Structure Elucidation of Major Galacto Oligosaccharides Formed by Growing Culture of *Trichoderma harzianum*

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A *Trichoderma harzianum* strain isolated from soil showed significantly high transgalactosylation activity during growth in a lactose-containing medium. In a 30% ethanol fraction obtained on an activated charcoal column, other than disaccharides, nine oligosaccharides comprised of about 81% trisaccharide, 16% tetrasaccharide, and 3% pentasaccharide were detected on a paper chromatogram. Structural characterization by chemical, methylation, and ^{13}C NMR spectral analysis revealed the four trisaccharides as *O*-β-D-galactopyranosyl-(1→4)-*O*-[β-D-galactopyranosyl-(1→6)]-D-glucose, *O*-β-D-galactopyranosyl-(1→6)-β-D-galactopyranosyl-(1→4)-D-glucose, *O*-β-D-galactopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-D-glucose, and *O*-β-D-galactopyranosyl-(1→4)-β-D-galactopyranosyl-(1→4)-D-glucose. The lone tetrasaccharide structurally determined was *O*-β-D-galactopyranosyl-(1→6)-β-D-galactopyranosyl-(1→6)-β-D-galactopyranosyl-(1→4)-D-glucose.

Galacto oligosaccharides are often reported in the hydrolysate and in fermented milk products during enzymatic hydrolysis of lactose by β-galactosidase (Huber et al., 1976; Asp et al., 1980; Toba et al., 1982, 1985, 1986). Such oligosaccharides are formed by the action of β-galactosidase, known as transgalactosylation. Functionally, certain oligosaccharides including those present in human milk play an important role as the bifidus factor in maintaining the normal intestinal microflora of infants. In an attempt to produce bifidus factor oligosaccharides in large amounts screening experiments showed that a soil isolate of *Trichoderma harzianum* could produce more than 30% oligosaccharides in high-lactose growth medium (Sai Prakash et al., 1987). In this study the structures of five major oligosaccharides formed in the medium during growth of *T. harzianum* are elucidated.

MATERIALS AND METHODS

Reagents. All reagents (AR) used were obtained from either WAKO Pure Chemicals or E. Merck. Allolactose [β-D-galactopyranosyl-(1→6)-D-glucose] and galactobiose [β-D-galactopyranosyl-(1→6)-D-galactose] were isolated in this laboratory (Toba and Adachi, 1978).

Culture Growth Conditions. The *T. harzianum* strain was isolated from the soil (Sai Prakash et al., 1987) and maintained on agar slants of growth medium containing 2% lactose.

The growth medium had the following composition (g/L): lactose, 150; cottage cheese whey (spray dried), 5; KH_2PO_4, 1; NH_4NO_3, 2.5; $MgSO_4 \cdot 7H_2O$, 0.2; $FeCl_3 \cdot 6H_2O$, 0.02; $CaCl_2$, 0.01; NaCl, 0.01. The pH of the medium was adjusted to 7. Culture inoculum on agar slant was transferred to 500-mL flasks containing 150 mL of medium and incubated aerobically at 30 °C in a shaker (100 rpm) for 2 weeks.

Separation of Oligosaccharides. The culture was centrifuged at 10000 rpm, filtered through a membrane filter (0.03 μm), and concentrated in a rotary evaporator at 40 °C to obtain the crude sample of oligosaccharides. Fractionation was carried out on an activated charcoal column (5.3 × 40 cm) by successive elution with 4 L of water and 2 and 30% ethanol. Elution with water was stopped when disaccharide was almost absent, which was tested by thin-layer chromatography using precoated silica gel G plates (Art. 5553, E. Merck) in an ethyl acetate–acetic acid–water (2:1:1, v/v/v) solvent system. Subsequently, elution with 30% ethanol was carried out when trisaccharide appeared in 2% ethanol eluate. Preparative paper chromatography of the 30% ethanol fraction containing oligosaccharides higher than disaccharide was carried out in a 1-butanol–pyridine–water (6:4:3, v/v/v) system for 10 ascents. Each oligosaccharide from paper strips was extracted in water and demineralized on Amberlite IR-120B (H^+) and Amberlite IRA-45 (OH^-) columns.

The molar proportion of oligosaccharides was determined by the phenol–sulfuric acid method (Dubois et al., 1956) using lactose as standard reducing sugar.

