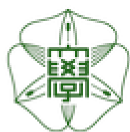
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Running title: Characterization of *R. marinus* MGP

Characterization of a Thermophilic 4-*O*-β-D-Mannosyl-D-glucose Phosphorylase from *Rhodothermus marinus*

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Abbreviations: β-mannanase, mannan endo-1,4-β-mannosidase; CE, cellobiose 2-epimerase; Man-Glc, 4-*O*-β-D-mannosyl-D-glucose; Man1*P*, α-D-mannosyl phosphate; MGP, 4-*O*-β-D-mannosyl-D-glucose phosphorylase; BfMGP, MGP from *Bacteroides fragilis* NCTC9343; RmMBP, *Rmar_2440*-encoded protein from *Rhodothermus marinus* ATCC43812; RaMP, MGP from *Ruminococcus albus* NE1; MES, morpholine ethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; Pi, inorganic phosphate

4-*O*-β-D-Mannosyl-D-glucose phosphorylase (MGP), found in anaerobes, converts 4-*O*-β-D-mannosyl-D-glucose (Man-Glc) to α-D-mannosyl phosphate and D-glucose. It participates in mannan metabolism with cellobiose 2-epimerase (CE), which converts β-1,4-mannobiose to Man-Glc. A putative MGP gene is present in the genome of the thermophilic aerobe *Rhodothermus marinus* (Rm) upstream of the gene encoding CE. Konjac glucomannan enhanced production by *R. marinus* of MGP, CE, and extracellular mannan endo-1,4-β-mannosidase. Recombinant RmMGP catalyzed the phosphorolysis of Man-Glc through a sequential bi-bi mechanism involving ternary complex formation. Its molecular masses were 45 kDa and 222 kDa under denaturing and nondenaturing conditions, respectively. Its pH and temperature optima were 6.5 and 75°C, and it was stable between pH 5.5–8.3 and below 80°C. In the reverse reaction, RmMGP had higher acceptor preferences for 6-deoxy-D-glucose and D-xylose than *R. albus* NE1 MGP. In contrast to *R. albus* NE1 MGP, RmMGP utilized methyl β-D-glucoside and 1,5-anhydro-D-glucitol as acceptor substrates.

Key words: *Rhodothermus marinus*; 4-*O*-β-D-mannosyl-D-glucose phosphorylase; mannan; substrate specificity; phosphorolysis

Plant mannan is a major component of hemicellulose in the cell wall. Its main chain is formed by β-1,4-linked D-mannosyl residues or a combination of β-1,4-linked D-mannosyl and D-glucosyl residues (glucomannan).[1)] Branches of single α-1,6-linked D-galactosyl residues are present in the backbones of mannan. The 2-OH or 3-OH groups of some D-mannosyl and D-glucosyl residues in the main chain are acetylated. Several microbial enzymes degrade mannan,[1-3)] including mannan endo-1,4-β-mannosidase (β-mannanase, EC 3.2.1.78), β-mannosidase (EC 3.2.1.25), and β-glucosidase (EC 3.2.1.21). β-Mannanase is an endo-acting enzyme that hydrolyzes internal β-1,4-mannosidic linkages to produce oligosaccharides. β-Mannosidase and β-glucosidase catalyze exo-hydrolysis of the non-reducing-end β-1,4-mannosidic and β-1,4-glucosidic linkages of the resulting oligosaccharides, respectively. α-Galactosidase (EC 3.2.1.22) removes the galactosyl branches to enhance the degradation of mannan. Acetyl mannan esterase (EC 3.1.1.6) contributes to the deacetylation of mannan.

A novel degradation pathway of β-1,4-mannooligosaccharides has been discovered in intestinal and ruminal anaerobes *Bacteroides fragilis* and *Ruminococcus albus* NE1.[4,5)] In this pathway, cellobiose 2-epimerase (CE, EC 5.1.3.11), which catalyzes the interconversion of D-glucose residues at the reducing end of β-1,4-linked oligosaccharides to D-mannose residues, epimerizes β-1,4-mannobiose to β-1,4-β-D-mannosyl-D-glucose (Man-Glc). Man-Glc is phosphorolyzed to α-D-mannosyl phosphate (Man1*P*) and D-glucose by 4-*O*-β-D-mannosyl-D-glucose phosphorylase (MGP, EC 2.4.1.281). In addition to MGP, *R. albus* NE1 produces a β-1,4-mannooligosaccharide phosphorylase (RaMP2) that catalyzes the sequential phosphorolysis of β-1,4-mannooligosaccharides longer than β-1,4-mannobiose to liberate Man1*P*. Based on their amino acid sequences,[6)] these mannoside phosphorylases are classified as glycoside hydrolase family 130 together with β-1,4-D-mannosyl-*N*-acetyl-D-glucosamine phosphorylase[7)] and unknown human gut bacterium Mannoside Phosphorylase which catalyzes the phosphorolysis of β-1,4-D-mannosyl-β-1,4-*N*-acetyl-D-glucosaminyl-β-1,4-*N*-acetyl-D-glucosamine[8)].

