



CHARACTERIZATION OF A RECOMBINANT L-RIBOSE ISOMERASE FROM GEODERMATOPHILUS OBSCURUS DSM 43160 AND APPLICATION OF THIS ENZYME TO THE PRODUCTION OF L-RIBOSE FROM L-ARABINOSE

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Key words: L-ribose isomerase, L-ribulose, L-arabinose isomerase, *Geodermatophilus obscurus*.

ABSTRACT

L-Ribose isomerase (L-RI) catalyzes the aldose-ketose isomerization between L-ribose and L-ribulose. In this study, a putative L-RI gene of *Geodermatophilus obscurus* DSM 43160 was cloned by PCR into pET-15b and pET-21b, respectively. The cloned target gene was expressed in *Escherichia coli*. The recombinant N-His-tagged and C-His-tagged proteins exhibited L-RI activity. Both N- and C-His-tagged L-RIs were purified from cell-free extracts by metal-affinity and ion-exchange chromatography. The purified N-His-tagged L-RI demonstrated its optimal activity at 30-40°C and pH of 9 (in glycine-NaOH buffer). The enzyme was stable at pH 7-9 and more than 90% activity was retained after incubation at 40°C for 2 h. Metal ions were not required for N-His-tagged L-RI activity to occur, but Hg²⁺ inhibited its activity completely. The conversion rates of L-arabinose to L-ribose by combining *Thermoanaerobacterium saccharolyticum* NTOU1 L-arabinose isomerase and *G. obscurus* DSM43160 N-His-tagged L-RI at 30°C and 40°C were 15.9% and 12.5% (mol mol⁻¹), respectively. Results obtained from this study suggest a potential application of this recombinant L-RI for the L-ribose production from L-arabinose.

I. INTRODUCTION

Rare sugars are monosaccharides that are not often found in nature, and have been used as food additives, antiviral and

anthelmintic agents, and cancer cell suppressors, in addition to numerous other applications (Choo et al., 2006; Naha et al., 2008; Sato et al., 2008; Milgrom et al., 2009; Mitani et al., 2009). For example, L-ribose can be used as a precursor for the synthesis of L-nucleoside analogs, which are widely used as antiviral and anticancer drugs (Graciet et al., 1999; Gumina et al., 2002; Okano, 2009). D-Tagatose is a tooth-friendly ingredient (Oh, 2007) that can reduce symptoms associated with type 2 diabetes, hyperglycemia, anemia, and hemophilia (Saunders et al., 1999). Another rare sugar, D-allose, suppresses the growth of Ca9-22 and DU145 cancer cell lines (Naha et al., 2008; Mitani et al., 2009), and confers rice plant resistance to the pathogen *Xanthomonas oryzae* pv. *oryzae* (Kano et al., 2010).

Although rare sugars have been applied in numerous areas, the scarcity and high price of these sugars continue to limit the pursuit of a wider range of applications and research. Therefore, efficient methods for producing rare sugars are necessary. Methods for the production of various rare sugars by using chemical synthesis have been developed (Yun et al., 2005; Okano, 2009). However, the use of enzymatic processes for the production of rare sugars is preferred because it offers high efficiency and stereoselectivity (Giffhorn et al., 2000; Granström et al., 2004; Helanto et al., 2009; Oh, 2007; Rhimi and Bejar 2006). Enzymatic processes have been used to produce D-tagatose and L-ribulose using L-arabinose isomerase (L-AI) (Yeom et al., 2008; Rhimi et al., 2009), D-allose using L-rhamnose isomerase (Menavuvu et al., 2006; Lin et al., 2010; Lin et al., 2011), and L-ribose using L-ribose isomerase (L-RI) (Helanto et al., 2009) and mannose-6-phosphate isomerase (Yeom et al., 2009a).

L-RI (EC 5.3.1.B3) catalyzes the reversible aldose-ketose isomerization between L-ribose and L-ribulose (Shimonishi and Izumori 1996). L-RIs can also catalyze conversions between other aldoses and their corresponding ketoses, such as D-mannose and D-fructose, D-lyxose and D-xylulose, and L-gulose and L-sorbose (Mizanur et al., 2001; Morimoto et al.,

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2013). This capability can potentially be used for producing various rare sugars. Only two L -RIs have been cloned and characterized: L -RIs from *Acinetobacter* sp. DL-28 and *Cellulomonas parahominis* MB426 (Mizanur et al., 2001; Morimoto et al., 2013). The amino acid sequence of L -RI derived from *C. parahominis* MB426 exhibited high homology with those of L -RI from *Acinetobacter* sp. DL-28 and a hypothetical protein from *Geodermatophilus obscurus* DSM 43160 (Morimoto et al., 2013). The crystal structure of L -RI still has not been resolved, and only the crystallization and preliminary X-ray diffraction analysis of L -RI from *Acinetobacter* sp. DL-28 have been reported (Yoshida et al., 2011).

The most important application of L -RI is the conversion reaction of L -ribulose to L -ribose. However, L -ribulose is expensive. Since L -AI mainly catalyzes the reversible isomerization between L -arabinose and L -ribulose (Kim, 2004), the production of L -ribose can start with the inexpensive L -arabinose, a common component of polymers of lignocellulosic biomass, by using L -AI to obtain L -ribulose first. The biotechnological production of L -ribose from L -arabinose was performed in resting bacterial cells containing endogenous L -AI and *Acinetobacter* sp. DL-28 L -RI or by using their cell-lysate precipitates (Helanto et al., 2009). Although the production of L -ribose from L -arabinose using purified L -AI and mannose-6-phosphate isomerase was reported (Yeom et al., 2009b), the usage of purified L -AI and L -RI for the same application has not been studied.

