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ORIGINAL RESEARCH PAPER

Characterization of a mannose-6-phosphate isomerase from *Geobacillus thermodenitrificans* that converts monosaccharides

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Abstract A recombinant mannose-6-phosphate isomerase from *Geobacillus thermodenitrificans* (GTMpi) isomerizes aldose substrates possessing hydroxyl groups oriented in the same direction at the C2 and C3 positions such as the D- and L-forms of ribose, lyxose, talose, mannose, and allose. The activity of GTMpi for D-lyxose isomerization was optimal at pH 7.0, 70°C and 1 mM Co^{2+}. Under these conditions, the k_{cat} and K_m values were 74,300 s^{-1} and 390 mM for D-lyxose and 28,800 s^{-1} and 470 mM for L-ribose, respectively. The half-lives of the enzyme at 60, 65, and 70°C were 388, 73, and 27 h, respectively. GTMpi catalyzed the conversion of D-lyxose to D-xylulose with a 38% conversion yield after 3 h, and converted L-ribose to L-ribulose with a 29% conversion yield.

Keywords Bioconversion · *Geobacillus thermodenitrificans* · Mannose-6-phosphate isomerase · L-Ribose · Substrate specificity

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Introduction

Recently, rare monosaccharides have attracted much attention in the food, nutritional, and pharmaceutical industries due to their many applications, including their use as low-calorie sweeteners, bulking agents, antioxidants, glycosidase inhibitors, and nucleoside analogs (Levin et al. 1995; Ahmed 2001; Granström et al. 2004). Many sugar isomerases that convert monosaccharides have been characterized (Pastinen et al. 1999; Mizanur et al. 2001; Leang et al. 2004; Kim and Oh 2005; Menavuvu et al. 2006; Cho et al. 2007). However, sugar isomerases themselves are sufficient to produce various types of monosaccharides more efficiently. Recently, sugar phosphate isomerases have been suggested as useful enzymes for producing various monosaccharides because they convert not only the authentic substrate sugar phosphate but also the substrate sugar that have a similar configuration (Park et al. 2007a, b; Yoon et al. 2009). Thus, we have proposed the use of mannose-6-phosphate isomerase (EC 5.3.1.8), which catalyzes the interconversion of mannose 6-phosphate and fructose 6-phosphate (DeRossi et al. 2006), as a tool to convert monosaccharides.

In this study, a gene encoding mannose-6-phosphate isomerase from *Geobacillus thermodenitrificans* (GTMpi) was cloned and expressed in *Escherichia coli*. Its substrate specificity and bioconversions of D-lyxose and L-ribose were investigated.

Materials and methods

Materials

Monosaccharides were purchased from Carbosynths (Newbury, UK) and Sigma. L-Ribulose was kindly provided by Professor Yoon-Mo Koo at Center for Advanced Bioseparation Technology of Inha University (Incheon, South Korea).

Bacterial strains, plasmid, and culture conditions

The genomic DNA from *Geobacillus thermodinitrificans*, *E. coli* ER2566, and pTrc 99A plasmid were used as the source of mannose-6-phosphate isomerase gene, host cells, and expression vector, respectively. The recombinant *E. coli* for protein expression of the enzyme was cultivated in a 2 l flask containing 500 ml of Luria-Bertani (LB) medium with 50 μg ampicillin ml^{-1} at 37°C with agitation at 200 rpm. When the OD_{600} reached 0.5, IPTG was added to 0.1 mM to induce GTMpi expression, and the culture was incubated with shaking at 200 rpm at 37°C for 4 h.

