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Purification, crystallization and preliminary X-ray study of β-xylosidase from *Trichoderma reesei*

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An extracellular multifunctional β-xylosidase was purified from a culture of the fungus *Trichoderma reesei*. The active 95 ± 5 kDa enzyme has been crystallized from sodium acetate buffer using PEG as a precipitant. The crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 67.75, b = 98.54, c = 227.25 Å, and diffract beyond 2.7 Å resolution. X-ray data were collected from frozen crystals on a synchrotron source.

1. Introduction

Hemicelluloses are the second most abundant polysaccharides in nature. The major constituents of hemicellulose found in grass, cereals and hardwoods are 1,4-β-D-xylans (Timell, 1967; Herrmann *et al.*, 1997). Xylan degradation is a multistep process involving a number of enzymatic activities (Dekker & Richards, 1976). Xylanases (1,4-β-D-xylan xylanohydrolases; E.C. 3.2.1.8) hydrolyse the internal β-1,4-xylosidic linkages of the xylan backbone. Xylosidases (1,4-β-D-xylan xylohydrolases; E.C. 3.2.1.37) in turn catalyse hydrolysis of 1,4-β-D-xylans, removing successive D-xylose residues from the non-reducing termini (Fig. 1), whereas arabinofuranosidases (α-L-arabinofuranoside arabinofuranohydrolase; E.C. 3.2.1.55) hydrolyse terminal non-reducing α-L-arabinofuranoside residues in α-L-arabinosides.

β-Xylosidases belongs to the glycosyl hydrolase family 39, which itself forms part of the wider GH-A clan (Henrissat, 1991; Henrissat & Bairoch, 1993, 1996). No crystallographic structures have been reported to date for the family 39 glycosyl hydrolases.

T. reesei β-xylosidase is active toward unbranched xylans, glucuronoxylans and β-1,4-xylooligosaccharides with different degrees of polymerization. The only resulting product of all these reactions is D-xylose, which characterizes β-xylosidase as an exoglycosidase (Herrmann *et al.*, 1997). A transglycosylation activity, apparent at high concentration of xylooligosaccharides or *p*-nitrophenyl β-xylopyranoside, may be used for the syntheses of xylopyranoside-containing compounds. The enzyme is a glycoprotein with mixed (*N*- and *O*-) types of glycosylation. The molecular weight estimated by SDS–PAGE is 95 ± 5 kDa and may differ depending on the fungus cultivation conditions and duration. The primary structure of the protein is known and exhibits considerable similarity to the primary structures of the *Aspergillus niger* and *A. nidulans* β-xylosidases and to the primary structures of the family 3 β-glucosidases (Margolles-Clark *et al.*, 1996; Perez-Gonzalez *et al.*, 1998). The molecular weight calculated from the amino-acid content (Margolles-Clark *et al.*, 1996) is 87 190 Da. The protein glycan content is consequently estimated to be 3–13%.

An additional α-L-arabinofuranosidase activity has been reported for the β-xylosidase from *T. reesei* (Margolles-Clark *et al.*, 1996; Herrmann *et al.*, 1997). This fact was checked thoroughly and cannot be explained by any contamination activities. The preliminary results of cross-inhibitor analysis have led to

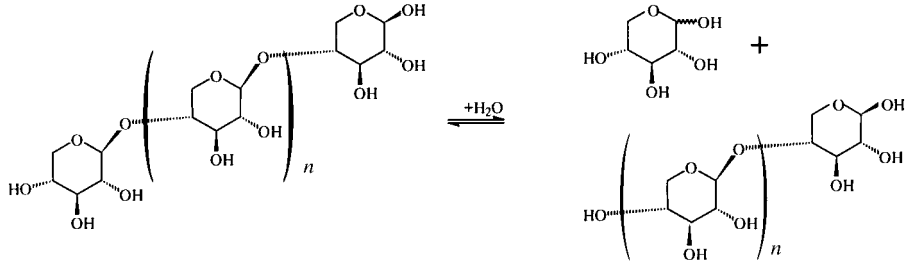

Figure 1
β-Xylosidase catalytic activity. β-Xylosidase catalyses hydrolysis of 1,4-β-D-xylans by the removal of successive D-xylose residues from non-reducing termini.

