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Characterization of a Metal-Dependent D-Psicose 3-Epimerase from a Novel Strain, *Desmospora* sp. 8437

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ABSTRACT: The rare sugar D-psicose is an ideal sucrose substitute for food products, due to having 70% of the relative sweetness but 0.3% of the energy of sucrose. It also shows important physiological functions. D-Tagatose 3-epimerase (DTEase) family enzymes can produce D-psicose from D-fructose. In this paper, a new member of the DTEase family of enzymes was characterized from *Desmospora* sp. 8437 (GenBank accession no. WP_009711885) and was named *Desmospora* sp. D-psicose 3-epimerase (DPEase) due to its highest substrate specificity toward D-psicose. *Desmospora* sp. DPEase was strictly metal-dependent and displayed maximum activity in the presence of Co^{2+}. The optimum pH and temperature were 7.5 and 60 °C, respectively. The enzyme was relatively thermostable below 50 °C, but easily lost initial activity when preincubated at 60 °C. The thermostability property was almost not affected by the addition of Co^{2+}. *Desmospora* sp. DPEase had relatively high catalysis efficiency for the substrates D-psicose and D-fructose, which were measured to be 327 and 116 mM^{-1} min^{-1}, respectively. The equilibrium ratio between D-psicose and D-fructose of *Desmospora* sp. DPEase was 30:70. The enzyme could produce 142.5 g/L D-psicose from 500 g/L of D-fructose, suggesting that the enzyme is a potential D-psicose producer for industrial production.

KEYWORDS: *D-psicose 3-epimerase, Desmospora sp., D-psicose, rare sugar, characterization*

INTRODUCTION

D-Psicose (D-ribo-2-hexulose or D-allulose), an epimer of D-fructose at the C3 position, is a novel ideal sucrose substitute,[1] which has 70% of the relative sweetness[2] but only 0.3% of the energy of sucrose,[3] and has a high solubility of 74% (w/w) at 25 °C.[4] Importantly, it has been recently announced as generally recognized as safe (GRAS) for food use by the U.S. Food and Drug Administration (FDA). The biological functions of D-psicose have been widely studied.[5] Typically, many studies have shown that D-psicose has hypoglycemic,[6,7] hypolipidemic,[8,9] neuroprotective,[10] and antioxidant activities[11] and an insulin resistance effect.[12,13] In addition, it improves gelling behavior and produces good flavor through the Maillard reaction with food proteins during food processing,[14,15] and it produces higher antioxidative Maillard reaction products,[16,17] which is helpful to maintain food quality during storage.[18]

D-Psicose is a member of rare sugars, which are defined by the International Society of Rare Sugars (ISIR) as monosaccharides and their derivatives existing in nature in limited quantities (First International Symposium of ISRS, Takamatsu, Japan, 2002). D-Psicose exists in wheat[19] and *Itea* plants[20] as a free sugar in trace amount and can be produced in very small amounts from fructose by nonenzyme reaction during heat treatment of food products.[2] D-Psicose can be chemically produced from D-fructose, but all of the chemical methods have disadvantages,[21,22] such as immoderate reaction conditions, chemical waste, complex byproducts, and difficult purification process. Therefore, biotechnological production of D-psicose by epimerization using D-tagatose 3-epimerase (DTEase) family enzymes has been focused on as a commercially attractive process.[1]

So far, there have been many kinds of DTEase family enzyme sources reported. DTEase was first characterized by Izumori et al. from *Pseudomonas cichorii* ST-24, showing C-3 epimerization activity of ketohexoses with the optimum substrate of D-tagatose.[23] Then, the D-psicose 3-epimerase (DPEase) from *Agrobacterium tumefaciens*[24] and the DTEase from *Rhodobacter sphaeroides* SK011[25] were characterized one after another, with optimum substrates D-psicose and D-fructose, respectively. Over the past 3 years, another five DTEase family enzymes were characterized with optimum substrate D-psicose, from *Clostridium cellulolyticum* H10,[26] *Ruminococcus* sp. 5_1_39BFAA,[27] *Clostridium scindens* ATCC 35704,[28] *Clostridium bolteae* ATCC BAA-613,[29] and *Clostridium* sp. BNL1100,[30] respectively. Metal ion has an important effect on the activity and thermostability of the family of enzymes. For examples, DPEases from *C. cellulolyticum*,[26] *C. scindens*,[28] *C. bolteae*,[29] and *Clostridium* sp. BNL1100[30] require metal ion as an essential cofactor for activity; metal ion significantly enhances the thermostability of DPEases from *C. cellulolyticum*,[26] *C. bolteae*,[29] and *Ruminococcus* sp. 5_1_39BFAA.[27]

In this study, a DPEase was characterized from a novel strain, *Desmospora* sp. 8437, showing the optimum substrate of D-psicose and the strict metal dependence for activity. The enzymatic properties and kinetic parameters were investigated in detail and were compared to other DTEase family enzymes. Phylogenetic analysis showed the encoding gene was completely novel and distinctive from known DTEase family enzyme genes.

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MATERIALS AND METHODS

Chemicals and Reagents. The resin for protein purification, Chelating Sepharose Fast Flow, was obtained from GE (Uppsala, Sweden). Electrophoresis reagents were purchased from Bio-Rad (Hercules, CA, USA). Isopropyl β-D-1-thiogalactopyranoside (IPTG) and all chemicals used for enzyme assays and characterization were of at least analytical grade obtained from Sigma (St. Louis, MO, USA) and Sinopharm Chemical Reagent (Shanghai, China).

