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Separation and Preparation of Galactosylmanno-Oligosaccharides from Copra Galactomannan by Mannanase from *Penicillium purpurogenum*

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Six kinds of oligosaccharides were obtained from the hydrolysate of copra galactomannan by a purified extracellular β-mannanase from *Penicillium purpurogenum*. These oligosaccharides were identified as M-M, M-M-M, M-M, M-M-M-M, M-M-M-M-M and M-M-M-M-M-M; where G- and M- repre-
G ⌊G⌋ ⌊G-G⌋ ⌊G-G-G⌋
sent α-1,6-D-galactosidic and β-1,4-mannosidic linkages, respectively. The mode of action of mannanase on galactomannan is discussed on the basis of the structure of these oligosaccharides.

We have previously reported the characteristic features of an α-galactosidase from *Penicillium purpurogenum* (5) and properties of the purified mannanase (6). There are many reports dealing with β-mannanase from various microorganisms (8) but only three kinds of the enzyme (from *Streptomyces* sp. No. 17 (2), *Leucaena glauca* (9) and *Bacilus subtilis* (9) have been studied for specificity of the enzyme to galactomannan.

The objectives of this investigation were to isolate oligosaccharides from the hydrolysate of copra galactomannan using a purified extracellular β-mannanase from *Penicillium purpurogenum* and, to study the structure of the oligosaccharides isolated, and simultaneously to comment on the specificity of β-mannanase for galactomannan based on the structure of the oligosaccharides.

MATERIALS AND METHODS

Galactomannan and β-1,4-Manno-oligosaccharides

Copra galactomannan was prepared (7) by alkali extraction from white copra meal. The galactomannan had a ratio of mannose to galactose of 14:1. The manno-oligosaccharides were prepared (3) from the partial hydrolysate of copra mannan using the mannanase from a species of *Streptomyces*.

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β-Mannanase

Purified β-mannanase (6) from *Penicillium purpurgenum* was used for the hydrolysis of copra galactomannan. The enzyme preparation was homogeneous on polyacrylamdie gel electrophoresis. In addition the enzyme did not hydrolzye cellulose powder or cellobiose.

β-Mannosidase

Purified β-mannosidase from *Aspergillus niger* 5-16 was used for establishing the structure of oligosaccharides. The enzyme hydrolzyed p-nitrophenyl-β-D-mannoside and β-1,4-mannobiose, but not cellobiose.

Thin-layer Chromatography

TLC was performed on a Merck TLC plate (200×200 mm) of DC-Fertigplatten Cellulose with a solvent system of 1-butanol:pyridine:water (6:4:3, v/v). Sugars on the plate were detected by heating at 130 to 140℃ for a few minutes after spraying with p-anisidine hydrochloride.

Hydrogenation of Sacchardies

Saccharides were hydrogenated into their corresponding sugar alcohols by treating aqueous solutions of the sugars with sodium borohydride for 2 hours at room temperature. The resultant sugar solutions were treated with Amberlite IR-200c(H^+) to decompose the excess sodium borohydride, and to remove the base, and then evaporated with methanol to remove boric acid.

Methylation Analysis

The sugar was methylated by the methods of Ciucanu *et al.* (1). The methylated sugar was hydrolzyed in 10%

trifluoroacetic acid, hydrogenated with sodium borohydride and acetylated with an equal mixture of pyridine and acetic anhydride. The resultant alditolacetate was analyzed by using a column of 3% ECNSS-M on Gas Chrom Q(Nippon Kuromato Kogyo, Japan) at 155℃, and a column of OV-210 on Spelcoport (Nippon Kuromato Kogyo, Japan) at 190℃.

Identification of Component Sugars

Oligosaccharides were hydrolyzed in 10% trifluoroa cetic acid (in an ampoule), by heating at 100℃ for 2 hours. The hydrolysate was evaporated to dryness on a rotary evaporator. The resultant sugars were converted into their alditol-acid tate derivatives and an alyzed by gasliquid chromatography (4) on a 3% ECNSS-M column.