1 *Rhodothermus marinus*, first isolated from submarine alkaline hot springs in Iceland,
2 is an obligatory aerobic, moderately halophilic, thermophilic Gram-negative
3 bacterium.[9] It produces thermophilic glycoside hydrolases, including endo-β-1,4-
4 xylanase (EC 3.2.1.8),[10] xylan 1,4-β-xylosidase (EC 3.2.1.37),[11] cellulase (EC
5 3.2.1.4),[12] endo-1,3(4)-β-glucanase (EC 3.2.1.6),[13] chitinase (EC 3.2.1.14),[14] β-
6 mannanase,[15] trehalase (EC 3.2.1.28),[16] α-glucosidase (EC 3.2.1.20),[17] pullulanase
7 (EC 3.2.1.41),[17] and α-amylase (EC 3.2.1.1).[17]. Recently we discovered that *R. marinus*
8 produces a CE that we subsequently characterized in detail.[18] Because this enzyme is
9 fully stable at high temperatures and prefers lactose over cellobiose, unlike other
10 enzymes, it is an attractive candidate for producing the prebiotic oligosaccharide
11 epilactose[19] from lactose. A system for the continuous production of epilactose by
12 means of immobilized *R. marinus* CE has been established.[20]
13 Analysis of the *R. marinus* R-10^T genome revealed that a putative MGP (RmMGP) is
14 encoded by *Rmar_2440*, which is located upstream of the gene, *Rmar_2439*, that
15 encodes CE.[21] The amino acid sequence of RmMGP is 68% and 61% identical to those
16 of BfMGP and *R. albus* NE1 MGP (RaMGP, called RaMP1 in reference 5),
17 respectively. Two genes encoding glycoside hydrolase family 26 β-mannanase
18 (*Rmar_0016*, ManA;[15] and *Rumal_0467*, a putative β-mannanase) are also present,
19 suggesting that *R. marinus* R-10^T also degrades mannan through a metabolic pathway
20 similar to those of *B. fragilis* NCTC9343 and *R. albus* NE1. In the present study, we
21 compared the activities of β-mannanase, CE, and MGP of *R. marinus* cultured in the
22 presence and the absence of konjac glucomannan, and investigated the enzymatic
23 properties of the recombinant Rmar_2440 protein produced in *Escherichia coli*.
24
25 **Materials and Methods**
26
27 *Bacterial strain. R. marinus* R-10^T (ATCC43812) was purchased from the American
28 Type Culture Collection (Manassas, VA).

1

2 *Culture conditions and protein extraction. R. marinus* was cultured in 50 mL of
3 Marine Broth 2216 (Becton Dickinson, Sparks, MD) with (2 mg/mL) and without
4 glucomannan (Shimizu Kagaku, Hiroshima, Japan) at 50°C for 60 h. Ten mL of culture
5 broth was harvested every 12 h, and the cells were harvested by centrifugation at 5,400
6 × *g* for 10 min at 4°C. The culture supernatant was assayed for β-mannanase activity.
7 Bacteria suspended in 1 mL of 10 mM potassium phosphate buffer (pH 7.0) were
8 disrupted using an Ultrasonic Disruptor UD-201 (Tomy, Tokyo). Cell debris was
9 removed by centrifugation at 19,000 × *g* for 5 min at 4°C. Protein concentrations and
10 the activities of CE, MGP, and β-mannanase were measured as described below.

11

12 *Preparation of an* Rmar_2440 *expression plasmid. Rmar_2440* was amplified by the
13 polymerase chain reaction with primer pair 5′-
14 AATGCATATGGAAGTGCGAATGGCACCGAC-3′ (*Nde*I site underlined) and 5′-
15 TTAACTCGAGTCACGGGCGCTTCAGCAGTT-3′ (*Xho*I site underlined). *R. marinus*
16 ATCC43812 genomic DNA was used as template.[18] The amplified DNA fragment was
17 cloned into the *Eco*RV site of pBluescript II SK (+) vector (Stratagene, La Jolla, CA). A
18 DNA fragment prepared by double-digestion with *Nde*I and *Xho*I was inserted into the
19 *Nde*I and *Xho*I sites of pET23a (Novagen, Darmstadt, Germany). The DNA sequence of
20 the inserted region was determined using an Applied Biosystems 3130 Genetic
21 Analyzer (Life Technologies, Carlsbad, CA).

22

23 *Production and purification of recombinant RmMGP. E. coli* BL21 (DE3),
24 transformed with the RmMGP expression vector, was cultured in 1 L of Luria-Bertani
25 medium containing 50 μg/mL of ampicillin at 37°C until the absorbance at 600 nm
26 reached 0.6. Protein expression was induced by adding 1 mL of 0.1 M isopropyl β-D-
27 thiogalactoside to the culture medium (final concentration, 0.1 mM), and incubation was
28 continued at 18°C for 24 h with vigorous shaking. Bacterial cells were harvested by

1 centrifugation at 8,400 × *g* at 4°C for 10 min, resuspended in 40 mL of 20 mM 4-
2 morpholine ethanesulfonic acid (MES)-NaOH buffer (pH 7.0), and disrupted by
3 sonication. A cell-free extract was prepared by centrifuging the suspension of the
4 disrupted cells at 13,000 × *g* at 4°C for 10 min, and the supernatant was subjected to
5 anion exchange chromatography using a Toyopearl DEAE-650 M column (i.d. 3.0 ×
6 10.5 cm, Tosoh, Tokyo). After a thorough washing the column with 20 mM MES-NaOH
7 buffer (pH 7.0), the adsorbed protein was eluted with a linear gradient of NaCl from 0
8 to 0.5 M (total elution volume, 250 mL). The active fractions were pooled and applied to
9 a Toyopearl Butyl-650M column (i.d. 3.0 × 10.5 cm, Tosoh) equilibrated with 10 mM
10 MES-NaOH buffer (pH 7.0) containing 300 g/L of ammonium sulfate. Non-adsorbed
11 protein was completely eluted with the same buffer, and the adsorbed protein was eluted
12 using a descending linear gradient of ammonium sulfate from 300 to 0 g/L (total elution
13 volume, 250 mL). The active fractions were pooled and concentrated to 3 mL with a
14 Vivaspin 20 (nominal molecular weight limit 30,000, Sartorius, Göttingen, Germany)
15 and subjected to Sephacryl S-300 column chromatography (i.d. 1.6 × 67 cm, GE
16 Healthcare Bio-Sciences, Uppsala, Sweden). The column was eluted with 10 mM MES-
17 NaOH buffer (pH 7.0) containing 0.2 M NaCl at a flow rate of 0.3 mL/min. The purity
18 of the fractions was confirmed by sodium dodecyl sulfate-polyacrylamide gel
19 electrophoresis (SDS-PAGE), and highly purified fractions were collected. The purified
20 enzyme was dialyzed against 10 mM MES-NaOH buffer (pH 7.0) and stored at –80°C.
21
22 *Protein assay.* The protein concentration of the cell-free extract was measured by the
23 Bradford method[22] with bovine serum albumin (Nacalai Tesque, Kyoto, Japan) as
24 standard. The protein concentrations of the column chromatography fractions were
25 determined using the UV method,[23] assuming an extinction coefficient of 1 mg/mL of
26 protein equal to 1.00. The concentration of the purified enzyme was calculated from the
27 amino acid concentrations of an acid hydrolysate (6 M HCl at 110°C for 24 h). The

