Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

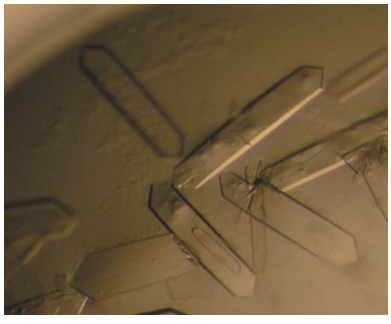
Crystallization and preliminary X-ray diffraction studies of L-rhamnose isomerase from *Pseudomonas stutzeri*

Hiromi Yoshida,[a] Poonperm Wayoon,[b] Goro Takada,[b] Ken Izumori[b] and Shigehiro Kamitori[a]*

[a]Molecular Structure Research Group, Information Technology Center and Faculty of Medicine, Kagawa University, 1750-1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793, Japan, and [b]Department of Biochemistry and Food Science, Faculty of Agriculture and Rare Sugar Research Center, Kagawa University, Miki-cho, Kagawa 761-0795, Japan

Correspondence e-mail: kamitori@med.kagawa-u.ac.jp

Received 2 March 2006
Accepted 1 May 2006



L-Rhamnose isomerase from *Pseudomonas stutzeri* (*P. stutzeri* L-RhI) catalyzes not only the reversible isomerization of L-rhamnose to L-rhamnulose, but also isomerization between various rare aldoses and ketoses. Purified His-tagged *P. stutzeri* L-RhI was crystallized by the hanging-drop vapour-diffusion method. The crystals belong to the monoclinic space group $P2_1$, with unit-cell parameters $a = 74.3$, $b = 104.0$, $c = 107.0$ Å, $\beta = 106.8^\circ$. Diffraction data have been collected to 2.0 Å resolution. The molecular weight of the purified *P. stutzeri* L-RhI with a His tag at the C-terminus was confirmed to be 47.7 kDa by MALDI–TOF mass-spectrometric analysis and the asymmetric unit is expected to contain four molecules.

1. Introduction

L-Rhamnose isomerase (L-RhI) catalyzes the reversible isomerization of L-rhamnose to L-rhamnulose and has been found to be an enzyme related to L-rhamnose metabolism in *Escherichia coli* (Wilson & Ajl, 1957; Moralejo *et al.*, 1993). It is also present in other microorganisms such as *Lactobacillus* (Domagk & Zech, 1963), *Salmonella* (Englesberg & Baron, 1959) and *Pseudomonas* (Bhuiyan *et al.*, 1997*a*). L-RhI from *Pseudomonas stutzeri* (*P. stutzeri* L-RhI, 430 amino-acid residues, 46 975 Da) shows a broader substrate specificity than *E. coli* L-RhI and is capable of catalyzing isomerizations between various aldoses and ketoses, as well as between L-rhamnose and L-rhamnulose (Bhuiyan *et al.*, 1997*b*; Leang, Takada, Fukai *et al.*, 2004; Leang, Takada, Ishimura *et al.*, 2004). Recently, *P. stutzeri* L-RhI was cloned and expressed in *E. coli* as recombinant His-tagged *P. stutzeri* L-RhI (Leang, Takada, Fukai *et al.*, 2004; Leang, Takada, Ishimura *et al.*, 2004), showing that His-tagged *P. stutzeri* L-RhI can efficiently catalyze isomerizations between L-mannose and L-fructose, D-allose and D-psicose, D-gulose and D-sorbose, L-talose and L-tagatose, and D-ribose and D-ribulose. Since some of these are so-called 'rare sugars' which exist in small amounts in nature, *P. stutzeri* L-RhI has been exploited for industrial applications in rare sugar production (Bhuiyan *et al.*, 1997*b*, 1998, 1999). However, little is known about the mechanism responsible for the broad substrate specificity of *P. stutzeri* L-RhI on the basis of the three-dimensional structure. Although the crystal structure of *E. coli* L-RhI has been determined, sequence homology between *P. stutzeri* and *E. coli* L-RhIs reveals only 17% similarity (Leang, Takada, Ishimura *et al.*, 2004; Korndörfer *et al.*, 2000), suggesting that *P. stutzeri* L-RhI may have a significantly different structure from *E. coli* L-RhI. The three-dimensional structure of the enzyme is expected to provide clues to elucidate the wide recognition of various substrates and to facilitate the design of engineered enzymes for controlled substrate specificity and more effective production of rare sugars. We report here the crystallization and preliminary X-ray diffraction studies of recombinant *P. stutzeri* L-RhI.