RESULTS

Enzymatic Hydrolysis of Copra Galactomannan

Eight hundred ninety *ml* of the purified mannanase solution (total activity; 400 units) and 890 *ml* of McIlvaine buffer solution (pH 5.) were added to 53.4g (48.1g as total sugars) of the galactomanan. The enzyme reaction was carried out at 55℃ for 24 hours in a 3-liter glass vessel with an agitator. At specific time intervals (0.5, 1, 3, 5, 8 and 24 hours) a small amount of the reaction mixture was withdrawn from the vessel and heated immediately to 100℃ for 5 minutes to inactive the enzyme. After the removal of insoluble materials from the resultant hydrolysate by centrifugation the supernatant liquid was subjected to TLC for the characterization of the hydrolysis products. The results are shown in Fig. 1. The chromatogram of the hydrolysis products demonstrated that mannobiose, unknown oligosaccharides, and small amounts of mannose were produced during the course of hydrolysis. These unknown oligosaccharides were tentatively designated as M_2, H_1, H_2, H_3 and H_4 in the order of R_f value.

Chromatographic Separation of Oligosaccharides

After a reaction time of 24 hours the enzymatic hydrolysate was heated to 100℃ for 5 minutes and then centrifuged to remove the insoluble materials. Sixteen hundred eighty *ml* of the sugar solution (34.2g as total sugar) was applied to a granular charcoal column (70× 80 mm, 600g of activated characoal for chromatography, Wako Pure Chemical Ind., Japan). The column was then washed with 10 liters of water to remove mannose and salts. The oligosaccharides in the column were eluted by a linear gradient of 5 to 30% ethanol (total volume; 40 liters). The eluent was collected in 500 *ml* fraciton tubes and the composition in each tube was examined by TLC. The contents in the fraction tubes having chromatographically identical sugars were combined, that is, M_2(frction tubes No. 22 to 35), H_1(40 to 45), H_2(61 to 65), H_3(70 to 72) and H_4(74 to 76). Four kinds of oligosaccharides (M_2, H_2, H_3 and H_4 separated by carbon column chromatography) revealed approximate homogeneity on TLC, but H_1 did not (Fig. 2).

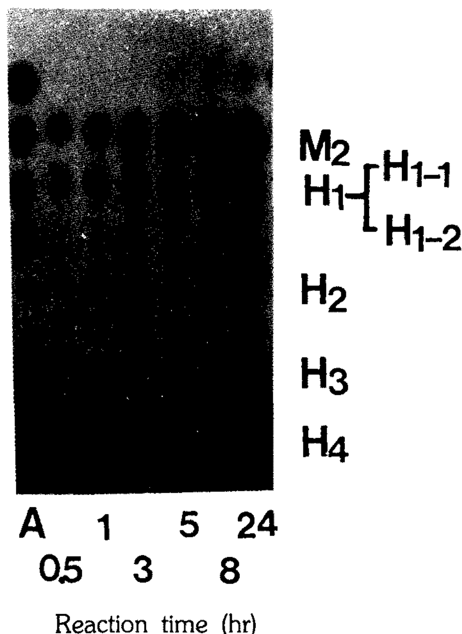

Fig. 1. TLC of time-course of enzymatic hydrolysis of copra galactomannan.
A, authentic mannose, mannobiose, mannotriose and mannotetraose from top to bottom.

Characterization of Oligosaccharides

Table 1 shows physical properties of oligosaccharides and Table 2 shows the results of methylation analysis of oligosaccharides isolated from enzymatic hydrolysate of copra galactomannan. The degree of polymerization of oligosaccharides was determined by the graphical method with reference samples (Fig. 3).

i) M_2; This sugar was composed only of mannose (Table 1) and the position of M_2 on TLC was the same as that of β-1,4-mannobiose (Fig. 2). Evidence supporting this structurl interpretation was also obtained by methylation analysis (Table 2).

ii) H_1; This sugar was a mixture of two kinds of oligosaccharides, named $H_{1\text{-}1}$ and $H_{1\text{-}2}$ in order of R_f value. The two were not seperated by gel filtration chromatography (TOYO PEARL HW-40F, Toyo Soda MFG, Co., Ltd., Toyko, Japan). We assumed that they had the same D.P., however, the position of $H_{1\text{-}1}$ on TLC was the same as that of β-1,4-mannotriose (Fig. 2). A β-mannosidase was used for studying the structure of $H_{1\text{-}2}$. The manno-

Fig. 2. TLC of oligosaccharides isolated from the enzymatic hydrolysate of copra galactomannan.
A, authentic mannose, mannobiose, mannotriose, mannotetraose, mannopentaose and mannohexaose from top to bottom.

sidase was purified from a culture filtrate of *Aspergillus niger* 5-16 and the purified enzyme preparation was free of both mannanase and α-galactosidase activities. The enzyme hydrolyzed β-1,4-mannobiose(also β-1,4-mannotriose) and p-nitrophenyl β-D-mannopyranoside.

