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Expression, Purification, and Crystallization of D-Psicose 3-Epimerase from *Agrobacterium tumefaciens*

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Abstract D-Psicose 3-epimerase (DPE) catalyzes the interconversion of D-fructose to D-psicose by epimerizing the carbon-3 position. The DPE from *Agrobacterium tumefaciens* was cloned and expressed in *Escherichia coli*. The expressed enzyme was purified by affinity chromatography on an IMAC, gel filtration chromatography on a Sephacryl S-300 HR, and anion-exchange chromatography on a RESOURCE Q. The molecular mass of the purified enzyme was estimated to be about 135 kDa by Superdex 200 gel filtration chromatography, corresponding to a homotetramer. The enzyme produced crystals suitable for X-ray diffraction to a 2.0 Å resolution at 100 K. The crystals were found to belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a=102.4, b= 113.0, and c=131.8 Å. In addition, the calculated packing parameter (V_m) was 2.79 Å^3/Da, the solvent content was 55.92%, and an asymmetric unit consisted of four monomers.

Key words: *Agrobacterium tumefaciens*, D-psicose 3-epimerase, crystallization, X-ray crystal structure

D-Psicose (D-ribo-2-hexulose), a carbon-3 epimer of D-fructose, is present in small quantities in commercial carbohydrates, such as D-glucose and D-fructose, obtained from the hydrolysis of sucrose or isomerization of D-glucose [10]. The sugar has zero energy, making D-psicose a useful sweetener for weight reduction [3, 11] based on its suppression of hepatic lipogenic enzyme activities, as previously shown in rats [12]. This rare sugar has already been commercially produced by the enzymatic epimerization of D-fructose using D-tagatose 3-epimerase (DTE) (EC 5.3.1) [6], an enzyme with a broad substrate specificity, yet sharing no sequence homology with any other sugar-related epimerases [4]. Thus, because of these characteristics, DTE has also been used to produce D-sorbose and D-psicose from D-tagatose and D-fructose, respectively [6, 7, 15], although details of its enzymatic properties are still unknown. Currently, limited information is only available on the DTE from *Pseudomonas cichorii* ST-24 used for D-psicose production [5], despite the identification of many DTEs from various sources (GenBank accession numbers: F72381, AL939126, BAB50266, AAL45542, NP_865388, BX294153, NC_005126, and NP_435986). In addition, L-arabinose isomerase was also recently found to produce D-tagatose [1], suggesting an alternative method of bioproduction to yield the rare sugar.

In a previous study by the present authors, a gene for a putative DTE was cloned from *Agrobacterium tumefaciens* [9]. Unexpectedly, the Mn^{2+}-dependent enzyme was more effective catalyzing the conversion of D-psicose into D-fructose, instead of the interconversion of D-tagatose and D-sorbose; thus it was renamed D-psicose 3-epimerase (DPE). Accordingly, this study reports on the expression, purification, crystallization, and preliminary X-ray crystallographic analysis of DPE, and the resulting structural information is expected to facilitate further investigation of its various enzymatic features.

The forward (5'-TTTTCATATGAAACACGGCATCT-ATTATTC-3') and reverse (5'-AACTCGAGTTAGCC-ACCAAGAACGAAG-3') primers used for the PCR amplification were designed to introduce an NdeI site (underlined) at the N-terminus and XhoI site (underlined) at the C-terminus, respectively. The DPE gene generated by a PCR using *A. tumefaciens* was cloned into a modified pET-15b vector (Novagen) containing a tobacco etch virus (TEV) protease cleavage site at the junction between a His_5-tag and a multiple cloning site. Recombinant *Escherichia coli* BL21(DE3) cells (Stratagene) transformed with the plasmid containing the DPE gene were grown on an LB

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broth up to an 0.8 O.D. (600 nm) at 37°C and 200 rpm. After being induced by 0.1 mM IPTG overnight at 16°C and 150 rpm, the expression of the DPE protein was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the activity assay described below.

