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Crystallization and preliminary X-ray crystallographic analysis of a *Trichoderma reesei* β-mannanase from glycoside hydrolase family 5

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Crystals of the catalytic core domain of a *Trichoderma reesei* β-mannanase belonging to glycoside hydrolase family 5 have been grown by the sitting-drop method at room temperature using ammonium sulfate as precipitant. The crystals grow as thin colourless plates and belong to space group $P2_1$, with unit-cell parameters $a = 50.0$, $b = 54.3$, $c = 60.2$ Å, $\beta = 111.3^\circ$, and have a single monomer of mannanase in the asymmetric unit. Native data to 2.0 Å resolution have been collected at room temperature using synchrotron radiation. Data for a platinum derivative have been collected to 1.65 Å at 110 K in a very short time at the CCLRC Daresbury synchrotron source, using a charge-coupled device (CCD) as detector.

1. Introduction

Hemicelluloses are linear or branched heteropolysaccharides which together with cellulose and lignin form the major structural components of plant cell walls (Zimmerman *et al.*, 1992). They are divided into different classes depending on the sugar composition, among which the two most important representatives are the hetero-1,4-β-D-xylans and the hetero-1,4-β-D-mannans. Heteromannans may be grouped into two types of polysaccharides: mannans, where the backbone consists only of β-1,4-linked mannopyranose units, and glucomannans, where there are also glucopyranose units within the backbone. The presence of D-galactose groups linked (by β-1,6-linkages) to the mannose or glucose units of the main polymer chain generates the galactoglucomannans.

Endo-1,4-β-D-mannanases (E.C. 3.2.1.78) catalyse random hydrolysis of the β-1,4-mannopyranosyl linkages within the main chain of mannans and heteromannans. In recent years, growing interest has been focused on the hemicellulases owing to their possible applications in the food and feed, and pulp and paper industries. A great deal of research has been devoted to finding alternative bleaching agents to replace chlorine-based chemicals. Hemicellulases such as xylanases and mannanases have been reported to improve bleaching results. Since the most advantageous working conditions for an enzyme which facilitates lignin removal whilst simultaneously not being harmful to the paper pulp are high temperature and alkaline conditions, enzymes able to function under these extreme conditions would be suited for technical processes. Therefore, knowledge of the tertiary structure of these mannanases is important for the development of engineered enzymes.

Mannanases are found in many of the 61 glycoside hydrolase families (Henrissat & Bairoch, 1996; Henrissat & Davies, 1997). They use acid/base catalytic machinery to promote glycosidic bond hydrolysis. The β-mannanase from *T. reesei* is a family 5 glycoside hydrolase (Stålbrand *et al.*, 1993, 1995). It is a particularly interesting enzyme in that it also possesses an additional domain which is separated from the catalytic domain by a flexible linker. This domain is highly homologous to fungal cellulase-binding domains (CBD) and also mediates binding of the mannanase to cellulose. The intact mannanase with CBD and linker appears to resist crystallization. In this paper, we present the construction and expression of a mannanase catalytic core domain, its crystallization and preliminary X-ray diffraction analysis. In addition, heavy-metal derivative data to 1.65 Å were collected using a charge-coupled device (CCD) detector in just 30 min, confirming the power of these devices for synchrotron applications.

2. Experimental methods

2.1. Extraction and purification

T. reesei strain ALKO4330 was constructed to express the core protein of *T. reesei* mannanase (amino acids 1–346; Stålbrand *et al.*, 1995) under the *cbh*1 promoter using methods described in Karhunen *et al.* (1993). The transformed strain overproduces mannanase core protein and is unable to produce two of the major cellulases of *Trichoderma* (CBHI and EGII) because of targeting of the expression cassette to the *cbh*1 locus of the parent

strain ALKO3620, which is already unable to produce EGII after prior replacement of the native *egl2* locus by a marker gene (Roal Oy, unpublished work). *T. reesei* ALKO4330 was cultivated in a fermentor on lactose-based medium, and the supernatant was separated from mycelium by centrifugation, concentrated by ultrafiltration and used for the purification of mannanase core protein.

The concentrate (750 ml, containing 69 g protein) was clarified and some of the impurities were removed by bentonite treatment at pH 4.0 (Zurbriggen *et al.*, 1990). The chromatographic purification started with anion-exchange chromatography on DEAE Sepharose FF (Pharmacia) equilibrated with 6 m*M* sodium phosphate pH 7.2. Before the DEAE run, buffer exchange of the enzyme preparation (750 ml) was carried out using Sephadex G-25c, and the conductivity and pH of the buffered preparation were adjusted to correspond exactly to those of the equilibration buffer. The sample (2.25 l) was applied to a DEAE Sepharose FF column (BP113, Pharmacia; 2 l volume and 20 cm bed height). The column was first eluted with equilibration buffer (12.6 l) and subsequently with a stepwise gradient of NaCl in equilibration buffer to a final concentration of 1 *M* to remove the adsorbed material. The fractions containing mannanase activity were pooled (7380 ml) and subjected to further purification by hydrophobic interaction chromatography.

