Note

Characterization of Mannose Isomerase from a Cellulolytic Actinobacteria *Thermobifida fusca* MBL10003

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Abstract: D-mannose isomerase was cloned and characterized from a newly isolated actinobacteria strain, *Thermobifida fusca* MBL10003. The structural gene (*manI*) is predicted to encode a polypeptide of 407 amino acids with an estimated molecular mass of 43,900. Although the identity of the deduced amino acid sequence is not so high (45.7% to *Salmonella enterica*, and 38.5% to *Agrobacterium radiobacter*), the catalytic center appears has an essential structure that is conserved in the characterized homologs. It was a dimeric enzyme composed of two active monomer units. The optimal temperature and pH were 60°C and 8.0, respectively. The enzyme was stable up to around 60°C, and between pH 4 and 11. It showed activity on D-mannose and also on D-lyxose, but not on N-acetyl D-glucosamine, suggesting that it is functionally different from N-acyl D-glucosamine 2-epimerase despite the sequence similarity. Although K_m value for D-mannose, 115 mM was similar to other mannose isomerase, k_{cat} , hence k_{cat}/K_m was much higher than those. The enzyme was significantly inhibited by such divalent metal ions as Cu^{2+} , Cd^{2+} or Ca^{2+} , but it was not a metal-required enzyme for activity emergence.

Key words: mannose isomerase, N-acyl D-glucosamine 2-epimerase, *Thermobifida fusca* MBL10003

Mannose isomerase (EC 5.3.1.7) is one of the aldo-keto isomerase that can catalyze isomerization between mannose and fructose. Reaction equilibrium is shifted to mannose at a ratio of *ca.* 70 to 30.¹⁾ This enzyme has been known inducibly produced by several microorganisms when D-mannose is supplied as a carbon source. Mannose isomerase is therefore, derepressed enzyme like xylose isomerase²⁾ or galactose dehydrogenase³⁾ that is acquired through a release of glucose catabolite repression.^{4,5)} A mutant of *Escherichia coli* K12 selected for growth on D-lyxose produced a D-mannose isomerase constitutively,⁶⁾ but D-mannose is normally transported into *E. coli* K12 by the phosphatetransferase system⁷⁾ as D-mannose-6-phosphate.⁸⁾ Hence, these enzymes were suggested to be possible evolutionary remnants.⁹⁾

Meanwhile, mannose has been paid attention from basic and practical aspect owing to its specific characteristics. Generally, sugar chains associated with molecular recognition of immune cells, erythrocytes and allergenic cross-reacting carbohydrate determinants consist of a significant amount of manno-oligosaccharides.¹⁰⁾ Mannose itself protects infection from *Salmonella thyphimillium* to chicken in poultry farming.^{11,12)} Mannose is thus potent aldo-hexose on medical, pharmaceutical and food-biochemical fields. However, expensiveness of this sugar compared with other

commercially available sugar is serious bottleneck for wide utilization at present. The enzymatic conversion from inexpensively available fructose by mannose isomerase is one of the promising methods for a practical scale production of mannose.

Until now, mannose isomerases from several microorganisms such as *Pseudomonas saccharophila*,¹⁾ *Streptomyces aerocolonigenes*,¹³⁾ *Escherichia coli*,¹⁴⁾ *Pseudomonas cepacia*,¹⁵⁾ or *Pseudomonas* sp.¹⁶⁾ have been reported, and also enzymatic conversion of mannose from fructose have been tried using the mannose isomerases from *P. cepacia*¹⁷⁾ or *Pseudomonas* sp.¹⁶⁾ On the other hand, there are only a few reports on genetic description of this enzyme.

Thermobifida fusca is a moderately thermophilic bacteria belonging to actinobacteria. It is known as a major degrader of plant cell wall material in rotting hay, compost heap, or manure piles since it can produces a wide variety of extracellular glycoside-hydrolyzing enzymes. Recently, the complete genome of the *T. fusca* YX strain has been sequenced and annotated.¹⁸⁾ To bring a new insight into the matter concerned, we have been studying on *T. fusca* MBL10003, a novel strain of *Thermobifida fusca* that was originally screened and isolated from soil sample by Meiji Co., Ltd. (Tokyo, Japan),^{19,20)} This actinobacteria was originally identified as mannose isomerase-producing microorganism that grew well at 50–60°C. Here, we characterized the mannose isomerase from *T. fusca* MBL10003 on molecular basis, and evaluated it in comparison to other mannose isomerases

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Abbreviation: *manI*, mannose isomerase gene.

derived from a variety of microbial sources.

