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Running title: Colorimetric quantification of Man_2 and Man-Glc

Full title: Colorimetric Quantification of β-(1→4)-Mannobiose and 4-*O*-β-D-Mannosyl-D-glucose

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Abbreviations: CE, cellobiose 2-epimerase; Man-Glc, 4-*O*-β-D-mannosyl-D-glucose; Man_2, β-(1→4)-mannobiose; MGP, 4-*O*-β-D-mannosyl-D-glucose phosphorylase; Man1*P*, α-D-mannose 1-phosphate; RaCE, CE from *Ruminococcus albus*; RaMP1, MGP from *Ruminococcus albus*; RmCE, CE from *Rhodothermus marinus*.

Spectrophotometric quantification method of carbohydrates is useful for processing multiple samples. In this study, we established colorimetric quantification for 4-*O*-β-D-mannosyl-D-glucose (Man-Glc) and β-(1→4)-mannobiose (Man_2). For quantification of Man-Glc, phosphorolysis of Man-Glc catalyzed by 4-*O*-β-D-mannosyl-D-glucose phosphorylase (MGP) was coupled with quantification of D-glucose by the glucose oxidase-peroxidase method. In addition to MGP, cellobiose 2-epimerase (CE) was added for quantification of Man_2. In both quantifications, a good linear relationship was obtained between A_{505} and the sample concentration (0–0.5 mM). The A_{505} values obtained at various concentrations of Man_2 and Man-Glc were almost identical to those with equivalent D-glucose concentrations. Kinetic parameters of *Ruminococcus albus* and *Rhodothermus marinus* CEs for the epimerization of Man_2 were determined using the quantification method for Man-Glc. Both enzymes showed 5–15-fold higher k_{cat}/K_m values than those for cellobiose and lactose, which supports the prediction that these enzymes utilize Man_2 as a substrate in the β-mannan metabolic pathway.

Keywords: oligosaccharide quantification; β-(1→4)-mannobiose; 4-*O*-β-D-mannosyl-D-glucose; cellobiose 2-epimerase; 4-*O*-β-D-mannosyl-D-glucose phosphorylase

β-(1→4)-Mannobiose (Man_2) is a potential prebiotic oligosaccharide, which is highly resistant to digestive enzymes and fermented to produce short chain fatty acids by human fecal bacteria.[1] This oligosaccharide possesses innate immune modulating activity.[2,3] It enhances antibacterial defenses in chicken macrophages, and oral administration of Man_2 prevents *Salmonella enteritidis* infection, which is a food-borne pathogen often found in broilers.[4]

In the metabolism of Man_2, this oligosaccharide is generally considered to be hydrolyzed by β-mannosidase (EC 3.2.1.25) to release D-mannose,[5] but we have found a new metabolic pathway involving epimerization and phosphorolysis in aerobic and anaerobic bacteria.[6-8] In this pathway, cellobiose 2-epimerase (EC 5.1.3.11, CE) epimerizes Man_2 to 4-*O*-β-D-mannosyl-D-glucose (Man-Glc), and Man-Glc is phosphorolyzed to α-D-mannose 1-phosphate (Man1*P*) and D-glucose by 4-*O*-β-D-mannosyl-D-glucose phosphorylase (EC 2.4.1.281, MGP), which is highly specific to Man-Glc.

Compared with chromatographic techniques such as high performance liquid chromatography, spectrophotometric methods can process a larger number of samples more easily. As D-glucose can be easily quantified by spectrophotometric methods,[9,10] colorimetric quantification methods of maltose and cellobiose were established by coupling the colorimetric quantification of D-glucose and phosphorolysis of these oligosaccharides with maltose phosphorylase (EC 2.4.1.8) and cellobiose phosphorylase (EC 2.4.1.20), respectively.[11-13] In this study, we established colorimetric quantification methods for Man-Glc and Man_2 by combination of the enzymatic quantification of D-glucose and reactions of MGP and CE.