In this paper, a third L -ri gene was cloned from *G. obscurus* DSM46160 and characterization of the recombinant L -RI was reported. The L -ri gene was PCR-cloned from the genomic DNA of *G. obscurus* DSM 43160 to an expression vector and overexpressed in *Escherichia coli*. In addition to characterizing the general properties of this recombinant L -RI, the production of L -ribose from L -arabinose by using purified L -RI from *G. obscurus* DSM 43160 and *Thermoanaerobacterium saccharolyticum* NTOU1 L -AI from our previous study (Hung et al., 2014) was investigated.

II. MATERIALS AND METHODS

1. Materials

G. obscurus DSM4316 was obtained from the Bioresource Collection and Research Center of the Food Industry Research and Development Institute (Hsinchu, Taiwan). *E. coli* DH5 α , *E. coli* BL21-CodonPlus (DE3)-RIL, and *PfuTurbo* DNA polymerase were obtained from Stratagene (La Jolla, CA). Plasmid pET-15b(+) and pET-21b(+) were obtained from Novagen (Madison, WI). DNazol reagent and NuPAGE 3-8% Tris-acetate gel (1.0 mm \times 10 wells) were purchased from Invitrogen Corp. (Carlsbad, CA). *T4* DNA ligase and restriction enzymes were supplied by Promega Corp. (Madison, WI). L -Ribose was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). L -Ribulose was acquired from Carbo-synth Ltd. (Berkshire, UK). L -Arabinose, L -cysteine hydrochloride monohydrate, carbazole crystalline, acrylamide, bo-

vine serum albumin (BSA), and isopropyl- β -d-thiogalactoside (IPTG) were obtained from Sigma-Aldrich (St. Louis, MO). A Microcon YM-3 centrifugal filter unit was acquired from Millipore Corp. (Bedford, MA). Low molecular weight standards, and HisTrap HP and HiPrep Q FF columns, were supplied by GE Healthcare Life Sciences (Piscataway, NJ). *T. saccharolyticum* NTOU1 L -arabinose isomerase (L -AI) was prepared as previously described (Hung et al., 2014).

2. Amplification of the Putative L -ri Gene and Construction of the Expression Vector

The genomic DNA of *G. obscurus* was extracted using DNazol reagent according to the manufacturer instructions. Two primers were designed based on the nucleotide sequence of a hypothetical protein from *G. obscurus* (GenBank accession no. CP001867, REGION: 2752243...2753004). To clone the putative L -ri gene into the pET-15b(+) and pET-21b(+) vectors, respectively, the *Nde*I and *Xho*I restriction sites were included in the forward and reverse primers, respectively.

The primer sequences are listed as follows: 5GORI (forward primer), 5'-GAA GGA GAT ATA **CAT ATG** GAG GGT CTC TTG GCA AG -3'; 3NGORI (reverse primer, for cloning the gene into pET-15b), 5'-GTG GTG GTG GTG **CTC GAG** CTA CTG GAT GGC GGT GCG CAA C -3'; 3CGORI (reverse primer, for cloning the gene into pET-21b), 5'-GTG GTG GTG GTG **CTC GAG** ACC CTG GAT GGC GGT GCG CAA C -3'; the *Nde*I and *Xho*I restriction sites are in boldface type. The reaction was performed in 50 μ L of reaction mixture containing *G. obscurus* genomic DNA, forward primer and primers, dNTP, *PfuTurbo* DNA polymerase, and *PfuTurbo* DNA polymerase buffer, and was performed using a TGradient PCR system (Biometra, Goettingen, Germany) in the following sequence: 95°C for 5 min, amplification, and a final extension at 68°C for 10 min. The amplification profiles were acquired using 34 cycles under the following conditions in sequence: 1 min at 95°C, 1 min at 55°C, and 2 min at 68°C.

The 0.8 kb PCR-amplified fragments were purified and then digested using *Nde*I and *Xho*I. The digested fragments were inserted into the pET-15b and pET-21b vectors, respectively, resulting in recombinant vectors designated as pET-15b-*Go-ri-NHis* and pET-21b-*Go-ri-CHis*, respectively. The amplified putative L -ri gene was fused in frame with the His-tag coding sequence on the pET-15b and pET-21b vectors; thus, the expressed recombinant proteins have a His-tag on the N-terminal or C-terminal region. The sequences of the entire L -ri gene and the fused His-tag coding region on pET-15b-*Go-ri-NHis* and pET-21b-*Go-ri-CHis* were confirmed by DNA sequencing, which was performed by Mission Biotech Corp. (Taipei, Taiwan).

3. Expressions and Purifications of the Recombinant Proteins by *E. coli*

The pET-15b-*Go-ri-NHis* and pET-21b-*Go-ri-CHis* vectors were both transformed into *E. coli* BL21-CodonPlus (DE3)-RIL to express the recombinant proteins. The culture conditions

and inductions of protein expressions were as previously described (Hung et al., 2014). Frozen cells weighing 4.05 and 4.82 g expressing the recombinant N-His-tagged and C-His-tagged L -RIs, respectively, were suspended in 12.2 and 14.5 mL, respectively, of lysis buffer containing 20 mM Tris-HCl (pH 7.9), 5 mM imidazole, 0.5 M NaCl, and 1 mM benzimidazole. The suspended cells were disrupted using a French Press disruptor (Sim-Aminco, Rochester, NY) and cell-free extracts were then prepared as previously described (Hung et al., 2014).

