Note

A Simple Method for Elucidating Structures of Galactomanno-oligosaccharides by Sequential Actions of β -Mannosidase and α -Galactosidase

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We reported in a previous paper¹⁾ that the hydrolysis of copra galactomannan by a β -mannanase of *Streptomyces* sp. No. 17 produced three kinds of 1,4- β -manno-oligosaccharides (DP; 2–4) and four kinds of galactomanno-oligosaccharides (Gal₁Man₄, Gal₁Man₅, Gal₂Man₅, and Gal₂Man₆. See the abbreviations in Table I). All of the galactomanno-oligosaccharides were composed of a main-chain of (1→4)-linked β -D-mannoses (4–6 residues) to which single α -D-galactosyl stubs (1 or 2 residues) were directly attached through the O-6 position. However, the precise location of the galactose stub was still unidentified, because we were unable to find the position of the stub using physico-chemical procedures.

This paper describes a simple method for elucidating structures of galactomanno-oligosaccharides using sequential actions β -mannosidase and α -galactosidase, and also a structural study of four kinds of galactomanno-oligosaccharides.

Galactomanno-oligosaccharides and 1,4- β -manno-oligosaccharides. Galactomanno-oligosaccharides (Gal₁Man₄, Gal₁Man₅, Gal₂Man₅, and Gal₂Man₆) were prepared by the method described in a previous paper,¹⁾ from a hydrolysate of copra galactomannan (Gal:Man $\approx$ 1:14) by the β -mannanase IV²⁾ of *Streptomyces* sp. No. 17. Manno-oligosaccharides were also prepared by the method described in a previous paper.³⁾

Enzymes. α -Galactosidase (EC 3.2.1.22) from *Mortierella vinacea*⁴⁾ was purchased from Seikagaku Kogyo Co.,

Ltd. The enzyme preparation hydrolyzes α -1,6-galactosidic linkage attached to non-reducing terminal mannose residue of 1,4- β -manno-oligosaccharide, but not the stub galactosidic linkage.⁵⁾

β -Mannosidase (EC 3.2.1.25) from *Aspergillus niger* 5–16 was purified by ion exchange and gel filtration chromatographies, and also by heat treatment (55°C for 5 min) to completely inactivate a trace of α -galactosidase contaminant. The enzyme preparation thus obtained was devoid of both α -galactosidase and β -mannanase (EC 3.2.1.78) activities. The preparation sequentially removed single mannosyl residue from the non-reducing terminal of (1→4)-linked β -manno-oligosaccharide, but was unable to cleave beyond a mannosyl residue to which galactose was attached at the O-6 position,⁶⁾ as well as the β -mannosidase of *Helix pomatia* (a snail).⁷⁾ For example, the enzyme could not liberate Gal α 1→6Man from galactomanno-oligosaccha-

A₁ S 1 2 3 4 A₁ A₂

Fig. 1. TLC of Hydrolysate of Gal₂Man₅ Obtained through the Sequential Actions of β -Mannosidase and α -Galactosidase.

TLC was done on Merck TLC plate silica gel 60 with the solvent system of 1-propanol–nitromethane–water (5:2:3, v/v). The sugars on the plate were detected by heating to 140°C for 5 min after spraying with 50% sulfuric acid–ethanol. A₁, authentic mannose to mannohexaose from top to bottom; A₂, authentic galactose; S, Gal₂Man₅; lanes 1 and 3, β -mannosidase actions; lanes 2 and 4, α -galactosidase actions.

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Table I. THE PROPOSED STRUCTURES OF GALACTOMANNO-OLIGOSACCHARIDES ISOLATED FROM ENZYMATIC HYDROLYSATE OF COPRA GALACTOMANNAN

Oligosaccharides ^a	Structures
Gal ₁ Man ₄	$\begin{array}{c} \text{Man}\beta 1 \rightarrow 4\text{Man}\beta 1 \rightarrow 4\text{Man}\beta 1 \rightarrow 4\text{Man} \\ \uparrow 6 \\ \text{Gal}\alpha 1 \end{array}$
Gal ₁ Man ₅	$\begin{array}{c} \text{Man}\beta 1 \rightarrow 4\text{Man}\beta 1 \rightarrow 4\text{Man}\beta 1 \rightarrow 4\text{Man}\beta 1 \rightarrow 4\text{Man} \\ \uparrow 6 \\ \text{Gal}\alpha 1 \end{array}$
Gal ₂ Man ₅	$\begin{array}{c} \text{Man}\beta 1 \rightarrow 4\text{Man}\beta 1 \rightarrow 4\text{Man}\beta 1 \rightarrow 4\text{Man}\beta 1 \rightarrow 4\text{Man} \\ \uparrow 6 \qquad \qquad \uparrow 6 \\ \text{Gal}\alpha 1 \qquad \text{Gal}\alpha 1 \end{array}$
Gal ₂ Man ₆	$\begin{array}{c} \text{Man}\beta 1 \rightarrow 4\text{Man}\beta 1 \rightarrow 4\text{Man}\beta 1 \rightarrow 4\text{Man}\beta 1 \rightarrow 4\text{Man}\beta 1 \rightarrow 4\text{Man} \\ \uparrow 6 \qquad \qquad \uparrow 6 \\ \text{Gal}\alpha 1 \qquad \text{Gal}\alpha 1 \end{array}$

^a The numbers on the right of sugar residues denote the molar number of the sugar residue in each oligosaccharide.

rides such as Gal α 1 $\rightarrow$ 6Man β 1 $\rightarrow$ 4Man, Gal α 1 $\rightarrow$ 6Man β 1 $\rightarrow$ 4Man β 1 $\rightarrow$ 4Man *etc.* One unit of β -mannosidase activity was defined as the amount releasing 1 μ mol of *p*-nitrophenol from *p*-nitrophenyl- β -D-mannopyranoside (Sigma Chemical Co.) per minute at pH 3.5 and 60°C.

