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Overexpression, purification, crystallization and preliminary X-ray crystal analysis of *Bacillus pallidus* D-arabinose isomerase

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D-Arabinose isomerase catalyzes the isomerization of D-arabinose to D-ribulose. *Bacillus pallidus* D-arabinose isomerase has broad substrate specificity and can catalyze the isomerization of D-arabinose, L-fucose, L-xylose, L-galactose and D-altrose. Recombinant *B. pallidus* D-arabinose isomerase was overexpressed, purified and crystallized. A crystal of the enzyme was obtained by the sitting-drop method at room temperature and belonged to the orthorhombic space group $P2_12_12$, with unit-cell parameters a = 144.9, b = 127.9, c = 109.5 Å. Diffraction data were collected to 2.3 Å resolution.

1. Introduction

D-Arabinose isomerase (D-AI) catalyzes the isomerization of D-arabinose (aldose) to D-ribulose (ketose) (Oliver & Mortlock, 1971). Since the enzyme also catalyzes the isomerization of L-fucose to L-fuculose, which is D-arabinose with a methyl group at the 6-position, it is called L-fucose isomerase when found in L-fucose metabolism in *Escherichia coli* (Chen *et al.*, 1987). *Bacillus pallidus* D-arabinose isomerase (*B. pallidus* D-AI; 595 amino-acid residues) shows broad substrate specificity and can catalyze isomerization between D-arabinose and D-ribulose, L-fucose and L-fuculose, L-xylose and L-xylulose, L-galactose and L-tagatose, and D-altrose and D-psicose (unpublished results). Some of these are the so-called 'rare sugars' which exist in small amounts in nature but have great significance not only in the food industry but also in the medicinal industry (Levin, 2002; Matsuo *et al.*, 2002, 2003). Recently, a complete strategy for the bioproduction of rare sugars has been developed (Granström *et al.*, 2004) and *B. pallidus* D-AI with its high thermostability is considered to have great potential for industrial applications in the bioproduction of rare sugars.

To date, two types of mechanism have been proposed for ketol isomerization. One type is the hydride-shift mechanism, which is supported for D-xylose isomerase (Whitlow *et al.*, 1991; Allen *et al.*, 1994) and L-rhamnose isomerase (Korndörfer *et al.*, 2000; Yoshida *et al.*, 2007) by their crystal structures. Another type is the ene-diol mechanism, which is supported in the cases of *E. coli* L-fucose isomerase (Seemann & Schulz, 1997) and *E. coli* L-arabinose isomerase (Manjasetty & Chance, 2006) by the crystal structures of these enzymes. The two types of enzymes are not related by convergent evolution (Banerjee *et al.*, 1995). The former type of enzyme adopts a $(\beta/\alpha)_8$-barrel and is a tetramer. The latter type of enzyme forms a relatively large hexamer and each subunit has a novel structure with three domains of almost equal size: a $(\beta/\alpha)_8$-barrel domain and two Rossmann-fold domains. A three-dimensional structure-homology search revealed that the type of folding found in *E. coli* L-fucose isomerase and *E. coli* L-arabinose isomerase has never been observed previously in proteins deposited in the PDB (Holm & Sander, 1996; Kawabata & Nishikawa, 2000). *B. pallidus* D-AI has a sequence identity of 63% to *E. coli* L-fucose isomerase,

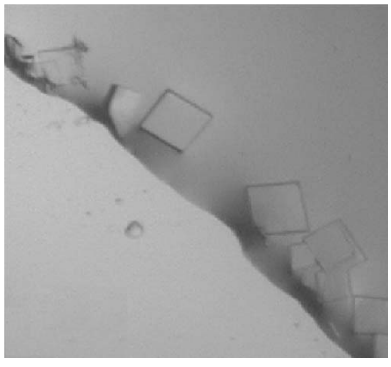

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution bin (2.38–2.30 Å).

Temperature (K)	100
Wavelength (Å)	1.0
Resolution range (Å)	50.0–2.3
No. of measured reflections	592546
Detector	Quantum 315
No. of images	180
Oscillation range (°)	1
No. of oscillations per image	1
Exposure time per image (s)	5
Crystal-to-detector distance (mm)	318.6
No. of unique reflections	91188
Completeness (%)	100 (100)
Mean $I/\sigma(I)$	8.8 (4.5)
$R_{\rm merge}$†	0.075 (0.333)
Space group	$P2_12_12$
Unit-cell parameters	
a (Å)	144.9
b (Å)	127.9
c (Å)	109.5
No. of monomers in ASU	3

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

suggesting that it belongs to the type of enzymes that undergo the ene-diol mechanism of ketol isomerization with structural similarity to *E. coli* L-fucose isomerase and *E. coli* L-arabinose isomerase.