the conclusion that the two active sites of the enzyme are different (Neustroev, unpublished data). Remarkably, a minor β-xylosidase activity of α-L-arabinofuranosidase from *T. reesei* toward *p*-nitrophenyl-β-D-xylopyranoside has also been reported (Margolles-Clark *et al.*, 1996).

In the present work, we describe the purification, crystallization and synchrotron X-ray diffraction data collection of the *T. reesei* β-xylosidase as a first step towards determining the X-ray structure of the enzyme. Detailed knowledge of the three-dimensional fold of the enzyme will help in understanding its activity and will provide a first example of the glycosyl hydrolase family 39 structure.

2. Protein purification, crystallization and data collection

The β-xylosidase was purified from a 10 l culture of *T. reesei* after 48–60 h of cultivation as previously described (Savel'ev *et al.*, 1997). After desalting and concentrating using hollow-fibre technology, the supernatant was applied to an SP-Sephadex column equilibrated with 50 m*M* sodium acetate buffer pH 4.10 and eluted with three steps of 0.2, 0.4 and 0.6 *M* sodium chloride in the same buffer. The β-xylosidase fraction was concentrated with an Amicon concentrator and a PM 50 membrane and further purified by gel-permeation chromatography on a Sephacryl S-300 column. Finally, the fractions containing β-xylosidase activity were pooled, concentrated and chromatographed on a MonoQ ion-exchange column equilibrated with 20 m*M* Tris–HCl buffer pH 7.4 and eluted with a linear gradient (0–0.6 *M*) of sodium chloride in the same buffer. A single peak corresponding to β-xylosidase activity was collected, diluted three times with eluting buffer and repurified under the same conditions. The resulting purified enzyme was dialysed against water and lyophilized. SDS–PAGE shows a single wide band typical of glycoproteins and corresponding to a MW of 95 kDa. Comparison of this value with data from analytical gel-permeation chromatography suggests that the enzyme exists as a monomer at pH 4–6. The activity of the enzyme during purification was monitored using *p*-nitrophenyl β-xylopyranoside as substrate (Eneyskaya *et al.*, 1997).

For crystallization trials, the lyophilized protein was dissolved in water at concentrations of 5–20 mg ml^{-1}; 5–10 µl drops of the protein solution were mixed with equal volume of 15% PEG 3350 (Sigma) in 50 m*M* sodium acetate pH 5.86. Hanging drops were equilibrated against 1 ml of the same PEG solution at room temperature. Bipyramidal crystals started to grow in 1–2 d and after 5 d reached a maximum size of 0.5 mm in each dimension. The size of the crystals strongly depended on the initial protein concentration. Protein solutions of initial concentration of 10–15 mg ml^{-1} gave crystals of maximum size.

For data collection, a single crystal was flash-frozen in a gaseous nitrogen flow at ~100 K (Oxford Cryosystems). X-ray data were collected by the oscillation method on a MAR Research 345 image-plate detector at the LNLS protein crystallography beamline (Polikarpov, Oliva *et al.*, 1998; Polikarpov, Perles *et al.*, 1998). The X-ray wavelength was set to 1.38 Å to maximize the signal-to-noise ratio and to optimize the speed of data collection (Polikarpov *et al.*, 1997; Teplyakov *et al.*, 1998). The crystal-to-detector distance was set to 200 mm and the oscillation range was 1°.

Table 1
Data-collection statistics.

Values in parentheses refer to the last resolution shell.

