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Expression, crystallization and preliminary X-ray crystallographic analysis of cellobiose 2-epimerase from *Dictyoglomus turgidum* DSM 6724

Cellobiose 2-epimerase epimerizes and isomerizes β -1,4- and α -1,4-glucosaccharides. *N*-Acyl-D-glucosamine 2-epimerase (DT_epimerase) from *Dictyoglomus turgidum* has an unusually high catalytic activity towards its substrate cellobiose. DT_epimerase was expressed, purified and crystallized. Crystals were obtained of both His-tagged DT_epimerase and untagged DT_epimerase. The crystals of His-tagged DT_epimerase diffracted to 2.6 Å resolution and belonged to the monoclinic space group $P2_1$, with unit-cell parameters $a = 63.9$, $b = 85.1$, $c = 79.8$ Å, $\beta = 110.8^\circ$. With a Matthews coefficient V_M of $2.18 \text{ Å}^3 \text{ Da}^{-1}$, two protomers were expected to be present in the asymmetric unit with a solvent content of 43.74%. The crystals of untagged DT_epimerase diffracted to 1.85 Å resolution and belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 55.9$, $b = 80.0$, $c = 93.7$ Å. One protomer in the asymmetric unit was expected, with a corresponding V_M of $2.26 \text{ Å}^3 \text{ Da}^{-1}$ and a solvent content of 45.6%.

1. Introduction

Sugar epimerases convert aldoses into epimeric aldoses and ketoses into epimeric ketoses, whereas sugar isomerases convert aldoses into ketoses. Isomerases such as L-rhamnose isomerase (Lin *et al.*, 2011; Leang *et al.*, 2004; Park *et al.*, 2010), L-arabinose isomerase (Yamanaka, 1975; Rhimi & Bejar, 2006) and D-xylose isomerase from many mesophilic, thermophilic and hyperthermophilic microorganisms (Brat *et al.*, 2009; Whitaker *et al.*, 1995) have been extensively studied, with a focus on their catalytic activity and structure. Although sugar epimerases, for example D-psicose 3-epimerase from *Agrobacterium tumefaciens* (Kim *et al.*, 2006) and D-tagatose-3-epimerases from *Pseudomonas cichorii* (Takeshita *et al.*, 2000) and *Rhodobacter sphaeroides* (Zhang *et al.*, 2009), have been studied, structural analyses of epimerases lag behind those of isomerases.

We have focused on cellobiose 2-epimerase (EC 5.1.3.11), which catalyzes the conversion of cellobiose to 4-O- β -D-glucopyranosyl-D-mannose (Ito *et al.*, 2008); the glucose moiety in the disaccharide cellobiose is then converted into its epimeric (mannose) moiety. The structure and mechanism of cellobiose 2-epimerase is similar to that of *N*-acyl-D-glucosamine 2-epimerase (Lee *et al.*, 2007). However, *N*-acyl-D-glucosamine 2-epimerase and cellobiose 2-epimerase show different substrate specificities, as thermostable cellobiose 2-epimerase from *Thermotoga maritima* performs isomerization after epimerization (Kim *et al.*, 2006; Park *et al.*, 2010). Furthermore, there is no structural information available for the cellobiose 2-epimerase enzyme. In this study, cellobiose 2-epimerase from *Dictyoglomus turgidum* was expressed, purified and crystallized and preliminary X-ray crystallographic analyses were performed.

