Article type: Note

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; Man1P, 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 (Man₂) 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 Man₂ prevents *Salmonella enteritidis* infection, which is a food-borne pathogen often found in broilers.⁴

In the metabolism of Man₂, 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.⁶,⁷ In this pathway, cellobiose 2-epimerase (EC 5.1.3.11, CE) epimerizes Man₂ to 4-O-β-D-mannosyl-D-glucose (Man-Glc), and Man-Glc is phosphorolysed to α-D-mannose 1-phosphate (Man1P) 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.¹¹,¹² In this study, we established colorimetric quantification methods for Man-Glc and Man₂ 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.\textsuperscript{7,14} Man\textsubscript{2} and Man-Glc were prepared as described elsewhere.\textsuperscript{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 RaMP\textsubscript{1} 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\textsubscript{2}, RaCE was also added to the
reaction mixture described above. Fifty microliters of 0–0.5 mM Man\textsubscript{2} was mixed with
100 μL of the enzyme solution, containing 1.3 μg/mL RaMP\textsubscript{1}, 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\textsubscript{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\textsubscript{2} were
consistent with those with equivalent concentrations of D-glucose, indicating that
Man-Glc and Man\textsubscript{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\textsubscript{2} indicates
that Man1\(P\) at \(\leq 0.5\) mM does not prevent the quantification reactions with the
indicated concentration of RaMP\textsubscript{1}, although Man1\(P\) at \(\geq 1\) mM causes product
inhibition in the phosphorolysis of Man-Glc.\textsuperscript{7} The colorimetric quantification methods
established here for Man-Glc and Man\textsubscript{2} are rapid and simple for quantification of many
samples.
As Man\textsubscript{2} and Man-Glc are the reaction products of mannan 1,4-mannobiosidase
(EC 3.2.1.100)\textsuperscript{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.\textsuperscript{16,17} A rapid and simple enzyme assay for CE is very helpful.

The kinetic parameters of RaCE and CE from \textit{Rhodothermus marinus} (RmCE) for the epimerization of Man\textsubscript{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\textsubscript{2}. Recombinant RmCE was prepared as described previously.\textsuperscript{18} Fifty microliters of the reaction mixture, containing 2.5–25 mM Man\textsubscript{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 \(\mu\text{L}\) of 0.1 M HCl and heating the sample at 100°C for 3 min. To neutralize the sample, 20 \(\mu\text{L}\) 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 \(\mu\text{L}\) of the enzyme solution, containing 2.2 \(\mu\text{g/mL}\) RaMP1 and 83 mM sodium phosphate buffer (pH 6.3), and 20 \(\mu\text{L}\) 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 $k_{\text{cat}}/K_m$ 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,\textsuperscript{16} $k_{\text{cat}}/K_m$ 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 $\beta$-mannan metabolic pathway.\textsuperscript{7,8}

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REFERENCES


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**Figure legends**

**Fig. 1.** Enzymatic quantification of Man-Glc and Man2.

Man-Glc, open triangles; Man2, 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>
<thead>
<tr>
<th>Origin</th>
<th>Man₂</th>
<th>Celllobiose</th>
<th>Lactose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(k_{\text{cat}})</td>
<td>(K_m)</td>
<td>(k_{\text{cat}}/K_m)</td>
</tr>
<tr>
<td></td>
<td>(s⁻¹)</td>
<td>(mM)</td>
<td>(s⁻¹mM⁻¹)</td>
</tr>
<tr>
<td>Ruminococcus albus</td>
<td>201 ± 1</td>
<td>8.74 ± 0.13</td>
<td>23.0</td>
</tr>
<tr>
<td>Rhodothermus marinus</td>
<td>102 ± 2</td>
<td>5.25 ± 0.27</td>
<td>19.4</td>
</tr>
</tbody>
</table>

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