The recombinant proteins were purified using the AK-TAprime plus chromatography system (GE Healthcare Life Sciences, Piscataway, NJ). The cell-free extract was loaded onto a HisTrap HP column (5 mL), which was previously equilibrated with binding buffer (30 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.4). The column was first washed with the binding buffer, then with a wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.4), and finally with a linear gradient of 60-1000 mM imidazole in 20 mM Tris-HCl (pH 7.4) and 0.5 M NaCl to elute the bound proteins. The eluted fractions containing L -RI activity were collected and dialyzed against 20 mM Tris-HCl buffer (pH 7), and were subsequently loaded onto a HiPrep QFF column (1.6 × 10 cm), which was preequilibrated with the same dialysis buffer. The column was first washed thoroughly with the same buffer until the absorbance of 280 nm reached the baseline. Finally, a linear gradient of 0-1.4 M NaCl in the aforementioned buffer was used to elute the bound proteins. The eluted fractions containing L -RI activity were collected and dialyzed against 20 mM Tris-HCl (pH 7). Protein concentration was determined according to Bradford's method (Bradford, 1976) and using BSA as the standard.

4. Enzyme Activity Assay and Determining the Effects of pH and Temperature on Enzyme Activity and Stability

L -RI activity was assayed at 30°C for 5 min using 0.8 mM L -ribulose as the substrate in 50 mM glycine-NaOH buffer (pH 9). The amount of unreacted L -ribulose in the reaction mixture was determined using a modified cysteine-carbazol method as previously described (Dische and Borenfreund 1951; Lin et al., 2010). One unit (U) of L -RI activity was defined as the amount of enzyme required to convert 1 μ mol of substrate to product in 1 min. In determining the effects of pH on enzyme activity and stability, the following buffers with a final concentration of 50 mM were used in various pH ranges: citrate phosphate buffer (pH 5-6.5), Tricine-HCl buffer (pH 7-9), Tris-HCl buffer (pH 7-9) and glycine-NaOH buffer (pH 9-11). For the determination of optimal pH, the enzymes (0.019 mg/mL) were assayed under the standard conditions except at various pH values. For the determination of pH stability, the enzymes (0.386 mg/mL) were incubated at various pH values at 4°C for 24 h, and the remaining activities were assayed after performing appropriate dilutions under the standard conditions. For the determination of optimal temperature, the enzymes (0.019 mg/mL) were assayed under the standard

conditions except at various temperatures. For the determination of thermostability, the enzymes (0.386 mg/mL) were incubated at various temperatures for 2 h, and the remaining activities were assayed after performing appropriate dilutions under the standard conditions.

5. Polyacrylamide Gel Electrophoresis

Nondenaturing electrophoresis (Native-PAGE) was performed using NuPAGE 3-8% Tris-acetate gel (1.0 mm × 10 wells) according to the manufacturer instructions. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, with 5% acrylamide as stacking gel and 12% acrylamide as running gel) was performed using the procedure described by Garfin (2009). The protein bands were stained with Coomassie Brilliant Blue R-250.

6. Molecular Weight Determination of L -RI

The molecular weights of the enzymes were estimated using a BioSep-SEC-S2000 column (300 × 7.8 mm), which is a silica-based gel filtration column. The mobile phase contained 20 mM Tris-HCl (pH 7.5) and 0.2 M NaCl, and the flow rate was controlled at 0.5 mL/min. The analysis was executed using a Hitachi High-Performance Liquid Chromatograph (HPLC) L-7000 (Tokyo, Japan) equipped with a UV detector. Mass spectrometric analysis was performed using a Microflex (Bruker Daltonics, Bremen, Germany) MALDI-TOF mass spectrometer, and was conducted by Mission Biotech Corp. (Taipei, Taiwan).

7. Enzyme Kinetics

The initial rates of conversion of L -ribose were determined at 30°C in 50 mM glycine-NaOH buffer at pH 9 using 8.3 μ g/mL of the purified enzyme at 8-10 substrate concentrations ranging from 5 to 600 mM. Samples taken at 6 time intervals were stopped by adding 600 μ L of 70% sulfuric acid into 100 μ L of reaction mixture. The amount of L -ribulose was determined using the cysteine-carbazol method (Dische and Borenfreund 1951; Lin et al., 2010). The values of k_{cat} and K_M were calculated by fitting the initial rates as a function of the substrate concentration to the Michaelis-Menten equation using SigmaPlot (Systat Software, San Jose, CA).

8. HPLC Analysis of the Production of L -Ribose and L -Ribulose

The isomerization products were measured using a Hitachi HPLC L-7000 (Tokyo, Japan) equipped with a Low-Temperature Evaporative Light-Scattering Detector (Sedex, Alfortville, France). The enzyme reaction was first performed at 60°C for 6 h in 50 mM glycine-NaOH buffer (pH 9) containing *T. saccharolyticum* NTOU1 L -AI (0.6 U/mL), L -arabinose (50 g/L), 0.1 mM Mn^{2+} , and 0.05 mM Co^{2+} . After 6 h of reaction, *G. obscurus* DSM4316 N-His-tagged L -RI (0.56 U/mL) was added to the reaction mixture; the reaction was continued at 30°C or 40°C for an additional 6 h. The reaction was stopped by incubating the mixture in boiling

Table 1. Comparison of the deduced amino acid sequence of putative L -RI from *G. obscurus* DSM 43160 with those of L -RIs from other microorganisms.

| source | accession no. ^a | no. of residues | identity ^b (%) |
|--------------------------------|----------------------------|-----------------|---------------------------|
| <i>G. obscurus</i> DSM 43160 | D2S5K0 | 253 | 100 |
| <i>Acinetobacter</i> sp. DL-28 | Q93UQ5 | 249 | 78 |
| <i>C. parahominis</i> MB426 | L0N3Y0 | 249 | 82 |

^aThe deduced amino acid sequences were obtained from UniProt Knowledgebase (<http://www.uniprot.org/uniprot/>).