Gene cloning

The gene encoding mannose-6-phosphate isomerase was amplified by PCR using the genomic DNA isolated from *G. thermodinitrificans* (Baek et al. 2004) as a template. The sequence of the oligonucleotide primers used for gene cloning was based on the DNA sequence of *G. thermodinitrificans* mannose-6-phosphate isomerase (GenBank accession number CP 000557). Forward (5′-TTTGAATTCATGCATCAAGAACCGATTTTTC-3′) and reverse primers (5′-TTTAAGCTTTTATTTGCTTGTCCGTGG-3′) were designed to introduce the *Eco*RI and *Hin*dIII restriction sites (underlined) for the gene cloning and were synthesized by Bioneer Co. (Daejon, Korea). The amplified DNA fragment obtained by PCR was purified and inserted into the pGEM-T easy vector (Promega, Madison, WI). *E. coli* Top10 strain was transformed with the ligation mixture and plated on LB agar containing 50 μg ampicillin ml^{-1}, 0.1 mM IPTG, and 80 μg X-gal ml^{-1}. The *Eco*RI–*Hin*dIII fragment from T-vector containing the gene encoding GTMpi was subcloned into the same sites of pTrc99A plasmid and then the resulting plasmid was obtained. The plasmid was transformed into *E. coli* ER2566 strain and grown on LB medium containing 50 μg ampicillin ml^{-1}.

Determination of molecular weight

The subunit and total molecular weights of GTMpi were estimated using SDS-PAGE and gel filtration chromatography of a Sephacryl S-300 preparative-grade HR 16/60 column (Amersham Biosciences, Uppsala, Sweden), respectively. The enzyme solution was applied to the column and eluted with 50 mM Tris/HCl (pH 7.5) buffer containing 150 mM NaCl at 1 ml min^{-1}. The column was calibrated with aldolase (158 kDa), albumin (67 kDa), chymotrypsinogen A (25 kDa), and ribonuclease (13.7 kDa) as reference proteins (Amersham Biosciences), and the total molecular weight was calculated by comparing with the migration length of reference proteins.

Effects of metal ions, pH, and temperature

To investigate the effect of metal ions on the activity of GTMpi, the enzyme assay was carried out after treatment with EDTA or addition of various metal ions (see Fig. 2 below).

To find optimal pH of the enzyme, pH was varied from 6.5 to 8.5 using 50 mM PIPES buffer (pH 6.5–7.5) and 50 mM EPPS buffer (pH 7.5–8.5). The effects of temperature on the enzyme activity and stability were evaluated in 50 mM PIPES (pH 7.0) buffer at temperatures ranging from 60 to 80°C. A sample was withdrawn at each time interval and the relative activity was determined after the reaction. One unit of enzyme activity was defined as the amount of enzyme required to increase 1 μmol D-xylulose per min at 70°C and pH 7.0.

Analytical methods

The concentrations of monosaccharides were determined by a Bio-LC system (Dionex ICS-3000, Sunnyvale, CA) with an electrochemical detector using a CarboPac PAI column. The column was eluted at 30°C with 200 mM NaoH at 1 ml min^{-1}.

Results and discussion

Gene cloning, expression, and purification of GTMpi

A gene of 963 bp encoding GTMpi, with the same sequence as that reported in GenBank (accession number CP000557), was cloned and expressed in *E. coli*. The enzyme was purified as a soluble protein from crude extract obtained from harvested cells by heat treatment and Hi-Trap ion-exchange chromatography (Table 1). GTMpi was purified with a final purification of 8.7-fold, a yield of 78%, and a specific activity of 890 U mg^{-1}. The molecular mass of the expressed protein analyzed by SDS-PAGE was about 36 kDa (Fig. 1), consistent with the calculated value of 36,452 Da based on the amino acid sequence of 321 residues. The molecular weight of the native enzyme estimated by gel-filtration chromatography was 36 kDa, corresponding to a monomer (data not shown).

