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Crystallization and preliminary X-ray crystallographic analysis of recombinant β -mannosidase from *Aspergillus niger*

β -Mannosidase (EC 3.2.1.25) is an important exoglycosidase specific for the hydrolysis of terminal β -linked mannoside in various oligomeric saccharide structures. β -Mannosidase from *Aspergillus niger* was expressed in *Pichia pastoris* and purified to clear homogeneity. β -Mannosidase was crystallized in the presence of D-mannose and the crystal diffracted to 2.41 Å resolution. The crystal belonged to space group *P*1, with unit-cell parameters $a = 62.37$, $b = 69.73$, $c = 69.90$ Å, $\alpha = 108.20$, $\beta = 101.51$, $\gamma = 103.20^\circ$. The parameters derived from the data collection indicate the presence of one molecule in the asymmetric unit.

1. Introduction

β -Mannosidase (β -D-mannoside mannohydrolase, EC 3.2.1.25) is an important glycoside hydrolase (GH) specific for the hydrolysis of terminal β -linked mannosides in various sugar chains. This enzyme plays an essential role in the complete hydrolysis of β -mannans to mannose and therefore β -mannosidases are very efficient in various industrial processes, such as hydrolysis of galactomannans used for the improved removal of drilled material in oil and gas drilling or coffee extraction, and as a bleach-boosting agent in the pulp and paper industries (Moreira & Filho, 2008). β -Mannosidases are also used in the synthesis of oligosaccharides or alkyl β -mannosides for medical and other purposes (Itoh & Kamiyama, 1995; Ademark *et al.*, 1999; Nashiru *et al.*, 2001).

There is a 29% sequence identity in the sequence alignment of β -mannosidase and another well described *exo*-acting glycoside hydrolase (also β -mannosidase), belonging to the 32nd family of the GH2 glycoside hydrolases (known structure as a homologue, pdb code 2je8, Tailford *et al.*, 2007). GH2 contains over 700 sequences encoding enzymes with a wide spectrum of different *exo*-acting β -glycosidase activities including β -galactosidase (EC 3.2.1.23), β -mannosidase (EC 3.2.1.25), β -glucuronidase (EC 3.2.1.31) and *exo*- β -glucosaminidase (EC 3.2.1.-). Recently, the structures of β -galactosidases from *Escherichia coli* (Skálová *et al.*, 2005), *exo*- β -glucosaminidase from *Amycolatopsis orientalis* (van Bueren *et al.*, 2009) and the β -glucuronidase from *Homo sapiens* (Jain *et al.*, 1996) were reported. Detailed structural information that could help in better understanding the functional role and the conformational behaviour of the substrate in the retaining hydrolase mechanism of β -mannosidase (Tailford *et al.*, 2008) is therefore really needed. Here, we report the crystallization and preliminary X-ray crystallographic analysis of recombinant β -mannosidase overexpressed in *Pichia pastoris*.

2. Materials and methods

2.1. Expression and purification

The recombinant extracellular β -mannosidase from *Aspergillus niger* (a gene product of the *A. niger* strain 513.88, accession no. XM_001394595) was prepared as described previously by Fliedrová *et al.* (2012) in *P. pastoris*. A signal peptide of wild-type β -mannosidase

was replaced by a signal peptide of the pPICZaC expression vector for extracellular production of the protein. No tag was appended to

the recombinant protein and no further post-translational modification has been observed (Fliedrová *et al.*, 2012).

Table 1

Data-collection statistics for β -mannosidase crystals.

Values in parentheses are for the highest-resolution shell.

	Native	I3C derivative
Space group	<i>P</i> 1	<i>P</i> 1
Unit-cell parameters ($\text{\AA}$, $^\circ$)	$a = 62.37$, $b = 69.73$, $c = 69.90$, $\alpha = 108.20$, $\beta = 101.51$, $\gamma = 103.20$	$a = 61.82$, $b = 65.23$, $c = 68.72$, $\alpha = 108.63$, $\beta = 101.06$, $\gamma = 103.06$
Wavelength ($\text{\AA}$)	0.9180	1.5419
Resolution ($\text{\AA}$)	40.91–2.41 (2.54–2.41)	40.28–2.44 (2.58–2.44)
$R_{\text{merge}}^\dagger$	0.188 (0.804)	0.125 (0.605)
$\langle I/\sigma(I) \rangle$	7.8 (2.2)	7.4 (2.0)
Completeness (%)	97.7 (91.8)	90.7 (88.5)
Multiplicity	3.9 (3.8)	3.9 (3.8)
No. of reflections	154978 (20449)	124224 (17536)
No. of unique reflections	39352 (5358)	31919 (4545)
Matthews coefficient ($\text{\AA}^3 \text{Da}^{-1}$)	2.56	2.34
Molecules in asymmetric unit	1	1

$$^\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl).$$

2.2. Crystallization

The purified protein was concentrated to 7.5 mg ml^{-1} and dialysed against 10 mM MOPS pH 7. The crystallization of β -mannosidase was carried out using the sitting-drop vapour-diffusion method at 290 K. Initial crystallization screening was performed using the screening kits Classics, Classics II, Classics Lite, PACT, PEGs and PEGs II (Qiagen). The protein solution with $250 \text{ } \mu\text{g ml}^{-1}$ of D-mannose was dispensed in three droplets (25, 50, 100 nl) with the help of the crystallization robot Mosquito (TTP LabTech Ltd, Melbourn, United Kingdom). The crystallization solution was added to the droplets such that the total volume of a droplet was 0.2 μl . After 10–14 d, non-coloured, transparent crystals were obtained. Crystals suitable for diffraction experiments were obtained under the following conditions: (i) 0.2 M magnesium chloride, 25% (w/v) polyethylene glycol 3350, 0.1 M bis-tris pH 6.5 (Classics II) and (ii) 0.2 M calcium chloride, 25% (w/v) polyethylene glycol 4000, 0.1 M Tris pH 8.5 (PEGs II). The growth of the crystals was observed not just in the visible region of light but also in the UV region (Rigaku Minstrel DT UV imager, Rigaku Corporation, Texas, USA) to distinguish the protein crystals from salt (Fig. 1).