Bacterial Strains and Culture Conditions. *Escherichia coli* strains DH5α and BL21(DE3) were purchased from Sangon Biological Engineering Technology and Services (Shanghai, China) and used as the host strains for DNA cloning and expression, respectively. They were routinely cultured in Luria−Bertani (LB) medium containing ampicillin (100 μg/mL) in a rotary shaker at 37 °C and 200 rpm. The plasmid pET-22b(+) was from Novagen (Darmstadt, Germany) and was used as the expression vector.

Gene Cloning and Protein Expression. The complete genome of *Desmospora* sp. 8437 chromosome has been released in GenBank (NCBI accession no. NZ_GL892032). The full-length nucleotide sequence of the putative DPEase-coding gene with locus_tag HMPREF9374_3821 from the strain *Desmospora* sp. 8437 was synthesized by Sangon Biological Engineering Technology and Services (Shanghai, China) and was incorporated with *Nde*I and *Xho*I sites in the 5′- and 3′-termini of the gene. Then the DNA was inserted into the corresponding restriction sites in the expression vector pET-22b(+), generating the recombinant plasmid pET-Desp-dpe. To facilitate the purification process of recombinant *Desmospora* sp. DPEase, an in-frame 6×histidine-tag sequence at the C-terminus was provided in the recombinant plasmid. The plasmid pET-Desp-dpe was transformed into the competent cells of the expression host *E. coli* BL21(DE3), and the *E. coli* harboring pET-Desp-dpe was screened for protein expression.

For protein expression, the recombinant *E. coli* was cultivated with shaking (200 rpm) in 500 mL of LB medium containing 100 μg/mL ampicillin at 37 °C. When the optical density at 600 nm reached 0.6, IPTG was added at a final concentration of 1 mM, and the recombinant DPEase was induced and overexpressed at 30 °C for 5 h. The cells were harvested by centrifugation at 4 °C for 10 min at 10000*g*. The enzyme was expressed as 6×histidine-tagged fusion protein, which was available for affinity chromatography.

Protein Purification. To purify the recombinant DPEase, the centrifuged cell pellets were washed with 50 mM Tris-HCl buffer (pH7.5) containing 100 mM NaCl, resuspended in lysis buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.5), and disrupted by sonication at 4 °C for 6 min (pulsations of 3 s, amplify 90) using a Vibra-Cell 72405 sonicator (Sonics & Materials, Newtown, CT, USA). After sonication, the cell lysates were centrifuged at 4 °C and 20000*g* for 20 min to remove the cell debris, and the supernatant was filtered through a 0.22 μm filter. The filtrate was loaded onto a Chelating Sepharose Fast Flow resin column (1.0 cm × 10.0 cm), previously chelating Ni^{2+}, and equilibrated with a binding buffer (50 mM Tris-HCl, 500 mM NaCl, pH 7.5). Unbound proteins were eluted from the column with a washing buffer (50 mM Tris-HCl, 500 mM NaCl, 50 mM imidazole, pH 7.5). Then, the recombinant DPEase was eluted from the column using an elution buffer (50 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole, pH 7.5). The active fractions were pooled and dialyzed overnight against 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM ethylenediaminetetraacetic acid (EDTA) for 24 h at 4 °C, to remove imidazole and divalent metal ions. Subsequently, the enzyme was dialyzed against 50 mM EDTA-free Tris-HCl buffer (pH 7.5) for 24 h at 4 °C to remove EDTA. After dialysis processes, the purified protein sample was applied to the different analysis, including the purity measurement, enzymatic properties, metal dependence, kinetic parameters, and the D-psicose-producing ability.

Enzyme Assay. The enzyme activity was measured by the determination of the amount of D-psicose produced from D-fructose. The reaction mixture of 1 mL contained D-fructose (50 g/L), Tris-HCl buffer (50 mM, pH 7.5), 1 mM Co^{2+}, and 0.5 μM enzyme. The reactions were performed at 60 °C for 5 min and were stopped after 10 min by boiling. The generated D-psicose was determined using high-performance liquid chromatography (HPLC). One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 μmol of D-psicose per minute at pH 7.5 and 60 °C.

Effect of Various Metallic Ions on Activity. To analyze the effects of metal ions on the activity of *Desmospora* sp. DPEase, the metal-free enzyme treated by EDTA dialysis was incubated at 4 °C for 1 h, in the different divalent metal ions reagents at 1 mM: Co^{2+}, Mn^{2+}, Mg^{2+}, Fe^{2+}, Ni^{2+}, Zn^{2+}, and Cu^{2+}, respectively. Then, the enzyme activities were assessed in the presence of corresponding metal ions at a final concentration of 1 mM.

Effect of pH on Enzyme Activity. The effect of pH on the activity of *Desmospora* sp. DPEase was measured in two buffer systems, sodium phosphate buffer (50 mM, pH 6.0−7.0) and Tris-HCl buffer (50 mM, pH 7.5−9.0).

Effect of Temperature on Activity and Stability. For determining the optimum temperature, enzyme activity was measured by assaying the enzyme samples over the range of 35−75 °C for 5 min. The thermal stability of the enzyme was studied by preincubating the enzyme in Tris-HCl buffer (50 mM, pH 7.5) at various temperatures for different incubation times. Relative and residual activities were measured in the same conditions used to determine the 3-epimerization activity, whereas the control activity of the un-preincubated sample was regarded as 100%.

Determination of Kinetic Parameters. Kinetic parameters of *Desmospora* sp. DPEase were determined in 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM Co^{2+} and 5−200 mM substrate. The enzyme reactions were stopped after 10 min by boiling, and the amount of monosaccharides was determined by HPLC assay. Kinetic parameters, such as the Michaelis−Menten constant (K_m) and turnover number (k_{cat}) values for D-fructose and D-psicose, were obtained using the Lineweaver−Burk equation and quantification of enzyme concentration.