In order to elucidate the catalytic mechanism of *B. pallidus* D-AI and to accumulate structural information about enzymes that undergo the ene-diol mechanism of ketol isomerization, determination of the three-dimensional structure of this enzyme is very important. Here, we present the overexpression, purification, crystallization and preliminary X-ray crystal analysis of *B. pallidus* D-AI.

2. Materials and methods

2.1. Overexpression and purification

Details of the cloning and expression of the *B. pallidus* D-AI gene from *B. pallidus* strain 14a (DDBJ accession No. AB429010) will be published elsewhere. The *B. pallidus* D-AI gene was subcloned from the previously constructed plasmid pUBDAI by the PCR method using the following designed primers: forward, 5′-GA**CCATGG**CA-AAAGATCCACGATATGTAG-3′, and reverse, 5′-AAGC**AGATC**-**T**TTTATAGATCGGGCCCAAAGTTTTTTAC-3′, including *Nco*I and *Bgl*II sites (bold), respectively. PCR products digested with *Nco*I and *Bgl*II were inserted into the expression vector pQE60 (Qiagen, Valencia, California, USA) linearized with *Nco*I and *Bgl*II. A clone was selected by D-AI activity assay. The constructed plasmid (pAI-02) was transformed into *E. coli* JM109 for *B. pallidus* D-AI overexpression. DNA sequencing was carried out using an ABI PRISM 310 Genetic Analyzer.

The cells were grown at 303 K in 2×YT medium containing 100 µg ml^{-1} ampicillin for 12 h. *B. pallidus* D-AI overexpression was initiated by the addition of 0.2 m*M* isopropyl β-D-1-thiogalactopyranoside (IPTG) and cultivation at 298 K for 12 h. The cells were harvested by centrifugation at 7300*g* for 10 min at 277 K. The pellet obtained from 1.0 l expression culture was resuspended in 30 ml buffer solution (10 m*M* HEPES pH 8.0) containing protease inhibitors (one tablet of Complete EDTA-free from Roche Diagnostics) and sonicated. The sonicated sample was centrifuged (20 400*g*, 20 min, 277 K) and the supernatant was loaded onto an anion-exchange column (20 × 85 mm, Q Sepharose High Performance; GE Healthcare Biosciences Corp., Piscataway, New Jersey, USA). After washing with buffer solution (10 m*M* HEPES pH 8.0), *B. pallidus* D-AI was eluted with a linear gradient of 0.0–1.0 *M* NaCl in 10 m*M* HEPES pH 8.0 at a flow rate of 5 ml min^{-1}. Fractions containing *B. pallidus* D-AI (27 ml) were treated with 2.0 *M* $(NH_4)_2SO_4$ and applied onto a Resource PHE hydrophobic interaction column (16 × 30 mm; GE Healthcare Biosciences Corp., Piscataway, New Jersey, USA). After washing with buffer solution [10 m*M* HEPES, 2.0 *M* $(NH_4)_2SO_4$ pH 8.0], *B. pallidus* D-AI was eluted by a linear gradient using the same buffer as before but without ammonium sulfate at a flow rate of 6 ml min^{-1}. Fractions containing *B. pallidus* D-AI (6 ml) were dialyzed against buffer solution (10 m*M* HEPES pH 8.0) and the protein solution was applied onto a Resource Q anion-exchange column (6.4 × 30 mm; GE Healthcare Biosciences Corp., New Jersey, USA). After washing with buffer solution (10 m*M* HEPES, 0.1 *M* NaCl pH 8.0), *B. pallidus* D-AI was eluted with a linear gradient of 0.1–0.25 *M* NaCl in 10 m*M* HEPES pH 8.0 at a flow rate of 3 ml min^{-1}. The fractions containing *B. pallidus* D-AI (3 ml) were dialyzed against buffer solution (10 m*M* HEPES pH 8.0) for 12 h and concentrated to 18 mg ml^{-1} using an Amicon Ultra-4 30 kDa Ultracel (Millipore, Billerica, Massachusetts, USA) for storage. The protein concentration was determined by measuring the absorbance at 280 nm and calculating the absorption coefficient. The purity was checked by SDS–PAGE analysis. Samples were loaded onto 10% SDS polyacrylamide gel and electrophoresed, applying a voltage of 200 V. Bench Mark Protein Ladder (Invitrogen Corp., California, USA) was used as molecular-weight markers.