2. Materials and methods

2.1. Protein preparation

Recombinant His-tagged L-RhI was expressed in *E. coli* JM109 harbouring a plasmid pOI-02 which was constructed previously

(Leang, Takada, Fukai *et al.*, 2004). Plasmid pOI-02 contains the L-RhI gene (DDBJ accession No. AB121136) with additional restriction-enzyme sites and a six-His tag from expression vector pQE60 (Qiagen, Valencia, CA, USA) at the C-terminus of L-RhI; consequently, the His-tagged L-RhI gene contains 1320 bp encoding a protein consisting of 440 amino-acid residues with a predicted molecular weight of 48 185 Da.

The cells were grown at 310 K in LB medium containing 100 µg ml^{-1} ampicillin until the culture reached an optical density of 0.7 at 600 nm and L-RhI overexpression was subsequently initiated by the addition of 1 m*M* isopropyl *β*-D-thiogalactopyranoside (IPTG). The cells were harvested after a further 4 h of cultivation and were then 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 Bio-Sciences Corp., Piscataway, NJ, 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 away the unbound protein with equilibration buffer, the protein was eluted with a linear gradient of 50 m*M* NaH_2PO_4, 300 m*M* NaCl, 250 m*M* imidazole pH 8.0. The purified protein was confirmed by SDS–PAGE, showing a single band with an estimated molecular weight of 43 kDa. After dialysis against the buffer solution (20 m*M* Tris–HCl pH 8.0) overnight, the purified His-tagged protein was concentrated to a final concentration of 40 mg ml^{-1} with a Centriprep YM-10 filter (Millipore, Billerica, MA, USA).

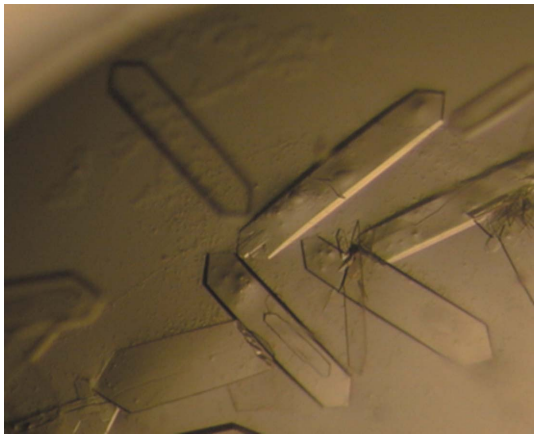


Figure 1
Crystals of His-tagged *P. stutzeri* L-RhI.

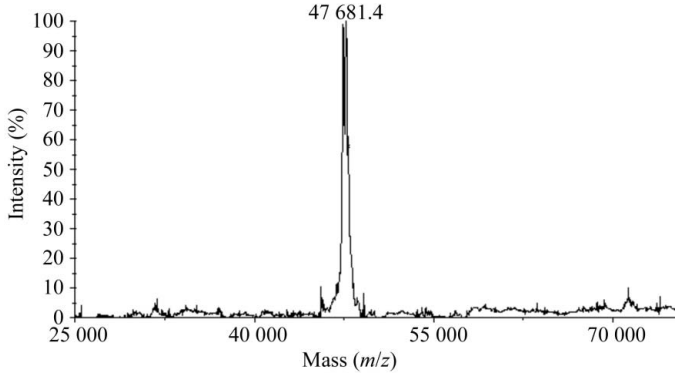


Figure 2
Mass spectrum of His-tagged *P. stutzeri* L-RhI.

2.2. Mass spectrometry

The protein sample for crystallization was mixed with an equal volume of 10 mg ml^{-1} sinapinic acid matrix in 50% acetonitrile and 0.1% trifluoroacetic acid. 1 µl of the mixture was applied to the target spot on a plate and dried in air. Using the MALDI time-of-flight mass spectrometer Voyager-DE STR (Applied Biosystems, CA, USA) in linear operation mode, MALDI–TOF mass spectra were recorded and calibrated with bovine serum albumin.