Analytical Methods. The concentrations of D-fructose and D-psicose were analyzed by HPLC equipped with a refractive index detector and a Ca^{2+}-carbohydrate column (Waters Sugar-Pak 1, Waters Corp., Milford, MA, USA). The column was eluted at 85 °C with water at a flow rate of 0.4 mL/min. Protein concentration was determined according to the method of Bradford using bovine serum albumin as a standard. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli. Gels (12% w/v polyacrylamide) were stained with Coomassie Brilliant Blue and destained with an aqueous mixture of 10% (v/v) methanol/10% (v/v) acetic acid.

RESULTS

Cloning, Expression, and Purification of *Desmospora* sp. DPEase. The whole genome shotgun sequence of the strain *Desmospora* sp. 8437 has been recently completed and deposited as GenBank accession no. NZ_GL892032. The protein with ProteinID WP_009711885.1 encoded by the hypothetical open reading frame HMPREF9374_3821 was supposed as the product dolichol monophosphate mannose synthase in the *Desmospora* sp. 8437 genome. However, the protein WP_009711885.1 was found relatively homologous in amino acid sequence with the DTEase family enzymes (Table 1). In this study, the protein was characterized and confirmed as a DTEase family enzyme with high substrate specificity for D-psicose and then was named as *Desmospora* sp. DPEase.

The recombinant enzyme was overexpressed by IPTG induction, and a strong protein band of approximately 33 kDa was exhibited on SDS-PAGE, compared to that of the control *E. coli* BL21(DE3) (data not shown). This was in agreement with the predicted molecular mass for the DPEase. Using a one-step purification process by nickel affinity column chromatography, the 6×histine-tagged *Desmospora* sp. DPEase was purified to electrophoretic homogeneity with a single band

Table 1. Amino Acid Sequence Homology of DTEase Family Enzymes

sequence A	length (Aa)	sequence B	length (Aa)	amino acid homology (%)
Desp-DPEase[a]	289	Psci-DTEase[b]	290	41.87
Desp-DPEase	289	Agtu-DPEase[c]	289	49.83
Desp-DPEase	289	Rhsp-DTEase[d]	295	29.41
Desp-DPEase	289	Clce-DPEase[e]	293	49.83
Desp-DPEase	289	Rusp-DPEase[f]	291	48.79
Desp-DPEase	289	Clsp-DPEase[g]	292	50.87
Desp-DPEase	289	Clsc-DPEase[h]	289	50.17
Desp-DPEase	289	Clbo-DPEase[i]	291	47.40
Psci-DTEase	290	Agtu-DPEase	289	38.75
Psci-DTEase	290	Rhsp-DTEase	295	31.03
Psci-DTEase	290	Clce-DPEase	293	41.38
Psci-DTEase	290	Rusp-DPEase	291	33.45
Psci-DTEase	290	Clsp-DPEase	292	41.03
Psci-DTEase	290	Clsc-DPEase	289	38.75
Psci-DTEase	290	Clbo-DPEase	291	36.21
Agtu-DPEase	289	Rhsp-DTEase	295	30.45
Agtu-DPEase	289	Clce-DPEase	293	60.90
Agtu-DPEase	289	Rusp-DPEase	291	50.52
Agtu-DPEase	289	Clsp-DPEase	292	61.25
Agtu-DPEase	289	Clsc-DPEase	289	58.82
Agtu-DPEase	289	Clbo-DPEase	291	49.48
Rhsp-DTEase	295	Clce-DPEase	293	28.67
Rhsp-DTEase	295	Rusp-DPEase	291	26.46
Rhsp-DTEase	295	Clsp-DPEase	292	28.42
Rhsp-DTEase	295	Clsc-DPEase	289	29.07
Rhsp-DTEase	295	Clbo-DPEase	291	28.87
Clce-DPEase	293	Rusp-DPEase	291	51.89
Clce-DPEase	293	Clsp-DPEase	292	94.18
Clce-DPEase	293	Clsc-DPEase	289	62.28
Clce-DPEase	293	Clbo-DPEase	291	52.58
Rusp-DPEase	291	Clsp-DPEase	292	52.58
Rusp-DPEase	291	Clsc-DPEase	289	52.60
Rusp-DPEase	291	Clbo-DPEase	291	62.54
Clsp-DPEase	292	Clsc-DPEase	289	62.98
Clsp-DPEase	292	Clbo-DPEase	291	53.61
Clsc-DPEase	289	Clbo-DPEase	291	51.90

[a]*Desmospora* sp. DPEase, GeneBank accession No: WP_009711885. [b]*P. cichorii* DTEase, GenBank accession no. BAA24429. [c]*A. tumefaciens* DPEase, GenBank accession no. AAL45544. [d]*R. sphaeroides* DTEase, GenBank accession no. ACO59490. [e]*C. cellulolyticum* DPEase, GenBank accession no. ACL75304. [f]*Ruminococcus* sp. DPEase, GenBank accession no. ZP_04858451. [g]*Clostridium* sp. DPEase, GenBank accession no. YP_005149214.1. [h]*C. scindens* DPEase, GenBank accession no. EDS06411.1. [i]*C. bolteae* DPEase, GenBank accession no. EDP19602.

on SDS-PAGE (Figure 1). On the other hand, the molecular weight of native enzyme was measured by the gel filtration experiment to be approximately 132 kDa (data not shown).

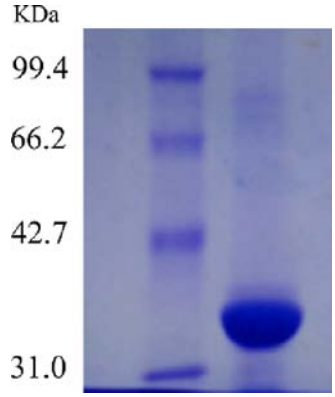

Figure 1. SDS-PAGE analysis of the purified *Desmospora* sp. DPEase.

