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Expression, purification and preliminary crystallographic analysis of the recombinant β-glucosidase (BglA) from the halothermophile *Halothermothrix orenii*

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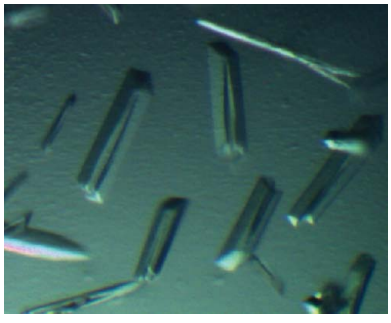
The β-glucosidase A gene (*bglA*) has been cloned from the halothermophilic bacterium *Halothermothrix orenii* and the recombinant enzyme (BglA; EC 3.2.1.21) was bacterially expressed, purified using metal ion-affinity chromatography and subsequently crystallized. Orthorhombic crystals were obtained that diffracted to a resolution limit of 3.5 Å. The crystal structure with two molecules in the asymmetric unit was solved by molecular replacement using a library of known glucosidase structures. Attempts to collect higher resolution diffraction data from crystals grown under different conditions and structure refinement are currently in progress.

1. Introduction

Halothermothrix orenii, which was isolated from a Tunisian salt lake, is one of the few thermophilic anaerobes that grow anaerobically under both thermophilic and halophilic conditions (with an optimum temperature of 333 K and preferred salt conditions of 5–10% NaCl; Cayol *et al.*, 1994). The genome of *H. orenii* has recently been sequenced (Mijts & Patel, 2001; Mavromatis *et al.*, 2009). In a bid to understand the adaptation of this organism to thermohalophilic conditions, we have previously characterized its amylase A (amyA), amylase B (amyB), sucrose phosphate synthase (SPS) and fructokinase (FRK) and determined the three-dimensional crystal structures of these enzymes (Li *et al.*, 2002; Tan *et al.*, 2003, 2008; Huynh *et al.*, 2005; Sivakumar *et al.*, 2006; Chua *et al.*, 2008, 2010). As part of our continuing studies on *H. orenii*, in this report we present the cloning, overexpression, purification and crystallization of a β-glucosidase (EC 3.2.1.21). β-Glucosidases act on β-1,1, β-1,2, β-1,4 and β-1,6 glucosidic bonds and are found in all members of Bacteria, Archaea and Eukarya (Parry *et al.*, 2001; Park *et al.*, 2005; Jeng *et al.*, 2010). They are known to play a fundamental role in biological processes ranging from the metabolism of cellulose and other carbohydrates to developmental regulation and chemical defence against pathogen invasion. Furthermore, these enzymes are of importance for various industrial applications such as biomass conversion, flavour enhancement and production of biodegradable nonionic surfactants (Park *et al.*, 2005). β-Glucosidases are classified as members of glycosyl hydrolase families 1 and 3 according to the Carbohydrate-Active Enzymes (CAZy) database (Cantarel *et al.*, 2009; http://www.cazy.org).



2. Material and methods

2.1. Expression and purification of recombinant β-glucosidase

The β-glucosidase A gene (NCBI No. YP_002509272.1; encodes 451 amino acids) was amplified by PCR using the upstream primer 5′-TTC**CATATG**ATGGCAAAAATAATATTTC-3′ (the *Nde*I restriction-enzyme site is shown in bold) and the downstream primer 5′-TA**CTCGAG**TCA*ATGATGGTGATGGTGATG*CTTTTAGTTAG-CTTCAACC-3′ (the *Xho*I restriction-enzyme site is shown in bold and the oligonucleotide expressing the hexahistidine fusion peptide is italicized). The PCR product was digested with *Xho*I and *Nde*I and the digested product was purified using a QIAquick gel-extraction kit (Qiagen, Germany), inserted into pET22b(+) expression vector

(Novagen) using T4 DNA ligase (New England Biolabs) and transformed into Rosetta 2 (DE3) competent cells (Novagen) according to the manufacturer's instructions. A clone designated rBGLA that expresses the recombinant protein (residues 1–451 plus the C-terminal His_6 tag) was selected for further studies. The rBGLA clone was grown at 310 K in 1 l LB broth supplemented with 100 µg ml^{-1} ampicillin and 35 µg ml^{-1} chloramphenicol to an OD_{650} value of 0.6. IPTG was added to a final concentration of 1 m*M* and the culture was incubated at 310 K for a further 6 h to induce expression of BglA. The cells were then harvested by centrifugation at 8000*g* for 10 min and resuspended in 5 ml 100 m*M* HEPES pH 7.0, 4 mg ml^{-1} lysozyme, 4 U ml^{-1} DNaseI (the amounts given are per gram of cells). The mixture was incubated at 310 K for 30 min and sonicated on ice (three bursts of 5 s with a 2 min rest on ice after each sonication). The resulting cell lysate was clarified by centrifugation at 18 000*g* for 30 min at 278 K and the supernatant was filtered using a low-protein-binding PVDF filter (Millipore, USA). The resulting filtrate was loaded onto an $NiSO_4$-charged Fractogel resin column (Merck) and incubated for 1 h at 278 K with gentle rotation on a tube rotator. The resin was loaded into a column and washed with 20 m*M* Tris–HCl pH 7.9 and 75 m*M* imidazole containing 500 m*M* NaCl. BglA was eluted with 100 and 350 m*M* imidazole and the resulting eluates were mixed. Removal of imidazole and concentration of BglA were achieved simultaneously using an ultrafiltration device with a cutoff of 30 kDa (U-tube concentrator, Novagen) at 5000*g* for 8 min at 298 K. The progress of the purification procedure was monitored by SDS–PAGE under reducing and denaturing conditions, where the recombinant protein appeared as a 53 kDa species, which was in agreement with its theoretical molecular mass. The protein was diluted to a concentration of 12 mg ml^{-1} (Bradford, 1976) in 20 m*M* HEPES pH 7.2, 100 m*M* NaCl and used in crystallization experiments.

