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Author(s)	Saburi, Wataru; Sato, Suzuka; Hashiguchi, Saki; Muto, Hirohiko; Iizuka, Takahisa; Mori, Haruhide
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1 **Enzymatic characteristics of D-mannose 2-epimerase, a new member of the acylglucosamine 2-**
2 **epimerase superfamily**

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5 Wataru Saburi,^{1,*} Suzuka Sato,¹ Saki Hashiguchi,¹ Hirohiko Muto,¹ Takahisa Iizuka,² and Haruhide
6 Mori^{1,*}

7 *¹Research Faculty of Agriculture, Hokkaido University, Kita 9, Nishi 9, Kita-ku, Sapporo 060-8589,*
8 *Japan*

9 *²Nihon Shokuhin Kako Co., Ltd., 30 Tajima, Fuji, Shizuoka 417-8530, Japan*

10

11 *Corresponding authors. Tel/Fax: +81-11-706-2508; E-mail: saburiw@chem.agr.hokudai.ac.jp (W.
12 Saburi). Tel/Fax: +81-11-706-2497; E-mail: hmori@chem.agr.hokudai.ac.jp (H. Mori).

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21 **Abstract**

22 Carbohydrate epimerases and isomerases are essential for the metabolism and synthesis of
23 carbohydrates. In this study, *Runella slithyformis* Runsl_4512 and *Dyadobacter fermentans* Dfer_5652
24 were characterized from a cluster of uncharacterized proteins of the acylglucosamine 2-epimerase (AGE)
25 superfamily. These proteins catalyzed the intramolecular conversion of D-mannose to D-glucose, whereas
26 they did not act on β -(1 \rightarrow 4)-mannobiose, *N*-acetyl-D-glucosamine, and D-fructose, which are substrates
27 of known AGE superfamily members. The k_{cat}/K_m values of Runsl_4512 and Dfer_5652 for D-mannose
28 epimerization were 3.89 and 3.51 $\text{min}^{-1}\text{mM}^{-1}$, respectively. Monitoring the Runsl_4512 reaction through
29 $^1\text{H-NMR}$ showed the formation of β -D-glucose and β -D-mannose from D-mannose and D-glucose,
30 respectively. In the reaction with β -D-glucose, β -D-mannose was produced at the initial stage of the
31 reaction, but not in the reaction with α -D-glucose. These results indicate that Runsl_4512 catalyzed the 2-
32 epimerization of the β -anomer substrate with a net retention of the anomeric configuration. Since ^2H was
33 obviously detected at the 2-C position of D-mannose and D-glucose in the equilibrated reaction mixture
34 produced by Runsl_4512 in $^2\text{H}_2\text{O}$, this enzyme abstracts 2-H from the substrate and adds another proton
35 to the intermediate. This mechanism is in accordance with the mechanism proposed for the reactions of
36 other epimerases of the AGE superfamily, that is, AGE and cellobiose 2-epimerase. Upon reaction with
37 500 g/L D-glucose at 50°C and pH 8.0, Runsl_4512 and Dfer_5652 produced D-mannose with a 24.4 and
38 22.8% yield, respectively. These D-mannose yields are higher than those of other enzyme systems, and
39 ME acts as an efficient biocatalyst for producing D-mannose.

40 **Keywords:** D-mannose; epimerase; cellobiose 2-epimerase; acylglucosamine 2-epimerase; D-mannose

41 isomerase

42

43 **Introduction**

44 Carbohydrates are the most abundant organic compounds in nature; they have diverse structures due
45 to presence of various monosaccharide units, linkages, and degrees of polymerization. To metabolize
46 carbohydrates, organisms utilize several intra- and extracellular enzymes, such as glycoside hydrolases,
47 glycoside phosphorylases, and sugar isomerases/epimerases. Moreover, these metabolizing enzymes have
48 great potential for application in carbohydrate synthesis. As only few carbohydrate types, including
49 starch, sucrose, and lactose, are abundantly available, enzymatic conversion of abundant sugars to rare
50 sugars is required to utilize rare sugars as foodstuffs and drugs.

51 Carbohydrate isomerases and epimerases catalyze the isomerization (conversion between aldose and
52 ketose) and epimerization (conversion between epimers) of carbohydrates, respectively. These enzymes
53 are essential in major carbohydrate metabolic pathways including glycolysis, oxidative/reductive pentose
54 phosphate pathways, and Leloir pathway, and provide suitable reactions for carbohydrate conversions.
55 For instance, isomerization of D-glucose to D-fructose by D-xylose isomerase (EC 5.3.1.5) is widely
56 applicable in the production of D-fructose-rich syrup (Bhosale et al. 1996). Furthermore, D-xylose
57 isomerase facilitates biofuel production from lignocellulosic biomass (Kuyper et al. 2004). Cellobiose 2-
58 epimerase (CE; EC 5.1.3.11) produces epilactose [β -D-galactopyranosyl-(1 \rightarrow 4)-D-mannose] from lactose
59 via 2-epimerization of reducing end sugar residue (Ito et al. 2008; Saburi et al. 2010). This disaccharide
60 has a prebiotic property (Watanabe et al. 2008) and enhances mineral absorption (Nishimukai et al. 2008;

61 Suzuki et al. 2010a; Suzuki et al. 2010b) and energy expenditure in the skeletal muscle and brown
62 adipose tissues in order to avoid obesity (Murakami et al. 2015). D-Tagatose 3-epimerase (EC 5.1.3.31)
63 isomerizes D-fructose to produce D-psicose (Takeshita et al. 2000). D-Psicose is not metabolized by
64 humans (Iida et al. 2010) and exhibits antiobesity activity by increasing the energy expenditure (Ochiai et
65 al. 2013; Ochiai et al. 2014). UDP-galactose 4-epimerase (EC 5.1.3.2) is applied to the enzymatic
66 production of lacto-*N*-biose I [β -D-galactopyranosyl-(1 \rightarrow 3)-*N*-acetyl-D-glucosamine], which is a part of
67 type I human milk oligosaccharide and has prebiotic property (Nishimoto et al. 2007; Kiyohara et al.
68 2009).

69 Acylglucosamine 2-epimerase (AGE) superfamily includes several carbohydrate epimerases and
70 isomerases: AGE (EC 5.1.3.8) (Itoh et al. 2000; Lee et al. 2007), CE (Saburi 2016), D-mannose isomerase
71 (MI; EC 5.3.1.7) (Kasumi et al. 2014; Saburi et al. 2018), and sulfoquinovose isomerase (SQI; EC
72 5.3.1.31) (Denger et al. 2014). These enzymes share an (α/α)₆-barrel folded catalytic domain (Itoh et al.
73 2000; Lee et al. 2007; Saburi et al. 2018; Fujiwara et al. 2013; Fujiwara et al. 2014; Itoh et al. 2008). Two
74 catalytic His residues on 8th and 12th α -helices of the catalytic domain and several residues for substrate
75 binding are completely conserved throughout the family. During epimerization of *N*-acetyl-D-
76 glucosamine and D-glucose residue of β -(1 \rightarrow 4)-disaccharides by AGE and CE, respectively, catalytic His
77 on the 12th α -helix abstracts the axial proton from 2-C of the substrate as general base catalyst in order to
78 generate the *cis*-endiolate intermediate, and His on the 8th α -helix donates the proton to the intermediate
79 as general acid catalyst for producing the epimerized product (Lee et al. 2007; Fujiwara et al. 2014).
80 During isomerization of D-mannose to D-fructose by MI, His on the 8th α -helix mediates intramolecular

81 proton transfer between 1-C and 2-C (Itoh et al. 2008; Saburi et al. 2018).

82 In this study, a new carbohydrate epimerase, D-mannose 2-epimerase (ME), which can catalyze the
83 2-epimerization of D-glucose and D-mannose, was found via the functional analysis of the *Runella*
84 *slithyformis* Runsl_4512 and *Dyadobacter fermentans* Dfer_5652 proteins present in an uncharacterized
85 cluster of AGE superfamily proteins (Fig. 1). Enzymatic properties of these enzymes and the mechanism,
86 through which they produce D-mannose from D-glucose, are described.

87

88 **Materials and methods**

89 *Phylogenetic analysis of AGE superfamily proteins*

90 Multiple sequence alignment of AGE superfamily enzymes was conducted using MAFFT version 7
91 (Kato et al. 2017), and a phylogenetic tree was prepared using all gap-free sites (69 amino acid
92 residues) through the neighbor joining method. The phylogenetic tree was visualized using a Figtree
93 program version 1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree/>).

