



Title	Functional reassignment of <i>Cellvibrio vulgaris</i> EpiA to cellobiose 2-epimerase and an evaluation of the biochemical functions of the 4-O-beta-d-mannosyl-d-glucose phosphorylase-like protein, UnkA
Author(s)	Saburi, Wataru; Tanaka, Yuka; Muto, Hirohiko; Inoue, Sota; Odaka, Rei; Nishimoto, Mamoru; Kitaoka, Motomitsu; Mori, Haruhide
Citation	Bioscience biotechnology and biochemistry, 79(6), 969-977 https://doi.org/10.1080/09168451.2015.1012146
Issue Date	2015-06-04
Doc URL	http://hdl.handle.net/2115/62061
Rights	This is an Accepted Manuscript of an article published by Taylor & Francis in Bioscience biotechnology and biochemistry on [date of publication], available online: http://www.tandfonline.com/doi/abs/10.1080/09168451.2015.1012146?journalCode=tbbb20
Type	article (author version)
File Information	70427 (mori) pdf.pdf



[Instructions for use](#)

1 Running title: Characteristics of *C. vulgaris* CE and MGP

2

3 **Functional Reassignment of *Cellvibrio vulgaris* EpiA to Cellobiose 2-Epimerase and an**
4 **Evaluation of the Biochemical Functions of the 4-*O*- β -D-Mannosyl-D-glucose**
5 **Phosphorylase-like Protein, UnkA.**

6

7 Wataru SABURI,¹ Yuka TANAKA,¹ Hirohiko MUTO,¹ Sota INOUE,¹ Rei ODAKA,¹ Mamoru

8 NISHIMOTO,² Motomitsu KITAOKA² and Haruhide MORI^{1,†}

9 ¹*Research Faculty of Agriculture, Hokkaido University, N-9, W-9, Sapporo 060-8589, Japan*

10 ²*National Food Research Institute, National Agriculture and Food Research Organization, 2-1-*
11 *12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan*

12 Received November 17, 2014; Accepted December 26, 2014

13 [†]To whom correspondence should be addressed. Tel/Fax: +81-11-706-2497; E-mail:

14 hmori@chem.agr.hokudai.ac.jp

15

16 *Abbreviations:* BSA, bovine serum albumin; CE, cellobiose 2-epimerase; Glc-Man, β -D-
17 glucosyl-(1 \rightarrow 4)-D-mannose; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; β -
18 mannanase, mannan endo-1,4- β -mannosidase; Man1P, α -D-mannose 1-phosphate; Man-Glc, β -
19 D-mannosyl-(1 \rightarrow 4)-D-glucose; MGP, 4-*O*- β -D-mannosyl-D-glucose phosphorylase; MES, 4-
20 morpholineethanesulfonic acid

21

1 The aerobic soil bacterium *Cellvibrio vulgaris* has a β -mannan-degradation gene cluster,
2 including *unkA*, *epiA*, *man5A*, and *aga27A*. Among these genes, *epiA* has been assigned to
3 encode an epimerase for converting D-mannose to D-glucose, even though the amino acid
4 sequence of EpiA is similar to that of cellobiose 2-epimerases (CEs). UnkA, whose function
5 currently remains unknown, shows a high sequence identity to 4-*O*- β -D-mannosyl-D-glucose
6 phosphorylase. In this study, we have investigated CE activity of EpiA and the general
7 characteristics of UnkA using recombinant proteins from *Escherichia coli*. Recombinant EpiA
8 catalyzed the epimerization of the 2-OH group of sugar residue at the reducing end of cellobiose,
9 lactose, and β -(1 \rightarrow 4)-mannobiose in a similar manner to other CEs. Furthermore, the reaction
10 efficiency of EpiA for β -(1 \rightarrow 4)-mannobiose was 5.5×10^4 -fold higher than it was for D-mannose.
11 Recombinant UnkA phosphorylated β -D-mannosyl-(1 \rightarrow 4)-D-glucose and specifically utilized
12 D-glucose as an acceptor in the reverse reaction, which indicated that UnkA is a typical 4-*O*- β -
13 D-mannosyl-D-glucose phosphorylase.

14

15 **Key words:** *Cellvibrio vulgaris*; cellobiose 2-epimerase; 4-*O*- β -D-mannosyl-D-glucose
16 phosphorylase; β -mannan; substrate specificity

17

1 β -Mannan is one of the major polysaccharides included in the hemicellulose found in all
2 plant cell walls. The main chain of β -mannan is formed exclusively of β -(1 \rightarrow 4)-linked D-
3 mannosyl residues, whereas that of glucomannan is formed of a combination of β -(1 \rightarrow 4)-linked
4 D-mannosyl and D-glucosyl residues.¹⁾ Galactomannan has branches composed of single α -
5 (1 \rightarrow 6)-linked D-galactosyl residues, which branch out from its β -mannan backbone. Some
6 glycosyl residues in the main chain of β -mannan are acetylated at the 2-OH or 3-OH position.

7 Several microbial enzymes have been reported to degrade β -mannan. For example, mannan
8 endo-1,4- β -mannosidase (β -mannanase, EC 3.2.1.78) hydrolyzes the internal β -(1 \rightarrow 4)-
9 mannosidic linkages to produce oligosaccharides. α -Galactosidase (EC 3.2.1.22) and acetyl
10 mannan esterase (EC 3.1.1.6) remove the galactosyl branches and acetyl groups, respectively,
11 which enhances the degradation of β -mannan.¹⁻³⁾ The resulting oligosaccharides are then
12 hydrolyzed to monosaccharides by β -mannosidase (EC 3.2.1.25) and β -glucosidase (EC
13 3.2.1.21). Senoura *et al.*⁴⁾ found a new pathway for the degradation of β -mannan in *Bacteroides*
14 *fragilis*, which was composed of several steps, including (1) the hydrolysis of β -mannan to β -
15 (1 \rightarrow 4)-mannobiose by β -mannanase; (2) the epimerization of β -(1 \rightarrow 4)-mannobiose to β -D-
16 mannosyl-(1 \rightarrow 4)-D-glucose (Man-Glc) by cellobiose 2-epimerase (CE, EC 5.1.3.11); and (3)
17 the phosphorolysis of Man-Glc to D-mannose 1-phosphate (Man1P) and D-glucose by 4-O- β -D-
18 mannosyl-D-glucose phosphorylase (MGP, EC 2.4.1.281). Similar mannan degradation systems
19 have been found in the other CE producing bacteria, including *Ruminococcus albus* and
20 *Rhodothermus marinus*.^{5,6)} In *R. albus*, β -(1 \rightarrow 4)-mannooligosaccharides longer than β -(1 \rightarrow 4)-
21 mannobiose are phosphorolyzed to β -(1 \rightarrow 4)-mannobiose and Man1P by the intracellular β -1,4-
22 mannoooligosaccharide phosphorylase (RaMP2) (EC 2.4.1.319).⁵⁾

23 Centeno *et al.*⁷⁾ reported that a gene cluster belonging to the aerobic soil bacterium *Cellvibrio*
24 *mixtus* (same strain as *Cellvibrio vulgaris*) was involved in the degradation of β -mannan. This
25 gene cluster is comprised of four genes, including *unkA*, *epiA*, *man5A*, and *aga27A*. *man5A*
26 encodes β -mannosidase, which liberates D-mannose from the non-reducing end of β -(1 \rightarrow 4)-
27 mannoooligosaccharides.⁸⁾ *aga27A* encodes α -galactosidase, which exhibits hydrolytic activity
28 towards the α -(1 \rightarrow 6)-galactosidic linkages in galactomannan.⁷⁾ EpiA has been assigned as an

1 epimerase, which is involved in the conversion D-mannose to D-glucose. However, the amino
2 acid sequence of EpiA shows a high sequence identity (32–42%) to those of several other
3 reported CEs.⁹⁻¹⁶⁾ UnkA, whose function currently remains unknown, has been classified into
4 Glycoside hydrolase family 130 based on its amino acid sequence.¹⁷⁾ Glycoside hydrolase
5 family 130 contains MGP, β -1,4-mannooligosaccharide phosphorylase and β -1,4-D-mannosyl-
6 *N*-acetyl-D-glucosamine phosphorylase (EC 2.4.1.320).¹⁸⁾ The sequence identity of UnkA to
7 other MGPs is high (57–66%), whereas that to *R. albus* β -1,4-mannooligosaccharide
8 phosphorylase and *Bacteroides thetaiotaomicron* β -1,4-D-mannosyl-*N*-acetyl-D-glucosamine
9 phosphorylase were low (21 and 17%, respectively). Both EpiA and UnkA are thought to be
10 intracellular enzymes because they are predicted to have no signal peptide by SignalP 4.1
11 server.¹⁹⁾ These sequence data suggest that EpiA and UnkA may have CE and MGP activities,
12 and that they could therefore be involved in the intracellular metabolism of the β -mannan found
13 in *B. fragilis*.⁴⁾ In this study, we have investigated the biochemical characteristics of EpiA and
14 UnkA using recombinant proteins from *Escherichia coli*.