A phenyl Sepharose FF column (Pharmacia; total volume 2 l and bed height 20 cm) was equilibrated with 20 m*M* sodium phosphate pH 6.0 containing 0.35 *M* ammonium sulfate. The conductivity of the pool from anion-exchange chromatography was adjusted to correspond to that of equilibration buffer by addition of $(NH_4)_2SO_4$. The sample was applied to the column, which was first eluted with equilibration buffer (15 l) and subsequently with 20 m*M* sodium phosphate buffer pH 6.0, distilled water and 6 *M* urea to remove the adsorbed material. The pool containing mannanase core protein was collected during the sample application and elution by equilibration buffer. This mannanase pool was applied in two batches (each 6.3 l) to the same phenyl Sepharose FF column equilibrated with 20 m*M* sodium phosphate pH 6.0 containing 0.75 *M* ammonium sulfate. In each batch the breakthrough was discarded, and the adsorbed mannanase eluted with 8 m*M* sodium acetate pH 4.0. The column was washed with distilled water and 6 *M* urea to remove the other adsorbed proteins. The pools containing mannanase core protein (total volume 7.2 l) were further purified by cation-exchange chromatography on CM Sepharose FF (Pharmacia).

Before the CM Sepharose run, the enzyme preparation buffer (750 ml) was changed to 10 m*M* sodium acetate by Sephadex G-25c, as the sample still contained some ammonium sulfate. The conductivity and pH of the buffered preparation were adjusted to correspond exactly to those of the equilibration buffer. The sample (14 l) was applied to a CM Sepharose FF column (BP113, Pharmacia; 2 l volume, 20 cm bed height) equilibrated with 10 m*M* sodium acetate pH 4.0. The column was first eluted with equilibration buffer (5 l) and subsequently with a 0–10 m*M* sodium acetate pH 4.8 gradient in equilibration buffer. The elution was continued with this buffer until mannanase activity had eluted out (16 l). The other adsorbed proteins were eluted with 1 *M* NaCl. The fractions containing mannanase activity were pooled (7.38 l, containing 15.6 g mannanase core protein) and the purity of the protein was confirmed by gel electrophoresis in denaturing conditions (SDS–PAGE).

Protein was assayed by the method of Lowry *et al.* (1951) using bovine serum albumin (Sigma) as the standard.

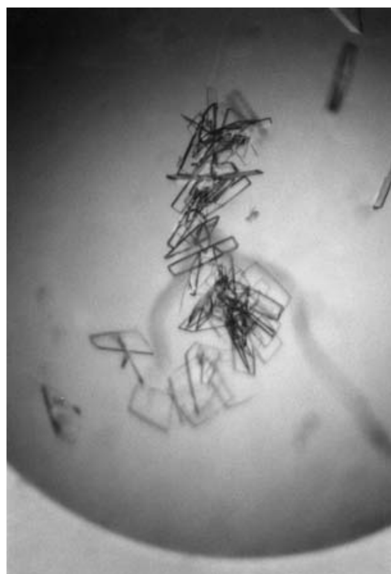

Figure 1
Crystals of the catalytic core domain of the *T. reesei* mannanase. Typical crystal dimensions are up to 0.7 × 0.4 × 0.05 mm.

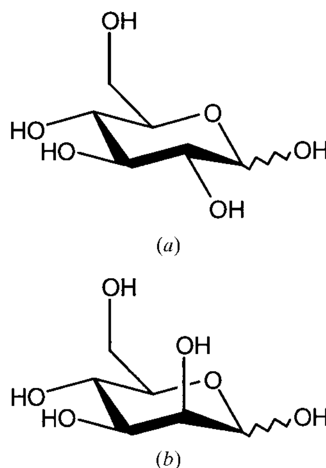

Figure 2
Schematic representation of (*a*) glucose and (*b*) its C2 epimer, mannose. Enzymes are found within glycoside hydrolase family 5 which are entirely specific for each epimer.