2.2. Crystallization and X-ray data collection

The initial crystallization conditions were screened using Crystal Screens I and II, Crystal Screen Cryo, PEG/Ion Screen (Hampton Research Corp., California, USA) and Wizard Screens I and II (Emerald BioSystems Inc., Washington, USA) by the sitting-drop method at 293 K. The storage protein solution described in the previous section was diluted with buffer solution (10 m*M* HEPES pH 8.0) to 10 mg ml^{-1}. Crystals appeared during equilibration of a droplet consisting of a mixture of the same volumes (1 µl) of protein and reservoir solution using Wizard Screen I condition No. 6 [100 m*M* citrate buffer (final pH 6.0), 20%(*w*/*v*) PEG 3000] against a reservoir containing 100 µl of the latter solution. To optimize the crystallization condition, Additive Screen (Hampton Research Corp., California., USA) was applied; the final reservoir solution was 100 m*M* citrate buffer (final pH 6.0), 20%(*w*/*v*) PEG 3000 and 100 m*M* potassium sodium tartrate.

Data collection was carried out using synchrotron radiation on beamline BL-5A at Photon Factory (Tsukuba, Japan). The crystal was soaked for a few seconds in cryosolution containing 20%(*v*/*v*) glycerol and then flash-cooled in a liquid-nitrogen gas stream. Diffraction data were then collected at 100 K using an ADSC/CCD detector system. Data processing was carried out using the program *HKL*-2000 (Otwinowski & Minor, 1997) at the beamline station. The data-collection statistics are listed in Table 1.

2.3. Enzyme assay

B. pallidus D-AI activity was assayed by measuring the increase in the keto sugar D-ribulose by the cysteine–sulfuric acid–carbazole method (Dische & Borenfreund, 1951). The reaction mixture contained 50 m*M* glycine–NaOH buffer pH 9.0, 1 m*M* $MnCl_2$ and 50 µl appropriately diluted enzyme. The enzymatic reaction was started by the addition of D-arabinose (to a final concentration of 5 m*M*). The mixture was incubated for 10 min at 328 K and the reaction was stopped by the addition of 50 µl 10% trichloroacetic

acid. After the enzyme reaction, 100 µl 1.5% cysteine hydrochloride solution, 3 ml 70% H_2SO_4 and 100 µl of a 0.12% alcoholic solution of carbazole were added to the reaction mixture, which was then incubated at 293 K for 20 min. After incubation, the production of the keto sugar was determined by measuring the absorption at 540 nm. One unit of enzyme activity represents the formation of 1 µmol D-ribulose in 1 min under these assay conditions.

3. Results and discussion

DNA sequencing of the constructed plasmids revealed two mutations. The first mutation occurs at codon 211 (5′-GAA-3′→5′-GGA-3′), leading to the replacement of a glutamate by a glycine. The second mutation is the insertion of an adenine nucleotide at codon 598 (shown in bold), leading to the replacement of seven residues owing to a frame shift at the C-terminal region; 5′-AAC TTT GGG CCG ATC TAT AAA TAA-3′→5′-AAA**A** CTT TGG GCC GAT CTA TAA-3′, giving Asn-Phe-Gly-Pro-Ile-Tyr-Lys-Stop→Lys-Leu-Trp-Ala-Asp-Leu-Stop. No other mutations or changes were found. Despite these mutations, the recombinant *B. pallidus* D-AI was successfully overexpressed in *E. coli* and purified using three columns, maintaining significant D-AI activity. A chromatogram of the final step, a Resource Q anion-exchange column, and the result of SDS–PAGE analysis are shown in Figs. 1 and 2, respectively.

A crystal suitable for X-ray data collection, which grew to dimensions of 0.12 × 0.10 × 0.01 mm in one week after set up, is shown in Fig. 3. The crystal of *B. pallidus* D-AI diffracted well to a resolution of 2.2 Å, as shown in Fig. 4, and a complete data set was successfully collected to 2.3 Å resolution, as summarized in Table 1. The crystal belongs to the orthorhombic space group $P2_12_12$, with unit-cell parameters a = 144.9, b = 127.9, c = 109.5 Å. The asymmetric unit is expected to contain three molecules, with a crystal volume per unit molecular weight V_M of 2.56 Å^3 Da^{-1}, corresponding to a solvent content of 52% (Matthews, 1968). If *B. pallidus* D-AI forms a hexamer like *E. coli* L-fucose isomerase, the two trimers in a hexamer must be related by a crystallographic twofold symmetry along the z

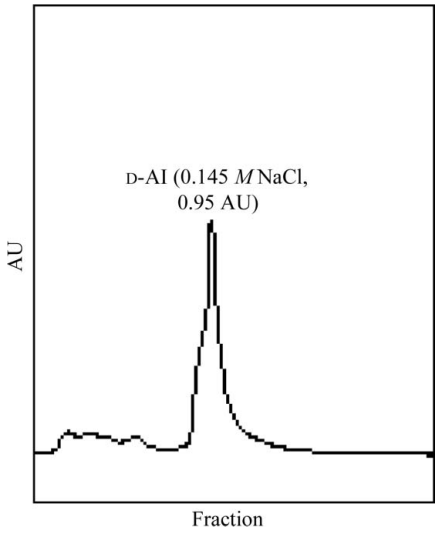

Figure 1
A chromatogram of D-AI on a Resource Q anion-exchange column.