94

95 *Preparation of expression plasmids*

96 The genes, encoding Runsl_4512 and Dfer_5652 (Genbank numbers, AEI50834.1 and ACT96842.1)
97 were amplified from the genomic DNA via PCR using Primestart HS DNA polymerase (Takara Bio,
98 Kusatsu, Japan). The genomic DNA of *R. slithyformis* DSM19594 and *D. fermentans* ATCC700827 were
99 purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany)
100 and the American Type Culture Collection (Manassas, VA, USA), respectively. The primer sequences are

101 listed in Table 1. Amplified PCR products were inserted into pET-23a vector (Novagen, Darmstadt,
102 Germany) via the restriction endonuclease sites: *Nde*I and *Xho*I sites for *Runsl_4512*, and *Eco*RI and *Xho*I
103 sites for *Dfer_5652*. The recombinant enzymes had extra eight residues, Leu-Glu-His-His-His-His-
104 His, at the C-terminal, and *Dfer_5652* also had 16 residues, Met-Ala-Ser-Met-Thr-Gly-Gly-Gln-Gln-Met-
105 Gly-Arg-Gly-Ser-Glu-Phe at the N-terminal. The DNA sequences of the insert and its flanking regions
106 were verified by sequence analysis using an Applied Biosystems 3130 Genetic Analyzer (Life
107 Technologies, Carlsbad, CA, USA).

108

109 *Preparation of recombinant enzymes*

110 Recombinant *Runsl_4512* and *Dfer_5652* were produced in transformant of *Escherichia coli* BL21
111 (DE3) carrying their expression plasmids. The transformants were grown in LB medium containing 100
112 µg/mL ampicillin, at 37 °C (1 L for *Runsl_4512* and 6 L for *Dfer_5652*). Recombinant proteins were
113 produced by adding 0.1 M isopropyl β-D-thiogalactopyranoside to a final concentration of 0.1 mM for
114 *Runsl_4512* and 0.05 mM for *Dfer_5652*, when absorbance at 600 nm reached 0.5. The induction was
115 carried out at 18 °C for 20 h for *Runsl_4512*, and at 37 °C for 4 h for *Dfer_5652*. Bacterial cells,
116 harvested via centrifugation at 6000 × *g* at 4 °C for 10 min, were suspended in 40 mL of 30 mM
117 imidazole-HCl buffer containing 0.5 M NaCl (pH 7.0; buffer A) for *Runsl_4512* and 30 mM imidazole-
118 HCl buffer containing 0.3 M NaCl (pH 8.0; buffer B) for *Dfer_5652*. The cells were disrupted by
119 sonication, and cell debris was removed via centrifugation at 18,000 × *g* at 4 °C for 10 min. The cell-free
120 extract obtained was applied onto a Ni²⁺-immobilized Chelating Sepharose Fast Flow column (2.8 cm i.d.

121 × 3.0 cm, GE Healthcare, Uppsala, Sweden) equilibrated with buffer A and B for Runsl_4512 and
122 Dfer_5652, respectively. The column was washed with the starting buffer, and the adsorbed protein was
123 eluted in a 30 to 500 mM linear gradient of imidazole (total elution volume, 200 mL). The fractions
124 containing highly purified protein, as demonstrated by SDS-PAGE, were pooled and dialyzed against a 10
125 mM Tris-HCl buffer (pH 7.0). The sample was concentrated to 4–10 mg/mL via ultrafiltration using
126 Vivaspin 20 centrifugal concentrators (nominal molecular weight limit 30,000 Da; Sartorius, Göttingen,
127 Germany). Runsl_4512 was stored at –20 °C in the presence of 50% (v/v) glycerol. Dfer_5652 was stored
128 at 4 °C without glycerol because this enzyme was fully stable under these conditions. Molar
129 concentrations of the purified enzymes were determined to be the average of the protein concentrations,
130 calculated, using Eq. 1, from the number of respective amino acids in one protein molecule and the
131 concentrations of respective amino acids after acid hydrolysis of the purified enzyme in 6 M HCl at
132 110 °C for 24 h.

133 Eq. 1: Protein concentration (μM) = [amino acid concentration, μM]/number of amino acid

134 Amino acid concentrations were measured using a JLC-500/V amino acid analyzer (JEOL, Tokyo,
135 Japan).

136 D187N mutation in Runsl_4512 was introduced with a Primestar Mutagenesis Basal Kit (Takara
137 Bio). The expression plasmid of Runsl_4512 as template and primers, as listed in Table 1, were used. The
138 mutant enzyme was prepared in a similar manner as the wild type.

139

140 *Blue native PAGE*

141 The purified Runsl_4512 and Dfer_5652 were evaluated by using blue native PAGE (BN-PAGE)
142 (Schägger et al. 1994). The analytical sample was prepared using a Native PAGE Sample Prep Kit (Life
143 Technologies). Native PAGE Novex 4–16% Bio-Tris Gels (Life Technologies) were used and
144 electrophoresis was performed at a constant 150 V for 115 min on ice. Native Mark Unstained Standard
145 (Life Technologies) was used as protein molecular size standards.

146

147 *Screening of substrates*

148 Activity of various substrates was examined via TLC and high-performance anion exchange
149 chromatography with pulsed amperometric detection (HPAEC-PAD). For TLC analysis, a reaction
150 mixture (5 μ L), containing 0.68–0.83 μ M enzyme, 50 mM substrate [D-lyxose, D-mannose, D-glucose, D-
151 tagatose, D-xylose (Wako Pure Chemical Industries, Osaka, Japan); D-talose, D-xylulose (Sigma, St.
152 Louis, MO, USA); D-fructose, D-galactose (Nacalai Tesque, Kyoto, Japan); β -(1 \rightarrow 4)-mannobiose,
153 prepared as described previously (Kawahara et al. 2012)], and 5 mM Tris-HCl buffer (pH 8.0), was
154 incubated at 37 $^{\circ}$ C for 4 h. About 1 μ L of the sample was spotted on a silica gel plate (TLC Silica gel 60
155 F₂₅₄ Aluminium sheet, Merck, Darmstadt, Germany) and developed by 2-propanose/1-butanol/water
156 (2/2/1, v/v/v). Sugars were detected by spraying a detection reagent [acetic acid/sulfuric
157 acid/anisaldehyde (100/2/1, v/v/v)] onto the plate, followed by heating of the plate. For HPAEC-PAD
158 analysis, a reaction mixture (50 μ L), containing 0.87–1.37 μ M enzyme, 50 mM substrate [*N*-acetyl-D-
159 glucosamine (Nacalai Tesque); *N*-acetyl-D-mannosamine, D-glucosamine (Tokyo Chemical Industry,
160 Tokyo, Japan); and D-mannosamine (Wako Pure Chemical Industries)], and 40 mM HEPES-NaOH buffer

161 (pH 8.0), was incubated at 37 °C for 12 h. The reaction mixture was analyzed using HPAEC-PAD under
162 following conditions: injection volume, 10 µL (10-fold diluted sample with water); column, Carbopac
163 PA1 (4 mm i.d. × 250 mm; Thermo Fischer Scientific, Waltham, MA, USA); eluent, 18 mM NaOH; flow
164 rate, 1 mL/min.

165

166 *ME activity assay*

167 The activity of epimerization to D-mannose was measured. A reaction mixture (100 µL) containing
168 enzyme, 10 mM D-mannose, and 40 mM HEPES-NaOH (pH 8.0) was incubated at 37 °C for 20 min. The
169 enzyme reaction was stopped by heating at 80 °C for 3 min, and 50 µL of the supernatant, obtained by
170 centrifuging the reaction mixture at 13,000 × g at 4 °C for 5 min, was mixed with 50 µL of a mixture
171 comprising 1 mM thio-NAD⁺ (Wako Pure Chemical Industries), 2 U/mL hexokinase (Nacalai Tesque), 2
172 U/mL glucose 6-phosphate dehydrogenase (Nacalai Tesque), 14 mM ATP (Sigma), 0.2 M Tris-HCl buffer
173 (pH 7.5), and 20 mM MgCl₂. This solution was incubated at 37 °C for 20 min, and its absorbance at 405
174 nm was measured. As a standard, 0–100 µM D-glucose containing 10 mM D-mannose was used. One unit
175 (U) of enzyme activity was defined as an enzyme amount producing 1 µmol of D-mannose in 1 min under
176 these conditions.

177 Kinetic parameters for the epimerization of D-mannose were determined from the reaction rates as
178 4–100 mM D-mannose. The Michaelis–Menten equation was fitted to the reaction rates by nonlinear
179 regression using the Grafit ver. 7.0.2 software (Erithacus Software, East Grinstead, UK).

