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Overexpression, crystallization and preliminary X-ray diffraction analysis of L-ribose isomerase from *Acinetobacter* sp. strain DL-28

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Acinetobacter sp. L-ribose isomerase (L-RI) catalyzes a reversible isomerization reaction between L-ribose and L-ribulose. To date, information on L-RI remains limited and its amino-acid sequence shows no similarity to those of any known enzymes. Here, recombinant His-tagged L-RI was successfully overexpressed, purified and crystallized. Crystals of His-tagged L-RI were obtained by the hanging-drop vapour-diffusion method at room temperature as two crystal forms which belonged to the monoclinic space group $C2$, with unit-cell parameters $a = 96.60$, $b = 105.89$, $c = 71.83$ Å, $\beta = 118.16^\circ$, and the orthorhombic space group $F222$, with unit-cell parameters $a = 96.44$, $b = 106.26$, $c = 117.83$ Å. Diffraction data were collected to 3.1 and 2.2 Å resolution, respectively.

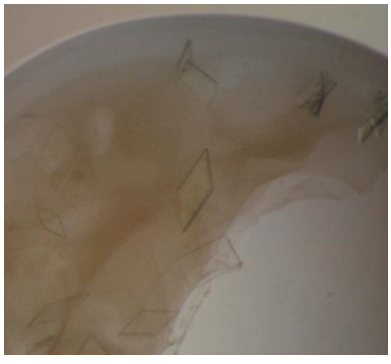
1. Introduction

L-Ribose can serve as a precursor for the synthesis of L-nucleoside analogues, which are widely used as pharmaceutical compounds such as antiviral and anticancer drugs (Doong *et al.*, 1991; Wang *et al.*, 1998; Gumina *et al.*, 2001; Yun *et al.*, 2005). There has been great interest in L-ribose as a plausible industrial material; nevertheless, L-ribose is not abundant in nature and is known as a 'rare sugar'. Helanto and coworkers have reported a novel strategy for producing L-ribose from L-arabinose for this purpose by introducing L-ribose isomerase activity into L-ribulokinase-deficient *Escherichia coli* UP1110 and *Lactobacillus plantarum* BPT197 strains (Helanto *et al.*, 2009).

L-Ribose isomerase (L-RI) from *Acinetobacter* sp., which is able to catalyze a reversible isomerization reaction between L-ribose and L-ribulose, has been reported as a new enzyme and cloned (249 amino-acid residues; Shimonishi & Izumori, 1996; Mizanur *et al.*, 2001). To date, information on L-RI is limited. The amino-acid sequence of *Acinetobacter* sp. L-RI shows no similarity to those of any known enzymes. A *BLAST* search against the PDB revealed that the highest homology was with human cystatin A (P25S; PDB entry 1gd4; Shimba *et al.*, 2000), with a Max Score of 28.9, a Query coverage of 13% and an *E* value of 2.0, followed by human stefin A (PDB entry 1n9j; Staniforth *et al.*, 2001), with a Max Score of 28.5, a Query coverage of 13% and an *E* value of 2.9, and λ repressor (PDB entry 3bdn; Stayrook *et al.*, 2008), with a Max Score of 27.7, a Query coverage of 25% and an *E* value of 4.7.

Although a mannose-6-phosphate isomerase from *Bacillus subtilis* has been reported to have similar properties to L-RI (Yeom *et al.*, 2009), the two enzymes are considered to be genetically different based on amino-acid sequence. To elucidate the unique substrate recognition and catalytic mechanism of L-RI, which are expected to differ from those of the well known isomerases, an X-ray structural analysis is essential.

Here, we present the overexpression, purification, crystallization and preliminary X-ray diffraction analysis of recombinant His-tagged L-RI.



