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Characterization of a Thermophilic L-Rhamnose Isomerase from *Caldicellulosiruptor saccharolyticus* ATCC 43494

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ABSTRACT: 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 *Caldicellulosiruptor saccharolyticus* ATCC 43494 and then expressed in *Escherichia coli*. A high yield of active L-RhI, 3010 U/g of wet cells, was obtained after 20 °C induction for 20 h. The enzyme was purified sequentially using heat treatment, nucleic acid precipitation, and ion-exchange chromatography. The purified L-RhI showed an apparent optimal pH of 7 and an optimal temperature at 90 °C. The enzyme was stable at pH values ranging from 4 to 11 and retained >90% activity after a 6 h incubation at 80 °C and pH 7−8. Compared with other previously characterized L-RhIs, the L-RhI from *C. saccharolyticus* ATCC 43494 has a good thermostability, the widest pH-stable range, and the highest catalytic efficiencies (k_{cat}/K_M) against L-rhamnose, L-lyxose, L-mannose, D-allose, and D-ribose, suggesting that this enzyme has the potential to be applied in rare sugar production.

KEYWORDS: L-rhamnose isomerase, rare sugar, D-allose, thermophilic enzyme, *Caldicellulosiruptor saccharolyticus* ATCC 43494, *E. coli*, overexpression

INTRODUCTION

L-Rhamnose isomerase (EC 5.3.1.14, L-RhI) catalyzes the reversible aldose−ketose isomerization between L-rhamnose and L-rhamnulose.[1] L-RhIs can also catalyze conversions between other aldoses and their corresponding ketoses, such as L-mannose and L-fructose, L-lyxose and L-xylulose, D-ribose and D-ribulose, and D-allose and D-psicose.[2] This capability has the potential for producing various rare sugars.[3,4] Several L-RhIs have been cloned and characterized, such as L-RhIs from *Bacillus halodurans* ATCC BAA-125, *Bacillus pallidus* Y25, *Escherichia coli* K12, *Pseudomonas stutzeri*, *Thermoanaerobacterium saccharolyticum* NTOU1, and *Thermotoga maritima* ATCC 43589.[5−10] The crystal structures of L-RhIs from *E. coli* and *P. stutzeri* have been investigated, and the catalytic mechanism of *P. stutzeri* L-RhI has also been revealed by inactive mutants and metal-bound structures.[11−13]

Rare sugars refer to monosaccharides that are rarely distributed in nature, such as D-allose and L-fructose.[14] Numerous studies reported the applications of rare sugars. For example, D-allose, a C3 epimer of D-glucose, is a nontoxic compound to rats.[15] D-Allose can suppress the growth of Ca9-22 and DU145 cancer cell lines[16,17] and confer rice plant resistance to the rice pathogen *Xanthomonas oryzae* pv *oryzae*.[18] Another rare sugar, L-fructose, has also been applied as a low-calorie sweetener, food preservative, and enzyme inhibitor.[19−21] Although rare sugars have been applied to many areas, the scarcity and high price of these sugars continue to limit the pursuit of wider applications and research. Therefore, an efficient way to produce rare sugars is desired.

Recently, many studies have been proposed to use enzymatic processes to produce rare sugars because the enzymatic processes have higher efficiency and stereoselectivity and can react under milder conditions than chemical processes. These processes include D-tagatose production using L-arabinose isomerase,[22] D-psicose production using tagatose 3-epimerase,[23] and D-allose production using L-RhI.[3] Sugar production using thermophilic enzymes has several advantages, such as higher reaction rate, lower viscosity in the reaction mixtures, and fewer risks of microbial contaminations.[24,25] There are four characterized thermostable L-RhIs, which are produced from *B. halodurans* ATCC BAA-125, *B. pallidus* Y25, *T. saccharolyticum* NTOU1, and *T. maritima* ATCC 43589, respectively.[5,6,9,10] However, their activities toward many substrates are lower than those of L-RhI from mesophile *P. stutzeri*.[8]

In this study, a thermostable and highly active L-RhI, which has the highest specific activities and catalytic efficiencies toward most tested substrates compared to those of other previously characterized L-RhIs, is reported. The *L-rhi* gene was PCR-cloned from the genomic DNA of *C. saccharolyticus* ATCC 43494 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.