Structural study of galactomanno-oligosaccharides. β -Mannosidase (100 μ l including 2 units in McIlvaine buffer solution without dilution, pH 3.5) was added to sugar solution (700 μ l including each 4 mg of galactomanno-oligosaccharides), and the mixture was incubated at 45°C for 12 hr. The enzyme reaction was stopped by heating at 100°C for 5 min. The resultant sugar solution (4 μ l) was chromatographed by TLC for the characterization of the hydrolysis products. Then, α -galactosidase (200 μ l including 2 units in 0.1 M phosphate buffer solution, pH 5.9) was added to the mannosidase-treated sugar solution, and the mixture (pH 5.1) was incubated at 40°C for 12 hr. The sample after heating was likewise used for TLC. The above procedures were repeated for characterizing the galactomanno-oligosaccharides.

As an example, Fig. 1 shows the results of structural study of Gal₂Man₅ by alternating β -mannosidase and α -galactosidase actions followed by the characterization of hydrolysis products by TLC. The oligosaccharide was

hydrolyzed to mannose and hexasaccharide by β -mannosidase (lane 1 in Fig. 1). The result indicates that an α -galactosyl residue on Gal₂Man₅ was located on the fourth mannosyl residue from the reducing end of mannopentaose. Next, α -galactosidase hydrolyzed the hexasaccharide to produce galactose and pentasaccharide (lane 2 in Fig. 1). Then, the resultant pentasaccharide was hydrolyzed to mannose and tetrasaccharide by β -mannosidase (lane 3 in Fig. 1). Finally, the treatment of the tetrasaccharide with α -galactosidase gave galactose and mannotriose (lane 4 in Fig. 1). The results (lanes 3 and 4 in Fig. 1) suggest that the other α -galactosyl residue on Gal₂Man₅ was located on the third mannosyl residue from the reducing end of mannopentaose. Therefore, the structure of Gal₂Man₅ is shown to be 6³,6⁴-di- α -D-galactosyl-(1 $\rightarrow$ 4)- β -D-mannopentaose.

Using the above procedure, the other galactomanno-oligosaccharides (Gal₁Man₄, Gal₁Man₅ and Gal₂Man₆) were characterized.

Gal₁Man₄ was shown to be 6³-mono- α -D-galactosyl- β -D-mannotetraose because the sugar was hydrolyzed by β -mannosidase to mannose and tetrasaccharide which produced galactose and 1,4- β -mannotriose by the treatment with α -galactosidase.

The hydrolysate of Gal₁Man₅ with β -mannosidase was composed of mannose and two kinds of oligosaccharides

(tetra- and penta-saccharides in the ratio of 1:3), and the subsequent treatment of the hydrolysate with α -galactosidase produced galactose, 1,4- β -mannotriose, and 1,4- β -mannotetraose. Accordingly, it is considered that Gal₁Man₅, though homogeneous on TLC, was a mixture of 6³-mono- α -D-galactosyl- β -D-mannopentaose and 6⁴-mono- α -D-galactosyl- β -D-mannopentaose.

The hydrolysis of Gal₂Man₆ by β -mannosidase gave hexa- and hepta-saccharides (1:1) which were separated on gel filtration for further characterization. On following sequential analysis, the hexasaccharide showed the same behavior as the oligosaccharide produced from Gal₂Man₅ by β -mannosidase. This indicates that one of Gal₂Man₆ was 6³,6⁴-di- α -D-galactosyl- β -D-mannohexaose. On the other hand, the heptasaccharide gave galactose and hexasaccharide by treatment with α -galactosidase, mannose and pentasaccharide by the subsequent treatment with β -mannosidase, and galactose and mannotetraose by the final treatment with α -galactosidase. So, another one of Gal₂Man₆ is 6⁴,6⁵-di- α -D-galactosyl- β -D-mannohexaose. Thus, Gal₂Man₆ (also homogeneous on TLC) from the above results is shown to be a mixture of 6³,6⁴-di- α -D-galactosyl- β -D-mannohexaose and 6⁴,6⁵-di- α -D-galactosyl- β -D-mannohexaose. Table I depicts the proposed structures of Gal₁Man₄, Gal₁Man₅, Gal₂Man₅ and Gal₂Man₆.

McCleary *et al.*⁸⁾ use NMR, enzymatic and chemical techniques to characterize the galactomanno-oligosaccharides produced on hydrolysis of galactomannan by β -mannanase. But the method needs many kinds of enzymes,

such as β -mannanases of *A. niger* and lucerne-seed, α -galactosidase and exo- β -mannanase of guar seed, and β -mannosidase of *Helix pomatia*, and is complicated compared with our method.

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