180

181 *Assaying the epimerization activity of D-mannose 6-phosphate*

182 A reaction mixture (200 μ L), containing enzyme, 10 mM D-mannose 6-phosphate disodium salt
183 (Sigma), and 40 mM HEPES-NaOH buffer (pH 8.0), was incubated at 37 $^{\circ}$ C for 30 min. Fifty μ L of the
184 reaction mixture was collected every 10 min and heated at 100 $^{\circ}$ C for 3 min to stop the reaction. D-
185 Glucose 6-phosphate was quantified as follows: 50 μ L of the sample was mixed with 20 μ L of mixture
186 containing 2.5 mM thio-NAD⁺ and 5 U/mL D-glucose 6-phosphate dehydrogenase, and was then
187 incubated at 37 $^{\circ}$ C for 30 min. Absorbance at 405 nm was measured, and D-glucose 6-phosphate
188 concentration was calculated from a standard curve of D-glucose 6-phosphate disodium salt (0–250 μ M;
189 Oriental Yeast, Tokyo, Japan).

190

191 *Effects of pH and temperature on activity and stability*

192 Optimum pH was determined from the activities at various pH values. The pH of the reaction was
193 varied using 100 mM Britton–Robinson buffer (pH 4.0–11.0) as reaction buffer. Optimum temperature
194 was evaluated based on the activity at 20–70 $^{\circ}$ C. Residual activities after the pH and heat treatments were
195 measured to evaluate the enzyme stability. During the pH treatment, the enzyme solution was incubated at
196 pH 2.3–12.1 using 50 mM Britton–Robinson buffer at 4 $^{\circ}$ C for 24 h; whereas, during the heat treatment,
197 the enzyme solution was incubated at 20–100 $^{\circ}$ C for 20 min. The pH and temperature ranges, in which
198 the enzyme retained more than 90% of its original activity, were regarded as stable ranges.

199

200 *Production of D-mannose from D-glucose*

201 A reaction mixture (1 mL) containing 500 g/L D-glucose, 0.25 U/mL Runsl_4512 or Dfer_5652, and
202 50 mM HEPES-NaOH (pH 8.0) was incubated at 50 °C for 48 h. One hundred μ L of the reaction mixture
203 was collected at a stipulated time, diluted with water 10 times, and heated at 100 °C for 5 min to stop the
204 reaction. The sugar content of the reaction mixture was analyzed via HPLC under the following
205 conditions: sample injection volume, 10 μ L; column, Hilic Pac VG50 4E (4.6 mm i.d. \times 250 mm;
206 Shodex, Tokyo, Japan); column temperature, 40 °C; eluent, 80% (v/v) acetonitrile; flow rate, 0.6 mL/min;
207 and detection, refractive index. Authentic D-glucose, D-mannose, and D-fructose were used as standards.

208

209 *NMR analysis of the reaction products of Runsl_4512*

210 The epimerization of D-mannose and D-glucose by Runsl_4512 was monitored by $^1\text{H-NMR}$. H_2O in
211 the enzyme solution was replaced with $^2\text{H}_2\text{O}$: the enzyme solution was diluted with 10 mM HEPES-
212 NaO^2H buffer in $^2\text{H}_2\text{O}$ (p ^2H 8.0) by 3-fold and was further concentrated to the original volume via
213 ultrafiltration as aforementioned. These steps were repeated four times in total. $^1\text{H-NMR}$ of a reaction
214 mixture (0.5 mL), containing Runsl_4512 (23 and 73 μM for the reactions with D-mannose and D-
215 glucose, respectively), 250 mM substrate, and 10 mM HEPES-Na buffer (p ^2H 8.0), was recorded at 27 °C
216 using Bruker AMX500 (Billerica, MA, USA). In the reactions with D-glucose and D-mannose, 22.5 mg of
217 substrate powder was dissolved in 50 μL of $^2\text{H}_2\text{O}$ and incubated at 25 °C for 24 h to obtain the
218 equilibrium mixture of α - and β -anomers. This substrate solution was mixed with 450 μL of the enzyme
219 solution in 10 mM HEPES- NaO^2H buffer (p ^2H 8.0), and this mixture was immediately subjected to the
220 $^1\text{H-NMR}$ analysis. In the reactions with α -D-glucose (Sigma) and β -D-glucose (Sigma), 22.5 mg of sugar

221 was dissolved with 0.5 mL of the enzyme solution in 10 mM HEPES-NaO²H buffer (p²H 8.0) just before
222 the reactions.

223 The reaction mixture with D-mannose was incubated at 25 °C for 24 h to prepare an equilibrium
224 reaction mixture. ¹H-NMR of this mixture was recorded, and the spectrum was compared with the
225 equimolar mixture (250 mM) of D-glucose and D-mannose. This reaction mixture was dried up under
226 reduced pressure, and the residue was dissolved in H₂O. ²H-NMR was recorded to detect ²H in the
227 reaction products.

228

229 **Results**

230 *Determination of catalytic activity of Runsl_4512 and Dfer_5652*

231 Runsl_4512 and Dfer_5652, belonging to a cluster of uncharacterized proteins in a phylogenetic
232 analysis of AGE superfamily enzymes (Fig. 1), were emphasized in this study. The amino acid sequence
233 of Runsl_4512 is 74% identical with that of Dfer_5652, and sequence identities of Runsl_4512 and
234 Dfer_5652 with characterized AGE superfamily members are as follows: *Ruminococcus albus* CE, 24%
235 and 24%; *Rhodothermus marinus* CE, 25% and 25%; *Anabaena* sp. AGE, 16% and 17%; *E. coli* SQI,
236 14% and 13%; *Marinomonas mediterranea* MI, 18% and 19%; and *Thermobifida fusca* MI, 12% and
237 17%, respectively.

238 To investigate the enzymatic functions of Runsl_4512 and Dfer_5652, recombinant enzymes were
239 produced in *E. coli* transformant, and purified. These proteins were displayed as single bands on SDS-
240 PAGE and BN-PAGE (Fig. 2). The molecular masses of Runsl_4512 and Dfer_5652 were estimated to be

241 45 kDa (theoretical molecular mass, 50 kDa) and 47 kDa (theoretical molecular mass, 52 kDa) on SDS-
242 PAGE, and 81 kDa and 101 kDa on BN-PAGE, respectively. These results suggested that Runsl_4512 and
243 Dfer_5652 exist as homodimers under nondenaturing conditions. The purified samples were then
244 incubated with various carbohydrates to find their substrates, and D-mannose and D-glucose were detected
245 as the reaction products from D-glucose and D-mannose, respectively (Fig. 3). This result indicates that
246 these proteins catalyzed the epimerization of D-mannose (ME reaction). No reaction products were
247 detected in the reactions with D-galactose, D-talose, D-xylose, D-lyxose, D-glucosamine, D-mannosamine,
248 *N*-acetyl-D-glucosamine, *N*-acetyl-D-mannosamine, β -(1 \rightarrow 4)-mannobiose, D-mannose 6-phosphate, D-
249 fructose, D-tagatose, and D-xylulose.

250 Enzymatic properties of Runsl_4512 and Dfer_5652 were investigated based on the ME activity. The
251 ME activity at pH 4.0–11.0 was measured to evaluate the optimum pH. Runsl_4512 and Dfer_5652 were
252 most active at pH 7.8 and 8.1, respectively. Optimum temperature was evaluated based on the activity at
253 20–70 °C. Both enzymes exhibited highest activity at 37 °C. Stable pH and temperature ranges of these
254 enzymes were pH 6.3–10 (4 °C for 24 h) and at \leq 40 °C (pH 8.0 for 20 min), respectively. The Michaelis–
255 Menten equation fitted well with the reaction rates of Runsl_4512 and Dfer_5652 at various D-mannose
256 concentrations (Fig. 4). The K_m values were determined from the concentration of D-mannose, including
257 both α - and β -anomers. The k_{cat} and K_m of Runsl_4512 were 286 min⁻¹ and 73.5 mM, and those of
258 Dfer_5652 were 233 min⁻¹ and 66.4 mM, respectively (Table 2).

259

260 *Production of D-mannose from D-glucose*

261 D-Mannose was enzymatically synthesized from 500 g/L D-glucose (Fig. 5). The concentrations of D-
262 mannose reached 122 g/L and 114 g/L, 48-h after reaction with Runsl_4512 and Dfer_5652, respectively.
263 Molar rates of conversion to D-mannose using Runsl_4512 and Dfer_5652 were 24.4% and 22.8%,
264 respectively. Faint D-fructose production was observed: D-fructose concentrations after 48-h reactions
265 with Runsl_4512 and Dfer_5652 were 8.5 and 7.9 g/L, respectively. These D-fructose concentrations were
266 consistent with those observed after a 48-h reaction without enzyme 8.3 g/L. Thus, the D-fructose
267 generated in ME reaction mixtures was regarded as a nonenzymatic reaction product. Nonenzymatic
268 epimerization was not observed under the reaction conditions.