MATERIALS AND METHODS

Materials. *C. saccharolyticus* ATCC 43494 (DSM 8903) was obtained from the American Type Culture Collection (Manassas, VA). *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

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purchased from Invitrogen Corp. (Carlsbad, CA). *T4* DNA ligase and restriction enzymes were supplied by Promega (Madison, WI). D-Allose, D-psicose, L-mannose, L-cysteine hydrochloride monohydrate, carbazole 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). D-Ribose was from Merck KGaA (Darmstadt, Germany). L-Rhamnose was from Fluka (Buchs SG, Switzerland). L-Lyxose was from MP Biomedicals (Solon, OH). Sephacryl S-200 HR and protein low molecular weight standards were from Amersham Pharmacia Biotech (Piscataway, NJ).

Amplification of the L-*rhi* Gene. The genomic DNA of *C. saccharolyticus* was extracted by using the DNAzol reagent according to the manufacturer's instructions. The L-*rhi* gene was amplified from the *C. saccharolyticus* genomic DNA by polymerase chain reaction (PCR). Two primers were designed on the basis of the L-*rhi* sequence of *C. saccharolyticus* (GenBank accession no. ABP66492). To clone the L-*rhi* gene into the pET-21b(+) vector, the *Nde*I and *Sal*I restriction sites were included in the forward and reverse primers, respectively.

The primer sequences are as follows: 5NdCSRhI (forward primer), 5′-CTT TAA GAA GGA GAT ATA **CAT ATG** GTT TTT GAT GGT GAG AA-3′; 3SalCSRhI (reverse primer), 5′-TGC GGC CGC AAG CTT **GTC GAC** TTA CAC CCT ATT TTT CAA AAC-3′, where the *Nde*I and *Sal*I restriction sites are in boldface. The reaction was carried out in 50 μL of reaction mixture containing *C. saccharolyticus* 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 *Sal*I. The digested fragment was inserted into the pET-21b(+) vector, resulting in a recombinant vector designated pET-21b-*Cs-rhi*. The sequence of the entire *Cs-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-*Cs-rhi* vector was transformed into *E. coli* BL21-CodonPlus (DE3)-RIL to express L-RhI. The culture conditions and induction of protein expression were as previously described.[9]

Purification of the Recombinant Enzyme. After cell harvest, the cells were disrupted by French press (Sim-Aminco, Rochester, NY), and the preparation of cell-free extract and the nucleic acid precipitation were as previously described.[9] Heat treatment was first used to precipitate most of the undesired proteins by incubating the cell-free extract in a 85 °C water bath for 30 min followed by centrifugation at 20000*g* for 30 min to remove the heat-labile proteins. The enzyme was then purified by Q-Sepharose ion-exchange chromatography as previously described.[9]

Enzyme Activity Assay. The L-RhI activity was assayed at 90 °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 a modified cysteine−carbazol method as previously described.[9] 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 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 the determination of optimal pH, the enzyme (74.2 ng/mL) was assayed under the standard conditions except at different pH values. For determination of the pH stability, the enzyme (7.42 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 the determination of optimal temperature, the enzyme (74.2 ng/mL) was assayed under the standard conditions except at different temperatures. For the determination of thermostability, the enzyme (6.79 μg/mL) was incubated at various temperatures at pH 7 and 1 mM Co^{2+} for 0−360 min, and the remaining activities were assayed after appropriate dilutions under the standard conditions.

Gel Filtration Chromatography Analysis of the Molecular Weight. The molecular weight was measured using a Sephacryl S-200 high-resolution column. The elution buffer contained 20 mM Tris−HCl (pH 7) and 0.2 M NaCl, and the flow rate was controlled at 0.5 mL/min. The molecular weight was determined by comparison with the calibration curve derived from the molecular weight standards.