2.2. Crystallization

Mannanase was concentrated using a Filtron 30K membrane in 10 m*M* Tris pH 7.5 buffer. Crystals were grown using sitting-drop vapour diffusion. 4 µl sitting drops containing 2 µl of protein (approximately 22 mg ml^{-1} in 10 m*M* Tris pH 7.5) and 2 µl of reservoir solution (0.1 *M* Tris–HCl pH 8.5, 2 *M* ammonium sulfate) were equilibrated at room temperature. Crystals grew within two weeks, producing single thin plates with dimensions up to 0.7 × 0.4 × 0.05 mm (Fig. 1). A potential platinum derivative was prepared by soaking a crystal in a stabilizing solution containing 5 m*M* platinum (II) (2,2′:6′,2″ terpyridine) chloride for 2 d.

2.3. Data collection and processing

Native data were collected at room temperature from a single crystal mounted in a capillary on beamline X11 at the EMBL Hamburg outstation. 174° of data were collected to 2.0 Å resolution. Data for a potential Pt derivative were collected at the Daresbury SRS beamline PX9.6 using the ADSC Quantum 4 CCD detector at a wavelength of 0.89 Å, during the initial testing of this device at the SRS. 180° of data were collected in order to permit complete coverage of the anomalous data. The images were integrated using the *HKL* suite (Otwinowski & Minor, 1997).

3. Results and discussion

Crystals of the *T. reesei* mannanase catalytic core are in monoclinic space group $P2_1$ with unit-cell parameters a = 50.0, b = 54.3, c = 60.2 Å, β = 111.3°. If we assume one molecule in the asymmetric unit, the resulting V_M is 1.90 Å^3 Da^{-1}, indicative of a solvent content of 35% (Matthews, 1968). Data, scaled and reduced using the *HKL* suite of programs (Otwinowski & Minor, 1997), have an $R_{\rm merge}$ of 0.030 to 2.0 Å

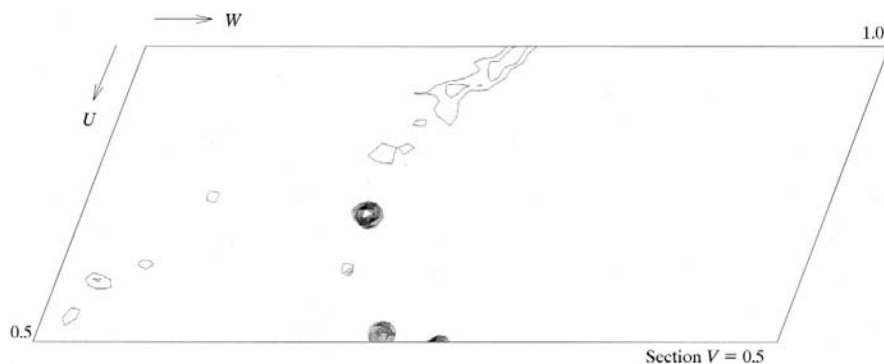

Figure 3
Harker section $V = 1/2$ of the anomalous difference Patterson for the platinum derivative of the *T. reesei* family 5 mannanase at 1.65 Å resolution. The map is contoured every 0.76σ, starting at 3σ.

resolution. In the outer resolution shell (2.03–2.00 Å), the $R_{\rm merge}$ is only 0.034 with an $I/\sigma(I)$ of 21, indicating that the true diffraction limit for these crystals is much better than 2 Å.

Glycoside hydrolase family 5 (Henrissat & Bairoch, 1996; Henrissat & Davies, 1997) contains many enzymes hydrolysing β-1,4 and β-1,3 glycosidic bonds, the majority of which are cellulases whose sequences are only distantly related to the *T. reesei* mannanase described here. Structures for a number of cellulases from this family are known (Ducros *et al.*, 1995; Dominguez *et al.*, 1995; Sakon *et al.*, 1996; Davies *et al.*, 1998), but none were successful as molecular-replacement search models for the *T. reesei* mannanase. For this reason, a search for heavy-metal derivatives was initiated. Data for a potential platinum derivative were collected to 1.65 Å at the Daresbury Synchrotron Radiation Source using a CCD as detector. Unfortunately, these data proved to be non-isomorphous with the native data, displaying a mean fractional isomorphous difference of over 0.40. However, the anomalous difference Patterson at 1.65 Å resolution shows two peaks on the Harker section $V = 1/2$ with peak heights of over 29σ (Fig. 3), which can readily be interpreted in terms of two sites. The rapid read-out of the CCD meant that these 180° of data were collected in just 35 min elapsed time on beamline PX 9.6, indicating the enormous power of these detectors for synchrotron applications.

Future work will concentrate on three directions. Firstly, we will continue to search for further isomorphous heavy-atom derivatives. Secondly, we will attempt to use the anomalous signal from the non-isomorphous Pt derivative to obtain initial phases for the protein. Thirdly, as the crystals clearly diffract well beyond 1.65 Å, we will extend the native data to as close as possible to atomic resolution.

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