Production of L-Ribose from L-Ribulose by a Triple-Site Variant of Mannose-6-Phosphate Isomerase from *Geobacillus thermodenitrificans*

Yu-Ri Lim, Soo-Jin Yeom, and Deok-Kun Oh

Department of Bioscience and Biotechnology, Konkuk University, Seoul, Republic of Korea

A triple-site variant (W17Q N90A L129F) of mannose-6-phosphate isomerase from *Geobacillus thermodenitrificans* was obtained by combining variants with residue substitutions at different positions after random and site-directed mutagenesis. The specific activity and catalytic efficiency (k_{cat}/K_m) for L-ribulose isomerization of this variant were 3.1- and 7.1-fold higher, respectively, than those of the wild-type enzyme at pH 7.0 and 70°C in the presence of 1 mM Co^{2+} . The triple-site variant produced 213 g/liter L-ribose from 300 g/liter L-ribulose for 60 min, with a volumetric productivity of 213 g liter⁻¹ h⁻¹, which was 4.5-fold higher than that of the wild-type enzyme. The k_{cat}/K_m and productivity of the triple-site variant were approximately 2-fold higher than those of the *Thermus thermophilus* R142N variant of mannose-6-phosphate isomerase, which exhibited the highest values previously reported.

L-Nucleosides have been widely used as antiviral nucleoside-analogue drugs in treatment of severe viral diseases because they have more potent biological activities and lower toxicities than the corresponding D-nucleosides (2). L-Ribose is a potential starting material for the synthesis of many antiviral drugs, such as L-nucleoside derivatives (1, 6, 14). Recently, a two-enzyme system consisting of L-arabinose isomerase and mannose-6-phosphate isomerase from *Geobacillus thermodenitrificans* (19) was used to produce L-ribose from L-arabinose via L-ribulose. A rate-limiting step in this process is the conversion of L-ribulose to L-ribose by mannose-6-phosphate isomerase (18–20). Thus, a genetically improved mannose-6-phosphate isomerase is essential for the industrial production of L-ribose.

In previous studies, L-ribulose has been converted from L-ribose using enzymes originated with various bacterial species, such as an L-ribose isomerase from an *Acinetobacter* sp. (13), an L-arabinose isomerase variant from *Escherichia coli* (4), a D-xylose isomerase variant from *Actinoplanes missouriensis* (16), and D-lyxose isomerases from *Cohnella laeviribosi* (3), *E. coli* (17), *Providencia stuartii* (9), and *Serratia proteamaculans* (15). This indicates that these enzymes can also be used to produce L-ribose from L-ribulose. Mannose-6-phosphate isomerases from *Bacillus subtilis* (18), *G. thermodenitrificans* (20), and *Thermus thermophilus* (21) have been used in the production of L-ribose from L-ribulose. Among all L-ribose-producing enzymes, mannose-6-phosphate isomerase from *T. thermophilus* exhibits the highest activity for L-ribose production. However, an enzyme with higher efficiency is required for the industrial production of L-ribose. Higher efficiency can be realized through genetic improvements via random mutagenesis and rational design. Of these two techniques, random mutagenesis, including methods such as error-prone PCR and DNA shuffling, is an easier way to improve enzyme activity because it does not require detailed structural information or accurate predictions for the residue substitutions (11, 22).

In this study, a triple-site (W17Q N90A L129F) variant of mannose-6-phosphate isomerase from *G. thermodenitrificans* was obtained by combining variants with residue substitutions at different positions after random and site-directed mutagenesis. The pH and temperature optima, thermostability, metal ion specificity, kinetic parameters for L-ribulose, and L-ribose production of the

variant were determined and compared with the corresponding values for the wild-type enzyme.

MATERIALS AND METHODS

Microorganisms, plasmid, medium, and culture conditions. *G. thermodenitrificans* as an isolated strain (8), *E. coli* ER2566, and the plasmids pET-28a(+) and pTrc99A were used as the source of genomic DNA of mannose-6-phosphate isomerase, host cells, and expression vectors, respectively. The recombinant *E. coli* cells for enzyme expression were cultured as described previously (18).