Therefore, *Desmospora* sp. DPEase was supposed to be a tetramer with four identical subunits.

Effect of Metal Ions on *Desmospora* sp. DPEase Activity. The relative activity of the metal-free *Desmospora* sp. DPEase was investigated in the presence of various divalent metal ions added at the final concentration of 1 mM. The enzyme was strictly metal-dependent and displayed no activity when without any metal. It showed the highest activity in the presence of Co^{2+}, and when Co^{2+} was replaced with Mn^{2+}, Fe^{2+}, and Ni^{2+}, the enzyme activity was reduced to 80, 49, and 14% of that in the presence of Co^{2+}, respectively. However, Mg^{2+}, Zn^{2+}, and Cu^{2+} could not excite the enzyme activity (Figure 2).

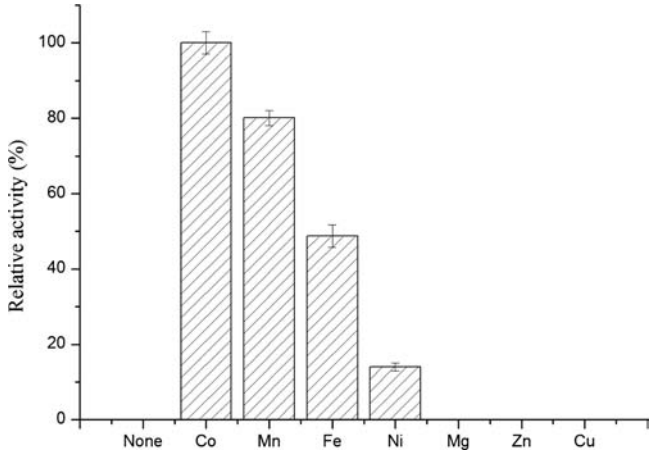

Figure 2. Effect of metal ions on the enzymatic activity of *Desmospora* sp. DPEase. Values are means of three replications ± standard deviation.

Effect of pH and Temperature on *Desmospora* sp. DPEase Activity. Figure 3A shows the pH profile for the *Desmospora* sp. DPEase acitivty. The maximal epimerization activity from D-fructose to D-psicose was given at pH 7.5. The enzyme displayed a relatively wide pH spectrum and showed >75% of relative activity from pH 6.5 to 9.0.

The temperature profile of purified *Desmospora* sp. DPEase was determined from 35 to 75 °C with D-fructose as substrate. As shown in Figure 3B, the enzyme exhibited an optimum temperature at 60 °C. The enzyme activity seemed to be relatively sensitive to temperature. It showed high relative activity with >90% of maximum activity at the range of 55−65 °C; however, the relative activity significantly decreased outside this range.

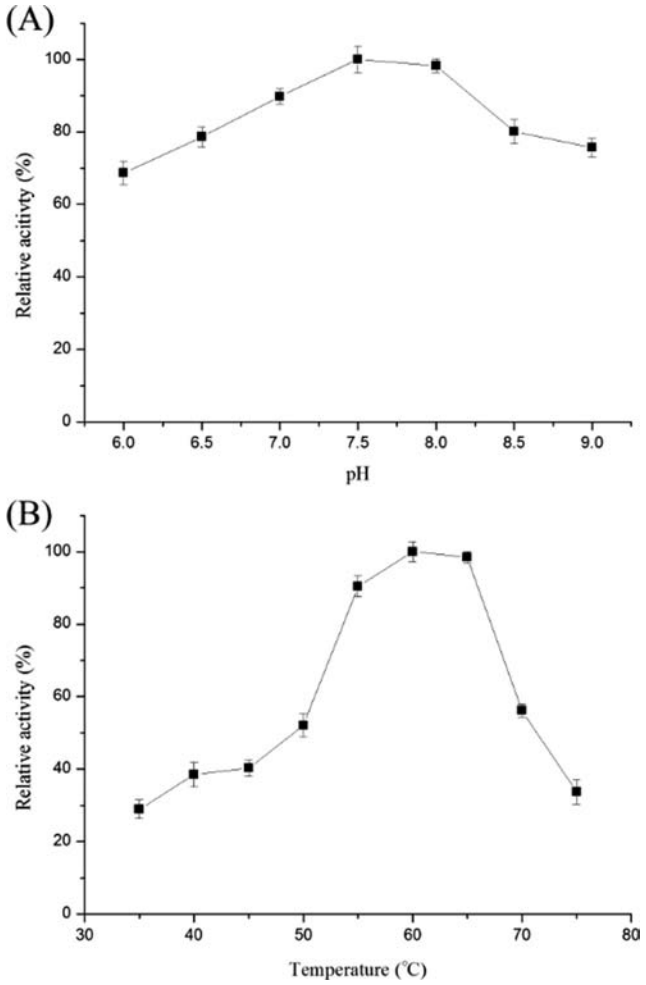

Figure 3. Effect of pH (A) and temperature (B) on the enzymatic activity of *Desmospora* sp. DPEase. Values are means of three replications ± standard deviation.

Effect of Temperature on Stability of *Desmospora* sp. DPEase. *Desmospora* sp. DPEase exhibited relatively high thermostability below 50 °C. It retained 95, 86, 73, and 58% of its initial activity after 0.5, 1, 1.5, and 2 h of exposure at 40 °C, respectively, and retained 94, 79, 70, and 54% after 0.5, 1, 1.5, and 2 h of exposure at 50 °C, respectively (Figure 4). However, when incubated at 60 °C, the enzyme lost all of the initial activity after 0.5 h of exposure (data not shown). In addition, adding the cofactor Co^{2+} during the incubation almost did not change the thermal stability of *Desmospora* sp. DPEase (Figure 4).

