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Expression, purification, crystallization and preliminary X-ray diffraction analysis of *Bifidobacterium adolescentis* xylose isomerase

Xylose isomerase (EC 5.3.1.5) is a key enzyme in xylose metabolism which is industrially important for the transformation of glucose and xylose into fructose and xylulose, respectively. The *Bifidobacterium adolescentis* *xylA* gene (NC_008618.1) encoding xylose isomerase (XI) was cloned and the enzyme was overexpressed in *Escherichia coli*. Purified recombinant XI was crystallized using the sitting-drop vapour-diffusion method with polyethylene glycol 3350 as the precipitating agent. A complete native data set was collected to 1.7 Å resolution using a synchrotron-radiation source. The crystals belonged to the orthorhombic space group $P2_12_12$, with unit-cell parameters $a = 88.78$, $b = 123.98$, $c = 78.63$ Å.

1. Introduction

The demand for biofuels based on renewable resources is growing and is expected to increase significantly in the future. Biomass is an important source of inexpensive and renewable sugars, which can be transformed into cellulosic ethanol in a biotechnological process which involves pre-treatment, enzymatic hydrolysis, fermentation and distillation (Wyman, 1994; Naik *et al.*, 2010). Hexoses (*e.g.* glucose) can be readily fermented by industrial *Saccharomyces cerevisiae* strains, whereas pentoses such as xylose cannot. To give an example, sugar-cane bagasse, an abundant agroindustrial residue in Brazil, is composed of 28% hemicellulose, of which xylose is a major component (Pandey *et al.*, 2000). *S. cerevisiae* cannot ferment xylose into ethanol, but it has the ability to utilize xylulose (Hahn-Hägerdal *et al.*, 2001; Wang *et al.*, 2004). Xylose isomerase (XI; EC 5.3.1.5), an intracellular enzyme which converts xylose to xylulose, is one of the key enzymes of xylose metabolism (Jeffries & Shi, 1999). It is widely present in most microorganisms as an important enzyme that addresses a nutritional requirement in bacteria and fungi that proliferate on decaying plant biomass. Generally, XI has two distinct molecular domains: an N-terminal catalytic domain and a C-terminal auxiliary domain. The tertiary structure of the catalytic domain of crystallized XIs revealed an architecture comprised of a globular fold consisting of an eight-stranded α/β -barrel, whereas the C-terminal domain is formed by a loop of interconnected helices which interacts with the N-terminal domain of another subunit. XIs form dimers and tetramers which display biological activity (Bhosale *et al.*, 1996).

The enzyme is widely utilized in the conversion of D-glucose to D-fructose, which is mostly used in the high-fructose corn syrup (HFCS) industry (Wiseman, 1975); hence its alternative name glucose isomerase (GI).

Thermostable XIs/GIs have been widely studied (Henrick *et al.*, 1989; Collyer & Blow, 1990; Chang *et al.*, 1999; Gérczei *et al.*, 1999; Sriprapundh *et al.*, 2003; Kluskens *et al.*, 2010) with the aim of discovering more stable enzymes that are capable of working at high temperatures, which might be advantageous in industrial settings (for example, to avoid bacterial contamination during HFCS production). However, for the fermentation of hemicellulose in second-generation ethanol production it is very important to match the best yeast

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fermentation conditions, pH 4.6–5.0 (Maia, 1989; Ratnam *et al.*, 2003) and a temperature of 305–312 K (Banat *et al.*, 1998), to the optimal conditions for catalytic activity of XIs. Thus, XIs from mesophiles could be attractive for such applications.

Bifidobacterium adolescentis xylose isomerase has 446 amino-acid residues and has less than 50% sequence identity to other XIs of known structure. Here, we report the cloning of the *xylA* gene and the expression, purification, crystallization and preliminary diffraction analysis of recombinant XI from *B. adolescentis*.

2. Materials and methods

2.1. Expression and purification

2.1.1. Bacterial strains, plasmids, enzymes and reagents. *Escherichia coli* DH5a and *E. coli* BL21 (DE3) cells (Invitrogen, Carlsbad, California, USA) were used for the cloning and expression of His-tagged xylose isomerase, respectively. The plasmid pPROEX-HTa (Invitrogen) was used to construct the ligation-independent cloning (LIC) vector for cloning. Phusion High Fidelity DNA Polymerase (Finnzymes, Espoo, Finland) was used for all of the amplifications performed in this work. The T4 DNA polymerase used in this work was obtained from Fermentas Life Sciences. The crystallization kits were purchased from Hampton Research and Qiagen.

