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

Purification, crystallization and preliminary X-ray diffraction studies of D-tagatose 3-epimerase from *Pseudomonas cichorii*

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Received 28 November 2006
Accepted 10 January 2007

D-Tagatose 3-epimerase (D-TE) from *Pseudomonas cichorii* catalyzes the epimerization of various ketohexoses at the C3 position. The epimerization of D-psicose has not been reported with epimerases other than *P. cichorii* D-TE and D-psicose 3-epimerase from *Agrobacterium tumefaciens*. Recombinant *P. cichorii* D-TE has been purified and crystallized. Crystals of *P. cichorii* D-TE were obtained by the sitting-drop method at room temperature. The crystal belongs to the monoclinic space group $P2_1$, with unit-cell parameters a = 76.80, b = 94.92, c = 91.73 Å, β = 102.82°. Diffraction data were collected to 2.5 Å resolution. The asymmetric unit is expected to contain four molecules.

1. Introduction

D-Tagatose 3-epimerase (D-TE) from *Pseudomonas cichorii* (290 amino-acid residues, 32 615 Da, 65 kDa dimeric enzyme) catalyzes the epimerization of various ketohexoses at the C3 position, interconverting D-fructose and D-psicose, D-tagatose and D-sorbose, L-fructose and L-psicose, and L-tagatose and L-sorbose, some of which exist in small amounts in nature and are known as 'rare sugars'; therefore, it is a key enzyme for the bioproduction of rare sugars (Granstrom *et al.*, 2004; Izumori, 2002, 2006). In particular, interconversion between D-fructose and D-psicose by epimerization has been focused on as an attractive enzymatic reaction for the commercial production of D-psicose (Itoh *et al.*, 1995; Takeshita *et al.*, 2000).

P. cichorii D-TE has been studied for over a decade. It was cloned and expressed in *Escherichia coli* as recombinant *P. cichorii* D-TE (Itoh *et al.*, 1994; Ishida, Kamiya, Itoh *et al.*, 1997; Ishida, Kamiya & Izumori, 1997). Focusing on the enzymatic properties of *P. cichorii* D-TE, major studies have concentrated on the bioproduction of rare sugars (Itoh *et al.*, 1995; Ishida, Kamiya, Itoh *et al.*, 1997; Ishida, Kamiya & Izumori, 1997; Takeshita *et al.*, 2000). To date, D-TE or related proteins have been reported from various sources, including *Agrobacterium tumefaciens* (SWISS-PROT accession No. Q8U6Q7), *Rhizobium loti* (Q98GF0), *Verminephrobacter eiseniae* (Q0XK57) and *Fulvimarina pelagi* (Q0FYP5); however, no structural and catalytic mechanistic details for D-TE have been reported until very recently. The crystal structure of D-psicose 3-epimerase (D-PE) from *A. tumefaciens* (283 amino-acid residues, 30 650 Da, 132 kDa tetrameric enzyme), which belongs to the D-TE family and is able to catalyze the interconversion of D-fructose and D-psicose, has recently been determined (Kim, Kim *et al.*, 2006). *P. cichorii* D-TE and *A. tumefaciens* D-PE show a sequence identity of 38% and the epimerization activities towards rare sugars and biochemical properties of the two enzymes differ significantly. *P. cichorii* D-TE shows higher activity towards D-tagatose, D-sorbose, D-ribose and D-xylulose than *A. tumefaciens* D-PE (Kim, Hyun *et al.*, 2006).

In order to elucidate the catalytic mechanism of *P. cichorii* D-TE and to accumulate structural information about the enzymes involved in rare sugar production, as well as to compare its active site with that of *A. tumefaciens* D-PE, we attempted to crystallize *P. cichorii* D-TE in order to determine its three-dimensional structure. Here, we present the purification, crystallization and preliminary X-ray diffraction studies of *P. cichorii* D-TE.

2. Materials and methods

2.1. Protein preparation

Recombinant D-TE was expressed in *E. coli* JM109 harbouring plasmid pIK-01, which was constructed previously (Ishida, Kamiya & Izumori, 1997). Plasmid pIK-01 contains the D-TE gene (DDBJ accession No. AB000361).