2.3. Crystallization

The initial crystallization screen for His-tagged *P. stutzeri* L-RhI was performed using Hampton Research Crystal Screens I and II by the sitting-drop method at room temperature. The initial crystals appeared on mixing the same volumes of protein and reservoir solution using Crystal Screen I condition No. 43 [30%(*w*/*v*) PEG 1500] or Crystal Screen II condition No. 22 [12%(*w*/*v*) PEG 20 000, 0.1 *M* MES pH 6.5]. After optimizing the crystallization condition using the hanging-drop vapour-diffusion method, crystals were obtained from a drop consisting of 2 µl protein solution (20 mg ml^{-1}) and 2 µl reservoir solution containing 7–8%(*w*/*v*) PEG 20 000 and 50 m*M* MES buffer pH 6.3 equilibrated against 500 µl reservoir solution at 293 K. Small crystals appeared within 1 d and reached maximum dimensions of 150 × 100 × 50 µm in a week (Fig. 1).

3. Results and discussion

The mass spectrum of His-tagged *P. stutzeri* L-RhI showed a molecular weight of 47 681 Da (Fig. 2) that differed from the molecular weight of 43 kDa previously estimated by SDS–PAGE and the theoretical molecular weight of 48 185 Da. However, there was no doubt unexpected cleavage or truncation of the produced recombinant His-tagged protein that resulted in 43 kDa.

A complete data set at 2.0 Å resolution was collected at the Photon Factory (Tsukuba, Japan) on beamline BL-6A using an ADSC

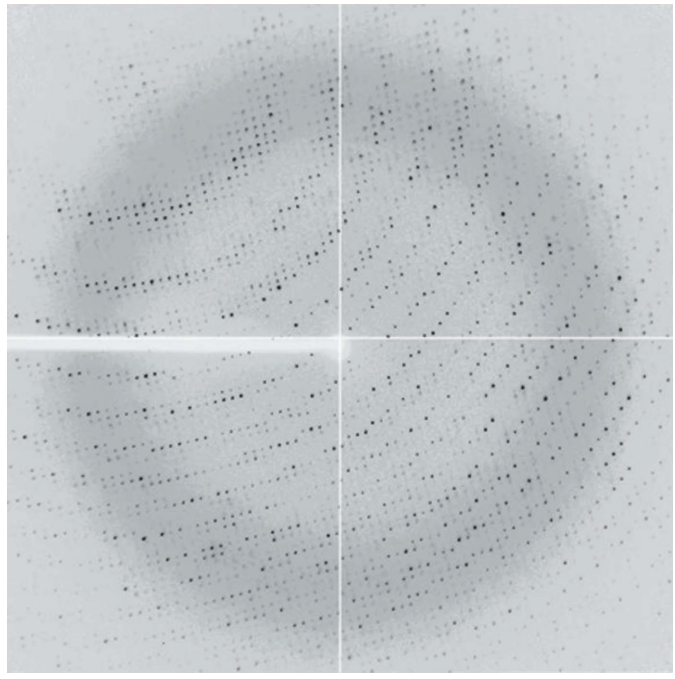


Figure 3
Diffraction images of His-tagged *P. stutzeri* L-RhI.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution bin (2.07–2.00 Å).

Temperature (K)	100
Wavelength (Å)	1.000
Resolution range (Å)	50–2.0
No. of measured reflections	647726
No. of unique reflections	99909
Completeness (%)	94.9 (95.0)
Mean $I/\sigma(I)$	13.3 (7.5)
R_{sym}† (%)	7.4 (29.8)
Space group	$P2_1$
Unit-cell parameters	94.9
a (Å)	74.3
b (Å)	104.0
c (Å)	107.0
β (°)	106.8

† $R_{sym} = \sum_h \sum_i [|I_i(h) - \langle I(h)\rangle|/\sum_h \sum_i I_i(h)]$, where I_i is the ith measurement and $\langle I(h)\rangle$ is the weighted mean of all measurements of $I(h)$.