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

Protein Concentration Measurement. Protein concentration was determined according to Bradford's method[26] 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 3 to 200 mM. The amounts of enzyme used were 0.268, 0.268, 0.447, 0.67, and 0.67 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.[27] 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-Psicose. The isomerization products of D-psicose 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 0−2 h in 50 mM potassium phosphate buffer (pH 7) containing 0.5 U/μL purified enzyme, 1 mM Co^{2+}, and 50 mM D-psicose. After reaction, each mixture was filtered through a Microcon centrifugal filter unit with a YM-3 membrane (MWCO 3000) to remove the enzyme. The filtrate was analyzed with a LiChroCART 250-4 NH_2 column (5 μm) (Merck KGaA, Darmstadt, Germany) with a mobile phase consisting of 25% (v/v) H_2O and 75% (v/v) acetonitrile.

RESULTS AND DISCUSSION

Comparison of Amino Acid Sequences of Several L-RhIs. *C. saccharolyticus* ATCC43494 L-RhI possesses 426 amino acids and a calculated molecular weight of 48294 Da, which was analyzed with the Compute pI/M_w tool at http://tw.expasy.ch/tools/pi_tool.html.[28] The amino acid sequence of L-RhI from *C. saccharolyticus* shares 69, 55, 52, 40, 19, and 12% identity with L-RhIs from *T. saccharolyticum*, *B. halodurans*, *E. coli*, *B. pallidus*, *T. maritima*, and *P. stutzeri*, respectively (Table 1). The crystal structure of *E. coli* L-RhI proposed that Val53, Leu63, Ile67, Ile105, Tyr106, and Phe336 can form a hydrophobic area to recognize the C-6 methyl group of L-rhamnose.[11] Except Leu63, the other five residues are conserved in L-RhI from *C. saccharolyticus*. Previously we reported that L-RhI from *T. saccharolyticum* has a substrate specificity similar to that of L-RhI from *E. coli* because they have the same active site and substrate recognition residues. These residues including Trp191, Glu232, Lys234, Asp265, His268, His292, Asp300, Asp302, and Asp332, which

Table 1. Comparison of the Deduced Amino Acid Sequence of Recombinant L-RhI from *C. saccharolyticus* ATCC 43494 with Those of L-RhIs from Other Microorganisms

source	accession no.[a]	no. of residues	identity[b] (%)
Caldicellulosiruptor saccharolyticus ATCC 43494	A4XHV7	426	100
Thermoanaerobacterium saccharolyticum NTOU1	D5LNE5	425	69
Bacillus halodurans ATCC BAA-125	Q9KCL9	418	55
Escherichia coli K12	P32170	419	52
Bacillus pallidus Y25	A8CEF6	412	40
Thermotoga maritima MSB8	Q9X0G0	383	19
Pseudomonas stutzeri	Q75WH8	430	12

[a] The deduced amino acid sequences were obtained from UniProt protein sequence database.[31]. [b] The identity values were analyzed by ClustalW program on the website http://www.ebi.ac.uk/Tools/msa/clustalw2/.[32]

Table 2. Summary of the Purification Steps of Recombinant L-RhI from *C. saccharolyticus* ATCC 43494 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[a]	1720	25400	100	14.8	1
heat treatment[b]	156	40900	161	262	17.7
Q-Sepharose	69	26200	103	379	25.6

[a] 8.44 g of frozen cells was suspended in 25 mL of lysis buffer. [b] The crude extract was heated at 85 °C for 0.5 h and then centrifuged to remove the precipitates.

are in *T. saccharolyticum* L-RhI numbering, are all conserved in L-RhI from *C. saccharolyticus*, suggesting that L-RhI from *C. saccharolyticus* might have enzymatic properties similar to those of L-RhI from *T. saccharolyticum*.

