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Characterization of a Thermophilic L-Rhamnose Isomerase from *Thermoanaerobacterium saccharolyticum* NTOU1

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L-Rhamnose isomerase (EC 5.3.1.14, L-RhI) catalyzes the reversible aldose−ketose isomerization between L-rhamnose and L-rhamnulose. In this study, the *L-rhi* gene encoding L-RhI was PCR-cloned from *Thermoanaerobacterium saccharolyticum* NTOU1 and then expressed in *Escherichia coli*. A high yield of the active L-RhI, 9780 U/g of wet cells, was obtained in the presence of 0.2 mM IPTG induction. L-RhI was purified sequentially using heat treatment, nucleic acid precipitation, and anion-exchange chromatography. The purified L-RhI showed an apparent optimal pH of 7 and an optimal temperature at 75 °C. The enzyme was stable at pH values ranging from 5 to 9, and the activity was fully retained after a 2 h incubation at 40−70 °C. L-RhI from *T. saccharolyticum* NTOU1 is the most thermostable L-RhI to date, and it has a high specific activity (163 U/mg) and an acceptable purity after heat treatment, suggesting that this enzyme has the potential to be used in rare sugar production.

KEYWORDS: L-Rhamnose isomerase; D-allose; thermophilic enzyme; *Thermoanaerobacterium saccharolyticum* NTOU1; *E. coli*; overexpression

INTRODUCTION

Rare sugars refer to those monosaccharides that are rarely distributed in nature (*1*). They and their derivatives have been used as food additives (*2*), antiviral and anthelmintic agents (*3*, *4*), and cancer cell suppressors (*5*, *6*) and applied to many other areas. However, the scarcity and high price of these sugars limit the pursuit of wider applications and research. Recently, many studies have been proposed to use enzymatic processes for the production of rare sugars because the enzymatic processes have high efficiency, stereoselectivity, and milder conditions. These processes include D-tagatose production using L-arabinose isomerase (*7*), D-psicose production using tagatose 3-epimerase (*8*), and D-allose production using L-rhamnose isomerase (*9*).

L-Rhamnose isomerase (EC 5.3.1.14, L-RhI), one of the L-rhamnose metabolic enzymes found in some plants and bacteria, catalyzes the reversible aldose−ketose isomerization between L-rhamnose and L-rhamnulose. The characteristics and crystal structures of L-RhIs from *Escherichia coli* and *Pseudomonas stutzeri* have been investigated (*10*−*13*). Although the L-RhIs from *E. coli* and *P. stutzeri* show different enzymatic properties, their catalytic mechanisms are similar. L-RhIs can also catalyze many other reactions, such as D-psicose to D-allose etc (**Figure 1**). This specific capability has the potential for the mass production of various rare and commercially expensive sugars, such as L-talose, D-gulose, and D-allose (*9*, *14*). D-Allose, a C3 epimer of D-glucose, has shown the potential to suppress the growth of many cancer cell lines, such as Ca9-22, DU145, HSC-3, and PC-3 (*5*, *6*), and resistance to the rice pathogen *Xanthomonas oryzae* pv. *oryzae* in transgenic plants (*15*). D-Allose and its precursor D-psicose can be produced from an abundant substrate, D-fructose, by enzymatic processes that first employ D-tagatose-3-epimerase to convert D-fructose to D-psicose and then use L-rhamnose isomerase to further convert D-psicose to D-allose (*16*).

The use of thermophilic enzymes to produce sugars has several advantages. The reaction is generally carried out at a high temperature, which can accelerate the reaction rate, reduce the viscosity in the reaction mixtures, and decrease the risks of microbial contaminations (*17*, *18*). However, the L-RhI from *Bacillus pallidus* Y25 is the only thermophilic L-RhI that had been studied. Recently, we have isolated a novel theromophile, *Thermoanaerobacterium saccharolyticum* NTOU1, from an acidic hydrothermal vent of Gueishandao Island in Taiwan (unpublished data). This bacterium is thermophilic, anaerobic, and Gram-negative and can grow at 45−78 °C and pH 5−9 (unpublished data). Functional genes of *T. saccharolyticum* NTOU1 were sequenced from genomic DNA by using GS20 FLX sequencer (454 Life Sciences, Branford, CT), and the L-rhamnose isomerase gene *L-rhi* was identified by genomic analysis (unpublished data).

In this study, the *L-rhi* gene was PCR-cloned from the genomic DNA of *T. saccharolyticum* NTOU1 to an expression vector and overexpressed in *E. coli*. In addition to characterizing the general properties of this recombinant L-RhI, we also investigated the substrate specificity and kinetic parameters of the enzyme on the isomerization reactions of various sugars.

