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Article

Separation and Identification of Galactosylmanno-oligosaccharides from Hydrolyzate of Brown Copra Meal by *Trichoderma* β-Mannanase

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Three kinds of oligosaccharides were obtained from the hydrolysate of brown copra meal galactomannan by a purified extracellular β-mannanase from Trichoderma sp. These oligosaccharides were identified as Man-Man, Gal^2Man_3 (6^2-mono-O-α-D-galactopyranosyl-4-O-β-D-mannotriose), and Gal^2Man_6 (6^2-mono- O-α-D-galactopyranosyl-4-O-β-D-mannohexaose), where Gal- and Man- represent α-1,6-D-galactosidic and β-1,4-mannosidic linkages, respectively. The mode of action of β-mannanase on brown copra meal galactomannan is described on the basis of the structure of these oligosaccharides.

Key words: *brown copra meal, β-mannanase,* Trichoderma *sp.*

In the process of extracting oil from copra, a large amount of the residual cake known as brown copra meal, containing 40-50% galactomannan (Gal:Man=1:14), is discharged as a by-product. By using the *Streptomyces* β-mannanase system, Kusakabe *et al.* [1983] prepared β-1,4-mannooligosaccharides for the application of functional mannooligosaccharides. In this study they further reported that direct hydrolysis of the brown copra meal by mannanase is easier and more economical for the preparation of mannooligosaccharides.

Although many reports have been made on β-mannanase from various microorganisms [Tipson and Horton, 1976], only three enzyme including *Streptomyces* sp. No. 17 [Kusakabe *et al.*, 1986], *Leucaena glauca* [Tipson and Horton, 1976], and *Bacillus subtilis* [Tipson and Horton, 1976] have been studied for the specificity of the enzymes to galactomannan. Park *et al.* [1991] previously reported the characteristic features of α-galactosidase from *Penicillium purpurogenum* and the properties of the purified mannanase.

The objectives of this study were to isolate oligosaccharides from the hydrolysate of the brown copra meal treated with the purified extracellular β-mannanase from *Trichoderma* sp., study the structure of the oligosaccharides isolated, and examine the specificity of β-mannanase for the brown copra meal galactomannan based on the structure of the oligosaccharides.

Materials and Methods

Galactomannan and β-1,4-manno-oligosaccharides. Brown copra meal galactomannan was prepared by alkali extraction from the copra cake [Takahashi *et al.*, 1983]. The galactomannan had a ratio of mannose to galactose of 14:1. The mannooligosaccharides were prepared [Kusakabe *et al.*, 1983] from the partial hydrolysate of the copra mannan using the mannanase from a *Streptomyces* species.

β-Mannanase. Purified β-mannanase [Park *et al.*, 1987] from *Trichoderma* sp. was used for the hydrolysis of the brown copra meal galactomannan.

β-Mannosidase. Purified β-mannosidase [Eriksson and Winell, 1968] from *Aspergillus niger* 5-16 was used for establishing the structure of oligosaccharides. The enzyme hydrolyzed *p*-nitrophenyl-β-D-mannoside and β-1,4-mannobiose, but not cellobiose.

Enzymatic hydrolysis. Five hundred milliliters of the purified mannanase solution (total activity, 225 units) and 500 mL of McIlvaine buffer solution (pH 4.5) were added to 30 g (27 g as total sugars) of the galactomannan. The enzyme reaction was carried out at 60°C for 24 h in a 3-L glass vessel with continuous agitation. At specific time

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Abbreviations: DP, degree of polymerization; TLC, thin-layer chromatography

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intervals, a small amount of the reaction mixture was withdrawn from the vessel and heated immediately to 100°C for 5 min to inactivate the enzyme. After the removal of the insoluble materials from the resultant hydrolysate by centrifugation, the supernatant liquid was subjected to TLC for the characterization of the hydrolysis products.

Thin-layer chromatography. TLC was performed on a Merck TLC plate (200×200 mm; Darmstadt, Germany) of DC-Fertigplatten Cellulose with a solvent system of 1-butano:pyridine:water (6:4:3, v/v/v). Sugars on the plate were detected by heating at 130 to 140°C for 5 min after spraying with *p*-anisidine hydrochloride.

Separation of oligosaccharides. After a reaction time of 24 h, the enzymatic hydrolysate was heated to 100°C for 5 min and then centrifuged to remove the insoluble materials. Three hundred milliliters of the sugar solution (15.5 g as total sugar) was applied to a granular charcoal column (70×80 mm, 500 g of activated charcoal for chromatography, Wako Pure Chemical Ind., Osaka, Japan). The column was then washed with 8 L of water to remove the mannose and the salts. The oligosaccharides in the column were eluted by a linear gradient of 5 to 30% ethanol (total volume; 30 L).

