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Crystallization and preliminary X-ray crystallographic analysis of L-rhamnose isomerase with a novel high thermostability from *Bacillus halodurans*

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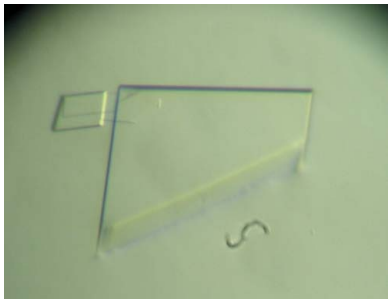


L-Rhamnose isomerases catalyze isomerization between L-rhamnose (6-deoxy-L-mannose) and L-rhamnulose (6-deoxy-L-fructose), which is the first step in rhamnose catabolism. L-Rhamnose isomerase from *Bacillus halodurans* ATCC BAA-125 (BHRI) exhibits interesting characteristics such as high thermostability and selective substrate specificity. BHRI fused with an HHHHHH sequence was purified and crystallized in order to elucidate the molecular basis of its unique enzymatic properties. The crystals were grown by the hanging-drop vapour-diffusion method and belonged to the monoclinic space group $P2_1$, with unit-cell parameters a = 83.2, b = 164.9, c = 92.0 Å, β = 116.0°. Diffraction data were collected to 2.5 Å resolution. According to a Matthews coefficient calculation, there are four monomers in the asymmetric unit with a V_M of 3.0 Å^3 Da^{-1} and a solvent content of 59.3%. The initial structure of BHRI has been determined by the molecular-replacement method.

1. Introduction

L-Rhamnose (6-deoxy-L-mannose) is an important component of polysaccharides in plants and is a construction component of cell walls in mycobacteria (Gottschalk, 1986). L-Rhamnose is also one of the rare sugars, with significant importance in the food industry as well as in the pharmaceutical and nutritional industries. These rare sugars, which exist in small amounts in nature, can be used as reduced-calorie sweeteners, inhibitors of microbial growth, bulking agents and so on (Bautista *et al.*, 2000; Lawson *et al.*, 2002; Levin *et al.*, 1964; Livesey & Brown, 1995). The first enzyme in rhamnose catabolism is L-rhamnose isomerase (L-RhI; EC 5.3.1.14), which reversibly converts L-rhamnose to L-rhamnulose (Wilson & Ajl, 1957; Fig. 1). Since rare sugars possess potential uses, isomerase enzymes such as L-RhI are becoming increasingly significant and play a pivotal role in their synthesis in large amounts (Izumori, 2002).

L-RhIs from *Escherichia coli* (Moralejo *et al.*, 1993), *Pseudomonas stutzeri* (Leang *et al.*, 2004) and *Bacillus pallidus* (Poonperm *et al.*, 2007) have been identified as enzymes with broad substrate specificities. Recently, L-RhI from *B. halodurans* (BHRI) was cloned and expressed in *E. coli* and the 6×His-tagged recombinant protein was characterized, demonstrating unique catalytic properties (Prabhu *et al.*, 2010). BHRI was also reported to use various aldoses as substrates in addition to L-rhamnose. However, it exhibited a less broad substrate specificity than *E. coli* L-RhI and *P. stutzeri* L-RhI. Interestingly, the enzyme showed the highest thermostability compared with the other reported L-RhIs. These advantages are useful for the industrial production of the rare sugar without contamination

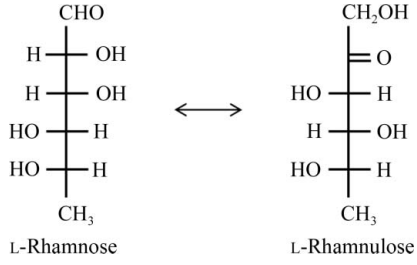


Figure 1
The reaction catalyzed by L-rhamnose isomerase.

(Mozhaev, 1993). Additionally, understanding of the mechanism based on the three-dimensional structure of the enzyme has shown promise for the development of engineered enzymes that can convert desired substrates into rare sugars with high efficiency. To date, crystal (tertiary) structures of *E. coli* L-RhI and *P. stutzeri* L-RhI have been determined. These enzymes showed differences in their substrate specificities as well as in their structures (Korndorfer *et al.*, 2000; Yoshida *et al.*, 2006, 2007). Therefore, it is expected that the BHRI structure will differ from those of other L-RhIs.