15

16 **Results and Discussion**

17 *Reaction of recombinant EpiA with disaccharide substrates*

18 Recombinant EpiA was produced in *E. coli*, and 37.9 mg of purified protein was obtained
19 from 1.0 L of culture broth. The resulting recombinant EpiA was incubated with cellobiose,
20 lactose, and β -(1→4)-mannobiose to investigate its CE activity. As shown in Fig. 1a–c, the
21 epimerized products of these substrates were detected by HPLC analysis. The reaction products
22 were then hydrolyzed under acidic conditions and the hydrolysates was subjected to HPLC
23 analysis, which showed that D-glucose and D-mannose were detected as the reaction products
24 from cellobiose and β -(1→4)-mannobiose, and D-galactose and D-mannose were detected as the
25 reaction products from lactose (Fig. 1d–f). The results of the reactions with cellobiose and β -
26 (1→4)-mannobiose suggested that Glc-Man or Man-Glc was produced in the reactions. On the
27 other hand, the result of the reaction with lactose clearly indicated that D-glucose residue at the
28 reducing end of lactose was epimerized to D-mannose residue. Because EpiA had epimerization

1 activity toward the 2-OH group of the sugar residue at the reducing end, the reaction products
2 from cellobiose and β -(1 \rightarrow 4)-mannobiose were thought to be Glc-Man and Man-Glc,
3 respectively. Recombinant EpiA did not act on any of the other disaccharides tested (i.e.,
4 maltose, isomaltose, sophorose, laminaribiose, gentiobiose, and β -(1 \rightarrow 4)-xylobiose) (data not
5 shown).

6 7 *Kinetic analysis of recombinant EpiA*

8 Recombinant EpiA exhibited its highest activity towards the disaccharide substrate β -D-
9 glucosyl-(1 \rightarrow 4)-D-mannose (Glc-Man) at pH 8.0 and a temperature of 40°C (Fig. 2), which was
10 similar to the results obtained using D-mannose as a substrate.⁷⁾ EpiA retained over 90% of its
11 activity at pH values in the range of 5.2 to 10.0 and temperatures below 40°C following the pH
12 (at 4°C for 24 h) and heat (at pH 8.0 for 30 min) treatment processes, respectively (Fig. 2).

13 The epimerization activities of EpiA towards several disaccharide substrates were compared
14 with its activity towards D-mannose based on their kinetic parameters that determined from
15 reaction rates at various substrate concentrations by non-linear regression to the Michaelis-
16 Menten equation (Fig. 3). Recombinant EpiA showed a significant preference for disaccharides
17 over D-mannose, and showed 1.4×10^3 – 5.5×10^4 -fold higher k_{cat}/K_m values for the disaccharide
18 substrates than it did for D-mannose (Table 1). The k_{cat}/K_m value for β -(1 \rightarrow 4)-mannobiose was
19 the highest of the disaccharide substrates tested, and was 16- and 39-fold higher than those for
20 cellobiose and lactose, respectively. EpiA exhibited weak epimerization activity to β -(1 \rightarrow 4)-
21 mannotriose. Reaction rate to 5 mM β -(1 \rightarrow 4)-mannotriose was $0.138 \pm 0.010 \text{ s}^{-1}$, which was
22 0.24% of that to 5 mM β -(1 \rightarrow 4)-mannobiose ($57.9 \pm 3.3 \text{ s}^{-1}$). EpiA had high epimerization
23 activity to β -(1 \rightarrow 4)-linked disaccharides, and β -(1 \rightarrow 4)-mannobiose is the best substrate of β -
24 (1 \rightarrow 4)-linked disaccharides as other reported CEs.^{4,13,16,20)} EpiA was consequently designated as
25 CvCE.

26 27 *Enzymatic characterization of UnkA*

1 Recombinant UnkA was produced in *E. coli*, which gave 168 mg of purified protein from 1.0
2 L of culture broth. The resulting recombinant UnkA showed phosphorolytic activity towards 2
3 mM Man-Glc in the presence of 100 mM sodium phosphate buffer (pH 8.0), and the specific
4 activity toward these substrates was 24.7 U/mg. The highest activity was obtained at pH 8.0 and
5 a temperature of 45°C (Fig. 4). Furthermore, greater than 90% of the initial activity of UnkA
6 was retained at pH values in the range of 3.9–10.6 and temperatures below 37°C after the pH (at
7 4°C for 24 h) and heat (at pH 8.0 for 20 min) treatment processes, respectively.

8 Recombinant UnkA was incubated with Man-Glc, β -(1→4)-mannobiose, β -(1→4)-
9 mannotriose, epimerized product of β -(1→4)-mannotriose by CvCE, cellobiose, and lactose. It
10 exhibited phosphorolytic activity only towards Man-Glc (Fig. 5). The spectrophotometric
11 analysis of phosphorolysis of cellobiose and lactose revealed that the activities to these
12 oligosaccharides were ≤ 0.07 U/mg, indicating that -1 subsite of UnkA is highly specific to
13 mannosyl residue. The acceptor specificity of the recombinant UnkA was investigated based on
14 its synthetic activity towards 10 mM Man1P and a variety of different sugars, including D-
15 glucose, D-mannose, D-allose, D-galactose, D-xylose, 1,5-anhydro-D-glucitol, 2-deoxy-D-
16 glucose, 3-deoxy-D-glucose, 6-deoxy-D-glucose, D-glucosamine, *N*-acetyl-D-glucosamine, D-
17 fructose, L-arabinose, D-glucitol, methyl α -D-glucoside, methyl β -D-glucoside, *N,N'*-
18 diacetylchitobiose, maltose, cellobiose, and β -(1→4)-mannobiose. UnkA exhibited synthetic
19 activity towards D-glucose, 6-deoxy-D-glucose, and D-xylose (Table 2). A comparison of the
20 $k_{\text{cat(app)}}/K_{\text{m(app)}}$ values of UnkA for 6-deoxy-D-glucose and D-xylose with the $k_{\text{cat(app)}}/K_{\text{m(app)}}$ value
21 of UnkA for D-glucose revealed that they were 5.1- and 34-fold lower, respectively. This result
22 indicates that UnkA has a high specificity for D-glucose in its +1 subsite (acceptor binding site)
23 than it does for 6-deoxy-D-glucose or D-xylose, which is similar to the results observed for
24 several other MGPs.^{5,6)} Based on these results, UnkA was designated as CvMGP.

25 The kinetic parameters of CvMGP for the phosphorolysis and synthesis of Man-Glc were
26 determined from its reaction rates at various substrate concentrations. In a similar manner to that
27 observed in several other MGPs,^{5,6)} the phosphorolysis and synthesis of Man-Glc by
28 recombinant CvMGP obeyed a sequential bi-bi mechanism, involving the formation of a ternary

1 complex composed of the enzyme and two substrates (Fig. 6). The kinetic parameters of the
2 recombinant CvMGP were as found to be $k_{\text{cat}} = 48.7 \pm 0.29 \text{ s}^{-1}$, $K_{\text{mA}} = 0.653 \pm 0.035 \text{ mM}$, $K_{\text{mB}} =$
3 $0.779 \pm 0.017 \text{ mM}$, and $K_{\text{iA}} = 2.42 \pm 0.11 \text{ mM}$ (A, Pi; B, Man-Glc) for the phosphorolysis; and
4 $k_{\text{cat}} = 97.9 \pm 9.5 \text{ s}^{-1}$, $K_{\text{mA}} = 0.685 \pm 0.013 \text{ mM}$, $K_{\text{mB}} = 11.3 \pm 3.7 \text{ mM}$, and $K_{\text{iA}} = 0.792 \pm 0.32 \text{ mM}$
5 (A, Man1P; B, D-glucose) for the synthesis.

