



Title	Metabolic Mechanism of Mannan in a Ruminant Bacterium, <i>Ruminococcus albus</i> , Involving Two Mannoside Phosphorylases and Cellobiose 2-Epimerase : Discovery of a New Carbohydrate Phosphorylase, β -1,4-Mannooligosaccharide Phosphorylase
Author(s)	Kawahara, Ryosuke; Saburi, Wataru; Odaka, Rei et al.
Citation	Journal of Biological Chemistry, 287(50), 42389-42399 https://doi.org/10.1074/jbc.M112.390336
Issue Date	2012-12-07
Doc URL	https://hdl.handle.net/2115/51063
Rights	This research was originally published in Journal of Biological Chemistry. Ryosuke Kawahara, Wataru Saburi, Rei Odaka, Hidenori Taguchi, Shigeaki Ito, Haruhide Mori and Hirokazu Matsui. Metabolic Mechanism of Mannan in a Ruminant Bacterium, <i>Ruminococcus albus</i> , Involving Two Mannoside Phosphorylases and Cellobiose 2-Epimerase : Discovery of a New Carbohydrate Phosphorylase, β -1,4-Mannooligosaccharide Phosphorylase. Journal of Biological Chemistry. 2012; Vol:42389-42399. © the American Society for Biochemistry and Molecular Biology.
Type	journal article
File Information	JBC287-50_42389-42399.pdf



Metabolic Mechanism of Mannan in a Ruminant Bacterium, *Ruminococcus albus*, involving two Mannoside Phosphorylases and Cellobiose 2-Epimerase: Discovery of a New Carbohydrate Phosphorylase, β -1,4-Mannooligosaccharide Phosphorylase*

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*Running title: Metabolic Mechanism of Mannan in *Ruminococcus albus*

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Keywords: mannoooligosaccharide phosphorylase; mannan; glycoside hydrolase family 130; phosphorolysis; *Ruminococcus albus*

Background: Characteristics of two 4-O- β -D-mannosyl-D-glucose phosphorylases from *Ruminococcus albus* were investigated.

Results: One enzyme was specific for 4-O- β -D-mannosyl-D-glucose, as observed for the *Bacteroides fragilis* enzyme, but the other showed high activity towards mannoooligosaccharides longer than β -1,4-mannobiose.

Conclusion: Two phosphorylases play distinct roles in the metabolism of mannan.

Significance: A new enzyme catalyzing the phosphorolysis of β -1,4-mannoooligosaccharides was identified.

SUMMARY

Ruminococcus albus is a typical ruminal bacterium digesting cellulose and hemicellulose. Cellobiose 2-epimerase (EC 5.1.3.11, CE), which converts cellobiose to 4-O- β -D-glucosyl-D-mannose, is a particularly unique enzyme in *R. albus*, but its physiological function is unclear. Recently, a new metabolic pathway of mannan involving CE was postulated

for another CE producing bacterium, *Bacteroides fragilis*. In this pathway, β -1,4-mannobiose is epimerized to 4-O- β -D-mannosyl-D-glucose (Man-Glc) by CE, and Man-Glc is phosphorolyzed to α -D-mannosyl 1-phosphate (Man1P) and D-glucose by Man-Glc phosphorylase (EC 2.4.1.281, MP). *Ruminococcus albus* NE1 showed intracellular MP activity, and two MP isozymes, RaMP1 and RaMP2, were obtained from the cell-free extract. These enzymes were highly specific for the mannosyl residue at the non-reducing end of the substrate, and catalyzed the phosphorolysis and synthesis of Man-Glc through a sequential bi bi mechanism. In a synthetic reaction, RaMP1 showed high activity only towards D-glucose and 6-deoxy-D-glucose in the presence of Man1P, while RaMP2 showed acceptor specificity significantly different from RaMP1. RaMP2 acted on D-glucose derivatives at the C2- and C3-positions including deoxy- and deoxyfluoro-analogues and epimers, but not on those substituted at the C6-position. Furthermore,

RaMP2 had high synthetic activity towards the following oligosaccharides: β -linked glucobioses, maltose, *N,N'*-diacetylchitobiose, and β -1,4-mannooligosaccharides. Particularly, β -1,4-mannooligosaccharides served as significantly better acceptor substrates for RaMP2 than D-glucose. In the phosphorolytic reactions, RaMP2 had weak activity towards β -1,4-mannobiose, but efficiently degraded β -1,4-mannooligosaccharides longer than β -1,4-mannobiose. Consequently, RaMP2 is thought to catalyze the phosphorolysis of β -1,4-mannooligosaccharides longer than β -1,4-mannobiose to produce Man1P and β -1,4-mannobiose.

Ruminococcus albus is a typical ruminal bacterium producing various cellulolytic enzymes including cellulase (EC 3.2.1.4) (1), β -glucosidase (EC 3.2.1.21) (2), and cellobiose phosphorylase (EC 2.4.1.20) (3). Cellobiose 2-epimerase (EC 5.1.3.11, CE), which converts cellobiose to 4-O- β -D-glucosyl-D-mannose, is a unique enzyme acting on cellulose-related carbohydrates in *R. albus* (4), but the physiological meaning of its epimerization of cello-oligosaccharides is unclear.

Once the CE gene of *R. albus* NE1 was cloned (5), the CE genes were identified based on the amino acid sequence similarities in various bacteria including a non-cellulolytic bacterium, *Bacteroides fragilis* NCTC9343 (6). In *B. fragilis*, the CE gene comprises the operon along with the genes encoding β -mannanase and 4-O- β -D-mannosyl-D-glucose phosphorylase (EC 2.4.1.281, MP), which specifically catalyzes the phosphorolysis of 4-O- β -D-mannosyl-D-glucose (Man-Glc) to α -D-mannosyl phosphate (Man1P) and D-glucose (7), implying that CE is involved in the metabolism of mannan, in which it converts β -1,4-mannobiose to Man-Glc for further phosphorolysis.

Known inverting carbohydrate phosphorylases catalyzing the phosphorolysis of a glycoside with anomeric inversion have catalytic domains that are structurally similar to inverting glycoside hydrolases (8, 9). Thus the inverting phosphorylases are classified into glycoside hydrolase (GH) families on the basis of the similarity of their amino acid sequences (10). Only limited information concerning MP from *B. fragilis* (BfMP) is available, but this enzyme was recently categorized into GH family

130 together with the putative proteins from the genome sequence of known bacterial species, the amino acid sequences of which are similar to that of BfMP. In this family, three-dimensional structures of *Thermotoga maritima* MSB8 TM1225 protein (PDB code, 1vkd), *Bacteroides thetaiotaomicron* VPI-5482 BT_4094 protein (3R67), and *Parabacteroides distansoni* ATCC 8503 BDI_3141 protein (3TAW) were elucidated, although their biochemical properties have not been examined thus far. These proteins are formed by five-bladed β -propeller folds, which are observed in the glycoside hydrolases belonging to GH families 32 (11, 12), 43 (13-18), and 68 (19, 20).

R. albus 7 has several putative β -1,4-mannanase genes and two MP-like genes (*Rumal_0099* and *Rumal_0852*) in the genome, indicating that this bacterium also has components necessary to metabolize mannan via epimerization and phosphorolysis as observed in *B. fragilis*. *Rumal_0852* and *Rumal_0099* encode proteins with 59% and 27% sequence identity to BfMP, respectively. In this study, two MP isozymes from *R. albus* NE1, which is phylogenetically close to *R. albus* 7 (5), were characterized in detail, and the physiological functions of these enzymes in the metabolism of mannan are discussed.

EXPERIMENTAL PROCEDURES

Preparation of Man-Glc – For the enzyme assay, highly-pure Man-Glc was prepared. A reaction mixture of 210 mL containing 100 mg/mL locust bean gum (Wako Pure Chemical Industries, Osaka, Japan), 1 U/mL β -mannanase from *Aspergillus niger* (Shin Nihon Chemical, Anzyo, Japan), and 10 mM sodium acetate buffer (pH 4.0) was incubated at 37°C for 24 h. The β -1,4-mannobiose produced was purified by carbon-celite column chromatography, as previously reported (21), and epimerized by CE as follows: a reaction mixture consisting of 50 mg/mL mannobiose, 0.1 U/mL CE from *R. albus* NE1 (22), and 20 mM sodium phosphate buffer (pH 7.5) was incubated at 37°C for 24 h. Man-Glc was purified, desalted, and freeze-dried, as described previously (21).

Identification of MP isozymes in the cell-free extract of *R. albus* NE1 – MP activity of the cell-free extract of *R. albus* NE1, obtained from 1.5 L of the culture

fluid (5), was detected by thin layer chromatography (TLC). Six μL of cell-free extract of *R. albus* NE1 was mixed with 2 μL of 100 mM Man-Glc, and 2 μL of 50 mM reaction buffer (pH 7.0), and incubated at 37°C for 3 h. Sodium phosphate buffer or MES-NaOH buffer was used as reaction buffer. The reaction mixture of 1 μL was analyzed by TLC, in which a developing solvent of acetonitrile/ethylacetate/1-propanol/water (85:20:50:30, v/v) was used. The chromatogram was visualized by spraying a detection reagent (acetic acid/sulfuric acid/anisaldehyde, 100:2:1, v/v) and heating. The cell-free extract was subjected to DEAE Sepharose CL-6B column chromatography ($\phi 1.5 \times 9.0$ cm, GE Healthcare, Uppsala, Sweden), in which adsorbed protein was eluted by a linear gradient of 0–0.5 M NaCl in 20 mM MES-NaOH buffer (pH 6.5). Enzyme activity was checked by TLC analysis as described above. Two active peaks (the enzymes eluted by high and low concentrations of NaCl were designated as RaMP1 and RaMP2 respectively) were further purified by Butyl Sepharose CL-6B ($\phi 1.5 \times 9.0$ cm, GE Healthcare), hydroxyapatite ($\phi 1.5 \times 12$ cm, Seikagaku, Tokyo), and Superdex 200 ($\phi 1.6 \times 60$ cm, GE Healthcare) column chromatography. Finally, the sample was separated by SDS-PAGE. The masses of the tryptic peptides derived from the approximately 40 kDa protein were measured and assigned to the theoretical values for Ruma1_0099 and Ruma1_0852 proteins, as described elsewhere (23).

Production and purification of recombinant RaMP1 and RaMP2 – The genomic DNA of *R. albus* NE1 as the template, Primstar HS DNA polymerase (Takara Bio, Otsu, Japan), and the primers listed in Table 1 were used in the PCR. The *RaMP1* and *RaMP2* genes were amplified by PCR and cloned into the *EcoRV* site of pBluescript II SK (+) vector (Stratagene, La Jolla, CA). The DNA sequences of the cloned genes were analyzed with the ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). These plasmids were used as the templates in the PCR to construct the expression plasmids. *NdeI* and *XhoI* sites were introduced to the 5'- and 3'-termini of the target gene respectively by PCR (primers listed in Table 1), and cloned into the *NdeI* and *XhoI* sites of the pET-23a vector (Novagen, Darmstadt, Germany).

The transformants of *Escherichia coli* BL21 (DE3) harboring the expression plasmid of RaMP1 or RaMP2 were cultured in 0.5 L and 1.5 L of LB broth containing 100 $\mu\text{g}/\text{mL}$ ampicillin respectively at 37°C until the A_{600} reached 0.6. The protein production was induced by the addition of isopropyl β -D-thiogalactoside at the final concentration of 0.1 mM, and the incubation was continued at 18°C for 16 h. The recombinant enzymes were purified from the *E. coli* cell-extract. The concentrations of the purified enzymes were determined based on the concentration of each amino acid after acid hydrolysis (24).

Enzyme assay – A reaction mixture of 50 μL consisting of the appropriate concentration of enzyme diluted with 20 mM MES-NaOH buffer (pH 6.5) containing 1 mg/mL bovine serum albumin, 100 mM sodium phosphate buffer (pH 6.5), and 2 mM Man-Glc was incubated at 37°C for 10 min. The enzyme reaction was terminated by boiling the mixture for 3 min after the addition of 25 μL of 4 M Tris-HCl buffer (pH 7.0). The D-glucose produced was measured by the glucose oxidase-peroxidase method (25). One U of enzyme activity was defined as the amount of enzyme producing 1 μmol of D-glucose in 1 min under these conditions.

To determine the optimum pH, the reaction buffer was changed to 100 mM sodium citrate buffer (pH 3.0–6.0), MES-NaOH buffer (pH 6.0–7.0), HEPES-NaOH buffer (pH 7.0–8.5), and glycine-NaOH buffer (pH 8.5–10.5). Sodium phosphate buffer (pH 6.5) was added as the substrate to the reaction mixture at the final concentration of 10 mM.

Temperature and pH stabilities were evaluated by the residual activity after temperature treatment (incubation at various temperatures for 20 min at pH 6.5) and pH treatment (incubation at various pH values for 24 h at 4°C) respectively. The values over which the enzymes retained more than 90% of their original activities were considered to be stable ranges.