This study describes the expression, purification and crystallization of BHRI, an interesting member of the isomerase family. Three-dimensional structural studies of BHRI will elucidate the molecular basis of its enzymatic reaction mechanism and will be useful for the design of an engineered enzyme for the industrial production of rare sugars.

2. Methods and results

2.1. Cloning

The *rhaA* gene was amplified by PCR from genomic DNA of *B. halodurans* ATCC BAA-125 using *Ex-Taq* DNA polymerase. The forward and reverse primers were incorporated with *Bam*HI and *Hin*dIII sites for easy cloning. The oligonucleotide primers were 5′-GC**G GAT CC**A TGA GCA TGA AAA GTC A-3′ and 5′-TT**A AGC TT**A TGG CGC TGG AGC AGC-3′ (*Hin*dIII and *Bam*HI restriction sites are shown in bold). The amplified PCR product with flanking restriction sites was initially cloned into pGEM-T Easy Vector and transformed into *E. coli* DH5α. The cloned *rhaA* gene was confirmed to be free of point mutations by DNA sequencing using the BigDye Terminator sequencing method and an ABI PRISM 3700 sequencer (Applied Biosystems, Foster City, California, USA). The insert was harvested by treating the recombinant plasmid DNA with *Bam*HI and *Hin*dIII restriction enzymes and ligating pQE-80L vector treated with the same enzymes to give pQE-*rhaA*. The recombinant vector is under the control of the T5 promoter and expresses BHRI as a tagged protein with the sequence HHHHHH attached directly to the N-terminus.

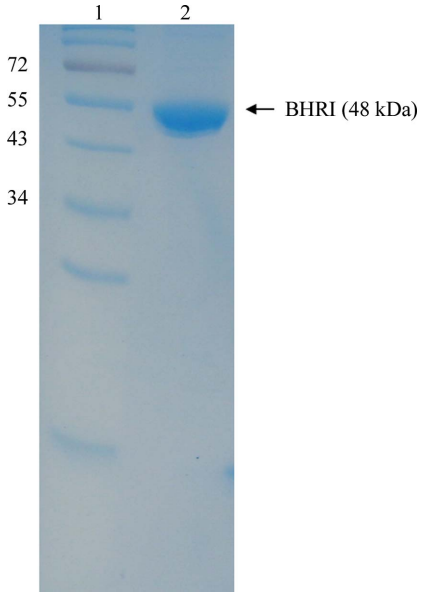


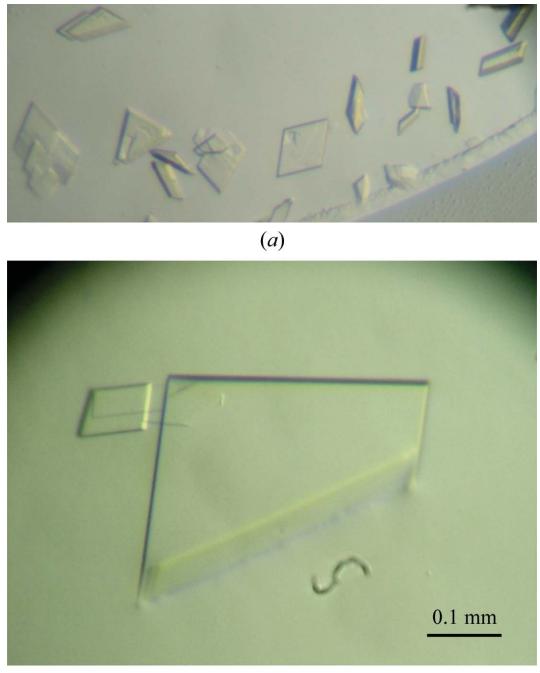
Figure 2
Purified BHRI shown on 15% SDS–PAGE. Lane 1, protein ladder (kDa); lane 2, BHRI.

