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Crystallization and preliminary X-ray diffraction study of a wheat (*Triticum aestivum* L.) TAXI-type endoxylanase inhibitor

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A TAXI-type endoxylanase inhibitor from *T. aestivum* L. wheat flour has been crystallized using the hanging-drop vapour-diffusion method. The needle-like crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 49.92, b = 66.45, c = 106.42 Å. From these crystals, a native data set and a gold-derivative data set were collected to 2.25 and 1.75 Å resolution, respectively. The heavy-atom derivative of this crystal form was obtained by the soaking method and allowed determination of the initial phases.

1. Introduction

Cereal grains contain three groups of important biopolymers: starch, proteins and non-starch polysaccharides. Starch and most of the proteins occur in the endosperm and serve as reserve material for the plant during germination and the initial stages of growth. The non-starch polysaccharides, which are to a large extent part of the cell walls, consist mainly of arabinoxylan and β-glucan. These components are hydrolyzed by xylanolytic and β-glucanolytic enzymes, respectively (Fincher & Stone, 1993). Degradation of the cell-wall polysaccharides during germination improves the accessibility of starch and protein to amylases and proteases (MacGregor & Fincher, 1993; Voragen *et al.*, 1987).

From a biotechnological point of view, endo-β-1,4-endoxylanases (also referred to as endoxylanases or xylanases; EC 3.2.1.8) are the most important xylanolytic enzymes, as they hydrolyze arabinoxylan internally. Endoxylanases from microbial origin are frequently used in biotechnological processes/applications such as bread-making, gluten/starch separation and animal feeding, with advantages for processing and/or product-quality parameters.

In this context, an important development is the discovery of proteinaceous inhibitors of endoxylanases in cereals such as wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.) and rye (*Secale cereale* L.) (Debyser *et al.*, 1997; Debyser, 1999). In the plant, these endoxylanase inhibitors may have a dual physiological function. They may possibly be important in the regulation of plant metabolism by inhibiting endogenous endoxylanases and/or they may play a role in plant defence by inhibiting the exogenous endoxylanases produced by microorganisms and predators (Gebruers *et al.*, 2001). Furthermore, in the biotechnological processes/applications mentioned above they may have a profound impact on the functionality of the endoxylanases used.

To date, two distinct types of endoxylanase inhibitors have been identified in cereals: TAXI (*T. aestivum* endoxylanase inhibitor) and XIP (xylanase-inhibiting protein) type inhibitors. The wheat TAXI-type inhibitors consist of at least two non-glycosylated xylanase inhibitors, *i.e.* TAXI I and TAXI II, both with molecular masses of 40 kDa. These isoforms show structural homology but differ from one another in pI (8.9 and at least 9.3, respectively) and endoxylanase specificity (Gebruers *et al.*, 2001, 2002). The XIP-type inhibitors isolated from wheat are monomeric glycosylated proteins with pI values of 8.7–8.9 and molecular masses of 29–32 kDa (McLauchlan *et al.*, 1999; Gebruers *et al.*, 2002). There is no sequence homology between the TAXI- and XIP-type endoxylanase inhibitors.

In a model of the molecular structure of the TAXI-type endoxylanase inhibitors, it is assumed that both isoforms, TAXI I and TAXI II, occur in two molecular forms, A and B (Debyser *et al.*, 1999). According to the model, after reduction with 2-mercaptoethanol form B dissociates into two fragments of approximately 10 and 30 kDa, whereas the molecular mass of form A is not affected by this treatment. Because the N-terminal sequences of the 30 and 40 kDa polypeptides are identical, the 10 and 30 kDa polypeptides of form B, held together by one or more disulfide bonds, are derived from form A by processing, possibly mediated by proteolytic enzymes. It is reasonable to speculate that form A is processed into form B in order to become more or less active. Processing of an α-amylase inhibitor from bean (*Phaseolus vulgaris* L.) seeds increases its activity (Santino *et al.*, 1992; Peuyo *et al.*, 1993).

In order to facilitate the study of the endoxylanase specificities and the inhibiting

mechanisms of the TAXI-type endoxylanase inhibitors, a crystallographic study of TAXI I, containing a mixture of forms A and B, has been undertaken. Here, we describe its crystallization and preliminary diffraction analysis.