The kinetic parameters for phosphorolysis and synthesis of Man-Glc were calculated from the initial velocities towards various concentrations of Man-Glc and inorganic phosphate, and Man1P and D-glucose, respectively by fitting to the equation for a sequential bi bi mechanism (26). Non-linear

regression was performed with Grafit version 7.0.2 (Erithacus Software, West Sussex, UK).

Product inhibition analysis was carried out to determine the order of substrate binding and product release. First, the rate of phosphorolysis catalyzed by rRaMP1 and rRaMP2 toward Man-Glc in the presence of 0–2 mM Man1P were measured at varying concentrations of Man-Glc and 10 mM sodium phosphate buffer (pH 6.5) or at varying concentrations of sodium phosphate buffer (pH 6.5) and 2 mM Man-Glc. Then, in the case of rRaMP2, the phosphorolysis of Man-Glc was measured in the presence of 0–100 mM cellobiose at varying concentrations of Man-Glc and 10 mM sodium phosphate buffer (pH 6.5) or at varying concentrations of sodium phosphate buffer (pH 6.5) and 2 mM Man-Glc. In the case of rRaMP1, initial velocities for the synthesis of Man-Glc were measured in the presence of 0–20 mM Man-Glc at varying concentrations of Man1P and 50 mM D-glucose or at varying concentrations of D-glucose and 5 mM Man1P.

The rates for the synthesis of Man-Glc was measured as follows: a reaction mixture of 50 μ L consisting of an appropriate concentration of enzyme, 50 mM MES-NaOH buffer (pH 6.5), 0.25–10 mM Man1P (sodium salt hydrate, Sigma, St. Louis, MO), and 10–200 mM D-glucose (Wako Pure Chemical Industries) were incubated at 37°C for 10 min, and heated at 80°C for 5 min. The released inorganic phosphate was measured following Lowry and Lopez (27). The apparent kinetic parameters for the synthetic reactions towards various acceptor substrates were determined by fitting the initial rates at various concentrations of acceptors in the presence of 10 mM Man1P to the Michaelis-Menten equation. The acceptor substrates tested were from the following suppliers: D-mannose, D-allose, 3-O-methyl-D-glucose, D-xylose, 1,5-anhydro-D-glucitol, methyl α -D-glucoside, and methyl β -D-glucoside were from Wako Pure Chemical Industries; 2-deoxy-D-glucose and D-glucosamine were from Tokyo Chemical Industry, Tokyo, Japan; 2-deoxy-2-fluoro-D-glucose, sophorose, and 6-deoxy-D-glucose were from Sigma; N-acetyl-D-glucosamine, maltose, cellobiose, and gentiobiose were from Nacalai Tesque, Kyoto, Japan; 3-deoxy-D-glucose, 3-deoxy-3-fluoro-D-glucose, and

6-deoxy-6-fluoro-D-glucose were from Carbosynth, Berkshire, UK; the series of mannooligosaccharides was from Megazyme; N,N'-diacetyl chitobiose and laminaribiose were from Seikagaku.

The phosphorolytic velocity of rRaMP2 towards β -1,4-mannooligosaccharides was determined based on the amount of β -1,4-mannooligosaccharides with reduced chain-length (D-mannose was measured in the reaction towards β -1,4-mannobiose) using HPLC. A reaction mixture of 200 μ L consisting of an appropriate concentration of enzyme, 10 mM sodium phosphate buffer (pH 6.5), and various concentrations of β -1,4-mannooligosaccharides was incubated at 37°C for 10 min, and heated at 80°C for 5 min. The reaction mixture was applied to HPLC under the following conditions: injection volume, 10 μ L; column, Sugar D ($\varnothing$ 4.6 $\times$ 250 mm, Nacalai Tesque); eluent, 65% acetonitrile; flow rate, 0.8 mL/min; detection, pulsed amperometry.

Analysis of reaction products towards β -1,4-mannooligosaccharides by rRaMP2 – Phosphorolytic activities of rRaMP2 towards β -1,4-mannotriose and β -1,4-mannotetraose were detected by TLC. A reaction mixture of 10 μ L containing 0.24 μ M rRaMP2, 20 mM β -1,4-mannotriose or β -1,4-mannotetraose, and 10 mM sodium phosphate buffer (pH 6.5) was incubated at 37°C. One μ L of the reaction mixture taken at 0, 30, 60, and 120 min was spotted on a TLC plate and dried immediately to stop the reaction. Developing solvent and detection conditions were as described above.

Structural analysis of the oligosaccharides produced by the synthetic reaction – The oligosaccharides produced by recombinant RaMP1 (rRaMP1) were prepared as follows: a reaction mixture of 0.2 mL consisting of 493 nM rRaMP1, 50 mM MES-NaOH buffer (pH 6.5), 250 mM D-glucose or D-xylose, and 250 mM Man1P was incubated at 37°C for 3 h. The reaction products were purified by HPLC as described previously (28). The synthetic reaction of recombinant RaMP2 (rRaMP2) was performed in a reaction mixture of 1 mL, consisting of 79 nM rRaMP2, 50 mM MES-NaOH buffer (pH 6.5), 100 mM Man1P, and 100 mM acceptor substrate. The reaction was carried out at 37°C for 12 h. Cellobiose,

β -1,4-mannobiose, and *N,N'*-diacetylchitobiose were used as acceptors. The reaction products were purified by gel filtration column chromatography under the following conditions: column, Bio-Gel P-2 ($\phi 1.8 \times 100$ cm, Bio-Rad, Hercules, CA); eluent, water; flow rate, 2.5 mL/h; fraction volume, 3 mL.

Electrospray ionization-mass spectrometry (ESI-MS) of the oligosaccharides produced was carried out using an Exactive (Thermo Scientific, San Jose, CA). The samples were introduced by flow injection. Methanol was used as a mobile phase solvent. The positive ion was detected under the following conditions: spray voltage, 3.00 kV; capillary temperature, 300°C. Nuclear magnetic resonance (NMR) spectra were recorded in D₂O (Wako Pure Chemical Industries) at 300 K using an ECP-400 (400 MHz, Jeol, Tokyo, Japan). Sodium 3-(trimethylsilyl)-1-propanesulfonate was used as the standard. A series of two-dimensional homo- and hetero-nuclear correlated spectra (COSY, HSQC, HSQC-TOCSY, and HMBC) were obtained.

RESULTS

Identification of RaMP1 and RaMP2 in the cell-free extract of R. albus NE1 – MP activity in the cell-free extract of *R. albus* NE1 was analyzed by TLC (Fig. 1). Man-Glc was degraded to D-glucose and Man1P only in the presence of inorganic phosphate, indicating that *R. albus* NE1 produces intracellular MP. *R. albus* MP was purified to identify the gene encoding it. MP activity was identified in two peaks, RaMP1 and RaMP2, eluted by high and low concentrations of NaCl respectively, via anion-exchange column chromatography using a DEAE Sepharose CL-6B column (data not shown). Two MPs were further purified, and the masses of the tryptic peptides were analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (Supplementary Fig. S1). The obtained masses of the digests of RaMP1 and RaMP2 matched with the theoretical values of the proteins encoded by *Rumal_0852* and *Rumal_0099* from *R. albus* 7, respectively. The analyzed tryptic fragments derived from RaMP1 and RaMP2 covered 27% and 36% of their entire sequences, respectively.

Production, purification, and general properties of recombinant RaMP1 and RaMP2 – The genes encoding RaMP1 and RaMP2 were

obtained from the genomic DNA of *R. albus* NE1 by PCR. The sequences of the amplified genes were completely identical to the corresponding genes of *R. albus* 7. These genes were overexpressed in *E. coli*, and the recombinant enzymes were purified to homogeneity. From 0.5 and 1.5 L of the culture broths of the *E. coli* transformants, 6.8 mg and 11.4 mg of the purified rRaMP1 and rRaMP2 (the specific activities were 55.2 U/mg and 2.03 U/mg) were obtained. RaMP1 and RaMP2 were 45 kDa and 38 kDa on SDS-PAGE respectively (data not shown), which coincided well with the theoretical masses from their amino acid sequences, but were 80 kDa and 209 kDa on a gel filtration column respectively. These results indicate that RaMP1 and RaMP2 exist as a homodimer and homohexamer in solution, respectively. Both RaMP1 and RaMP2 showed highest activity at pH 6.5, and their optimum temperatures were 50°C and 45°C respectively. RaMP1 retained original activity in a pH range of 4.5–10.5 and below 45°C, whereas RaMP2 was stable at pH 3.5–9.5 and below 40°C.

Kinetic mechanisms for phosphorolysis and synthesis of Man-Glc by RaMP1 and RaMP2 – Initial reaction velocities for phosphorolysis at various concentrations of Man-Glc and inorganic phosphate were measured to investigate the kinetic mechanisms of RaMP1 and RaMP2. The lines obtained for both enzymes from double reciprocals plots of $1/v$ versus $1/[\text{Man-Glc}]$ at various concentrations of inorganic phosphate were linear and crossed at a certain point (Fig. 2). This indicates that these enzymes catalyze the phosphorolysis of Man-Glc through a sequential bi bi mechanism involving the formation of a ternary complex. To investigate the orders of substrate binding and product release, product inhibition analysis was carried out (Fig. 3). Man1P acted as a competitive inhibitor of rRaMP1 and rRaMP2 against both Man-Glc and inorganic phosphate as observed for several cellobiose phosphorylases (3, 29), indicating that these substrates bind to the enzymes in random order, and Man1P can be the second product (the first substrate in the reverse reaction). Therefore, a possible kinetic mechanism for these enzymes is a random order bi bi mechanism or a random-ordered bi bi mechanism (3, 29). In the reverse reaction of rRaMP1, Man-Glc served as a competitive inhibitor

against both Man1P and D-glucose, indicating that rRaMP1 catalyzes phosphorolysis of Man-Glc through a random order bi bi mechanism. In the case of rRaMP2, inhibition of phosphorolysis towards Man-Glc by cellobiose which is not phosphorolyzed by rRaMP2 but acts as an acceptor substrate of this enzyme (described later) was analyzed, because very high concentration of Man-Glc is required for the inhibition analysis of the synthetic reaction. Uncompetitive inhibition by cellobiose was observed, when the concentration of inorganic phosphate was varied, presumably because of the formation of an enzyme-phosphate-cellobiose complex, which does not react or reacts very slowly. On the other hand, cellobiose competitively inhibited the phosphorolysis of Man-Glc at various concentrations of Man-Glc. This inhibition occurred by binding of cellobiose to +1 and +2 subsites or to -1 and +1 subsites. The former case indicates D-glucose (cellobiose) also can be the second product, and the kinetic mechanism of the phosphorolysis of Man-Glc by rRaMP2 is a random order bi bi mechanism as rRaMP1. The possibility of the latter case makes order of release (binding) of Man1P and D-glucose (cellobiose) ambiguous. However in the kinetic analysis of the synthetic reaction towards cellobiose and Man1P, no substrate inhibition by cellobiose was observed, indicating that cellobiose acts only as an acceptor substrate, and does not bind to -1 and +1 subsites. The kinetic parameters for the phosphorolysis and synthesis of Man-Glc are summarized in Table 2. In the phosphorolytic reaction of Man-Glc, rRaMP1 had a 4.8-fold higher k_{cat} and 91-fold lower K_{mMG} compared with rRaMP2. The K_{mPi} of rRaMP2 was considerably lower (12-fold) than that of rRaMP1.

Kinetic parameters for the synthesis of Man-Glc with rRaMP1 and rRaMP2 were determined from the initial reaction rates for various concentrations of Man1P and D-glucose (Table 2). rRaMP1 had a 6.6-fold higher k_{cat} value for the synthetic reaction than rRaMP2 as it did for the phosphorolytic reaction. The K_{mGlc} value of rRaMP1 was 3.7-fold lower than that of rRaMP2, indicating that the +1 subsite of RaMP1 is more suitable for binding the D-glucose moiety than RaMP2, consistent with the higher affinity of rRaMP1 towards Man-Glc than that of rRaMP2. The rRaMP2 K_{mMan1P} value was 18-fold lower than that

of rRaMP1, corresponding with the observation that RaMP2 has higher affinity toward inorganic phosphate than RaMP1 during the phosphorolysis of Man-Glc.

