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RESEARCH NOTE

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Specificity of β-Mannanase from *Trichoderma* sp. for *Amorphophallus konjac* Glucomannan

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Abstract Five oligosaccharides were isolated from the hydrolysate of *konjac* (*Amorphophallus konjac*) glucomannan by a purified β-mannanase from *Trichoderma* sp. These oligosaccharides were identified as M-M, G-M, M-G-M, M-G-M-M, and M-G-G-M; where G- and M- represent β-1,4-D-glucopyranosidic and β-1,4-D-mannopyranosidic linkages, respectively. The mode of action of the mannanase on the glucomannan is discussed on the basis of the structure of the above oligosaccharides.

Keywords: *konjac* glucomannan, *Trichoderma* sp., β-mannanase

Introduction

In previous papers, the preparation (1) of β-1,4-mannobiose from white copra meal by a β-mannanase from *Penicillium purpurogenum* No. 618 and some properties (2) of the purified mannanase have been reported. In addition, crystalline β-1,4-mannotriose has been prepared from poonac using an enzyme system and yeast fermentation (3). There are many reports dealing with β-mannanase from various microorganisms, (4) but only three of these enzymes, from *konjac* tubers (5), *Tyromyces sp.* (6) and *Streptomyces* sp. (7), have been studied for the specificity of the enzyme toward glucomannan.

The objectives of this investigation are to isolate the oligosaccharides from the hydrolysate of *konjac* (*Amorphophallus konjac*) glucomannan by a purified extracellular β-mannanase from *Trichoderma* sp. and to study the structure of the isolated oligosaccharides, simultaneously commenting on the specificity of the β-mannanase for glucomannan based on the structure of the oligosaccharides.

Materials and Methods

***Konjac* glucomannan and β-1,4-mannooligosaccharides** These were a gift from Tsuruta Shokuhin Kôgyô Co., Ltd. (Gunma-ken, Japan). The ratio of mannose to glucose in the glucomannan was 1.0:0.6. The manno-oligosaccharides were prepared by previously described methods (8).

β-Mannanase Purified β-mannanase from *Trichoderma* sp. was used for the hydrolysis of *konjac* glucomannan. The enzyme preparation was homogeneous according to polyacrylamide gel electrophoresis analysis and had a specific activity of 22.8 units/mg protein. In addition, the enzyme did not hydrolyze cellulose powder or cellobiose.

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β-Mannosidase Purified β-mannosidase from *Aspergillus niger* 5-16 was used to establish the structures of the oligosaccharides. The enzyme hydrolyzed *p*-nitrophenyl-β-D-mannoside and β-1,4-mannobiose, but not cellobiose.

Thin-layer and paper chromatography Thin-layer chromatography (TLC) was performed on a Merck TLC plate (200×200 mm) with DC-Fertigplatten Cellulose in a solvent system of 1-butanol-pyridine-water (6:4:3, v/v); sugars on the plate were detected by heating at 130-140°C for a few minutes after spraying with *p*-anisidine hydrochloride. Paper chromatography was done on Tôyô-Roshi No. 526 filter paper for preparative chromatography by the method described above.

Identification of component sugars Oligosaccharides were hydrolyzed in 10% trifluoroacetic acid (in an ampoule) by heating at 100°C for 2 hr. 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 (9, 15) on a 3% ECNSS-M column.

Hydrogenation of saccharides Saccharides were hydrogenated into the corresponding sugar alcohols by treating an aqueous solution of the sugars with 40 mg of sodium borohydride for 2 hr at room temperature. The resultant sugar solution was treated with Amberlite IR-200c(H^+) to decompose the excess sodium borohydride and to remove the resin. The residue was then evaporated in the presence of methanol to remove boric acid.

Methylation analysis Samples of the parent oligosaccharides or the oligosaccharides obtained after hydrogenation with sodium borohydride were methylated by the method of Ciucanu and Kerek. (10). The methylated sugars were hydrolyzed under the conditions described above, and their alditol-acetate derivatives were analyzed by gas-liquid chromatography (9).

