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# Overexpression, crystallization and preliminary X-ray crystallographic analysis of D-ribose-5-phosphate isomerase from *Clostridium thermocellum*

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Rare sugars are used for many industrial and medical purposes and are produced by the interconversion between aldoses and ketoses catalyzed by sugar and sugar-phosphate isomerases. Recently, *Clostridium thermocellum* D-ribose-5-phosphate isomerase (CTRPI), an aldose–ketose isomerase, was cloned in order to synthesize D-allose and its substrate specificity was further characterized for industrial usage. CTRPI has a novel substrate specificity that differs from those of other isomerases, which have broad substrate specificities. CTRPI prefers aldose substrates such as L-talose, D-ribose and D-allose. CTRPI was purified and crystallized in order to determine its three-dimensional structure and thus to elucidate its enzymatic reaction mechanism and understand its substrate specificity. The crystal belonged to the trigonal space group $P3_221$, with unit-cell parameters $a = b = 69.5$, $c = 154.4$ Å, and diffracted to 1.9 Å resolution. According to Matthews coefficient calculations, the crystallographic structure consists of a dimer in the asymmetric unit, with a $V_M$ of 3.2 Å$^3$ Da$^{-1}$ and a solvent content of 61.7%.

## 1. Introduction

Rare sugars have many industrial and medical usages, including uses as low-calorie sweeteners and bulking agents (Levin *et al.*, 1995; Livesey & Brown, 1996; Levin, 2002; Matsuo *et al.*, 2002), as immunosuppressants (Hossain *et al.*, 2000), as potential inhibitors of various glycosidases (Muniruzzaman *et al.*, 1996), in the treatment of ischaemia/reperfusion injury of the liver (Hossain *et al.*, 2004) and in segmented neutrophile production (Murata *et al.*, 2003). They are produced by the interconversion between aldoses and ketoses catalyzed by sugar and sugar-phosphate isomerases. For industrial purposes, many efforts have been made to identify sugar isomerases with unique specificity which can be used to produce rare sugars efficiently. Most known sugar isomerases have broad substrate specificities. D-Arabinose isomerase from *Klebsiella pneumonia* converts D-arabinose, L-xylose, D-mannose and L-lyxose to D-ribulose, L-xylulose, D-fructose and L-xylulose, respectively (Menavuvu *et al.*, 2006). L-Rhamnose isomerase from *Pseudomonas stutzeri* converts D-psicose to D-allose and D-altrose, converts L-xylulose to L-xylose and L-lyxose, converts L-fructose to L-mannose and L-glucose and converts D-ribulose to D-arabinose and D-ribose (Leang *et al.*, 2004). Galactose-6-phosphate isomerase (LacAB) from *Lactococcus lactis* catalyzes the interconversion D-allose ↔ D-psicose ↔ D-altrose (Park, Park *et al.*, 2007).

D-Ribose-5-phosphate isomerase (Rpi; EC 5.3.1.6) is an aldose–ketose isomerase. Rpi is involved in D-allose metabolism by converting D-ribose-5-phosphate to D-ribulose-5-phosphate and *vice versa* (Fig. 1) in a branch of the pentose phosphate pathway (Grochowski *et al.*, 2005). Rpis are divided into two types, namely RpiA and RpiB. RpiA is present in all organisms but RpiB is only present in a few bacterial and eukaryotic species. Recently, RpiB from *Clos-*

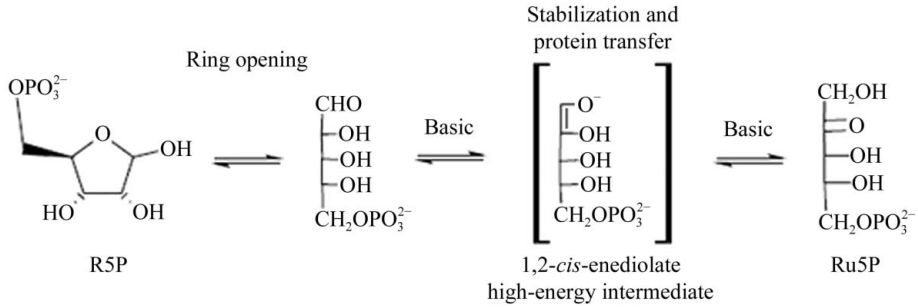


**Figure 1**
The isomerization catalyzed by Rpi proteins.

*tridium thermocellum* has been used to produce D-allose from D-psicose (Park, Yeom *et al.*, 2007). RpiA and RpiB have similar functions but vary in their sequence and molecular size. Structurally, RpiA is a dimer with two different active sites, while RpiB has an active-site interface between the dimer structures (Zhang *et al.*, 2003).