The internal consistency of the kinetic parameters of rRaMP1 and rRaMP2 was confirmed with the Haldane relationship for a sequential bi bi mechanism ($K = (k_{cat}^P K_{sMan1P} K_{mGlc}) / (k_{cat}^S K_{sMan-Glc} K_{mPi})$, k_{cat}^P and k_{cat}^S are k_{cat} for phosphorolytic and synthetic reactions, respectively) (30). Values of 0.435 ± 0.062 and 0.658 ± 0.137 were obtained for K of rRaMP1 and rRaMP2, respectively, from the kinetic parameters in Table 2. These values were similar in magnitude to the value 0.272 ± 0.021 experimentally obtained with rRaMP1 at pH 6.5 at 37°C for the thermodynamic equilibrium constant (K) calculated from following equation:

$$K = ([Man1P][Glc]) / ([Man-Glc][phosphate])$$

Substrate specificity of RaMP1 and RaMP2 in phosphorolytic reactions – D-Glucose was not detected in the reaction carried out in the presence of rRaMP1 or rRaMP2 and cellobiose or lactose even at a 10-fold higher enzyme concentration than used for the enzyme assay towards Man-Glc, indicating that these enzymes are highly specific to the mannosyl residue at the non-reducing end of the substrate.

Acceptor specificity of RaMP1 in synthetic reaction – The acceptor specificity of rRaMP1 was investigated based on the initial reaction rates towards Man1P and various acceptor substrates. rRaMP1 showed a very narrow acceptor specificity. Almost all the oligosaccharides tested were not recognized as acceptor substrates. Only laminaribiose served as a poor acceptor substrate for rRaMP1. The apparent kinetic parameters, k_{catapp} and K_{mapp} , for laminaribiose, determined in the presence of 10 mM Man1P, were 7.6-fold lower and 6.0-fold higher than those for D-glucose respectively (Table 3).

rRaMP1 was not active towards D-glucose derivatives with changes at the C2-position, and it was less active towards derivatives with changes at the C3-position compared with D-glucose. The monodeoxygenation at the 6-OH position was less effective for RaMP1 than at the other positions. The k_{catapp} and K_{mapp} values of rRaMP1 for

6-deoxy-D-glucose were 1.4- and 4.6-fold higher than those for D-glucose, respectively. On the other hand, 6-deoxy-6-fluoro-D-glucose and D-xylose were considerably less favorable acceptor substrates than 6-deoxy-D-glucose. This enzyme had a 3.3-fold lower k_{catapp} and 6.0-fold higher K_{mapp} for 6-deoxy-6-fluoro-D-glucose than those for D-glucose, and a saturation curve was not obtained for D-xylose because of its very high K_{mapp} . These results indicate that the 6-OH partially contributes to the enzyme-substrate interaction as a hydrogen bond donor, and that the methylene group of D-glucose is also involved in stabilization of the transition state presumably through the hydrophobic interaction.

Structures of oligosaccharides produced by the synthetic reaction of rRaMP1 with D-glucose and D-xylose as acceptors were investigated. A single oligosaccharide was produced in each reaction, and 2.0 and 4.9 mg of product was obtained by HPLC, respectively. The structures of these oligosaccharides were elucidated by ESI-MS and NMR. The oligosaccharides produced from the reactions with D-glucose and D-xylose gave signals at 365.11 and 335.09 m/z $[M + Na]^+$, respectively. Each oligosaccharide had a correlation peak between the 4C of the acceptor and the 1H of the D-mannosyl residue at the non-reducing end. The $^1J_{\text{CH}}$ values for the D-mannosyl residues at the non-reducing ends of these oligosaccharides in ^{13}C -NMR (insensitive nuclei enhanced by polarization transfer) were 161 Hz, indicating that D-mannosyl residues were bound to the acceptor substrates through a β -linkage (31). On the basis of these results, the oligosaccharides produced in the synthetic reactions in the presence of D-glucose and D-xylose were identified as Man-Glc and 4-O- β -D-mannosyl-D-xylose, respectively. The chemical shifts of ^1H - and ^{13}C -NMR analyses are summarized in supplementary Table S1.

Acceptor specificity of RaMP2 in synthetic reaction – rRaMP2 was not active when D-glucose was derivatized at the C6-position, but showed very broad acceptor specificity (Table 3). rRaMP2 showed the highest $k_{\text{catapp}}/K_{\text{mapp}}$ towards *N*-acetyl-D-glucosamine among the 2-OH derivatives of D-glucose, even though *N*-acetyl-D-glucosamine has a bulky acetamide group at the C2 position. All the D-glucose derivatives with changes at the C3-position acted as

acceptor substrates of rRaMP2. The k_{catapp} and K_{mapp} values for these derivatives were considerably lower than for D-glucose. The K_{mapp} value for 3-deoxy-D-glucose was too low to determine the value.

rRaMP2 had $k_{\text{catapp}}/K_{\text{mapp}}$ values for 1,5-anhydro-D-glucitol and methyl α -D-glucoside similar to that for D-glucose, and the $k_{\text{catapp}}/K_{\text{mapp}}$ for methyl β -D-glucoside was 2.1-fold higher than for D-glucose. All the oligosaccharides acted as acceptor substrates of rRaMP2. The $k_{\text{catapp}}/K_{\text{mapp}}$ values for β -linked glucobioses were 1.4–4.5-fold higher than for D-glucose due to low K_{mapp} values for these oligosaccharides. Cellobiose was the best acceptor substrate for rRaMP2 among the β -linked glucobioses in terms of its $k_{\text{catapp}}/K_{\text{mapp}}$ value, followed by gentiobiose. rRaMP2 also had a 3.4-fold lower K_{mapp} for *N,N'*-diacetylchitobiose. The $k_{\text{catapp}}/K_{\text{mapp}}$ for maltose was 50% of that for D-glucose, thus the acceptor site of rRaMP1 efficiently accommodates even an α -linked oligosaccharide. D-Mannose was a poor acceptor substrate of rRaMP2 compared with D-glucose, but rRaMP2 showed 7.3–20-fold higher $k_{\text{catapp}}/K_{\text{mapp}}$ values for β -1,4-mannooligosaccharides than for D-glucose. The $k_{\text{catapp}}/K_{\text{mapp}}$ values for β -1,4-mannooligosaccharides increased depending on the degree of polymerization (DP) up to β -1,4-mannotetraose, while those for substrates longer than β -1,4-mannotetraose slightly decreased with increasing substrate chain-length, although a lower K_{mapp} was observed for longer β -1,4-mannooligosaccharides.

Structural analysis of oligosaccharides produced in the synthetic reactions of rRaMP2 towards cellobiose, β -1,4-mannobiose, and *N,N'*-diacetylchitobiose were performed. In contrast to rRaMP1, rRaMP2 produced several oligosaccharides with different DPs. The resulting reaction mixtures were subjected to gel filtration column chromatography (data not shown). The yields of the oligosaccharides purified were as follows: from cellobiose, DP3, 9.5 mg; DP4, 2.7 mg; DP5, 0.8 mg; and DP6, 0.4 mg; from β -1,4-mannobiose, DP3, 4.5 mg; DP4, 2.8 mg; DP5, 0.8 mg; and DP6, 0.3 mg; from *N,N'*-diacetylchitobiose, DP3, 2.4 mg; DP4, 1.3 mg; DP5, 0.8 mg; and DP6, 0.6 mg. Molecular masses of the obtained oligosaccharides were analyzed by

ESI-MS. The masses were as follows: the products from cellobiose, 527.16, 689.26, 851.27, and 1013.32 m/z $[M + Na]^+$; from β -1,4-mannobiose, 527.16, 689.21, 851.27, and 1013.32 m/z $[M + Na]^+$; from N,N' -diacetylchitobiose, 609.21, 771.27, 933.36, and 1095.37 m/z $[M + Na]^+$. These results indicate that rRaMP2 successively transferred mannosyl residues to the synthesized oligosaccharides. The chemical structures of the trisaccharides synthesized in the reactions in the presence of cellobiose and N,N' -diacetylchitobiose by rRaMP2 were analyzed as described above, and determined to be 4-*O*- β -D-mannosyl-cellobiose and 4-*O*- β -D-mannosyl- N,N' -diacetylchitobiose, respectively (supplementary Table S1). ^{13}C - and 1H -NMR spectra of the trisaccharides produced from β -1,4-mannobiose were completely identical to those of β -1,4-mannotriose.

Phosphorolysis of β -1,4-mannooligosaccharides by rRaMP2 – The kinetic analysis of the synthetic reaction of rRaMP2 indicated that this enzyme mainly catalyzes the phosphorolysis of β -1,4-mannooligosaccharides rather than that of Man-Glc. In the phosphorolytic reaction, rRaMP2 degraded β -1,4-mannotriose and β -1,4-mannotetraose to Man1P and shorter β -1,4-mannooligosaccharides (Fig. 4). β -1,4-Mannobiose was accumulated in the late stage of the reaction with β -1,4-mannotriose. The apparent kinetic parameters of rRaMP2 for Man-Glc and β -1,4-mannooligosaccharides, which was measured in the presence of 10 mM sodium phosphate buffer (pH 6.5), were compared (Table 4). The k_{catapp} values for β -1,4-mannooligosaccharides longer than β -1,4-mannobiose were similar to that for Man-Glc, but the K_{mapp} values were 3.6–8.8-fold lower, consistent with the acceptor specificity for the synthetic reactions. On the other hand, β -1,4-mannobiose was a very poor substrate for rRaMP2. The k_{catapp}/K_{mapp} value was 22–65-fold lower than those for the longer β -1,4-mannooligosaccharides.

DISCUSSION

In *B. fragilis*, the metabolic pathway of mannan has been postulated as follows: i) hydrolysis of mannan by extracellular β -1,4-mannanase, ii) epimerization of the resulting β -1,4-mannobiose to Man-Glc by CE, iii) phosphorolysis of Man-Glc to

D-glucose and Man1P by MP (7). Phosphomannose mutase and mannose-6-phosphate isomerase convert Man1P to fructose 6-phosphate, which is then metabolized through glycolysis. *R. albus* NE1 is also a CE producer (5), and two MP homologous genes were encoded in the genome of *R. albus* 7. This bacterium also has putative proteins involved in the metabolism of mannan, eight extracellular β -1,4-mannanases belonging to GH family 26, a phosphomannomutase, phosphomannose isomerase, and sugar transport protein similar to sugar/cation symporter (Table 5). MP activity was detected in the cell-free extract of *R. albus* NE1, and two MP isozymes, the deduced amino acid sequences of which are completely identical to the corresponding genes of *R. albus* 7, were found. Consequently, these MP isozymes are predicted to be physiologically functional and *R. albus* NE1 (*R. albus* 7) is thought to degrade mannan through a similar pathway as postulated for *B. fragilis* (Fig. 5).

Both rRaMP1 and rRaMP2 catalyzed phosphorolytic and synthetic reactions of Man-Glc through a random bi bi mechanism in contrast to other known inverting carbohydrate phosphorylases (3, 32–34). Both rRaMP1 and rRaMP2 specifically catalyzed the phosphorolysis of a β -1,4-mannosidic linkage at the non-reducing end of a substrate, but they had acceptor specificities which were clearly different from each other. Consistent with the acceptor specificity of BfMP, which shows high sequence similarity to RaMP1 (7), rRaMP1 was highly active only towards D-glucose and 6-deoxy-D-glucose, but rRaMP2 recognized a significantly wider variety of sugars including oligosaccharides as the acceptor substrates. rRaMP2 was more active towards β -1,4-mannooligosaccharides than D-glucose, and successive formations of β -1,4-mannosidic linkages were observed in the synthetic reactions. In the phosphorolytic reactions, β -1,4-mannooligosaccharides were degraded to Man1P and shorter oligosaccharides (Fig. 4). β -1,4-Mannooligosaccharides longer than β -1,4-mannobiose were considerably better substrates for rRaMP2 than Man-Glc (Table 4). Hence the physiological role of RaMP2 is not phosphorolysis of Man-Glc, but is phosphorolysis of β -1,4-mannooligosaccharides of $\geq DP3$ to β -1,4-mannobiose. On the basis of the substrate

preference, RaMP2 should be named β -1,4-mannooligosaccharide phosphorylase rather than 4-O- β -D-mannosyl-D-glucose phosphorylase. The low activity of rRaMP2 towards β -1,4-mannobiose clearly indicates the importance of the processes catalyzed by CE and RaMP1 as described above. *B. fragilis* also has a RaMP2-like protein, BF1316, (NCBI reference sequence, YP_210978.1), which has 64.6% sequence identity to RaMP2. It is possible that this bacterium also degrades mannoooligosaccharides longer than β -1,4-mannobiose by the RaMP2-type phosphorylase. Furthermore, the aerobic CE-producing bacterium, *Rhodothermus murinus* (28), also has both RaMP1- and RaMP2-type proteins (YP_003291706 and YP_003291875 respectively), and hence, the metabolic pathway of mannan postulated here is not likely limited to anaerobic bacteria.

