2.2 Å were collected at 291 K with a Rigaku R-AXIS II image-plate X-ray detector mounted on a MAC Science SRA M18XH1 rotating-anode X-ray generator operating at 47 kV and 60 mA with focusing optics. A total exposure time of 40 min of 1° oscillations was used, with a crystal-to-detector distance of 180 mm and a 2θ offset of 10°.

3. Results and discussion

After approximately 100 d, a single large crystal was observed in the hanging drop over the 1.7 *M* ammonium sulfate reservoir. The crystal's dimensions were approximately 0.8 × 0.4 × 0.6 mm and it was truncated bi-pyramidal in shape (Fig. 1). During the crystallization experiments the use of the Hepes–NaOH buffer and the inclusion of 1.2%(*w/v*) ethylene glycol 400 were found to be essential ingredients of the hanging drop. When the ethylene glycol 400 was omitted, amorphous precipitates of the enzyme often formed. Following the collection of the X-ray data, the single crystal was deposited in a hanging drop in which no precipitate or crystals were evident. This induced the growth of 100–150 microcrystals which were used to seed other hanging drops. Microcrystals (3–4) were transferred to new hanging drops of identical composition, and within 3 weeks they grew in size to about 0.35 × 0.25 × 0.25 mm.

The microcrystals (10–20) were also checked for enzyme activity. They were transferred from the hanging drop on a rabbit whisker, essentially free of solvent, to 50 µl 20 m*M* sodium acetate buffer, pH 5.25. The microcrystals dissolved immediately and retained high levels of activity against laminarin, barley (1,3;1.4)-β-glucan and 4-nitrophenyl-β-glucoside. Specific activity ratios of 20:1:2 against the three substrates, respectively, were very similar to those reported for the original enzyme after its purification from barley seedlings (Hrmova *et al.*, 1996). SDS–polyacrylamide gel electrophoresis of the re-dissolved crystals revealed a single polypeptide band of apparent M_r 69–70 kDa (data not shown).

The space group of the barley β-glucan exohydrolase isoenzyme ExoI crystals is $P4_12_12$ or $P4_32_12$ and the unit-cell dimensions are $a = b = 102.1$ and $c = 184.5$ Å, consistent with one monomer per asymmetric unit and a solvent content of about 63% (Matthews, 1968). The crystals diffract to about 2.2 Å with 93° data collected in 1° frames (Fig. 2). The data set was 86% complete to 2.30 Å, and 64% complete in the resolution shell of 2.45–2.30 Å. No data beyond 2.20 Å was observed and only 32% of the data in the 2.30–2.20 Å resolution shell was collected, because of radiation damage to the crystal.

An $R_{\rm merge}$ of 5.4% (14.2% in shell 2.30–2.20 Å) over 148 526 total observations reducing to 39 235 independent reflections (>1σ) was obtained with $\langle I\rangle/\langle\sigma(I)\rangle$ of 12.3.

In summary, we have shown that we can obtain useful X-ray diffraction data from crystals of barley β-glucan exohydrolase isoenzyme ExoI. In addition, platinum derivatives of the crystals have been obtained and mercury derivatives are currently being sought. If these derivatives are successfully generated, it should be possible to solve the first three-dimensional structure for a family 3 glycosyl hydrolase.

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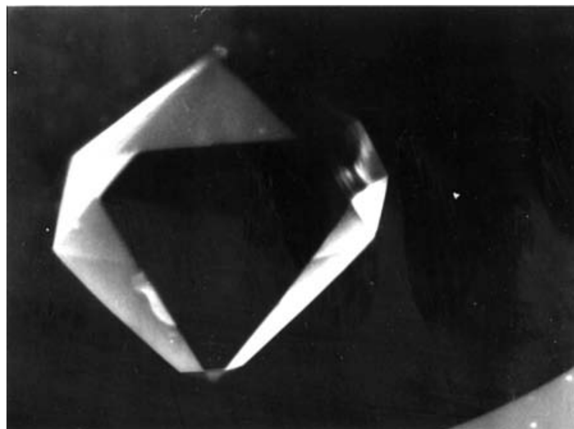

Fig. 1. Micrograph of a single crystal of barley β-glucan exohydrolase isoenzyme ExoI. The dimensions of the crystal are approximately 0.8 × 0.4 × 0.6 mm.

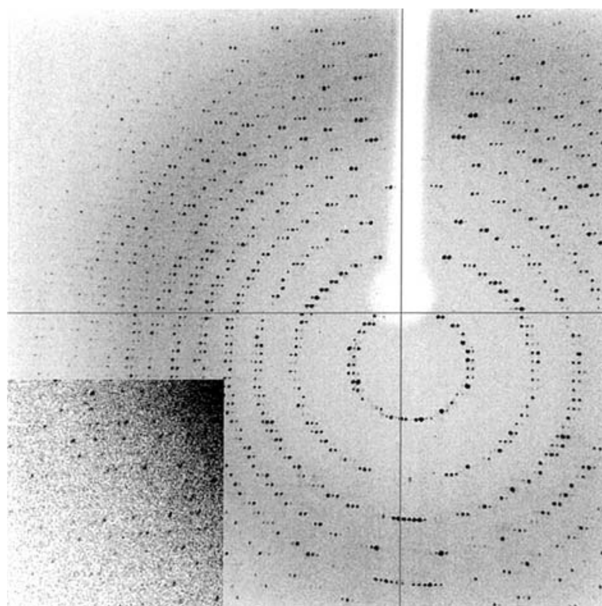

Fig. 2. Diffraction image of the barley β-glucan exohydrolase crystal. The X-rays are in a^* direction, b^* is the vertical (spindle axis) and c^* is horizontal. The left edge is 2.5 Å in length, the bottom corner is 2.2 Å, and the inset in the bottom left-hand corner shows diffraction intensities to 2.2 Å.

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