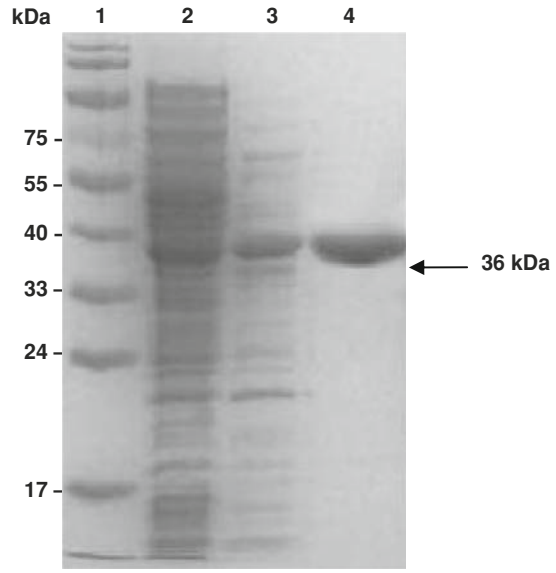

Fig. 1 SDS-PAGE analysis of GTMpi from each purification step. Lanes 1, molecular weight markers; lane 2, crude extract; lane 3, supernatant after heat treatment at 70°C for 5 min; lane 4, Hi-trap column product (purified enzyme)

Effects of metal ions, pH, and temperature on the activity of GTMpi

To investigate the effect of metal ions on the activity of GTMpi, the metal ions were added without prior EDTA treatment. Among the metal ions tested, Co^{2+} was the most effective metal ion for isomerization of D-lyxose by GTMpi, resulting in an approx 2.4-fold increase in activity in relative to the control (Fig. 2). Its optimal concentration was 1 mM (data not shown). Thus, all subsequent experiments were performed in the presence of 1 mM Co^{2+}. Typically, phosphate sugar isomerases are metal-independent (Park et al. 2007a, b; Yoon et al. 2009), but mannose-6-phosphate isomerases require a metal cofactor such as Zn^{2+} for activity (Wu et al. 2002).

The activity of GTMpi for isomerization of D-lyxose was maximal at pH 7.0 and 70°C (data not shown). The activity of D-lyxose isomerases from *Cohnella laevoribosii* for D-lyxose isomerization was maximal at pH 6.5 and 70°C with 1 mM Mn^{2+} (Cho et al. 2007). The thermostability was examined by measuring the activity at temperatures ranging from 60 to 80°C. Thermal inactivation of GTMpi followed first-order kinetics and the half-lives of the enzyme at 60, 65, 70, 75, and 80°C were 338, 73, 27, 17, and 6 h, respectively (Fig. 3).

Substrate specificity of GTMpi for aldoses

The specific activity of GTMpi was investigated with the D- and L-forms of all pentoaldoses and hexoaldoses.

Table 1 Purification of GTMpi

Step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification (fold)
Crude extract	300	30600	102	100	1
Heat treatment	111	25300	228	83	2.2
Hi-Trap	27	24000	890	78	8.7

Cells were harvested from culture broth, and then resuspended in buffer which composed of 50 mM Tris/HCl buffer (pH 7.5) and 0.1 mM PMFS. The resuspended cells were disrupted by ultrasonication. Enzymes from the disrupted cells were purified by heat treatment 70°C for 10 min and Hi-Trap Q HP (Amersham Biosciences). The active fraction was dialyzed in 50 mM PIPES (pH 7.0) buffer

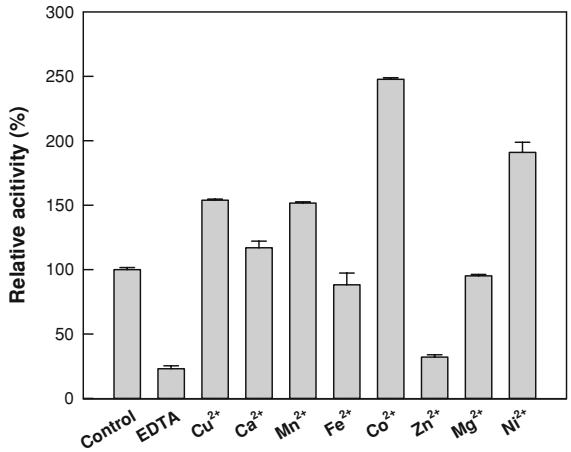

Fig. 2 Effect of metal ions on the activity of GTMpi. The metal ions were added without prior EDTA treatment and the reactions were performed in 50 mM PIPES buffer (pH 7.0) containing 10 mM D-lyxose and 1 mM of each metal ion at 70°C for 10 min. 'Control' is an enzyme solution obtained after heat treatment without treatment of EDTA. Data represent the means of three separate experiments. The relative activity of 100% was 125 U enzyme ml^{-1}