β -1,4-Mannotetraose was the best acceptor substrate for RaMP2 among the β -1,4-mannooligosaccharides tested in terms of the k_{catapp}/K_{mapp} (Table 3), while the k_{catapp}/K_{mapp} for longer β -1,4-mannooligosaccharides slightly decreased

with an increase of DP. The acceptor site of RaMP2 is likely composed of at least four subsites. Hence RaMP2 presumably has a cleft-type substrate binding site to accommodate a long-chain acceptor substrate unlike RaMP1, which recognizes only monosaccharides as the acceptors. In the case of RaMP2, a β -1,4-glycosidic linkage is preferred in subsites +1 and +2, but the specificity for the acceptor substrates is very loose and even maltose and *N,N'*-diacetylchitobiose were acceptable.

Herein, we described in detail the biochemical properties of two MP isozymes from *R. albus* NE1 belonging to GH family 130. Both enzymes were specific to the β -1,4-mannosidic linkage at the non-reducing end, but RaMP1 and RaMP2 showed narrow and broad acceptor specificities in synthetic reactions. RaMP2 is the first enzyme known to efficiently participate in the phosphorolysis and synthesis of β -1,4-mannooligosaccharides longer than β -1,4-mannobiose. Structural analysis of an enzyme-substrate complex is required for further understanding of the structure-function relationship of GH family 130 enzymes.

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FOOTNOTES

*This work was partially supported by Kakenhi, the Grants-in-Aid for Young Scientists (B) (24780091) of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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The abbreviations used are: CE, cellobiose 2-epimerase; MP, 4-O- β -D-mannosyl-D-glucose phosphorylase; Man-Glc, 4-O- β -D-mannosyl-D-glucose; Man1P, α -D-mannosyl phosphate; BfMP, *Bacteroides fragilis* MP; GH, glycoside hydrolase; TLC, thin layer chromatography; RaMP1, *Ruminococcus albus* MP1 (Rumal_0852); RaMP2, *R. albus* MP2 (Rumal_0099); rRaMP1, recombinant RaMP1; rRaMP2, recombinant RaMP2; ESI-MS, electrospray ionization-mass spectrometry; NMR, nuclear magnetic resonance

FIGURE LEGENDS

FIGURE 1. Detection of MP activity in the cell-free extract of *R. albus* NE1. Phosphorolytic activity of the cell-free extract of *R. albus* NE1 towards Man-Glc in the presence or absence of inorganic phosphate was analyzed by TLC. S1, Man-Glc; S2, glucose; S3, Man1P. Man-Glc was degraded only in sodium phosphate buffer.

FIGURE 2. Double reciprocal plot for the phosphorolysis of Man-Glc by rRaMP1 and rRaMP2. The initial velocities for phosphorolysis of Man-Glc at various concentrations of Man-Glc and inorganic phosphate were measured. (A) rRaMP1. Concentrations of inorganic phosphate were 2.5 mM (open circle), 5.0 mM (filled circle), 10 mM (open triangle), and 20 mM (filled triangle). (B) rRaMP2. Concentrations of inorganic phosphate were 0.125 mM (open circle), 0.25 mM (filled circle), 0.5 mM (open triangle), 1 mM (filled triangle), and 3 mM (filled square). Data are the mean $\pm$ SD for three independent experiments.

FIGURE 3. Product inhibition analyses of rRaMP1 and rRaMP2.

Product inhibition analyses of rRaMP1 and rRaMP2 are shown in panel A-D and E-H, respectively. A and B (E and F for rRaMP2) are inhibition of the phosphorolysis of Man-Glc by Man1P against Man-Glc and inorganic phosphate, respectively. C and D are inhibition of the synthesis of Man-Glc by Man-Glc against Man1P and D-glucose, respectively. G and H are inhibition of the phosphorolysis of Man-Glc by cellobiose against Man-Glc and inorganic phosphate, respectively. Black circle, triangle square, and diamond indicate 0, 1, 2, and 2.5 mM, respectively. Gray circle, triangle, and square indicate 0, 10, and 20 mM, respectively. Open circle, triangle, and square are 0, 50, and 100 mM, respectively. Data are the mean $\pm$ SD for three independent experiments.

FIGURE 4. Phosphorolysis of β -1,4-mannooligosaccharides by rRaMP2. Phosphorolytic reactions of rRaMP2 towards β -1,4-mannotriose and β -1,4-mannotetraose were analyzed by TLC. Reaction time

Metabolic Mechanism of Mannan in *Ruminococcus albus* is indicated below the figure. S1 and S2 are standards of Man1P and β -1,4-mannobiose, respectively. M2, β -1,4-mannobiose; M3, β -1,4-mannotriose; M4, β -1,4-mannotetraose; M1P, Man1P.

FIGURE 5. **Metabolic pathway of mannan in *R. albus* NE1.** Metabolic pathway of mannan involving two MPs and CE is illustrated. CE, cellobiose 2-epimerase; PMM, phosphomannose mutase; PMI mannose 6-phosphate isomerase.

Table 1. **Primers used in this study.** Single- and double-underlines indicate restriction sites for *NdeI* and *XhoI* respectively.

Name	Sequence (5'→3')	Orientation	Purpose
RaMP1 7 N	GATAAATATGAGTATTATCCGTTTCCATAG	Sense	Amplification of RaMP1 gene
RaMP1 7 C	CTTTCACCTTAACAAAATAAAGAATCTATC	Antisense	Amplification of RaMP1 gene
RaMP1 sen1	GGAACGTTTAGGTATAAACGCAGTATT	Sense	Sequence analysis
RaMP1nde	GAGCGACATATGATACACGAAAAATAC	Sense	Preparation of expression plasmid
RaMP1 xho nonhis	CAATGTAATCCTCGAGATATCAGATATC	Antisense	Preparation of expression plasmid
RaMP2 N	CTACCGAATGACCAAAAGCTG	Sense	Amplification of RaMP2 gene
RaMP2 C3	GGTGAAAGATCAGATGCGAGTACG	Antisense	Amplification of RaMP2 gene
RaMP2 nde	GGTGGAATT <u>CATATGA</u> AGACACAG	Sense	Preparation of expression plasmid
RaMP2 xho nonhis	CTTTTCTCTTGAT <u>CTCGAG</u> ATATTATATC	Antisense	Preparation of expression plasmid
T7	TAATACGACTCACTATAGGG	Sense	Sequence analysis
T3	GCAGCAATTATAGCACAACGATG	Antisense	Sequence analysis
T7 terminator	GCTAGTTATTGCTCAGCGG	Antisense	Sequence analysis

Table 2. **Kinetic parameters for the phosphorolysis and synthesis of Man-Glc by rRaMP1 and rRaMP2.** Substrate concentrations were as follows: phosphorolysis of rRaMP1, 0.25-2.0 mM Man-Glc and 2.5-20 mM inorganic phosphate; synthesis of rRaMP1, 0.5-5.0 mM Man1P and 10-50 mM D-glucose; phosphorolysis of rRaMP2, 1.0-20 mM Man-Glc and 0.125-3.0 mM inorganic phosphate; synthesis of rRaMP2, 0.25-2.0 mM Man1P and 25-200 mM D-glucose. a, the values were calculated from the relationships of kinetic parameters, $K_{s\text{Man-Glc}}K_{m\text{Pi}} = K_{s\text{Pi}}K_{m\text{Man-Glc}}$. N.D., not determined.

		rRaMP1	rRaMP2
Phosphorolysis	$k_{\text{cat}} (\text{s}^{-1})$	83.3 ± 5.8	17.5 ± 1.3
	$K_{m\text{Man-Glc}} (\text{mM})$	0.304 ± 0.070	27.7 ± 2.7
	$K_{m\text{Pi}} (\text{mM})$	5.34 ± 0.47	0.444 ± 0.108
	$K_{s\text{Man-Glc}} (\text{mM})$	7.70 ± 0.24	30.3 ± 6.0
	$K_{s\text{Pi}} (\text{mM})^a$	135	N.D.
Synthesis	$k_{\text{cat}} (\text{s}^{-1})$	204 ± 20	30.9 ± 1.9
	$K_{m\text{Glc}} (\text{mM})$	38.2 ± 5.2	142 ± 12
	$K_{m\text{Man1P}} (\text{mM})$	5.80 ± 0.93	0.324 ± 0.097
	$K_{s\text{Glc}} (\text{mM})$	7.65 ± 1.45	51.5 ± 25.7
	$K_{s\text{Man1P}} (\text{mM})$	1.16	N.D.

Table 3. **Apparent kinetic parameters for the synthetic reaction of rRaMP1 and rRaMP2.** Kinetic parameters were calculated by fitting the initial velocities towards various concentrations of acceptor substrates in the presence of 10 mM Man1P to the Michaelis-Menten equation. Only k_{cat}/K_m values are provided when saturation curves were not obtained due to high K_m values (estimated to be higher than 200 mM). Data are mean $\pm$ SD for three independent experiments. N.D., not determined. a, reaction velocity at 40 mM. b, reaction velocity was completely saturated at 0.05 mM, which is lower limit of substrate concentration to determine accurate reaction velocity.

Substrate	rRaMP1			rRaMP2		
	k_{catapp} (s ⁻¹)	K_{mapp} (mM)	k_{cat}/K_{mapp} (s ⁻¹ mM ⁻¹)	k_{catapp} (s ⁻¹)	K_{mapp} (mM)	k_{cat}/K_{mapp} (s ⁻¹ mM ⁻¹)
D-Glucose	126 $\pm$ 1	25.9 $\pm$ 0.1	4.86	34.5 $\pm$ 0.2	172 $\pm$ 5	0.201
2-Deoxy-D-glucose	N.D.	N.D.	N.D.	11.7 $\pm$ 0.8	90.7 $\pm$ 8.0	0.129
2-Deoxy-2-fluoro-D-glucose	N.D.	N.D.	N.D.	5.71 $\pm$ 0.16	48.7 $\pm$ 1.8	0.117
D-Mannose	N.D.	N.D.	N.D.	5.12 $\pm$ 0.05	45.6 $\pm$ 0.3	0.112
N-Acetyl-D-glucosamine	N.D.	N.D.	N.D.	15.8 $\pm$ 1.7	107 $\pm$ 13	0.148
3-Deoxy-D-glucose	N.D.	N.D.	N.D.	0.412 $\pm$ 0.002 ^a	<0.05 ^b	N.D.
3-Deoxy-3-fluoro-D-glucose	N.D.	N.D.	0.0526	0.940 $\pm$ 0.015	6.44 $\pm$ 0.68	0.146
D-Allose	N.D.	N.D.	N.D.	0.667 $\pm$ 0.018	6.14 $\pm$ 0.71	0.109
3-O-Methyl-D-glucose	N.D.	N.D.	0.0764	0.763 $\pm$ 0.010	7.82 $\pm$ 0.34	0.0976
6-Deoxy-D-glucose	177 $\pm$ 21	119 $\pm$ 18	1.49	N.D.	N.D.	N.D.
6-Deoxy-6-fluoro-D-glucose	42.0 $\pm$ 3.4	156 $\pm$ 14	0.269	N.D.	N.D.	N.D.
D-Xylose	N. D.	N. D.	0.180	N.D.	N.D.	N.D.
1,5-Anhydro-D-glucitol	N.D.	N.D.	N.D.	20.7 $\pm$ 1.2	79.2 $\pm$ 5.3	0.261
Methyl β -D-glucoside	N.D.	N.D.	N.D.	35.2 $\pm$ 3.9	85.2 $\pm$ 12.4	0.413
Methyl α -D-glucoside	N.D.	N.D.	N.D.	20.1 $\pm$ 0.9	85.3 $\pm$ 5.1	0.236
Sophorose	N.D.	N.D.	N.D.	13.8 $\pm$ 1.1	49.8 $\pm$ 6.3	0.277
Laminaribiose	16.6 $\pm$ 1.4	156 $\pm$ 16	0.106	11.6 $\pm$ 1.0	29.1 $\pm$ 4.6	0.399
Cellobiose	N.D.	N.D.	N.D.	20.2 $\pm$ 0.7	22.2 $\pm$ 1.4	0.910
Gentiobiose	N.D.	N.D.	N.D.	26.1 $\pm$ 1.1	60.8 $\pm$ 4.4	0.429
Maltose	N.D.	N.D.	N.D.	5.07 $\pm$ 0.23	50.4 $\pm$ 3.5	0.101
N,N'-diacetylchitobiose	N.D.	N.D.	N.D.	6.34 $\pm$ 0.15	49.9 $\pm$ 2.0	0.127
β -1,4-Mannobiose	N.D.	N.D.	N.D.	45.1 $\pm$ 1.1	30.7 $\pm$ 1.5	1.47
β -1,4-Mannotriose	N.D.	N.D.	N.D.	34.6 $\pm$ 0.3	13.3 $\pm$ 0.3	2.60
β -1,4-Mannotetraose	N.D.	N.D.	N.D.	31.2 $\pm$ 0.1	7.59 $\pm$ 0.16	4.11
β -1,4-Mannopentaose	N.D.	N.D.	N.D.	20.5 $\pm$ 0.3	5.29 $\pm$ 0.12	3.87
β -1,4-Mannohexaose	N.D.	N.D.	N.D.	16.0 $\pm$ 0.4	4.48 $\pm$ 0.26	3.57