269

270 *NMR analysis of the reaction products by Runsl_4512*

271 The anomeric configuration of the products of reactions carried out using Runsl_4512 was analyzed
272 through ¹H-NMR. In the reactions with D-mannose and D-glucose (equilibrium mixture of α - and β -
273 forms), the 1-H signals of β -D-glucose (4.63 ppm) and β -D-mannose (4.88 ppm) were detected from the
274 initial stage of the reactions whereas the 1-H signals of α -D-glucose (5.22 ppm) and α -D-mannose (5.18
275 ppm) were not (Fig. 6A and B). This result indicates that β -D-glucose and β -D-mannose were produced
276 from D-mannose and D-glucose, respectively. Production of the epimerized product from α - and β -D-
277 glucose was monitored (Fig. 6C and D). The 1-H signal of β -D-mannose was detected at the initial stage
278 of the reaction with β -D-glucose, but in the reaction with α -D-glucose, this signal was scarce and was only
279 observed in the later stages, along with the production of β -D-glucose. Thus, Runsl_4512 used β -D-
280 glucose as the substrate.

281 The equilibrium reaction mixture, produced from D-mannose in $^2\text{H}_2\text{O}$, was analyzed by ^1H - and ^2H -
282 NMR analyses (Fig. 6E). In the ^1H -NMR spectrum of the equilibrium reaction mixture, 2-H signals of D-
283 mannose (3.9 ppm) and D-glucose (3.2 ppm and 3.5 ppm for β - and α -D-glucose, respectively) were not
284 detected, whereas ^2H signals were clearly detected at the corresponding chemical shifts of the 2-H of D-
285 mannose and D-glucose by ^2H -NMR. This result indicates that Runsl_4512 abstracts 2-H from the
286 substrates and adds ^2H from the reaction solvent.

287

288 *Site-directed mutagenesis at Asp187 of Runsl_4512*

289 Multiple sequence alignment of AGE superfamily enzymes was carried out to compare the amino
290 acid sequences of MEs with those of the other members (Fig. 7). His273 and His404 of Runsl_4512 and
291 Dfer_5652 correspond to the catalytic His residues of known enzymes, which are situated on the 8th and
292 12th α -helices of the catalytic (α/α)₆-barrel domain. Arg60, Tyr119, His191, Glu276, and Trp336 of
293 Runsl_4512 and Dfer_5652 also correspond to the substrate-binding residues of CE, MI, and SQI, which
294 act on the substrates harboring 2-OH group. Nevertheless, in the ME sequences, Asp187 was observed as
295 the equivalent residue of Asn on α -helix 6 of CE, MI, and SQI, which has a hydrogen bonding interaction
296 with 2-OH of substrates (Fujiwara et al. 2014, Itoh et al. 2008). D187N mutant of Runsl_4512 was
297 prepared to evaluate the importance of Asp187. The activity of this mutant enzyme to 10 mM D-mannose
298 was not detectable (<0.002 U/mg), while that of wild type was 0.61 U/mg. Thus, Asp187 is essential for
299 the catalytic activity of Runsl_4512.

300

301 **Discussion**

302 Carbohydrate epimerization and isomerization are essential reactions to convert abundant
303 sugars into rare sugars. In this study, reversible 2-epimerization activity between D-mannose and D-
304 glucose was observed in Runsl_4512 and Dfer_5652 proteins belonging to a yet uncharacterized protein
305 cluster of AGE superfamily (Fig. 1). This activity was also detected in several CEs (Park et al. 2011;
306 Saburi et al. 2015); however, the k_{cat}/K_m values of Runsl_4512 and Dfer_5652 were 4.1–140-fold higher
307 than CEs from *Caldicellulosiruptor saccharolyticus* and *Cellvibrio vulgaris* (Table 2). In contrast to CEs,
308 Runsl_4512 and Dfer_5652 did not present any detectable epimerization activity to β -(1→4)-linked
309 disaccharides.

310 Sequence comparison between MEs and function-known AGE superfamily enzymes revealed that
311 the MEs have residues corresponding to two catalytic His residues and a majority of residues important
312 for substrate-binding in MI, SQI, and CE (Fig. 7); ME presumably shares the mechanisms of substrate
313 binding and catalysis with these enzymes. Among the possible residues involved in the substrate binding,
314 only Asp187 of Runsl_4512 and Dfer_5652 differs from the corresponding residue of CE and MI as
315 aforementioned. As Asp187 was confirmed to be essential for the catalytic activity through D187N
316 mutation in Runsl_4512, it might have specific function for ME activity. Multiple-sequence alignment
317 suggests that ME has a longer loop connecting the 7th and 8th α -helices ($\alpha 7 \rightarrow \alpha 8$ loop) than any
318 characterized AGE superfamily member (Fig. 7). In MI, the corresponding loop is predicted to cause
319 steric hindrance upon binding to the disaccharide substrate (Saburi et al. 2018). Therefore, long $\alpha 7 \rightarrow \alpha 8$
320 loop of ME might be important for the monosaccharide specificity. Three-dimensional structure analysis

321 of MEs would provide essential information to understand the structure–function relationship of this
322 enzyme.

323 Analysis of anomeric configuration of the reaction product by $^1\text{H-NMR}$ revealed that ME forms the
324 product of β -anomer. This anomeric configuration of the reaction product is in accordance with that of β -
325 (1 \rightarrow 4)-disaccharides bound to CE (Fujiwara et al. 2014), and the isomerized product, D-mannose, from
326 D-fructose by MI (Saburi et al. 2018). In the reaction with β -D-glucose, β -D-mannose was formed from
327 the initial stage of the reaction; however, this was not observed in the reaction with α -D-glucose. Thus,
328 ME utilizes β -glucose as a substrate, that is, the anomeric configuration of the substrate is retained in the
329 product. Slight production of β -D-mannose in the later stage of the reaction with α -D-glucose is
330 presumably attributable to the reaction with β -D-glucose, generated from α -D-glucose via mutarotation.
331 Retention of β -anomer in the substrate and product is presumably suitable for the ring opening and
332 closure mechanism catalyzed by His on the 12th α -helix as general acid/base catalyst, which forms
333 hydrogen bonds with 1-O and 5-O of the substrate of β -anomer (Fujiwara et al. 2014).

334 The NMR analysis of the equilibrated reaction product indicated that 2-H of the substrate was
335 substituted with ^2H from solvent $^2\text{H}_2\text{O}$, indicating that ME epimerizes the substrate via proton abstraction
336 and addition mechanisms as postulated in CE and AGE (Lee et al. 2007; Fujiwara et al. 2014). Based on
337 the sequence comparison between ME and CE, it is predicted that His273 of Runsl_4512 and Dfer_5652
338 serves as general base catalyst to abstract 2-H from D-mannose and produce *cis*-endiolate intermediate,
339 and His404 serves as general acid catalyst to donate proton to the intermediate and produce D-glucose
340 (Fig. 8).

341 D-Mannose has several beneficial physiological properties, such as preventing urinary tract
342 infections (Kranjčec et al. 2014) and treating phosphomannose isomerase deficient induced congenital
343 disorders of glycosylation (de Lonlay and Seta 2009), and it could be widely applied as an important
344 intermediate for synthesizing immunostimulatory agents (Ranta et al. 2012), vitamin (Chen et al. 2007),
345 and D-mannitol (Ghoreishi and Shahrestani 2009). Furthermore, D-mannose is useful as an animal feed
346 for suppressing *Salmonella* contamination (Oyofe et al. 1989). Since the decomposition of an abundant
347 naturally occurring D-mannose polymer, β -mannan, requires rigorous conditions, the enzymatic
348 production of D-mannose from other monosaccharides, especially D-glucose, is useful. Isomerization of
349 D-fructose by MI (Hirose et al. 2003; Hu et al. 2016), D-lyxose isomerase (Park et al. 2010a), and L-
350 rhamnose isomerase (Park et al. 2010b) and the epimerization of D-glucose to D-mannose by CE (Park et
351 al. 2011) have been reported to date. The production yield of D-mannose from 200–500 g/L D-fructose
352 through isomerization by the abovementioned isomerases at 45–85°C and pH 7.0–8.0 was 20–35%
353 (molar ratio). As D-fructose is produced from D-glucose by enzymatic isomerization at 60°C and pH 6.0–
354 8.0 using D-xylose isomerase, at approximately 40% yield (molar ratio; Bhosale et al. 1996), the
355 production yield of D-mannose from D-glucose through two-step isomerization, is estimated to be 8–14%
356 (molar ratio). In fact, that of D-mannose by the coupling reaction of D-xylose isomerase and D-lyxose
357 isomerase with 400 g/L D-glucose at 55°C and pH 6.5 was reported to be 16% (Huang et al. 2018). In D-
358 mannose production from 500 g/L D-glucose at 75°C and pH 7.5 by *C. saccharolyticus* CE, the yield of
359 D-mannose from D-glucose was only 15% (molar ratio) due to its isomerization activity (yield of D-
360 fructose was 9.5%) (Park et al. 2011). Isomerization activity was not detected in MEs under the analytical

361 conditions, and the production yield of D-mannose from 500 g/L D-glucose at 50°C and pH 8.0 by ME
362 (23–24%, molar ratio) was higher than that observed for the reactions previously reported. Thus, ME is a
363 useful biocatalyst for enzymatic D-mannose production, and further study of ME for application in D-
364 mannose production could provide a new and efficient synthetic pathway for D-mannose.