*C. thermocellum* D-ribose-5-phosphate isomerase (CTRPI) has a novel narrow substrate specificity for rare sugars and reversibly converts aldose substrates containing hydroxyl groups oriented in the same direction to one of the corresponding ketoses (Yoon *et al.*, 2009). Of the D- and L-forms of all pentose and hexoses, including ribose, talose, allose, ribulose, tagatose and psicose, the CTRPI enzyme shows the highest specificity for L-talose. CTRPI prefers aldose substrates to ketose substrates and prefers aldoses with the C2, C3 and C4 hydroxyl groups in the right-handed configuration (Fischer projections), such as L-talose, D-ribose and D-allose, compared with those with left-handed hydroxyl groups, such as L-allose, L-ribose and D-talose. A similar trend is observed for ketose substrates, in which ketoses with right-handed hydroxyl groups at C3 and C4, such as D-ribulose, D-psicose and L-tagatose, were preferred to those with left-handed C3 and C4 hydroxyl groups, such as L-ribulose, L-psicose and D-tagatose.

In order to obtain a better understanding of the substrate specificity and preference of sugar isomerases, the target CTRPI was chosen as a prototype. It was cloned, expressed, purified and crystallized and preliminary X-ray crystallographic studies were carried out with the aim of understanding its structural conformation and substrate specificity. The atomic resolution structure of CTRPI complexed with substrates may provide information on its ligand specificity and for engineering of the enzyme to enhance its activity for industrial purposes.

## 2. Materials and methods

### 2.1. Cloning

The ribose-5-phosphate isomerase (*rpiB*) gene was amplified from the genomic DNA of *C. thermocellum* by PCR using *Pfu* DNA polymerase (Daemyung Science, Seoul, Republic of Korea). The primer sequences for gene cloning were engineered based on the DNA sequence of the *C. thermocellum rpiB* gene (GenBank accession No. ZP 00503831). Forward (5′-C**CCATGG**AGGAAAGTATGAAAATTGG-3′) and reverse (5′-C**CTGCAG**ATCAACGGATGATCCATAAC-3′) primers were designed (Bioneer Co., Daejon, Republic of Korea) to introduce *Nco*I and *Pst*I restriction sites (shown in bold). The PCR-amplified DNA fragment was purified and digested with *Nco*I and *Pst*I endonucleases (Promega, Madison, Wisconsin, USA). The resulting DNA fragment was extracted and then inserted into the pBluescript II SK(+) plasmid digested with the same restriction enzymes. The resultant plasmid (pBCTrpiB) was digested with *Nco*I and *Pst*I and the fragment obtained containing the *rpiB* gene was treated using the Klenow fragment (Takara, Shiga, Japan). Finally, the fragment was inserted into pET28a(+) plasmid

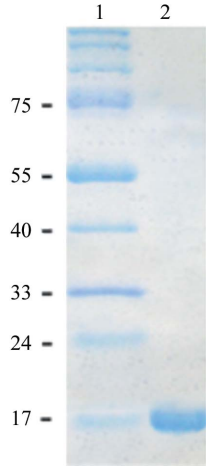


**Figure 2**
SDS–PAGE analysis of purified ribose-5-phosphate isomerase from *C. thermocellum*. The proteins were analyzed using 15% SDS–PAGE and stained with Coomassie Blue. Lane 1, molecular-weight markers (kDa); lane 2, purified CTRPI enzyme.

**Figure 3**
Crystal of CTRPI protein obtained by the sitting-drop vapour-diffusion method after three weeks using Crystal Screen condition No. 6 (0.05 *M* Tris pH 7.0, 10% PEG 8000, 0.15 *M* $MgCl_2$ and 0.2 *M* KCl).

and fully digested with *Nde*I and *Xho*I to obtain the pETrpiB plasmid containing an N-terminal six-His tag.