A sequence of the gene encoding *T. fusca* MBL10003 mannose isomerase was determined as follows. First, a 453 bp gene fragment was obtained by PCR using forward and reverse primer designed from N-terminal amino acid sequences of the enzyme purified with sequential five step (DEAE-Sepharose, Resource ISO, Resource PHE, DEAE-COSMOgel and Superdex 200HR, Pharmacia AB., Uppsala, Sweden) column chromatography, and also its end-proteinase Glu-C digested fragment.²¹⁾ Then, unknown 5'- and 3'-regions were amplified by three step thermal asymmetric interlaced PCR (TAIL-PCR)^{22,23)} using combination of three sets of forward and reverse long specific primers, and three short arbitrary degenerate primers designed from the gene fragment sequence above, MBL10003 genomic DNA as template prepared with an Insta-Gene Matrix DNA preparation kit (Bio-Rad Laboratories Inc., Hercules, USA), TaKaRa LA Taq Polymerase, and GC buffer (Takara Bio Inc., Tokyo, Japan).

Finally, PCR was performed using forward (5'-CCTCCAC-GCGGCGCGGTGCGCGTATGCCGTTTC-3') and reverse (5'-CCATTCCCCGGCCTGCCCGGCGGGGCGCGC-GG-3') primer designed according to the sequence of TAIL-PCR fragment, genomic DNA as template, TaKaRa LA Taq Polymerase, and LA PCR buffer (Takara Bio Inc.). The DNA fragment obtained thus, was confirmed as a whole *T. fusca* MBL 10003 mannose isomerase gene (*manI*) through sequence analysis with ABI sequence analyzer (PerkinElmer Japan Co., Ltd., Tokyo, Japan). To obtain recombinant *manI* gene, *NcoI* and *XhoI* cleavage sites were inserted in upstream and downstream of the gene respectively, using PCR amplification with the specific primer set,

MBL10003 genomic DNA as template, TaKaRa LA Taq Polymerase, and GC buffer for 15 amplification cycles that consisted of denaturation at 98°C for 1 min, annealing at 74°C for 1.5 min, and extension at 72°C for 10 min. The resulting PCR products were ligated to the pET-30a (+) vector (Novagen Inc., Madison, USA) between the *NcoI* and *XhoI* site, yielding p30MITF, a plasmid carrying the *manI* gene of *T. fusca* MBL10003. The plasmid was transferred into *E. coli* BL21 Gold (Stratagene Inc., La Jolla, USA) by electroporation with an *E. coli* Pulser (Bio-Rad Laboratories Inc.) in a 0.1 cm cuvette at 1.80 kV. Transformants were cultured at 37°C in LB medium containing 50 µg/mL kanamycin until OD₆₀₀ reached approximately 0.5. After addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.1 mM, the cultures were grown for 21 h at 20°C on a rotary shaker at 150 rpm.

The deduced amino acid sequence encoded by the mannose isomerase gene, *manI*, from *Thermobifida fusca* MBL10003 was compared to homologs from other microbial strains (Fig. 1). The gene consists of 1,221 bp that is predicted to encode 407 amino acids with an estimated molecular mass of 43,900. The GC content, 72%, was considerably high, as is the case of actinobacteria.

In the *T. fusca* YX genome data base, the gene homologous to mannose isomerase is assigned as a putative protein.¹⁸⁾ Comparing the sequence obtained from *T. fusca* MBL10003 with the published sequence, there was only one amino acid substitution (Ser-26 in YX is substituted with Ala-26 in MBL10003) between these two genes. Hence, both genes are predicted to encode almost the same amino acid sequence. The accession number of *manI* gene of *T. fusca* MBL10003 was assigned as AB761401 in DDBJ/

Fig. 1. Comparison of amino acid sequences of mannose isomerases from several bacteria.

○, catalytic residue; ●, substrate binding residue. (1) *Thermobifida fusca* MBL 10003; (2) *Thermobifida fusca* YX (assigned as a putative protein in genome data base); (3) *Salinispora arenicola*; (4) *Salmonella enterica*; (5) *Pseudomonas syringae*; (6) *Enterobacter*; (7) *Escherichia coli*.