Parameter	Experimental results
Number of frames	162
Space group	$P2_12_12_1$
Unit-cell dimensions at 85 K (Å, °)	a = 67.736, b = 99.049, c = 227.276, $\alpha = \beta = \gamma = 90$
Resolution range (Å)	30.0–2.7 (2.76–2.7)
Total number of reflections	116360 (6187)
Number of unique reflections	40004 (2489)
Redundancy	2.9 (2.5)
R_{sym}† (%)	9.1 (32.0)
Completeness (%)	93.6 (90.1)
$\langle I/\sigma(I)\rangle$	12.6 (2.9)

† $R_{sym} = \sum(I - \langle I\rangle)/\sum I$.

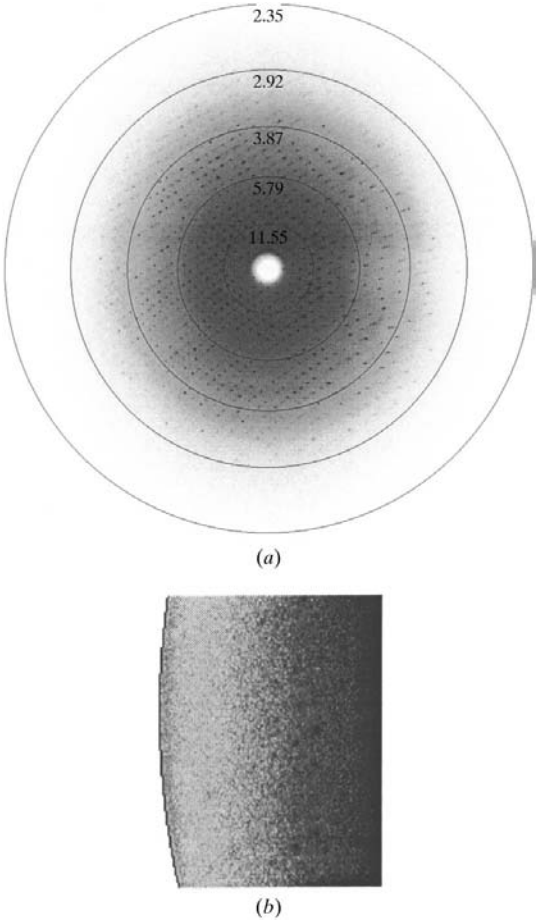

Figure 2
Representative diffraction image. (*a*) Full-view example of the collected frames. (*b*) Enhanced contrast close-up of the frame border (to the left to the beamstop).

3. Results and discussion

Among the many factors affecting the crystallization of glycoproteins, the main ones are the degree of glycosylation and the heterogeneity of the glycan content. It has been shown (Neustroev *et al.*, 1993) that during microorganism cultivation the sugar components of glycoproteins change dramatically, causing pronounced changes in the biophysical properties of the glycoprotein, particularly its solubility. The cultivation conditions for *T. reesei* growth were optimized to obtain a form of the glycoprotein most suitable for crystallization. At short cultivation times, a high degree of glycosylation did not allow the precipitation of β-xylosidase with PEG, whereas when long cultivation times were used the protein lost its solubility easily at low ionic strength. Slow culture-growth conditions resulted in a protein that was readily crystallizable. Remarkably, the first crystals of highly glycosylated β-xylosidase only diffracted to 3.8 Å resolution. Optimization of the cultivation conditions permitted us to significantly extend the diffraction limit of the crystals (Fig. 2).

The crystals were found to belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 67.75, b = 98.54, c = 227.25 Å. Useful X-ray data were collected to 2.7 Å resolution. X-ray diffrac-

tion data were processed using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The data-collection statistics are presented at Table 1. Freezing the crystals improved their stability in the X-ray beam significantly, allowing us to improve the data-collection statistics. Since no suitable model for molecular replacement has been found despite exhaustive sequence comparison searches, we will have to use the heavy-atom replacement method for the structure solution. Screening for heavy-atom derivatives is under way.

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