Expression and Purification of the Recombinant L-RhI. The protein expression levels of the recombinant L-RhI were similar under 0−0.5 mM IPTG induction. The yields of active L-RhI expressed in *E. coli* were 38000 U/L and 3010 U/g of wet cells, which were higher than the previously reported yields of 36500, 20000, and 2400 U/L for the *B. pallidus*, *P. stutzeri*, and *E. coli* L-RhIs expressed in *E. coli*, respectively.[6−8] The L-RhI was purified from the cell-free extract of *E. coli* sequentially by heat treatment, nucleic acid precipitation, and Q-Sepharose ion-exchange chromatography (Table 2). After analysis by gel electrophoresis, the expressed L-RhI was mostly soluble and appeared in the cell-free extract (Figure 1, lanes 1−3). Heat treatment was noted to effectively remove most of the undesired proteins (Figure 1, lane 4). Surprisingly, the total activity increased 61% after heat treatment (Table 2). This may due to the interference of the other heat-labile enzymes in the crude extract or due to the heat activation after heat treatment, which has also been proposed in some enzymes from hyperthermophilic bacteria.[29] After ion-exchange chromatography, the purified L-RhI showed a single band on SDS-PAGE, indicating that a high purity of protein was obtained. The apparent molecular mass of the L-RhI under

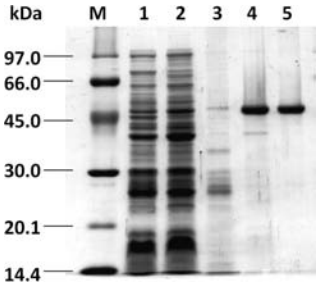

Figure 1. SDS-PAGE analysis of purifications of L-RhI from *C. saccharolyticus* ATCC 43494 expressed in *E. coli* BL21-CodonPlus (DE3)-RIL carrying pET-21b-*Cs-rhi*. Lanes: M, molecular weight standards; 1, total cellular proteins of the *E. coli* cells carrying pET-21b-*Cs-rhi*; 2, crude cell-free extracts 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 ion-exchange chromatography.

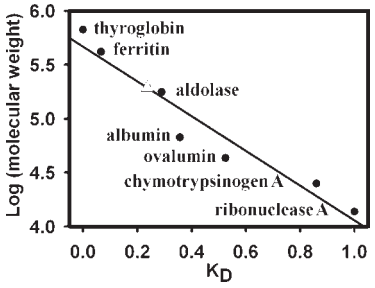

Figure 2. Determination of the molecular weight of L-RhI from *C. saccharolyticus* ATCC 43494 by gel filtration chromatography. The protein standards (●) included thyroglobin (669 kDa), ferritin (416 kDa), aldolase (176 kDa), albumin (67 kDa), ovalumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa). *C. saccharolyticus* L-RhI (△) was plotted with the protein standards. The K_D values were calculated using the equation $K_D = (V_e - V_0)/(V_T - V_0)$, where V_e is the elution volume, and V_T and V_0 represent the total liquid volume and void volume of the column, respectively.

denaturing conditions by SDS-PAGE was around 48 kDa, which is in good agreement with the calculated molecular mass (Figure 1, lane 5). The molecular mass of purified L-RhI under nondenaturing conditions was then analyzed by gel filtration chromatography. The purified L-RhI had a peak at 93.97 min (data not shown). By comparison with the calibration curve derived from the molecular mass standards, the molecular mass of the L-RhI under nondenaturing conditions was around 193.4 kDa (Figure 2). Because the theoretical molecular mass of one subunit of the L-RhI is around 48.3 kDa, the estimated 193.4 kDa corresponded to four subunits. These results suggest that the purified L-RhI is a homotetramer, which is the same with L-RhIs from *E. coli*, *P. stutzeri*, and *T. maritima*.[10−12] The molecular weight of the purified L-RhI was also analyzed by MALDI-TOF mass spectrometry. It showed a molecular mass of 48292 Da, which is in good agreement with the calculated molecular weight (48294 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 6 or increased to above 8 (Figure 3a). To investigate the effect of pH on enzyme stability, the purified enzyme was incubated at various pH values at room temperature for 24 h. The results showed that the enzyme