6

7 *Metabolic pathway of β -mannan in C. vulgaris*

8 In this study, we have clearly shown that EpiA (CvCE) and UnkA (CvMGP) are CE and
9 MGP enzymes, respectively. Considering the functions of these enzymes, *C. vulgaris* probably
10 degrades β -(1 \rightarrow 4)-mannobiose through epimerization and phosphorolysis pathways in the same
11 way as *B. fragilis*,⁴⁾ *R. albus*,⁵⁾ and *R. marinus*⁶⁾ (Fig. 7). D-Mannose could be produced
12 intracellularly through the β -mannosidase (Man5A)-catalyzed hydrolysis of β -(1 \rightarrow 4)-
13 mannoooligosaccharides. It could be subsequently metabolized *via* D-glucose as postulated by
14 Centeno *et al.*,⁷⁾ although the epimerization activity of CvCE towards D-mannose is very low.
15 Alternatively, D-mannose could be converted to D-fructose 6-phosphate through sequential
16 reactions with hexokinase (EC 2.7.1.1) and mannose-6-phosphate isomerase (EC 5.3.1.8) to
17 allow for further metabolism through glycolysis. Man5A shows 670-fold higher $k_{\text{cat}}/K_{\text{m}}$ values
18 for β -(1 \rightarrow 4)-mannotriose and β -(1 \rightarrow 4)-mannotetraose than it does for β -(1 \rightarrow 4)-mannobiose.⁸⁾
19 The substrate chain-length preference observed in these cases is similar to that of *R. albus* β -1,4-
20 mannoooligosaccharide phosphorylase.⁵⁾ The low activity of these enzymes towards β -(1 \rightarrow 4)-
21 mannobiose therefore suggests that the reactions of CE and MGP are particularly important to
22 the metabolism of this oligosaccharide. CvCE has weak epimerization activity to β -(1 \rightarrow 4)-
23 mannotriose. However the epimerization of this oligosaccharide unlikely occurs in the
24 metabolism, because β -(1 \rightarrow 4)-mannotriose could be rapidly degraded to (1 \rightarrow 4)-mannobiose
25 and D-mannose by Man5A. Compared with an obligatory anaerobe *R. albus*, *C. vulgaris*, which
26 is an aerobic bacterium, more efficiently generates the ATP required for the phosphorylation of
27 monosaccharides. Consistent with differences in the availability of oxygen to these bacteria, in
28 that *C. vulgaris* uses β -mannosidase to release “free” D-mannose to facilitate the degradation of

1 β -(1→4)-mannooligosaccharide, whereas *R. albus* uses phosphorylase to allow for the direct
2 release of a sugar phosphate.

4 **Experimental**

5 *Bacterial strain.* *C. vulgaris* NCIMB8633 was purchased from NCIMB (Aberdeen, UK).
6 This bacterium was aerobically cultured according to supplier's instruction.

7
8 *Preparation of recombinant CvCE.* The CvCE gene was amplified by PCR with the
9 following primers 5'-AAACCATATGAACGCCGCATGCTTCCTTCA-3' (sense, *NdeI* site
10 underlined) and 5'-AAAGAATTCTTAAACCAAATCCTTCATTA-3' (antisense, *EcoRI* site
11 underlined), and then cloned into the *NdeI* and *EcoRI* sites of pET23a (Novagen, Darmstadt,
12 Germany). Primestar HS DNA polymerase (Takara Bio, Otsu, Japan) was used for the PCR
13 process. Genomic DNA of *C. vulgaris*, which was prepared using a Bacteria Genomic Prep
14 Mini Spin Kit (GE Healthcare, Uppsala, Sweden), was used as template. The DNA sequence of
15 the inserted region was analyzed using an Applied Biosystems 3130 Genetic Analyzer (Life
16 Technologies, Carlsbad, CA, USA). *E. coli* BL21 (DE3), which had been transformed with the
17 CvCE expression vector, was cultured in 1 L of Luria-Bertani medium containing 100 μ g/mL of
18 ampicillin at 37°C until the A_{600} value reached 0.5. Production of the recombinant protein was
19 induced by the addition of 1 mL of 0.1 M isopropyl β -D-thiogalactoside to the culture medium
20 (final concentration, 0.1 mM), and the resulting mixture was incubated with vigorous shaking at
21 18°C for 16 h. Bacterial cells, which had been harvested by centrifugation at 6,000 $\times g$ for 10
22 min at 4°C, were suspended in 30 mL of 10 mM sodium phosphate buffer (pH 7.0) and
23 disrupted by sonication. The cellular debris was removed by centrifugation at 8,000 $\times g$ for 10
24 min at 4°C, and the resulting supernatant was applied to a Toyopearl DEAE-650M column (2.8
25 I.D. \times 6.5 cm, Tosoh, Tokyo), which had been equilibrated with 10 mM sodium phosphate
26 buffer (pH 7.0). The adsorbed protein was eluted with a linear gradient of NaCl from 0 to 0.5 M
27 (total elution volume, 400 mL). Ammonium sulfate was added to the pooled fractions up to 30%
28 saturation, and the sample was applied to a Toyopearl Butyl-650M column (1.6 I.D. \times 2.0 cm,

1 Tosoh), which had been equilibrated with 10 mM sodium phosphate buffer (pH 7.0) containing
2 30% saturation ammonium sulfate. The adsorbed protein was eluted with a descending linear
3 gradient of ammonium sulfate from 30 to 0% saturation (total elution volume, 200 mL). The
4 collected fractions were dialyzed against 5 mM sodium phosphate buffer (pH 7.0), and applied
5 to a hydroxyapatite Bio-Gel HTP column (2.8 I.D. × 2.0 cm, Bio-Rad, Hercules, CA, USA).
6 Non-adsorbed fractions were collected (contaminant protein was adsorbed on the resin).
7 Purified recombinant CvCE was mixed with an equal volume of glycerol and stored at -20°C.

8
9 *Preparation of recombinant CvMGP.* The CvMGP gene was inserted into pET23a as
10 described above. Primers 5'-TTGACCATATGAGTAGTTTTAAAGAAAAAGC-3' (sense, *NdeI*
11 site underlined) and 5'-CGTTGAAATTCTTAAACCAAATCCTTCATTA-3' (antisense, *EcoRI*
12 site underlined) were used for the PCR process. The production of recombinant CvMGP was
13 carried out in the same fashion as that of the recombinant CvCE, and the recombinant protein
14 was extracted from bacterial cells using a 20 mM 4-morpholineethanesulfonic acid (MES)-
15 NaOH buffer (pH 6.5) solution by sonication. The cell-free extract was subjected to purification
16 by Toyopearl DEAE-650M column chromatography (2.8 I.D. × 25 cm), where the adsorbed
17 protein was eluted using the conditions described above (equilibrium buffer, 20 mM MES-
18 NaOH buffer, pH 6.5). The pooled fractions were further purified by Toyopearl Butyl-650M
19 column chromatography (2.8 I.D. × 12 cm), where a 20 mM MES-NaOH buffer (pH 6.5)
20 solution containing a 30% saturation of ammonium sulfate was used to equilibrate the column.
21 The adsorbed protein was eluted according to the procedure described above. Purified
22 recombinant CvMGP was dialyzed against a 20 mM MES-NaOH buffer (pH 6.5) solution and
23 stored at -80°C.

24
25 *Protein assay.* The concentrations of protein in the fractions collected by column
26 chromatography were measured using a UV method (A_{280}),²¹⁾ assuming that an extinction
27 coefficient of 1.00 mg/mL of protein was equal to 1.00. The concentration of the purified
28 enzyme was determined from the amino acid concentrations of the acid hydrolysate (6 M HCl at

1 110°C for 24 h), which were measured by the ninhydrin colorimetric method using a JLC-500/V
2 amino acid analyzer (JEOL, Tokyo, Japan).²²⁾

3
4 *Analysis of reaction products of CvCE.* A reaction mixture (1 mL) containing 6.4 μM
5 recombinant CvCE, 100 mM substrate, and 10 mM sodium phosphate buffer (pH 8.0) was
6 incubated at 37°C for 48 h. β-(1→4)-Mannobiose (Megazyme, Wicklow, Ireland), cellobiose
7 (Sigma, St. Louis, MO, USA), and lactose (Nacalai Tesque, Kyoto, Japan) were used as
8 substrates. The enzyme reaction was stopped by heating the sample at 100°C for 3 min. The
9 reaction products were purified by HPLC under the following conditions: column, Sugar
10 SP0810 (8.0 I.D. × 300 mm, Shodex, Tokyo); column temperature, 80°C; eluent, water; flow
11 rate, 0.8 mL/min; and detection method, refractive index. The purified reaction product (40 μg)
12 was hydrolyzed in 2 M trifluoroacetic acid at 100°C for 3 h. The reaction mixture was
13 evaporated to dryness to give a residue, which was dissolved in 25 μL of water. The resulting
14 solution was then analyzed by HPLC as follows: injection volume, 5 μL; columns, two tandem
15 Sugar SP0810 columns, column temperature, 70°C; eluant, water; flow rate, 0.5 mL/min; and
16 detection method, pulsed amperometry. D-Mannose (Wako Pure Chemical Industries, Osaka,
17 Japan), D-glucose (Wako Pure Chemical Industries), and D-galactose (Nacalai Tesque) were
18 used as standards.

