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Crystallization and preliminary crystallographic analysis of two β-mannanase isoforms from *Thermomonospora fusca* KW3

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Abstract

Three β-mannanase isoforms were isolated from the supernatant of a thermophilic actinomycete culture from *Thermomonospora fusca* KW3. Two of the isoforms (Q1, Q1.1) were crystallized by the hanging-drop method at room temperature using ammonium sulfate as a precipitant. The isoforms form rod-shaped colorless crystals. Both belong to the orthorhombic space group $P2_12_12_1$. The cell dimensions are $a = 46.7$, $b = 61.1$, and $c = 128.2$ Å for isoform Q1, and $a = 43.8$, $b = 46.2$, and $c = 132.8$ Å for isoform Q1.1. The asymmetric unit of either isoform contains one mannanase molecule. Native data have been collected to 2.2 Å resolution for Q1 and to 1.65 Å resolution for Q1.1 using synchrotron radiation.

1. Introduction

Hemicelluloses are linear or branched polysaccharides mostly found as heteroglycans in higher terrestrial plants. Depending on the sugar composition they are divided into xylans, mannans, arabinogalactans and arabinans. The two most important representatives are the hetero-1,4-β-D-xylans and the hetero-1,4-β-D-mannans (galacto- and glucomannans). Together with cellulose and lignin they form the most important structural components of plant cell walls (Zimmermann, 1991).

Hemicellulose-degrading microorganisms are frequently found in soil or compost and in the rumen of animals. To completely hydrolyze (hetero)-mannans fungi and bacteria have to produce at least a mannanase (E.C. 3.2.1.78), a β-mannosidase (E.C. 3.2.1.25) and an α-galactosidase (E.C. 3.2.1.22) (McCleary, 1983). Mannanases (1,4-β-D-mannanmannanohydrolases) hydrolyze the 1,4-β-mannanopyranose bond in galacto-, gluco- and galactoglucomannans to manno-oligomers, mannobiose and mannose. The degree of hydrolysis of galactomannan decreases with increasing substitution by galactose.

High-temperature and broad-pH tolerant glycosyl hydrolases such as xylanases enhance bleaching of pulps in the pulp and paper industries thereby reducing the consumption of chlorine bleaching chemicals (Zimmermann, Davies, Winter & Zhou, 1992; Ciba-Geigy, 1995). In this process the addition of mannanases could improve the bleaching results. However, peroxide- and ozone-based procedures are still more frequently used. To offer environmentally safe alternatives competitive with conventional chemical bleaching compounds, optimized and cheap enzymes are necessary. Enzymes possessing thermostable properties would be particularly suited for technical processes since usually elevated temperatures are required. Therefore, the knowledge of the tertiary structure together with the availability of the gene sequence of these mannanases are essential for the development of engineered enzymes.

The thermophilic actinomycete *Thermomonospora fusca* secretes three β-mannanases with very similar properties. All three mannanases have an isoelectric point around 3.9, the same temperature optimum of 352 K and a pH optimum between 6 and 8. They are glycoproteins, and no differences in their mode of action have been found so far (Tajana & Zimmermann, 1995). However, matrix-assisted laser desorption ionization mass spectroscopy (LDI-MS) experiments revealed different molecular weights (Q1 38.1, Q1.1 37.9 and Q2 47 kDa), and an analysis of the amino-acid composition shows a very similar but not completely identical amino-acid distribution for all three forms. As deduced from N-terminal amino-acid sequence, the first 42 amino acids of all three forms are identical (Tajana, 1993; Tajana & Zimmermann, 1996). Therefore, the difference in the molecular weights is likely to result from a different degree of glycosylation. However, other processing, *e.g.*, proteolytic cleavage, could also contribute to molecular weight heterogenity. Initial genomic analysis also indicates that the isoforms are derived from a single gene (unpublished results).

The *T. fusca* mannanases share 52.4% amino-acid sequence identity with *Streptomyces lividans* β-mannanase in the N-terminal 42 amino acids. According to the classification of glycosyl hydrolases (Henrissat, 1991; Henrissat & Bairoch, 1993) the β-mannanase from *S. lividans* belongs to the cellulase family A (glycosyl hydrolase family 5). Recently the structure of two representatives, cellulases CelC and CelCCA, of this family has been reported (Dominguez *et al.*, 1995; Ducros *et al.*, 1995) showing a TIM barrel as the common motif.

We report here the crystallization and a preliminary X-ray diffraction study of two mannanase isoforms from *T. fusca* KW3 as the first step towards structure determination.