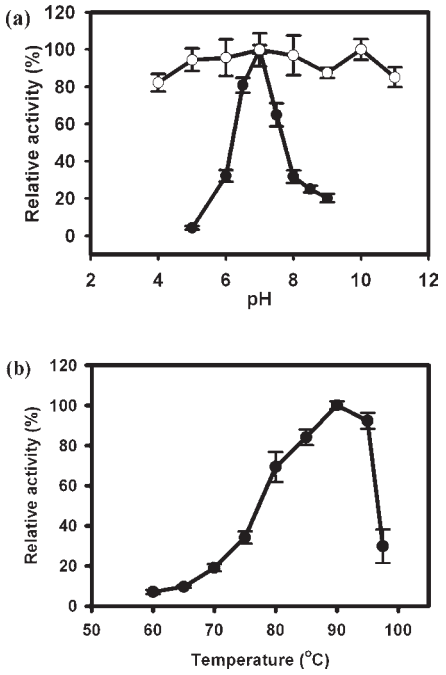

Figure 3. Effects of pH and temperature on the activity (●) and stability (○) of *C. saccharolyticus* L-RhI. (a) Effects of pH. For the determination of optimal pH, the enzyme activity was assayed at 90 °C and different pH values. For the determination of pH stability, the enzyme was incubated at various pH values at room temperature for 24 h, and the remaining activities were assayed at 90 °C and pH 7. (b) Effects of temperature on activity. The enzyme activity was assayed at pH 7 and different temperatures. Data represent the mean ± SD from triplicate experiments.

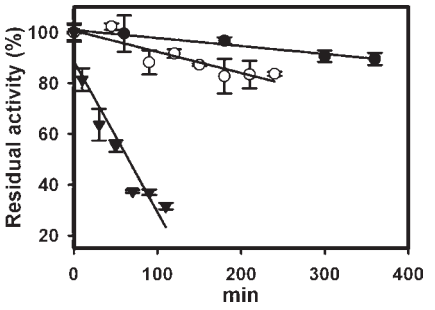

Figure 4. Thermostabilities of *C. saccharolyticus* L-RhI at 80 °C (●), 85 °C (○), and 90 °C (▼) in the presence of 1 mM Co^{2+} at pH 7. Data represent the mean ± SD from triplicate experiments.

Table 3. Specific Activities (U/mg) of Some Recombinant L-RhIs for Various Aldoses

substrate	*C. saccharolyticus*[a]	*T. saccharolyticum*[b]	*B. pallidus*[c]	*T. maritima*[d]	*P. stutzeri*[e]
L-rhamnose	380	203	77.2	55	244
L-lyxose	65	130	19.3	20	110
L-mannose	38	6.0	4.5	15	81.6
D-allose	21	5.7	2.6	6.7	7.5
D-ribose	4.9	1.6	6.3	2.8	16

[a] This study. Experiments were carried out at 90 °C and pH 7 in the presence of 1 mM Co^{2+}. [b] Lin et al.[9] Experiments were carried out at 65 °C in the presence of 1 mM Co^{2+}. [c] Poonperm et al.[6] Experiments were carried out at 65 °C in the presence of 1 mM Mn^{2+}. [d] Park et al.[10] Experiments were carried out at 85 °C. [e] Leang et al.[8] Experiments were carried out at 50 °C in the presence of 1 mM Mn^{2+}.