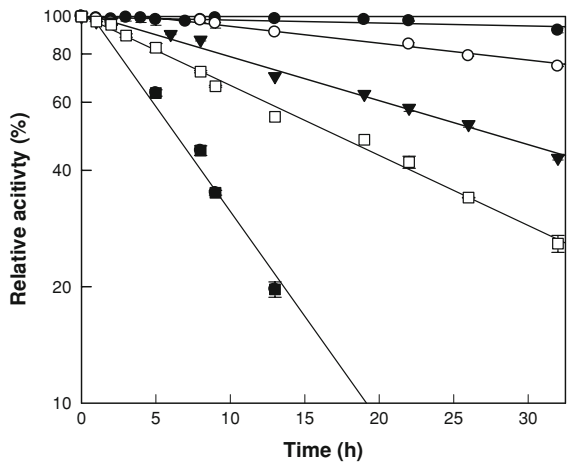

Fig. 3 Thermal inactivation of the activity of GTMpi at temperatures of 60 (*black circle*), 65 (*white circle*), 70 (*black down-pointing triangle*), 75 (*white square*), and 80°C (*black square*). Data represent the means of three separate experiments. The relative activity of 100% was 298 U enzyme ml^{-1}

Among the aldose substrates, the specific activity was the highest for D-lyxose, followed by L-ribose, D-talose, D-ribose, D-allose, L-lyxose, D-mannose, L-allose, L-talose, and L-mannose (Table 2). The aldose substrates with hydroxyl groups oriented in the same direction at C2 and C3 were converted reversibly by the enzyme to the corresponding ketoses,

Table 2 Specific activity and conversion ratio of GTMpi for aldoses

Substrate	Product	Specific activity (U mg^{-1})
D-Lyxose	D-Xylulose	298 ± 1.2
L-Ribose	L-Ribulose	82 ± 0.5
D-Talose	D-Tagatose	77 ± 0.1
D-Ribose	D-Ribulose	71 ± 0.3
D-Allose	D-Psicose	63 ± 0.5
L-Lyxose	L-Xylulose	60 ± 0.4
D-Mannose	D-Fructose	47 ± 0.1
L-Allose	L-Psicose	28 ± 0.1
L-Talose	L-Tagatose	26 ± 0.1
L-Mannose	L-Fructose	5 ± 0.0

To measure the specific activity for aldoses and ketoses, the enzyme reactions were performed in 50 mM PIPES (pH 7.0) buffer at 70°C with 10 mM substrates. The reaction was stopped by adding HCl to the reaction mixture at a final concentration of 200 mM. Data represent the means of three separate experiments

suggesting that GTMpi has novel substrate specificity for monosaccharides. The specific activity of the L-ribose isomerase from *Acinetobacter* sp. follows the order L-ribose>D-lyxose>D-mannose, whereas the specific activity of D-lyxose isomerase from *C. laevoribosii* follows the order D-lyxose>D-mannose>L-ribose (Mizanur et al. 2001; Cho et al. 2007). No activity of either of these enzymes was observed for D-talose, L-allose, D-ribose, L-talose, D-allose, L-lyxose or L-mannose, demonstrating the different substrate specificity of GTMpi.