2. Materials and methods

2.1. Cloning

The genomic DNA from *D. turgidum* was extracted using a genomic DNA extraction kit (Qiagen, Hilden, Germany). The gene (1173 bp) encoding a putative *N*-acyl-D-glucosamine 2-epimerase (DT_epimerase) was amplified by PCR using the genomic DNA isolated from *D. turgidum* as a template. The sequences of the

oligonucleotide primers used for gene cloning were based on the DNA sequence of *D. turgidum* *N*-acyl-D-glucosamine 2-epimerase (GenBank accession No. NC_011661.1). Forward (5'-AAC **ATA TGG** ATT TAA AAG TTT TAA AAA GTG-3') and reverse (5'- TTC **TCG AGT** TAA ATC CTT TTT ATT ACC TCA AGA-3') primers were designed to introduce the *Nde*I and *Xho*I restriction sites (shown in bold), respectively. The PCR-amplified DNA fragments were purified using a QIAquick gel-extraction kit (Qiagen, Hilden, Germany) and inserted into pET-28a vector digested with the same restriction enzymes. The resulting plasmid was transformed into *Escherichia coli* ER2566 and plated on Luria-Bertani (LB) agar containing kanamycin (50 $\mu\text{g ml}^{-1}$). A kanamycin-resistant colony was selected and plasmid DNA from the transformant was isolated using a plasmid-purification kit (Promega, Madison, Wisconsin, USA).

We subcloned the DT_epimerase gene from the pET-28a-DT_epimerase vector into pET-29b-His-Tev vector by PCR using forward (5'-CCC CCC **ATA TGG** ATT TAA AAG TTT TAA AAA GTG-3') and reverse (5'-CCC CCC **CCA TGG** TTA AAT CCT TTT TAT TAC CTC-3') primers containing *Nde*I and *Bam*HI restriction sites (shown in bold), respectively. The amplified gene was inserted into the expression vector pET-29b-His-Tev which was modified to have seven histidine residues and a TEV cleavage site at the N-terminus of the gene product from the original pET-29b vector (Novagen) in order to facilitate protein purification. The resulting plasmid was transformed into *E. coli* NEB Turbo (New England Biolabs) and plated onto LB agar containing kanamycin (50 $\mu\text{g ml}^{-1}$). A kanamycin-resistant colony was selected and plasmid DNA from the transformant was isolated using a plasmid-purification kit (FavorPrep, Favorgen, Taiwan). All DNA sequencing was carried out at Macrogen (Seoul, Republic of Korea).

2.2. Overexpression and purification

The pET28a and pET29b-His-TEV expression vectors containing the coding sequence for the DT_epimerase gene were introduced into *E. coli* BL21(DE3) cells. Cells were grown at 288 K until the OD_{600} reached 0.6 in LB medium containing 50 $\mu\text{g ml}^{-1}$ kanamycin.

Figure 1
Purified His-tagged DT_epimerase and untagged DT_epimerase are shown on a 10% SDS-PAGE gel. Lane M1, His-tagged DT_epimerase (~48 kDa); lane M2, untagged DT_epimerase (~46 kDa); lane P, Prestained Protein Ladder (Fermentas).

Protein expression was induced by the addition of isopropyl β -D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM and the cells were cultured at the same temperature for a further 16 h. Cultured cells were harvested by centrifugation for 30 min at 6000g (Vision VS24-SMTi V5006A rotor) at 277 K and then resuspended in 25 mM Tris-HCl pH 7.5, 300 mM NaCl, 10 mM imidazole on ice. A sonicator was used to disrupt the resuspended cells (Sonomasher, S&T Science, Korea). The crude lysate was centrifuged for 30 min at 21 000g (Vision VS24-SMTi V508A rotor) at 277 K. An SDS-PAGE experiment indicated that 10% of the total expressed DT_epimerase was soluble. The supernatant containing soluble DT_epimerase protein was applied onto Ni-NTA resin (Novagen) and affinity purification was performed based on the manufacturer's protocol at 277 K. The elution buffer containing 250 mM imidazole was used to elute the 7 $\times$ His-tagged DT_epimerase. The eluate was dialyzed for 8 h at 277 K against dialysis buffer (25 mM Tris-HCl pH 7.5, 15 mM NaCl). The 7 $\times$ His tag was removed by treatment with TEV protease overnight at 277 K. Further purification was carried out on an UNO 6Q column (Bio-Rad). The homogeneity of the purified protein was

(a)

(b)