^bThe identity values were analyzed by ClustalW program on the website <http://www.ebi.ac.uk/Tools/msa/clustalw2/> (Chenna et al., 2003).

water for 10 min; the mixtures were then filtered through a Microcon centrifugal filter unit with a YM-3 membrane to remove the enzymes. The filtrate was analyzed using a Shodex SUGAR SC1011 column (8 × 300 mm) with deionized H₂O (0.6 mL/min) as the mobile phase.

III. RESULTS AND DISCUSSION

1. Protein Homology Analyses among the Putative *G. obscurus* L -RI and Other L -RIs

The hypothetical protein from *G. obscurus* DSM 43160 possessed 253 amino acids and a calculated molecular weight of 28181 Da, which was analyzed using the Compute pI/Mw tool available at http://tw.expasy.ch/tools/pi_tool.html (Wilkins et al., 1999). The deduced amino acid sequence of the hypothetical protein from *G. obscurus* DSM 43160 shared 72% and 78% identities with L -RIs from *Acinetobacter* sp. strain DL-28 and *C. parahominis* MB426, respectively (Table 1). This high percentage of shared identities suggests that the hypothetical protein from *G. obscurus* DSM 43160 was an L -RI.

2. Expression and Purification of the Recombinant N-His-tagged and C-His-tagged L -RIs

The expressed recombinant N-His-tagged and C-His-tagged proteins demonstrated L -RI activity, and the expression levels of N-His-tagged and C-His-tagged L -RIs were higher in the presence of 0.2 and 0.1 mM IPTG, respectively (data not shown). The yield of active N-His-tagged L -RI expressed in *E. coli* was 114 U/L and in 16.5 U/g of wet cells, which was higher than the yield of 8.92 U/L and 1.11 U/g of wet cells for the C-His-tagged L -RI expressed in *E. coli*. The considerable difference between the yields of recombinant L -RIs was also observed in the SDS-PAGE analysis results (Fig. 1, Lanes 1 and 4). Both recombinant L -RIs were purified from the cell-free extract of *E. coli* sequentially by using metal-affinity chromatography and ion-exchange chromatography (Table 2). However, the SDS-PAGE results indicated that ion-exchange chromatography did not facilitate purity improvement (Fig. 1,

Table 2. Summary of purification steps of recombinant L -RIs from *G. obscurus* DSM 43160 expressed in *E. coli* BL21-CodonPlus (DE3)-RIL^a.

| purification step | Total protein (mg) | Total activity (U) | Activity recovery (%) | Specific activity (U/mg) | Purification fold |
|----------------------------|--------------------|--------------------|-----------------------|--------------------------|-------------------|
| N-His-tagged | | | | | |
| crude extract ^b | 305 | 68.3 | 100 | 0.22 | 1 |
| HisTrap TM HP | 61.8 | 50.1 | 73 | 0.81 | 3.62 |
| HiPrep TM QFF | 33.7 | 35.4 | 52 | 1.05 | 4.69 |
| C-His-tagged | | | | | |
| crude extract ^c | 248 | 5.35 | 100 | 0.022 | 1 |
| HisTrap TM HP | 4.35 | 2.82 | 52.7 | 0.648 | 32.5 |
| HiPrep TM QFF | 1.74 | 0.60 | 11.2 | 0.344 | 17.0 |

^aActivity assays were carried out at 30°C for 5 min by using 0.8 mM L -ribulose as substrate in 50 mM glycine-NaOH buffer (pH 9).

^b4.05 g of frozen cells were suspended in 12.2 mL of lysis buffer.

^c4.82 g of frozen cells were suspended in 14.5 mL of lysis buffer.

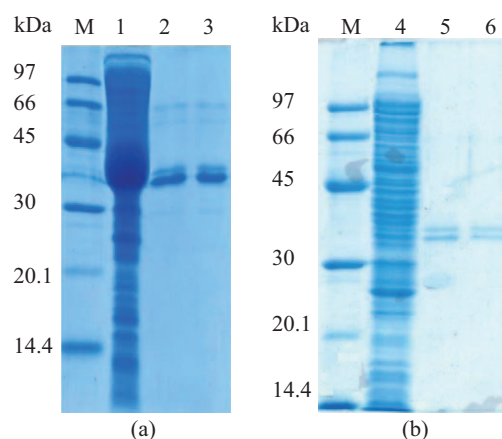


Fig. 1. SDS-PAGE analysis of purifications of the recombinant N-His-tagged and C-His-tagged L -RIs from *G. obscurus* DSM4316 expressed in *E. coli* BL21-CodonPlus (DE3)-RIL carrying the pET-15b-*Go-ri-NHis* (a) and pET-21b-*Go-ri-CHis* (b), respectively. Lane M: molecular weight standards; Lane 1: cell-free extracts of the *E. coli* cells carrying the pET-15b-*Go-ri-NHis*; lane 2: collected fraction of the recombinant N-His-tagged L -RI after metal-affinity chromatography; lane 3: collected fraction of the recombinant N-His-tagged L -RI after ion-exchange chromatography; lane 4: cell-free extracts of the *E. coli* cells carrying the pET-21b-*Go-ri-CHis*; lane 5: collected fraction of the recombinant C-His-tagged L -RI after metal-affinity chromatography; lane 6: collected fraction of the recombinant C-His-tagged L -RI after ion-exchange chromatography.