The cells were harvested from the culture broth by centrifugation at 6,000 ×*g* for 30 min at 4°C, washed twice with 0.85% NaCl, and resuspended in a phosphate buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0) containing 1 mg/ml lysozyme, and then the mixtures were stood in ice for 30 min. Next, the resuspended cells were disrupted by passing them through a French press at 15,000 lb/in^2, the cell debris removed by centrifugation at 13,000 ×*g* for 20 min at 4°C, and the resulting supernatant filtered through a 0.45-μm pore size filter. This filtrate was then used as the crude extract. All the purification steps using columns were carried out in a cold room with a fast protein liquid chromatography (FPLC™) system (Amersham Biosciences). The filtrate was applied to an immobilized metal affinity chromatography column (IMAC, HisTrap™ HP column) equilibrated with a 50 mM NaH_2PO_4 buffer containing 300 mM NaCl and 10 mM imidazole (pH 8.0). The column was then washed extensively with the same buffer, and the bound protein eluted with a linear gradient of 10 to 200 mM imidazole in the same buffer at a flow rate of 1 ml/min. The fractions containing a high DPE activity were collected and loaded onto a gel filtration column (Sephacryl S-300™ HR column, Amersham Biosciences) previously equilibrated with 50 mM Tris-HCl (pH 8.0). The buffer used for all the additional purification steps below was 50 mM Tris-HCl (pH 8.0). The loaded protein was eluted at a flow rate of 0.5 ml/min, and the resulting pooled enzyme solution further purified using an anion-exchange chromatography column (RESOURCE™ Q column, Amersham Biosciences) equilibrated with 50 mM Tris-HCl (pH 8.0). The column was then washed extensively and the bound DPE protein eluted with a linear gradient of 500 mM NaCl at a flow rate of 3 ml/min. After dialysis against 50 mM Tris-HCl (pH 8.0) at a concentration up to 14 mg/ml using a Centricon® YM-10 ultrafiltration membrane (Millipore), the resulting solution was used as the purified DPE enzyme for the crystallization trials (Fig. 1). Since the N-terminal His-tag was not removed, all the experiments in this report were carried out using the fusion DPE with a His-tag.

The DPE activity was determined by measuring the D-fructose as the substrate. The reactions were carried out at pH 7.5 and 50°C in a 50 mM PIPES buffer containing 1.0% (w/v) D-fructose for 20 min, and stopped by heating at 100°C for 5 min. The enzyme solution was then centrifuged, filtered, and used for the substrate and product analyses as described below. One unit (U) of DPE activity was defined as the amount of the enzyme producing

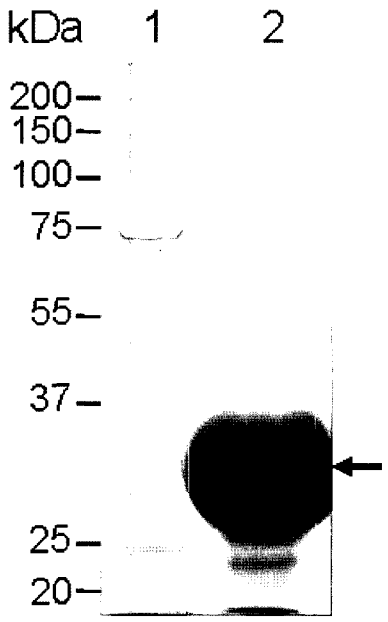

Fig. 1. SDS-PAGE analysis of *A. tumefaciens* DPE.
The DPE purified by the RESOURCE™ Q was concentrated up to 14 mg/ml. Lane 1, molecular marker; Lane 2, concentrated DPE protein at the arrow.

1 μmole D-psicose per min at pH 7.5 and 50°C. The protein concentrations were determined using the Bradford method with bovine serum albumin as the standard protein [2]. The concentrations of D-fructose and D-psicose were determined by HPLC (Shimadzu SCL-10A) equipped with a Shimadzu RID-10A detector and BP-100 Ca^{2+} carbohydrate column (Benson Polymeric Inc.). The column was eluted at 80°C with water at a flow rate of 0.5 ml/min.