remained stable with >80% remaining activity in the pH range from 4 to 11 (Figure 3a). The enzyme was further incubated at 80 °C for 6 h in the pH range from 4 to 9 to determine the effect of pH on thermostability. The enzyme activity was fully retained at pH 7−8, 32% remaining at pH 9, but became inactivated in the pH range from 4 to 6. The results indicate that the enzyme was stable after a 6 h incubation at 80 °Cand pH 7−8. When the enzyme activity was measured at different temperatures, the activity increased with increasing temperature and reached an optimal activity at 90 °C (Figure 3b). The data of Figure 3b 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 98.0 kJ mol^{-1} at pH 7. The enzyme was stable at 80 °C for 6 h and 85 °C for 4 h. However, the enzyme activity dropped sharply to 30% after 2 h at 90 °C, and the half-life at 90 °C was around 65 min (Figure 4). The good thermostability of this enzyme indicated that the purified recombinant L-RhI might be well folded. The data of Figure 4 were replotted on a semilogarithmic scale as ln(residual activity) versus T (h) to determine the inactivation rate coefficients (k), and then the activation energy for enzyme inactivation was obtained by the plot of ln(k) versus $1/T$. The activation energy for enzyme inactivation was 367 kJ mol^{-1} at pH 7.

Effect of Metal Ions on the Activity of the Recombinant L-RhI. The effects of 1 mM Co^{2+}, Mn^{2+}, Na^+, Mg^{2+}, Ca^{2+}, Cu^{2+}, K^+, and Zn^{2+} on the activity of L-RhI were investigated. In previous findings, the enzyme activities ofL-RhIs from many sources were enhanced in the presence of metal ions.[5,6,8−11] L-RhI from *C. saccharolyticus* showed maximum activity in the presence of Co^{2+}, 58% of the maximum activity in the presence of Mn^{2+}; however, it showed null activity in the presence of Na^+, Mg^{2+}, Ca^{2+}, Cu^{2+}, K^+, and Zn^{2+} or in the absence of any ions. These results suggested that metal ions, especially Co^{2+} and Mn^{2+}, are required for the *C. saccharolyticus* L-RhI activity.

Substrate Specificity and Enzyme Kinetics. L-Rhamnose, L-lyxose, L-mannose, D-allose, and D-ribose were used to study the substrate specificity and enzyme kinetics of the recombinant *C. saccharolyticus* L-RhI (Tables 3 and 4). *C. saccharolyticus* L-RhI shows the highest specific activity toward L-rhamnose and a moderate specific activity toward L-lyxose and L-mannose, but a very minimal activity toward D-ribose. This substrate specificity is similar to those of other L-RhIs. Among all of the characterized L-RhIs, *C. saccharolyticus* L-RhI shows the highest specific activity toward L-rhamnose and D-allose. Besides, the specific activities against all tested sugars are almost higher than those of other thermostable L-RhIs, such as L-RhIs from *T. saccharolyticum*, *B. pallidus*, and *T. maritima* (Table 3).[5,6,8−10] The kinetic parameters (k_{cat} and K_M) of L-RhI from *C. saccharolyticus* are given in Table 4. The k_{cat} values of *C. saccharolyticus* L-RhI against L-rhamnose, L-lyxose, and L-mannose are 99.9, 186, and 78.7 s^{-1}, respectively, which are almost lower than those of all previously characterized L-RhIs. The k_{cat} values of *C. saccharolyticus* L-RhI against D-allose and D-ribose are 68.1 and 118 s^{-1}, respectively, which are higher than those of the previously characterized L-RhIs. Notably, the K_M values toward all tested sugars are lower than those of the other characterized L-RhIs, so that the catalytic

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Table 4. Comparison of Kinetic Parameters of the Recombinant L-RhIs from *P. stutzeri*, *B. pallidus*, *T. maritima*, *B. halodurans*, *T. saccharolyticum*, and *C. saccharolyticus* for Isomerization of L-Rhamnose, L-Lyxose, L-Mannose, D-Allose, and D-Ribose, Respectively