The Michaelis-Menten constant (K_m), turnover number (k_{cat}), and catalytic efficiency (k_{cat}/K_m) of GTMpi for D-lyxose was 390 mM, 74,300 s^{-1}, and 191 mM^{-1} s^{-1}, respectively; and those for L-ribose were 470 mM, 28,800 s^{-1}, and 61 mM^{-1} s^{-1}, respectively. The k_{cat}/K_m value of the enzyme for D-lyxose was 2.3-fold higher than that of the D-lyxose isomerase from *C. laevoribosii* (Cho et al. 2007). L-Ribose is a potential starting material for the synthesis of many L-nucleoside-based pharmaceutical compounds (Ahmed 2001). This enzyme displayed a k_{cat}/K_m value for L-ribose that was significantly higher (310- and 1.2 × 10^6-fold) than those of the D-lyxose isomerase from *C. laevoribosii* and the D-xylose isomerase mutant from *Actinoplanes missouriensis*, respectively (Santa et al. 2005; Cho et al. 2007). Thus, GTMpi has the highest k_{cat}/K_m for

L-ribose among the L-ribose-converting enzymes reported to date, suggesting that GTMpi is a potential L-ribose producer.

Bioconversion of D-lyxose and L-ribose by GTMpi

GTMpi exhibited the highest and second highest specific activity for D-lyxose and L-ribose, respectively, among the aldose substrates. Thus, the bioconversions of D-lyxose and L-ribose by GTMpi were performed using 10 mM of each substrate (Fig. 4).

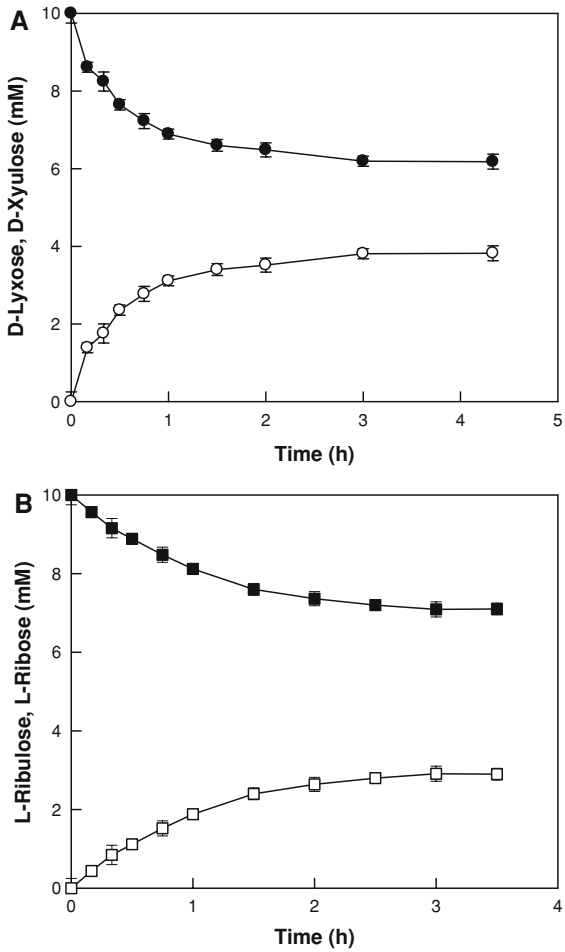

Fig. 4 Isomerization reactions of GTMpi. **a** Conversion of the aldose D-lyxose (*black circle*) to the ketose D-xylulose (*white circle*). **b** Conversion of the aldose L-ribose (*black square*) to the ketose L-ribulose (*white square*). The bioconversions of D-lyxose to D-xylulose and L-ribose to L-ribulose by GTMpi were investigated in 50 mM PIPES (pH 7.0) buffer containing 10 mM substrate at 70°C. A sample was withdrawn at each time interval and then assayed

D-Lyxose was converted to D-xylulose with a 38% conversion yield after 3 h, whereas L-ribose was converted to L-ribulose with a 29% conversion yield, similar to that obtained (30%) with the L-ribose isomerase from *Acinetobacter* sp. (Ahmed et al. 1999).

GTMpi shows more activity for D-lyxose than D-lyxose isomerase, and can be used for the more efficiently conversion of D-lyxose to D-xylulose. Moreover, the enzyme is a potential L-ribose producer. Thus, in the future, we will attempt to convert L-arabinose to L-ribose via a two-step reaction using a two-enzyme system in which L-arabinose will first be transformed to L-ribulose by L-arabinose isomerase (Yeom et al. 2008), and then L-ribulose will be transformed to L-ribose by GTMpi.

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