### 2.2. Overexpression and purification

CTRPI was overexpressed in *Escherichia coli* ER2566 cells. The cells were grown at 310 K to an $OD_{600}$ of 0.6 in Luria–Bertani medium containing 50 µg ml$^{-1}$ kanamycin. Protein expression was induced by the addition of 0.5 m*M* isopropyl *β*-D-1-thiogalactopyranoside (IPTG). Cell growth was continued at 310 K for 6 h after IPTG induction. The recombinant cells were harvested from the culture broth by centrifugation at 6000*g* for 30 min at 277 K, washed twice with 0.85% NaCl and then resuspended in buffer *A* (50 m*M* sodium monophosphate pH 8.0, 300 m*M* NaCl, 10 m*M* imidazole with 0.1 m*M* phenylmethylsulfonyl fluoride as a protease inhibitor). The resuspended cells were disrupted by ultrasonication (Fisher Scientific, Pittsburgh, Pennsylvania, USA) on ice. The cell debris was removed by centrifugation at 13 000*g* for 20 min at 277 K and the supernatant was filtered through a 0.45 µm filter. The filtrate was applied onto a HisTrap HP chromatography column (Amersham Biosciences, Uppsala, Sweden) equilibrated with buffer *A*. The bound protein was eluted with a linear gradient of 10–250 m*M* imidazole in buffer *A* using a BioLogic Duoflow QuadTec system (Bio-Rad, Hercules, California, USA) in a cold room. The homogeneity of the purified protein was assessed by SDS–PAGE (Fig. 2). The active fraction was dialyzed at 277 K overnight against 50 m*M* Tris–HCl buffer pH 7.5 and the resulting solution was concentrated to 7 mg ml$^{-1}$ for crystallization. The native PAGE (not shown) of CTRPI supported its existence as a dimer with a molecular mass of around 35 kDa.

### 2.3. Crystallization and X-ray data collection

Initial crystallization was conducted at 283 K using the sitting-drop vapour-diffusion method in a 24-well Cryschem plate using the Hampton Research screening kits Crystal Screen and Crystal Screen 2. The sitting drops were made up of 1 µl protein solution mixed with 1 µl reservoir solution and were positioned over 1 ml reservoir solution. The plate was sealed with Crystal Clear sealing tape. Initially, very tiny and poorly shaped crystals were observed. After optimization, bipyramid-shaped crystals with adequate thickness were observed with a reservoir solution containing 0.05 *M* Tris pH 7.0, 10% PEG 8000, 0.15 *M* $MgCl_2$ and 0.2 *M* KCl (Crystal Screen condition No. 6) over a period of three weeks (Fig. 3). The fully grown crystal was flash-cooled at 100 K in liquid nitrogen with a cryoprotectant solution containing 65 m*M* Tris pH 7.0, 13% PEG 8000, 195 m*M* $MgCl_2$, 260 m*M* KCl and 20% glycerol. X-ray diffraction data were collected from the flash-cooled crystals using a