365

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372

373 **Compliance with ethical standards**

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379

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502

503 Figure caption

504 **Fig. 1** Phylogenetic analysis of AGE superfamily proteins

505 Proteins of functionally known AGE superfamily enzymes are presented. AGE, acylglucosamine 2-

506 epimerase; CE, cellobiose 2-epimerase; MI, D-mannose isomerase; and SQI, sulfoquinovose isomerase.

507

508 **Fig. 2** SDS-PAGE and BN-PAGE of Runsl_4512 and Dfer_5652

509 Recombinant Runsl_4512 and Dfer_5652, purified from *E. coli* transformant, were analyzed on SDS-

510 PAGE and BN-PAGE. Lane M, R, and D indicate molecular mass standard, Runsl_4512, and

511 Dfer_5652.

512

513 **Fig. 3** TLC analysis of the reaction products by Runsl_4512 and Dfer_5652

514 Plus and minus lanes indicate the reactions with and without the enzyme, respectively. A, Runsl_4512; B,

515 Dfer_5652.

516

517 **Fig. 4** The s-v plot for D-mannose epimerization by Runsl_4512 and Dfer_5652

518 Close and open circles indicate Runsl_4512 and Dfer_5652, respectively. Values and error bars are

519 average and standard deviation of three independent experiments.

520

521 **Fig. 5** Time course of production of D-mannose from D-glucose

522 Black circle, D-glucose; white circle, D-mannose; and black triangle, D-fructose. Values indicate mean \pm
523 standard deviation for three independent experiments.

524

525 **Fig. 6** NMR analysis of ME reaction products

526 Time course of the ME reaction was monitored using $^1\text{H-NMR}$. A, reaction with mixture of α -D-mannose
527 and β -D-mannose; B, reaction with mixture of α -D-glucose and β -D-glucose; C, reaction with α -D-
528 glucose; and D, reaction with β -D-glucose. Reaction time (min) is presented on the left side of the figures.
529 E, $^1\text{H-NMR}$ and $^2\text{H-NMR}$ of equilibrated reaction mixture of ME. The spectrum of the equimolar mixture
530 of D-glucose and D-mannose (Man + Glc) is shown as standard.

531

532 **Fig. 7** Multiple sequence alignment of AGE superfamily enzymes

533 Multiple sequence alignment was performed using MAFFTFFTash (Standley et al. 2007). The
534 secondary structure is depicted above the sequence. Amino acid residues involved in substrate binding
535 (reducing end part of disaccharides in CE) are presented in bold face. Catalytic His residues are indicated
536 by black circles. Asp187 of Runsl_4512 and Dfer_5652 is indicated by black triangle. RaCE,
537 *Ruminococcus albus* CE; RmCE, *Rhodothermus marinus* CE; MmMI, *Marinomonas mediterranea* MI;
538 TfMI, *Thermobifida fusca* MI; EsSQI, *Escherichia coli* SQI; and AspAGE, *Anabaena* sp. AGE.

539

540 **Fig. 8** Predicted reaction mechanism of ME

541 Arrows indicate electron transfer processes that convert β -D-mannose to β -D-glucose. The bond
542 between 2-C and 3-C would rotate after acceptance of a proton from the general acid catalyst, His404, in
543 the epimerization of D-mannose, according to the reaction mechanism of CE (Fujiwara et al. 2014).

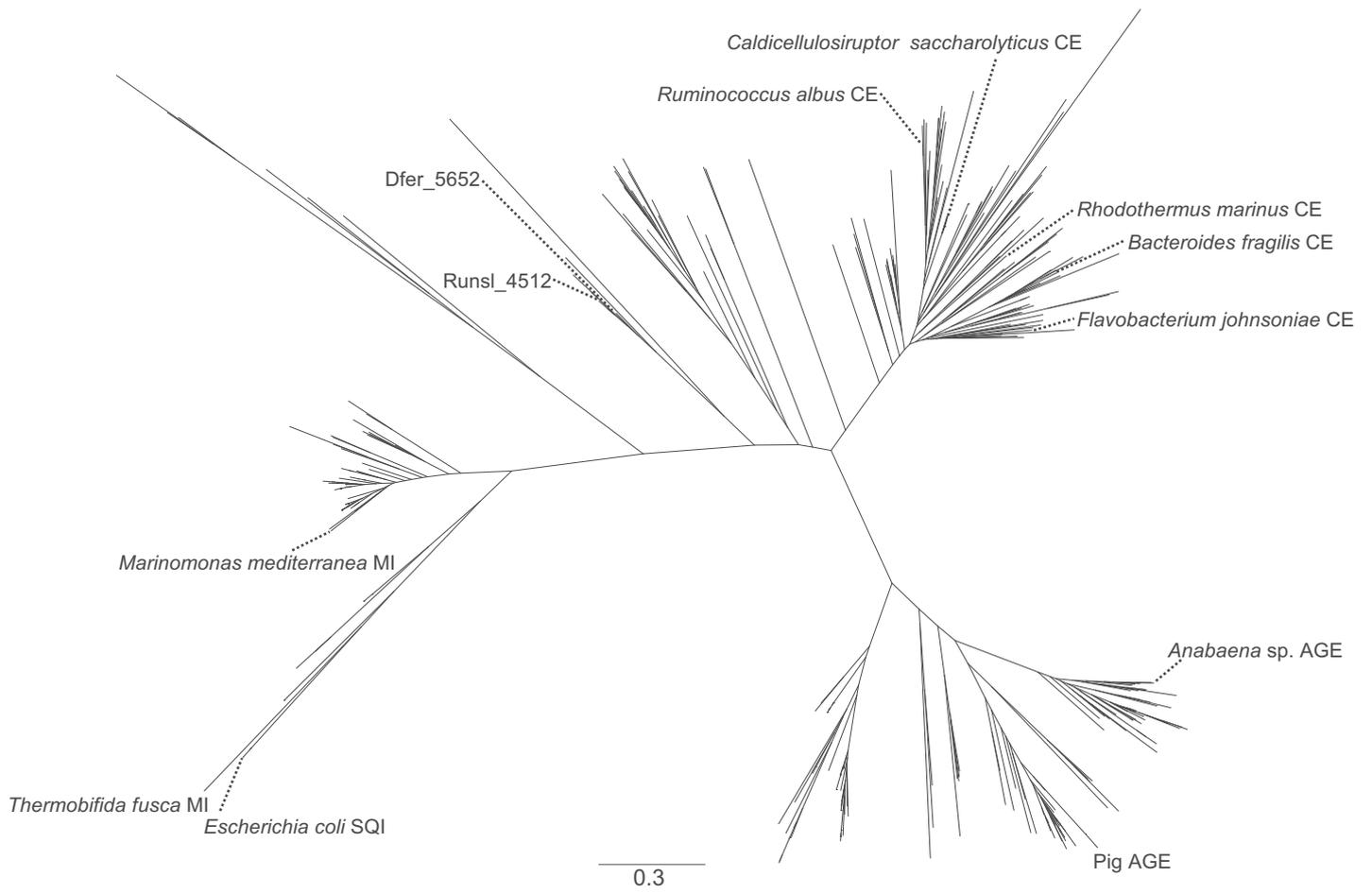


Fig. 1, Saburi et al.

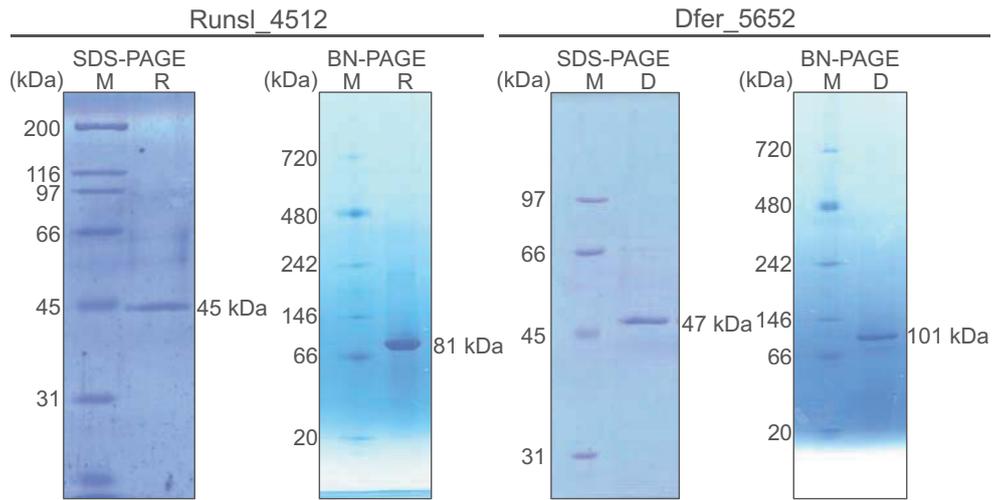


Fig. 2., Saburi et al.