Gene cloning and enzyme purification. The gene (963 bp) encoding mannose-6-phosphate isomerase was obtained from genomic DNA isolated from *G. thermodenitrificans*. The sequences of the primers used for gene cloning were based on the DNA sequence of the gene encoding mannose-6-phosphate isomerase from *G. thermodenitrificans* (GenBank accession number CP000557). The forward (5'-TTTCATATGGACCTTGAACCGATTCTTCTCA-3') and reverse (5'-TTTGAATTCTTATTTGCTTTCCGTGGCCA-3') primers were designed to introduce the NdeI and EcoRI restriction sites (underlined). The amplified DNA fragment obtained by PCR was cloned into pET-28a(+) (20). The NdeI-EcoRI fragment from the pET-28a(+) vector containing the mannose-6-phosphate isomerase gene was subcloned into the same site of pET-28a(+) and transformed into *E. coli* ER2566. The obtained mannose-6-phosphate isomerase was purified as described previously (18).

Error-prone PCR and site-directed mutagenesis. A mutant library of mannose-6-phosphate isomerase from *G. thermodenitrificans* was constructed by carrying out an error-prone PCR with a PCR mutagenesis kit (ClonTech Laboratories, Palo Alto, CA) with a mutation rate of 2 to 4 mutations per 1,000 bp using pTrc99A. The PCR products, which were obtained by using the same primers as those used for cloning the gene, were transformed into *E. coli* ER2566. Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit and protocol (Stratagene, Beverly, MA). DNA sequencing was performed at the DNA sequencing facility of Macrogen (Seoul, Republic of Korea).

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Address correspondence to Deok-Kun Oh, deokkun@konkuk.ac.kr.

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Construction of mutant library and selection of mutant cells. *E. coli* ER2566 cells, which were transformed with the pTrc99A plasmid containing a mutant gene for *G. thermodenitrificans* mannose-6-phosphate isomerase, were cultured in a 96-well plate. Each well had 200 μ l of Luria-Bertani (LB) medium containing 50 μ g/ml of ampicillin. The plate was incubated at 37°C on a plate shaker with shaking at 500 rpm for 6 h. Subsequently, in another 96-well plate, 10 μ l of the above culture broth was added in each well with 200 μ l of LB medium containing 50 μ g/ml of ampicillin, along with 0.1 mM isopropyl β -D-thiogalactopyranoside (IPTG) for inducing enzyme expression. The plate was then incubated at 37°C with shaking at 500 rpm for 6 h. The cultured mutant cells were then heated at 70°C for 10 min for cell lysis. After heating, 100 μ l of the culture broth was transferred to each well of another 96-well plate with 50 μ l L-ribose at a final concentration of 10 mM, and the plate was incubated at 70°C for 30 min. The L-ribulose concentration of the reaction mixture was determined by a cysteine-carbazole method as a ketose assay method (5). We first selected the mutant cells in which the L-ribose-converting activity was 1.2-fold higher than that in wild-type cells. The mutant cells from the first selection were cultured in a 20-ml test tube containing 3 ml of LB medium with 50 μ g/ml of ampicillin at 37°C with shaking at 200 rpm for 6 h. Subsequently, IPTG was added to a final concentration of 0.1 mM to induce enzyme expression, and the culture was incubated at 37°C with shaking at 200 rpm for another 6 h. The cells were harvested and disrupted on ice using a sonicator. Cell debris was removed by centrifugation at $13,000 \times g$ for 20 min at 4°C. The crude extracts obtained as the supernatants were incubated at 70°C for 10 min in 50 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 7.0) containing 10 mM L-ribulose. The mutant cells in which L-ribose-producing activity using crude extracts was 1.2-fold higher than that in wild-type cells were then selected using a Bio-LC system (ICS-3000; Dionex, Sunnyvale, CA). The mannose-6-phosphate isomerase mutant genes in pTrc99A of the mutant cells from the second selection were transferred to pET-28a(+). The transformed cells were cultivated in a 2-liter flask with 500 ml of LB medium containing 25 μ g/ml of kanamycin at 37°C with shaking at 200 rpm. When the optical density of the bacterial solution reached 0.6 at an absorbance of 600 nm, IPTG was added to a final concentration of 0.1 mM, and the culture was incubated at 16°C with shaking at 150 rpm for 16 h. The enzymes from mutant cells were purified as described previously (18), and each variant was observed as a single band in SDS-PAGE. The mutant cells were finally selected on the basis of the L-ribose-producing activities of their purified enzymes.