1 amino acids concentrations were measured by the ninhydrin colorimetric method with a
2 JLC-500/V (JEOL, Tokyo, Japan).[24)]
3
4 *Enzyme activity assays.* MGP activity: The reaction mixture (50 μL) contained 2 mM
5 Man-Glc, 100 mM sodium phosphate buffer (pH 6.5), 4 mM MES-NaOH buffer (pH
6 6.5), 0.2 mg/mL of bovine serum albumin, and an appropriate concentration of enzyme.
7 After incubation at 50°C for 10 min, the reaction was stopped by adding 100 μL of 2 M
8 Tris-HCl buffer (pH 7.0) and incubated immediately at 100°C for 3 min. The liberated
9 D-glucose was measured using a Glucose CII test (Wako Pure Chemical Industries,
10 Osaka, Japan). Man-Glc was prepared as described previously.[5)] The enzyme was
11 diluted with 20 mM MES-NaOH buffer (pH 6.5) containing 1 mg/mL of bovine serum
12 albumin. One unit (U) of enzyme activity was defined as the amount of enzyme that
13 produces 1 μmol of D-glucose from Man-Glc in 1 min.
14 β-Mannanase activity: The reaction mixture (100 μL) containing 1 mg/mL of
15 glucomannan, 25 mM sodium phosphate buffer (pH 6.0), and the enzyme was incubated
16 at 60°C for 10 min. The reducing sugar product was quantified by the Somogyi-Nelson
17 method[25)] with 0–1 mM D-mannose as standard. One U of enzyme activity was defined
18 as the amount of enzyme that produces 1 μmol of reducing sugar in 1 min.
19 CE activity: The reaction mixture (100 μL), containing 100 mM β-1,4-mannobiose
20 (Megazyme, Wicklow, Ireland), 20 mM potassium phosphate buffer (pH 7.0), and the
21 enzyme, was incubated at 50°C for 1 h, and the reaction was stopped by incubation at
22 100°C for 5 min. The yield of the reaction product Man-Glc was measured by high-
23 performance liquid chromatography under the conditions as follows: injection volume,
24 5 μL; columns, two tandem Sugar SP0810 columns (i.d. 8.0 × 300 mm × 2; Shodex,
25 Tokyo), column temperature, 70°C; eluant, water; flow rate, 0.5 mL/min. One U of
26 enzyme activity was defined as the amount of enzyme that produces 1 μmol of Man-Glc
27 in 1 min.
28

1 *Effects of pH and temperature on the activity and stability of RmMGP.* Enzyme
2 activity was determined over a pH range of 3.1–9.7 and a temperature range of 30–
3 100°C. To determine the optimum pH, reaction mixtures containing 74.8 nM RmMGP,
4 2 mM Man-Glc, 10 mM sodium phosphate, and 100 mM reaction buffer (sodium citrate
5 buffer, MES-NaOH buffer, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
6 (HEPES)-NaOH buffer, and glycine-NaOH buffers at pH values of 3.1–6.4, 6.5–6.9,
7 7.3–8.1, and 8.2–9.7, respectively) were incubated at 50°C for 10 min. The
8 concentrations of D-glucose were measured as described above. To determine the
9 optimum temperature, a reaction mixture containing 150 nM RmMGP, 2 mM Man-Glc,
10 10 mM sodium phosphate, and 100 mM MES-NaOH buffer (pH 6.5) was incubated at
11 30–90°C at 5°C increments for 10 min. The concentrations of D-glucose were measured
12 as described above.
13 The pH stability and thermostability of RmMGP were evaluated by measuring
14 residual activity after pH and heat treatment, respectively. To determine enzyme
15 stability as a function of pH, mixtures (100 μL) containing 3.74 μM RmMGP and 80
16 mM buffers described above (pH 3.1–9.7) were incubated at 4°C for 24 h. To determine
17 enzyme stability as a function of temperature, mixtures (40 μL) containing 93.5 nM
18 RmMGP, 12.5 mM sodium phosphate, and 125 mM MES-NaOH buffer (pH 6.5) were
19 incubated at 30–90°C for 20 min. The enzyme was considered stable under conditions
20 that caused the retention of ≥90% of activity before treatment.
21

22 *Kinetic analysis of MGP.* Kinetic parameters for the phosphorolysis of Man-Glc
23 were determined from reaction rates at various concentrations of Man-Glc and inorganic
24 phosphate (Pi). A reaction mixture (50 μL) containing 37 nM RmMGP, 1–20 mM Man-
25 Glc, 1–10 mM sodium phosphate, and 100 mM MES-NaOH buffer (pH 6.5) was
26 incubated at 50°C for 10 min, and D-glucose was measured as described above. The

1 kinetic parameters were calculated by fitting the reaction rates to the equation for a
2 sequential bi-bi mechanism,[26] as follows:

3 $$v = k_{cat}[A][B]/(K_{iA}K_{mB} + K_{mB}[A] + K_{mA}[B] + [A][B])$$
4 (A = Man-Glc, B = Pi)