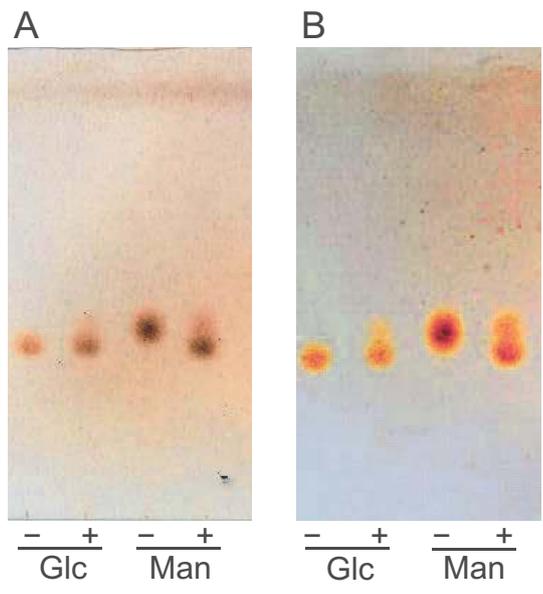


Fig. 3., Saburi et al.

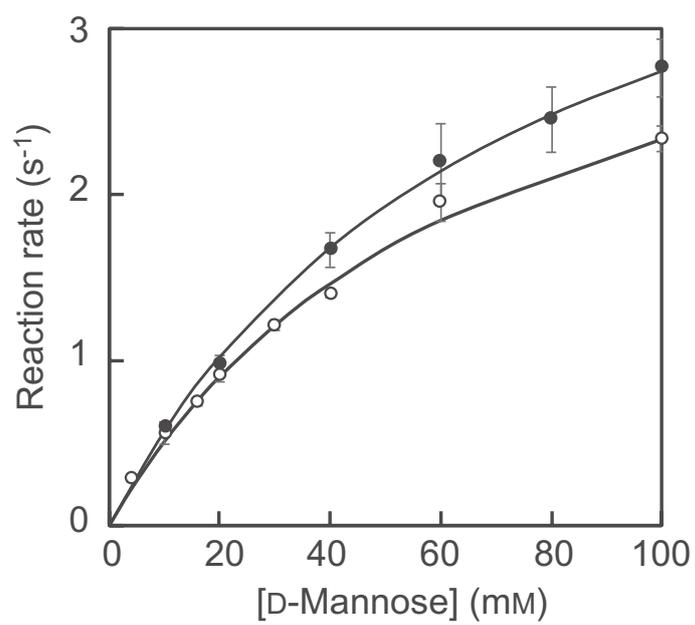


Fig. 4., Saburi et al.

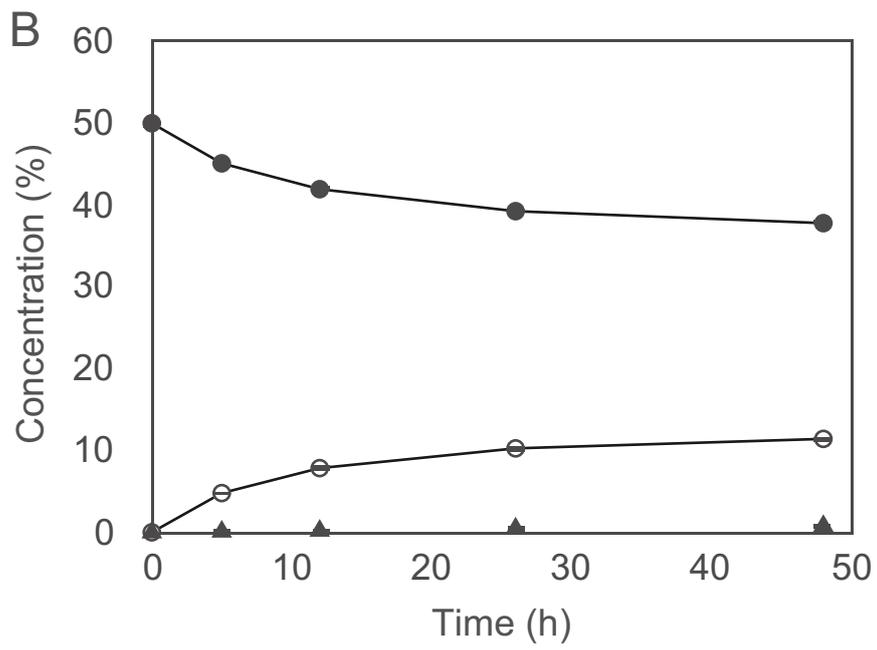
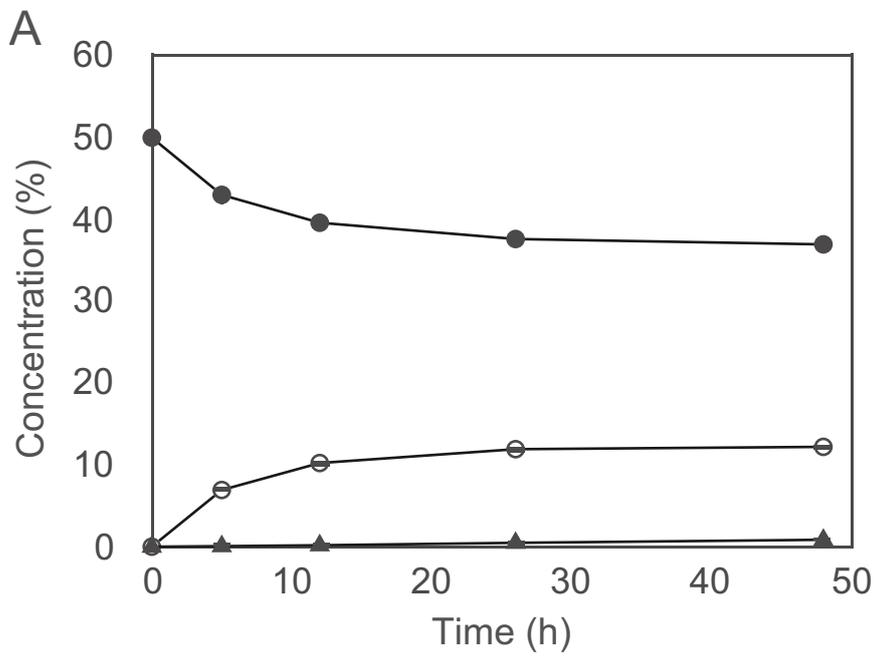


Fig. 5., Saburi et al.