19
20 *Substrate specificity of CvCE.* A reaction mixture (5 μL), containing 13.8 μM recombinant
21 CvCE, 40 mM substrate, and 20 mM sodium phosphate buffer (pH 8.0) was incubated at 37°C
22 for 24 h. Several substrates were tested, including maltose (Nihon Shokuhin Kako, Tokyo),
23 gentiobiose (Nacali Tesque), lactose, isomaltose (Seikagaku, Tokyo), cellobiose, sophorose
24 (Sigma), β-(1→4)-mannobiose, laminaribiose (Megazyme), and β-(1→4)-xylobiose (Wako
25 Pure Chemical Industries). One microlitre of the reaction mixture was spotted onto a TLC plate
26 (Silica Gel 60 F₂₅₄, Merck, Darmstadt, Germany), and the plate was developed using a 2:2:1
27 (v/v/v) mixture of 2-propanol/1-butanol/water. The developed TLC plate was visualized by

1 spraying it with a detection reagent composed of a 100:2:1 (v/v/v) mixture of acetic
2 acid/sulfuric acid/anisaldehyde followed by heating.

3
4 *Enzyme assay of CvCE.* CE activity was assayed based on the epimerization of Glc-Man to
5 cellobiose. Glc-Man was prepared from α -D-glucose 1-phosphate and D-mannose through the
6 reverse reaction of *R. albus* cellobiose phosphorylase.²³⁾ A reaction mixture (50 μ L) containing
7 enzyme, 10 mM Glc-Man, 42 mM sodium phosphate buffer (pH 8.0), and 0.2 mg/mL bovine
8 serum albumin (BSA) was incubated at 37°C for 10 min. The reaction was then terminated by
9 heating the sample at 100°C for 3 min, and the amount of cellobiose produced by the reaction
10 was measured as described previously.²⁴⁾ Briefly, cellobiose was phosphorylated with
11 cellobiose phosphorylase, and the resulting D-glucose was measured using a Glucose CII-Test
12 (Wako Pure Chemical Industries). This enzyme assay method was used to investigate effects of
13 pH and temperature.

14 Epimerization activity to β -(1 \rightarrow 4)-mannotriose was determined from the amount of D-
15 glucose liberated from the epimerization product by the enzymatic degradation. A reaction
16 mixture (50 μ L) containing 641 nM CvCE, 5 mM β -(1 \rightarrow 4)-mannotriose (Megazyme), 40 mM
17 sodium phosphate buffer (pH 8.0), and 0.2 mg/mL BSA was incubated at 37°C for 10 min. The
18 reaction was stopped by heating the sample at 100°C for 5 min. The sample was mixed with 100
19 μ L of the enzyme mixture, containing 1.3 μ g/mL *R. albus* MGP (RaMP1), 0.65 mg/mL RaMP2,
20 and 50 mM sodium phosphate buffer (pH 7.0), and 20 μ L of D-glucose quantification reagent
21 described previously.²⁰⁾ After incubation of the sample at 37°C for 30 min, A_{505} was measured.
22 As standard, 0-0.5 mM D-glucose was used.

23 The kinetic parameters for the epimerization reactions of D-mannose, β -(1 \rightarrow 4)-mannobiose,
24 lactose, and cellobiose were determined experimentally. The rates of reaction were determined
25 at various substrate concentrations and the resulting data were fitted to the Michaelis-Menten
26 equation using the version 7.0.2 of the Grafit program (Erithacus Software, East Grinstead, UK)
27 to calculate kinetic parameters. For the reaction with D-mannose, 50 μ L of a reaction mixture
28 containing 9.22 μ M recombinant CvCE (from which the glycerol had been removed by dialysis),

1 50–500 mM D-mannose and 12 mM sodium phosphate buffer (pH 8.0) was incubated at 37°C
2 for 2 h. The reaction was stopped by the addition of 25 µL of 0.1 M HCl, and the resulting
3 mixture was immediately heated at 100°C for 3 min. The pH of the mixture was then adjusted to
4 8.0 by adding 50 µL of 200 mM sodium phosphate buffer (pH 8.0). The amount of D-glucose
5 produced by this reaction was determined with a D-glucose determination kit (Roche, Darmstadt,
6 Germany). For the reaction with lactose and cellobiose, 100 µL of a reaction mixture containing
7 86.5 nM recombinant CvCE, 5–40 mM substrate, and 12 mM sodium phosphate buffer (pH 8.0)
8 was incubated at 37°C for 20 min. The enzymatic reaction was then stopped by the addition of
9 50 µL of 0.1 M HCl, and the resulting mixture was immediately heated at 100°C for 3 min. The
10 progress of the reaction (product formation) was measured by HPLC using two tandem Sugar
11 SP0810 columns as described above. Authentic Glc-Man²³⁾ and epilactose²⁵⁾ were used as
12 standards. The kinetic parameters for β-(1→4)-mannobiose were determined as previously
13 reported.²⁰⁾ A reaction mixture (50 µL) containing 8.01 nM recombinant CvCE, 1.0–15 mM β-
14 (1→4)-mannobiose, and 40 mM sodium phosphate buffer (pH 8.0) was incubated at 37°C for 10
15 min. The enzymatic reaction was then terminated by the addition of 20 µL of 0.1 M HCl, and the
16 resulting mixture was immediately heated at 100°C for 3 min. The sample was neutralized with
17 20 µL of 0.1 M NaOH, and the resulting Man-Glc was quantified using a colorimetric
18 quantification method.²⁰⁾

19
20 *Enzyme assay of CvMGP.* MGP activity was determined by measuring the phosphorolytic
21 velocity towards 2 mM Man-Glc. A reaction mixture (50 µL) containing enzyme, 2 mM Man-
22 Glc, 100 mM sodium phosphate buffer (pH 8.0), 4 mM 4-(2-hydroxyethyl)piperazine-1-
23 ethanesulfonic acid (HEPES)-NaOH buffer (pH 6.5), and 0.2 mg/mL BSA was incubated at
24 37°C for 10 min. The enzymatic reaction was then terminated by adding 100 µL of 2 M Tris-
25 HCl buffer (pH 7.0) and immediately heating the resulting mixture at 100°C for 3 min. The
26 liberated D-glucose was measured using a Glucose CII test.

27 The acceptor specificity of the recombinant CvMGP in the reverse reaction was investigated
28 by measuring its synthetic activity towards 10 mM Man1P and various acceptor substrates. A

1 reaction mixture (20 μ L) containing recombinant CvMGP (10 nM for D-glucose and 300 nM for
2 the other substrates), 10 mM Man1P, 10 mM acceptor, 104 mM HEPES-NaOH buffer (pH 8.0),
3 and 0.2 mg/mL BSA was incubated at 37°C for 10 min. The enzymatic reaction was stopped by
4 heating the sample at 80°C for 5 min, and the amount of inorganic phosphate generated by the
5 reaction was measured according to the method published by Lowry and Lopez.²⁶⁾ Man1P
6 (dicyclohexylamine salt) was prepared according to the method of Liu *et al.*²⁷⁾ Several acceptor
7 substrates were tested, including D-glucose, D-mannose, D-allose (Wako Pure Chemical
8 Industries), D-galactose, D-xylose (Wako Pure Chemical Industries), 1,5-anhydro-D-glucitol
9 (Wako Pure Chemical Industries), methyl α -D-glucoside (Wako Pure Chemical Industries),
10 methyl β -D-glucoside (Wako Pure Chemical Industries), 6-deoxy-D-glucose (Sigma), cellobiose,
11 *N,N'*-diacetylchitobiose (Sigma), 2-deoxy-D-glucose (Tokyo Chemical Industries, Tokyo), D-
12 glucosamine (Tokyo Chemical Industries), D-fructose (Nacalai Tesque), D-glucitol (Nacalai
13 Tesque), *N*-acetyl-D-glucosamine (Nacalai Tesque), 3-deoxy-D-glucose (Carbosynth, Berkshire,
14 UK), L-arabinose (Kanto Chemical, Tokyo), maltose, and β -(1 \rightarrow 4)-mannobiose. The apparent
15 kinetic parameters for the acceptor substrates were calculated by fitting the reaction rates at
16 various concentrations of the acceptor substrates (1.25–25 mM) and 10 mM Man1P to the
17 Michaelis-Menten equation.