Table 4. **Apparent kinetic parameters for phosphorolysis of Man-Glc and β -1,4-mannooligosaccharides by rRaMP2.** Kinetic parameters were calculated by fitting the initial velocities towards various concentrations of substrates in the presence of 10 mM inorganic phosphate to the Michaelis-Menten equation. Data are mean $\pm$ SD for three independent experiments.

	k_{catapp} (s ⁻¹)	K_{mapp} (mM)	$k_{\text{catapp}}/K_{\text{mapp}}$ (s ⁻¹ mM ⁻¹)	relative $k_{\text{catapp}}/K_{\text{mapp}}$ (-fold)
Man-Glc	20.7 $\pm$ 0.7	28.4 $\pm$ 1.6	0.729	1
β -1,4-mannobiose	7.06 $\pm$ 1.52	44.5 $\pm$ 11.0	0.159	0.218
β -1,4-mannotriose	27.5 $\pm$ 6.1	7.94 $\pm$ 3.30	3.46	4.75
β -1,4-mannotetraose	33.1 $\pm$ 10.3	3.21 $\pm$ 1.66	10.3	14.1
β -1,4-mannopentaose	31.9 $\pm$ 12.4	4.55 $\pm$ 2.76	7.01	9.62

Table 5. **Putative mannan metabolizing enzymes of *R. albus* 7.**

Enzyme name	Locus	Localization	Homologous protein of <i>B. fragilis</i>
β -Mannanase	Rumal_0299, Rumal_0317, Rumal_0327, Rumal_0484, Rumal_0863, Rumal_1368, Rumal_2064, Rumal_3152	Extracellular	BF0764, BF0771
4-O- β -D-Mannosyl-D-glucose phosphorylase (RaMP1)	Rumal_0852	Intracellular	BF0772
β -1,4-Mannooligosaccharide phosphorylase (RaMP2)	Rumal_0099	Intracellular	BF1316
Cellobiose 2-epimerase	Rumal_0019	Intracellular	BF0774
Phosphomannomutase	Rumal_2924	Intracellular	BF3668
Phosphomannose isomerase	Rumal_2865	Intracellular	BF1664
Sugar transport protein	Rumal_0598	Intracellular	BF0773

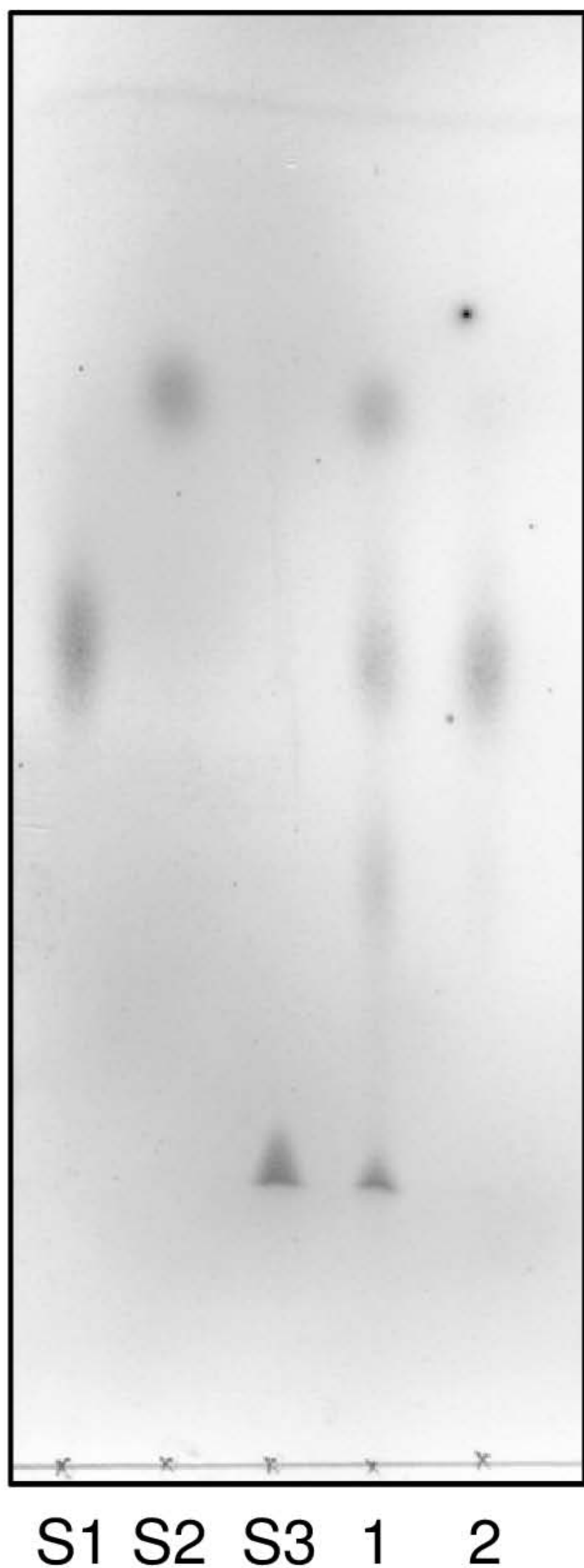


Fig. 1, Kawahara et al

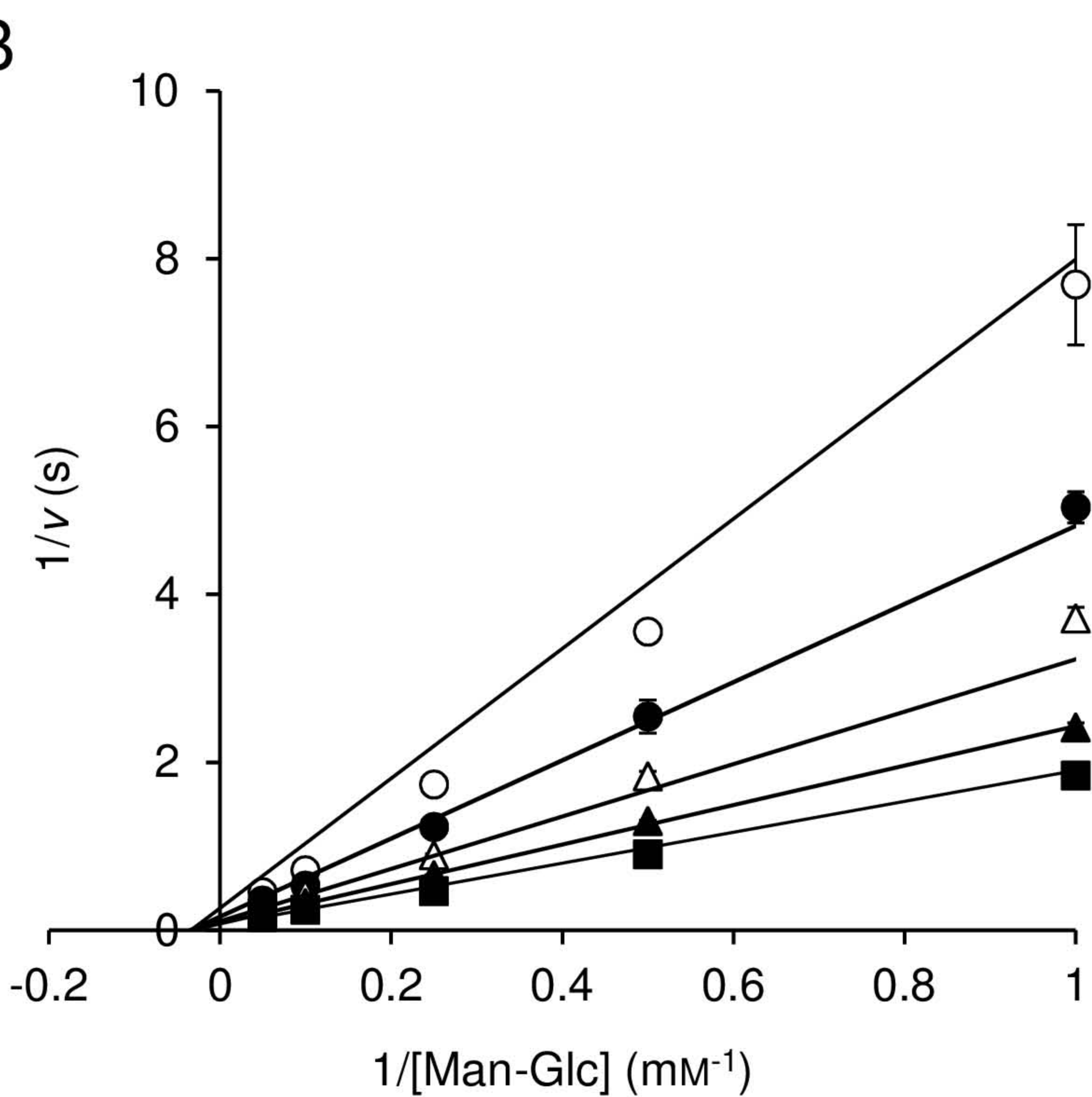
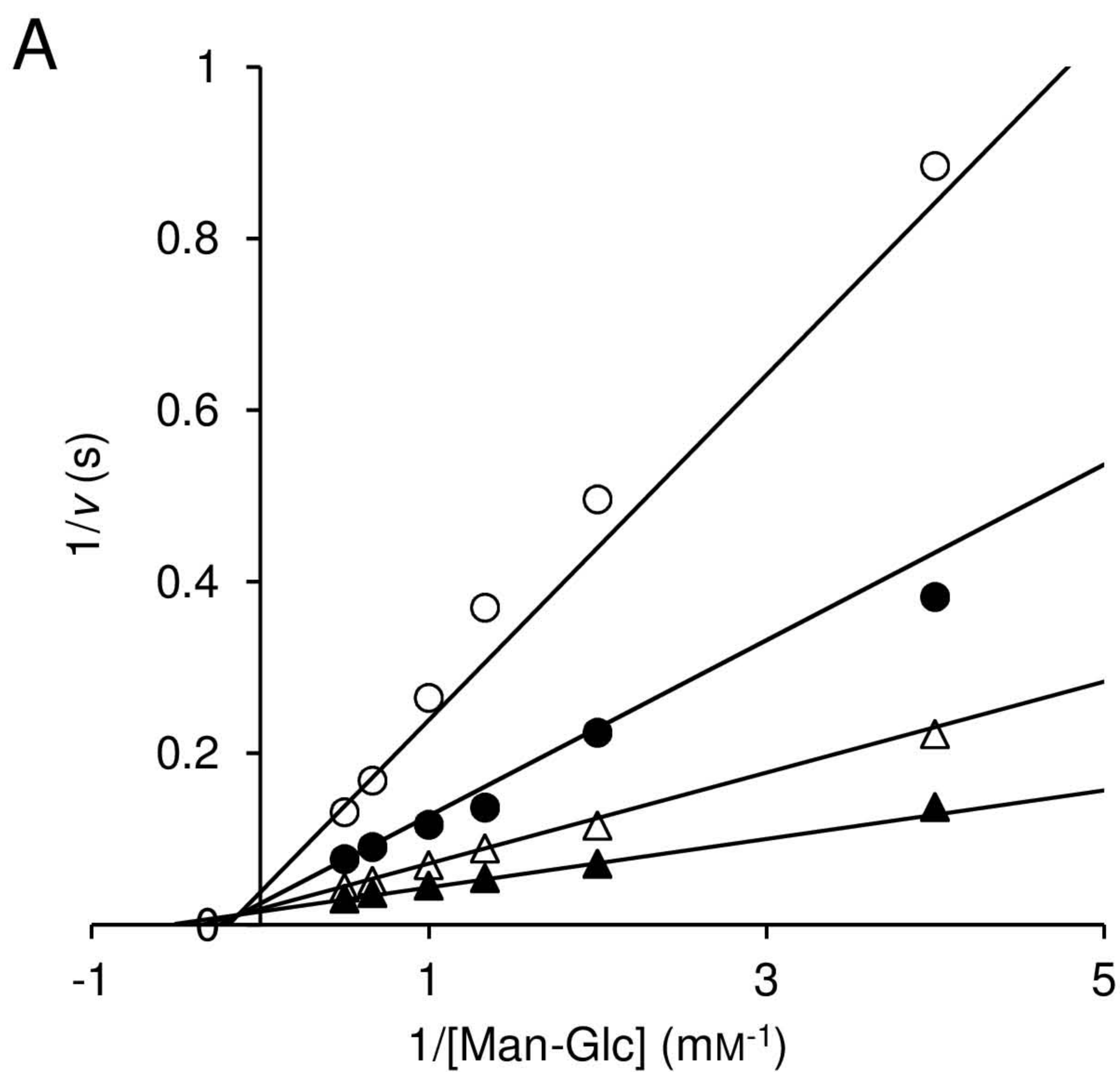


Fig. 2. Kawahara et al.