	L-rhamnose	L-lyxose	L-mannose	D-allose	D-ribose
C. saccharolyticus[a]					
k_{cat} (s^{-1})	99.9 ± 2.4[f]	186 ± 3	78.7 ± 0.3	68.1 ± 0.5	118 ± 2
K_M (mM)	1.03 ± 0.20	3.65 ± 0.25	3.64 ± 0.08	14.3 ± 0.3	33.5 ± 1.3
k_{cat}/K_M (mM^{-1} s^{-1})	97.1 ± 17.3	50.8 ± 2.7	21.6 ± 0.4	4.77 ± 0.08	3.54 ± 0.09
T. saccharolyticum[b]					
k_{cat} (s^{-1})	180	240	30.0	33.9	21.5
K_M (mM)	3.53	45.2	58.9	121	148
k_{cat}/K_M (mM^{-1} s^{-1})	50.8	5.30	0.51	0.28	0.15
P. stutzeri[c]					
k_{cat} (s^{-1})	172	100	89.7	5.02	15.8
K_M (mM)	11.9	55.5	61.7	42.0	38.5
k_{cat}/K_M (mM^{-1} s^{-1})	14.4	1.81	1.45	0.12	0.41
B. pallidus[c]					
k_{cat} (s^{-1})	68	31	76.8	34.5	14.2
K_M (mM)	4.89	16.1	28.9	41.8	34.9
k_{cat}/K_M (mM^{-1} s^{-1})	13.9	1.93	2.66	0.83	0.41
T. maritima[d]					
k_{cat} (s^{-1})	146	462	442		
K_M (mM)	37	69	76		
k_{cat}/K_M (mM^{-1} s^{-1})	3.95	6.7	5.82		
B. halodurans[e]					
k_{cat} (s^{-1})	150	216	88.9		
K_M (mM)	528	771	119		
k_{cat}/K_M (mM^{-1} s^{-1})	0.28	0.28	0.74		

[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-RhI from *T. saccharolyticum* were from Lin et al.[9] Experiments were carried out at 65 °C and pH 7 in the presence of 1 mM Co^{2+}. [c] The kinetic parameters of L-RhIs from *B. pallidus* and *P. stutzeri* were recalculated from the data reported by Poonperm et al.[6] Experiments were carried out at 65 and 37 °C, respectively, in the presence of 1 mM Mn^{2+}. [d] The kinetic parameters of L-RhI from *T. maritima* were recalculated from the data reported by Park et al.[10] Experiments were carried out at 85 °C and pH 8. [e] The kinetic parameters of L-RhI from *B. halodurans* were recalculated from the data reported by Prabhu et al.[5] Experiments were carried out at 70 °C and pH 7 in the presence of 1 mM Mn^{2+}. [f] Standard error from the curve fitting.

efficiencies (k_{cat}/K_M) of *C. saccharolyticus* L-RhI against all tested sugars are higher than those of the previously characterized L-RhIs (Table 4). Like the L-RhI from *T. saccharolyticum*, the K_M values of *C. saccharolyticus* L-RhI toward D-allose and D-ribose are much higher than those of L-form substrates, indicating that *C. saccharolyticus* L-RhI also exhibits lower affinities toward D-form sugar substrates.[9] Among the five tested substrates, *C. saccharolyticus* L-RhI has the lowest K_M value toward L-rhamnose. Because L-RhIs from *E. coli* and *C. saccharolyticus* have almost the same substrate-recognizing residues, which had been proposed to form a hydrophobic area to recognize the methyl group of L-rhamnose in *E. coli* L-RhI,[11] the results suggest that *C. saccharolyticus* L-RhI might have a similar hydrophobic area to increase the affinity to L-rhamnose.

Conversion of D-Psicose to D-Allose. To further examine the isomerization products and equilibrium ratio of the L-RhI from *C. saccharolyticus* against D-psicose, the enzyme reaction was carried out at 65 °C for 0−2 h. As shown in Figure 5, *C. saccharolyticus* L-RhI isomerizes D-psicose to D-allose without forming any detectable byproducts after 2 h. The lack of a byproduct in the sugar conversion catalyzed by *C. saccharolyticus* L-RhI would be industrially welcome for further downstream purification processes. Around 28% of D-allose was formed after 0.25 h, and around 33% of D-allose was formed when the reaction was approaching equilibrium (0.5 h). To clarify the equilibrium ratio within different L-RhIs, we further examined the equilibrium ratio of the *C. saccharolyticus* L-RhI against L-rhamnose at 65 and 90 °C. Around 51 and 65% of L-rhamnulose were formed when the reactions were approaching equilibrium at 65 and 90 °C, respectively. The equilibrium ketose ratios of *C. saccharolyticus*