18 Kinetic parameters for phosphorolysis and synthesis of Man-Glc were determined from the
19 reaction rates at various substrate concentrations by fitting the results to the following equation
20 for a sequential bi bi mechanism²⁸⁾ using version 7.0.2 of the Grafit program:

$$21 \quad v = k_{\text{cat}}[A][B]/(K_{iA}K_{mB} + K_{mB}[A] + K_{mA}[B] + [A][B])$$

22 (For phosphorolysis, A = inorganic phosphate, B = Man-Glc; for synthesis, A = Man1P, B =
23 D-glucose)

24 The phosphorolysis of Man-Glc was carried out in a 50 μ L reaction mixture containing 13–
25 38 nM recombinant CvMGP, 0.5–10 mM sodium phosphate buffer (pH 8.0), 0.25–1.5 mM Man-
26 Glc, 104 mM HEPES-NaOH buffer (pH 8.0), and 0.2 mg/mL BSA. The reaction rates for the
27 synthesis of Man-Glc were determined from the amount of inorganic phosphate liberated by the

1 reaction using a reaction mixture (20 μ L) consisting of 5.0 nM recombinant CvMGP, 0.5–2.0
2 mM Man1P, 5.0–40 mM D-glucose, 104 mM HEPES-NaOH buffer (pH 8.0), and 0.2 mg/mL
3 BSA. The enzymatic reactions and processes involved in the subsequent quantification of the
4 reaction products were carried out as described above.

5
6 *Effects of the pH and temperature on the activity and stability.* The optimum pH values for
7 CvCE and CvMGP were investigated based on the enzyme activities at various pH values. The
8 reaction buffer for CvCE was changed to Britton-Robinson buffer (pH 6.0–9.5). Sodium citrate
9 buffer (pH 3.0–6.0), MES-NaOH buffer (pH 6.0–7.0), HEPES-NaOH buffer (pH 7.0–8.5), and
10 glycine-NaOH buffers (pH 8.5–10.5) were used as the reaction buffers for CvMGP. The
11 optimum temperatures for CvCE and CvMGP were determined from the activities at various
12 temperatures. The stable pH and temperature ranges were evaluated by measuring the residual
13 activities of the enzymes after the pH and heat treatment processes, respectively. Mixtures (100
14 μ L) containing the enzyme and 100 mM Britton-Robinson buffer (pH 3.0–12.5) were incubated
15 at 4°C for 24 h to determine the enzyme stability as a function of pH. The stability of the
16 enzyme as a function of temperature was investigated as follows: For CvCE, mixtures (30 μ L)
17 containing enzyme, 67 mM sodium phosphate buffer (pH 8.0), and 0.33 mg/mL BSA were
18 incubated at 20–50°C for 30 min, whereas mixtures (100 μ L) containing enzyme, 20 mM
19 HEPES-NaOH buffer (pH 8.0), and 1 mg/mL BSA, were incubated at 20–50°C for 20 min for
20 CvMGP. The enzyme was considered stable under conditions that allowed for the retention of
21 $\geq 90\%$ of the enzymes initial activity prior to the treatment.

22
23 *Analysis of substrate specificity of CvMGP in the phosphorolysis.* A reaction mixture (5 μ L)
24 containing 189 nM CvMGP, 5 mM substrate, 5 mM sodium phosphate buffer (pH 8.0), and 0.25
25 mg/mL BSA was incubated at 37°C for 4 h. One microlitre of the reaction mixture was analyzed
26 by TLC in which the plate was developed two times using a 12:3:4 (v/v/v) mixture of 2-
27 propanol/1-butanol/water and the chromatogram was visualized as described above. Man-Glc,
28 β -(1 \rightarrow 4)-mannobiose, β -(1 \rightarrow 4)-mannotriose, epimerized β -(1 \rightarrow 4)-mannotriose by CvCE,

1 cellobiose, and lactose were tested as substrate. The epimerized β -(1 \rightarrow 4)-mannotriose was
2 prepared as follows: a reaction mixture (25 μ L) containing 1.3 μ M CvCE, 10 mM β -(1 \rightarrow 4)-
3 mannotriose, 20 mM sodium phosphate buffer (pH 8.0), and 0.4 mg/mL BSA was incubated at
4 37°C for 20 h. The reaction was stopped by heating the sample at 100°C for 3 min, and the
5 reaction mixture was desalted with Amberlite MB-4 (Roam and Haas, Philadelphia, PA, USA).
6 Resulting sample was used as the substrate of CvMGP.

7 The phosphorolytic activity of CvMGP to cellobiose and lactose was measured based on the
8 amount of D-glucose liberated. A reaction mixture (50 μ L) containing 151 nM CvMGP, 2 mM
9 cellobiose or lactose, 100 mM sodium phosphate buffer (pH 8.0), and 0.2 mg/mL BSA was
10 incubated at 37°C for 10 min. D-Glucose liberated was measured as described above.

11

12 **Acknowledgments**

13 Part of this work was supported by JSPS KAKENHI with a Grants-in-Aid for Young
14 Scientists (B) (Grant No 26850059). We would like to thank Tomohiro Hirose of the
15 Instrumental Analysis Division, Equipment Management Center, Creative Research Institute,
16 Hokkaido University, and the staff of the DNA Sequencing Facility of the Research Faculty of
17 Agriculture, Hokkaido University for amino acid and DNA sequence analyses, respectively.

18

19 **References**

20

- 21 1) Moreira LR, Filho EX. An overview of mannan structure and mannan-degrading enzyme
22 systems. *Appl. Microbiol. Biotechnol.* 2008;79:165-178.
- 23 2) Shallom D, Shoham Y. Microbial hemicellulases. *Curr. Opin. Microbiol.* 2003;6:219-228.
- 24 3) Chauhan PS, Puri N, Sharma P, Gupta N. Mannanases: microbial sources, production,
25 properties and potential biotechnological applications. *Appl. Microbiol. Biotechnol.*
26 2012;93:1817-1830.

- 1 4) Senoura T, Ito S, Taguchi H, Higa M, Hamada S, Matsui H, Ozawa T, Jin S, Watanabe J,
2 Wasaki J, Ito S. New microbial mannan catabolic pathway that involves a novel
3 mannosylglucose phosphorylase. *Biochem. Biophys. Res. Commun.* 2011;408:701-706.
- 4 5) Kawahara R, Saburi W, Odaka R, Taguchi H, Ito S, Mori H, Matsui H. Metabolic
5 mechanism of mannan in a ruminal bacterium, *Ruminococcus albus*, involving two
6 mannoside phosphorylases and cellobiose 2-epimerase: discovery of a new carbohydrate
7 phosphorylase, β -1,4-mannooligosaccharide phosphorylase. *J. Biol. Chem.*
8 2012;287:42389-42399.
- 9 6) Jaito N, Saburi W, Odaka R, Kido Y, Hamura K, Nishimoto M, Kitaoka M, Matsui H, Mori
10 H. Characterization of a thermophilic 4-*O*- β -D-mannosyl-D-glucose phosphorylase from
11 *Rhodothermus marinus*. *Biosci. Biotechnol. Biochem.* 2014;78:263-270.
- 12 7) Centeno MS, Guerreiro CI, Dias FM, Morland C, Tailford LE, Goyal A, Prates JA, Ferreira
13 LM, Caldeira RM, Mongodin EF, Nelson KE, Gilbert HJ, Fontes CM. Galactomannan
14 hydrolysis and mannose metabolism in *Cellvibrio mixtus*. *FEMS Microbiol. Lett.*
15 2006;261:123-132.
- 16 8) Dias FM, Vincent F, Pell G, Prates JA, Centeno MS, Tailford LE, Ferreira LM, Fontes CM,
17 Davies GJ, Gilbert HJ. Insights into the molecular determinants of substrate specificity in
18 glycoside hydrolase family 5 revealed by the crystal structure and kinetics of *Cellvibrio*
19 *mixtus* mannosidase 5A. *J. Biol. Chem.* 2004;279:25517-25526.
- 20 9) Ito S, Hamada S, Yamaguchi K, Umene S, Ito H, Matsui H, Ozawa T, Taguchi H,
21 Watanabe J, Wasaki J, Ito S. Cloning and sequencing of the cellobiose 2-epimerase gene
22 from an obligatory anaerobe, *Ruminococcus albus*. *Biochem. Biophys. Res. Commun.*
23 2007;360:640-645.
- 24 10) Taguchi H, Senoura T, Hamada S, Matsui H, Kobayashi Y, Watanabe J, Wasaki J, Ito S.
25 Cloning and sequencing of the gene for cellobiose 2-epimerase from a ruminal strain of
26 *Eubacterium cellulosolvens*. *FEMS Microbiol. Lett.* 2008;287:34-40.