2. Materials and methods

2.1. Overexpression and purification

The L-RI gene of *Acinetobacter* sp. strain DL-28 (DDBJ accession No. AB062121; UniProt accession No. Q93UQ5) was subcloned from the previously constructed plasmid p8IR (Mizanur *et al.*, 2001) by the PCR method using as primers forward 1 (5′-GG**CCATGG**CAAGGACGTCGATTACTCGTCGCGAGTATG-3′) and reverse 1 (5′-C-C**GGATCC**GCTGATCGCGGTCTGAAGCCGCCCGGTGTT-3′) containing *Nco*I and *Bam*HI sites (bold), respectively, and forward 2 (5′-CC**GGATCC**GCAAGGACGTCGATTACTCGTCGCGAGTAT-3′) and reverse 2 (5′-CC**AAGCTT**CTAGCTGATCGCGGTCTGAAGCCGCCCGGT-3′) containing *Bam*HI and *Hin*dIII sites (bold), respectively. The first PCR products digested with *Nco*I and *Bam*HI were inserted into the expression vector pQE60 (Qiagen, Valencia, California, USA) linearized with *Nco*I and *Bgl*II. In the same way, the second PCR products digested with *Bam*HI and *Hin*dIII were inserted into the expression vector pQE30 (Qiagen, Valencia, California, USA) linearized with *Bam*HI and *Hin*dIII. The newly constructed plasmid pQE60LRI or pQE30LRI was introduced into *E. coli* JM109 cells and was capable of producing a C-terminally His-tagged L-RI or an N-terminally His-tagged L-RI, respectively. DNA sequencing was performed with an ABI PRISM 310 genetic analyzer.

For both N-terminally and C-terminally His-tagged L-RI the cells were grown at 310 K in 2×YT medium containing 100 µg ml^{-1} ampicillin until the culture reached an optical density of 0.4–0.5 at 600 nm. L-RI overexpression was initiated by the addition of 0.1 m*M* isopropyl *β*-D-1-thiogalactopyranoside (IPTG) and the cells were cultivated with the addition of 1 m*M* $MnCl_2$ at 293 K overnight. The cells harvested by centrifugation at 5000*g* for 10 min at 277 K were resuspended and sonicated in buffer (50 m*M* NaH_2PO_4, 300 m*M* NaCl pH 8.0). The sonicated sample was centrifuged (20 400*g*, 20 min, 277 K) and the resultant cell-free extract was applied onto an affinity column (HisTrap HP 5 ml, GE Healthcare Biosciences, Piscataway, New Jersey, USA) equilibrated with sodium phosphate buffer (50 m*M* NaH_2PO_4, 300 m*M* NaCl, 20 m*M* imidazole pH 8.0). After washing with the equilibration buffer, the protein was eluted with a linear gradient of 50 m*M* NaH_2PO_4, 300 m*M* NaCl, 500 m*M* imidazole pH 8.0. The purity of the eluted sample was confirmed by SDS–PAGE. After dialysis against buffer solution (5 m*M* Tris–HCl pH 7.5) overnight, the L-ribose isomerase activity of both His-tagged L-RIs was assayed as reported previously (Shimonishi & Izumori, 1996). L-RI activity was determined by measuring the increase in a keto sugar, L-ribulose, using the cysteine–sulfuric acid–carbazole method (Dische & Borenfreund, 1951). The enzymatic reaction was started by the addition of 50 µl 50 m*M* L-ribose (final concentration of 5 m*M*) to a reaction mixture consisting of 350 µl 50 m*M* glycine–NaOH buffer pH 9.0, 50 µl 10 m*M* $MnCl_2$ and 50 µl of the enzyme solution. The mixture was incubated for 10 min at 303 K and the reaction was stopped by the addition of 50 µl trichloroacetic acid (10%). The amount of keto sugar in the reaction mixture was determined by the cysteine–sulfuric acid–carbazole method. One unit of activity represents the formation of 1 µmol L-ribulose within 1 min under the assay conditions. L-Ribose isomerase activity was only detected for the N-terminally His-tagged L-RI (referred to in the following as His-tagged L-RI). The purified His-tagged L-RI was concentrated to 13 mg ml^{-1} using an Amicon Ultra-4 10 kDa Ultracel (Millipore, Billerica, Massachusetts, USA). Protein concentrations were determined by measuring the absorbance at 280 nm and using the absorption coefficient of 38 390 M^{-1} cm^{-1} calculated on the basis of the numbers of tryptophan, tyrosine and cysteine residues in the enzyme (Pace *et al.*, 1995).