2. Experimental

2.1. Purification

Wheat whole meal (cultivar Soissons, 5.0 kg) was extracted with 0.1%(*w*/*v*) ascorbic acid solution (25 l). After centrifugation (10 000*g*, 30 min, 280 K), 2.0 g l^{-1} $CaCl_2$ was added to the supernatant and the pH was raised to 8.5 with 2.0 *M* NaOH, which resulted in the formation of a precipitate. The resulting extract was centrifuged (10 000*g*, 30 min, 280 K) and the precipitate was discarded. The pH of the supernatant was adjusted to 5.0 with 2.0 *M* HCl. From the resulting solution, a concentrated fraction containing endoxylanase inhibitors was isolated by performing SP Sepharose Fast Flow (Amersham Biosciences, Uppsala, Sweden) cation-exchange chromatography at pH 5.0; proteins were eluted in one step with 0.5 *M* NaCl solution. Subsequently, the TAXI-type endoxylanase inhibitors were captured from the concentrated fraction by performing affinity chromatography with *Bacillus subtilis* endoxylanase immobilized on an *N*-hydroxysuccinimide-activated Sepharose 4 Fast Flow matrix (Amersham Biosciences, Uppsala, Sweden). Elution was performed by raising the pH from 5.0 (the optimal pH for binding of the TAXI-type inhibitors to the affinity column) to 12 (the pH at which dissociation of the inhibitor–enzyme complexes on the affinity column occurs). Portions of the resulting TAXI-type inhibitor fraction were then separated at pH 6.0 on a MonoS HR5/5 cation-exchange column (Amersham Biosciences, Uppsala, Sweden) using a linear salt gradient. The TAXI-type inhibitor eluting between 0.040 and 0.065 m*M* NaCl, *i.e.* TAXI I, was used for crystallization. The purification steps above are described in more detail in Gebruers *et al.* (2002).

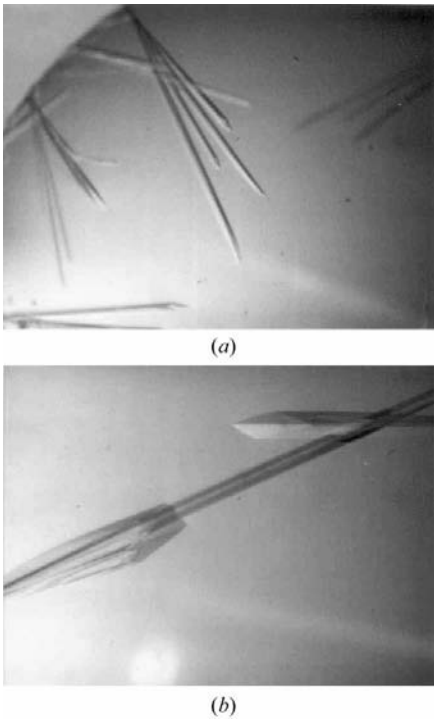

Figure 1
Needle-like crystals of TAXI I (*a*) before and (*b*) after optimization. The typical dimensions of diffraction-quality crystals are approximately 1.0 × 0.3 × 0.2 mm.

2.2. Crystallization

Prior to crystallization trials, the protein solution was further concentrated to approximately 10 mg ml^{-1} by ultrafiltration using a Microcon concentrator (Amicon) with a 3 kDa cutoff. To determine the initial crystallization conditions, Hampton Research Crystal Screens I and II (Jancarik & Kim, 1991; Cudney *et al.*, 1994) were applied using the hanging-drop vapour-diffusion geometry with Linbro multiwell tissue plates stored at room temperature. Each well was filled with 700 µl of reservoir solution and drops consisting of 1.5 µl protein solution and 1.5 µl of reservoir solution were placed on cover slips and equilibrated against the reservoir solutions. Several conditions containing polyethylene glycol and ammonium sulfate favoured crystal growth. However, the dimensions of these crystals could be improved substantially by optimizing the conditions. A solution containing 0.15 *M* ammonium sulfate, 0.1 *M* sodium acetate buffer pH 4.6 and 23%(*w*/*v*) polyethylene glycol 4000 yielded good crystals (Fig. 1).