Enzyme Kinetics. Using D-fructose and D-psicose as substrates, the kinetic parameters of the purified *Desmospora* sp. DPEase were investigated at pH 7.5 and 60 °C. From the double-reciprocal plots of the reaction rate against the substrate concentrations, the K_m and k_{cat} of *Desmospora* sp. DPEase for substrate D-psicose were estimated to be 81.3 mM and 5157.8 min^{-1}, respectively, and the parameters for substrate D-fructose were 549 mM and 63573 min^{-1}, respectively. The catalytic efficiency (k_{cat}/K_m) value of the enzyme was calculated to be 327 and 116 mM^{-1} min^{-1} for the substrates D-psicose and D-fructose, respectively. The results suggested that D-psicose acted as the optimum substrate.

Production of D-Psicose by *Desmospora* sp. DPEase. The equilibrium ratio between D-psicose and D-fructose of

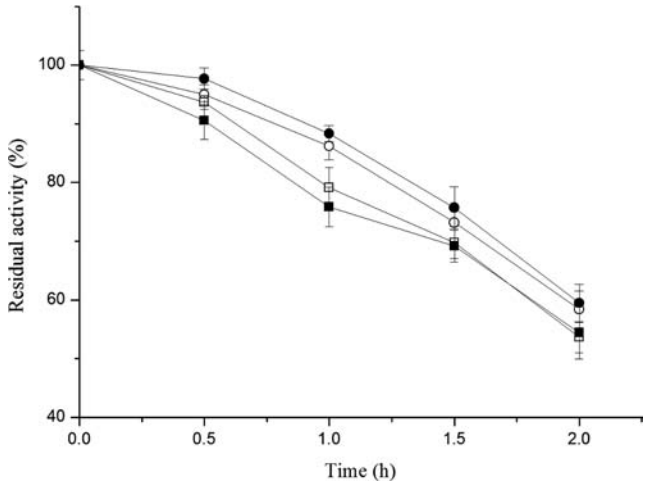

Figure 4. Effect of temperature on the stability of *Desmospora* sp. DPEase. Thermal stability was investigated by exposing the enzyme without metal ion at 40 (○) and 50 °C (□) and with 1 mM Co^{2+} at 40 (●) and 50 °C (■) for different time intervals at pH 7.5. Values are means of three replications ± standard deviation.

Desmospora sp. DPEase at 60 °C and pH 7.5 was determined to be 30:70. As for the large-scale D-psicose production by purified *Desmospora* sp. DPEase with a final concentration of 1 μM, when substrate D-fructose was added at final concentrations of 100, 300, and 500 g/L, the maximal conversion rate of D-fructose into D-psicose was achieved at approximately 28.5% after reaction at pH 7.5 and 55 °C, for 15, 40, and 60 min, respectively, giving the D-psicose productivity with 28.5, 85.5, and 142.5 g/L, respectively (Figure 5).

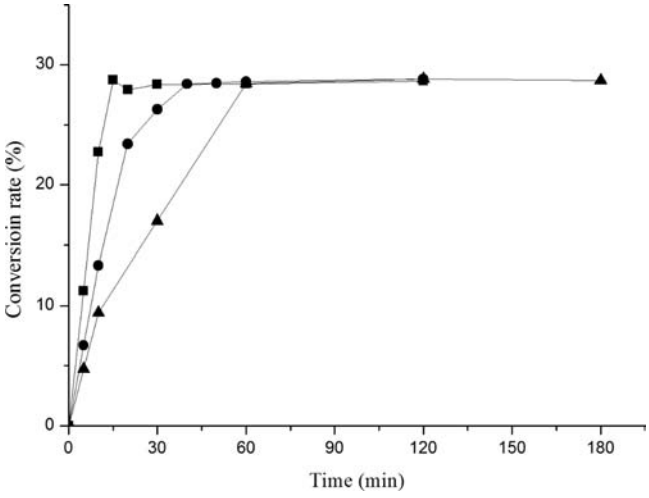

Figure 5. Bioconversion of D-fructose into D-picose by *Desmospora* sp. DPEase. D-Psicose was produced by 1 μM *Desmospora* sp. DPEase at pH 7.5 and 55 °C from three concentrations of D-fructose, 100 (■), 300 (●), and 500 g/L (▲).

■ DISCUSSION

DTEase family enzymes catalyze the C-3 epimerization of ketoses[1] and ketose derivatives[31,32] and play an important role in the biotransformation of functional rare sugars and deoxyhexoses, especially in the biotechnological production of D-psicose.[1] From 1993 to 2009, three DTEase family enzymes from different genera of microorganisms were identified one

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after another, which were *P. cichorii*,[23] *A. tumefaciens*,[24] and *R. sphaeroides*.[25] D-Psicose, D-tagatose, and D-fructose acted as substrates with highest specificity for *P. cichorii* DTEase,[23] *A. tumefaciens* DPEase,[24] and *R. sphaeroides* DTEase,[25] respectively. Over the past 3 years, DPEases were characterized from one *Ruminococcus* genus strain, *Ruminococcus* sp. 5_1_39BFAA,[27] and four *Clostridium* genus strains including *C. cellulolyticum* H10,[26] *C. scindens* ATCC 35704,[28] *C. bolteae* ATCC BAA-613,[29] and *Clostridium* sp. BNL1100.[30] In addition, in a recent patent, Maruta et al. reported that DTEase family enzymes could be produced from a wide range of microorganisms of the genus *Rhizobium*, such as *Rhizobium leguminosarum*, *Rhizobium fredii*, and *Rhizobium meliloti*.[33] In this study, a new member of the DTEase family of enzymes was characterized from a novel genus of microorganism strain, *Desmospora*.