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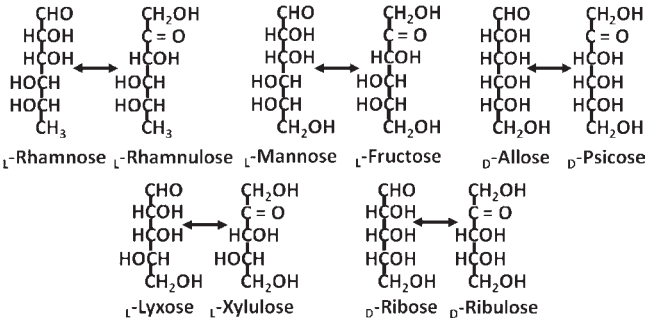

Figure 1. Schematic diagram of several aldose−ketose conversions catalyzed by L-RhI.

MATERIALS AND METHODS

Materials. *T. saccharolyticum* NTOU1 was enriched and isolated from acidic hydrothermal fluids of Gueishandao Island in Taiwan with 0.5% starch and 0.5% maltose as the carbon source. *T. saccharolyticum* NTOU1 has been submitted to the Bioresource Collection and Research Center (BCRC) in Taiwan and assigned a strain number of BCRC 910455. *E. coli* DH5α and *E. coli* BL21-CodonPlus (DE3)-RIL were obtained from Stratagene (La Jolla, CA). Plasmid pET-21b(+) was from Novagen (Madison, WI). *Pfx* DNA polymerase and DNAzol reagent were purchased from Invitrogen Corp. (Carlsbad, CA). *T4* DNA ligase and restriction enzymes were supplied by Promega (Madison, WI). D-Allose, L-mannose, L-cysteine hydrochloride monohydrate, cabazole crystalline, acrylamide, bovine serum albumin (BSA), and isopropyl-β-D-thiogalactoside (IPTG) were from Sigma (St. Louis, MO). A Microcon YM-3 centrifugal filter unit was obtained from Millipore (Bedford, MA). Low molecular weight standards were from Amersham Pharmacia Biotech (Piscataway, NJ). D-Ribose was from Merck KGaA (Darmstadt, Germany). L-Rhamnose was from Fluka (Buchs SG, Switzerland). L-Lyxose was from MP Biomedicals (Solon, OH).

Amplification of the *L-rhi* Gene. The genomic DNA of *T. saccharolyticum* NTOU1 was extracted by using the DNAzol reagent according to the manufacturer's instructions. The *L-rhi* gene was amplified from the *T. saccharolyticum* NTOU1 genomic DNA by Polymerase Chain Reaction (PCR). Two primers were designed on the basis of the *L-rhi* sequence of *T. saccharolyticum* NTOU1 (GenBank accession no. GU951509). To clone the *L-rhi* gene into the pET-21b(+) vector, the *Nde*I and *Hin*dIII restriction sites were included in the forward and reverse primers, respectively. The primer sequences are as follows: 5NdRhI (forward primer), 5′-TAA GAA GGA GAT ATA **CAT ATG** TAC GAA GTA GAA AAA GAT AAA-3′; 3HiRhI (reverse primer), 5′-GAG TGC GGC CGC **AAG CTT** TCA ATC TCT ATA TTT TAG AAT TTC-3′, where the *Nde*I and *Hin*dIII restriction sites are in boldface. The reaction was carried out in 50 μL of reaction mixture containing *T. saccharolyticum* NTOU1 genomic DNA, two primers, dNTP, *Pfx* DNA polymerase, and *Pfx* DNA polymerase buffer and was performed by using a TGradient PCR system (Biometra, Goettingen, Germany) according to the following conditions in sequence: 95 °C for 5 min, an amplification, and a final extension at 68 °C for 10 min. The amplification profile was 35 cycles of the following conditions in sequence: 1 min at 95 °C, 1 min at 55 °C, and 2 min at 68 °C.

Construction of the Expression Vector for L-RhI. The 1.3 kb PCR-amplified fragment was purified and then digested with *Nde*I and *Hin*dIII. The digested fragment was inserted into the pET-21b(+) vector, resulting in a recombinant vector designated pET-21b-*rhi*. The sequence of the entire *L-rhi* gene was confirmed by DNA sequencing, which was carried out by Mission Biotech Corp. (Taipei, Taiwan).

Expression of L-RhI by *E. coli*. The pET-21b-*rhi* vector was transformed into *E. coli* BL21-CodonPlus (DE3)-RIL to express L-RhI. One single colony from a newly transformed culture plate was inoculated at 37 °C into the terrific broth (TB) medium supplemented with 100 μg/mL ampicillin plus 34 μg/mL chloramphenicol and grown at 37 °C until the OD_{600} reached around 0.6. Cells were collected by centrifugation and resuspended in 4 mL of fresh medium. A volume of 3 mL of the resuspended culture was then added to 600 mL of fresh TB medium containing 100 μg/mL of ampicillin plus 34 μg/mL chloramphenicol. The culture was grown until the OD_{600} reached around 0.6 and induced in the presence of 0.2 mM IPTG. After a further culture at 20 °C for 20 h, the cells were then harvested by centrifugation and stored at −80 °C before further processing.