5 Non-linear regression was carried out using Grafit version 7.0.2 (Erithacus Software,
6 West Sussex, UK).
7
8 *Acceptor specificity of RmCE in the synthetic (reverse) reaction.* Twenty-five μL of a
9 reaction mixture containing the enzyme, 10 mM Man1*P*, 10 mM acceptor substrate, and
10 50 mM MES-NaOH buffer (pH 6.5) was incubated at 50°C for 10 min. The enzyme
11 reaction was stopped by adding 62.5 μL of 100 g/L of ascorbic acid solution, and Pi was
12 measured following the method published by Lowry and Lopez.[27] Man1*P*
13 (dicyclohexylamine salt) was synthesized from D-mannose and ATP using *N*-
14 acetylhexosamine 1-kinase.[28] The acceptor substrates were as follows: D-glucose, D-
15 mannose, D-allose, D-xylose, 1,5-anhydro-D-glucitol, methyl α-D-glucoside, and methyl
16 β-D-glucoside (Wako Pure Chemical Industries); 6-deoxy-D-glucose and cellobiose
17 (Sigma, St. Louis, MO); D-glucosamine (Tokyo Chemical Industries, Tokyo); D-glucitol
18 and *N*-acetyl-D-glucosamine (Nacalai Tesque); and β-1,4-mannobiose. Apparent kinetic
19 parameters were determined by fitting the reaction rates at varying concentrations of
20 acceptor substrates (1.25–40 mM) and 10 mM Man1*P* to the Michaelis-Menten equation.
21
22 *Estimation of molecular mass by gel filtration column chromatography.* One hundred
23 μL of 0.42 mg/mL of RmMGP was subjected to gel filtration column chromatography
24 as follows: column, Superose 12 10/300 GL (i.d. 1.0 x 30 cm, GE Healthcare Bio-
25 Sciences); buffer, 20 mM MES-NaOH buffer (pH 7.0) containing 0.2 M NaCl; flow rate,
26 0.5 mL/min; detection, absorbance at 280 nm. A gel filtration molecular mass standard
27 (Bio-Rad, Hercules, CA) was used to generate a calibration curve.

Results and Discussion

CE, MGP, and β-mannanase activities of a cell-free extract of R. marinus cultured in the presence and the absence of mannan

The growth of *R. marinus* and the production of mannan-metabolizing enzymes in the presence (2 mg/mL) and the absence of glucomannan were compared (Fig. 1). Growth was more abundant in the presence of glucomannan as carbon source than in its absence. Thus the absorbance at 600 nm of the culture broth with glucomannan at 60 h was higher than that of the control by a factor of approximately 2. The production of extracellular β-mannanase in the presence of glucomannan reached a maximum in the early phase of cell growth (36 h).β-Mannanase activity at 36 h was 0.087 U/mL, higher by a factor of 37 than that in the absence of glucomannan.β-Mannanase activity in the presence of glucomannan was maintained until 48 h, but had decreased significantly by 60 h. The reason for a decrease in extracellular β-mannanase activity remains unclear. ManA is fully stable at a culture temperature 50°C,[15)] and heat inactivation does not appear to occur during a culture. At a late stage of culture, extracellular enzymes might be degraded proteolytically as nitrogen source. The intracellular MGP and CE activities of cells grown in the presence of glucomannan were significantly higher than for the control. The maximum activities of intracellular MGP and CE in the cells cultured in the presence of glucomannan were higher by factors of 6.1 (60 h) and 34 (48 h), respectively, than in the control.

Fig. 1

Production and purification of recombinant RmMGP

Recombinant RmMGP was successfully produced in *E. coli* BL21 (DE3) in soluble form and was purified to homogeneity at a yield of 3.32 mg/L (Fig. 2). The recombinant enzyme catalyzed the phosphorolysis of Man-Glc, and its specific activity was 7.17 U/mg in the presence of 2 mM Man-Glc and 100 mM Pi at pH 6.5 at 50°C. The

1 molecular mass of RmMGP was estimated as 45 kDa by SDS-PAGE, which is
2 consistent with 45,295.67 Da calculated from its amino acid sequence, and is close to
3 those of BfMGP and RaMGP.[4,5] Under nondenaturing conditions, the molecular mass
4 of RmMGP was estimated as 222 kDa, indicating that RmMGP forms a homopentamer.
5 In contrast, RaMGP and BfMGP form homodimers and homohexamers,
6 respectively.[5,29]

Fig. 2

7

8 *Effects of pH and temperature on enzyme activity and stability*

9 RmMGP activity was highest at pH 6.5, similarly to those of other MGPs (Fig.
10 3a),[4,5] and was stable at pH 5.5–8.3. Activity was highest at 75°C, similarly to those of
11 other *R. marinus* enzymes.[9-18] RmMGP retained more than 90% of its activity before
12 treatment when incubated for 20 min at temperatures up to 80°C (Fig 3b). The optimum
13 temperature of RmMGP is higher than those of other MGPs, which are most active at
14 50°C.[4,5] The Pro residue content of RmMGP (8.3%) is approximately twice as high as
15 those of BfMGP (4.9%) and RaMGP (3.6%). Moreover, the Pro residue content of *R.*
16 *marinus* CE is higher than those of other CEs with mild optimum temperatures.[18] Pro
17 residues situated in loops connecting adjacent secondary structures might account for
18 the high thermostability of *Bacillus thermoglucosidasius* KP1006 oligo-1,6-
19 glucosidase.[30] Compared with RaMGP and structure known BfMGP,[29] RmMGP
20 apparently has more Pro residues on several loops (Fig. 4). Hence these Pro residues
21 might contribute to the rigidity and thermostability of RmMGP as well.