2.1.2. Construction of the expression plasmids. LIC (Aslanidis & de Jong, 1990) was used to construct the recombinant vector pPROEX-*xylA*, which carries the *xylA* gene.

Firstly, the pPROEX vector was entirely amplified using Phusion High Fidelity DNA Polymerase to create a linear pPROEX vector. This linear product was treated with T4 DNA polymerase (Sambrook & Russell, 2001), which has 3'–5' exonuclease activity, and dTTP nucleotides. This treatment was necessary to create the LIC free and cohesive ends. These ends were created after amplification from *B. adolescentis* genomic DNA, with the primer sequences sense, 5'-CAGGGCGCCATGGGTCTGTGGGATATCG-3', and antisense, 5'-GACCCGACGCGGTACGCCTCGGCCAGGG-3', and treated with T4 DNA polymerase plus dATPs. Finally, the pPROEX vector and the gene inserts were combined at room temperature, generating the recombinant plasmid pPROEX-*xylA*, without ligase in the reaction. The resulting plasmid was designed to express the gene with a histidine affinity-tag sequence. The *E. coli* DH5a cells were transformed by electroporation as a propagation step. *E. coli* BL21 (DE3) cells were then transformed by heat shock to generate the xylose isomerase expression strain.

2.1.3. Production and purification. The cells harbouring the recombinant vector were aerobically cultured at 310 K in Luria–Bertani (LB) medium (1% bactotryptone, 0.5% yeast extract, 1% NaCl) containing 100 mg ml⁻¹ ampicillin (final concentration). After 4 h, when the OD (absorbance at 600 nm) had reached 0.6–0.8, *xylA* was induced by the addition of 0.5 mM IPTG (final concentration). Subsequently, the temperature was decreased to 291 K and the culture was spun at 150 rev min⁻¹ for 16 h. The cells were harvested by centrifugation (2000g, 35 min) and resuspended in 20 ml buffer A [50 mM HEPES pH 8.0, 300 mM NaCl, 5 mM imidazole, 5% glycerol, 1 mM PMSF, 1 mM DTT, 0.6% (m/v) sarkosyl] per litre of culture. Lysozyme was added to the suspension to a final concentration of 250 µg ml⁻¹ and it was left for 60 min. The lysate was then sonicated in ice bath using a 550 Sonic Dismembrator Sonifier (Fisher Scientific). Cell debris was removed by centrifugation (6000g, 30 min) and the soluble fraction was loaded onto equilibrated (50 mM HEPES pH 8.0, 300 mM NaCl, 5 mM imidazole) Talon Superflow Metal Affinity Resin (BD Biosciences Clontech, Palo Alto, California, USA) and

incubated for 60 min. The suspension was then packed into a column and eluted using an imidazole gradient (5–300 mM). The fraction containing the purified protein was used in the next purification step, which involved size-exclusion chromatography (SEC). All purification procedures were performed at 277 K or in an ice bath.

SEC was performed on a Superdex 75 HR 16/60 (GE Healthcare) column attached to an ÄKTAexplorer 10 (GE Healthcare) chromatographic system. 50 mM HEPES, 150 mM NaCl pH 7.5 was used as the running buffer at a flow rate of 0.5 ml min⁻¹. The His tag was cleaved with TEV (tobacco etch virus) protease (Kapust *et al.*, 2002) at 277 K overnight. The fraction containing the purified xylose isomerase from *B. adolescentis* was concentrated by centrifugation using a Centricon (Millipore, 10 kDa cutoff).

2.1.4. Crystallization and X-ray diffraction data collection. Initial crystallization screening was performed at 292 K using a crystallization robot (Honeybee, Digilab) and commercial crystallization kits. After 5 d, small plate-shaped crystals appeared in a condition consisting of 0.2 M CaCl₂, 20% polyethylene glycol (PEG) 3350. This condition was reproduced using the hanging-drop vapour-diffusion method, in which 1 µl xylose isomerase at a concentration of 15 mg ml⁻¹ in 50 mM HEPES pH 7.5, 150 mM NaCl was mixed with 1 µl of the crystallization condition described above. A single crystal was harvested in a nylon loop and transferred into a cryoprotectant solution consisting of 0.2 M CaCl₂, 20% PEG 3350, 15% (m/v) ethylene glycol for 1 min. X-ray diffraction data collection was performed on the MX2 beamline of the Brazilian National Synchrotron Light Laboratory (LNLS), Campinas, Brazil (Guimarães *et al.*, 2009) using synchrotron radiation of wavelength 1.46 Å. Diffraction images were collected using a MAR Mosaic 225 CCD detector (MAR USA). Data covering 225° were collected to a maximum resolution of about 1.7 Å using a 0.5° oscillation step. Autoindexing and strategy determination were performed with *iMosflm* (Battye *et al.*, 2011) and integration of diffraction intensities and data reduction were performed with *XDS* (Kabsch, 2010) and *SCALA* (Winn *et al.*, 2011), respectively.