Figure 1

(a) Classics II condition: 0.2 M magnesium chloride, 25% (w/v) polyethylene glycol 3350, 0.1 M bis-tris pH 6.5 (droplet with β -mannosidase crystals in visible and UV region of light). (b) PEGs II condition: 0.2 M calcium chloride, 25% (w/v) polyethylene glycol 4000, 0.1 M Tris pH 8.5 (droplet with β -mannosidase crystals in visible and UV region of light).

2.3. Data collection and analysis

The crystals used for native data collection and single-wavelength anomalous dispersion (SAD) data collection (Fig. 2) were taken from the optimized condition which consisted of 0.2 M magnesium chloride, 25%(w/v) polyethylene glycol 3350, 0.1 M bis-tris pH 6.5 (hanging drop). Native data were collected with the protein co-crystallized with D-mannose. In the case of the SAD experiment, 0.1 M 5-amino-2,4,6-triiodoisophthalic acid was used as a co-crystallization agent (I3C derivative; Beck *et al.*, 2008). In order to collect data at a cryogenic temperature, crystals that had been soaked in cryoprotectant (40% PEG 400 in the crystallization solution) were cooled in liquid nitrogen and mounted on the goniostat under a nitrogen-gas stream at 100 K. Diffraction data were collected on BL14.1 and BL14.2 operated by the Helmholtz-Zentrum Berlin (HZB) at the BESSY II electron storage ring (Berlin-Adlershof, Germany) (Mueller *et al.*, 2012). A complete native data set for β -mannosidase was collected to 2.41 Å resolution at an X-ray wavelength of 0.918 Å. The SAD data set was collected to 2.44 Å resolution at an X-ray wavelength of 1.542 Å. For the data sets, 360 frames of 6.0 s (10.0 s SAD experiment) exposure time and 1.0° oscillation were collected. Diffraction images from the SAD experiment were indexed and integrated using the program *iMOSFLM* (Battye *et al.*, 2011) and the diffraction images from the native data collection were indexed and integrated using the program *XDS*

(Kabsch, 2010). Scaling was carried out using *SCALA* in the *CCP4* program package (Winn *et al.*, 2011). Molecular replacement and initial refinement for the native data set were carried out using the programs *MOLREP* (Vagin & Teplyakov, 2010) and *REFMAC5* (Murshudov *et al.*, 2011), respectively, from the *CCP4* program package. The data-collection statistics and preliminary results obtained are summarized in Table 1.

(a)

(b)

Figure 2

Drops from optimization step with 0.1 M bis-tris pH 6.5, 20%(w/v) of PEG 3350 and 0.25 M MgCl₂; native crystals of β -mannosidase. (b) Drops from optimization step with 0.1 M bis-tris pH 6.5, 20%(w/v) of PEG 3350 and 0.3 M MgCl₂; crystals of β -mannosidase co-crystallized with I3C derivative.

(a)

(b)

Figure 3

Diffraction patterns of (a) native data collection and (b) SAD data collection for β -mannosidase.

3. Results and discussion

The initial screening showed two conditions for obtaining the crystals: (i) 0.2 M magnesium chloride, 25% (w/v) polyethylene glycol 3350, 0.1 M bis-tris pH 6.5 and (ii) 0.2 M calcium chloride, 25% (w/v) polyethylene glycol 4000, 0.1 M Tris pH 8.5. The best crystals were produced by further optimization using the hanging-drop vapour-diffusion method. The main precipitants PEG 3350 and PEG 4000 were changed in small concentration steps. The trials showed the best possible concentration was 20% (w/v) for PEG 3350 and PEG 4000. The long needles were observed to be more compact with a small increase in the salt concentration (MgCl_2 or CaCl_2); the effective range of the concentration is 0.2–0.4 M. The crystals of the β -mannosidase were typically needles with dimensions $0.25 \times 0.01 \times 0.08$ mm. The crystals belonged to space group *P*1. The β -mannosidase in the native data set diffracted to 2.41 Å resolution (Fig. 3) and had unit-cell parameters $a = 62.37$, $b = 69.73$, $c = 69.90$ Å, $\alpha = 108.20^\circ$, $\beta = 101.51^\circ$, $\gamma = 103.20^\circ$. The calculated Matthews coefficient (V_M) (Matthews, 1968) of $2.56 \text{ Å}^3 \text{ Da}^{-1}$ with a solvent content of 52.06% indicates the presence of one molecule in the asymmetric unit. The molecular-replacement method was performed with the structure of the β -mannosidase from *Bacteroides thetaiotaomicron* (PDB entry 2je8) as a model, but a suitable solution was not found. The β -mannosidase in the SAD data set diffracted to 2.44 Å resolution (Fig. 3) and had unit-cell parameters $a = 61.82$, $b = 65.23$, $c = 68.72$ Å, $\alpha = 108.63^\circ$, $\beta = 101.06^\circ$, $\gamma = 103.06^\circ$. The calculated Matthews coefficient (V_M) of $2.34 \text{ Å}^3 \text{ Da}^{-1}$ with a solvent content of 47.42% indicates the presence of one molecule in the asymmetric unit. Experimental phasing, model fitting and refinement are in progress.

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