Effects of metal ions, pH, and temperature. One unit of mannose-6-phosphate isomerase activity was defined as the amount of enzyme required to produce 1 μ mol of L-ribose per min at 70°C and pH 7. Unless otherwise stated, the reaction was carried out at 70°C in 50 mM PIPES buffer (pH 7) containing 10 mM L-ribulose, 0.05 U/ml of enzyme, and 1 mM Co^{2+} for 10 min. After EDTA was added to the purified enzyme with a final concentration of 10 mM, the solution was incubated at 4°C for 6 h and was dialyzed at 4°C for 16 h against 50 mM PIPES buffer (pH 7) without metal ions to remove EDTA. The metal ion-treated enzyme was prepared by adding 1 mM metal ions, such as Co^{2+} , Cu^{2+} , or Mn^{2+} , to the EDTA-treated enzyme. The reactions were carried out in 50 mM PIPES buffer (pH 7) containing 0.05 U/ml of enzyme and 1 mM metal ion at 70°C for 10 min. To examine the effects of pH and temperature on the activity of mannose-6-phosphate isomerase from *G. thermodenitrificans*, the pH was varied from 6 to 8 using 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 6.0 to 6.5), 50 mM PIPES buffer (pH 6.5 to 7.5), and 50 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(3-propanesulfonic acid) (EPPS) buffer (pH 7.5 to 8.0), and the temperature was varied from 60 to 80°C. The influence of temperature on enzyme stability was monitored at temperatures from 60 to 80°C at pH 7 after 12 h.

Homology modeling. Homology modeling of the *G. thermodenitrificans* mannose-6-phosphate isomerase was performed using Discovery Studio 3.1 (Accelrys, San Diego, CA) based on the X-ray structure of mannose-6-phosphate isomerase from *B. subtilis* (Protein Data Bank

FIG 1 L-Ribulose isomerization activities of the wild-type and variant mannose-6-phosphate isomerases from *G. thermodenitrificans*.

[PDB] entry 1QWR). The homologous search and sequence alignment were conducted using sequence analysis and multiple sequence alignment modules, respectively. Based on the alignment between the sequences of the target protein and its template, 5 comparative models of the target sequence were conducted with the MODELLER software program (12) by applying the default model building routine "model" with fast refinement. This procedure has an advantage in that one can select the best model from several candidates. Furthermore, the variability among the models can be used to evaluate modeling reliability. Energy minimization was performed using the consistent valence force field and DS CHARMM with the steepest descent and conjugated gradient algorithms. The qualities of these models were analyzed by PROCHECK software (10). L-Ribulose was docked in the model of *G. thermodenitrificans* mannose-6-phosphate isomerase using the CDOCKER module. The binding energy values of the wild-type and variant enzymes for L-ribulose were calculated using the Calculate Binding Energies protocol with default parameters except that ligand minimization was performed to consider the flexibility of residues within binding sites and the implicit solvent model was set to the Generalized Born method (7).

Analytical methods. The concentrations of L-ribulose and L-ribose were determined using a Bio-LC system equipped with an electrochemical detector and a CarboPac PA1 column. The column was eluted at 30°C with 200 mM sodium hydroxide at a flow rate of 1 ml/min. The monosaccharides were purchased from Carbosynth (Newbury, Berkshire, United Kingdom).

RESULTS AND DISCUSSION

Preparation of variants containing only one amino acid residue substitution based on L-ribose-producing activity. The catalytic efficiency (k_{cat}/K_m) of mannose-6-phosphate isomerase from *G. thermodenitrificans* at 70°C was 2.4-fold lower than that of the enzyme from *T. thermophilus* at 75°C, whereas the half-lives of the enzyme at 75 and 80°C were 3- and 15-fold longer, respectively, than those of the enzyme from *T. thermophilus* (20, 21). The low thermostability of mannose-6-phosphate isomerase from *T. thermophilus* renders it highly unsuitable for long-term use in the industrial production of L-ribose. Thus, we chose the mannose-6-phosphate isomerase from *G. thermodenitrificans* for this study.

FIG 2 Effects of metal ions on the specific activities of the wild type (black) and L129F (light gray), N90A-L129F (dark gray), and W17Q-N90A-L129F (palest gray) variant mannose-6-phosphate isomerases from *G. thermodenitrificans*. Data represent the means for three separate experiments.