Figure 1

Purification of *B. adolescentis* xylose isomerase monitored by SDS–PAGE. Lane M, molecular-weight markers (labelled in kDa): bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), bovine trypsinogen (24 kDa), soybean trypsin inhibitor (20 kDa) and bovine α -lactalbumin (14 kDa). Lane 1, crude extract obtained from *E. coli* BL21 (DE3) cells harbouring the recombinant plasmid pPROEX-*xylA* after induction with 0.5 mM IPTG. Lane 2, the fraction eluted from metal-affinity chromatography using 300 mM imidazole. Lane 3, purified fraction from size-exclusion chromatography.

Table 1

X-ray data-collection statistics.

Values in parentheses are for the outermost resolution shell.

Beamline	MX2, LNLS
Wavelength (Å)	1.46
Space group	$P2_12_12$
Unit-cell parameters (Å)	$a = 88.78, b = 123.98, c = 78.63$
Resolution range (Å)	48.68–1.70 (1.79–1.70)
No. of images	265
$\Delta\varphi$ (°)	0.50
Mosaicity (°)	0.22
Unique reflections	63389
Total reflections	485458
Multiplicity	5.1 (4.7)
Completeness (%)	99.7 (98.4)
$R_{\text{merge}}^{\dagger}$	0.069 (0.543)
$\langle I/\sigma(I) \rangle$	15.4 (2.7)
Average B factor (Å ²)	20.2

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the i th observation of reflection hkl and $\langle I(hkl) \rangle$ is the average intensity of reflection hkl .

Figure 2

Crystals of *B. adolescentis* xylose isomerase. The black bar corresponds to 300 μm .

Figure 3

Diffraction pattern of *B. adolescentis* xylose isomerase. The resolution limit at the edge of the detector is 1.7 Å.

3. Results and discussion

To obtain xylose isomerase (XI) for structural studies, we cloned the *xylA* gene encoding XI from *B. adolescentis* and constructed an overproducing *E. coli* strain. The recombinant *B. adolescentis* XI was purified to homogeneity. Fig. 1 shows 15% SDS–PAGE analysis of XI purification from protein extraction using affinity and molecular-exclusion chromatography steps. The purified enzyme has a molecular mass of 51 kDa (Fig. 1). In the molecular-exclusion purification step, XI migrated as a multimer with a molecular mass of approximately 200 kDa, which indicates that the native enzyme forms tetramers (data not shown). The protein was purified to a high purity and crystallized (Fig. 2). X-ray diffraction data were collected at a synchrotron source and extended to 1.7 Å resolution (Fig. 3).

A total of 485 458 measured reflections were merged into 63 389 unique reflections with an R_{merge} of 6.9%; the completeness of the diffraction data set was 99.7%. The space group was found to be $P2_12_12$ on the basis of systematic absences and symmetry and the crystal unit-cell parameters were $a = 88.78, b = 123.98, c = 78.63$ Å (Table 1). The calculated Matthews coefficient (Matthews, 1968) of $2.16 \text{ Å}^3 \text{ Da}^{-1}$ indicated that there were two molecules in the asymmetric unit and that the solvent content of the crystal was about 43%. The structure was solved by molecular replacement with the program *Phaser* (Winn *et al.*, 2011) using PDB entry 1a0e (O. Gallay, R. Chopra, E. Conti, P. Brick, R. Jackson, B. Hartley, C. Vieille, J. G. Zeikus & D. Blow, unpublished work) as a template. This process resulted in a single solution with suitable statistics (translation function Z -score of 41.9 and log-likelihood gain of 34 377), confirming that the space group is indeed $P2_12_12$. The asymmetric unit cell of the crystal contained two monomers of the protein. Model refinement of xylose isomerase from *B. adolescentis* is in progress.

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