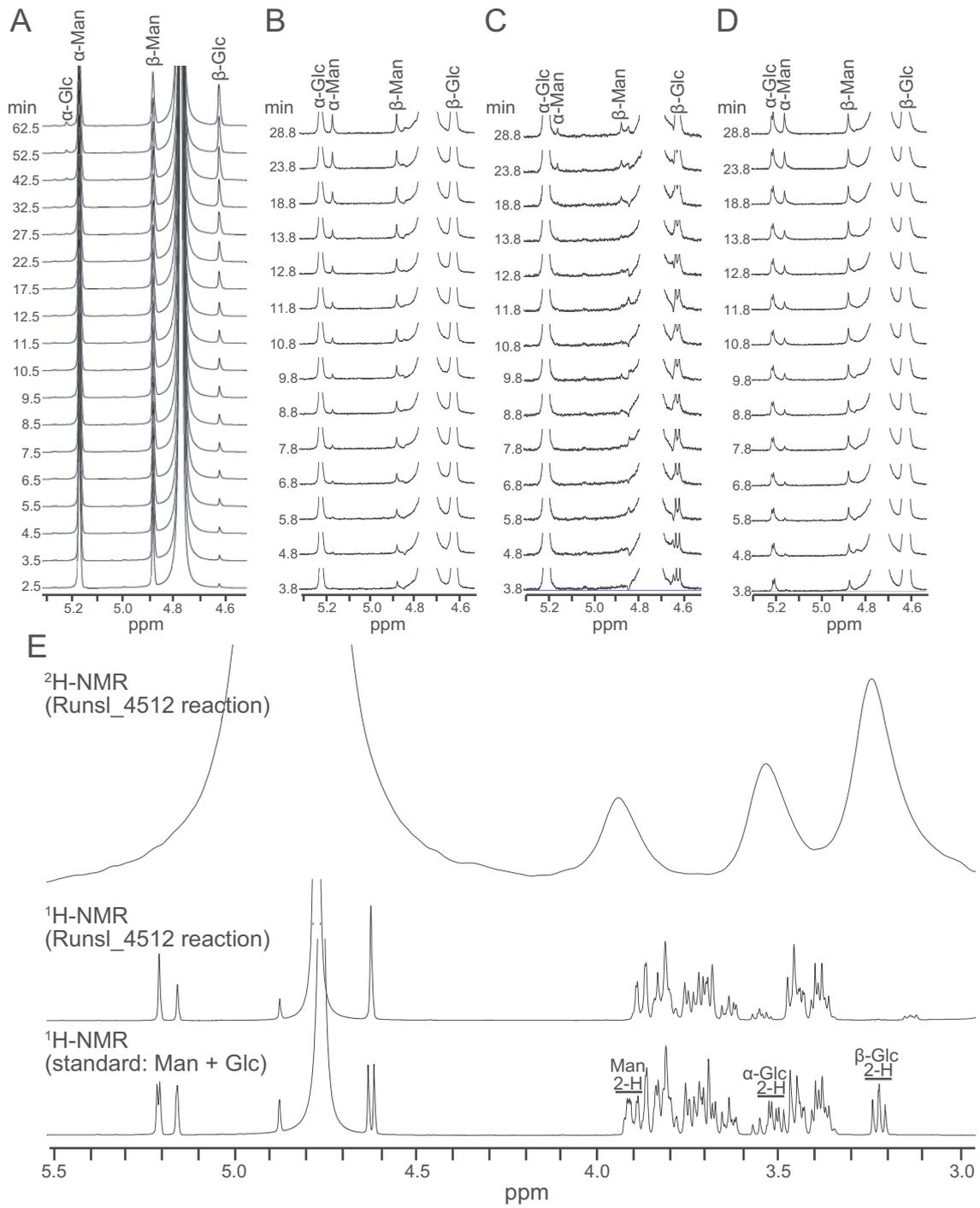


Fig. 6. Saburi et al.

	α-helix 1	α-helix 2							
Runsl_4512	-----MTSEKIASLRQEIETYLTNTGLLPFWITRTVTDKENGGFLLTHFDQFGNDSGEDEKSLIAQSR	RSVFYTYSSAHRAGYG	74						
Dfer_5652	-----MNVDQLKSYREEIRQHLTSELLPFWENRAVDRENGGFI THFDNAGNDSGEDEKSLIAQTR	RTVYTFSSAHRAGYG	74						
RaCE	-----MMISEIRQELTDHIIIPFWNK-LRDDENGFFGYGLSYGLGLDKKADKGVILHSR	ILWLFYSNAYM-TLG	65						
RmCE	MSTETIPDVRRLRALQAEVHEELTENILKFWATRTHDPVHGGFVGRVGPDRPHPEAPRGAILNAR	ILWTFAAAYR-QLG	79						
MmMI	-----MSYPAFDSKTFLEAHIE-KTMAFYFPTCIDP-EGGFFQFFKDDGSVYDPNTRHLVSSST	RFIFNFAQAYL-HTN	70						
TfMI	-----MTLWTAARAAHRAWLDAEAR-RLVDFAA--AADHPEHGF-AWLDGSGAPLPEQGVHTWITC	RVTHVAALAHL--EG	69						
EcSQI	-----MKWFNTLSHNRWLEQETD-RIFDFGK-NSVV--PTGF-GWLGKNGQIKKEEMGTHLWITAR	MHLVYSVAAA--MG	67						
AspAGE	-----MGKNLQALAQLYKNALLNDVLPFWENHSLDS-EGGYFTCLDRQGVY-DTKFIWLQNR	QVWTFMSLCNQLK	71						
	α-helix 3	α-helix 4	α-helix 5						
Runsl_4512	GGVLAEMARHGVDYLINNMWDNEHGGFYWMTNRKGEVTDQKIVY	GLSFCIYLSSEYTLATGDP	PRGREYAEKTFD	LLQKY	154				
Dfer_5652	EGRYADIARHGVDFLINKMWDNEYGGFYWLMDRKGNVNI	DEKIVYGH	SFAIYSLAEYTLATGDP	RGLEYAEKVF	DLQKH	154			
RaCE	GDELLDNAKHAYEFIKNNCIDYEGGVYWMDFEGKPADTMKHT	YNI	AFAIYALSSY	YRASGDKEALALAYR	PFEDIEKN	145			
RmCE	TPLYREMAERAYRYFVRHFVDAEHGGVYWMVAADGRPLDTRKHV	YAQ	SFAIYALSEWHRATG	GEAALALARSY	DLIETH	159			
MmMI	IAEYKHAHVHGIQYLRQRH-QSQSGGYVWLLD-GGTNLDET	NHCY	GLAFVILAYS	SNALQ-IGLSEAEV	WIEVYTDLLETH	147			
TfMI	IPGASALADHGLRALAGPLRDPEDHGWFTALDSRGTVADSRKEA	YQHAFVLLAAASATV-AGRPGARELLD	AAA	AVIEQR		148			
EcSQI	RPGAYSLVDHGKAMNGALRDKKYGWYACVN-DEGVVDASKQ	GYQHFFALLGAASAVT-TGHPEARKLLD	YTI	IEI	IEKY	145			
AspAGE	RENWLKIARNGAKFLAQHGRD-DEGNWYFALTRGGEPLVQ	PNIFSDCFAAMAFSQYALASGE	EWAQKDVAMQ	AYNNVLR		150			
	α-helix 6	α-helix 7							
Runsl_4512	AVDTHYGGYFEMFNRDWTLKG-----PGAAGGRKTL	DVHMHLMEAYTTLYECTGQ-EI	HRRKLETTIELLVNKMVH-PE	227					
Dfer_5652	GADTYGGYFEMFHRNWDLKG-----PGAAGGRKTL	DAHMHLEAFTTLYEASGK-QV	HRRKLV EIRLLINKIMH-PQ	227					
RaCE	TLYEY--GYREAFDRQWRLV-DNEALSENGLKADKTM	NAIHLHLEI	EAYTELYKADGN-EKVADR	LKFQLGQMRD	IVYT-PD	220			
RmCE	CADR VHGGYVEACDRAWRPLEDARL-SAKDAPEPRSM	NTHLHVLEAYANLYRVWPE-TE	LAARLQALIELFLRAIYH-PA	236					
MmMI	FWENKHGLYLDEISSDWKTV-----SPYRQ	NANMHMCEALMSAF	DATQN-PKYLDRAKLLAKNICQ	KQASLSN	215				
TfMI	FWEEETGRCRESWDAAWHAD-----EPYR	GANSNMHLVEAFLA	AFD	TGD-RVWAER	ALRIA	HFFVHEVAA-PR	215		
EcSQI	FWSEEEQMCLESWDEAFSKT-----EYR	GGNANMHAVEAFLI	VYDVTHD-KKWLDR	AIRVASVI	IHDVAR-NN	212			
AspAGE	-KDNPKGKYTKYPT-----RPMKAL	AVPMILANLTLEM-EWLLPQ	ETLENVLAATVQ	EVMGDFLD-QE	212				
	α7→α8 Loop	α-helix 8	α-helix 9						
Runsl_4512	YGTGIPQFWADWSVAPQIKFDIVGWDRFNP	DGLKSAEDNTSYGHNSEFAWLLMH	ALDILGL----	PYDTYREQITKSY	303				
Dfer_5652	YRTGIPQFWEDWSVAPQIKFDI IWGWDRF	TEDEGVKSSAEDNTSYGHNVEFAWLLMH	ALDIAGI----	PYDEYHDQLKASY	303				
RaCE	TNALKVFFDTAF----NLV-----GD-----	IHSYGHDI	EATWLM	DRACDVLGD--EDLKKQ	FAEMDLKIS	275			
RmCE	TGHLILFFDERW----RPR-----SR-----	AVSFGHDI	EASWLLLE	AVDVLGQ--ATLR	PRVQOASLHLA	291			
MmMI	SNEVWEHYTNDW----QID-----WDYNK	NDPKHLFRPWG	FQPGHQTEWAK	LLLMLDK-----	RSPENWYLPKAKYLF	279			
TfMI	DWRLPEHFTPDW----QVV-----ADYNT	DDRAPHFRPYGVT	VGHVLEWARLLVH	VEAAL----	PDPPSWLLADAEAMF	281			
EcSQI	HYRVNEHFDTQW----NPL-----PDYNK	DNPAHRFRAFGGT	PGHWIEWGR	MLMHIHA	ALEARCEQP	PAWLLLEDAKGLF	282		
AspAGE	QGLMYENVAPDGHIDCFE-----GR-----	LINPGHIEAMWF	IMDIARRKN-----	DSKTINQ	AVD	267			
	α-helix 10	α-helix 11							
Runsl_4512	THAVENGVDW-EFGGVYVEGSHAG----QVYDKEKE	FWQQAEMLIGMLDAYRFLKDEKYLQAYENI	HRFVFDKMINHSL-	377					
Dfer_5652	DHAVEYGIDW-EFGGVYVEGSHAG----EVDREKE	FWQQA EVIIGMLDAYRVYVGDEKYLKAYDATH	RFVFDKMIHHSV-	377					
RaCE	HNIQDIALE--D-GALNNERD-KN-----EID	KTRVWVWQAEAVVGF	INAYQHS	GDEKFL	ESAKSVWENI	KEYI	IDKREG	346	
RmCE	RATLAEGRA--PDGSLYIEIGEQG-----HLD	TDRHWVWQAEALVGF	LNAYQES	GEVLFYEAE	ADVWRY	IRERQR	TRG-	363	
MmMI	DLAYKKAWDT-KKGLLHYGYAPDG----TVCD	PKYFWVQAESFAAAWLLY	KATKDETY	KQYLT	LWFE	SWNHMI	DHTF-	353	
TfMI	AAAVARGWSVDGTEGFVYTLDYDD----TPV	VRSRMHVVAEAI	SAAAVL	QRTGDER	YEHWYRT	WWDHAAT	YFVDTVQ-	356	
EcSQI	NATVRDAWAPDGADGIVYTVDWEG----KPV	VRVRVWPIVEAMGT	AYSLSY	TVTGDR	QYETWYQ	TWWEYCI	KYLM	DYEN-	357
AspAGE	LNILNFAWDN-EYGGLYYFMDAAGHP	PQQLLEWDQKLVHLES	LVALAMGYRLTGR	DACAWAYQ	KMHDY	SWQHF	ADPEY-	345	
	α-helix 12								
Runsl_4512	GEWWPLMTREGVPIW-K---HMSHSWKIN	YHDVRSMTQSI	VRLDKIAKGV-----	423					
Dfer_5652	GEWLPLLTRQGEPIW-K---HMSHSWKVNY	HSVRSMVQGI	VRLNKLIGNK-----	423					
RaCE	GEWYSEVTFDHTPHDYK---ETVGP	WKCPYHNGRMC	MEVITRGVDI-----	389					
RmCE	GEWFARVRDDGAPYP-D---DKVDF	WKGPYHNGRAC	EAIQRLRHLEH	VRSR-----	412				
MmMI	GAWYRILDENNAQYD-N---NKSPAG	KTDYHTMGAC	YEVLLKTLTL-----	394					
TfMI	GSWHHELDP	TLAPPPG	TWSGKP----DVYHAY	QATRLPLPLAPSL	AGALATVG-----	407			
EcSQI	GSWWQELDADNKVTT-KVWDGKQ	---DIYHLLH	CLV	IPRIPLAPG	MAPAVAAGLLD	INAK	413		
AspAGE	GEWFGYLNRRGEVLL-N---LKGGK	WKGCFHVPRAMY	LCWQQFEALS-----	388					