Chemical Analysis. The identification and quantification of constituent sugars were done after complete hydrolysis of oligosaccharide (1 mg) with 2 N trifluoroacetic acid (1 mL) at 105 °C for 8 h. The hydrolyzed products were analyzed by gas–liquid chromatography as trimethylsilyl (TMS) derivatives on a 2% Dexsil 300 GC (500 × 3 mm) column programmed at 150–350 °C, 10 °C/min in a Hitachi 163 gas chromatograph. The carrier gas was N_2 at a flow rate of 40 mL/min, and the injector/detector temperature was set at 250 °C.

FAB Mass Spectrometry. Positive-ion fast atom bombardment mass spectra were recorded in a JMS DX 300 mass spectrometer (JEOL, Tokyo, Japan). The sample and stainless steel probe tip were introduced into the FAB source and bombarded by a 3-keV argon atom beam. The oligosaccharide sample (~5 mg) was dissolved in about 5% sodium acetate and loaded on a glycerol-coated probe tip.

^{13}C NMR Analysis. Proton-decoupled ^{13}C NMR spectra were obtained at 25 MHz with a JEOL FX 100 operated in the pulsed Fourier transform mode at 30 °C. D_2O was used as solvent, and the chemical shifts obtained were referenced to an external standard of 2,2,3,3-tetradeuterio-4,4-dimethyl-4-silapentanoate (TSP-d_4). The spectrum of each oligosaccharide was evaluated as suggested by Bradbury and Jenkins (1984) and compared with the standard disaccharides, viz. lactose, allolactose, and galactobiose.

Methylation Analysis. The methylation was carried out by the method of Hakomori (1964). The acetylated alditol of partially methylated oligosaccharide was prepared (Toba et al., 1985), and the alditol acetates were

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0021-8561/89/1437-0334$01.50/0

Table I. Molar Proportion of Oligosaccharides in 30% Ethanol Fraction in Activated Charcoal Column Chromatography[a]

trisaccharide	%	tetrasaccharide	%	pentasaccharide	%
TR 1	23.2	TE 1	7.7	P 1	1.9
TR 2	49.9	TE 2	5.3	P 2	1.4
TR 3	5.5	TE 3	2.6		
TR 4	2.5				

[a] Excluding disaccharides. Trisaccharide:tetrasaccharide:pentasaccharide ≈ 25:5:1.

Table II. Characteristics of Oligosaccharides Formed by *T. harzianum*

oligosaccharide	R_{glc}[a]	molar ratio of Gal	molar ratio of Glc	reducing[b] end unit	$(M + Na)^+$
TR 1	0.3	1.97	1.07	Glc	527
TR 2	0.34	2.00	1.09	Glc	527
TR 3	0.38	2.12	0.97	Glc	527
TR 4	0.41	2.03	0.84	Glc	527
TE 1	0.11	3.23	0.92	Glc	689

[a] For four ascents. [b] Determined by ^{13}C NMR analysis.

injected into a JEOL DX 300 gas chromatograph–mass spectrometer attached with a fused silica capillary column (Supelco. Inc.; column length 60 m; i.d. 0.33 mm; phase SPB-1; carrier gas He; injection temperature 250 °C; column temperature programmed from 150 to 250 °C at 5 °C/min; ionization voltage of EI mode 25 eV).

RESULTS AND DISCUSSION

Paper chromatography of the 30% ethanol fraction eluted from activated charcoal column revealed nine oligosaccharides (Figure 1). These included four trisaccharides (TR), three tetrasaccharides (TE), and two pentasaccharides (P), numbered in increasing order of mobility. The observed R_{glc} (mobility relative to glucose) values for oligosaccharides ranged from 0.06 to 0.41 after four ascents of 48 h each. The data on the quantitative determination of oligosaccharides are given in Table I. The trisaccharide concentration was higher than that of tetra- and pentasaccharides. As the medium contained 15% lactose and a small amount of monosaccharide, the formation of a large amount of trisaccharide is attributed to the abundance of disaccharide available as an acceptor for transgalactosylation. Among four trisaccharides isolated, TR 2 constituted about 60%, the highest in total oligosaccharides. TR 1 was also detected in substantial quantity (23.2%).

The results of the chemical analyses of four tri- and one tetrasaccharide isolated from the growth medium of *T. harzianum* are shown in Table II. The gas–liquid chromatography of hydrolysates of oligosaccharides showed that each one contained one glucose residue and the remaining monosaccharide units were galactose. From the ^{13}C NMR analysis, the presence of two chemical shifts (~94 and ~98 ppm, respectively) in the anomeric region corresponding to C1α and C1β of glucose suggested the location of glucose at the reducing end in all oligosaccharides. The number of constituent monosaccharides was determined by FAB mass spectrometry. The quasi-molecular ions $(M + Na)^+$ of oligosaccharides were detected at m/z 527 and 689, which could account for tri- and tetrasaccharide, respectively.