From the phylogenetic tree of the genes encoding for DTEase family enzymes, the DPEase gene from *Desmospora* sp. 8437 showed a much closer relationship with the DPEase genes than with the DTEase genes (Figure 6). According to the

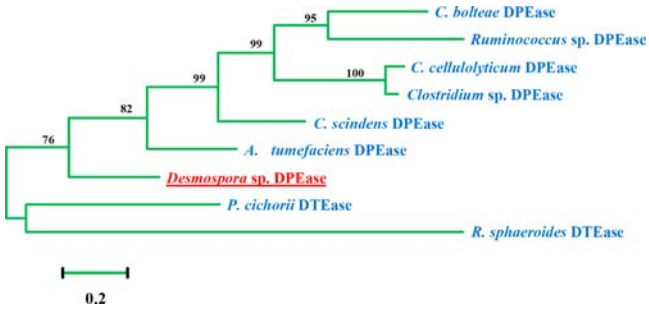

Figure 6. Phylogenetic tree of the genes encoding for DTEase family enzymes. The tree was created by the neighbor-joining method in the ClustalW program. *Bar* represents the k_{nuc} value (the length corresponding to 0.2 base substitutions per position). The DNA sequence sources were the genes encoding for *Desmospora* sp. DPEase (WP_009711885), *Clostridium* sp. DPEase (YP_005149214.1), *C. scindens* DPEase (EDS06411.1), *C. cellulolyticum* DPEase (ACL75304), *C. bolteae* DPEase (EDP19602), *Ruminococcus* sp. DPEase (ZP_04858451), *A. tumefaciens* DPEase (AAL45544), *R. sphaeroides* DTEase (ACO59490), and *P. cichorii* DTEase (BAA24429).

amino acid sequence homology analysis, the *Desmospora* sp. DPEase exhibited only around 30−50% identities with other reported DTEase family enzymes and showed higher homology with DPEases than with DTEases (Table 1); specifically, the enzyme had 51, 50, 50, 50, 49, 47, 42, and 29% of amino acid sequence identity with *Clostridium* sp. DPEase, *A. tumefaciens* DPEase, *C. cellulolyticum* DPEase, *C. scindens* DPEase, *Ruminococcus* sp. DPEase, *C. bolteae* DPEase, *P. cichorii* DTEase, and *R. sphaeroides* DTEase, respectively. Although the amino acid sequence identity was not much higher among all of the DTEase family enzymes, the enzymes showed a very high conservative property in amino acid residues responsible for metal coordination, epimerization catalysis, and molecular interaction between enzyme and substrate (Figure 7).

Interestingly, the DTEase family enzymes showed different activity changes affected by EDTA and divalent metal ions. *Desmospora* sp. DPEase was a kind of metal-dependent enzyme and did not exhibit activity when without any divalent metal ion; even if the metal ion was added, *Desmospora* sp. DPEase had no activity when EDTA existed as a metal chelator. All of the reported DPEases from the *Clostridium* genus were also metal-dependent[26,28−30] and showed similar results with the effect of EDTA and metal ions on *Desmospora* sp. DPEase (Table 2). Among the metal-independent DTEase family enzymes, EDTA could significantly inhibit the activities of *A. tumefaciens* DPEase[24] and *R. sphaeroides* DTEase,[25] but showed no effect on the *P. cichorii* DTEase activity.[23] Except for *P. cichorii* DTEase, all of the family enzymes could be obviously activated by Co^{2+} and Mn^{2+}, but could be significantly inactivated by Zn^{2+} and Cu^{2+}. The mechanism for the effect of metal ion on different DTEase family enzymes is still not clear. A suggested strategy is to compare the structures of DPEase bound to different metal ions, which has recently been used for analyzing the metal roles in D-xylose isomerase.[34]

According to the demand for industrial biotransformation of monosaccharides, D-psciose production requires a slightly acidic pH condition to reduce the nonspecific side and browning reactions;[1] unfortunately, all of the reported DTEase family enzymes show weakly alkaline pH optimum ranging from 7.5 to 9.0, except the *C. bolteae* DPEase with neutral pH optimum. *Desmospora* sp. DPEase showed optimum pH at 7.5; however, it exhibited a relatively wide pH spectrum and displayed >75% of relative activity at pH 6.5.

Desmospora sp. DPEase was suggested to be sensitive to temperature and displayed high relative activity at the range of 55−65 °C, with optimum temperature at 60 °C. It exhibited relatively high thermostability below 50 °C, but easily lost initial activity under preincubation at 60 °C. Supplementing the cofactor Co^{2+} could not change the thermal stability of *Desmospora* sp. DPEase. By comparison, the binding of metal cofactor could significantly improve the thermostability of the DPEases from *C. cellulolyticum*,[26] *C. bolteae*,[29] and *Ruminococcus* sp.[27] It was reported that metal cofactor improved the structural stability of *C. scindens* DPEase during both heat- and urea-induced unfolding through circular dichroism analysis.[28]

The k_{cat}/K_m of *Desmospora* sp. DPEase for the substrates D-psicose and D-fructose was 327 and 116 mM^{-1} min^{-1}, respectively. By comparison, the k_{cat}/K_m of other DTEase family enzymes was much less than 300 mM^{-1} min^{-1} and much less than 100 mM^{-1} min^{-1} for the substrates D-psicose and D-fructose, respectively (Table 3). The catalysis efficiency of *Desmospora* sp. DPEase was much higher than that of other DTEase family enzymes; therefore, it had potential advantage for D-psicose production.