Cells were grown at 291 K in LB medium containing 100 µg ml^{-1} ampicillin for 12 h and D-TE expression was induced by the addition of 0.1 m*M* isopropyl *β*-D-thiogalactopyranoside (IPTG). After cultivation for 12 h, the cells were harvested by centrifugation, resuspended in buffer (20 m*M* Tris–HCl pH 8.0) and sonicated. The sonicated sample was centrifuged (20 400*g*, 20 min, 277 K) and the resultant cell-free extract was applied onto an anion-exchange column (Q Sepharose FF, GE Healthcare Biosciences Corp., Piscataway, NJ, USA) equilibrated with the same buffer (20 m*M* Tris–HCl pH 8.0). The protein was eluted with a linear gradient to 20 m*M* Tris–HCl, 1 *M* NaCl pH 8.0. Fractions containing D-TE were pooled and treated by 45% saturated ammonium sulfate precipitation. After removing the precipitate, the protein-sample solution was applied onto a hydrophobic interaction chromatography column (Phenyl FF, GE Healthcare Biosciences Corp., Piscataway, NJ, USA) equilibrated with 20 m*M* Tris–HCl, 45% saturated ammonium sulfate pH 8.0. After washing, D-TE was eluted with a linear gradient to the same buffer without ammonium sulfate. The purified D-TE was identified by SDS–PAGE, showing a single band with an estimated molecular weight of 30 kDa (Fig. 1). After dialysis against buffer solution (20 m*M* Tris–HCl pH 8.0) overnight, the purified protein was concentrated to 3.5 mg ml^{-1} with a Amicon Ultra-15 10k (Millipore, Billerica, MA, USA) for crystallization.

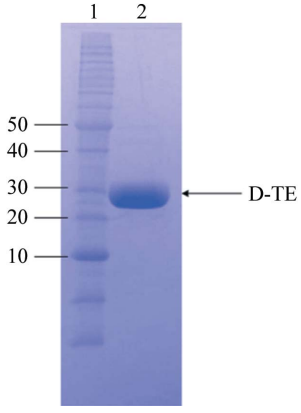

Figure 1
SDS–PAGE analysis of purified *P. cichorii* D-TE. Lane 1, purified *P. cichorii* D-TE. Lane 2, molecular-weight markers (kDa).

Figure 2
Crystal of *P. cichorii* D-TE with dimensions of 0.15 × 0.15 × 0.15 mm.

2.2. Crystallization

Initial crystallization screening for *P. cichorii* D-TE was performed by the sitting-drop method at room temperature using Hampton Research Crystal Screens I and II. Small crystals (Fig. 2) appeared on mixing 2.5 µl each of protein solution (3.5 mg ml^{-1} in 20 m*M* Tris–HCl pH 8.0) and reservoir solution [20%(*w*/*v*) PEG 10 000, 0.1 *M* HEPES pH 7.5; Crystal Screen II condition No. 38]. After additional screening using Hampton Research Additive Screen, crystals suitable for X-ray diffraction experiment were obtained within 10–14 d using reservoir solution containing 20%(*w*/*v*) PEG 10 000, 0.1 *M* HEPES pH 7.5 and 10 m*M* $SrCl_2$.

2.3. Enzyme assay

The purified protein sample was incubated with the same volume of 200 m*M* D-fructose at 303 K for 30 min, followed by heat treatment at 373 K for 5 min. The reaction sample was assayed by high-performance liquid chromatography (HPLC) using a Hitachi L-7490

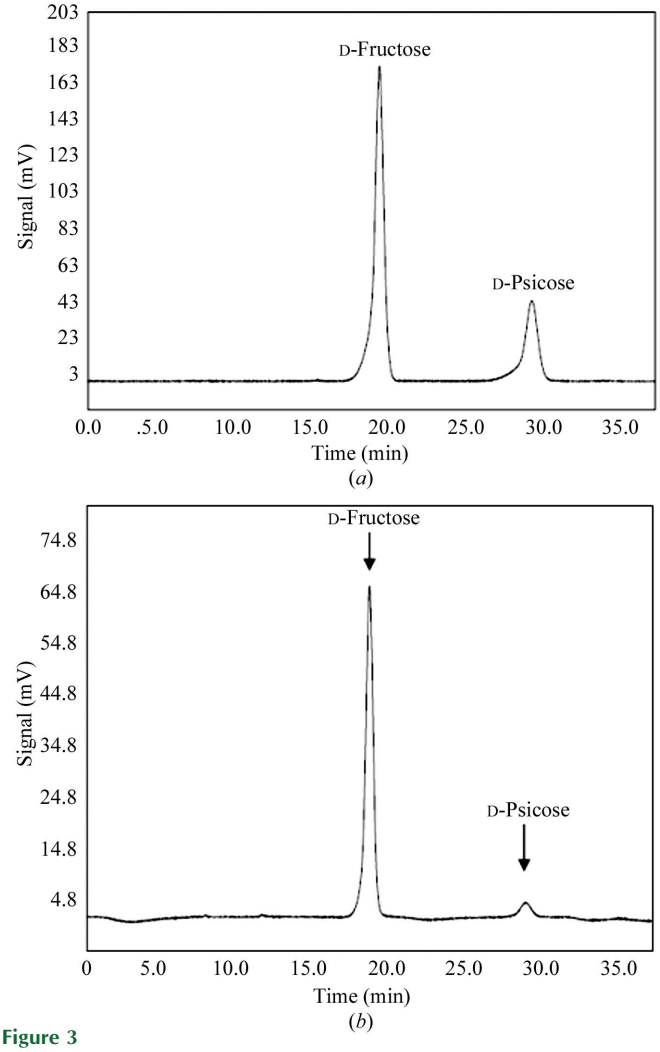

Figure 3
HPLC analysis of D-psicose converted from D-fructose (*a*) by purified *P. cichorii* D-TE and (*b*) by dissolved crystals of *P. cichorii* D-TE.