Purification of the Recombinant Enzyme. The frozen cells (4.65 g) expressing L-RhI were suspended in 14 mL of lysis buffer containing 20 mM Tris-HCl (pH 8.5), 1 mM Co^{2+}, and 1 mM benzamidine. The suspended cells were disrupted using a French press disruptor (Sim-Aminco, Rochester, NY) at 20000 psi (1360 atm). The cell-free extract was then prepared by removing the insoluble fractions from the supernatant of the above mixture by centrifugation at 10000*g* for 30 min. Heat treatment was first used to precipitate most of the undesired proteins by incubating the cell-free extract in a 60 °C water bath for 30 min followed by centrifugation to remove the heat-labile proteins. A 10% (w/v) streptomycin sulfate stock solution was then added to a final concentration of 1% (w/v) to precipitate the nucleic acids. After centrifugation at 10000*g* for 30 min, the supernatant was dialyzed against 20 mM Tris-HCl buffer (pH 8.5) and was subsequently loaded onto a Q-Sepharose column (1.6 × 10 cm), which was pre-equilibrated with the same dialysis buffer. The column was first washed thoroughly with the same buffer until the absorbance of 280 nm reached baseline. Finally, a linear gradient of 0−0.5 M NaCl in the above buffer was used to elute the bound proteins. The eluted fractions containing enzyme activity were collected and dialyzed against 20 mM Tris-HCl (pH 8.5).

Mass Spectrometric Analysis. The mass spectrometric analysis was performed on a Microflex (Bruker Daltonics, Bremen, Germany) MALDI-TOF mass spectrometer and carried out by Mission Biotech Corp.

Enzyme Activity Assay. The L-RhI activity was assayed at 65 °C for 10 min by using 10 mM L-rhamnose as substrate in 50 mM potassium phosphate buffer (pH 7) and 1 mM Co^{2+}. The amount of L-rhamnulose in the reaction mixture was determined by using the cysteine−carbazol method (*19*). First, 600 μL of 70% sulfuric acid was added into the 100 μL reaction mixture to stop the enzyme reaction. Then 20 μL of 1.5% cysteine hydrochloride and 20 μL of 0.12% carbazole solutions were added into the reaction mixture in turn. After the resulting mixture was further incubated at 35 °C for 20 min, the absorbance at 560 nm was measured to determine the amount of L-rhamnulose. One unit (U) of L-RhI activity was defined as the amount of enzyme required to produce 1 μmol of L-rhamnulose in 1 min. In determining the effects of pH and temperature on the activity and stability of enzyme, the following buffers with a final concentration of 50 mM were used in different pH ranges: citrate−phosphate buffer (pH 3−5), potassium phosphate buffer (pH 6−7.5), Tris-HCl buffer (pH 8−9), and glycine−NaOH buffer (pH 10−11). For determination of the optimal pH, the enzyme (0.577 μg/mL) was assayed under the standard conditions except at different pH values. For determination of the pH stability, the enzyme (57.7 μg/mL) was incubated at various pH values at room temperature for 24 h, and the remaining activities were assayed after appropriate dilutions under the standard conditions. For determination of the optimal temperature, the enzyme (0.577 μg/mL) was assayed under the standard conditions except at different temperatures. For determination of the thermostability, the enzyme (57.7 μg/mL) was incubated at various temperatures at pH 7 for 2 h, and the remaining activities were assayed after appropriate dilutions under the standard conditions. For determination of the effects of metal ions on the activity, the enzyme (0.577 μg/mL) was assayed at 65 °C and pH 7 as in the standard conditions except in the absence or presence of 1 mM concentrations of different metal ions.

Protein Concentration Measurement. Protein concentration was determined according to Bradford's method (*20*) with BSA as standard.

Enzyme Kinetics. The initial rates of conversion of L-rhamnose, L-lyxose, L-mannose, D-allose, and D-ribose were determined at 65 °C in 50 mM potassium phosphate buffer and 1 mM Co^{2+} at pH 7 by using 8−10 substrate concentrations ranging from 2 to 300 mM. The amounts of enzyme used were 0.537, 0.560, 5.77, 11.5, and 5.77 μg/mL for the substrates L-rhamnose, L-lyxose, L-mannose, D-allose, and D-ribose, respectively. Samples taken at six different time intervals were stopped by adding 600 μL of 70% sulfuric acid into 100 μL of reaction mixture. The amounts of ketoses were determined according to the cysteine−carbazol method (*19*). Values of k_{cat} and K_M were calculated by fitting the initial rates as a function of substrate concentration to the Michaelis−Menten equation using Enzfitter software (Elsevier-Biosoft).