Lanes 3 and 6) and resulted in decreased specific activity for C-His-tagged L -RI and only slightly increased activity for N-His-tagged L -RI (Table 2). After ion-exchange chromatography, the SDS-PAGE results of both recombinant L -RIs indicated two major bands rather than a single band (Fig. 1, Lanes 3 and 6). And these major bands had an apparent molecular mass of approximately 37 kDa, which was larger than the theoretical molecular mass of approximately 30 kDa for

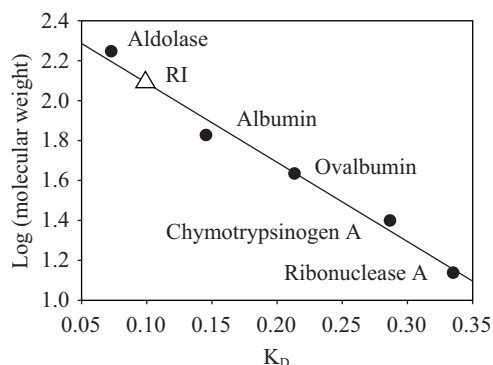


Fig. 2. Determination of the molecular weight of the recombinant N-His-tagged L -RI from *G. obscurus* DSM 43160 by gel filtration chromatography. The protein standards (●) included: aldolase (176 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa). The N-His-tagged L -RI (Δ) from *G. obscurus* DSM4316 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.

these two recombinant L -RIs. The molecular weight of the recombinant L -RIs from *Acinetobacter* sp. strain DL-28 estimated using SDS-PAGE was 32 kDa; this value was also larger than the theoretical value (27473Da) (Mizanur et al., 2001). The differences in expression level and specific activity between C-His-tagged and N-His-tagged L -RIs suggest that the position of the additional His-tag influenced the expression and activity of *G. obscurus* DSM 43160 L -RI. Because the active enzyme yield and specific activity of C-His-tagged L -RI were both less than those of the N-His-tagged L -RI (Table 2), further studies on the N-His-tagged L -RI alone were performed.

The molecular weight of N-His-tagged L -RI was analyzed using MALDI-TOF mass spectrometry; the results revealed a single peak with a molecular mass of 30158 Da, which was consistent with the theoretical molecular weight (30195 Da) of the N-His-tagged L -RI with the N-terminal methionine removed (data not shown). The single peak suggested that the purity of the purified N-His-tagged L -RI was high, thereby contradicting the two major bands observed in the SDS-PAGE analysis results. Therefore, native-PAGE analysis was performed to determine the purity of the N-His-tagged L -RI. The enzyme showed a single band (data not shown), which was consistent with the high purity suggested by the MALDI-TOF mass spectrometry results. The molecular mass of purified N-His-tagged L -RI under nondenaturing conditions was then analyzed by performing HPLC using a BioSep-SEC-S4000 gel filtration column. The purified N-His-tagged L -RI had a peak at 13.65 min (data not shown). In comparison with the calibration curve derived from the molecular mass standards, the molecular mass of the N-His-tagged L -RI under nondenaturing conditions was determined to be approximately 120.5 kDa (Fig. 2). Because the theoretical molecular mass of one

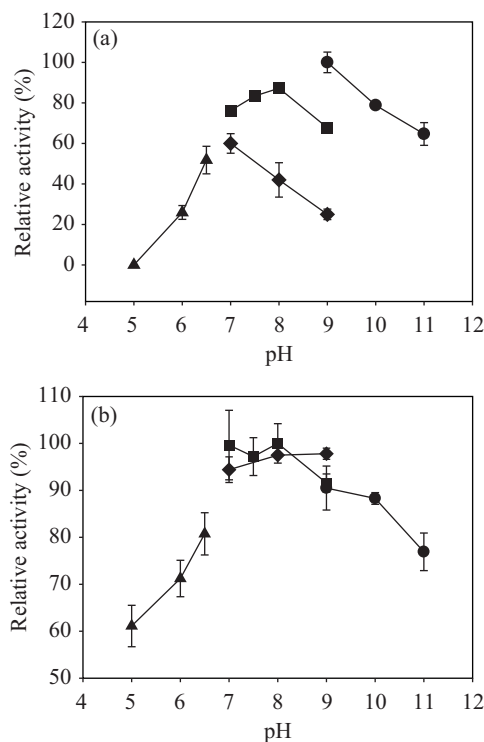


Fig. 3. Effects of pH on the activity (a) and stability (b) of the recombinant N-His-tagged L -RI from *G. obscurus* DSM4316. For the determination of optimal pH, the enzyme activity was assayed at 30°C and different pH values. For the determination of pH stability, the enzyme was incubated at various pH values at 4°C for 24 h, and the remaining activities were assayed at 30°C and pH 9. Data represent the mean \pm S.D. from triplicate experiments. The following buffers with a final concentration of 50 mM were used in different pH ranges: citrate phosphate buffer (pH 5-6.5) (▲), Tricine-HCl buffer (pH 7-9) (■), Tris-HCl buffer (pH 7-9) (◆) and glycine-NaOH buffer (pH 9-11) (●).

subunit of L -RI was approximately 30.2 kDa, the estimated 120.5 kDa corresponds to 4 subunits. These results suggest that the purified N-His-tagged L -RI from *G. obscurus* DSM 43160 is a homotetramer, as is the L -RI derived from *Acinetobacter* sp. strain DL-28 (Shimonishi and Izumori, 1996).