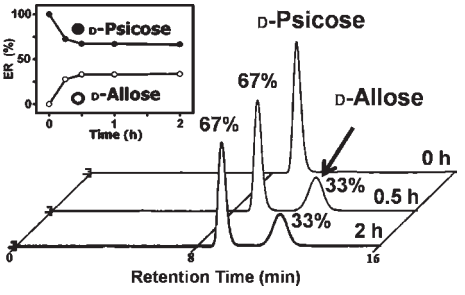

Figure 5. HPLC analysis of the isomerization products of *C. saccharolyticus* L-RhI against D-psicose. 0 h indicates 50 mM D-psicose containing enzyme inactivated by boiling; 0.5 and 2 h indicate enzyme reaction after 0.5 and 2 h, respectively. (Inset) Equilibrium ratio (ER) between D-psicose and D-allose. These ratios are molar ratios calculated from the standard curves of D-psicose and D-allose.

Table 5. Comparison of Some Enzymatic Properties of Characterized Thermostable L-RhIs

property	*C. saccharolyticus*[a]	*T. saccharolyticum*[b]	*B. pallidus*[c]	*B. halodurans*[d]	*T. maritima*[e]
optimum temperature (°C)	90	75	65	70	85
thermostability (°C)	80, 6 h (90%)	70, 2 h (90%)	65, 1 h (50%)	60, 15 h (90%)	95, 65 h (50%)
optimum pH	7.0	7.0	7.0	7.0	8.0
pH stability	4−11	5−9	7−10	6−10	NR[f]
cofactor	Mn^{2+}, Co^{2+}	Mn^{2+}, Co^{2+}	Mn^{2+}, Co^{2+}	Mg^{2+}, Mn^{2+}, Co^{2+}	Mn^{2+}
molecular weight of monomer	48294	48961	47636	48178	45527
quaternary structure	tetramer	NR	NR	dimer	tetramer

[a] This study. [b] Lin et al.[9]. [c] Poonperm et al.[6]. [d] Prabhu et al.[5]. [e] Park et al.[10]. [f] NR, Not recorded in the published literature.

L-RhI against L-rhamnose and D-allose at 65 °C were 51 and 67%, respectively, which were higher than those from *B. pallidus* carried out at 50 °C. The results suggest that the different equilibrium ratios might be due to different reaction temperatures, according to the Le Chatelier principle.[30]

Table 5 lists a further comparison of some enzymatic properties of the characterized thermostable L-RhIs. In general, the molecular weights of the monomers of these denatured L-RhIs are between 45 and 49 kDa, whereas the native quaternary structures are dimer or tetramer, and the presence of Mn^{2+} can increase the enzyme activity. In addition, L-RhI from *B. halodurans*, *T. saccharolyticum*, and *C. saccharolyticus* present wide pH stability.

In this study, *C. saccharolyticus* 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 L-RhIs from other bacteria. One of the important applications of L-RhI is to convert D-psicose to D-allose. However, this enzyme reaction catalyzed by *P. stutzeri* L-RhI resulted in the D-altrose byproduct.[3] No byproduct in the conversion catalyzed by *C. saccharolyticus* L-RhI would be more industrially welcome. In addition, *C. saccharolyticus* L-RhI has a good thermostability, highest activity against L-rhamnose and D-allose, highest catalytic efficiencies, and widest pH stable range among all characterized L-RhIs, suggesting that this L-RhI can be used to produce rare sugars, especially D-allose at high temperatures.

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ABBREVIATIONS USED

L-RhI, L-rhamnose isomerase; IPTG, isopropyl-β-D-thiogalactoside; LT-ELSD, low-temperature evaporative light scattering detection.

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