HPLC Analysis of the Isomerization Products of D-Allose. The isomerization products of D-allose were measured by a Hitachi HPLC L-7000 (Tokyo, Japan) equipped with an LT-ELSD detector (Sedex, Alfortville, France). The enzyme reactions were carried out at 65 °C for

Table 1. Comparison of the Deduced Amino Acid Sequence of Recombinant L-RhI from *T. saccharolyticum* NTOU1 with Those of L-RhIs from Other Microorganisms

source	accession no.[a]	no. of residues	identity[b] (%)
Thermoanaerobacterium saccharolyticum NTOU1	GU951509	425	100
Caldicellulosiruptor saccharolyticus DSM 8903	A4XHV7	426	69
Anaerocellum thermophilum DSM 6725	B9MQ29	426	68
Clostridium beijerinckii NCIMB 8052	A6LQK4	418	60
Escherichia coli K12	P32170	419	53
Bacillus pallidus Y25	A8CEF6	412	42
Pseudomonas stutzeri	Q75WH8	430	13

[a] The deduced amino acid sequences were obtained from the UniProt protein sequence database (*25*). [b] The identity values were analyzed by the ClustalW program on the Website http://www.ebi.ac.uk/Tools/clustalw2/index.html (*24*).

0−36 h in 50 mM potassium phosphate buffer (pH 7) containing 2 U/μL purified enzyme and 1 mM Co^{2+} by using 100 mM D-allose as substrate. The reaction was stopped by incubating the mixture in boiling water for 10 min, and then the mixtures were filtered through a Microcon centrifugal filter unit with a YM-3 membrane (MWCO 3000) to remove the enzyme. The filtrate was analyzed by a Vercopak Nucleosil 5 μm NH_2 column (4.6 × 250 mm) with a mobile phase consisting of 30% (v/v) H_2O and 70% (v/v) acetonitrile.

RESULTS AND DISCUSSION

Gene Analysis and Comparison of Amino Acid Sequences of Several L-RhIs. *T. saccharolyticum* NTOU1 L-RhI possesses 425 amino acids and a calculated molecular weight of 48960 Da, which was deduced from the nucleotide sequence of the *L-rhi* gene and analyzed by the Compute pI/Mw tool at http://tw.expasy.ch/tools/pi_tool.html (*21*), respectively. *T. saccharolyticum* NTOU1 L-RhI shares 69% identity with L-RhI from *Caldicellulosiruptor saccharolyticus* DSM 8903, 68% identity with L-RhI from *Anaerocellum thermophilum* DSM 6725, 60% identity with L-RhI from *Clostridium beijerinckii* NCIMB 8052, 53% identity with L-RhI from *E. coli* K12, and 42% identity with L-RhI from *B. pallidus* Y25, but only 13% identity with L-RhI from *P. stutzeri* (**Table 1**). Although the sequence identity between the L-RhIs from *E. coli* and *P. stutzeri* is only 17%, their crystal structures show that the catalytic and metal binding residues are highly conserved (*13*). **Figure 2** shows the sequence alignment of several L-RhIs. The residues involved in the active site of *E. coli* L-RhI (*12*) are highly conserved between these L-RhIs (**Figure 2**). In addition, the crystal structure of *E. coli* L-RhI has shown that Val53, Leu63, Ile67, Ile105, Tyr106, and Phe336 can form a hydrophobic area to recognize the methyl group on the C6 of L-rhamnose (*12*). As shown in **Figure 2**, six putative substrate-recognizing residues of *E. coli* L-RhI (*12*) are all preserved in *T. saccharolyticum* NTOU1 L-RhI, indicating that the substrate recognition of *T. saccharolyticum* NTOU1 L-RhI might be similar to that of *E. coli* L-RhI.

Expression and Purification of the Recombinant L-RhI. The protein expression level of the recombinant L-RhI was higher in the presence of 0.2 mM IPTG than in the absence of IPTG or in the presence of 0.5 mM IPTG induction. The yield of active L-RhI expressed in *E. coli* was 111000 U/L and 9780 U/g of wet cells, which was much higher than the previously reported yields of 36500, 20000, and 2400 U/L for the *B. pallidus* Y25, *P. stutzeri*, and *E. coli* K12 L-RhIs expressed in *E. coli*, respectively (*10*, *11*, *22*).