2.2. Crystallization and X-ray data collection

Initial crystallization screening for His-tagged L-RI was performed using Index Screen (Hampton Research, California, USA) and Wizard I, II and III (Emerald BioSystems, Washington, USA) by the

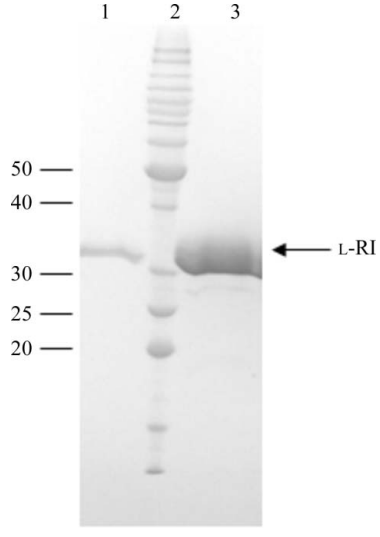
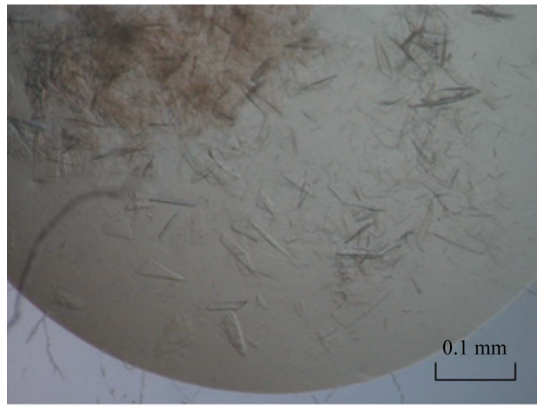
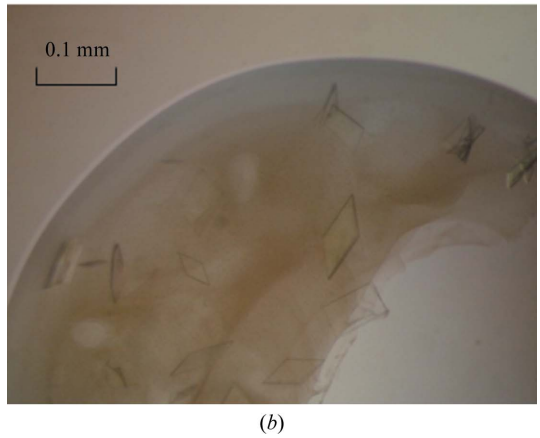


Figure 1
SDS–PAGE of purified N-terminally/C-terminally His-tagged L-RI. Lane 1, purified C-terminally His-tagged L-RI; lane 2, molecular marker (labelled in kDa); lane 3, purified N-terminally His-tagged L-RI.