- 1 11) Senoura T, Taguchi H, Ito S, Hamada S, Matsui H, Fukiya S, Yokota A, Watanabe J,
2 Wasaki J, Ito S. Identification of the cellobiose 2-epimerase gene in the genome of
3 *Bacteroides fragilis* NCTC 9343. *Biosci. Biotechnol. Biochem.* 2009;73:400-406.
- 4 12) Ojima T, Saburi W, Sato H, Yamamoto T, Mori H, Matsui H. Biochemical characterization
5 of a thermophilic cellobiose 2-epimerase from a thermohalophilic bacterium, *Rhodothermus*
6 *marinus* JCM9785. *Biosci. Biotechnol. Biochem.* 2011;75:2162-2168.
- 7 13) Park CS, Kim JE, Choi JG, Oh DK. Characterization of a recombinant cellobiose 2-
8 epimerase from *Caldicellulosiruptor saccharolyticus* and its application in the production of
9 mannose from glucose. *Appl. Microbiol. Biotechnol.* 2011;92:1187-1196.
- 10 14) Kim JE, Kim YS, Kang LW, Oh DK. Characterization of a recombinant cellobiose 2-
11 epimerase from *Dictyoglomus turgidum* that epimerizes and isomerizes β -1,4- and α -1,4-
12 gluco-oligosaccharides. *Biotechnol. Lett.* 2012;34:2061-2068.
- 13 15) Ojima T, Saburi W, Yamamoto T, Mori H, Matsui H. Identification and characterization of
14 cellobiose 2-epimerases from various aerobes. *Biosci. Biotechnol. Biochem.* 2013;77:189-
15 193.
- 16 16) Park CS, Kim JE, Lee SH, Kim YS, Kang LW, Oh DK, Characterization of a recombinant
17 manno-2-epimerase from *Spirochaeta thermophila* that is suggested to be a cellobiose
18 2-epimerase. *Biotechnol. Lett.* 2013;35:1873-1880.
- 19 17) Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B. The
20 Carbohydrate-Active EnZymes (CAZy): an expert resource for Glycogenomics. *Nucleic*
21 *Acids Res.* 2009;37:D233-D238.
- 22 18) Nihira T, Suzuki E, Kitaoka M, Nishimoto M, Ohtsubo K, Nakai H. Discovery of β -1,4-D-
23 mannosyl-*N*-acetyl-D-glucosamine phosphorylase involved in the metabolism of *N*-glycans.
24 *J. Biol. Chem.* 2013;288:27366-27374.
- 25 19) Petersen TN, Brunak S, von Heijne G, Nielsen H. SignalP 4.0: discriminating signal
26 peptides from transmembrane regions. *Nat. Methods* 2011;8:785-786.
- 27 20) Jaito N, Saburi W, Muto H, Matsui H, Mori H. Colorimetric quantification of β -(1 \rightarrow 4)-
28 manno-2-epimerase and 4-*O*- β -D-mannosyl-D-glucose. *J. Appl. Glycosci.* 2014;61:117-119.

- 1 21) Dayhoff MO, Perlmann GE, MacInnes DA. The partial specific volumes, in aqueous
2 solution, of three proteins. J. Am. Chem. Soc. 1952;74:2515-2517.
- 3 22) Moore S, Stein WH. Photometric ninhydrin method for use in the chromatography of amino
4 acids. J. Biol. Chem. 1948;176:367-388.
- 5 23) Hamura K, Saburi W, Matsui H, Mori H. Modulation of acceptor specificity of
6 *Ruminococcus albus* cellobiose phosphorylase through site-directed mutagenesis.
7 Carbohydr. Res. 2013;379:21-25.
- 8 24) Sawano T, Saburi W, Hamura K, Matsui H, Mori H. Characterization of *Ruminococcus*
9 *albus* cellodextrin phosphorylase and identification of a key phenylalanine residue for
10 acceptor specificity and affinity to the phosphate group. FEBS J. 2013;280:4463-4473.
- 11 25) Saburi W, Yamamoto T, Taguchi H, Hamada S, Matsui H. Practical preparation of
12 epilactose produced with cellobiose 2-epimerase from *Ruminococcus albus* NE1. Biosci.
13 Biotechnol. Biochem. 2010;74:1736-1737.
- 14 26) Lowry OH, Lopez JA. The determination of inorganic phosphate in the presence of labile
15 phosphate esters. J. Biol. Chem. 1946;162:421-428.
- 16 27) Liu Y, Nishimoto M, Kitaoka M. Facile enzymatic synthesis of sugar 1-phosphate as
17 substrates for phosphorylase using anomeric kinases. Carbohydr. Res. in press.
- 18 28) Cleland WW. The kinetics of enzyme-catalyzed reactions with two or more substrates or
19 products. I. Nomenclature and rate equations. Biochim. Biophys. Acta. 1963;67:104-137.
20
21

1 **Figure legends**

2 **Fig. 1.** HPLC analysis of the products resulting from the reaction of recombinant EpiA (CvCE)
3 with various disaccharide substrates.

4 Panels, a, b, and c show the HPLC chromatograms of reaction mixtures with β -(1 \rightarrow 4)-
5 mannobiose, cellobiose, and lactose, respectively. S and P indicate substrate and product,
6 respectively. The glycerol in this figure was from the enzyme solution. Reaction products from
7 β -(1 \rightarrow 4)-mannobiose, cellobiose, and lactose, were hydrolyzed with trifluoroacetic acid, and
8 analyzed by HPLC (panels d, e, and f, respectively).

9

10 **Fig. 2.** Effects of pH and temperature on activity and stability of EpiA (CvCE).

11 Epimerization activity of EpiA (CvCE) was measured using 10 mM Glc-Man as substrate. a,
12 pH activity profile. Epimerization activity was measured at indicated pH values at 37°C. b, pH
13 stability. Residual activity was measured after incubation at indicated pH values at 4°C for 24 h.
14 c, Temperature activity curve. Activity was measured at indicated temperatures at pH 8.0. d,
15 Temperature stability. Residual activity was measured after incubation at indicated temperatures
16 at pH 8.0 for 30 min. Error bars indicate standard deviation from three independent experiments.

17

18 **Fig. 3.** [s]-v plots for epimerization of EpiA (CvCE) towards various substrates.

19 a, β -(1 \rightarrow 4)-Mannobiose (closed circles); b, cellobiose (open circles) and lactose (closed
20 triangles); and c, D-mannose (open triangles). Error bars indicate standard deviation from three
21 independent experiments.

22

23 **Fig. 4.** Effects of pH and temperature on activity and stability of UnkA (CvMGP).

24 Phosphorolytic activity of UnkA (CvMGP) was measured using 2 mM Man-Glc as substrate.
25 a, pH activity profile. Epimerization activity was measured at indicated pH values at 37°C.
26 Closed circles, sodium citrate buffer (pH 3.0–6.0); open circles, MES-NaOH buffer (pH 6.0–
27 7.0); closed triangles, HEPES-NaOH buffer (pH 7.0–8.5); and open triangles, glycine-NaOH
28 buffers (pH 8.5–10.5). b, pH stability. Residual activity was measured after incubation at

1 indicated pH values at 4°C for 24 h. c, Temperature activity curve. Activity was measured at
2 indicated temperatures at pH 8.0. d, Thermal stability. Residual activity was measured after
3 incubation at indicated temperatures at pH 8.0 for 20 min. Error bars indicate standard deviation
4 from three independent experiments.

5

6 **Fig. 5.** Specificity of UnkA (*Cv*MGP) in the phosphorolytic reaction.

7 Plus and minus mean the reaction with and without the enzyme, respectively. S1, Man1P;
8 S2, D-glucose; MG, Man-Glc; M2, β -(1→4)-mannobiose; M3, β -(1→4)-mannotriose; M3E,
9 epimerized β -(1→4)-mannotriose by EpiA (*Cv*CE); C, cellobiose; and L, lactose.

10

11 **Fig. 6.** Double reciprocal plots for phosphorolysis and synthesis of Man-Glc catalyzed by UnkA
12 (*Cv*MGP).

13 a, Double reciprocal plots for phosphorolysis of Man-Glc. The concentrations of Man-Glc
14 were 0.25 mM (closed circles), 0.50 mM (open circles), 0.75 mM (closed triangles), 1.0 mM
15 (open triangles), and 1.5 mM (closed squares). b, Double reciprocal plots for synthesis of Man-
16 Glc. Concentrations of D-glucose were 5.0 mM (closed circles), 10 mM (open circles), 20 mM
17 (closed triangles), 30 mM (open triangles), and 40 mM (closed squares). Error bars indicate
18 standard deviation from three independent experiments.