22

Fig. 3

23 *Kinetic analysis of the phosphorolysis of Man-Glc*

Fig. 4

24 The initial velocities of phosphorolysis of Man-Glc at various concentrations of Pi
25 and Man-Glc were determined (Fig. 5). The curves obtained by plotting 1/*v* *versus*
26 1/[Man-Glc] at various Pi concentrations were linear, and intersected at the same point.
27 These data indicate that RmMGP catalyzed the phosphorolysis of Man-Glc through a
28 sequential bi-bi mechanism involving the formation of a ternary complex, as observed

1 for RaMGP and other inverting carbohydrate phosphorylases.[5,6,31-37] The calculated
2 kinetic parameters are as follows: k_{cat} = 20.5 ± 0.1 s^{-1}, K_{mA} = 0.994 ± 0.051 mM, K_{mB} =
3 1.07 ± 0.03 mM, and K_{iA} = 5.78 ± 0.43 mM (A, Man-Glc; B, Pi).
4 Fig. 5

5 *Acceptor specificity of the synthetic reaction*

6 The acceptor specificity of the synthetic reaction was determined by measuring
7 initial reaction rates at a single concentration (10 mM) of Man1*P* and various substrates.
8 D-Glucose, methyl β-D-glucoside, 1,5-anhydro-D-glucitol, 6-deoxy-D-glucose, and D-
9 xylose served as acceptors. No synthetic activity was detected in the presence of D-
10 allose, D-mannose, methyl α-D-glucoside, cellobiose, D-glucosamine, *N*-acetyl-D-
11 glucosamine, or β-1,4-mannobiose. The apparent kinetic parameters for acceptors were
12 determined from the reaction rates at various concentrations of acceptors and 10 mM
13 Man1*P* (Table 1). The value of $k_{cat(app)}/K_{m(app)}$ of RmMGP for 6-deoxy-D-glucose is
14 similar to that of D-glucose, although for the synthetic reaction of RaMGP,
15 monodeoxygenation at the 6-OH position results in large decreases in $k_{cat(app)}/K_{m(app)}$
16 caused by an increase in $K_{m(app)}$.[5] Because $k_{cat(app)}/K_{m(app)}$ of RaMGP for D-xylose is
17 12% of that for 6-deoxy-D-glucose,[5] the methylene group of acceptor substrates is
18 important for activity. However, for RmMGP, $k_{cat(app)}/K_{m(app)}$ for D-xylose is 36% of that
19 for 6-deoxy-D-glucose. This finding indicates that the 6-OH and C-6 methylene groups
20 of acceptor substrates are less important for the recognition of acceptor substrate by
21 RmMGP compared with RaMGP. Table 1

22 In the complex of BfMGP and Man-Glc, hydrophobic interactions with the C-6
23 methylene group of the reducing-end D-glucose residue are provided by Phe214 and
24 Ile215.[29] The positions of these residues in RmMGP and RaMGP correspond to those
25 of BfMGP (Fig. 4). Hence, the difference in synthetic activity towards the 6-OH
26 derivatives of D-glucose between RmMGP and RaMGP can be attributed to differences
27 in indirect interactions with the acceptors. In contrast to RaMGP, RmMGP exhibited
28 synthetic activity towards 1-OH glucose derivatives 1,5-anhydro-D-glucitol and methyl

1 β-D-glucoside. In view of the fact that the absence of the 1-OH group did not result in a
2 complete loss of synthetic activity, in contrast to RaMGP, the 1-OH group of the
3 acceptor substrate is less important for the synthetic activity of RmMGP than for
4 RaMGP. In the BfMGP structure, Arg94 forms a hydrogen bond with the 1-OH group
5 of the β-D-glucose residue of Man-Glc,[29] but this Arg is conserved in RaMGP and
6 RmMGP (Fig. 4). The synthetic activity of RmMGP towards methyl β-D-glucoside
7 suggests that the orientation of Arg101, corresponding to BfMGP Arg94, differs from
8 that of BfMGP, because the space in the acceptor binding site accommodates a β-linked
9 methyl group.

10

11 In summary, we report here the first enzymatic characterization of an MGP of the
12 aerobic bacterium *R. marinus*. The presence of MGP in an aerobic bacterium suggests
13 that the degradation of mannan mediated by CE and MGP is not limited to anaerobes
14 such as *B. fragilis* NCTC9343 and *R. albus* NE1. The acceptor specificity of RmMBP
15 differs from that of RaMGP. Insight into their structural differences will be provided by
16 determining the three-dimensional structures of these MGPs.

17

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23

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25

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1 **Figure legends**

2 **Fig. 1.** Growth Curve of *R. marinus* ATCC43812 and Production of Mannan-Degrading
3 Enzymes.
4 Growth in the presence (black circles) and absence (white circles) of glucomannan as
5 a carbon source, respectively. a, Growth curve; b, extracellular β-mannanase activity; c,
6 MGP activity; d, CE activity.
7
8 **Fig. 2.** SDS-PAGE Analysis of Purified RmMGP.
9 Lane M, size marker; lane S, purified RmMGP (1 μg). The molecular masses of
10 standard proteins are indicated on the left. The proteins were visualized using
11 Coomassie Brilliant Blue.
12
13 **Fig. 3.** Effects of Temperature and pH on Enzyme Activity and Stability.
14 MGP activity was measured at various pH values (a, closed circles) and temperature
15 (b, closed circles) to determine optimal conditions. Activity was measured after pH (a,
16 open circles) and heat treatment (b, open circles) to evaluate stability. The enzyme
17 solution was incubated at various pH values at 4°C for 24 h and at various temperatures
18 at pH 6.5 for 20 min.
19
20 **Fig. 4.** Multiple Alignment of Amino Acid Sequences of RmMGP, RaMGP, and
21 BfMGP.
22 Multiple alignment was constructed using Clustal W program
23 (http://clustalw.ddbj.nig.ac.jp/). Secondary structures of BfMGP are shown above
24 sequence alignment using ESPript 2.2 program (http://espript.ibcp.fr/ESPript/ESPript/).
25 α and β indicate α-helix and β-strand, respectively. Conserved amino acid residues are
26 shown by black shade. Inverted black triangles indicate non-conserved Pro residues of
27 RmMGP, situated on the loops connecting adjacent secondary structures. Black circles
28 indicate amino acid residues forming the acceptor binding site.