After the analysis by gel electrophoresis, the expressed L-RhI was mostly soluble and appeared in the cell-free extract (**Figure 3**, lanes 1−3). The L-RhI was purified from the cell-free extract of *E. coli* sequentially by heat treatment, nucleic acid precipitation, and anion-exchange chromatography (**Table 2**). Heat treatment was noted to effectively remove most of the undesired proteins (**Figure 3**, lane 4) and retain most of the enzyme activity (**Table 2**). After the anion-exchange chromatography, the purified L-RhI showed a single band with a molecular mass of 48 kDa on SDS-PAGE, indicating that a high purity of L-RhI was obtained (**Figure 3**, lane 5). The molecular weight of the purified L-RhI was also analyzed by MALDI-TOF mass spectrometry. It showed a

Figure 2. Sequence alignment of L-RhIs from *B. pallidus* Y25 (B.pall), *E. coli* (E.coli), *P. stutzeri* (P.stut), and *T. saccharolyticum* NTOU1 (T.ntou). The alignment was performed using the ClustalW program (*24*). Amino acid residues that are identical in all of the displayed sequences are labeled by asterisks (∗), strongly conserved or weakly conserved residues are marked by colons (:) or dots (·), respectively. The residues involved in the active site and the putative substrate-recognizing residues of L-RhI from *E. coli* are highlighted in black and gray, respectively. These putative substrate-recognizing residues are located outside the active site.

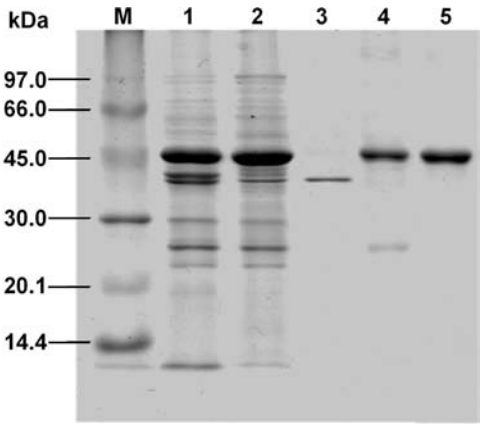

Figure 3. SDS-PAGE analysis of purifications of L-RhI from *T. saccharolyticum* NTOU1 in *E. coli* BL21-CodonPlus (DE3)-RIL carrying the pET-21b-*rhi*. Lanes: M, molecular weight standards; 1, total cellular proteins of the *E. coli* cells carrying the pET-21b-rhi; 2, crude cell-free extracts of the *E. coli* cells; 3, insoluble proteins after cell lysis; 4, partially purified fraction of recombinant L-RhI after heat treatment; 5, purified fraction of recombinant L-RhI after anion-exchange chromatography.

Table 2. Summary of Purification Steps of Recombinant L-RhI from *T. saccharolyticum* NTOU1 Expressed in *E. coli* BL21-CodonPlus (DE3)-RIL

purification step	total protein (mg)	total activity (U)	activity recovery (%)	specific activity (U/mg)	purification fold
crude extract	693	45500	100	65.6	1
heat treatment[a]	231	37700	82.8	163	2.48
Q Sepharose	117	23100	50.8	197	3.00

[a] The crude extract was heated at 60 °C for 0.5 h and then centrifuged to remove the precipitates.

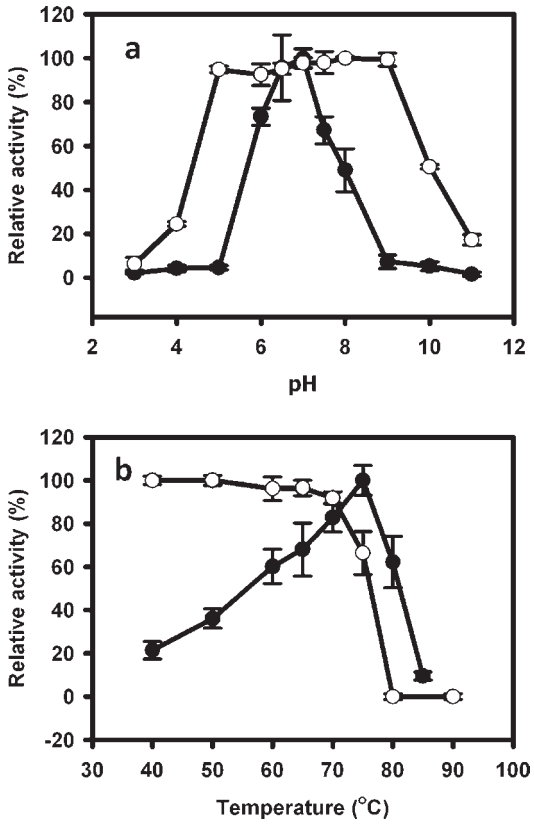

Figure 4. Effects of pH and temperature on the activity (●) and stability (○) of NTOU1 L-RhI. Panel **a** shows the effects of pH. For determination of the optimal pH, the enzyme activity was assayed at 65 °C and different pH values. For determination of the pH stability, the enzyme was incubated at various pH values at room temperature for 24 h, and the remaining activities were assayed at 65 °C and pH 7. Panel **b** shows the effects of temperature. For determination of the optimal temperature, the enzyme activity was assayed at pH 7 and different temperatures. For determination of the thermostability, the enzyme was incubated at various temperatures at pH 7 for 2 h, and the remaining activities were assayed at 65 °C and pH 7. Data represent the mean ± SD from triplicate experiments.

molecular mass of 48930 Da, which is in good agreement with the calculated molecular weight (48961 Da).