2.3. Heavy-atom derivative search

More than ten different heavy-atom reagents at concentrations of 1.0 and 1.5 m*M*, directly injected into the drop several hours before data collection, were screened in-house in the heavy-atom derivative search. A derivative was successfully obtained when crystals were soaked in 1.5 m*M* $KAuCl_4$ solution for 20 h.

Table 1
Data-collection and reduction statistics.

Values in parentheses indicate data in the highest resolution shell.

	TAXI I ($KAuCl_4$ derivative)	TAXI I (native)
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters (Å)		
a	49.92	50.42
b	66.45	66.80
c	106.42	106.14
Wavelength used (Å)	0.94	1.54
Resolution limit (Å)	1.75 (1.78–1.75)	2.25 (2.29–2.25)
Total observations	356729	47340
Unique reflections	36679 (1797)	16333 (598)
Redundancy	9.72	2.90
Completeness (%)	99.8 (99.9)	92.4 (68.3)
Completeness ($I > 2\sigma$) (%)	91.7 (71.2)	86.8 (57.0)
Mean $I/\sigma(I)$	31.8 (6.5)	20.2 (6.86)
R_{merge} (%)	5.0 (34.1)	5.9 (19.8)

2.4. X-ray diffraction study

Collection of diffraction data from the native and derivative crystals was performed under liquid-nitrogen cryoconditions at 100 K (Oxford Cryosystems Cryostream). Crystals, cryoprotected by soaking for 30 s in reservoir solution containing 20% glycerol, were mounted in cryoloops and flash-cooled by plunging into liquid nitrogen. In-house diffraction data were collected on a MAR Research image-plate detector (345 mm) mounted on a Rigaku rotating-anode generator operating at 50 kV and 80 mA with Cu $K\alpha$ radiation and equipped with MSC mirrors. Crystals generally diffracted well. Good-quality diffraction to 2.25 Å could be obtained by employing macromolecular crystal annealing (Harp *et al.*, 1998), by simply blocking the nitrogen stream for 5 s.

Following the successful heavy-atom derivative search, a highly redundant SAD data set to a resolution limit of 1.75 Å was collected using synchrotron radiation (BW7A beamline of the DESY synchrotron, Hamburg).

3. Discussion

All data processing was performed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The space group was assigned as $P2_12_12_1$, with unit-cell parameters a = 49.92, b = 66.45, c = 106.42 Å. The final $KAuCl_4$ SAD data set is 99.8% complete and is characterized by an R_{sym} value of 5.0% and 9.7-fold redundancy. Further statistics are summarized in Table 1. According to Matthews coefficient calculations (Matthews, 1974), the asymmetric unit should consist of a single monomer, with a corresponding V_M of 2.21 $Å^3$ Da^{-1} and a solvent content of 47%. This high-

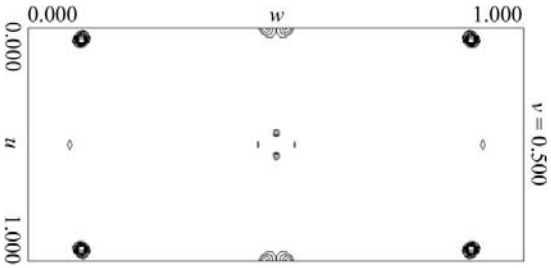

Figure 2
$v = 0.5$ section of the heavy-atom difference Patterson map of the gold derivative.

data set was used to locate the Au-atom site, considering that at the wavelength used (0.94 Å) the Au anomalous contribution to the scattering factor (f'') is 8.55 e^-. An anomalous difference Patterson map calculated in the resolution range 30–1.75 Å with *PHASES* (Furey & Swaminathan, 1997) clearly showed coherent peaks in the three Harker sections at $u = 0.5$, $v = 0.5$ and $w = 0.5$ (Fig. 2). The TAXI I structure solution using the SAD strategy based on the Au-atom position, confirmed and refined with *SHARP* (de Fortelle & Bricogne, 1997), is currently in progress.

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