1

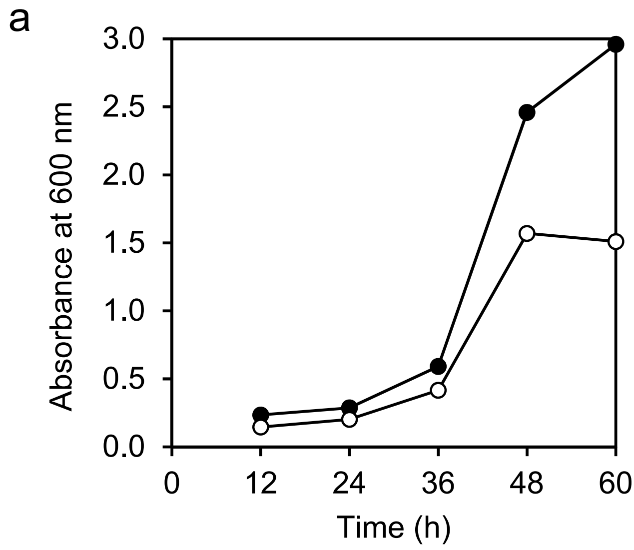
2 **Fig. 5.** Double-Reciprocal Plot of the Phosphorolysis of Man-Glc by RmMGP.

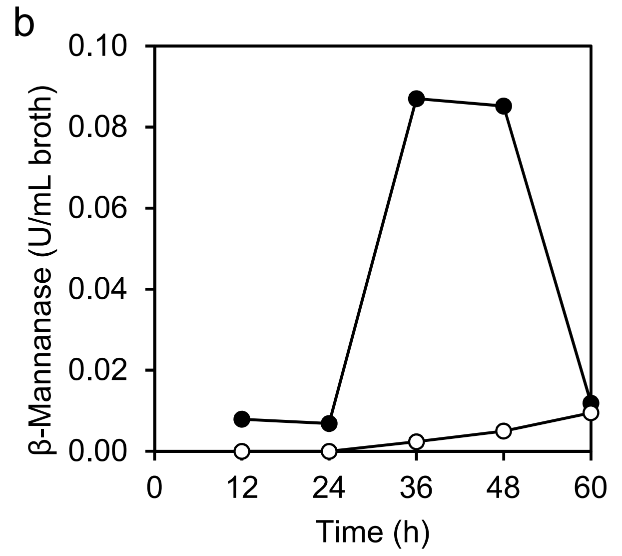
3 The initial velocities for the phosphorolysis of Man-Glc of various concentrations of

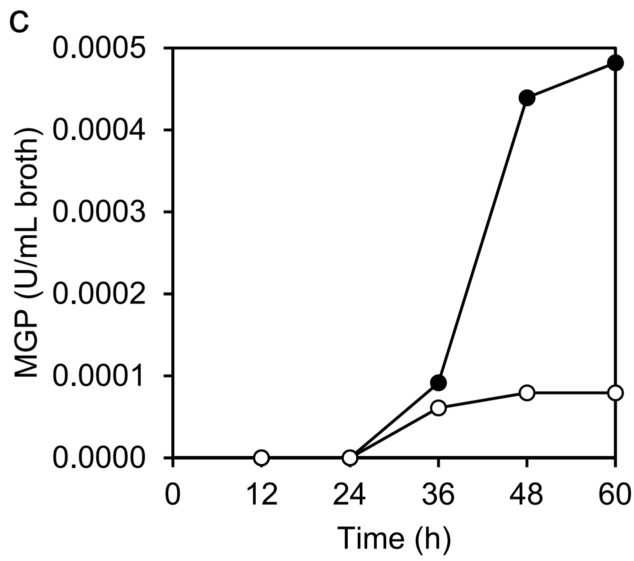
4 Man-Glc and Pi were determined. The concentrations of Pi were 1 (open circles), 2.5

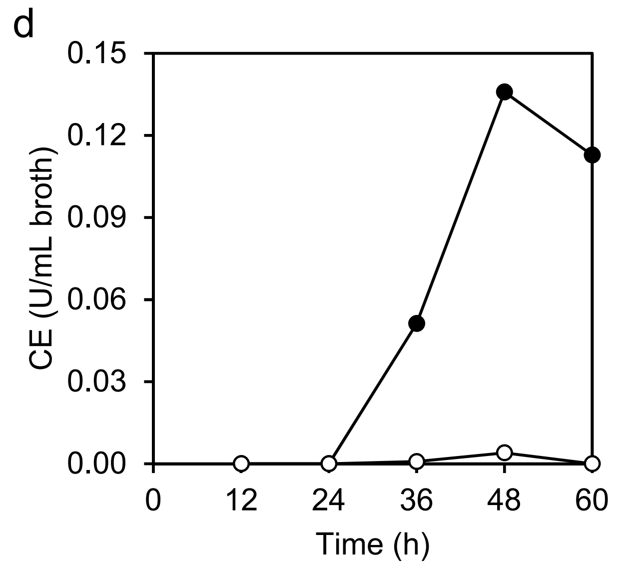
5 (filled circles), 5 (open triangles), and 10 mM (filled triangles). Data represent mean ±

6 standard deviation (error bars) for three independent experiments.