Results and Discussion

Enzymatic hydrolysis of *konjac* glucomannan Approxi-

mately 500 mL of the purified mannanase solution (total activity: 998 units) was added to 15 g (12.5 g as anhydro-glucomannose) of the glucomannan. The enzyme reaction was performed at 60°C and pH 4.5 in a glass vessel with agitation. After 24 hr, the reaction mixture was withdrawn from the vessel and heated in a boiling water bath for 5 min to inactive the enzyme. Insoluble materials were then removed from the resultant hydrolysate by centrifugation, and the supernatant liquid was chromatograghed by TLC in order to characterize the hydrolysis products (Fig. 1). At the final stage of the reaction, the chromatogram had four spots. Glucose was not observed at any time during the entire course of the hydrolysis.

Separation of oligosaccharides After 24 hr of reaction time, the enzymatic hydrolysate was heated in a boiling water bath for 10 min, and then centrifuged to remove the small amounts of insoluble materials from the hydrolysate. The supernatant solution, containing 12.8 g of total sugar content, was put on a granular-charcoal column (70×350 mm, 250 g of activated charcoal for chromatography; Wakô Pure Chemical Ind., Tokyo, Japan). The column was then washed with 3 L of water to remove mannose and salts. The oligosaccharides in the column were eluted by a linear gradient of 13 L of water in a mixing bucket and 13 L of 30% aqueous ethanol at a flow rate of 300 mL per hr. The eluent was fractionated into 400 mL fractions using a preparative fraction collector. The sugar composition of each fraction was examined by TLC, and the fractions containing chromatographically identical sugars were combined.

***M*$_2$:** This sugar had the same Rf value as β-1,4-mannobiose on TLC. Fractions 18 to 22 from the charcoal column chromatography were combined and concentrated to a syrup with a concentration of approximately 80% ethanol; upon cooling, crystallization occurred. The resultant crystals were separated by centrifugal filtration from the mother liquor and dried in a vacuum desiccator over silica gel. Thus, 791 mg of crystalline M_2 was obtained.

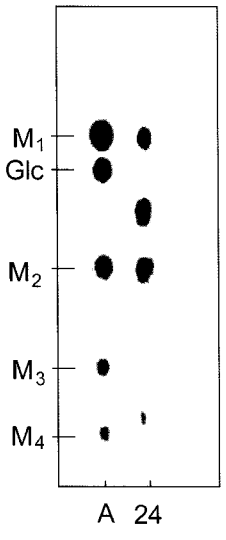

Fig. 1. TLC of the enzymatic hydrolysis of *konjac* glucomannan. A: authentic mannose, glucose, mannobiose, mannotriose, and mannotetraose from top to bottom; 24: hydrolysate after 24-hr reaction time.

***F*$_1$:** This sugar was located between mannose and mannobiose on TLC. Fractions 26 to 36 were combined and concentrated to a syrup. The sugar in the syrup was crystallized by the method described above, and 3128 mg of crystalline F_1 was obtained.

***F*$_{2\text{-}1}$, *F*$_{2\text{-}2}$, *and F*$_{2\text{-}3}$:** Fractions 59 to 68 (F_2 fraction) were combined and concentrated; the concentrate was then used in preparative paper chromatography on No. 526 filter paper sheets. After development with the solvent system described in the Material and Methods section, the sugars in the F_2 fraction were separated into three oligosaccharides, tentatively named $F_{2\text{-}1}$, $F_{2\text{-}2}$, and $F_{2\text{-}3}$ according to the fraction and their corresponding Rf values.

Chromatographically identical sugar zones were extracted from the filter paper with water, and each extract was decolorized with activated charcoal and deionized with cation and anion exchange resins. The purified sugar solution thus obtained was concentrated to an approximately 1% sugar solution. $F_{2\text{-}1}$, $F_{2\text{-}2}$, and $F_{2\text{-}3}$ contained 90, 65, and 89 mg of sugar, respectively, measuring the sugars by the phenol-sulfuric acid method (11).

Figure 2 summarizes the procedure and yield for obtaining oligosaccharides from the enzymatic hydrolysate of *konjac* glucomannan, and Fig. 3 shows the TLC of the oligosaccharides.

Characterization of oligosaccharides Table 1 summarizes the results of the methylation analyses of the oligosaccharides and their hydrogenated derivatives, including reference samples.

***M*$_2$:** The position of M_2 on TLC (Fig. 3) was the same as that of β-1,4-mannobiose. Complete hydrolysis of M_2 yielded only mannose. The $[\alpha]^{20}_D$ of M_2 was -7°. Based on the preceding results, M_2 is proposed to have the structure of β-1,4-mannobiose. Further evidence supporting this structure was also obtained from the methylation analysis (Table 1).