19

20 **Fig. 7.** Metabolic pathway of β -(1→4)-mannooligosaccharides in *C. vulgaris*.

21 HK, hexokinase; GPI, glucose phosphate isomerase; PMM, phosphomannomutase; PMI,
22 mannose 6-phosphate isomerase.

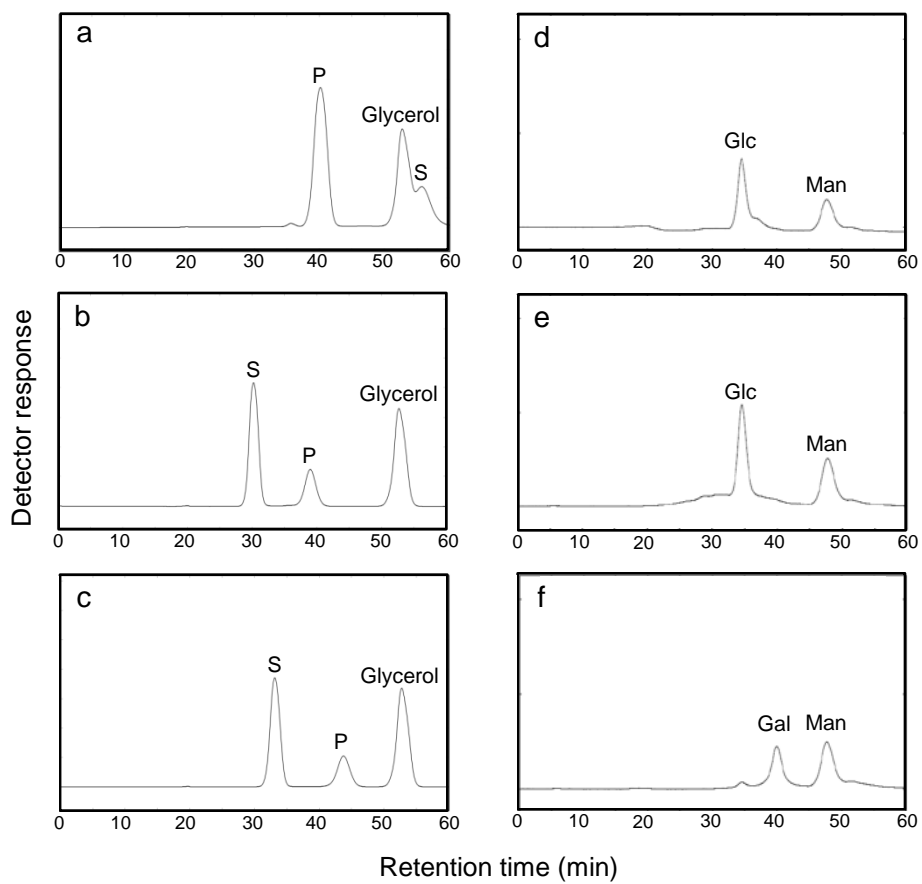


Fig. 1, Saburi *et al.*

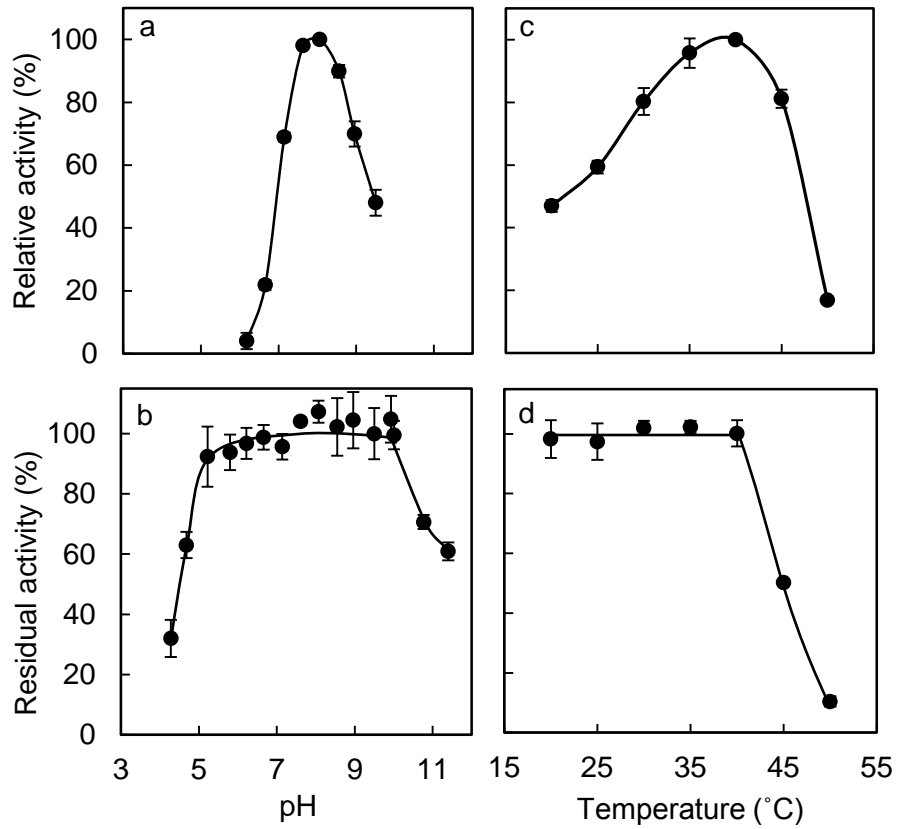


Fig. 2, Saburi *et al.*

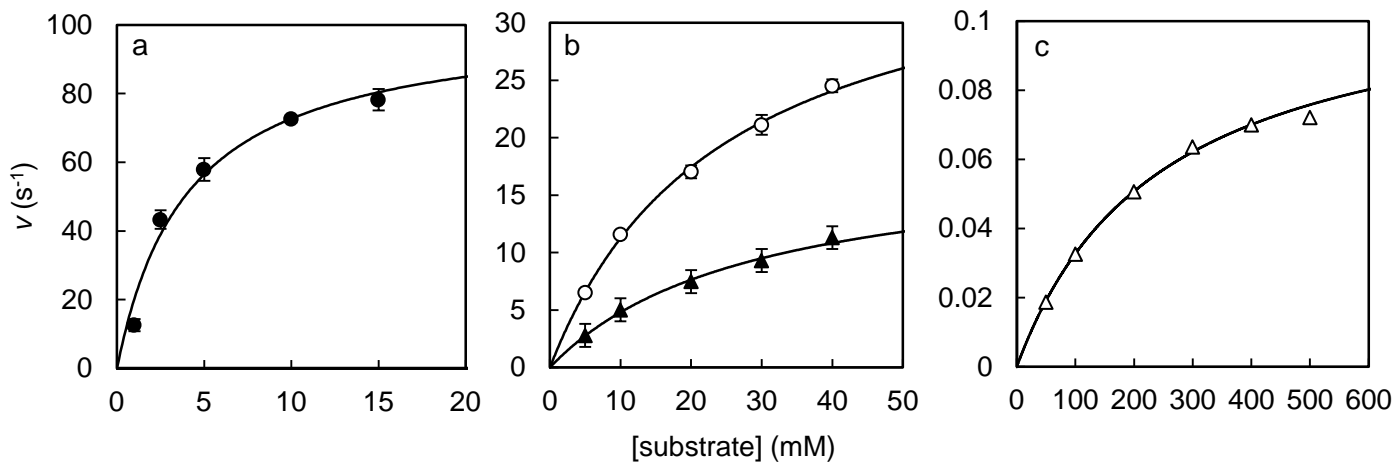


Fig. 3, Saburi *et al.*

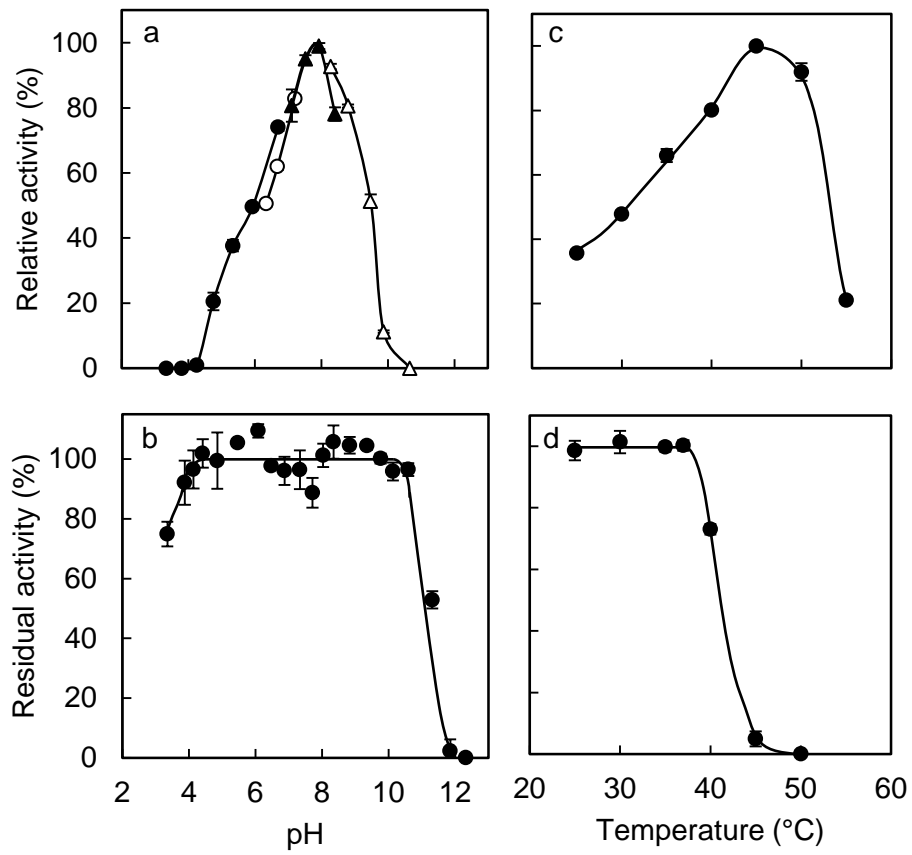


Fig. 4, Saburi *et al.*

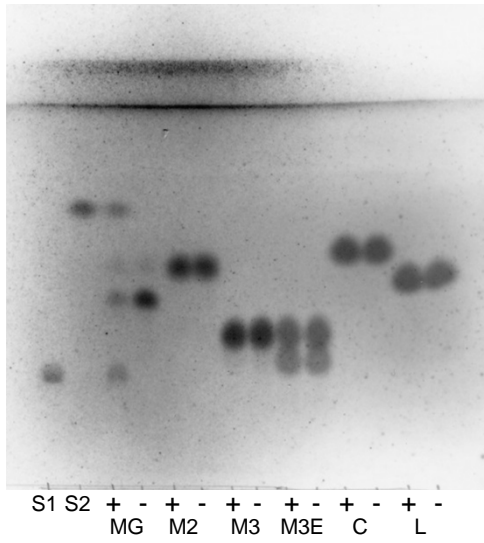


Fig. 5, Saburi *et al.*

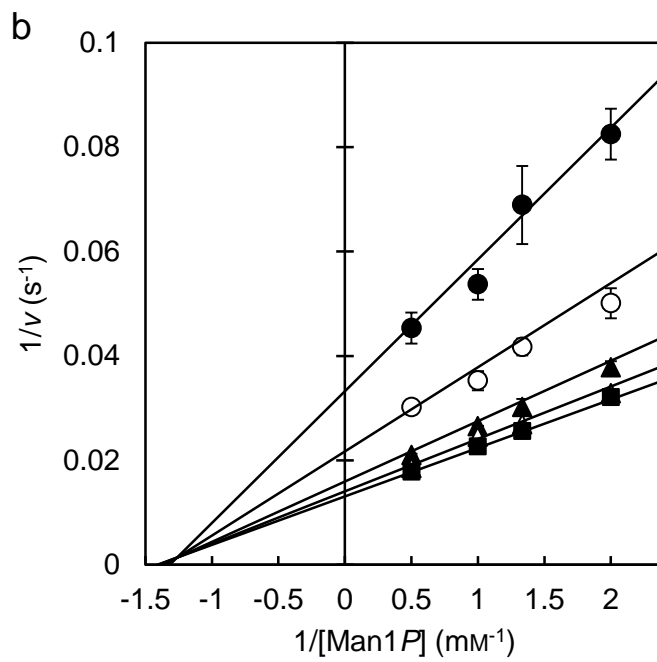
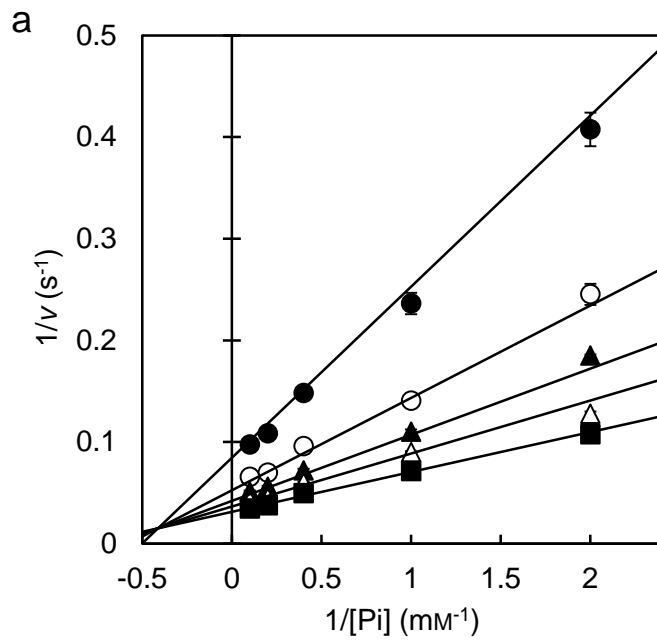


Fig. 6, Saburi *et al.*

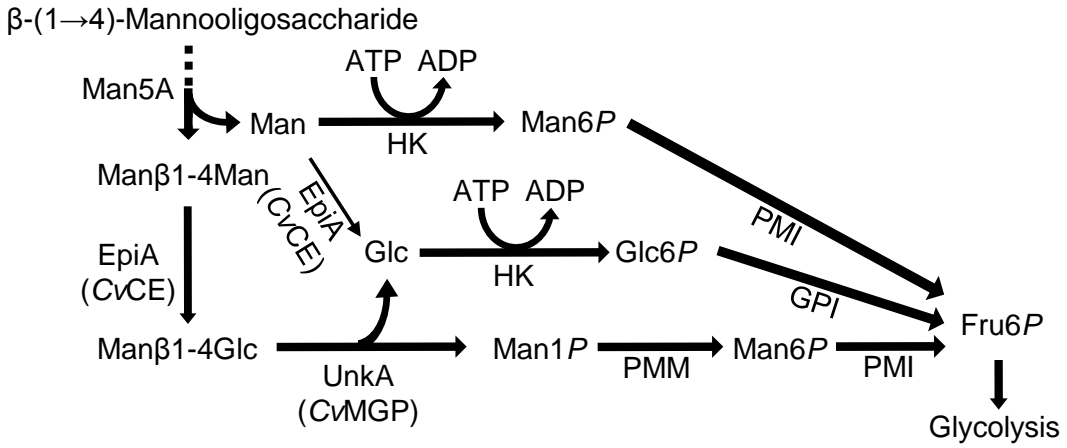


Fig. 7, Saburi *et al.*

Table 1. Kinetic Parameters of Recombinant EpiA (CvCE).