β-mannosidase was added to the H_1 solution and it was then incubated at pH 3.5 and 60℃ for 24 horus. The resultant hydrolysate was subjected to TLC for characterization of hydrolysis products and developed twice with a solvent system of chloroform:methanol:water (90:65:15, v/v). As shown in Fig. 4 the mannosidase reaction led to the disappearence of H_1 (both $H_{1\text{-}2}$ and $H_{1\text{-}2}$), mannose, and the unknown sugar which was located between mannobiose and mannotriose. This suggests that the unknown sugar was 6-α-galactosylmannose, and

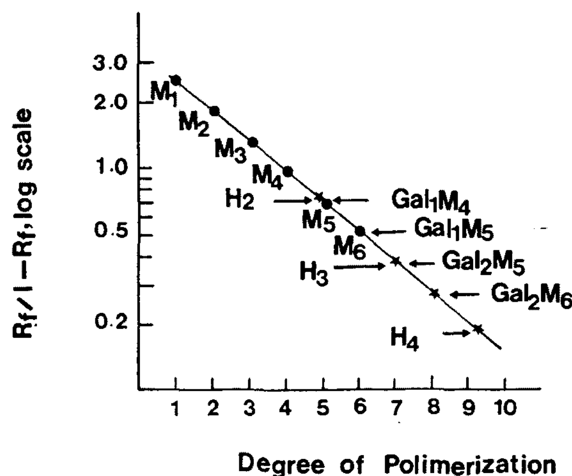

Fig. 3. Relation between the degree of polymerization of identified oligosaccharides and their log $R_f/1\text{-}R_f$.
Gal_1M_4, Gal_1M_5, Gal_2M_5 and Gal_2M_6 were obtained from the hydrolysate of copra galactomannan using *Streptomyces* sp. No. 17 mannanase.

that $H_{1\text{-}2}$ has the same structure as 6^1-α-galactosyl-mannobiose, because the β-mannosidase hydrolyzed 6^3-α-galactopyranosylmannotriose in an equimolar riato.

iii) H_2; This sugar produced galactose and mannose on complete hydrolysis in the molar ratio of 1:3.78 (Table 1). This ratio is considered to be 1:4 because of a D.P. of 5 (Table 1). The methylation analysis (Table 2) of H_2 revealed the 2,3,4,6-Me-Man (1 mol) 2,3,6-Me-Man (2 mol), 2,3-Me-Man (1 mol) and 2,3,4,6-Me-Gal (1 mol). The methylation of the corresponding hydrogenated derivative revealed the appearence of 1,2,3,5,6-Me-Mannitol (1 mol) with the disappearence of 2,3,6-Me-Man (1 mol) (Table 2). These results indicate that H_2 has one non-reducing-end D-mannosyl residue, two 4-substituted D-mannosyl residues, one 4,6-di-substituted D-mannosyl residue, and one non-reducing-end D-galactosyl residue linked at the o-6 of the mannosyl residue. Based on the above results the proposed structure for H_2 is shown in Fig. 5. However, the exact loca-

Table 1. Yield and properties of oligosaccharides resulting from enzymatic hydrolysate of copra galactomannan, fractionated by charcoal column chromatography.

Fraction (Fraction tubes No.)		M_2 (22~35)	H_1 (40~45)	H_2 (61~65)	H_3 (70~72)	H_4 (74~76)
Total sugar content(g)		0.51	4.25	0.83	0.41	0.20
Component	Galactose	0	N.D.	1	1	1
Suagr	Mannose	only	N.D.	3.78	2.38	2.01
D.P.		2	N.D.	5	7	9

N.D; not determined.

Table 2. Methylation analysis of oligosaccharides and their hydrogenated derivatives isolated from the enzymatic hydrolysate of copra galactomannan.

Alditol acetate Retention time (min)		1,2,3,5,6-Penta-O-Me-D-Mannitol 1.8	2,3,4,6-Tetra-O-Me-D-Man 5.5	2,3,4,6-Tetra-O-Me-D-Gal 7	2,3,6-Tri O-Me-D-Man 12.7	2,3-Di-O-Me-D-Man 30.6
References	Sample					
Mannotriose	A		+		++	
	B	+		+		+
Gal_1M_4	A		+	+	++	+
	B	+	+	+	+	+
Gal_1M_5	A		+	+	+++	+
	B	+	+	+	++	+
Gal_2M_5	A		+	++	++	++
	B	+	+	++	+	++
Gal_2M_6	A		+	++	+++	++
	B	+	+	++	++	++
M_2	A		+		+	
	B	+	+			
H_2	A		+	+	++	+
	B	+	+	+	+	+
H_3	A		+	++	++	++
	B	+	+	++	+	++
H_4	A		+	+++	++	+++
	B	+	+	+++	+	+++

A, original sugar; B, after hydrogenation with $NaBH_4$; +, 1 mol; ++, 2 mol; +++, 3 mol.