2.2. Crystallization

Initial crystallization conditions were screened manually by the sitting-drop vapour-diffusion technique using 576 different conditions from a selection of >1000 conditions available in-house. 96-well plates (Molecular Dimensions, UK) were set up manually using 2 µl recombinant BglA (12 mg ml^{-1}) mixed with 2 µl reservoir solution and were equilibrated against 100 µl reservoir solution at 289 K. An optimization strategy was designed to grow the crystals by the hanging-drop method in 24-well plates using conditions that had previously yielded crystals in the 96-well plates.

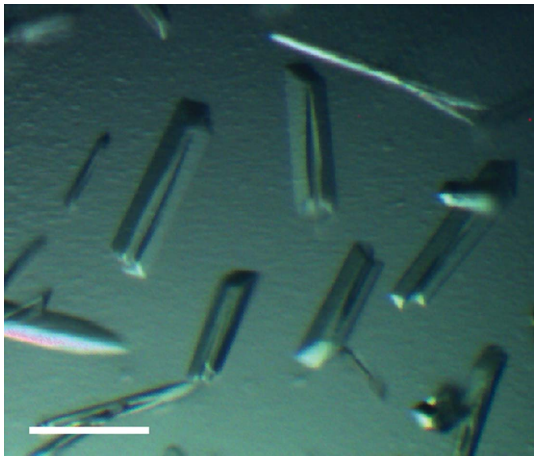


Figure 1
Rod-shaped crystals of *H. orenii* β-glucosidase with typical dimensions of 0.5 × 0.1 × 0.1 mm. The white bar indicates 0.5 mm.

Table 1
Diffraction data statistics.

Values in parentheses are for the highest resolution shell.

X-ray source	Rigaku MicroMax-007 HF
Detector	R-AXIS IV^{++}
Wavelength (Å)	1.5418
Temperature (K)	130
Sample-to-detector distance (mm)	300
Rotation per image (°)	1
Total rotation (°)	180
Exposure time (s)	120
No. of crystals	1
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 95.51, b = 104.60, c = 105.40
Resolution range (Å)	25–3.5 (3.69–3.50)
Wilson B factor (Å^2)	37
Mosaicity (°)	0.22
Total No. of measurements	98832 (14470)
No. of unique reflections	13826 (1977)
Multiplicity	7.1 (7.3)
Completeness (%)	100 (100)
R_{merge}†	0.173 (0.338)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity and $\langle I(hkl)\rangle$ is the average intensity obtained from multiple observations of symmetry-related reflections after rejections.

2.3. Data collection and analysis

Crystals were picked up from the crystallization drop using nylon loops and cryoprotected with a mixture of Paratone-N (Hampton, US) and mineral oil (Oxoid, Australia) in a 1:1(*v*:*v*) ratio (Hope, 1988). X-ray diffraction data were obtained using an in-house diffractometer consisting of a Rigaku MicroMax-007 HF generator, VariMax optics and an R-AXIS IV^{++} detector. The data were processed with *XDS* (Kabsch, 2010) and *SCALA* from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994). Self-rotation functions were calculated with *GLRF* (Tong & Rossmann, 1990).

2.4. Structure solution

The structure was solved by molecular replacement using *PHENIX* (Adams *et al.*, 2010) and a library of search models. The library consisted of 29 known structures of glucosidases, each of which was present as a wild-type and a polyalanine model, yielding a total of 58 individual models. The best solution for two molecules in the asymmetric unit was obtained with a log-likelihood score of 810 for the wild-type model of β-glucosidase B from an uncultured bacterium (52% sequence identity; PDB code 3fj0; K. Y. Hwang & K. H. Nam, unpublished work). The second best solution was obtained for β-glucosidase from *Streptomyces* sp. (47% sequence identity; PDB code 1gnx; A. Guasch, M. Vallmitjana, R. Perez, E. Querol, J. A. Perez-Pons & M. Coll, unpublished work) with a score of 777. Visual inspection and manual model building was carried out with *O* (Jones *et al.*, 1991) and *Coot* (Emsley & Cowtan, 2004).