Figure 2
Crystals of His-tagged and untagged DT_epimerase. (a) The optimized crystal of His-tagged DT_epimerase obtained using the reservoir condition 23%(w/v) PEG 3350, 0.2 M magnesium chloride hexahydrate, 0.1 M bis-tris pH 6.0 and (b) the optimized crystal of untagged DT_epimerase obtained using the reservoir condition 26.0%(v/v) 2-propanol, 10.0%(w/v) PEG 8000, 0.1 M imidazole-HCl pH 7.0.

checked *via* SDS-PAGE (Fig. 1). Approximately 30 mg DT_epimerase was purified from 1 l of culture. The purified protein was concentrated to 7.5 mg ml⁻¹ using Amicon Ultra-15 centrifugal filter units (Merck Millipore) and was stored as aliquots at 193 K. The protein was thawed and used for crystallization.

2.3. Crystallization and X-ray data collection

The sitting-drop vapour-diffusion method was used for initial crystallization at 287 K in 96-well Intelli-Plates (Art Robbins) using a

Table 1

Data-collection statistics of His-tagged and untagged DT_epimerase crystals.

Values in parentheses are for the outer shell.

	His-tagged DT_epimerase	Untagged DT_epimerase
X-ray source	XU-32, SPring-8	5C-SBII, PLS
Detector type	CMOS	ADSC Q315r
Wavelength (Å)	0.97951	0.97951
Unit-cell parameters (Å, °)	$a = 63.9, b = 85.1, c = 79.8,$ $\alpha = \gamma = 90, \beta = 110.8$	$a = 55.9, b = 80.0, c = 93.7,$ $\alpha = \beta = \gamma = 90.0$
Space group	$P2_1$	$P2_12_12_1$
Resolution range (Å)	50.0–2.61 (2.66–2.61)	50.0–1.85 (1.88–1.85)
No. of observations	53514	253777
No. of unique reflections	21114	36358
Multiplicity	2.5 (2.5)	7.0 (7.2)
Completeness (%)	95.2 (97.1)	99.6 (100.0)
$R_{\text{merge}}^{\dagger}$ (%)	10.3 (35.5)	6.7 (37.9)
$\langle I/\sigma(I) \rangle$	16.3 (3.2)	46.9 (8.3)
V_M (Å ³ Da ⁻¹)	2.18	2.26
Solvent content (%)	43.74	45.59

$\dagger R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$, where $I(hkl)$ is the intensity of reflection hkl , $\sum_{hkl}$ is the sum over all reflections and $\sum_i$ is the sum over i measurements of reflection hkl .

Figure 3
Diffraction images of (a) His-tagged and (b) untagged DT_epimerase. Diffraction resolutions are marked as circles.

Hydra II eDrop automated pipetting system (Matrix) and screening kits from Hampton Research (Index, Crystal Screen, Crystal Screen Cryo, Crystal Lite and PEG Rx), Emerald BioSystems (Wizard and Wizard Precipitant Synergy) and Molecular Dimensions (Morpheus). Two kinds of DT_epimerase (with and without the 7×His tag) at 7.5 mg ml⁻¹ concentration were set up for crystallization. 0.5 µl protein solution was mixed with 0.5 µl reservoir solution and equilibrated against 50 µl reservoir solution.

After 2 d, crystals of His-tagged DT_epimerase protein were observed in condition G10 of Index [25%(w/v) polyethylene glycol 3350, 0.2 M magnesium chloride hexahydrate, 0.1 M bis-tris pH 5.5]. This condition was used to reproduce and optimize crystals by the hanging-drop method using drops consisting of 0.9 µl protein solution mixed with 0.9 µl of reservoir solution equilibrated against 1 ml reservoir solution. Optimization was achieved by varying the concentration of PEG 3350 [22–27%(w/v)] and the pH of the 0.1 M bis-tris (4.5–6.0). Tetragonal shaped crystals of His-tagged DT_epimerase were obtained after 1 week using a reservoir solution consisting of 23%(w/v) PEG 3350, 0.2 M magnesium chloride hexahydrate, 0.1 M bis-tris pH 6.0 (Fig. 2a). The fully grown crystals (0.05 × 0.10 × 0.02 mm) were flash-cooled at 100 K in liquid nitrogen using 20%(v/v) 2-methyl-2,4-pentanediol, 23%(w/v) PEG 3350, 0.2 M magnesium chloride hexahydrate, 0.1 M bis-tris pH 6.0.