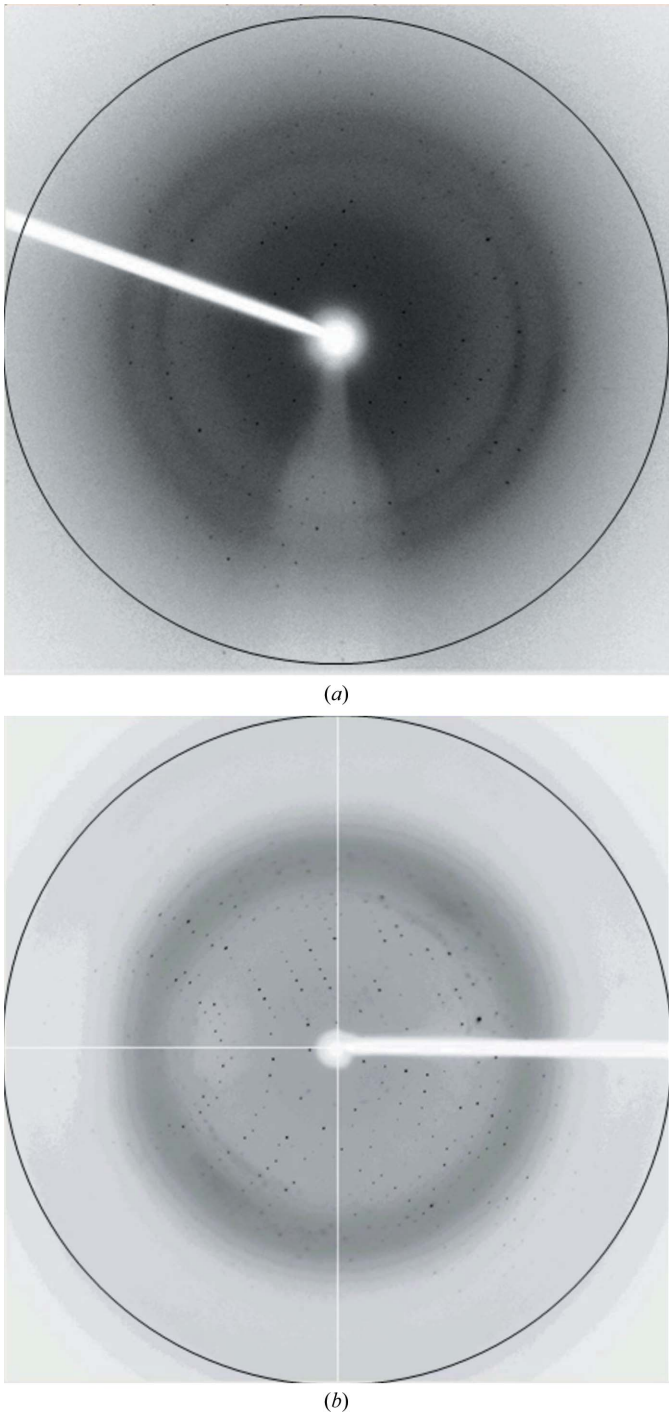
(*a*)



(*b*)

Figure 2
Crystals of His-tagged L-RI. (*a*) Form 1 in the absence of hexamminecobalt(III) chloride. (*b*) Form 2 in the presence of hexamminecobalt(III) chloride.

sitting-drop vapour-diffusion method in 96-well plates (Corning, New York, USA) at 293 K. Two crystal forms of His-tagged L-RI were found (forms 1 and 2). Data for form 1 were collected using a Rigaku R-AXIS VII imaging system on a Rigaku MicroMax-007 HF rotating-anode (Cu $K\alpha$) X-ray generator with ValiMax optics (40 kV, 30 mA) at 100 K (Rigaku, Japan). Data collection for form 2 was carried out at KEK PF-AR NW12A (Tsukuba, Japan) at a wavelength of 1.0 Å using an ADSC Quantum 210 CCD detector at 100 K. Both crystals were directly flash-cooled in a cryostream of liquid nitrogen without any additional cryoprotectant. Data were processed using *CrystalClear* (Rigaku, Japan) and/or *HKL*-2000 (Otwinowski & Minor, 1997).



(*a*)

(*b*)

Figure 3
Diffraction of His-tagged L-RI. (*a*) Form 1 with 3.1 Å resolution marked, (*b*) form 2 with 2.1 Å resolution marked.

Table 1
Data-collection statistics for His-tagged L-RI.

Values in parentheses are for the high-resolution bin.

	Form 1	Form 2
X-ray source	MicroMax-007 HF	PF-AR NW12A
Temperature (K)	100	100
Wavelength (Å)	1.54178	1.0
Detector	R-AXIS VII imaging plate	Quantum 210 CCD
Crystal data		
Space group	*C*2	*F*222
Unit-cell parameters		
a (Å)	96.60	96.44
b (Å)	105.89	106.26
c (Å)	71.83	117.83
β (°)	118.16	
Solvent content (%)	55.6	52.4
V_M (Å^3 Da^{-1})	2.8	2.6
No. of molecules per asymmetric unit	2	1
Data collection		
Resolution range (Å)	66.37–3.10 (3.21–3.10)	50.00–2.20 (2.28–2.20)
No. of measured reflections	37234	79673
No. of unique reflections	11573	15886
Completeness (%)	99.4 (98.3)	94.5 (80.6)
Multiplicity	3.2 (3.1)	5.3 (4.3)
R_{merge}† (%)	21.0 (38.6)	8.4 (31.5)
Mean $I/\sigma(I)$	3.7 (1.9)	12.4 (3.7)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith measurement and $\langle I(hkl)\rangle$ is the weighted mean of all measurements of $I(hkl)$.