Effects of pH and Temperature on the Activity and Stability of the Recombinant L-RhI. The recombinant enzyme showed an optimal activity at pH 7 and very minimal activity when the pH either dropped below 5 or increased to above 9 (**Figure 4a**). A further treatment of the purified enzyme was carried out at various pH values at room temperature for 24 h to investigate the effect of pH on enzyme stability. The results showed that the enzyme remained stable in the pH range from 5 to 9 (**Figure 4a**).

When the enzyme activity was measured at different temperatures, the activity increased with increasing temperature, reached an optimal activity at 75 °C, and then dropped with a steeper slope to a minimal level at 85 °C (**Figure 4b**). The data of **Figure 4b** were also replotted on a semilogarithmic scale as ln(activity) versus $1/T$ to determine the activation energy, E_a, of the isomerase reaction by the Arrhenius equation (data not shown). The activation energy of the isomerase reaction calculated from the Arrhenius plot was 39.7 kJ mol^{-1} at pH 7. The enzyme was incubated at various temperatures for 2 h in an effort to examine the effect of temperature on stability. The enzyme activity decreased very slightly to 90% as the incubation temperature increased to 70 °C and dropped sharply to a very minimal level at 80 °C (**Figure 4b**). The good thermostability of this enzyme over the wide temperature range (40−70 °C) indicated that the purified recombinant L-RhI might be well folded and should have the same structure as that produced directly from *T. saccharolyticum* NTOU1.

Table 3. Effect of Various Metal Ions on Recombinant L-RhI from *T. saccharolyticum* NTOU1

metal ion	relative activity (%)	metal ion	relative activity (%)
none	ND[a]	Zn^{2+}	2
Co^{2+}	100	Ca^{2+}	1
Mn^{2+}	61	Cu^{2+}	ND
Na^{+}	19	K^{+}	ND
Mg^{2+}	9		

[a] ND, not detected.

Table 4. Specific Activities (U/mg) of Some Recombinant L-RhIs for Various Aldoses

substrate	*T. saccharolyticum* NTOU1[a]	*B. pallidus* Y25[b]	*P. stutzeri*[c]	*E. coli*[c]
L-rhamnose	203	77.2	244	65.8
L-lyxose	130	19.3	110	8.1
L-mannose	6.0	4.5	81.6	2.5
D-allose	5.7	2.6	7.5	0.2
D-ribose	1.6	6.3	16	0.7

[a] This study. Experiments were carried out at 65 °C and pH 7 in the presence of 1 mM Co^{2+}. [b] Poonperm et al. (*22*). Experiments were carried out at 65 °C in the presence of 1 mM Mn^{2+}. [c] The specific activities of L-RhIs from *P. stutzeri* and *E. coli* were the recalculated results from Leang et al. (*11*). Experiments were carried out at 50 °C in the presence of 1 mM Mn^{2+}.

Effect of Metal Ions on the Activity of the Recombinant L-RhI. The effects of metal ions on the activity of L-RhI were investigated (**Table 3**). In the previous findings, the enzyme activities of *E. coli*, *P. stutzeri*, and *B. pallidus* L-RhIs were enhanced in the presence of metal ions (*12*, *13*, *22*). *T. saccharolyticum* NTOU1 L-RhI also showed a high activity in the presence of Co^{2+}; however, it showed null activity in the absence of any ions. Furthermore, when Co^{2+} was replaced with Mn^{2+} or Na^{+}, the activity was reduced to only 61 and 19% of that in the presence of Co^{2+}, respectively. For other ions, such Mg^{2+}, Ca^{2+}, Cu^{2+}, K^{+}, and Zn^{2+}, the enzyme activity was barely detected. The crystal structure of *E. coli* L-RhI showed that Zn^{2+} and Mn^{2+} are required for the activity. Our results also suggested that metal ions, especially Co^{2+} and Mn^{2+}, are also required for the enzyme activity of *T. saccharolyticum* NTOU1 L-RhI.