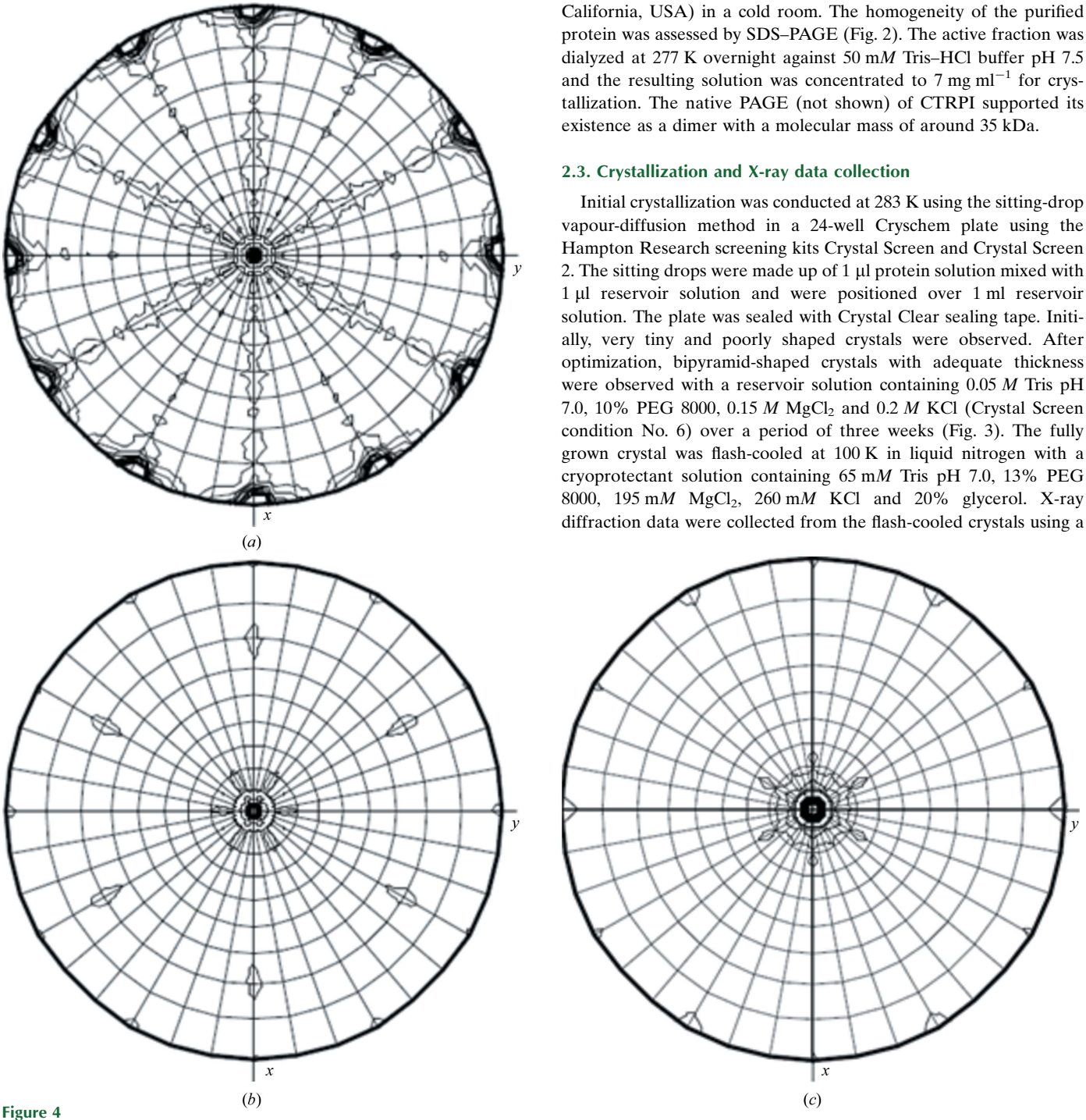


**Figure 4**
(*a*) $\chi = 180^\circ$, (*b*) $\chi = 120^\circ$ and (*c*) $\chi = 60^\circ$ sections of the self-rotation function calculated with *MOLREP* (Vagin & Teplyakov, 1997) using data from 50 to 4 Å resolution.

**Table 1**
Data-collection statistics.

Values in parentheses are for the last resolution shell.

| | |
|---|---|
| Synchrotron beamline | PAL 6C1 |
| Wavelength (Å) | 0.96418 |
| Unit-cell parameters | |
| $a = b$ (Å) | 69.5 |
| $c$ (Å) | 154.4 |
| $\alpha = \beta$ (°) | 90 |
| $\gamma$ (°) | 120 |
| Space group | $P3_22_1$ |
| Resolution (Å) | 50.00–1.90 (1.97–1.90) |
| Total No. of reflections | 32228 |
| No. of unique observations | 2036 |
| Redundancy | 10.5 (10.2) |
| Completeness (%) | 92.2 (59.3) |
| Molecules per ASU | 2 |
| $V_M$ (Å$^3$ Da$^{-1}$) | 3.2 |
| Solvent content (%) | 61.7 |
| $R_{merge}$† (%) | 0.051 (0.477) |
| $I/\sigma(I)$ | 63.1 (5.6) |

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

Bruker Proteum 300 CCD on beamline 6C1 at Pohang Light Source (PLS), Republic of Korea. The crystal was rotated by 1.0° per frame and data were collected to 1.9 Å resolution. The data sets were integrated and scaled using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997), respectively. Based on autoindexing with *DENZO*, the CTRPI crystal belonged to the trigonal space group $P3_22_1$, with unit-cell parameters $a = b = 69.5$, $c = 154.4$ Å, $\alpha = \beta = 90$, $\gamma = 120$°. The final statistics of data collection and processing details are summarized in Table 1. According to Matthews coefficient calculations, the crystal contains two monomers in the asymmetric unit, with a volume per unit molecular weight of the protein of 3.2 Å$^3$ Da$^{-1}$ and a calculated solvent content of 61.7% (Matthews, 1968). Initial structure solution was carried out using the program *MOLREP* (Vagin & Teplyakov, 1997) in space groups $P3_22_1$ and $P3_12_1$. Of these space groups, $P3_22_1$ ($R$ and $R_{free}$ of 39.7% and 42.3%, respectively) showed more reliable $R$ factors and correlation coefficient than $P3_12_1$ ($R$ and $R_{free}$ of 42.1% and 46.5%, respectively). Self-rotation functions were calculated at $\chi = 180$, 120, 90 and 60° to detect twofold, threefold, fourfold and sixfold rotation axes. Based on this self-rotation calculation (Fig. 4), the presence of twofold, threefold and sixfold rotation axes was confirmed. Molecular replacement was performed using the *E. coli* ECRPI structure (PDB code 2vvr; Roos *et al.*, 2008) as a model. Our structural information on CTRPI will provide an insight into its enzymatic mechanism and substrate preference and will also be useful for the development of an improved enzyme for industrial purposes.

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