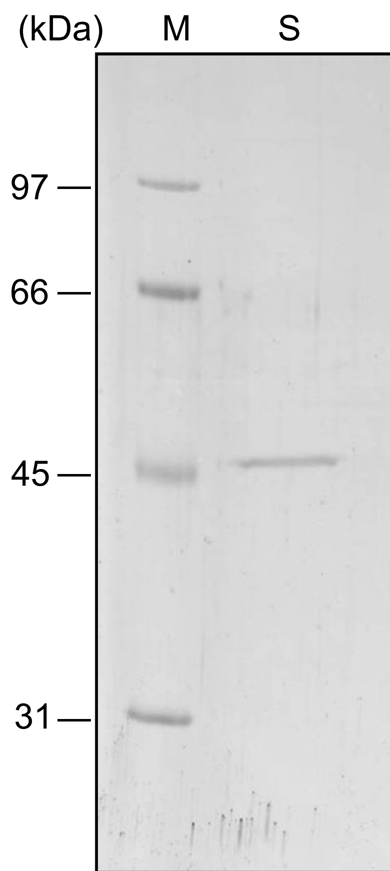








Jaito et al., Fig. 1



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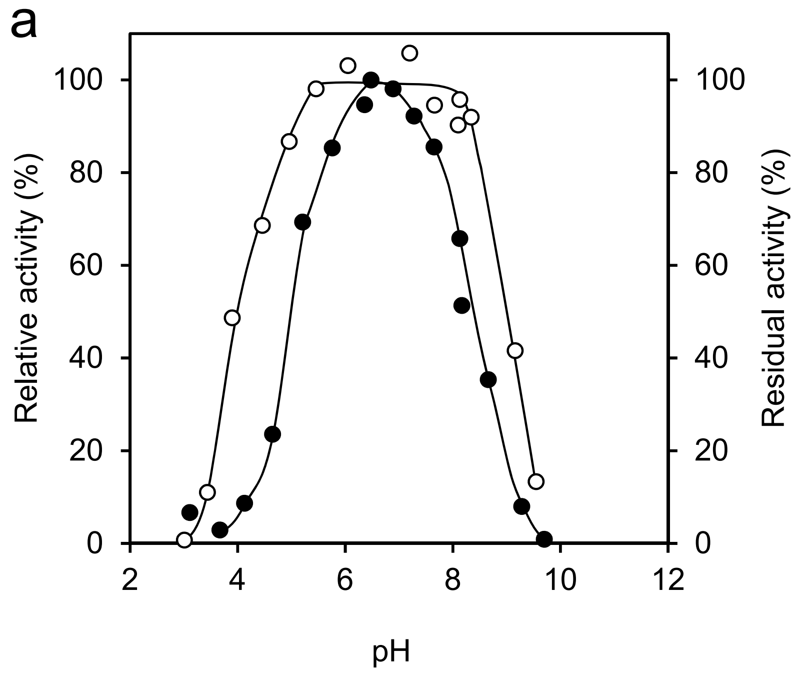

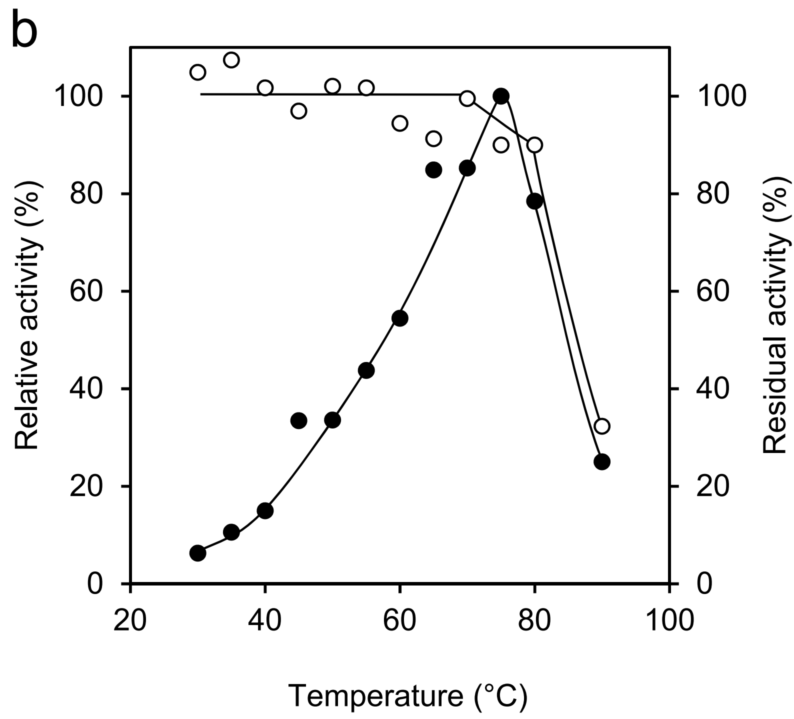

Jaito et al, Fig. 3

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BfMGP   α1  β1  η1  α2
        1         10        20        30        40        50        60
BfMGP   .......MSLFNDKVAKLLAGHEALLMRKNEPVEEGNGVITRYRYPVLTAAHTPVFWRYDLNEETNPFLM
RmMGP   MEVRMAPTEAFALRLKRLEAAYEELITRPNEPLPDGNGIITRYRYPVLTAAHTPIFWRYDLNPETNPYLM
RaMGP   .........MIHEKYTEMRNEQEALLSRKNTKTSFYNGIYDRYEHPVLTREHIPLHWRYDLNKETNPFFQ

BfMGP   β2  β3  β4  β5  β6
          70        80        90        100       110       120       130
BfMGP   ERIGMNATLNAGAIKWDGKYLMLVRVEGADRKSFFAVAESPNGIDNFRFWEYPVTLPEDVVPATNVYDMR
RmMGP   ERMGINAVFNPGAIKLGDRYLLVARVEGVDRKSFFAVAESPNGIDNFRFWDEPIVLPETDDPDVNVYDMR
RaMGP   ERLGINAVFNAGAIKLNDRYCLVARVEGNDRKSFFAVAESDKGTEGFRFRQYPVCLPALTDDETNVYDMR

BfMGP   β7  β8  β9  β10  β11
          140       150       160       170       180       190       200
BfMGP   LTAHEDGWIYGIFCAERHDDNAPIGDLSSATATAGIARTKDLKNWERLPDLKTKS..QQRNVVLHPEFVD
RmMGP   LTLHEDGWIYGVFCTERKDPKAPPTDTTAAMAQCGIARTKDLKTWERLPDLKTPSP.QQRNCVLHPEFVD
RaMGP   LTQHEDGWIYGVFCVEKS...AGTADLSEAVASAGIARTKDLTNWERLPDLVTLRSPQQRNVTLLPEFVD