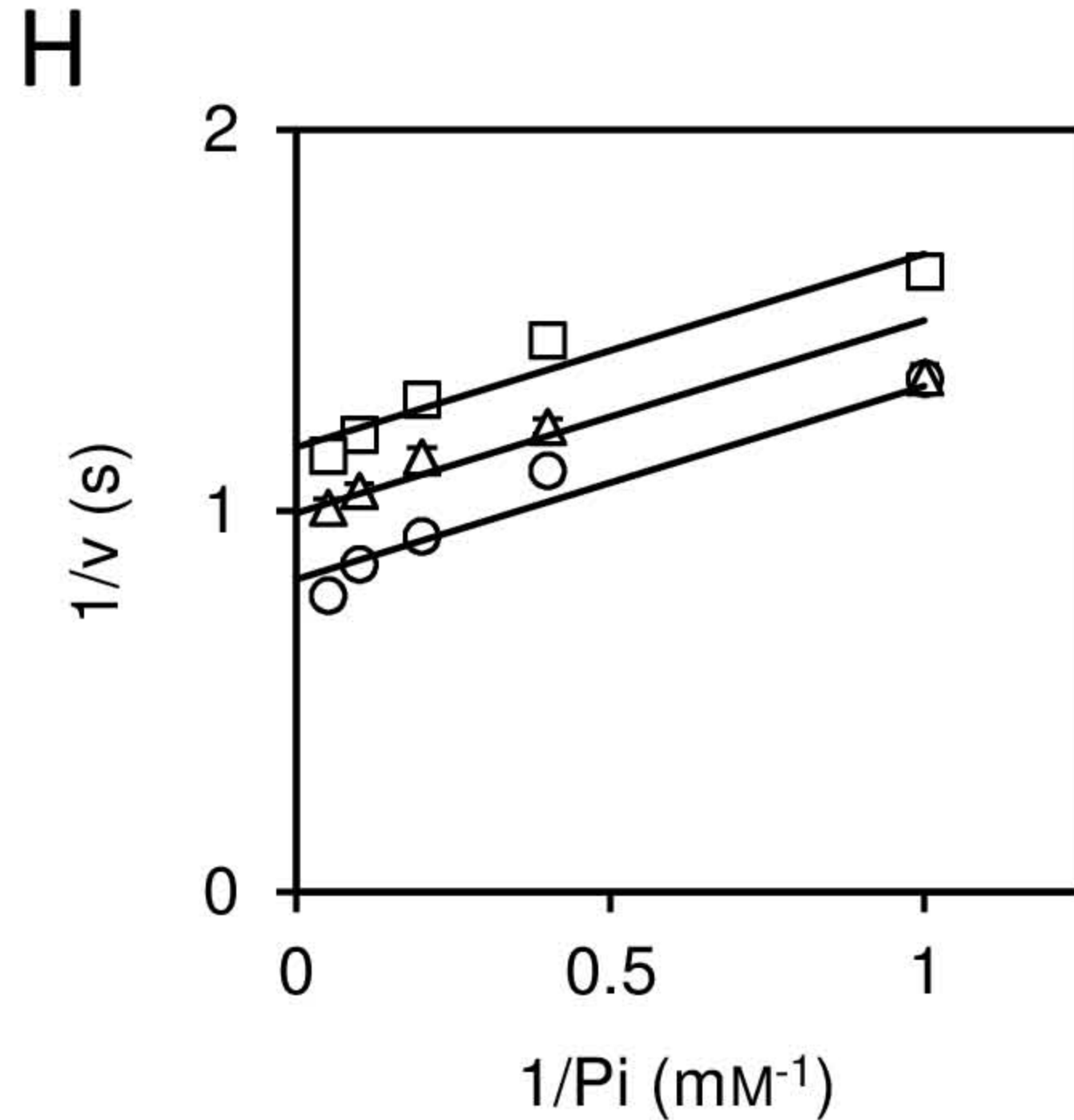
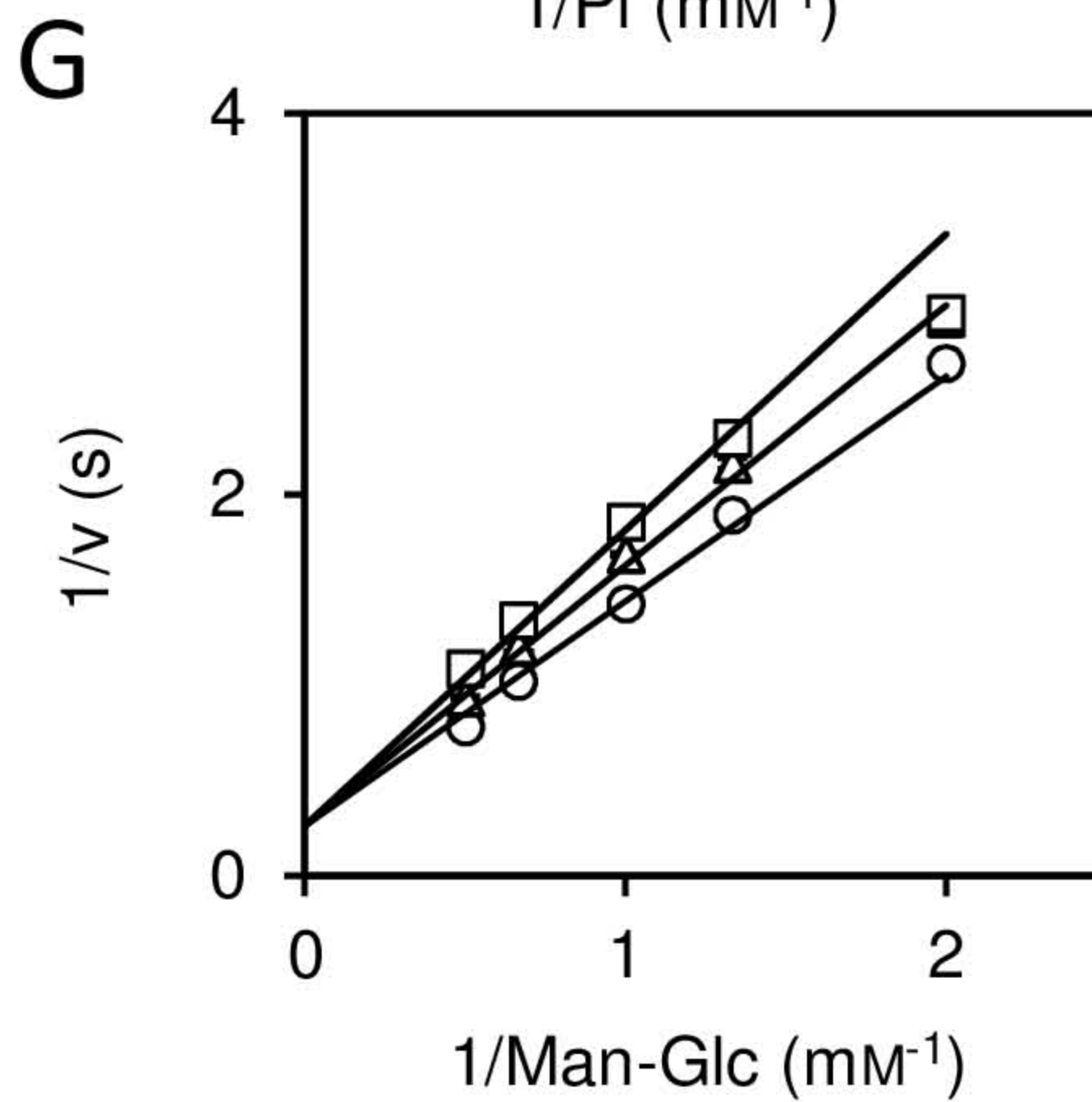
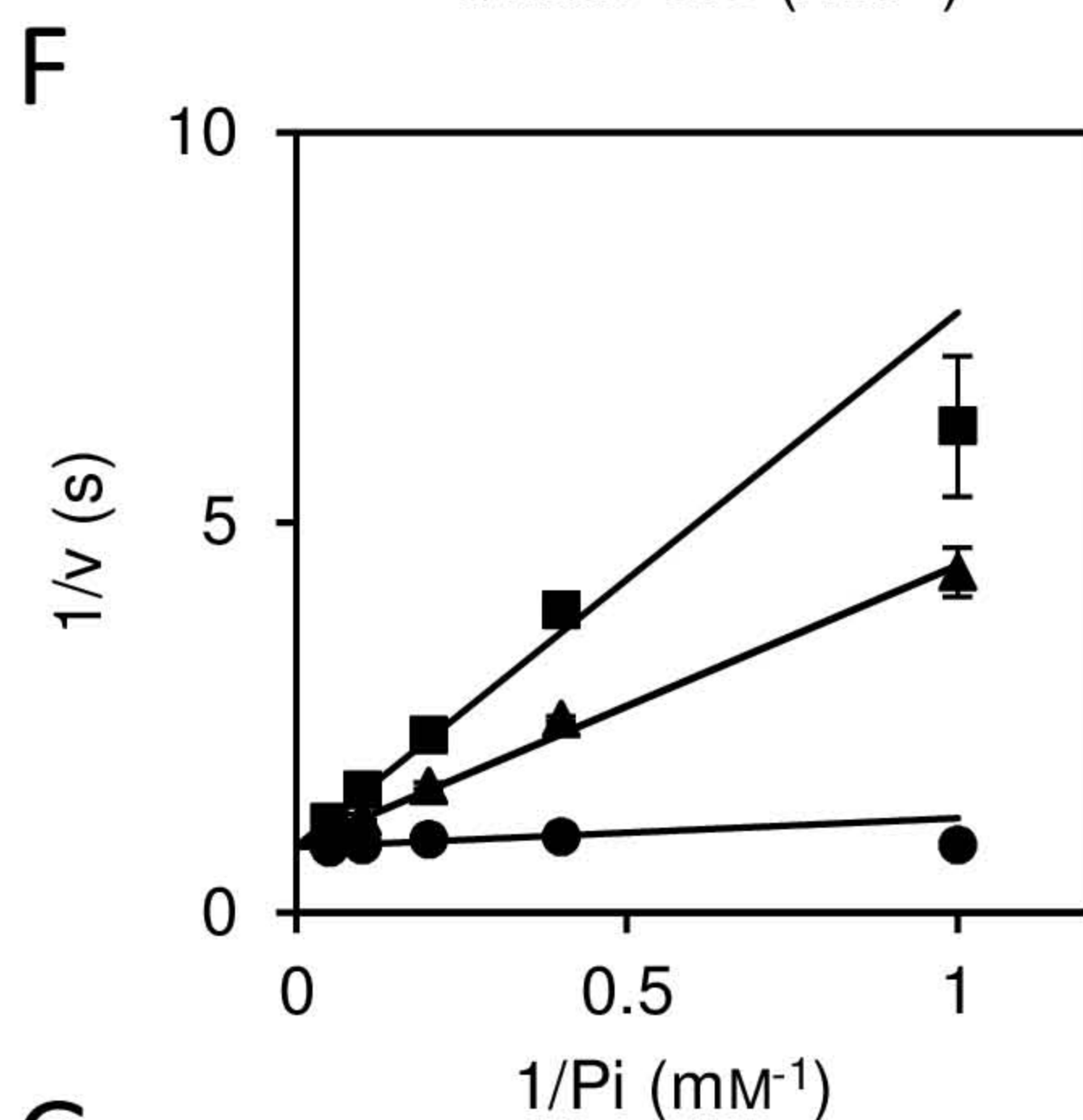
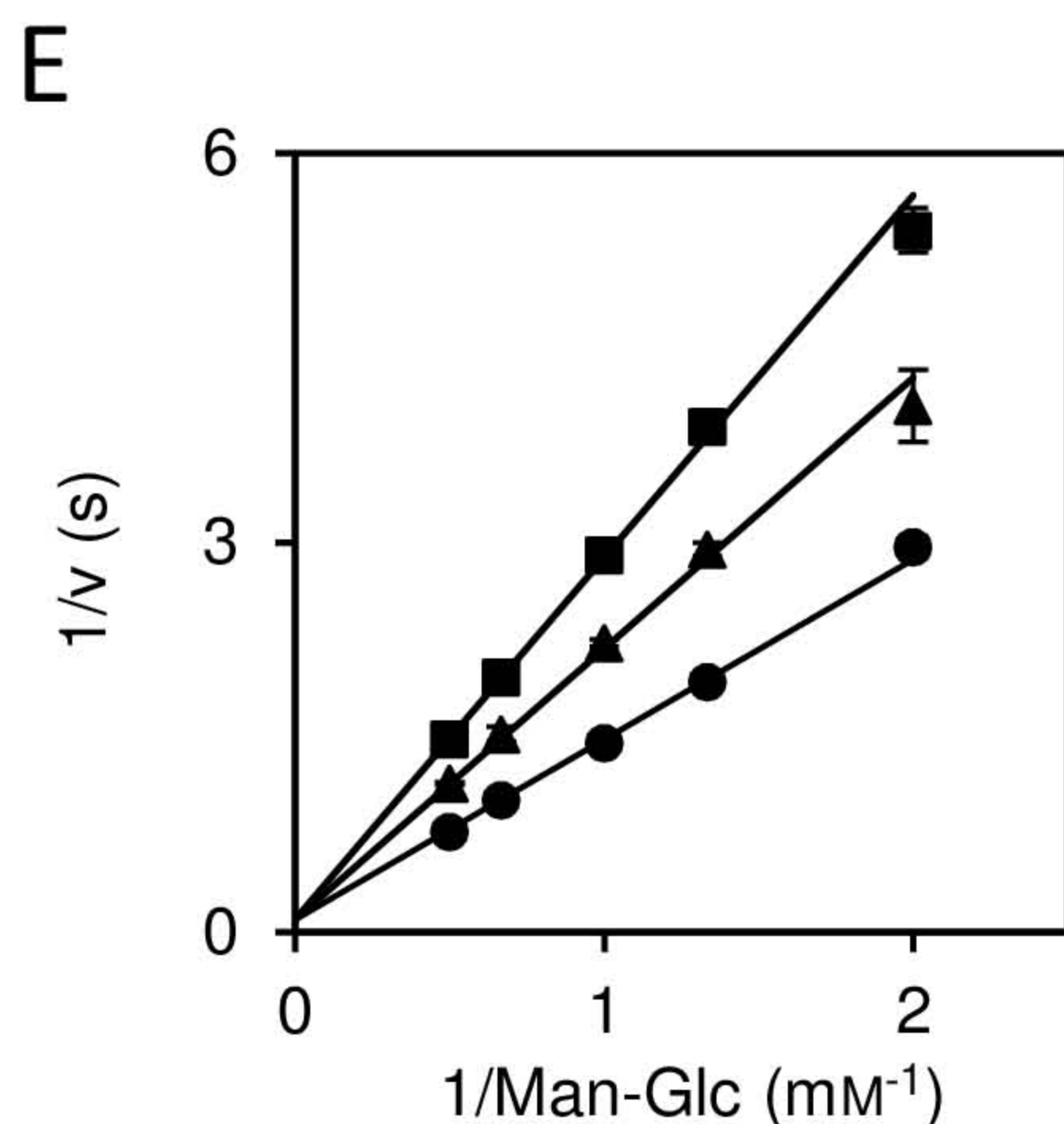