Crystals of untagged DT_epimerase appeared after 2 d in crystallization condition No. 101 from Wizard Precipitant Synergy kit 5 [26.8%(v/v) 2-propanol, 10.05%(w/v) PEG 8000, 0.1 M imidazole-HCl pH 6.5]. We tried to reproduce and optimize the crystals by screening the pH range 5.5–7.5. Orthorhombic shaped crystals were obtained in a reservoir solution consisting of 26.0%(v/v) 2-propanol, 10.0%(w/v) PEG 8000, 0.1 M imidazole-HCl pH 7.0 (Fig. 2b). The fully grown crystals (0.2 × 0.15 × 0.3 mm) were cooled to 100 K in liquid nitrogen using 15%(v/v) glycerol, 26%(v/v) 2-propanol, 10%(w/v) PEG 8000, 0.1 M imidazole-HCl pH 7.0.

X-ray diffraction data were collected from the cryoprotected crystals on beamline 5C SBII of the Pohang Light Source (PLS), Republic of Korea and on beamline XU32 of SPring-8, RIKEN, Japan. Crystals diffracted to 2.6 Å (His tagged) and 1.8 Å (untagged) resolution, respectively (Fig. 3). The diffraction data were integrated and scaled using *DENZO* and *SCALEPACK*, respectively (Otwinowski & Minor, 1997).

3. Results and discussion

His-tagged DT_epimerase crystals belonged to space group $P2_1$, with unit-cell parameters $a = 63.9$, $b = 85.1$, $c = 79.8$ Å, $\beta = 110.8^\circ$. The space group was assigned by auto-indexing (Otwinowski & Minor, 1997) and data-collection statistics are provided in Table 1. According to the Matthews coefficient calculation (Matthews, 1968), there are probably two molecules in the asymmetric unit, corresponding to a V_M of 2.18 Å³ Da⁻¹ and a solvent content of 43.74%. *MOLREP* (Vagin & Teplyakov, 2010) from the *CCP4* program package (Winn *et al.*, 2011) with *N*-acetylglucosamine 2-epimerase from *Xylella fastidiosa* (PDB entry 3gt5; Lang *et al.*, 2012; 22% sequence identity) as a search model was successful and showed dimers in the asymmetric unit. Although the initial *R* factor from the molecular-replacement search was 55.0%, the resulting electron-density maps were clear and no clashes were found between molecules. After rigid-body and initial restrained refinement by *REFMAC5* (Murshudov *et al.*, 2011), the *R* factor decreased to 31.7% and the R_{free} was 44.8%.

The untagged DT_epimerase crystals belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 55.9$, $b = 80.0$, $c = 93.7$ Å. The space group was assigned by auto-indexing (Otwinowski & Minor, 1997) and data-collection statistics are provided in Table 1. According to the Matthews coefficient calculation (Matthews, 1968), there is one molecule in the asymmetric unit, corresponding to a V_M of 2.26 Å³ Da⁻¹ and a solvent content of 45.59%. *MOLREP* from the *CCP4* program package with the same search model was successful and showed a monomer in the asymmetric unit. The initial *R* factor from the molecular-replacement search was 48.3%; the resulting electron-density maps were of high quality and no clashes were found between molecules. After rigid-body and initial restrained refinement by *REFMAC5* (Murshudov *et al.*, 2011), the *R* factor decreased to 24.7% and the R_{free} was 27.8%.

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