BfMGP   β12  β13  β14  η2  β15  β16  β17
          210       220       230       240       250       260       270
BfMGP   GKYALYTRPQDGFIDTGSGGGIGWALIDDITHAEVGEEKIIDKRYYHTIKEVKNGEGPHPIKTPQGWLHL
RmMGP   GKYAFYTRPQDDFIEAGSGGGIGWGLCADITNPVITEERIVDPRVYHTIKEAKNGLGPPPIKTPHGWLHL
RaMGP   GKYAFYTRPMDGFIETGSGGGIGFGLADDITHAVIDEERMTSIRRYHTITESKNGAGATPIKTERGWLNI

BfMGP   β18  β19  β20  η3  β21  β22
          280       290       300       310       320       330       340
BfMGP   AHGVRNCAAGLRYVLYMYMTSLDDPTRLIASPAGYFMAPVGEERIGDVSNVLFSNGWIADDDGKVFIYYA
RmMGP   AHGVRNTAAGLRYVLYLFMTALDEPWRVIYAPGGYFMAPEGEERVGDVSNVLFCNGWILDDDGRVLIYYA
RaMGP   AHGVRNTAAGLRYVIYCFVTDLSEPWKVIAEPGGYLIAPFKDERVGDVSNVVFTNGAIVDDNGDVYIYYA

BfMGP   β23  α3  α4
          350       360       370       380       390
BfMGP   SSDTRMHVATSTIERLVDYCLHTPQDGFSSSASVEILKNLIERNLRLMK..
RmMGP   SSDTRCHVATSTLDRLLDYVMHTPPDGGRSAVCVQQRLELIRRNLQLLKRP
RaMGP   SSDTRLHVAVSSIDKLLDYAFNTPADALRTAECVKQRCDLIKRNIELL...
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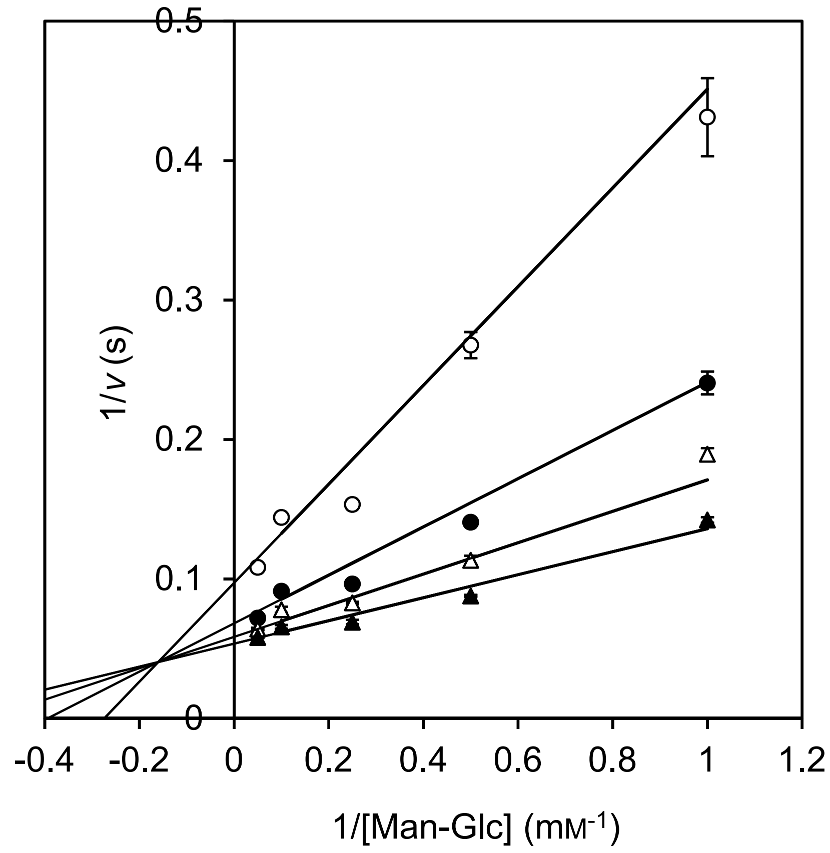
Jaito, et al., Fig. 4



Jaito et al, Fig. 5

Table 1. Comparison of Apparent Kinetic Parameters for Synthetic Reactions between RmMGP and RaMGP

Substrate	RmMGP			RaMGP[5)]		
	$k_{cat(app)}$ (s^{-1})	$K_{m(app)}$ (mM)	$k_{cat(app)}/K_{m(app)}$ ($s^{-1}mM^{-1}$)	$k_{cat(app)}$ (s^{-1})	$K_{m(app)}$ (mM)	$k_{cat(app)}/K_{m(app)}$ ($s^{-1}mM^{-1}$)
1,5-Anhydro-D-glucitol	1.62 ± 0.18	47.5 ± 7.6	0.0341	N.D.	N.D.	N.D.
6-Deoxy-D-glucose	42.3 ± 3.7	65.7 ± 9.0	0.644	177 ± 21	119 ± 18	1.49
D-Glucose	29.6 ± 0.7	42.1 ± 1.1	0.703	126 ± 1	25.9 ± 0.1	4.86
Methyl β-D-glucoside	2.02 ± 0.26	58.0 ± 8.9	0.0348	N.D.	N.D.	N.D.
D-Xylose	N.D.	N.D.	0.229*	N.D.	N.D.	0.180

Apparent kinetic parameters were determined from reaction rates at 1.25-40 mM an acceptor and 10 mM Man1*P*. Values are mean ± SD for three independent experiments. N.D., not determined. *Determined from the slope of s-*v*