Hydrogenation of saccharides. Saccharides were hydrogenated into their corresponding sugar alcohol forms by treating the aqueous solutions of the sugars with sodium borohydride for 2 h 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. The resultants were then evaporated with methanol to remove the boric acid.

Methylation analysis. The sugar was methylated using the method of Ciucanu and Kerek [1984]. The methylated sugar was hydrolyzed in 10% trifluoroacetic acid, hydrogenated with sodium borohydride, and acetylated with an equal mixture of pyridine and acetic anhydride. The resultant alditolacetate was analyzed using a column of 3% ECNSS-M on Gas Chrom Q (Nippon Kuromato Kogyo, Japan) at 155°C and a column of OV-210 on Spelcoport (Nippon Kuromato) at 190°C.

Identification of component sugars. Oligosaccharides were hydrolyzed in 10% trifluoroactic acid (in an ampoule), by heating at 100°C for 2 h. The hydrolysate was evaporated to dryness on a rotary evaporator. The resultant sugars were converted into their alditol-acetate derivatives and analyzed by gas liquid chromatography on a 3% ECNSS-M column using the method of Kusakabe *et al.* [1977].

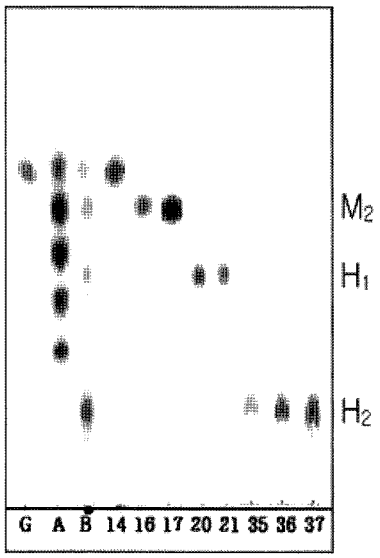

Fig. 1. TLC of oligosaccharides isolated from the enzymatic hydrolysate of brown copra meal galactomannan. A, authentic mannose, mannobiose, mannotriose, mannotetraose, and mannopentose in the order of top to bottom; G, authentic galactose; B, enzymatic hydrolysates; 14-37, fraction numbers.

Results and Discussion

Enzymatic hydrolysis of brown copra meal galactomannan. The chromatogram of the hydrolysis products demonstrated that mannobiose, unknown oligosaccharides, and small amounts of mannose were produced in Fig. 1-B. The unknown oligosaccharides were tentatively designated as M_2, H_1, and H_2 in the order of increasing R_f value. Used enzyme preparation was homogeneous on SDS-polyacrylamide gel electrophoresis. In addition, the enzyme did not hydrolyze cellulose powder or cellobiose.

Chromatographic separation of oligosaccharides. The eluent was collected in 500-mL fraction tubes, and the composition of the eluent in each tube was examined by TLC. The contents in the fraction tubes having chromatographically identical sugars were combined; that is, M_2 (fraction tubes No. 16 to 17), H_1 (20 to 21), and H_2 (35 to 37). These three oligosaccharides, separated by carbon column chromatography, revealed approximate homogeneity on TLC (Fig. 1).

Characterization of oligosaccharides. Tables 1 and 2 show the physical properties of oligosaccharides and the results of methylation analysis of the oligosaccharides isolated from the enzymatic hydrolysate of the brown copra meal galactomannan. The degree of polymerization

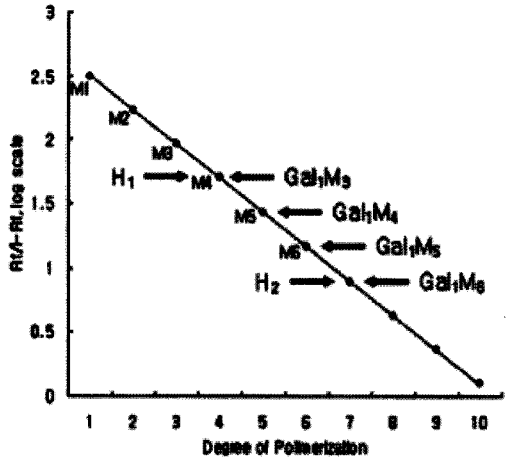

Fig. 2. Relationship between the degree of polymerization of identified oligosaccharides and their log $R_f/1\text{-}R_f$. Gal_1M_3, Gal_1M_4, Gal_1M_5, and Gal_1M_6 were obtained from the hydrolysate of the copra galactomannan using *Streptomyces* sp. no. 17 mannanase.

of oligosaccharides was determined by the graphical method with the reference sample (Fig. 2).

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. 1). Evidence supporting this structural interpretation was also obtained by methylation analysis (Table 2).