Substrate Specificity and Enzyme Kinetics. Because L-RhIs can also react with other sugars in addition to L-rhamnose (*22*, *23*), several substrates were used to study the substrate specificity and enzyme kinetics of the recombinant *T. saccharolyticum* NTOU1 L-RhI (**Tables 4** and **5**). In comparisons with other sources of L-RhIs (*22*, *23*), *T. saccharolyticum* NTOU1 L-RhI shows higher specific activity than those of *E. coli* and *B. pallidas* L-RhIs, but lower specific activity than that of *P. stutzeri* L-RhI toward almost all of the tested sugars (**Table 4**). Like other L-RhIs, *T. saccharolyticum* NTOU1 L-RhI has the highest specific activity toward L-rhamnose,

Table 5. Comparison of Kinetic Parameters of the Recombinant L-RhIs from *P. stutzeri*, *B. pallidus* Y25, *E. coli*, and *T. saccharolyticum* NTOU1 for Isomerization of L-Rhamnose, L-Lyxose, L-Mannose, D-Allose, and D-Ribose, Respectively

	T. saccharolyticum NTOU1[a]			*B. pallidus* Y25[b]			*P. stutzeri*[b]			*E. coli*[c]
substrate	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (mM^{-1} s^{-1})	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (mM^{-1} s^{-1})	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (mM^{-1} s^{-1})	K_M (mM)
L-rhamnose	180 ± 4[d]	3.53 ± 0.39	50.8 ± 5.0	68.0	4.89	13.9	172	11.9	14.4	2.0
L-lyxose	240 ± 3	45.2 ± 1.8	5.30 ± 0.16	31.0	16.1	1.93	100	55.5	1.81	5.0
L-mannose	30.0 ± 0.5	58.9 ± 2.3	0.51 ± 0.01	76.8	28.9	2.66	89.7	61.7	1.45	5.0
D-allose	33.9 ± 1.3	121 ± 10	0.28 ± 0.02	34.5	41.8	0.83	5.02	42.0	0.12	ND[e]
D-ribose	21.5 ± 0.9	148 ± 13	0.15 ± 0.01	14.2	34.9	0.41	15.8	38.5	0.41	ND

[a] This study. Experiments were carried out at 65 °C and pH 7 in the presence of 1 mM Co^{2+}. [b] The kinetic parameters of L-RhIs from *B. pallidus* and *P. stutzeri* were the recalculated results from Poonperm et al. (*22*). Experiments were carried out at 65 and 37 °C, respectively, in the presence of 1 mM Mn^{2+}. [c] The K_M values of L-RhI from *E. coli* were from Poonperm et al. and Badia et al. (*22*, *26*). The k_{cat} and k_{cat}/K_M values of L-RhI from *E. coli* were not reported. [d] Standard error from the curve fitting. [e] ND, not detectable.

Table 6. Comparison of Some Enzymatic Properties of L-RhIs from *P. stutzeri*, *E. coli*, *B. pallidus* Y25, and *T. saccharolyticum* NTOU1

	natural	recombinant		
property	*E. coli*[a]	*P. stutzeri*[b]	*B. pallidus* Y25[c]	*T. saccharolyticum* NTOU1[d]
optimum temperature (°C)	60	60	65	75
thermostability (°C)	50 (10 min)	50 (10 min)	60 (60 min)	70 (2 h)
optimum pH	7.6	9.0	7.0	7.0
pH stability	NR[e]	5−11	7−10	5−9
cofactor	Mn^{2+}, Zn^{2+}	Mn^{2+}	Mn^{2+}, Co^{2+}	Mn^{2+}, Co^{2+}
molecular weight	47199	46946	47636	48961
equilibrium ratio between D-allose and D-psicose	NR	14:75 (10 mM,[f] 72 h)	35:65 (10 mM, 48 h)	29:71 (100 mM, 24 h)
byproduct	NR	Yes	No	No

[a] Korndorfer et al. (*12*), Poonperm et al. (*22*). [b] Leang et al. (*11*, *23*). [c] Poonperm et al. (*22*). [d] This study. [e] NR, not recorded in the published literature. [f] Initial D-allose concentration in the enzyme reaction.

a moderate specific activity toward L-lyxose, but a very minimal activity toward L-mannose, D-allose, and D-ribose.

A comparison of kinetic parameters (k_{cat} and K_M) of L-RhIs from different organisms is given in **Table 5**. The catalytic efficiencies (k_{cat}/K_M) of *T. saccharolyticum* NTOU1 L-RhI against L-rhamnose and L-lyxose are higher than those of *P. stutzeri* and *B. pallidas* L-RhI. The k_{cat} values of *T. saccharolyticum* NTOU1 L-RhI are similar or higher than those of *P. stutzeri* and *B. pallidas* L-RhI, except for the conversion of L-mannose. Like other L-RhIs, *T. saccharolyticum* NTOU1 L-RhI has the lowest K_M value toward L-rhamnose. Because six putative substrate-recognizing residues of *E. coli* L-RhI (*12*) are all preserved in *T. saccharolyticum* NTOU1 L-RhI, the low K_M value toward L-rhamnose suggests that *T. saccharolyticum* NTOU1 L-RhI might have a similar hydrophobic area to recognize the methyl group of L-rhamnose. The K_M values of *T. saccharolyticum* NTOU1 L-RhI toward L-lyxose and L-mannose are similar to those of *P. stutzeri* L-RhI, but the K_M values toward D-allose and D-ribose are much higher than those of other L-RhIs. These results indicate that *T. saccharolyticum* NTOU1 L-RhI exhibits lower affinities toward D-form sugar substrates.