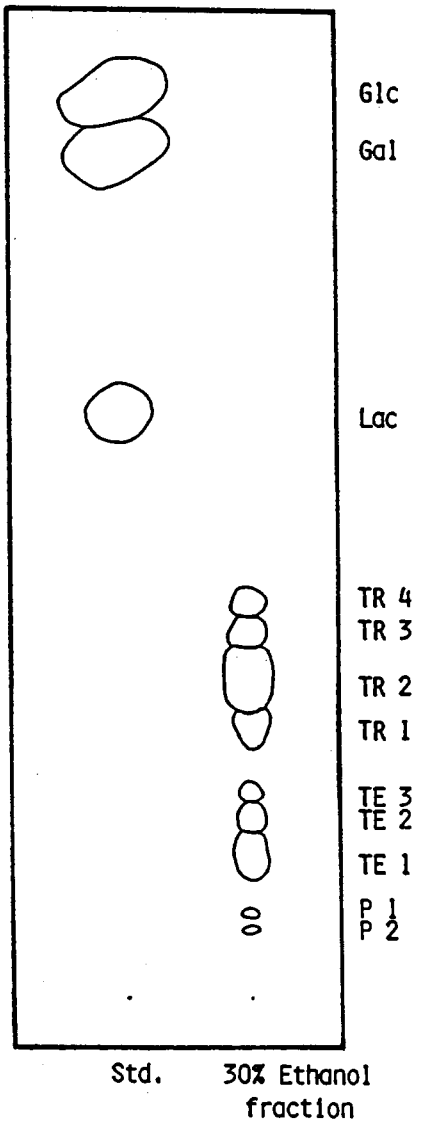

Figure 1. Paper chromatogram of oligosaccharides formed by *T. harzianum*.

The oligosaccharides as partially methylated alditol acetates were analyzed by GLC/MS. The results are recorded in Table III. Except for TR 1, all oligosaccharides showed the presence of 4-*O*-acetyl-1,2,3,5,6-penta-*O*-methylglucitol. In TR 1 the presence of 4,6-di-*O*-acetyl-1,2,3,5-tetra-*O*-methylglucitol and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol in the ratio of 1:2 suggests that the reducing-end glucose is linked with two nonreducing galactose units. The TR 2 is a trisaccharide containing a central galactose residue substituted at C6. Similarly in TE 1 the proportion of 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-

Table III. *O*-Acetyl-*O*-methylalditols of Oligosaccharides Formed by *T. harzianum*

peracetyl derivative	ratio of peak integrals obtained in GLC TR 1	TR 2	TR 3	TR 4	TE 1	prominent peaks
4-Ac-1,2,3,5,6-Me_5Glc		1.12	0.81	1.19	1.00	205, 173, 134, 131, 129, 113, 101 (base), 89, 59, 45, 43
4,6-Ac_2-1,2,3,5-Me_4Glc	0.9					233, 173, 133, 131, 129, 117, 102, 99, 87, 85, 57, 43 (base)
1,5-Ac_2-2,3,4,6-Me_4Gal	2.19	1.00	1.13	1.30	1.01	205, 163, 161, 145, 129, 118, 102, 101, 89, 88, 87, 85, 75, 71, 43 (base)
1,5,6-Ac_3-2,3,4-Me_3Gal		1.03			1.83	234, 233, 190, 173, 162, 161, 131, 129, 118, 113, 102, 99, 87, 75, 71, 59, 43 (base)
1,3,5-Ac_3-2,4,6-Me_3Gal			0.97			234, 202, 161, 129, 118, 102, 88, 71, 59, 45, 43 (base)
1,4,5-Ac_3-2,3,6-Me_3Gal				0.80		233, 173, 162, 142, 134, 131, 129, 118 (base), 113, 102, 99, 87, 75, 71, 57, 45, 43

Table IV. ^{13}C NMR Chemical Shifts for Oligosaccharides Isolated from the Growth Medium of *T. harzianum*[a]