The crystallization was performed by the hanging-drop vapor-diffusion method using 24-well tissue-culture VDX™ plates (Hampton Research) at 295 K. Each hanging drop was prepared by mixing 2 μl of the protein solution with 2 μl of the reservoir solution. The initial crystallization conditions followed the sitting-drop vapor-diffusion method using CrystalClear strips™ (Hampton Research) with depressions and Crystal screen I, II, Grid screen & PEG/ION (Hampton Research), and Wizard I & II (Emerald Biostructures). For data collection, the crystals were briefly soaked in a liquor containing a cryoprotectant [10% (v/v) glycerol, 17% (w/v) PEG 1000, 0.1 M imidazole pH 8.0, 0.25 M calcium acetate], and then flash-frozen in liquid propane. Several native data sets were collected at a PAL 6B MX beamline (Pohang Accelerator Laboratory, Pohang, Korea).

The recombinant DPE from *A. tumefaciens* with twenty residues (MGSSHHHHHSSGENLYFQGH) at the N-terminus, including five histidines and a TEV cleavage site, was overexpressed in *E. coli* in a soluble form. Typically, a total of ~6.4 mg of DPE was purified per liter of culture (Table 1). To obtain large protein quantities for

Table 1. Purification steps for *A. tumefaciens* D-psicose 3-epimerase.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	103.7	484	1.55	100	1.00
HisTrap HP	37.3	248	2.22	51.2	1.43
Sephacryl S-300 HR	7.7	205	8.89	42.4	5.74
Resource Q	6.4	202	10.5	41.8	6.80

crystallization, the fermentation was performed with 3 l of the LB broth. The crude enzyme was then purified by the sequential application of affinity chromatography on an IMAC using a HisTrap™ HP column, gel filtration on a Sephacryl™ S-300 HR, and anion-exchange chromatography on a RESOURCE™ Q. The proteins from each purification step were separated and analyzed by SDS-PAGE. Unlike the other steps, the gel filtration step revealed two peaks on the chromatogram (data not shown). However, the enzyme activity was only associated with one peak at 136 kDa, suggesting that the other peak for a higher molecular mass was an unfolded or aggregated form. The enzymatically active DPE was purified 6.8-fold with a yield of 41.8% and final specific activity of 10.54 U/mg (Table 1). After three chromatographic steps, the concentrated DPE (14 mg/ml) exhibited a single major band at 33 kDa in SDS-PAGE with some impurities at a lower molecular mass, which was consistent with the calculated value of 33,834 Da based on the 289 amino acids and additional N-terminal residues (Fig. 1). Two different approaches to measuring the molecular mass indicated that the DPE was a homotetramer, as the nondenaturing PAGE and gel filtration of the Superdex™ 200 GL (Amersham Biosciences) estimated that the molecular masses of the DPE were 135 kDa and 136 kDa, respectively. Therefore, the molecular mass of the native DPE from *A. tumefaciens* was determined to be 135 kDa, representing a tetramer of about 33,834 Da subunits.

After an extensive search through 362 precipitant conditions, initial crystals were finally formed at 295 K in 20% (w/v) PEG 1000, 0.1 M imidazole pH 8.0, and 0.2 M calcium acetate (Fig. 2A).

Crystals suitable for further crystallographic analysis were grown in hanging drops with 14–15% (w/v) PEG 1000, 0.1 M imidazole pH 8.0, and 0.2 M calcium acetate within 5 days to overall dimensions of 0.4 mm×0.28 mm×0.28 mm (Fig. 2B). Diffraction data were collected at a PAL 6B MX beamline (Pohang Accelerator Laboratory, Pohang, Korea) under cryoconditions at 100 K. The best data to 2.0 Å were collected at an X-ray wavelength of 0.97900 Å and processed using the *HKL2000* suite (Table 2) [14].