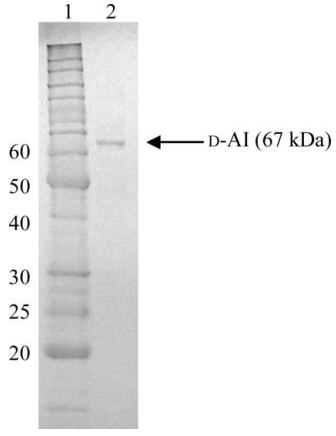

Figure 2
SDS–PAGE analysis of purified *B. pallidus* D-AI. Lane 1, molecular-weight markers. Lane 2, purified *B. pallidus* D-AI for crystallization.

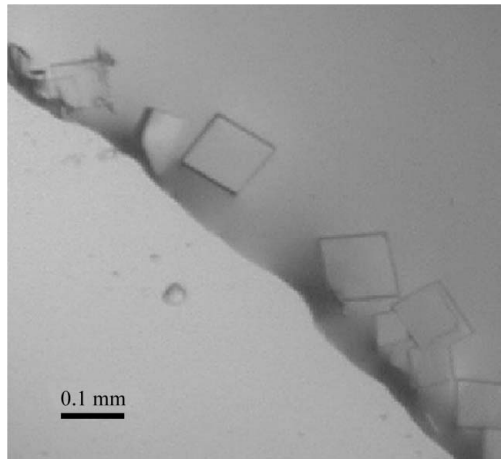

Figure 3
Crystals of *B. pallidus* D-AI with dimensions of 0.12 × 0.10 × 0.01 mm.

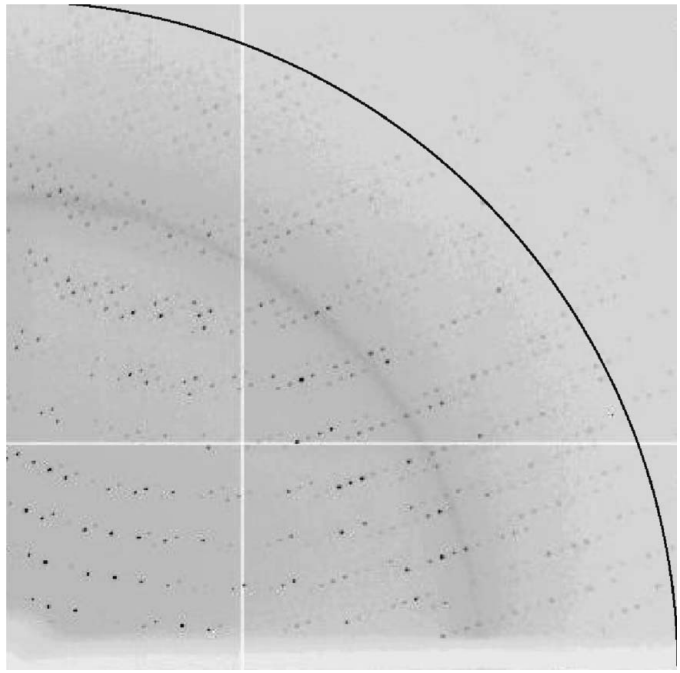

Figure 4
Diffraction images of *B. pallidus* D-AI with 2.2 Å resolution marked.

axis. This was supported by calculation of the self-rotation function using the program *POLARRFN* from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994), which revealed a clear local threefold axis perpendicular to the z axis: $\omega = 90.0^\circ$ (the inclination to the z axis within the yz plane), $\varphi = 72.8^\circ$ (within the xy plane), $\kappa = 120^\circ$.

We are currently refining the crystallization conditions in order to obtain a better diffracting crystal and attempting to solve the structure by the molecular-replacement method using the structure of *E. coli* L-fucose isomerase (PDB code 1fui) as a probe model. In addition, overexpression and purification of recombinant *B. pallidus* D-AI with the same amino-acid sequence as the wild-type enzyme have been carried out, but crystals have not yet been obtained.

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