3. Results and discussion

The β-glucosidase A gene of *H. orenii* was successfully cloned and overexpressed in Rosetta 2 (DE3) cells and purified using the IMAC method. The enzyme was confirmed to be active at high temperatures (ranging from 318 to 333 K; data not shown).

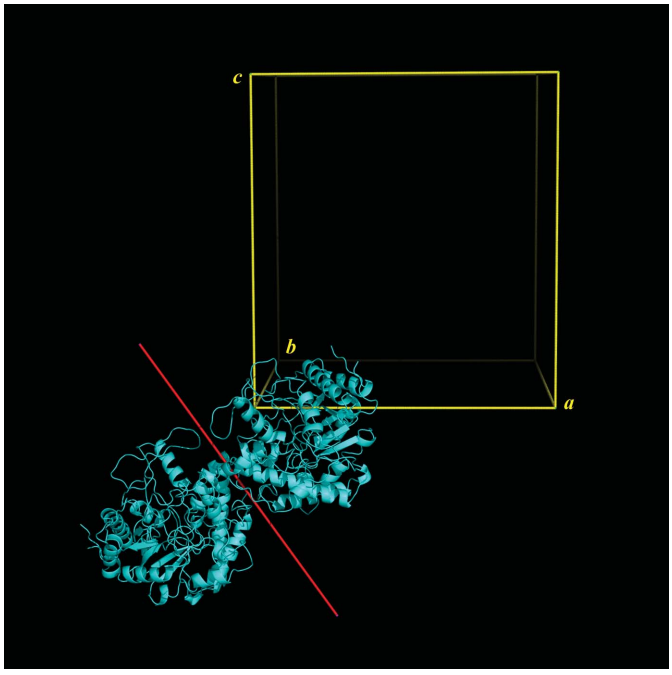


Figure 2
Location of the noncrystallographic twofold axis with respect to the unit cell (yellow). The two monomers in the asymmetric unit (cartoon model in blue) are related by a twofold NCS axis (red) which makes angles of 54° with *a*, 36° with *c* and 90° with *b*.

A total of 61 of the 576 conditions in the 96-well plates yielded crystals. Their shape varied from small squares to large rods; more needle-like crystals were observed at high PEG concentrations.

As a large number of crystals were observed in conditions with PEG (PEG 1500, 3400, 3500 and 6000) and monovalent salts (such as sodium formate, sodium acetate, NaBr, NaI, $NaNO_3$, NaF *etc.*) in the pH range 5.0–8.5, 0.2 *M* sodium formate, 20%(*w*/*v*) PEG 3400, 0.1 *M* bis-tris propane pH 7.5 was selected for optimization. For this, a PEG 3400 concentration gradient from 15 to 30% on one axis and a pH of 6.5–9.0 on the second axis was used with the 24-well hanging-drop method. Crystals appeared in less than 24 h using a solution consisting of 20%(*w*/*v*) PEG 3400, 0.1 *M* bis-tris propane pH 7.5, 0.1 *M* sodium formate (Fig. 1).

The crystals from the 96-well sitting-drop plates were of lesser quality and diffracted X-rays to a maximum of 4.5 Å resolution, whereas the crystals from the 24-well plate hanging-drop method appeared to be of better quality; they diffracted X-rays to 3.5 Å resolution and belonged to an orthorhombic space group. Crystal parameters and data-collection statistics are summarized in Table 1. Based on the presence of serial extinction patterns on [*h*00], [0*l*0] and [00*k*], space group $P2_12_12_1$ was assumed and was subsequently confirmed by the results from molecular replacement, which was carried out in all possible orthorhombic space groups.

A Matthews coefficient (Matthews, 1968) of 2.7 $Å^3\,Da^{-1}$ with a corresponding solvent content of 54% made two molecules in the asymmetric unit the most likely scenario. Additionally, the self-rotation function for $\kappa = 180°$ yielded four noncrystallographic peaks with $\varphi = 54°$, 36°, 126° and 144° and with $\psi = 90°$ for all peaks. This suggested the presence of a noncrystallographic twofold axis perpendicular to [010]. Using the model obtained by molecular replacement, the rotation axis relating the two monomers in the asymmetric unit was calculated using *SYMMETRY* (Hofmann & Wlodawer, 2002). The axis is visualized in Fig. 2 and makes angles of 54° with the *a* axis, 35° with the *c* axis and 90° with the *b* axis, which is in agreement with the peaks found in the self-rotation function.

Refinement of the structure of β-glucosidase A from *H. orenii* is currently in progress (R = 0.260, R_{free} = 0.382). Since it proved difficult to obtain crystals of the apoenzyme with better diffraction properties, efforts are currently under way to grow ligand-bound crystals (including cellobiose, lactose *etc.*) in order to obtain high-resolution data.

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