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

Fraction (Fraction tubes No.)	M_2 (16~17)	H_1 (20~21)	H_2 (35~37)
Total sugar content(g)	0.66	0.81	0.64
Component Galactose	0	1	1
Sugar Mannose	only	2.86	5.67
DP	2	4	7

DP, Degree of Polymerization

H_1 produced galactose and mannose upon complete hydrolysis with the molar ratio of 1:2.86 (Table 1). This ratio was considered as 1:3 due to DP of 4 (Table 1). The methylation analysis (Table 2) of H_1 revealed the presence of 2,3,4,6-Me-Man (1 mol) 2,3,6-Me-Man (1 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 appearance of 1,2,3,5,6-Me-Mannitol (1 mol) with the disappearance of 2,3,6-Me-Man (1 mol) (Table 2). The proposed structure of H_1 based on the above results is shown in Fig. 3.

Based upon the comparison with H_1, it was deduced that H_2 had the structure shown in Fig. 3. The properties

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

Alditol acetate	1,2,3,5,6-Penta-*O*-Me-D-Mannitol	2,3,4,6-Tetra -*O*-Me-D-Man	2,3,4,6-Tetra -*O*-Me-D-Gal	2,3,6-Tri -*O*-Me-D-Man	2,3-Di -*O*-Me-D-Man
Retention time (min)	2.1	5.8	7.5	13.2	31.9
References Sample					
M_3 A		+		++	
B	+	+		+	
Gal_1M_3 A		+	+	+	+
B	+	+	+		+
Gal_1M_4 A		+	+	++	+
B	+	+	+	+	+
Gal_1M_5 A		+	+	+++	+
B	+	+	+	++	+
Gal_1M_6 A		+	+	++++	+
B	+	+	+	+++	+
M_2 A		+		+	
B	+	+			
H_1 A		+	+	+	+
B	+	+	+		+
H_2 A		+	+	++++	+
B	+	+	+	+++	+

A, original sugar, B, after hydrogenation with NaBH4; +, 1 mol; ++, 2 mol; +++, 3 mol; ++++, 4 mol.

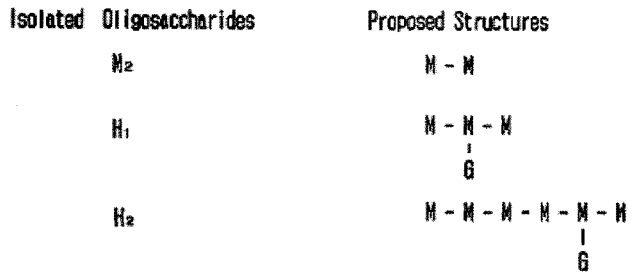

Fig. 3. The proposed structure of oligosaccharides isolated from the enzymatic hydrolysates of copra galactomannan. G, D-galactopyranose; M, D-mannopyranose; M-M, β-1,4-mannosidic linkage; G, α-1,6-galactosidic linkage.

(Table 1) of the sugars and the results of the methylation analyses (Table 2) support this interpretation. In addition, the mannosidic linkages and the galactosyl branches of the two galactomanno-oligosaccharides obtained in the present study are thought to have β- and α-configurations, respectively [Kusakabe *et al.*, 1977]. All mannosyl and galactosyl linkages in the brown copra meal galactomannan are known to have these configurations. Moreover, the copra galactomannan was hydrolyzed into galactose, mannose, and mannobiose [Kusakabe *et al.*, 1986].

Kusakabe *et al.* [1986] proposed a structure of the brown copra meal galactomannan, where galactomannan gives galactose and mannose at 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 a single α-D-galctosyl branch is 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 fundamental chemical structure of galactomannan bears a close resemblance to the structure proposed herein. However, Park and Chang [1992] proposed that three brachings of galactose, at some parts of the galactomannan chain, are contiguous with each other. The isolation of DP 7 galactosylmanno-oligosaccharide is an evidence for this interpretation. In the present study, oligosaccharide having three branches in its structure was not identified. Thus, the specificity of the mannanase to the galactomannan is propose as follows: firstly, the enzyme does not cleave the D-galactosyl branching from the galactomannan; secondly, the enzyme has a specificity to the D-mannosyl residues of the main-chain devoid of an α-galactosyl branching, thereby forming 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 the α-galactosyl branches.

The present study determined the structures of three galactosylmanno-oligosaccharides by the methylation method and the sequential actions of the purified mannanse, mannosidase, and galactosidase (data not shown), as well as the position of galactose. Two oligosaccharides were identified as Gal^2Man_3 (6^2-mono-*O*-α-D-galactopyranosyl-4-*O*-β-D-mannotriose), and Gal^2Man_6 (6^2-mono-*O*-α-D-galactopyranosyl-4-*O*-β-D- mannohexaose), where Gal- and Man- represent α-1,6-D-galactosidic and β-1,4-mannosidic linkages, respectively.

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