2.2. Overexpression and purification

The pQE-*rhaA* plasmid was transformed into *E. coli* BL21 (DE3) grown in Luria–Bertani (LB) medium supplemented with ampicillin (100 µg ml^{-1}) at 310 K. Expression of the recombinant enzyme was performed using 0.4 m*M* isopropyl β-D-1-thiogalactopyranoside. The induced cells were harvested by centrifugation at 277 K for 15 min at 10 000*g* and rinsed with phosphate-buffered saline. The cell pellet was resuspended in binding buffer (50 m*M* NaH_2PO_4, 300 m*M* NaCl pH 8.0) and incubated on ice for 30 min in the presence of 1 mg ml^{-1} lysozyme. Cell disruption was carried out by sonication at 277 K for 5 min and the lysate was centrifuged at 14 000*g* for 20 min at 277 K to remove cell debris. The filtrate was applied onto an Ni–NTA Superflow column (3.4 × 13.5 cm, Qiagen) previously equilibrated with binding buffer. Unbound proteins were washed out from the column with washing buffer (50 m*M* NaH_2PO_4, 300 m*M* NaCl, 20 m*M* imidazole pH 8.0). BHRI protein was eluted from the column with elution buffer (50 m*M* NaH_2PO_4, 300 m*M* NaCl, 250 m*M* imidazole pH 8.0). Enzyme fractions were analyzed by 12% SDS–PAGE and visualized by staining with Coomassie Blue R250. The purified protein showed a molecular weight of 48 kDa on SDS–PAGE (Fig. 2) and the molecular weight was confirmed by gel filtration (Prabhu *et al.*, 2010). The fractions containing pure protein were dialyzed overnight at 277 K against 25 m*M* Tris–HCl pH 7.5. The purified protein was concentrated to 8 mg ml^{-1} using a 30 000 Da molecular-weight cutoff concentrator.

2.3. Crystallization and X-ray data collection

The initial crystallization conditions for BHRI were screened at 283 K by the sitting-drop vapour-diffusion method using Hampton Research Crystal Screen Lite and Index. Crystal Screen Lite condition No. 28 (15% PEG 8000, 0.1 *M* sodium cacodylate pH 6.5 and 0.2 *M* sodium acetate), in which crystals clearly appeared (Fig. 3*a*), was selected for optimization. For optimization, hanging drops made



(*a*)

(*b*)

Figure 3
(*a*) Crystals of BHRI from Crystal Screen Lite condition No. 28 and (*b*) a large crystal from the optimized condition. The large crystal reached maximum dimensions of 0.35 × 0.35 × 0.03 mm in a week.

up of 1 µl protein solution mixed with 1 µl reservoir solution were equilibrated against 1 ml reservoir solution. Good-quality crystals were observed after one week using a reservoir solution consisting of 10%(*w*/*v*) PEG 8000, 0.1 *M* HEPES pH 6.0 and 0.2 *M* sodium acetate. Prior to data collection, a fully grown crystal with maximum dimensions of 0.35 × 0.35 × 0.03 mm (Fig. 3*b*) was flash-cooled to 100 K in liquid nitrogen using a cryoprotectant consisting of the reservoir solution plus 20%(*v*/*v*) ethylene glycol. A complete data set was collected to 2.5 Å resolution at Pohang Light Source (PLS, Republic of Korea) using a Buker Proteum 300 CCD detector and a wavelength of 1.23999 Å. The data were integrated and scaled *via* the *DENZO* and *SCALEPACK* crystallographic data-reduction routines (Otwinowski & Minor, 1997). The data-collection statistics are summarized in Table 1. The asymmetric unit is likely to contain four single-chain BHRI molecules with a volume per unit molecular weight of the protein of 3.0 Å^3 Da^{-1}, corresponding to a solvent content of 59.3% (Matthews, 1968). The crystallographic space group $P2_1$ was derived by autoindexing (Otwinowski & Minor, 1997), with unit-cell parameters a = 83.2, b = 164.9, c = 92.0 Å, β = 116.0°. Self-rotation functions were computed in the resolution range 50–4 Å. Analysis of the self-rotation peaks (Fig. 4) revealed the presence of four twofold rotation axes, including one crystallographic twofold axis and one fourfold rotation axis. The initial structure of BHRI was determined by molecular replacement using *Phaser* (McCoy *et al.*, 2005) and *MOLREP* (Vagin & Teplyakov, 1997) from the *CCP*4 program package (Collaborative Computational Project, Number 4, 1994), with L-rhamnose isomerase from *E. coli* (PDB code 1d8w; sequence identity 57.0%; Korndorfer *et al.*, 2000) as the template. The initial *R* factor from the resulting model was 39.3%; the initial reliable *Z* score from *MOLREP* was 0.742. The resulting electron-density maps are of high quality and show the presence of the tetramer in the asymmetric unit. The structural details will be described in a separate paper. Our structural model of BHRI will provide an insight into its thermostability and substrate specificity and will be useful for the development of improved enzymes for the industrial production of large quantities of pure rare sugars.