3. Effects of pH and Temperature on the Activity and Stability of the Recombinant N-His-tagged L -RI

The recombinant N-His-tagged L -RI exhibited optimal activity at a pH of 9 in glycine-NaOH buffer, and no activity when the pH was reduced to 5 (Fig. 3(a)). At a pH value of 9, the enzyme exhibited only 25% and 68% activity in the Tris-HCl and Tricine-HCl buffers, respectively (Fig. 3(a)). To investigate the effect of pH on enzyme stability, the purified enzyme was incubated at various pH values at 4°C for 24 h. The results indicated that the enzyme remained stable, and retained more than 90% activity, at pH 7-9 (Fig. 3(b)). Enzyme activity was fully retained at pH 7-8; 77% activity was retained at a pH of 11, and 61% activity was retained at a pH of 5. When the enzyme activity was measured at various temperatures, optimal activity was observed at 30-40°C (Fig. 4(a)).

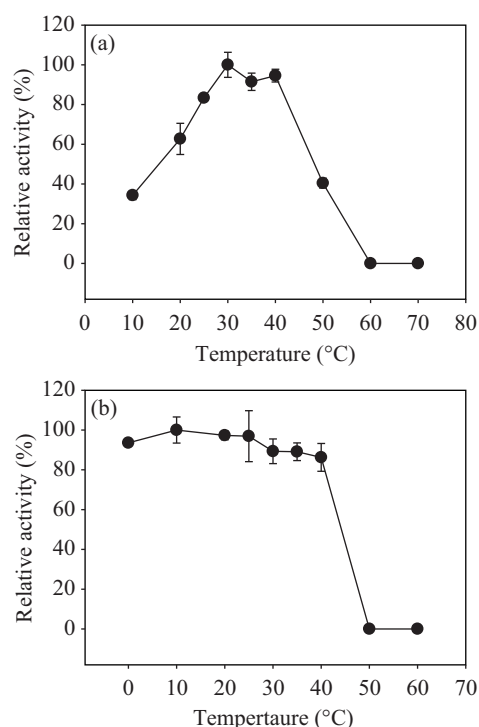


Fig. 4. Effects of temperature on the activity (a) and stability (b) of the recombinant N-His-tagged *L*-RI from *G. obscurus* DSM4316. For the determination of optimal temperature, the enzyme activity was assayed at pH 9 and different temperatures. For the determination of thermostability, the enzyme was incubated at various temperatures in pH 9 for 2 h, and the remaining activities were assayed at 30°C and pH 9 after appropriate dilutions.

The data in Fig. 4(a) were replotted on a semilogarithmic scale as $\ln(\text{activity})$ versus $1/T$ to determine the activation energy, E_a , of the isomerase reaction by applying the Arrhenius equation (data not shown). The activation energy of the isomerase reaction calculated using the Arrhenius plot was 39.7 kJ mol^{-1} at pH 9. The enzyme was stable at 0–40°C for 2 h. However, enzyme activity dropped sharply to zero after 2 h at 50°C (Fig. 4(b)).

4. Effect of Metal Ions on the Activity of the Recombinant N-His-tagged *L*-RI

The effects of 0.5 mM EDTA, Cu^{2+} , Co^{2+} , Mn^{2+} , Ni^{2+} , Zn^{2+} , Mg^{2+} , Ba^{2+} , Ca^{2+} , and Hg^{2+} on the activity of N-His-tagged *L*-RI were investigated. Hg^{2+} strongly inhibited *L*-RI activity. The addition of EDTA, Cu^{2+} , Co^{2+} , Mn^{2+} , Ni^{2+} , Zn^{2+} , Mg^{2+} , and Ba^{2+} exerted no activating or inhibitory effect on *L*-RI activity; however, the addition of Ca^{2+} slightly inhibited enzyme activity (Table 3). These results suggest that metal ions are not required for the activity of N-His-tagged *L*-RI from *G. obscurus* DSM 43160. In a previous study, the enzyme activity of recombinant *L*-RI from *C. parahominis* MB426 was observed to not be enhanced in the presence of various metal ions, but was strongly inhibited by Li^+ , Ca^{2+} , Cu^{2+} , Ni^{2+} , Zn^{2+} , Ba^{2+} , Ag^+ , and Fe^{2+} (Morimoto et al., 2013). By contrast, Mn^{2+} highly

Table 3. Effects of various metal ions and EDTA on the activity of N-His-tagged *L*-RI.

| Metal ions (0.5 mM) | Relative activity ^a (%) |
|---------------------|------------------------------------|
| None | 100 ± 8 |
| EDTA | 97.0 ± 4.1 |
| Cu^{2+} | 83.6 ± 8.5 |
| Co^{2+} | 88.9 ± 5.2 |
| Mn^{2+} | 81.8 ± 5.1 |
| Ni^{2+} | 96.5 ± 2.4 |
| Zn^{2+} | 85.1 ± 2.5 |
| Mg^{2+} | 88.9 ± 6.2 |
| Ba^{2+} | 90.3 ± 4.9 |
| Ca^{2+} | 70.6 ± 5.0 |
| Hg^{2+} | ND ^b |

^a Enzyme activity was assayed at 30°C in 50 mM glycine-NaOH buffer (pH 9) under the presence of 0.5 mM various metal ions and EDTA, respectively.

^b ND: Not detectable.