EMBL/Genbank data base. The predicted amino acid sequence of mannose isomerase was 45.7% identical to amino acid sequence of YihS (mannose isomerase) from *Salmonella enterica*²⁴⁾ and 38.5% identical to *A. radiobacter*.²⁵⁾ Based on the three-dimensional structure shown in *S. enterica* mannose isomerase,²⁴⁾ five amino acid residues in *T. fusca*, Trp-53, Tyr-114, Phe-118, Tyr-299, Tyr-382 are demonstrably involved in substrate binding, and contributing to the formation of deep cleft. Five amino acid residues, His-251, His-383, Arg-57, Glu-254, Glu-319 are essential for enzyme catalysis, and His-251 is thought to function as a base catalyst responsible for transferring the proton from C1 to C2 position in the isomerization step. Similar to *S. enterica* mannose isomerase, metal binding residues were not observed in amino acid sequence of *T. fusca* mannose isomerase. In *S. enterica*, the transient intermediate between aldo-keto interconversion was proposed as a *cis*-enediol due to a lack of metal binding residues. However, we obtained different results as to the enzyme from *T. fusca* (data not shown). His-383 may act for pyranose ring opening through the deprotonation of O1 atom and protonation of O5 atom in corporation with Glu-254. Thus, despite the overall level of amino acid identity among mannose isomerases, the catalytic center appears have an essential structure that is conserved in the characterized homologs.

E. coli transformants harboring a plasmid encoding *T. fusca manI* (p30XITF) were harvested by centrifugation and then disrupted by sonication for 3 min. Cell-free extract was collected by centrifugation of the sonicate at $8,000 \times G$ for 10 min, and then salted out with 80% NH_4SO_4 . The preparation was dialyzed against 50 mM phosphate buffer, and a crude recombinant mannose isomerase preparation was applied to a 5×50 mm Ni-nitrilotriacetic acid (NTA) agarose column (Qiagen GmbH, Hilden, Germany) for further purification. Mannose isomerase was eluted from the column with a linear gradient of 0.04 to 0.2 M imidazole in 50 mM phosphate buffer, pH 7.3 at a flow rate of 1 mL/min. Recombinant *T. fusca* mannose isomerase was purified to homogeneity from the *E. coli* extracts by NTA affinity chromatography, and proteins in each preparation or fraction were separated by SDS-PAGE. Through one-step affinity column chromatography, the enzyme was purified to a single

band on SDS-PAGE of 10% gel, the protein had an estimated molecular mass of 41 kD, the same value calculated from amino acid component. While, molecular mass obtained from gel filtration using Superdex 75 (GE Healthcare Bio-Sciences Corp., Piscataway, USA) was 89 kD, indicating that *T. fusca* mannose isomerase was a dimer. One other peak appeared in gel filtration chromatography at molecular mass of 48 kD was estimated as a monomeric enzyme, which had 60–70% mannose isomerase activity compared with dimer fraction. *E. coli* enzyme has been reported to be a tetramer and dissociated into a dimer with NaSO_4 treatment.¹⁵⁾ Contrast to this, dissociation into a monomer with NaSO_4 was not observed in *T. fusca* enzyme.

The reaction mixture used to measure recombinant mannose isomerase activity consists of 50 μL of 0.4 M mannose (final concentration 50 mM), 100 μL of 0.2% bovine serum albumin, 35 μL of 4 mM MgCl_2 , 200 μL of 50 mM phosphate buffer, pH 7.5, an appropriate amount of enzyme preparation, and distilled water in a total volume of 400 μL . Following incubation at 50°C for 10 min, the reaction mixture was immediately put on a heating block to stop the reaction. After removal of the precipitate by centrifugation at $12,000 \times G$ for 3 min, the supernatant was assayed for fructose content using a cysteine-carbazole-sulfuric acid method.²⁶⁾ One unit of enzyme activity was defined as one micromole of fructose formed per minute. In case of *N*-acetyl glucosamine 2-epimerase activity, *N*-acetyl mannosamine formed from *N*-acetyl glucosamine as substrate was measured using ICS-3000 (DIONEX) chromatographic system with a Carbo pac PA-1 column.

The enzyme activity of sonicated cell suspension of transformant *E. coli*, 7,550 U/g dry cell was 755 times as higher as that of the wild *T. fusca* MBL10003, 10 U/g dry cell. The temperature dependence of the enzyme activity was measured at each temperature from 30 to 90°C. The thermal stability of the enzyme was determined by measuring the residual activity following pre-incubation in the range from 30 to 90°C. The thermal profile and pH dependences of the *T. fusca* mannose isomerase are shown in Figs. 2 and 3, respectively. The optimum reaction temperature was approximately 60°C, and the optimum reaction pH appeared to be around pH 8.0. The enzyme was stable up to 60°C, and also stable between pH 4 and 11. These features

Fig. 2. Effect of temperature on mannose isomerase.

(a) Effect of temperature on enzyme activity: activity was measured over 10 min at each temperature at pH 7.5. (b) Heat stability: after pre-incubation at each temperature, residual activity over 10 min at 50°C was measured at pH 7.5.

Fig. 3. Effect of pH on mannose isomerase.