***F*$_1$:** This sugar was composed of glucose and mannose in the molar ratio of 1:1.03. The $[\alpha]^{20}_D$ value of the sugar was +5°. Based on the methylation analysis (Table 1), the proposed structure for F_1 is 4-*O*-β-D-glucopyranosyl-D-mannopyranose (epicellobiose).

***F*$_{2\text{-}1}$, *F*$_{2\text{-}2}$, *and F*$_{2\text{-}3}$:** $F_{2\text{-}1}$, $F_{2\text{-}2}$, and $F_{2\text{-}3}$ were composed of glucose and mannose in the following molar ratios: 1:1.9, 1:2.8, and 1:1.1, respectively. From the results of the methylation analyses (Table 1), these three isolated oligosaccharides were proposed to have the following structures: $F_{2\text{-}1}$ is *O*-D-mannopyranosyl-(1 → 4)-*O*-D-mannopyranose, while the structure of $F_{2\text{-}2}$ is either *O*-D-mannopyranosyl-(1 → 4)-*O*-D-glucopy-ranosyl-(14)-*O*-D-mannopyranosyl-(1 → 4)-D-manno-pyranose or *O*-D-mannopyranosyl-(1 → 4)-*O*-D-manno-pyranosyl-(14)-*O*-D-glucopyranosyl-(1 → 4)-D-manno-pyranose. However, β-manno-sidase hydrolyzed $F_{2\text{-}2}$ to produce mannose and 4-*O*-β-D-glucopyranosyl-mannobiose. Therefore, $F_{2\text{-}2}$ is proposed to have the former structure rather than the latter. $F_{2\text{-}3}$ is *O*-D-mannopyranosyl-(1→4)-*O*-D-glucopyranosyl-(1 →4)-*O*-D-gluco-pyranosyl-(1 →4)-D-mannopyranose. Moreover, the glycosidic linkages on all three oligo-saccharides are presumed to have a β-configuration, because all glucose

Table 1. Methylation analysis of the oligosaccharides and their hydrogenated derivatives isolated from the enzymatic hydrolysate of *konjac* glucomannan[1)]

Alditol acetate of Retention time (min)		2,3,4,6-O-Me-Glc 155°C 5.2 140°C 11.4	2,3,6-O-Me-Glc 14.0 34.6	2,3,4,6-O-Me-Man 5.2 11.9	2,3,6-O-Me-Man 12.4 30.0	1,2,3,5,6-O-Me-Mannitol 1.7 3.4	1,2,3,5,6-O-Me-Glucitol 2.0 3.9
Oligosaccharides			+				+
Cellobiose	A	+					
	B	+					
Mannobiose	A			+	+	+	
	B			+			
M_2	A			+	+	+	
	B			+			
F_1	A	+			+	+	
	B	+					
F_{2-1}	A		+	+	+	+	
	B		+	+			
F_{2-2}	A		+	+	++	+	
	B		+	+	+		
F_{2-3}	A		++	+	+	+	
	B		++	+			

[1)]A: precursor, B: after hydrogenation, Me: methyl, Glc: D-glucopyranose, Man: D-mannopyranose, +:1 mol, ++: 2 mol.

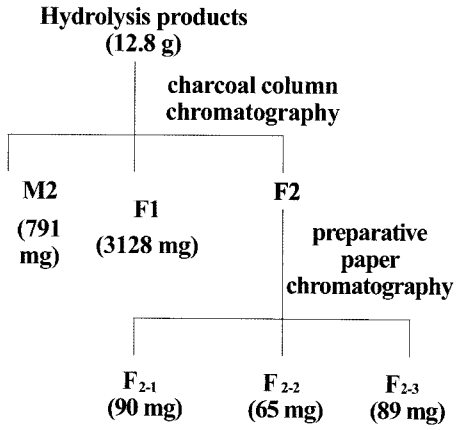

Fig. 2. Procedure and yield for obtaining oligosaccharides from enzymatic hydrolysate of *konjac* glucomannan.