3. Results and discussion

The purity of the purified samples was confirmed by SDS–PAGE, showing a single band with an estimated molecular mass of 28.4 kDa for C-terminally His-tagged L-RI (257 amino-acid residues; eight residues, GSHHHHHH, were added at the C-terminus) and 28.7 kDa for N-terminally His-tagged L-RI (260 amino-acid residues; the initial two residues MT were replaced by 13 residues MRGSHHHHHHG-SA) (Fig. 1). However, L-ribose isomerase activity was only detected for the N-terminally His-tagged L-RI. Thus, we used N-terminally His-tagged L-RI (His-tagged L-RI) for crystallization.

Small thin crystals appeared in a few days in a droplet consisting of 1 µl protein solution (13 mg ml^{-1} in 5 m*M* Tris–HCl pH 7.5) and 1 µl reservoir solution [0.2 *M* NaCl, 30%(*v*/*v*) PEG 400, 0.1 *M* HEPES pH 7.5; Wizard II condition No. 42] equilibrated against 100 µl reservoir solution. For further optimization of the conditions, Additive Screen (Hampton Research, California, USA) was applied. Crystals with dimensions of 0.1 × 0.1 × 0.03 mm that were suitable for X-ray diffraction experiments were grown within a few days from a droplet consisting of 2 µl protein solution (10–13 mg ml^{-1} in 5 m*M* Tris–HCl pH 7.5) and 2 µl reservoir solution consisting of 18–23%(*v*/*v*) PEG 400, 0.1 *M* HEPES pH 7.5, 0.16–0.2 *M* NaCl with or without 10–20 m*M* hexamminecobalt(III) chloride equilibrated against 450 µl reservoir solution by the hanging-drop vapour-diffusion method in a 24-well plate (TPP, Switzerland), as shown in Fig. 2.

Two crystal forms of His-tagged L-RI were found (forms 1 and 2). Form 1 was obtained in the absence of hexamminecobalt(III) chloride, while form 2 was obtained in the presence of hexamminecobalt(III) chloride. Forms 1 and 2 of His-tagged L-RI diffracted to resolutions of 3.1 and 2.2 Å, respectively, without any cryoprotectant, as shown in Fig. 3.

Data sets for form 1 (3.1 Å resolution) and form 2 (2.2 Å resolution) were successfully collected as summarized in Table 1. The crystals belonged to the monoclinic space group $C2$, with unit-cell parameters a = 96.60, b = 105.89, c = 71.83 Å, β = 118.16°, and the orthorhombic space group $F222$, with unit-cell parameters a = 96.44, b = 106.26, c = 117.83 Å, respectively. The asymmetric units of crystal forms 1 and 2 are expected to contain two and one molecules, with crystal volumes per unit molecular weight (V_M) values of 2.8 and 2.6 Å^3 Da^{-1}, corresponding to solvent contents of 55.6 and 52.4%, respectively (Matthews, 1968). L-RI has been reported to be a tetrameric enzyme in solution (Shimonishi & Izumori, 1996) and our preliminary X-ray diffraction analysis showed that L-RI possibly forms a tetramer with 222 symmetry in the crystals. N-terminally His-tagged L-RI was successfully overexpressed and showed enzymatic activity; however, C-terminally His-tagged L-RI did not maintain enzymatic activity despite being adequately expressed. This may suggest that the C-terminal region is involved in the formation of the active enzyme.

Since there is no X-ray structure with significant sequence homology to L-RI, molecular replacement could not be applied. In order to determine the initial phase, we are currently preparing selenomethionine-substituted L-RI.

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