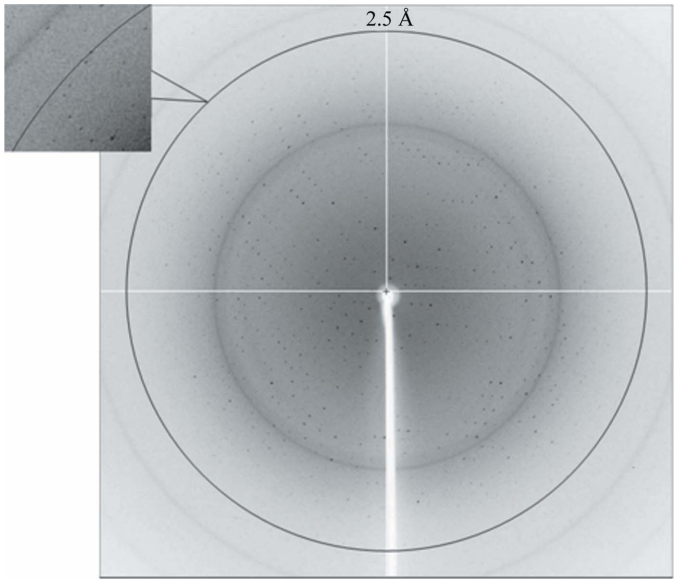

Figure 4
Diffraction images of *P. cichorii* D-TE with 2.5 Å resolution marked. An expanded portion of the diffraction at 2.5 Å is also shown.

refractive-index detector, D-2500 chromato-integrator and GL-C611 HPLC column. Chromatography was carried out at 333 K using 2 m*M* NaOH solution at a flow rate of 1.0 ml min^{-1} and showed the equilibrium ratio of D-fructose (substrate) and D-psicose (product). An enzyme assay of the crystal sample of *P. cichorii* D-TE was carried out in the same manner after washing the crystals with reservoir solution and dissolving them in 20 m*M* Tris–HCl buffer (Fig. 3).

3. Results and discussion

The results of the SDS–PAGE analysis are shown in Fig. 1. D-TE assays of the purified protein and the dissolved crystals are shown in Figs. 3(*a*) and 3(*b*), respectively. The dissolved crystals also showed D-TE activity, although at a lower detection level.

A complete data set was collected to 2.5 Å resolution at Photon Factory BL-6A (Tsukuba, Japan) at a wavelength of 0.978 Å using an ADSC Quantum 4R CCD detector (Fig. 4). The crystal was directly cooled in a stream of evaporating nitrogen at 100 K without an additional cryoprotectant. All data were processed using *HKL*-2000 (Otwinowski & Minor, 1997). Data-collection statistics are summarized in Table 1. The crystal belongs to the monoclinic space group $P2_1$, with unit-cell parameters a = 76.80, b = 94.92, c = 91.73 Å, β = 102.82°. The asymmetric unit is expected to contain four molecules, with a crystal volume per unit molecular weight V_M of 2.5 Å^3 Da^{-1}, corresponding to a solvent content of 50.4% (Matthews, 1968). The molecular-replacement method was applied using the *MOLREP* program with a monomer of *A. tumefaciens* D-PE (PDB code 2hk0), which belongs to the D-TE family, as the model. Another small *P. cichorii* D-TE crystal grown in the same reservoir solution containing 100 m*M* potassium sodium tartrate diffracted to 1.4 Å, but data processing was unsuccessful because it was highly twinned. We are currently refining the crystallization conditions in order to obtain better diffracting crystals and are attempting to prepare crystals of the enzyme–substrate complex.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution bin (2.59–2.50 Å).

Temperature (K)	100
Wavelength (Å)	0.978
Resolution range (Å)	50–2.5
No. of measured reflections	193386
No. of unique reflections	44789
Completeness (%)	99.9 (100)
Mean $I_o/\sigma(I_o)$	16.1 (4.7)
R_{sym}† (%)	8.6 (29.0)
Space group	$P2_1$
Unit-cell parameters	
a (Å)	76.8
b (Å)	94.9
c (Å)	91.7
β (°)	102.8

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

This study was supported in part by the National Project on Protein Structural and Functional Analyses and the 'intellectual-cluster' of 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.

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