The equilibrium ratio between D-psicose and D-fructose of *Desmospora* sp. DPEase was 30:70, which was similar to that of all reported DPEases but was significantly higher than that of DTEases (Table 3). When the large-scale D-psicose production by purified *Desmospora* sp. DPEase was carried out from D-fructose with an initial concentration of 500 g/L, 142.5 g/L D-psicose was produced, giving 28.5% of turnover ratio; by comparison, the *Clostridium* sp. DPEase[30] and *Ruminococcus* sp.[27] DPEase produced 120 and 125 g/L D-psicose from the same initial concentration of D-fructose (Table 3).

The crystal structures of *A. tumefaciens* DPEase,[35] *P. cichorii* DTEase,[36] and *C. cellulolyticum* DPEase[37] have been reported. In future work, based on the known structure information, research on the relationship between structure and function of *Desmospora* sp. DPEase will be carried out, especially the mechanism of high catalytic efficiency, and protein modification will be needed to improve the thermostability, pH stability, and substrate specificity. In addition, for practical application of the

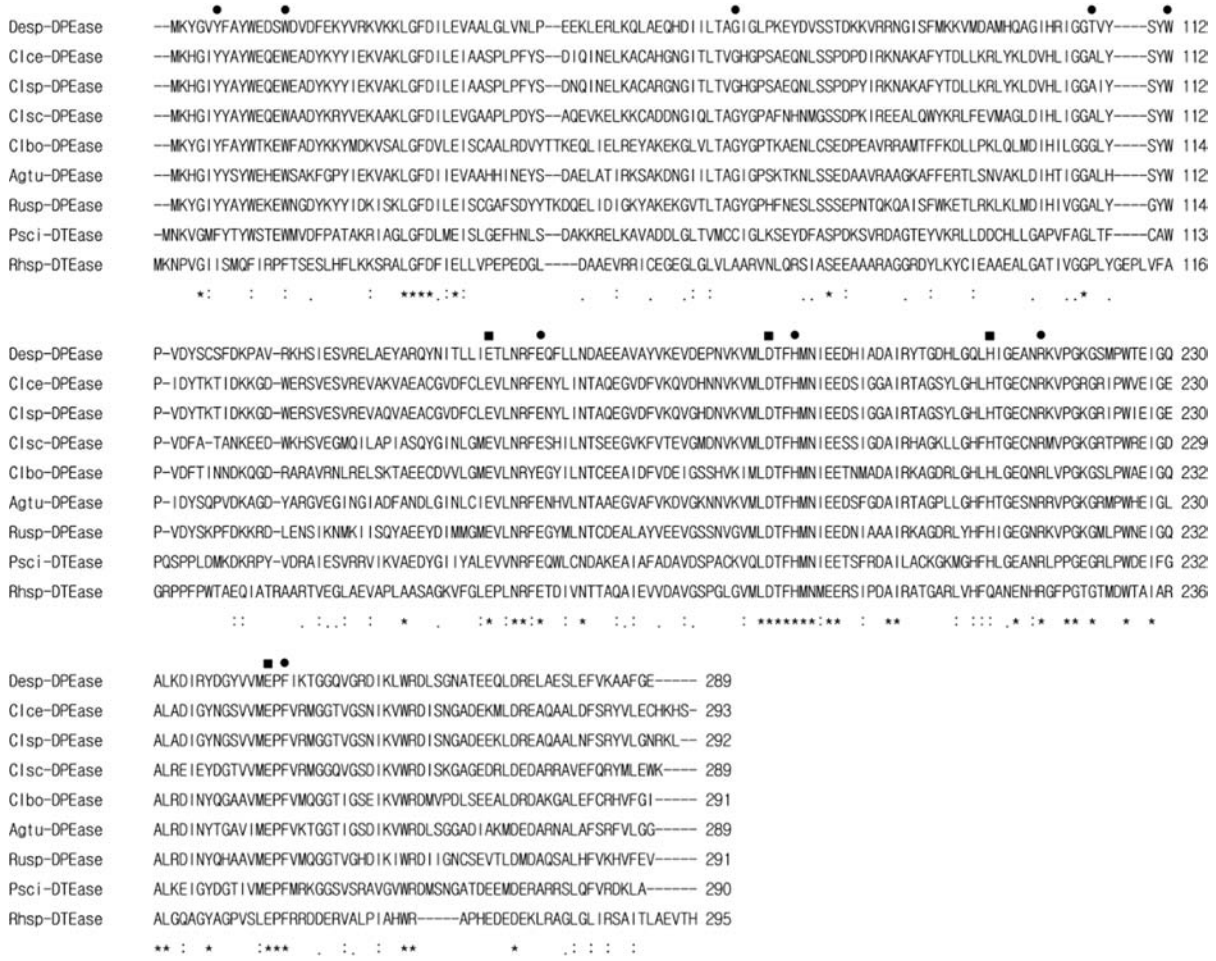

Figure 7. Alignment of the amino acid sequences of DTEase family enzymes. The alignment was performed using the ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Amino acid residues that are identical in all of the displayed sequences are marked by asterisks (*); strongly conserved or weakly conserved residues are indicated by colons (:) or dots (.), respectively. The origins of DTEase family enzymes and their GenBank accession numbers are as follows: *Desmospora* sp. DPEase (Desp-DPEase; GenBank accession no. WP_009711885), *Clostridium* sp. DPEase (Clsp-DPEase; YP_005149214.1), *C. scindens* DPEase (Clsc-DPEase; EDS06411.1), *C. cellulolyticum* DPEase (Clce-DPEase; ACL75304), *C. bolteae* DPEase (Clbo-DPEase; EDP19602), *Ruminococcus* sp. DPEase (Rusp-DPEase; ZP_04858451), *A. tumefaciens* DPEase (Agtu-DPEase; AAL45544), *R. sphaeroides* DTEase (Rhsp-DTEase; ACO59490), and *P. cichorii* DTEase (Psci-DTEase; BAA24429). The residues involved in both the metal coordinating sites and catalytic center (■) and those responsible for the interaction between the enzyme and substrate D-fructose (●) are symbolized according to the crystal structures of *A. tumefaciens* DPEase,[35] *P. cichorii* DTEase,[36] and *C. cellulolyticum* DPEase.[37]