2. Materials and methods

To purify β-mannanases from *T. fusca* KW3 42 l of cultivation medium were centrifuged. Then, the supernatant was lyophilized. About 10 g of lyophilisate were dissolved in double-distilled water, and the mannanases were precipitated in the 40–70% ammonium sulfate fraction. After desalting, the proteins were redissolved and the mannanases were prepurified by an anion-exchange chromatography run (MonoQ HR 16/10, Pharmacia). The mannanases were finally purified by affinity chromatography with galactomannan as a substrate and the isoforms were separated by an additional anion-exchange chromatography run (MonoQ HR 16/10, Pharmacia). During purification, the activity was monitored using an enzyme assay with galactomannan as substrate (Sinner & Puls, 1978; Tajana, 1993). Mannanase-containing fractions were pooled, desalted by PD-10 columns (Pharmacia) and concentrated to 4–7 mg ml^{-1} with Centricon-30 spin columns (Amicon). First crystallization trials were performed following a fast-screening

protocol (Jancarik & Kim, 1991) by mixing 5 µl protein solution with the same volume of reservoir solution using the hanging-drop method at room temperature.

Native data were collected for Q1 and for Q1.1 crystals at room temperature using synchrotron radiation from beamline X31 ($\lambda = 0.99$ Å) at the EMBL outstation at Hamburg. The data were processed with *DENZO* and *SCALEPACK* (Otwinowski, 1993).

3. Results and discussion

Affinity chromatography and a single anion-exchange chromatography step (MonoQ HR 16/10) allowed to separate Q1 and Q1.1 almost completely, although they differ only slightly in their p*I*. Intitial crystallization conditions of the proteins purified in this way were found (0.1 *M* Tris pH 8.5, 2.0 *M* ammonium sulfate), but these crystals were exclusively intergrown. Further improvement of the protein quality was achieved by including an ammonium sulfate precipitation step and an additional anion-exchange step. A small variation of the initial crystallization conditions yielded long rod-like crystals using the purer protein. The final conditions were 0.1 *M* Hepes pH 7, 1.8 *M* ammonium sulfate for Q1 and 0.1 *M* sodium citrate pH 6.5, 2.0–2.2 *M* ammonium sulfate for Q1.1. Exposing these crystals to X-ray radiation during 24 h revealed high crystal stability. Precession photographs and the results of the diffraction data processing showed that the crystals from both isoforms belong to the orthorhombic space group $P2_12_12_1$ (Fig. 1). The cell dimensions are $a = 46.7$, $b = 61.1$, and $c = 128.3$ Å for the isoform Q1, and $a = 43.8$, $b = 46.2$, and $c = 132.8$ Å for the isoform Q1.1. The cell volumes are consistent with one molecule in the asymmetric unit with V_m of 2.40 Å^3 Da^{-1} and a solvent content of 49% by volume for Q1 and V_m of 1.81 Å^3 Da^{-1} and a solvent content of 32% by volume for Q1.1. These values are in the usual range for proteins (Matthews, 1968). We do not yet know whether this difference in packing density is due to the slightly different crystallization conditions used or reflects structural differences between the two isoforms.

Table 1. *Data-collection statistics*

		Isoform Q1	Isoform Q1.1
Resolution (Å)		100–2.2	100–1.65
No. of reflections	Total	78492	152207
	Unique	15908	30041
Completeness (%)	Overall	82.2	89.7
$R_{linear}(I)$	Overall	0.062	0.089

The results of the data collection applying no σ-cutoff are summarized in Table 1. A search for heavy-atom derivatives and the cloning of the respective mannanase gene(s) are in progress. Furthermore, we also attempt the solution of the mannanase crystal structure(s) by molecular replacement.

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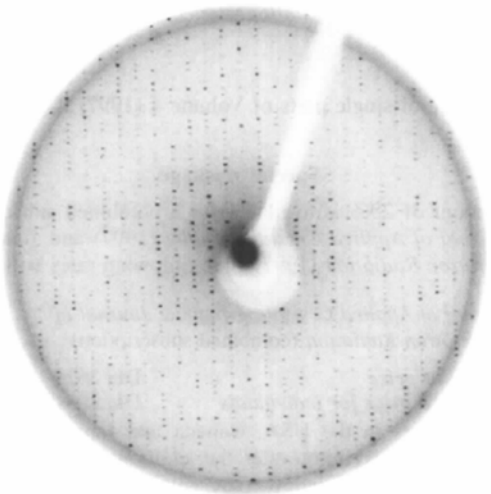

Fig. 1. Precession photograph (*h*0*l* zone) of a β-mannanase (Q1) crystal utilizing Cu $K\alpha$ X-rays. The precession angle is 13° and the crystal-to-film distance is 75 mm.

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