Determination of the Equilibrium Ratio of Products between D-Allose and D-Psicose. One of the important applications of L-RhI is to convert D-psicose to D-allose. To further examine the isomerization reaction and equilibrium ratio between D-psicose and D-allose, the enzyme reactions were carried out at 65 °C for 0−36 h by using D-allose as substrate. The enzyme inactivated by boiling was added to mimic the components of reaction mixture at time zero. There is no difference between 0 and 6 h after the inactivated enzyme was added, indicating no nonenzymatic catalytic conversion of the substrate (data not shown). As shown in **Figure 5**, *T. saccharolyticum* NTOU1 L-RhI isomerizes D-allose to D-psicose without forming any detectable byproducts at 100 mM D-allose. Around 66% of D-psicose was formed after 6 h, and around 71% of

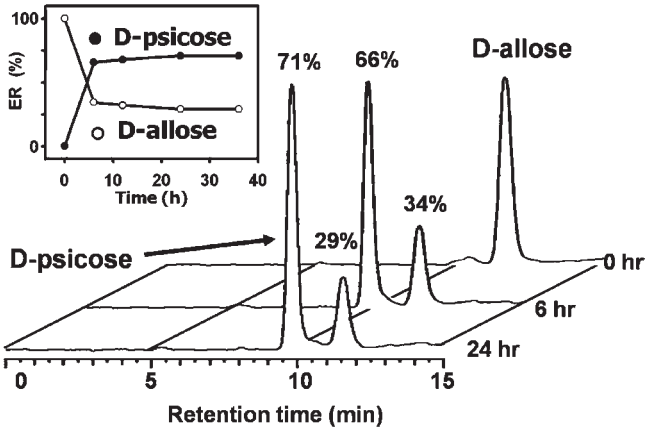

Figure 5. HPLC analysis of isomerization products and equilibrium ratio between D-allose and D-psicose. 0 h indicates 100 mM D-allose containing enzyme inactivated by boiling; 6 and 24 h indicate enzyme reaction after 6 and 24 h, respectively. (Inset) Equilibrium ratio (ER) between D-allose and D-psicose. These ratios are molar ratios calculated from the standard curves of D-allose and D-psicose.

D-psicose was formed when the reaction was approaching equilibrium (24 h). The same result was observed when the substrate was replaced by D-psicose (data not shown). *P. stutzeri* L-RhI had been used in a large-scale production of D-allose (*16*); however, its poor enzyme fidelity resulted in the D-altrose byproduct. Removing the byproduct from the reaction mixture is laborious. The lack of a byproduct in the conversion between D-psicose and D-allose catalyzed by *T. saccharolyticum* NTOU1 L-RhI is industrially welcome.

Table 6 lists a further comparison of some enzymatic properties of L-RhIs from different sources. In general, the molecular weights of these L-RhIs are between 47 and 49 kDa per monomer,

and Mn^{2+} can increase the enzyme activity. L-RhI from *P. stutzeri* presents wide pH stability. In addition, *B. pallidus* and *T. saccharolyticum* NTOU1 L-RhIs show better thermostability and higher D-allose equilibrium ratios. In industrial processes, thermostable enzymes can provide a higher reaction rate and process yield, higher solubilities of substrates and products, and less contamination. *T. saccharolyticum* NTOU1 L-RhI is the most thermostable L-RhI to date. Furthermore, *T. saccharolyticum* NTOU1 L-RhI presents high specific activity (163 U/mg) and acceptable purity after heat treatment, suggesting that this enzyme might have the potential to be used in the commercial production of rare sugars.

In this study, *T. saccharolyticum* NTOU1 L-RhI was cloned and expressed in *E. coli* with a high yield of active enzyme. We characterized the enzymatic properties of this recombinant enzyme and compared these properties with those of L-RhIs from other bacteria. Except the effects of metal ions and molecular weight, the cloned L-RhI was quite different from other natural or recombinant L-RhIs, specifically with regard to thermostability. Among the reported studies to date, *T. saccharolyticum* NTOU1 L-RhI is the most thermostable L-RhI among all characterized L-RhIs. The results from this study suggest that this L-RhI can be used to produce D-allose at high temperatures.

ABBREVIATIONS USED

L-RhI, L-rhamnose isomerase; IPTG, isopropyl-β-D-thiogalactoside; MALDI-TOF, matrix-assisted laser desorption inoization−time-of-flight; LT-ELSD, low-temperature evaporative light scattering detection.

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Supporting Information Available: Additional procedural details. This material is available free of charge via the Internet at http://pubs.acs.org.

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