Table 2. Effect of EDTA and Divalent Metallic Ions on the Enzyme Activity of Different DTEase Family Enzymes

	relative activity (%)								
DTEase family enzyme	none	EDTA	Co^{2+}	Mn^{2+}	Fe^{2+}	Mg^{2+}	Ni^{2+}	Zn^{2+}	Cu^{2+}
Desmospora sp. DPEase	0	0	100 ± 3	80 ± 2	49 ± 3	0	14 ± 1	0	0
Clostridium sp. DPEase[30]	0	0	100	66	NR[a]	22	31	2	0
C. scindens DPEase[28]	0	0	92	100	70	41	69	NR	NR
C. cellulolyticum DPEase[26]	0	0	100	92	67	38	65	0	0
C. bolteae DPEase[b, 29]	100	0	225	165	80	25	91	37	17
Ruminococcus sp. DPEase[27]	100	NR	~92	~160	NR	~115	~19	0	0
A. tumefaciens DPEase[24]	100	20	268	274	NR	NR	NR	4.4	2.2
R. sphaeroides DTEase[25]	100	31	NR	163	NR	NR	NR	23	13
P. cichorii DTEase[23]	100	~100	NR	NR	NR	NR	NR	~40	~40

[a]NR, not reported. [b]The enzyme was purified without EDTA pretreatment.

enzyme, D-psicose production may be tried from D-glucose using a two-enzyme system containing *Desmospora* sp. DPEase and glucose isomerase, and research on the D-psicose production by recycling of the residual D-fructose will be focused on.

In conclusion, *Desmospora* sp. DPEase (GenBank accession no. WP_009711885) was characterized to be a new member of the DTEase family of enzymes. The enzyme was strictly metal-dependent with optimum metal ion cofactor Co^{2+} and displayed maximum activity at pH 7.5 and 60 °C. The catalysis

Table 3. Comparison of Biochemical Properties and Kinetic Parameters of DTEase Family Enzymes

DTEase family enzyme	optimum pH	optimum temperature (°C)	optimum metal ion	highest specificity	k_{cat} (min^{-1})		K_m (mM)		k_{cat}/K_m (mM^{-1} min^{-1})		equilibrium ratio between D-psicose and D-fructose	production of D-psicose (g/L)
					D-psicose	D-fructose	D-psicose	D-fructose	D-psicose	D-fructose		
Desmospora sp. DPEase	7.5	60	Co^{2+}	D-psicose	5157.8 ± 108	63573 ± 472	81.3 ± 2.1	549 ± 18	327 ± 19	116 ± 11	30:70 (60 °C)	142.5 (55 °C, pH 8.0)[a]
Clostridium sp. DPEase[30]	8.0	65	Co^{2+}	D-psicose	32185	16372	227.6	279	141.4	58.7	28:72 (65 °C)	120 (55 °C, pH 8.0)[a]
C. scindens DPEase[28]	7.5	60	Mn^{2+}	D-psicose	1827	350	28.3	40.1	64.5	8.72	28:72 (50 °C)	NR[b]
C. cellulolyticum DPEase[26]	8.0	55	Co^{2+}	D-psicose	3243.5	3354.5	17.4	53.5	186.4	62.7	32:68 (55 °C)	218 (55 °C, pH 8.0)[c]
C. bolteae DPEase[29]	7.0	55	Co^{2+}	D-psicose	2940[d]	3540[d]	27.4	59.8	107[d]	59[d]	32:68 (60 °C)	216 (55 °C, pH 7.0)[c]
Ruminococcus sp. DPEase[27]	7.5 − 8.0	60	Mn^{2+}	D-psicose	2427	3562	48	216	51	16	28:72 (60 °C)	125 (50 °C, pH 8.0)[a]
A. tumefaciens DPEase[24]	8.0	50	Mn^{2+}	D-psicose	2381	2068	12	24	205	85	33:67 (40 °C)	230 (50 °C, pH 8.0)[e]
R. sphaeroides DTEase[25]	9.0	40	Mn^{2+}	D-fructose	NR	NR	NR	NR	NR	NR	23:77 (40 °C)	118 (40 °C, pH 9.0)[e]
P. cichorii DTEase[23]	7.5	60	none	D-tagatose	NR	NR	NR	NR	NR	NR	20:80 (30 °C)	150 (55 °C, pH 8.0)[e]

[a]Produced from D-fructose with an initial concentration of 500 g/L. [b]NR, not reported. [c]Produced by whole cells from D-fructose with an initial concentration of 750 g/L. [d]Values were converted from the original reference after exchanging the units. [e]Produced from D-fructose with an initial concentration of 750 g/L.

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efficiency (k_{cat}/K_m) for substrate D-fructose was measured to be 116 mM^{-1} min^{-1}, which was much higher than the values of other reported DTEase family enzymes. The *Desmospora* sp. DPEase could produce 142.5 g/L D-psicose from 500 g/L D-fructose, giving a 28.5% turnover ratio. These findings showed that *Desmospora* sp. DPEase could be a good candidate to produce D-psicose industrially.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

DTEase, D-tagatose 3-epimerase; DPEase, D-psicose 3-epimerase; LB, Luria–Bertani; IPTG, isopropyl β-D-1-thiogalactopyranoside; HPLC, high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid

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