		C_1	C_2	C_3	C_4	C_5	C_6
TR 2	β-Gal-(1→6)-	106.1	73.7	75.5	71.3	77.9	63.8
	β-Gal-(1→4)-	105.9	74.4	75.3	71.4	76.6	71.8
	Glc α	94.7	74.4	73.9	82.0	72.8	63.0
	Glc β	98.5	76.8	77.3	82.3	77.5	63.0
TR 3	β-Gal-(1→3)-	106.9	74.0	75.2	71.2	77.6	63.6
	β-Gal-(1→4)-	105.2	72.8	84.5	71.0	77.6	63.6
	Glc α	94.4	74.0	73.7	80.9	72.8	62.8
	Glc β	98.4	76.5	77.0	80.9	77.4	62.8
TR 4	β-Gal-(1→4)-	106.8	74.1	75.5	71.3	77.8	63.7
	β-Gal-(1→4)-	105.5	75.2	75.6	79.8	77.0	63.4
	Glc α	94.5	74.1	73.8	81.1	72.8	62.7
	Glc β	98.4	76.5	77.1	81.1	77.4	62.7
TE 1	β-Gal-(1→6)-	106.1	73.6	75.4	71.4	78.1	63.8
	β-Gal-(1→6)-	105.8	73.5	75.3	71.4	77.3	71.9
	β-Gal-(1→4)-	105.8	73.5	75.3	71.4	76.6	71.9
	Glc α	94.6	72.8	73.5	81.9	74.2	63.0
	Glc β	98.5	76.6	77.5	82.1	77.9	63.0

[a] Key: Gal = D-galactose; Glc = D-glucose.

Table V. Structure of Oligosaccharides Formed by *T. harzianum*

TR 1	β-D-Gal-(1→6) D-Glc β-D-Gal-(1→4)
TR 2	β-D-Gal-(1→6)-β-D-Gal-(1→4)-D-Glc
TR 3	β-D-Gal-(1→3)-β-D-Gal-(1→4)-D-Glc
TR 4	β-D-Gal-(1→4)-β-D-Gal-(1→4)-D-Glc
TE 1	β-D-Gal-(1→6)-β-D-Gal-(1→6)-β-D-Gal-(1→4)-D-Glc

methylgalactitol was 2 times than in TR 2, indicating that both central galactose residues are substituted at position 6. Galactose forms (1→3) and (1→4) linkages with reducing-end lactose in the linear structures of TR 3 and TR 4, respectively.

The anomeric configuration and linkage positions were conformed by ^{13}C NMR spectral analysis (Table IV; Figure 2). Glucose is located at the reducing end in all oligosaccharides. The only existence of β-configuration for the galactose anomeric carbon was decided by the presence of a chemical shift at 105–106 ppm. In TR 3, C1 of central galactose was assigned the signal at 105.2 ppm, which is 1.7 ppm upfield as compared to the nonreducing terminal galactose and is attributed to the involvement of C3 in linkage (Collins et al., 1981). For TR 1, the signal corresponding to free C6 of glucose was absent (Figure 2) whereas the intensity of glucose C6 resonance shift relative to C1 of glucose was 128, 157, 154, and 161% for TR 2, TR 3, TR 4, and TE 1, respectively. This could be explained by assignment of 69.8 ppm to glucose C6, suggesting that TR 1 is a digalactosylglucose. The chemical shift of 79.8 ppm for TR 4 indicates that (1→4) galactosidic linkage occur in the nonreducing-end portion.

Trichoderma is known for high cellulolytic activity and is used for the production of many glycosidic enzymes (e.g., glucosidase, xylanase, and CMCase) that are important in cellulose degradation (Mandels et al., 1971; Kyriacou et al., 1987). The structures of five oligosaccharides (Table V) isolated from growth medium of *T. harzianum* are established. The five galacto oligosaccharides structurally determined in this study have been reported in various lactose-β-galactosidase systems (Ballio and Russi, 1960; Gorin et al., 1964; Asp et al., 1980; Toba et al., 1985). In this study, for the first time, high β-galactosidase activity, especially transgalactosylation, has been detected in *T. harzianum*. In total oligosaccharides, β(1→6) linkage was about 90%, and the order of linkage formation was β(1→6) > β(1→3) > β(1→4) although oligosaccharides with the