Table 4. Substrate specificity of N-His-tagged *L*-RI.

| Substrate (10 mM) | Activity ^a ($\mu\text{mol}/\text{min}/\text{mg}$) |
|---------------------|--|
| Pentose | |
| <i>L</i> -arabinose | ND ^b |
| <i>D</i> -lyxose | 8.00 ± 1.75 |
| <i>L</i> -ribose | 23.4 ± 1.42 |
| <i>L</i> -ribulose | 15.4 ± 1.79 |
| Hexose | |
| <i>D</i> -fructose | ND |
| <i>D</i> -mannose | ND |
| <i>L</i> -sorbitose | 9.00 ± 0.66 |
| <i>D</i> -tagatose | ND |

^a Enzyme activity was assayed at 30°C in 50 mM glycine-NaOH buffer (pH 9) using 10 mM different substrates.

^b ND: Not detectable.

increased the enzyme activity of recombinant *L*-RI from *Acinetobacter* sp. strain DL-28, whereas the addition of NiCl_2 , FeCl_3 , CuSO_4 , ZnSO_4 , and AlCl_3 caused a complete loss of activity (Mizanur et al., 2001).

5. Substrate Specificity and Enzyme Kinetics

L-arabinose, *D*-lyxose, *D*-mannose, *L*-ribose, *L*-ribulose, *L*-sorbitose, and *D*-tagatose were used to study the substrate specificity of the recombinant N-His-tagged *L*-RI from *G. obscurus* DSM 43160 (Table 4). With the exception of *L*-arabinose, the other 6 tested sugars could be isomerized to their corresponding aldoses and ketoses, respectively, by the recombinant *L*-RI from *C. parahominis* MB426 (Morimoto et al., 2013). N-His-tagged *L*-RI demonstrated high activity toward *L*-ribose and *L*-ribulose, moderate activity toward *D*-lyxose and *L*-sorbitose, and no activity toward *L*-arabinose, *D*-fructose, *D*-mannose, or *D*-tagatose. Similar to other characterized *L*-RIs, *L*-ribose and *L*-ribulose were the most favorable substrates

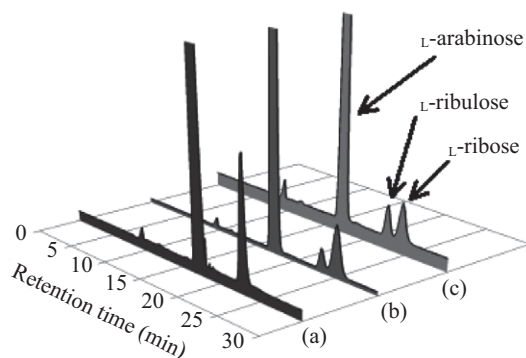


Fig. 5. HPLC profiles of the isomerization products using *T. saccharolyticum* NTOU1_{L-AI} and *G. obscurus* DSM4316 N-His-tagged_{L-RI}. The isomerization of _L-arabinose (50 g/L) was first catalyzed by *T. saccharolyticum* NTOU1_{L-AI} at 60°C for 6 h (a). After 6 h _L-AI reaction, *G. obscurus* DSM4316 N-His-tagged_{L-RI} (0.56 U/mL) was added to the reaction mixture and the reaction was further proceeded at 30°C (b) or 40°C (c) for additional 6 h.

(Morimoto et al., 2013; Shimonishi and Izumori 1996). However, the lack of activity toward _D-fructose, _D-mannose, and _D-tagatose demonstrated by N-His-tagged_{L-RI} from *G. obscurus* DSM 43160 differentiated it from _L-RIs from *Acinetobacter* sp. DL-28 (Shimonishi and Izumori 1996) and *C. parahominis* MB426 (Morimoto et al., 2013). The results suggest that the substrate binding site of recombinant N-His-tagged_{L-RI} from *G. obscurus* DSM 43160 might be different from those of the other characterized _L-RIs, and that it has difficulty in accommodating hexoses with _D-form conformation.

The kinetic parameters of the recombinant N-His-tagged_{L-RI} from *G. obscurus* DSM 43160 were determined using _L-ribose as a substrate. The V_{max} , k_{cat} , K_M , and catalytic efficiency (k_{cat}/K_M) of the recombinant N-His-tagged_{L-RI} against _L-ribose were 39.2 $\mu\text{mol}/\text{min}/\text{mg}$, 1224 min^{-1} , 51.6 mM, and 21.8 $\text{min}^{-1}\cdot\text{mM}^{-1}$, respectively.

6. Production of _L-Ribose and _L-Ribulose from _L-Arabinose by Using *T. saccharolyticum* NTOU1_{L-AI} and *G. obscurus* DSM4316 N-His-tagged_{L-RI}

To examine the production of _L-ribose from _L-arabinose, the enzyme reactions were performed using *T. saccharolyticum* NTOU1_{L-AI} and *G. obscurus* DSM4316 N-His-tagged_{L-RI}. *T. saccharolyticum* NTOU1_{L-AI} is a thermophilic enzyme that demonstrates better activity at 60-75°C (Hung et al., 2014), but *G. obscurus* DSM4316 N-His-tagged_{L-RI} is mesophilic, and exhibits better activity at 30-40°C. Therefore, _L-arabinose isomerization was first performed at 60°C for 6 h using *T. saccharolyticum* NTOU1_{L-AI}. After 6 h of the _L-AI reaction, N-His-tagged_{L-RI} was added, and the reaction was continued at 30°C or 40°C for an additional 6 h. No byproducts were observed in either the sugar conversion catalyzed using *T. saccharolyticum* NTOU1_{L-AI} alone or combined with *G. obscurus* DSM4316 N-His-tagged_{L-RI} (Fig. 5). The lack of