We obtained 5,000 mutant clones containing the mutant gene for *G. thermodenitrificans* mannose-6-phosphate isomerase by error-prone PCR. Through the three selection steps using cells, crude extracts, and purified enzymes, we obtained 4 mutant clones. These cell clones contained the mutant K21E N74T M134R, W17R E67G T238I, K124R L129F, and N90K K105R R142C genes, and their L-ribose-producing activities were 1.2-, 1.3-, 1.3-, and 1.2-fold higher, respectively, than that of the wild-type cells. The 11 variants containing only one amino acid residue substitution, such as W17R, K21E, E67G, N74T, N90K, K105R, K124R, L129F, M134R, R142C, and T238I, were created by site-directed mutagenesis. The activities of the W17R, N90K, and L129F variants were 1.2-, 1.3-, and 2.0-fold higher, respectively, than that of the wild-type cells, whereas the activities of the other 8 variants were almost the same as that of the wild-type enzyme (data not shown). Thus, the W17R, N90K, and L129F variants were prepared, since they showed high L-ribose-producing activities. The Trp17, Asn90, and Leu129 residues in the homology model of mannose-6-phosphate isomerase from *G. thermodenitrificans* were located at more than 6 Å from the substrate (distances of 6.4, 10.5, and 13.5 Å, respectively), suggesting that these residues may not be involved in the active site of the enzyme.

Generation of an effective variant by combining variants with residue substitutions at different positions after site-directed mutagenesis at positions 17, 90, and 129. To find mannose-6-phosphate isomerase variants that had higher activity than the W17R, N90K, and L129F variants, the residue at position 17, 90, or 129 was replaced with the nonpolar residue Gly, Ala, Val, Ile, Leu, or Gln, the positively charged residue Lys or Arg, the negatively charged residue Glu, or the aromatic residue His, Phe, or Tyr. The specific activities of the variants at positions 17, 90, and 129 were in the following order, from greatest to least: W17Q variant, W17L variant, W17I variant, W17E variant, W17V variant, W17R variant, W17A variant, wild-type enzyme, W17G variant, W17Y variant, W17F variant; N90A variant, N90V variant, N90L variant, N90K variant, wild-type enzyme, N90E variant, N90F variant, N90G variant, N90Q variant;

FIG 3 Thermostability of the wild type (●) and L129F (□), N90A-L129F (■), and W17Q-N90A-L129F (○) variant mannose-6-phosphate isomerases from *G. thermodenitrificans* for L-ribulose isomerization. Data represent the means for three experiments, and error bars represent standard deviations.

and L129F variant, L129Q variant, L129E variant, L129A variant, wild-type enzyme, L129Y variant, L129H variant, L129K variant, L129W variant, respectively (Fig. 1). The aromatic rings of Trp, Tyr, and Phe reduced the activity of the enzyme. The replacement of Trp with Gln, which may be the optimal size of amino acid, may have increased the enzyme activity. If the Asn residue at position 90 is replaced with any smaller residue except Gly, the enzyme activity increases. The activities of the L129F, L129Y, and L129W variants were 204%, 94%, and 10% of the activity of the wild-type enzyme, respectively, indicating that the type of aromatic ring of the residue at position 129 affected critically enzyme activity. Therefore, the W17Q, N90A, and L129F mutations were used for generating double- and triple-site variants (Fig. 1). As a result, the triple-site variant (W17Q N90A L129F) showed the highest activity, which was 3.1-fold higher than that of the wild-type enzyme.

Effects of metal ions, pH, and temperature on activities of wild-type and variant mannose-6-phosphate isomerases from *G. thermodenitrificans*. L-Ribulose isomerization by mannose-6-phosphate isomerases from *B. subtilis* (18), *G. thermodenitrificans* (20), and *T. thermophilus* (21) was significantly stimulated by Co²⁺ or Mn²⁺, Co²⁺, and Cu²⁺ or Co²⁺ ions, respectively. The specific activities on L-ribulose of the purified, EDTA-treated enzymes supplied with Co²⁺, Mn²⁺, or Cu²⁺ were investigated using the wild-type, single-site (L129F), double-site (N90A L129F), and triple-site (W17Q N90A L129F) variant mannose-6-phosphate isomerases from *G. thermodenitrificans* (Fig. 2). Addition of Co²⁺ ions significantly enhanced the activities of the wild-type and variant enzymes in the following order, from greatest to least enhancement: triple-site variant, double-site variant, single-site variant, wild-type enzyme. However, there was no significant increase with the addition of Mn²⁺ or Cu²⁺ ions. Thus, our results indicated that the addition of Co²⁺ was essential for increasing the activities of mannose-6-phosphate isomerase variants.