Fig. 7., Saburi et al.

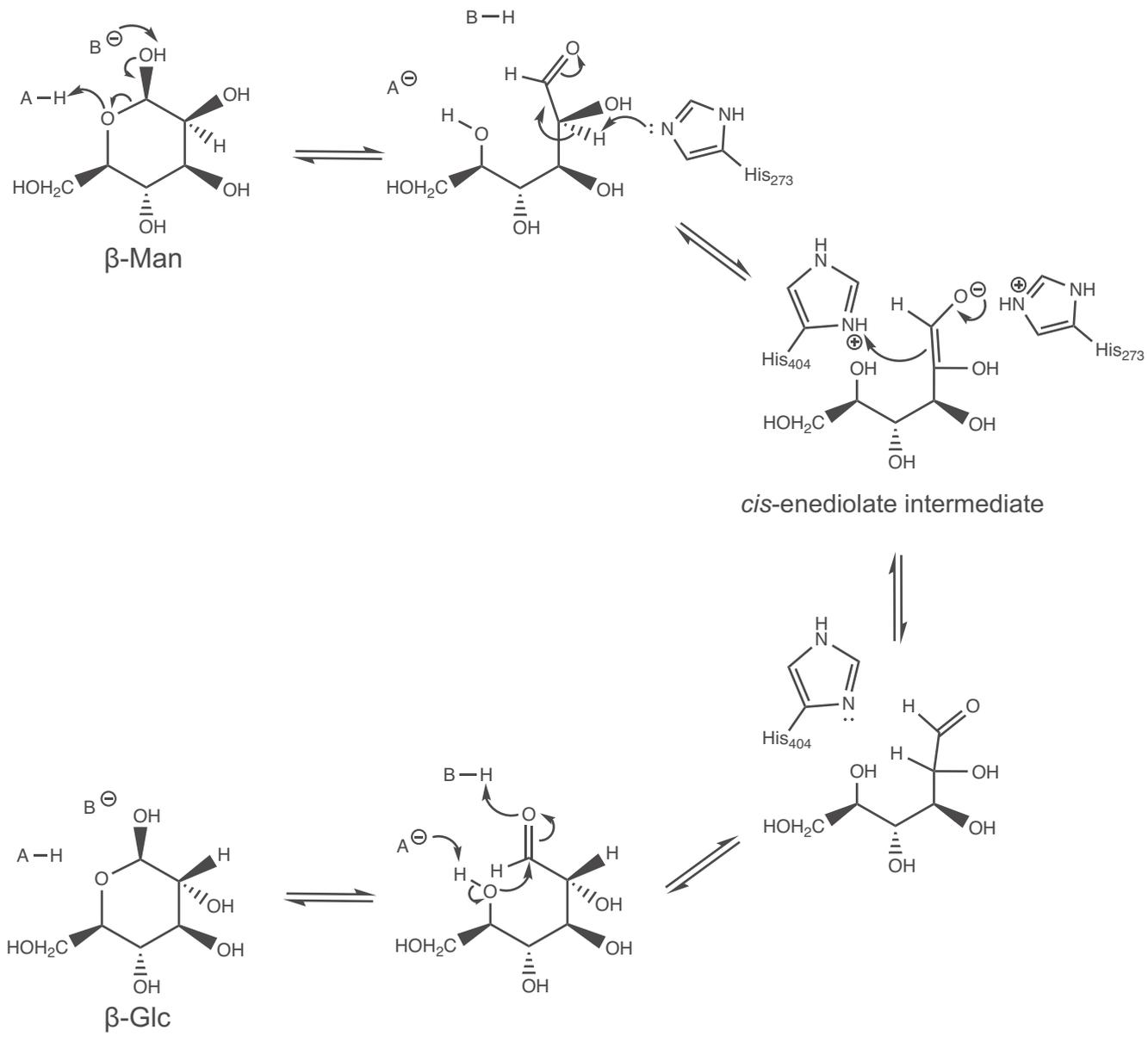


Fig. 8., Saburi et al.

Table 1. Sequences of primers used in this study.

Name	Sequence (5' to 3')	Purpose
Runsl_4512_s	TATATTTT CATATG ACTTCTGAAAAAATT	Preparation of expression plasmid
Runsl_4512_a	AAATTATTCCTCGAGCACCCCTTTTGCAAT	Preparation of expression plasmid
Dfer_5652_s	CCATATTTTGAATTCATGAATGTAGATCAA	Preparation of expression plasmid
Dfer_5652_a	TCCAAATTCCTCGAGCTTATTTCCAATCAG	Preparation of expression plasmid
D187N_s	ACCCTCAATGTACACATGCACCTCATG	Mutagenesis in Runsl_4512
D187N_a	GTGTACATTGAGGGTCTTGCGGTCACC	Mutagenesis in Runsl_4512

Recognition sites of restriction endonucleases are underlined. Substituted nucleotides are shown in bold face.

Table 2. Kinetic parameters of Runsl_4512 and Dfer_5652 for D-mannose.

Enzyme	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m ($\text{min}^{-1} \text{mM}^{-1}$)
Runsl_4512	286 ± 20	73.5 ± 6.7	3.89
Dfer_5652	233 ± 25	66.4 ± 11.1	3.51
CsCE	44.3 ± 0.6	51.8 ± 1.2	0.855
CvCE	6.78 ± 0.18	245 ± 10	0.0277

Values are mean \pm standard deviation for three independent experiments. CsCE, *Caldicellulosiruptor saccharolyticus* CE (Park et al. 2011); CvCE, *Cellvibrio vulgaris* CE (Saburi et al. 2015).