The crystals belonged to the space group $P2_12_12_1$ with unit-cell parameters a=102.4, b=113.0, and c=131.8 Å. The asymmetric unit contained four monomers of DPE, giving a crystal volume per protein mass (V_m) of 2.79 Å^3/Da and solvent content of 55.92% [8, 13]. The structural determination of the DPE from *A. tumefaciens* is now underway using crystals from a selenomethionine-substituted DPE enzyme (28 selenomethionines per asymmetric unit), and preliminary results using SOLVE [16] indicated at least 24 identifiable seleniums and four monomers in an asymmetric unit. Details of the DPE structure will be

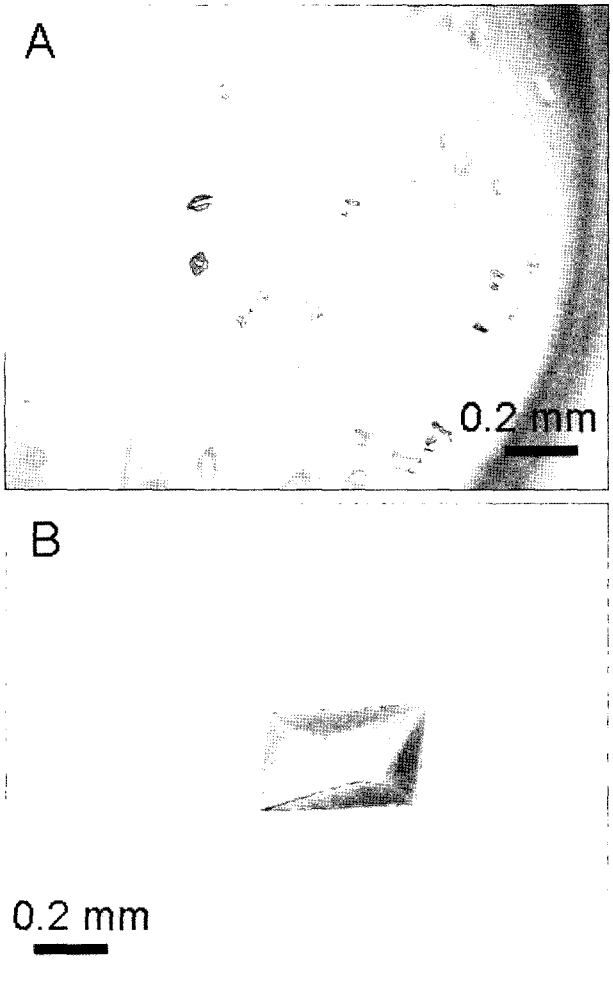

Fig. 2. Crystals of *A. tumefaciens* DPE grown at 295 K.
A. The initial crystals were grown using sitting-drop vapor-diffusion, and the crystallization solution was 20% PEG 1000, 0.1 M imidazole pH 8.0, and 0.2 M calcium acetate. **B.** Diffraction-quality crystals were produced using hanging-drop vapor-diffusion, where the crystallization solution was 14–15% PEG 1000, 0.1 M imidazole pH 8.0, and 0.2 M calcium acetate, and the crystal dimensions were 0.4 mm×0.28 mm×0.28 mm.

Table 2. X-ray data collection statistics.

Space group	$P2_12_12_1$
Wavelength (Å)	0.97900
Cell dimension (Å)	a=102.4, b=113.0, c=131.8 Å and $\alpha=\beta=\gamma=90$
Resolution range (Å)	30-2.0 (2.07-2.0)
Observed reflections	1,438,592
Unique reflections	99,936 (9,800)
Redundancy	14.4
Completeness (%)	99.6 (99.0)
R_{sym} (%)	9.8 (84.9)
I/sigma (I)	21
No. molecules in AU	4
V_m (Å^3/Da)	2.79

$R_{sym}=\Sigma_{hi}|I_{hi}-<I_h>|/\Sigma_{hi}I_{hi}$, where h denotes hkl and i counts through all the symmetry-related reflections. The values for the last shell are in parentheses.

published elsewhere and should enhance the present understanding of the epimerization reaction.

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