(a) Effect of pH on enzyme activity: activity over 10 min was measured at each pH. (b) pH stability of enzyme: after pre-incubation at each pH for 10 min, residual enzyme activity was measured over 10 min at pH 7.5. ◆, MacILvaine; ■, Na phosphate; ▲, Tris-HCl; ●, Glycine-HCl; concentration of each buffer is 50 mM.

are similar to those of most mannose isomerases described to date.

We examined seven sugars as potential substrates of *T. fusca* mannose isomerase. Among sugars tested, D-mannose (69.2 U/mg) and D-lyxose (18.0 U/mg) acted as substrates of *T. fusca* mannose isomerase. In contrast, the other sugars (hexoses: L-glucose, D-galactose, *N*-acetyl D-glucosamine, or pentoses: L-arabinose, D-xylose) little acted as substrates for this enzyme. These results suggested that the *T. fusca* enzyme is functionally different from *N*-acyl D-glucosamine 2-epimerase in spite of homologous amino acid sequence. Substrate specificity of the *T. fusca* enzyme was similar to other microbial mannose isomerases; however, the kinetic parameters of the enzyme were clearly different. The kinetic parameters of recombinant mannose isomerase for mannose and lyxose are summarized in the Table 1. The K_m values for mannose and lyxose; 115 and 537 mM, respectively, were almost the same as those of *E. coli* mannose isomerase; 108 and 405 mM, respectively.²⁴⁾ In contrast, the k_{cat} and, therefore, k_{cat}/K_m of the *T. fusca* mannose isomerase for mannose was approximately 30-fold larger than those of *E. coli* mannose isomerase. Thus, *T. fusca* mannose isomerase is demonstrated to be highly active than the other homologs with respect to the rate constant rather than the affinity constant. *T. fusca* MBL10003 is a strain originally exploited and isolated as mannose isomerase-producing microorganism, hence, the results obtained might be reasonable. The enzyme activity was considerably inhibited by the addition of divalent metal ions such as Cu^{2+} ; 37%, Cd^{2+} ; 45%, and Ca^{2+} ; 20%. Other metal ions examined; Mg^{2+} , Co^{2+} , Mn^{2+} , Zn^{2+} , Pb^{2+} , and also EDTA showed no significant effect on the enzyme activity. It was therefore considered that *T. fusca* mannose isomerase was not a metal-required enzyme. This can be successfully supported by amino acid sequence analysis, in which metal binding residues are not demonstrably found similar to the enzyme from *S. enterica*.²⁴⁾

The reaction equilibrium of enzyme-catalyzed isomerization between mannose and fructose was determined using each sugar as a substrate over a long time span. The reactions proceeded with time and finally reached equilibrium points of approximately mannose; 25% and fructose; 75% both from fructose and mannose as starting substrates. This findings indicated that the *T. fusca* mannose isomerase had

Table 1. Substrate specificity of mannose isomerase.

Substrate	Relative activity (%)
D-Mannose	100
D-Lyxose	26.6
D-Galactose	2.0
D-Arabinose	1.8
D-Xylose	<1.0
D-Glucose	<1.0
<i>N</i> -Acetyl glucosamine	<1.0

Ketose formed from corresponding aldose through enzymatic activity was measured colorimetrically using a cysteine-carbazole H_2SO_4 method. In case of *N*-acyl glucosamine 2-epimerase activity, *N*-acetyl mannamine formed from *N*-acetyl glucosamine as substrate was measured using ICS-3000(DIONEX) chromatographic system with a Carbo pac PA-1 column.

Table 2. Kinetic constants of mannose isomerase.

Kinetic parameter	D-Mannose	D-Lyxose
K_m (mM)	115 ± 15	537 ± 12
V_{max} ($mM \cdot s^{-1}$)	0.0116 ± 0.0006	0.00186 ± 0.00019
k_{cat} (S^{-1})	788 ± 40	63.3 ± 6.5
k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)	6.85 ± 0.20	0.118 ± 0.009

higher affinity toward fructose than mannose, similar to other mannose isomerase hitherto described.

The low conversion ratio of mannose like this, seems to be one of the bottlenecks for applicable production of mannose with mannose isomerase. It is therefore necessary to increase the mannose content of the product following enzymatic isomerization process. For this purpose, separation of mannose and fructose has been tried using membrane bioreactor combined to resin.²⁷⁾ Continuous reaction process with immobilized enzyme preparation has also been proposed to increase efficiency of the enzyme.^{17,27)} Anyway, In addition to basic enzymatic characterization, bio-process technologies like these are expected to develop for a wide variety of utilization of this promising sugar.

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