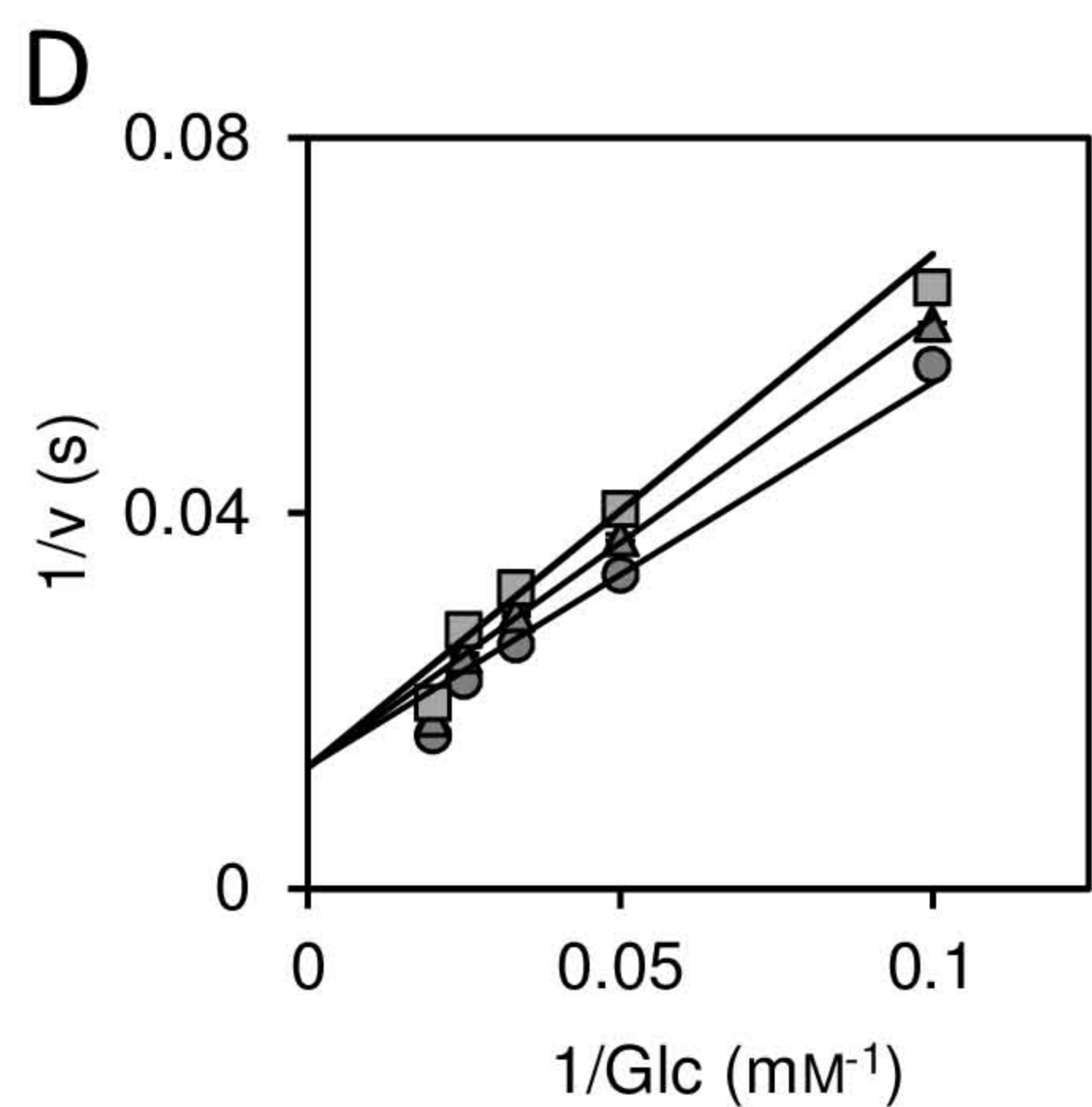
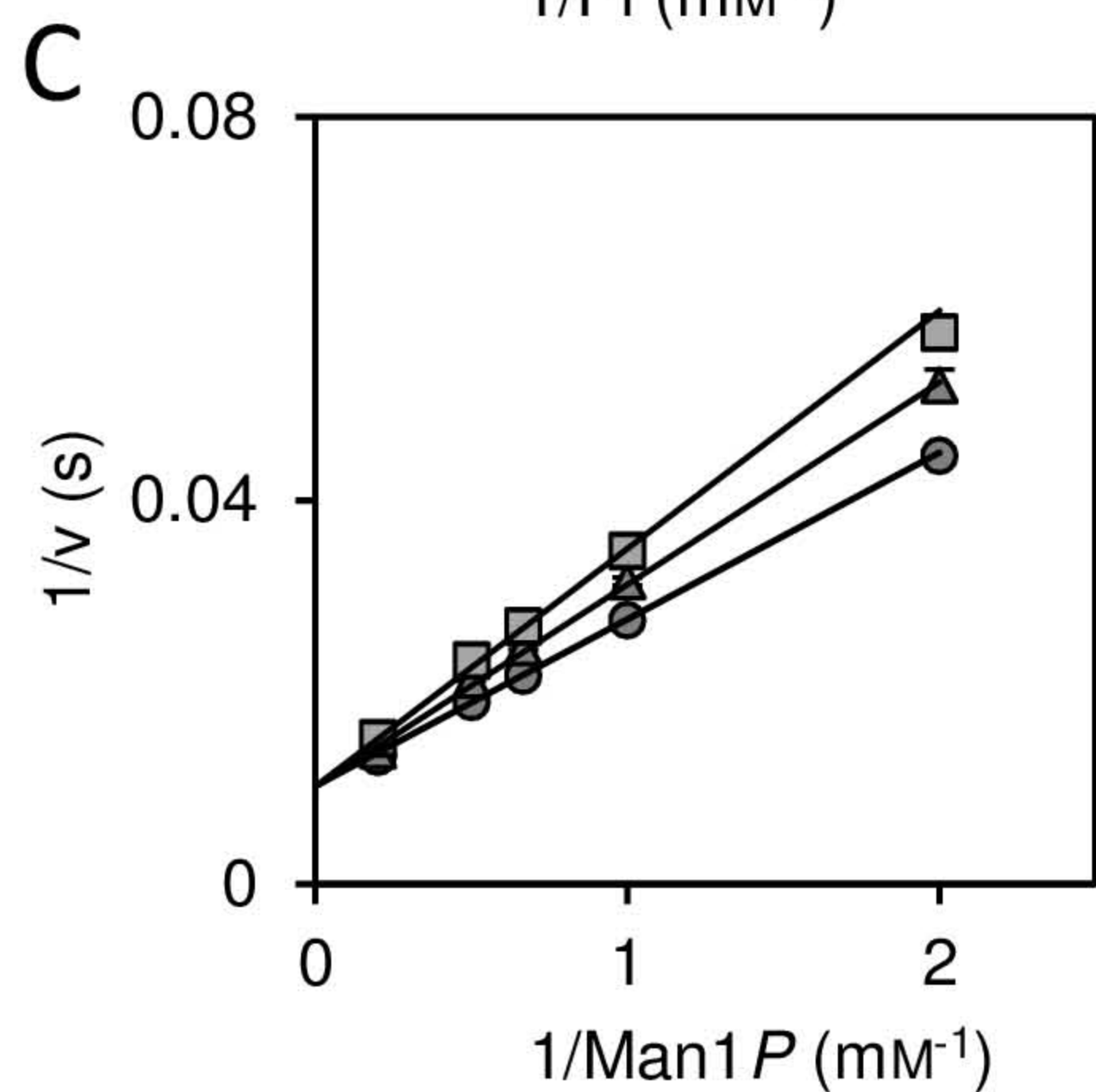
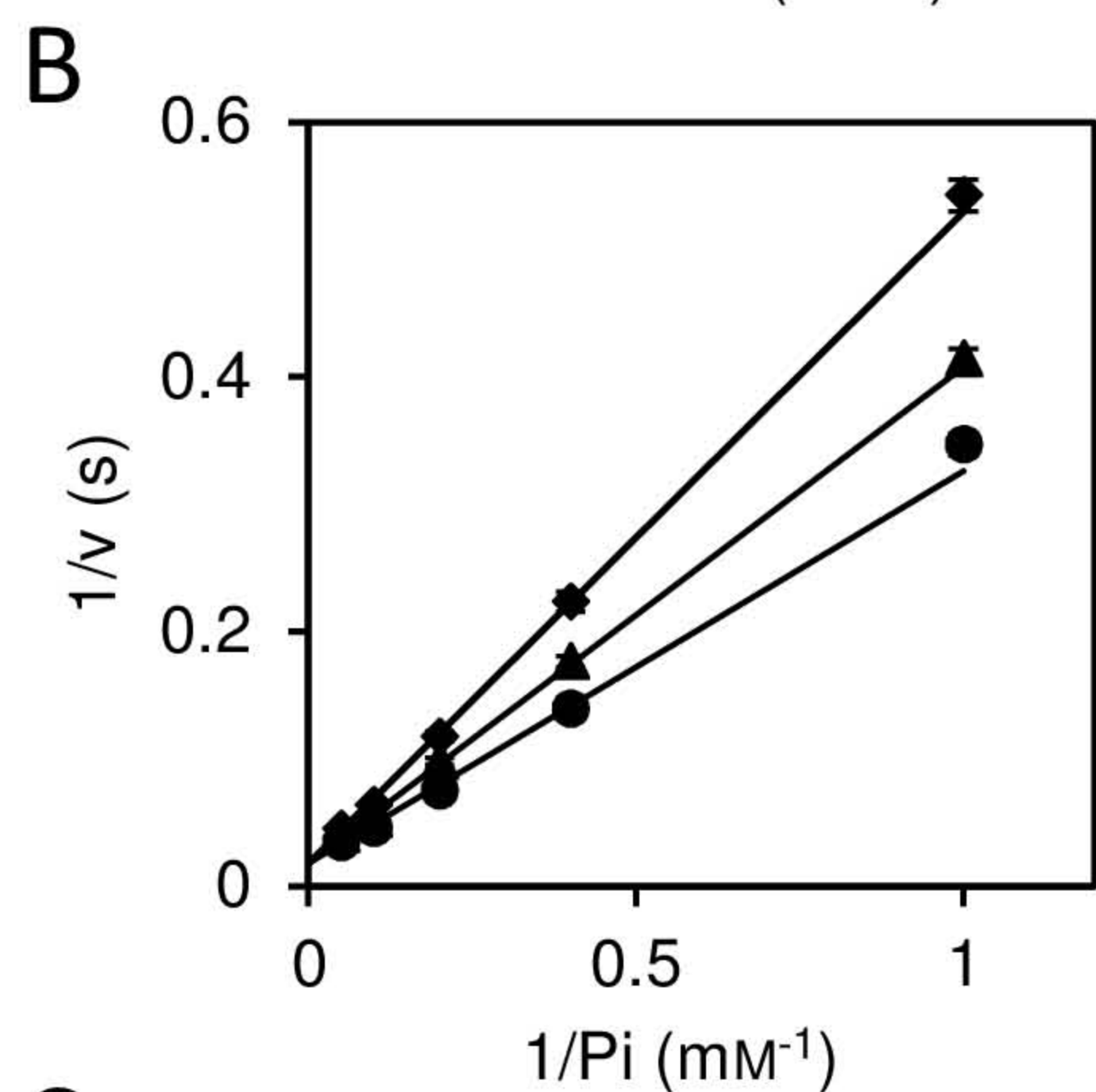
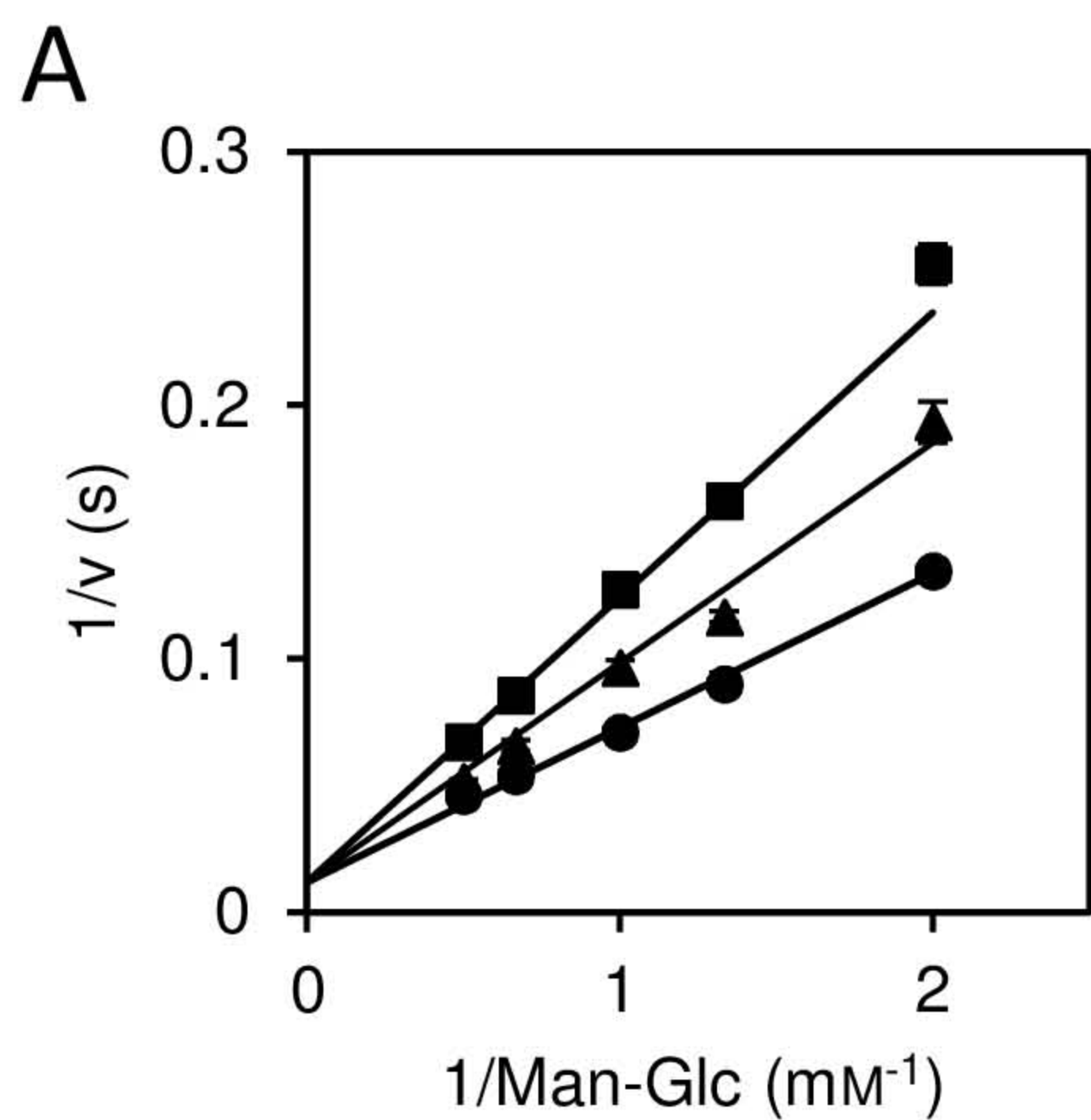