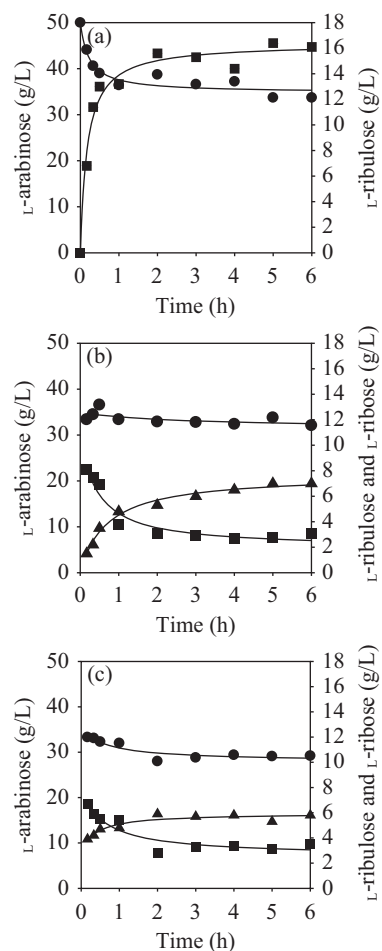


Fig. 6. The changes of sugar concentrations during isomerization using *T. saccharolyticum* NTOU1_{L-AI} and *G. obscurus* DSM4316 N-His-tagged_{L-RI}. The isomerization was performed as in Fig. 5. Symbols: \blacktriangle , _L-ribose; \blacksquare , _L-ribulose; \bullet , _L-arabinose.

byproducts is industrially valuable for further downstream purification processes. Changes in sugar concentrations that occurred during isomerization using *T. saccharolyticum* NTOU1_{L-AI} and *G. obscurus* DSM4316 N-His-tagged_{L-RI} are shown in Fig. 6. *T. saccharolyticum* NTOU1_{L-AI} isomerized _L-arabinose to _L-ribulose, and 17g/L _L-ribulose was formed when the reaction approached equilibrium (6 h) (Fig. 6(a)). When N-His-tagged_{L-RI} was added and the reaction was performed at 30°C or 40°C for an additional 6 h, the amount of _L-arabinose decreased slightly during the period; this indicated that the *T. saccharolyticum* NTOU1_{L-AI} was still active at 30°C and 40°C, and especially at 40°C (Figs. 6(b) and 6(c)). The conversion of _L-ribulose to _L-ribose at 40°C quickly approached equilibrium (2 h), while the concentration of _L-ribose continued to increase at 30°C. After N-His-tagged_{L-RI} was added to isomerize _L-ribulose to _L-ribose for 6 h, 7.0 and 5.8 g/L _L-ribose were formed at 30°C and 40°C, respectively (Figs. 6(b) and 6(c)). The conversions of _L-arabinose to _L-ribose achieved by using *T. saccharolyticum* NTOU1_{L-AI} and the N-His-tagged_{L-RI} were 15.9% and

Table 5. Comparison of the enzymatic properties of L -RIs from different sources.

| Properties | Recombinant | | | Natural |
|--------------------------|---|--|---|---|
| | <i>G. obscurus</i> DSM 43160 ^a | <i>C. parahominis</i> MB426 ^b | <i>Acinetobacter</i> sp. DL-28 ^c | <i>Acinetobacter</i> sp. DL-28 ^d |
| Optimum temperature (°C) | 30~40 | 40 | 30 | 30 |
| Thermostability | 40°C, 2 h | 40°C, 1 h | 30°C, 10 min | 30°C, 10 min |
| Optimum pH | 9 | 9 | 9 | 9 |
| pH stability | 7 - 9 | 7- 9 | 7 - 9 | 7 - 9 |
| Metal ion activation | No | No | Mn ²⁺ | NR ^e |
| Preferred substrate | L -ribose | L -ribulose | L -ribose | L -ribose |
| MW (SDS-PAGE) | 37 kDa | 32 kDa | 32 kDa | 32 kDa |
| MW (theoretical) | 30.2 kDa | NR | 27.5 kDa | NR |
| MW (mass spectrometry) | 30.2 kDa | NR | NR | NR |
| MW (gel filtration) | 120.5 kDa | NR | NR | 120 kDa |

^a This study.^b Morimoto et al. (2013).^c Mizanur et al. (2001).^d Shimonishi and Izumori (1996).^e NR: Not recorded in the published literature.^b ND: Not detectable.

12.5% (mol mol⁻¹) at 30°C and 40°C, respectively.

Table 5 further compares several enzymatic properties of the characterized L -RIs. In general, these L -RIs have the same optimal pH and similar pH stability. The effects of temperature on the activity and thermostability of these L -RIs are somewhat different. The thermostability of L -RI from *G. obscurus* DSM4316 is superior to those of *Acinetobacter* sp. DL-28 (Shimonishi and Izumori 1996) and *C. parahominis* MB426 (Morimoto et al., 2013).

IV. CONCLUSION

In this study, a gene of rarely studied L -RI was cloned and expressed in *E. coli*. The thermostability of L -RI from *G. obscurus* DSM4316 is superior to other characterized L -RIs. In addition, the production of L -ribose from L -arabinose was performed in this study. The lack of a byproduct in the conversion of L -ribose from L -arabinose catalyzed using *T. saccharolyticum* NTOU1 L -AI and *G. obscurus* DSM4316 N-His-tagged L -RI is industrially valuable. The results of this study suggest that this recombinant N-His-tagged L -RI could be used to produce L -ribose from L -arabinose.

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