Recombinant CE and MGP from *Ruminococcus albus* (RaCE and RaMP1, respectively) were produced in *Escherichia coli* and purified as described

previously.[7,14] Man_2 and Man-Glc were prepared as described elsewhere.[7] A bottle of the coloring reagent Glucose CII-Test Wako (Wako Pure Chemical Industries, Osaka, Japan) was dissolved in 175 mL of 100 mM sodium phosphate buffer (pH 6.0) containing 2.8 mM 4-aminoantipyrine and 40 mM phenol as a substitute for 350 mL of the original buffer in the kit. This mixture was used as the D-glucose quantification reagent. In the quantification of Man-Glc, 50 μL of 0–0.5 mM Man-Glc was mixed with 100 μL of the enzyme solution, containing 1.3 μg/mL RaMP1 and 70 mM sodium phosphate buffer (pH 6.3), and 20 μL of the D-glucose quantification reagent, and incubated at 37°C for 30 min. For quantification of Man_2, RaCE was also added to the reaction mixture described above. Fifty microliters of 0–0.5 mM Man_2 was mixed with 100 μL of the enzyme solution, containing 1.3 μg/mL RaMP1, 12 μg/mL RaCE, and 70 mM sodium phosphate buffer (pH 6.3), and 20 μL of the D-glucose quantification reagent. As a control sample, D-glucose in equivalent concentrations was used. After the incubation, A_{505} of the sample was measured. In the reactions for both Man-Glc and Man_2, a good linear relationship between the concentration of the sample and A_{505} was obtained (Fig. 1). The results with various concentrations of Man-Glc and Man_2 were consistent with those with equivalent concentrations of D-glucose, indicating that Man-Glc and Man_2 were completely consumed in this reaction system. No difference of A_{505} values obtained in the experiments of D-glucose, Man-Glc, and Man_2 indicates that Man1*P* at ≤0.5 mM does not prevent the quantification reactions with the indicated concentration of RaMP1, although Man1*P* at ≥1 mM causes product inhibition in the phosphorolysis of Man-Glc.[7] The colorimetric quantification methods established here for Man-Glc and Man_2 are rapid and simple for quantification of many samples.

As Man_2 and Man-Glc are the reaction products of mannan 1,4-mannobiosidase

(EC 3.2.1.100)[15] and CE, the quantification methods of these oligosaccharides can be utilized for activity assays. CE is a useful enzyme for the production of a prebiotic oligosaccharide, epilactose.[16,17] A rapid and simple enzyme assay for CE is very helpful.

The kinetic parameters of RaCE and CE from *Rhodothermus marinus* (RmCE) for the epimerization of Man_2 have not been determined thus far, although the involvement of these enzymes in the metabolism of mannan was predicted as described above. Using the assay method for the quantification of Man-Glc, we determined the kinetic parameters for the epimerization of Man_2. Recombinant RmCE was prepared as described previously.[18] Fifty microliters of the reaction mixture, containing 2.5–25 mM Man_2, 40 mM sodium phosphate buffer (pH 7.0), and the enzyme, was incubated at 37°C (RaCE) or 60°C (RmCE) for 10 min. The concentrations of RaCE and RmCE in the reaction mixture were 7.48 nM and 13.7 nM, respectively. The reactions were terminated by addition of 20 μL of 0.1 M HCl and heating the sample at 100°C for 3 min. To neutralize the sample, 20 μL of 0.1 M NaOH was added. As HCl and NaOH were added to the sample, following procedures for determination of Man-Glc were modified (concentrations of phosphate and RaMP1 were not changed significantly from the experiments described above). The sample was mixed with 60 μL of the enzyme solution, containing 2.2 μg/mL RaMP1 and 83 mM sodium phosphate buffer (pH 6.3), and 20 μL of the D-glucose quantification reagent, and incubated at 37°C for 30 min. The concentration of Man-Glc produced was determined based on the A_{505} using a standard curve of D-glucose. The kinetic parameters were calculated by fitting the velocities at various substrate concentrations to the Michaelis-Menten equation. Non-linear regression was carried out using Grafit version 7.0.2 (Erithacus Software, West Sussex, UK). As summarized in Table 1, RaCE and RmCE showed 5–15-fold

higher k_{cat}/K_m values than those for cellobiose and lactose. The reaction conditions of RaCE for Man_2 (pH 7.0, 37°C) are different from those for cellobiose and lactose (pH 7.5, 30°C). As epimerization activity of RaCE at pH 7.0 at 37°C is slightly lower than that obtained at pH 7.5 at 30°C,[16] k_{cat}/K_m value for Man_2 at pH 7.5 at 30°C is estimated to be higher than the value shown in Table 1. High preference of RaCE and RmCE for Man_2 supports the prediction that these enzymes epimerize Man_2 to Man-Glc for further phosphorolysis by MGP in the β-mannan metabolic pathway.[7,8]