The effects of pH and temperature on enzyme activity were also investigated, and all enzymes, including the wild-type, single-site,

TABLE 1 Kinetics parameters of mannose-6-phosphate isomerases from *B. subtilis*, *G. thermodenitrificans*, and *T. thermophilus* in conversion of L-ribulose to L-ribose and binding energy values after docking L-ribulose of the wild-type and variant enzymes from *G. thermodenitrificans*^a

Microorganism	Enzyme description	Temp (°C)	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ mM ⁻¹)	ΔE_{bind} ^b (kcal/mol)	Reference
<i>Bacillus subtilis</i>	Wild type	40	849	3,694	44		15
<i>Thermus thermophilus</i>	Wild type	75	136	50,644	374		18
	R142N	75	140	81,063	579		18
<i>Geobacillus thermodenitrificans</i>	Wild type	70	149	23,546	158	-30.9	This study
	L129F	70	243	55,123	227	-32.4	
	N90A L129F	70	89	85,715	965	-34.6	
	W17Q N90A L129F	70	100	112,098	1,120	-35.7	

^a The reactions with the wild-type and variant enzymes of mannose-6-phosphate isomerases from *G. thermodenitrificans* were incubated in 50 mM PIPES buffer (pH 7.0) containing various amounts of L-ribulose (10 to 1,000 mM) at 70°C for 10 min.

^b ΔE_{bind} , change in binding energy after docking L-ribulose.

double-site, and triple-site variant enzymes, had maximal activities at pH 7, 70°C (data not shown). The activities of the wild-type, single-site, double-site, and triple-site variant enzymes remained for up to almost 12 h at 60°C and 65°C but were reduced to 10%, 13%, 26%, and 32% of their maximal activities at 80°C, respectively (Fig. 3). Thus, the thermostability of the enzymes was in the following order, from greatest to least: triple-site variant, double-site variant, single-site variant, and wild-type enzyme.

Kinetic parameters and production of L-ribose from L-ribulose with wild-type and variant mannose-6-phosphate isomerases from *G. thermodenitrificans*. The kinetic parameters of the wild-type, single-site, double-site, and triple-site variant enzymes for L-ribulose as a substrate are presented in Table 1. The catalytic efficiency (k_{cat}/K_m) of the triple-site variant mannose-6-phosphate isomerase was 7.1-, 4.9-, and 1.2-fold higher, respectively, than those of the wild-type, single-site, and double-site variant enzymes and 26-, 3-, and 2-fold higher, respectively, than those of *B. subtilis* wild-type, *T. thermophilus* wild-type, and *T. thermophilus* R142N variant enzymes (18, 21). In the homology model, L-ribulose as the substrate was docked to the wild-type and variant enzymes, and then their energy values were calculated to explain

how the variants changed the activity of the enzyme. The binding energy values of the wild-type, W17Q, N90A, L129F, W17Q N90A, W17Q L129F, N90A L129F, and W17Q N90A L129F variant enzymes were -30.9, -31.0, -31.3, -32.4, -33.2, -34.1, -34.6, and -35.7 kcal/mol, respectively. The results indicated that the binding energy after docking L-ribulose decreased proportionally with increasing activity of the enzyme. Therefore, the improved activity of the variant may be attributable to increased binding interaction, and the triple-site variant may bind the most efficiently to the substrate among the wild-type and variant mannose-6-phosphate isomerases.

For practical biotechnological application, time course reactions by the same concentrations of the wild-type, single-site, double-site, and triple-site variant enzymes were performed with L-ribulose as a substrate. The reaction conditions were pH 7, 70°C, 300 g/liter L-ribulose, 1 mM Co²⁺, and 2 mg/ml of enzyme. Under these conditions, the wild-type enzyme produced 213 g/liter L-ribose after 270 min, with a conversion yield of 70% and a volumetric productivity of 47 g liter⁻¹ h⁻¹ (Fig. 4). The single-site, double-site, and triple-site variants produced the same concentration of L-ribose after 180, 90, and 60 min, respectively, with volumetric productivities of 71, 142, and 213 g liter⁻¹ h⁻¹, respectively. Thus, the productivity of the triple-site variant was 4.5-fold higher than that of the wild-type enzyme.

In summary, a W17Q N90A L129F variant of mannose-6-phosphate isomerase from *G. thermodenitrificans* was obtained by combining variants with residue substitutions at different positions after random and site-directed mutagenesis. The specific activity, k_{cat}/K_m , and productivity of this variant were 3.1-, 7.1-, and 4.5-fold higher than those of the wild-type enzyme, respectively, and are the highest values reported thus far for any L-ribose-producing enzyme. These results suggest that the triple-site mutant of mannose-6-phosphate may be useful for the industrial production of L-ribose.

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FIG 4 Time course reactions for L-ribose production from L-ribulose by the wild-type (●) and L129F (□), N90A-L129F (■), and W17Q N90A L129F (○) variant mannose-6-phosphate isomerases from *G. thermodenitrificans*. Data represent the means for three experiments, and error bars represent standard deviations.

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