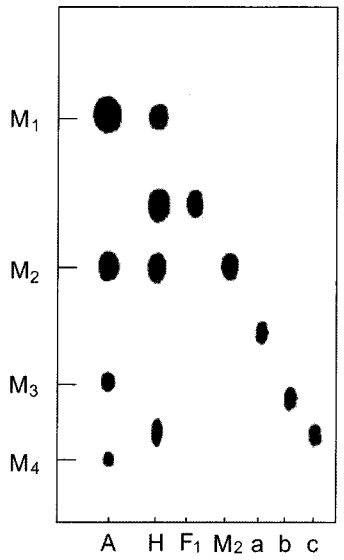

Fig. 3. TLC of oligosaccharides isolated from the enzymatic hydrolysate of *konjac* glucomannan. A: authentic mannose, mannobiose, mannotriose, and mannotetraose from top to bottom; H: enzymatic hydrolysate of konjac glucomannan; a: F_{2-1}; b: F_{2-2}; c: F_{2-3}.

and mannose residues in konjac glucomannan are known to have this configuration (5, 12, 13). The structures of the oligosaccharides obtained in this study are depicted in Fig. 4.

In summary, five types of oligosaccharides (Fig. 4) were isolated from the hydrolysate of *konjac* glucomannan by a purified β-mannanase from *Trichoderma* sp. The oligosaccharides are believed to have been produced by the enzyme as follows.

i) The enzyme produced M_2 (mannobiose). It is suggested that β-1,4-linked β-D-mannopyranose residues in the glucomannan were hydrolyzed at the beginning of the reaction, and the resultant mannooligosaccharides were further hydrolyzed to produce mannobiose. The enzyme β-mannanase is capable of hydrolyzing both mannotriose and mannotetraose to mannose and mannobiose, but can not hydrolyze mannobiose further (2).

ii) The enzyme produced glucomanno-oligosaccharides having a glucose residue at the non-reducing terminal. Moreover, the enzyme did not produce free glucose and cellobiose. These results substantiate the observation that the enzyme does not have either cellulase or β-glucosidase activity.

iii) The enzyme produced glucomanno-oligosaccharides having a mannose residue at the non-reducing terminal. This confirms that the enzyme does not have β-mannosidase activity.

Saccharides	Structures
M_2	M-M
F_1	G-M
F_{2-1}	M-G-M
F_{2-2}	M-G-M-M
F_{2-3}	M-G-G-M

Fig. 4. The proposed structure of oligosaccharides isolated from the enzymatic hydrolysate of *konjac* glucomannan. G-, β-1,4-D-glucopyranosidic linkage; M-, β-1,4-D-mannopyranosidic linkage.

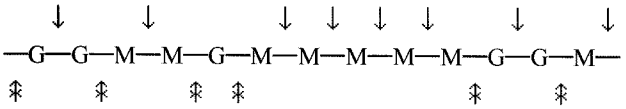

Fig. 5. Partial structure of *konjac* glucomannan (14) and possible action of a mannanase from *Trichoderma* sp. G-: β-1,4-D-glucopyranosidic linkage; M-: β-1,4-D-mannopyranosidic linkage. ↓ and ⇞ indicate the mannanase hydrolyzable and non-hydrolyzable linkages, respectively.

Based on the five oligosaccharides isolated in this study, the proposed specificity of the mannanase for *konjac* glucomannan is shown in Fig. 5.

Table 2 compares the degradation products that arise from the action of other mannanases on glucomannan. It seems that these four enzymes have many points of similarity to each other with regard to substrate specificity. However, there are slight differences between three of these enzymes (from *konjac* tubers, *Trichoderma*, and *Tyromyces*) and that from *Streptomyces*, in that the former produced glucomanno-oligosaccharides in which glucose or cellobiose are interposed between two mannose residues.

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Table 2. Oligosaccharides isolated from enzymatic digestion of glucomannan

Enzyme substrate	*Konjac* tubers(5) (crude enzyme) *konjac* glucomannan	*Trichoderma* sp. *konjac* glucomannan	*Tyromyces palustris*(6) *larch* glucomannan	*Streptomyces* sp.(7) *konjac* glucomannan
Oligosaccharide	M-M M-M-M M-M-M-M M-M-M-M-M G-M G-M-M G-M-M-M G-M-M-M-M M-G-M G-G G-G-M G-G-M-M G-M-M-G	M-M G-M M-G-M M-G-M-M M-G-G-M	M-M M-M-M M-M-M-M G-M G-M-M G-M-M-M M-G-M G-G	M-M M-M-M M-M-M-M G-M G-M-M G-G-M G-G-M-M