Substrate	k_{cat} (s^{-1})	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ ($\text{mM}^{-1}\text{s}^{-1}$)
β -(1 \rightarrow 4)-Mannobiose	102 \pm 5	4.03 \pm 0.72	25.3
Cellobiose	38.8 \pm 1.0	24.5 \pm 0.8	1.58
Lactose	18.6 \pm 1.7	28.7 \pm 6.3	0.648
D-Mannose	0.113 \pm 0.003	245 \pm 10	4.61×10^{-4}

Each value represents the mean \pm standard deviation of three independent experiments.

Table 2. Apparent Kinetic Parameters of Recombinant UnkA (CvMGP) for Various Acceptor Substrates.

Substrate	$k_{\text{cat}(\text{app})}$ (s^{-1})	$K_{\text{m}(\text{app})}$ (mM)	$k_{\text{cat}(\text{app})}/K_{\text{m}(\text{app})}$ ($\text{mM}^{-1}\text{s}^{-1}$)
D-Glucose	91.3 \pm 3.4	14.1 \pm 1.5	6.48
6-Deoxy-D-glucose	48.1 \pm 6.7	37.5 \pm 5.1	1.28
D-Xylose	8.63 \pm 1.05	45.6 \pm 8.6	0.189

Each value represents the mean \pm standard deviation of three independent experiments.