Fig. 3. Kawahara et al

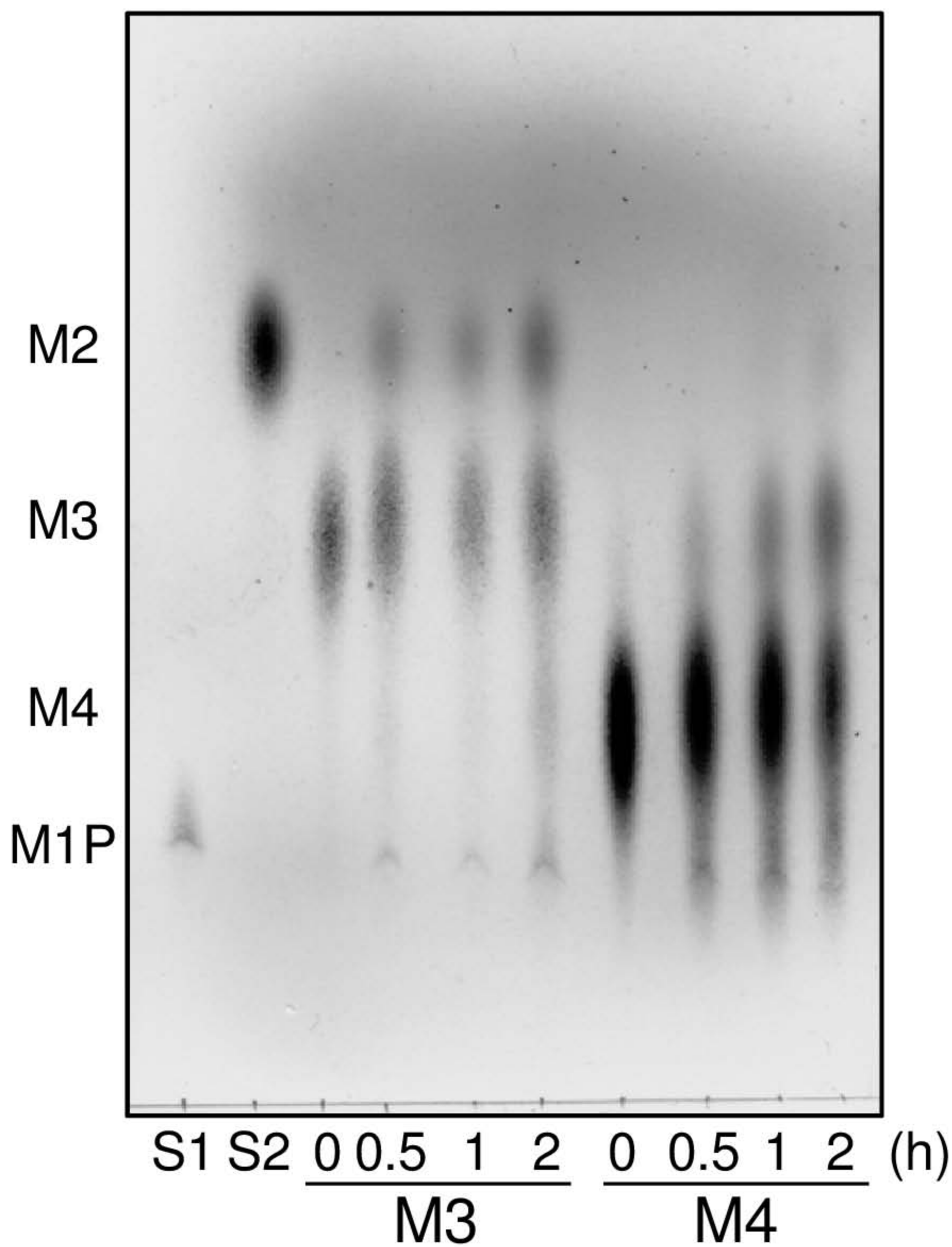


Fig. 4, Kawahara, et al.

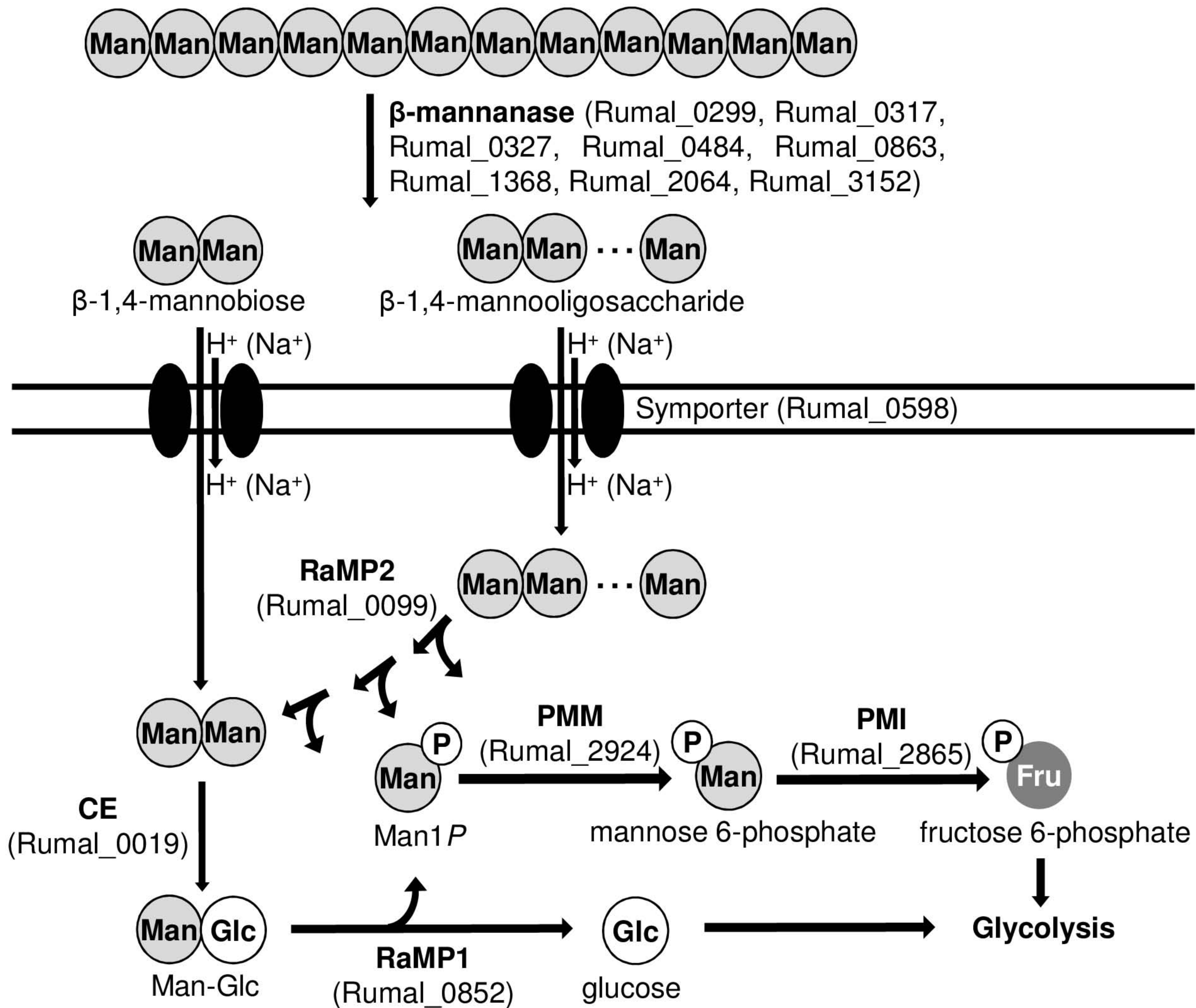


Fig. 5, Kawahara, et al.