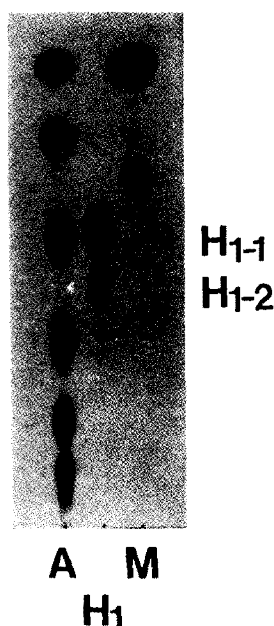

Fig. 4. TLC of hydrolysate obtained through the action of the purified β-mannosidase on H_1.

A, authentic mannose, mannobiose, mannotriose, mannotetraose, mannopentaose and mannohexaose from top to bottom; H_1, mixture of $H_{1\text{-}1}$ and $H_{1\text{-}2}$; M, products from H_1 by hydrolysis with the mannosidase.

Isolated Oligosaccharides		Proposed Structures
M_2		M-M
H_1	$H_{1\text{-}1}$	M-M-M
	$H_{1\text{-}2}$	M-M (G on second M)
H_2		M-M-M-M (G branch)
H_3		M-M-M-M-M (G----G branches)
H_4		M-M-M-M-M-M (G---G--G branches)

Fig. 5. The proposed structure of oligosaccharides isolated from the enzymatic hydrolysate of copra galactomannan.

G, D-galactopyranose; M, D-mannopyranose; M-M, β-1,4-mannosidic linkage; G, α-1,6-galactosidic linkage; ----, unknown linkage.

tion of the galactose branch is still undetermined.

iv) H_3 and H_4; Based upon comparison with H_2 it was deduced that H_3 and H_4 had the structures shown in Fig. 5. The properties (Table 1) of the sugars and the results of these methylation analyses (Table 2) support this interpretation. In addition, the mannosidic linkages and the galactosyl branchings, on the three kinds of galactomanno-oligosaccharides obtained in this study,

are thought to have a β-configuration (4) and α-configuration (4), respectively. All mannosyl and galactosyl linkages in copra galactomannan are known to have these configurations. Moreover, copra galactomannan was hydrolyzed to galactose, mannose and mannobiose (2). However, the precise points at which branching occurs are yet to be determined.

DISCUSSION

Kusakabe *et al.* (2) proposed a structure of copra galactomannan where galactomannan gives galactose and mannose in an average ratio of 1 to 14. The galactomannan is composed of a main chain of (1→4)-linked β-D-mannosyl residues, to which single α-D-galactosyl branchings are directly linked through the o-6 of some of the mannosyl residues. The distribution of the branching is irregular and the distance between two branchings on some parts of the galactomannan chain is short. This fundmental chemical structure of galactomannan bears a close resemblance to the structure proposed herein. However, we propose that three branchings of galactose, at some parts of the galactomannan chain, are contiguous with each other. The isolation of H_4 is evidence for this interpretation. Kusakabe *et al.* did not identify an oligosaccharide having three branchings in its structure.

We propose the specificity of the mannanase to the galactomannan as follows:

1) The enzyme dose not cleave the D-galactosyl branching from the galactomannan.

2) The enzyme has a specificity to the D-mannosyl residues of the main-chain devoid of an α-galactosyl branching, thereby forming the three galactomanno-oligosaccharides which have no α-galactosyl branching at either the non-reducing-end or the reducing-end. In other words the enzyme is supposed to show stiffness toward the hydrolysis of both sides of the mannosyl residues having α-galactosyl branchings. The isolation of galactomanno-oligosaccharides (H_2, H_3 and H_4) is evidence for this interpretation.

3) The enzyme produced one galactomanno-oligosaccharide having a galactosyl branch at the reducing-end ($H_{1\text{-}2}$). It seems that this sugar was a secondary hydrolysis product because the sugar was not observed in the initial stages of the reaction (0.5 to 1 hours). The sugar appeared 8 to 24 hours late. We consider that $H_{1\text{-}2}$ was produced from primary hydrolysis products, such as H_2, H_3 and H_4.

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