Table 1
Data-collection statistics.

Values in parentheses are for the outer shell.

X-ray source	6C1, Pohang Light Source
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 83.2, b = 164.9, c = 92.0, β = 116
Wavelength (Å)	1.23999
Resolution (Å)	50.0–2.5 (2.54–2.5)
No. of observed reflections	912353
No. of unique reflections	76165
Completeness (%)	98.2 (99.3)
Molecules per asymmetric unit	4
V_M (Å^3 Da^{-1})	3.0
Solvent content (%)	59.3
R_{merge}†	0.112 (0.568)
$R_{p.i.m.}$‡	0.083 (0.502)
Mean $I/\sigma(I)$	11.0 (1.9)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$. ‡ $R_{p.i.m.} = \sum_{hkl}[1/(N-1)]^{1/2} \times \sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$. $I(hkl)$ 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. N is the redundancy of the measurement of reflection hkl.

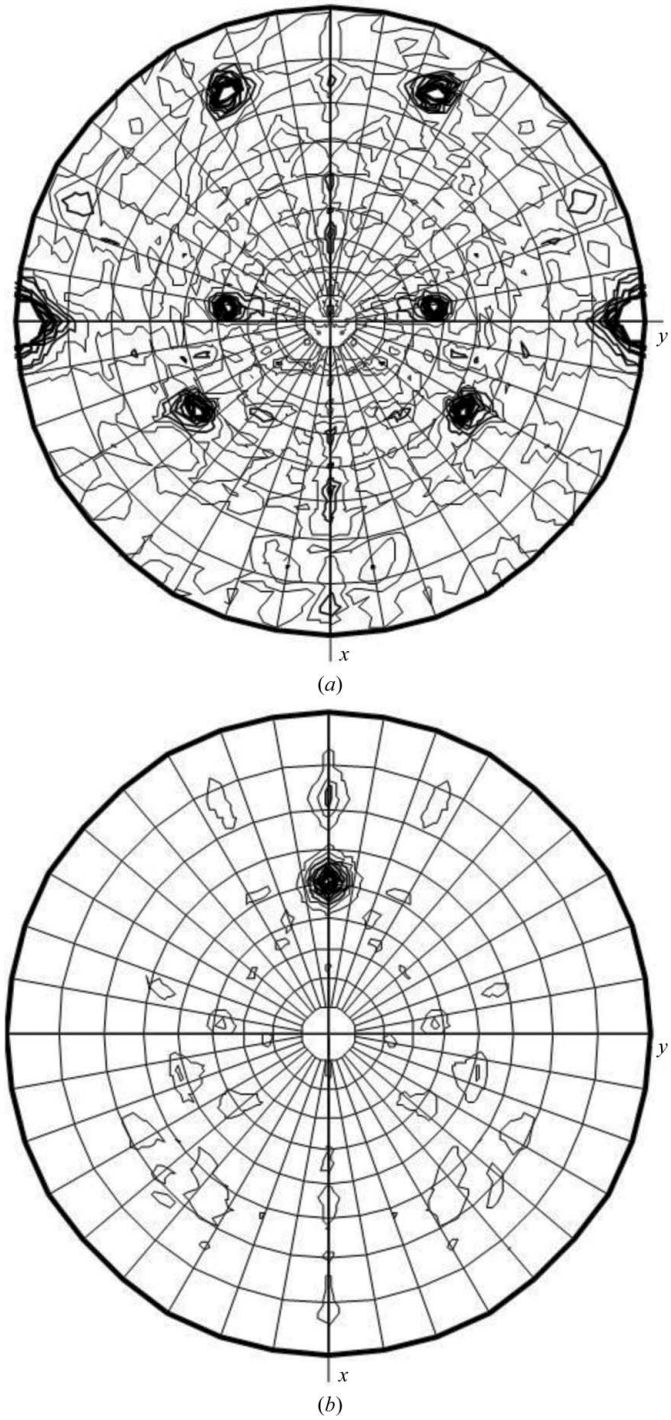


Figure 4
(*a*) The χ = 180° and (*b*) the χ = 90° sections of the self-rotation function calculated with *MOLREP* (Vagin & Teplyakov, 1997) using data from 50 to 4 Å resolution.

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