Quantum 4R CCD detector at a wavelength of 1.0 Å (Fig. 3). Prior to data collection, crystals were soaked for a few seconds in cryosolution containing 15%(*w*/*v*) PEG 20 000 and 50%(*v*/*v*) glycerol, 0.1 *M* MES pH 6.3, followed by flash-cooling in a gas stream from liquid nitrogen. All data were processed with *HKL*2000 (Otwinowski & Minor, 1997). The data-collection statistics are summarized in Table 1. The asymmetric unit is likely to contain four single chains of His-tagged *P. stutzeri* L-RhI molecules with a crystal volume per unit molecular weight V_M of 2.1 Å^3 Da^{-1}, corresponding to a solvent content of 41.1% (Matthews, 1968). In the *E. coli* L-RhI crystal structure, the enzyme forms a homotetramer of 188 kDa (47 kDa per monomer containing 430 residues) related by non-crystallographic 222 symmetry (Korndörfer *et al.*, 2000). Although we tried to find non-crystallographic symmetry elements between subunits by self-rotation function calculations, a clear solution could not be obtained. Also, cross-rotation function calculations using the structures of a homotetramer and/or homodimer extracted from *E. coli* L-RhI and/or xylose isomerases of known structure showing 17–25% similarity to *P. stutzeri* L-RhI as the probe structure [PDB codes 1de6; Korndörfer *et al.*, 2000), 2xin (Jenkins *et al.*, 1992) and 4xis (Whitlow *et al.*, 1991)] have been tried without success. We are in the process of identification of the initial phases of *P. stutzeri* L-RhI, also trying heavy-atom soaking and preparing crystals of selenomethionine-substituted protein.

This study was supported in part by the National Project on Protein Structural and Functional Analyses and the 'intellectual cluster' from the Ministry of Education, Culture, Sports, Science and Technology of Japan. This research was performed with the approval of the Photon Factory Advisory Committee and the National Laboratory for High Energy Physics, Japan (proposals 2003S2002 and 2003G305).

References

Bhuiyan, S. H., Itami, Y. & Izumori, K. (1997*a*). *J. Ferment. Bioeng.* **84**, 319–323.
Bhuiyan, S. H., Itami, Y. & Izumori, K. (1997*b*). *J. Ferment. Bioeng.* **84**, 558–562.
Bhuiyan, S. H., Itami, Y., Rokui, Y., Katayama, T. & Izumori, K. (1998). *J. Ferment. Bioeng.* **85**, 539–541.
Bhuiyan, S. H., Itami, Y., Takada, G. & Izumori, K. (1999). *J. Ferment. Bioeng.* **88**, 567–570.
Domagk, G. F. & Zech, R. (1963). *Biochem. Z.* **339**, 145–153.
Englesberg, E. & Baron, L. S. (1959). *J. Bacteriol.* **78**, 675–686.
Jenkins, J., Janin, J., Rey, F., Chiadmi, M., van Tilbeurgh, H., Lasters, I., De Maeyer, M., Van Belle, D., Wodak, S. J., Lauwereys, M., Stanssens, P., Mrabet, N. T., Snauwaert, J., Matthyssens, G. & Lambeir, A.-M. (1992). *Biochemistry*, **31**, 5449–5458.
Korndörfer, I. P., Fessner, W. D. & Matthews, B. W. (2000). *J. Mol. Biol.* **300**, 917–933.
Leang, K., Takada, G., Fukai, Y., Morimoto, Y., Granstrom, T. B. & Izumori, K. (2004). *Biochim. Biophys. Acta*, **1674**, 68–77.
Leang, K., Takada, G., Ishimura, A., Okita, M. & Izumori, K. (2004). *Appl. Environ. Microbiol.* **70**, 3298–3304.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Moralejo, P., Egan, S. M., Hidalgo, E. & Aguilar, J. (1993). *J. Bacteriol.* **175**, 5585–5594.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Whitlow, M., Howard, A. J., Finzel, B. C., Poulos, T. L., Winborne, E. & Gilliland, G. L. (1991). *Proteins*, **9**, 153–173.
Wilson, D. M. & Ajl, S. (1957). *J. Bacteriol.* **73**, 410–414.