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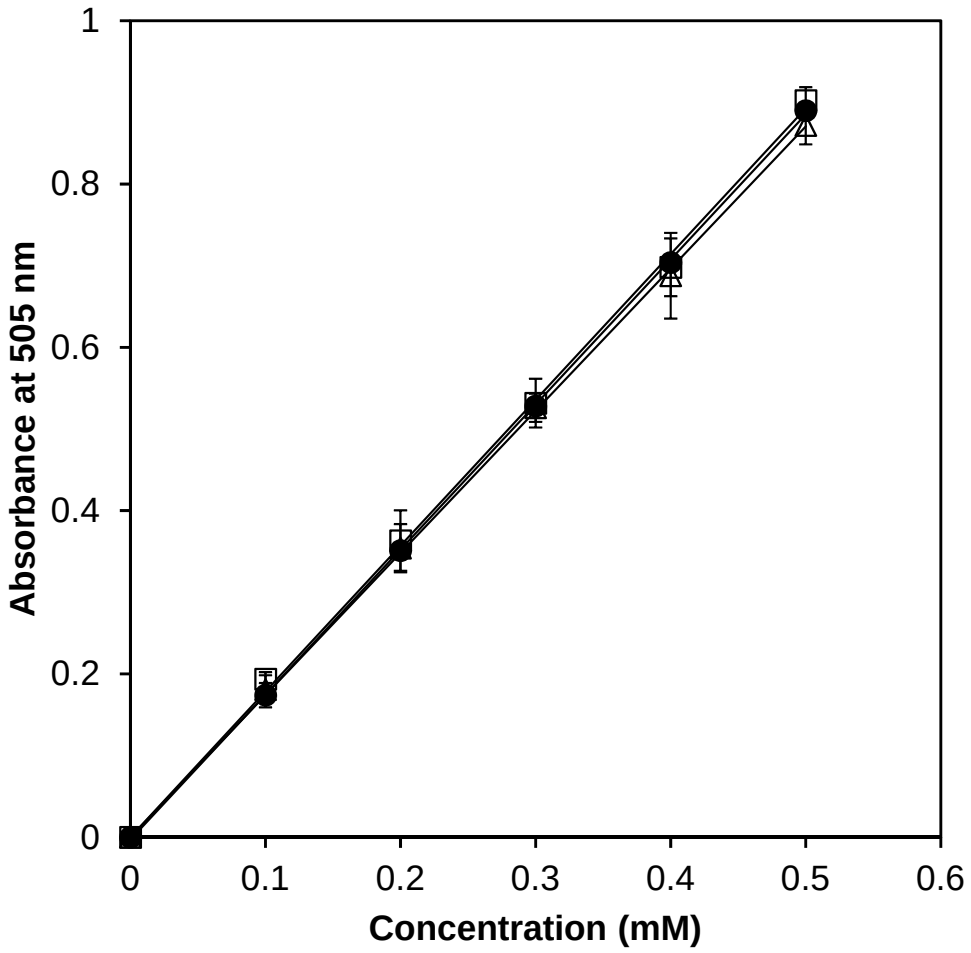
Figure legends

Fig. 1. Enzymatic quantification of Man-Glc and Man_2.

Man-Glc, open triangles; Man_2, open squares; D-glucose (control), filled circles. Data represent the mean ± standard deviation (error bars) for three independent experiments.



Jaito et al., Fig. 1

Table 1. Comparison of kinetic parameters of cellobiose 2-epimerases from *Ruminococcus albus* and *Rhodothermus marinus.*

Origin	Man_2			Cellobiose			Lactose		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}mM^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}mM^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}mM^{-1}$)
Ruminococcus albus	201 ± 1	8.74 ± 0.13	23.0	63.8[16)]	13.8[16)]	4.62[16)]	52.1[16)]	33.0[16)]	1.58[16)]
Rhodothermus marinus	102 ± 2	5.25 ± 0.27	19.4	80.8[19)]	27.2[19)]	2.97[19)]	111[19)]	28.8[19)]	3.85[19)]

Data are mean ± S.D. for three independent experiments. N.D., not determined. Kinetic parameters of *R. albus* CE for cellobiose and lactose were determined at 30°C in 100 mM sodium phosphate buffer (pH 7.5). Those of *R. marinus* CE for cellobiose and lactose were measured at 60°C in 30 mM sodium phosphate buffer (pH 7.0)