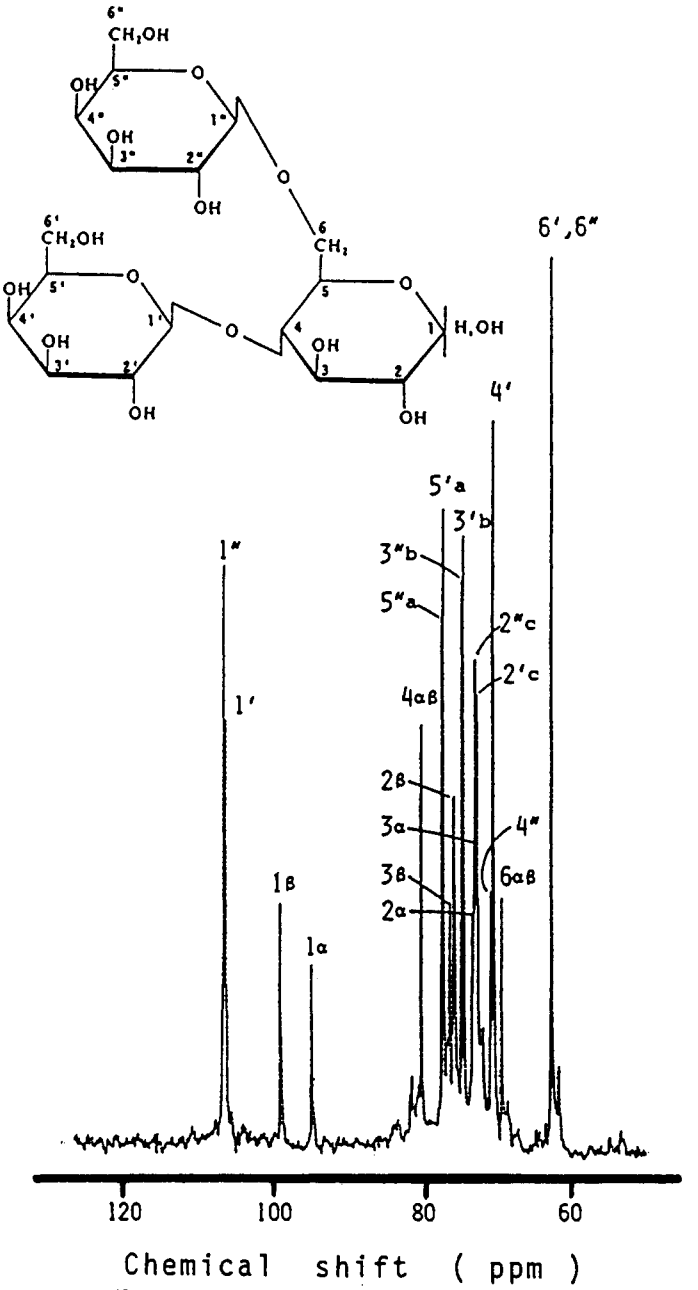

Figure 2. ^{13}C NMR spectra and chemical shift assignments of TR 2. (a–c) Chemical shifts may be reversed.

latter two types of linkages are produced in small quantity. Importantly, high production of *O*-β-D-Gal-(1→6)-β-D-Gal-(1→4)-D-Glc is of significant interest as it occurs in human milk (Yamashita and Kobata, 1974) and is an effective growth promoter for many of the *Bifidobacterium* strains in vivo (Tanaka et al., 1983).

ACKNOWLEDGMENT

We thank Dr. S. Hara, National Institute of Animal Industry, Japan, for identification of mold culture and to A. Yamaji for technical assistance in the microbiological experiment. B.S.P. acknowledges the award of a scholarship by the Ministry of Education, Science and Culture, Japan, during the study.

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Received for review July 20, 1987. Revised manuscript received July 13, 1988. Accepted October 5, 1988.

Organic Acids and Calcium Oxalate in Tropical Root Crops

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Oxalate, malate, citrate, and succinate contents of tropical root crops were determined by HPLC. Water extraction gave soluble oxalates, and extraction with acid gave total oxalates. The difference between them equaled the amount of calcium oxalate. Total calcium was determined by atomic absorption, and free calcium (calcium not present as calcium oxalate) was readily calculated. Taro (*Colocasia esculenta*) leaves, from edible and nonedible cultivars (because of their acrid nature), showed no differences in their amounts of total oxalate or of calcium oxalate. This showed that acridity of taro leaves was not due solely to calcium oxalate raphides present. Stems of giant swamp taro (*Cyrtosperma chamissonis*), elephant foot yam (*Amorphophallus campanulatus*), skin of giant taro (*Alocasia macrorrhiza*), and taro leaves contained about 400 mg/100 g fresh weight of calcium oxalate, about 10 times the amount present in sweet potato, cassava, taro *Colocasia* and *Xanthosoma*, and yam. The free calcium content was 0–20 mg/100 g fresh weight and would be adequate for all root crops, except taro *Xanthosoma*.

Oxalate is widely distributed in plants in a readily water-soluble form as potassium, sodium, and ammonium oxalate and as insoluble calcium oxalate (Fassett, 1973; Connor, 1977; Smith, 1982). Since calcium may also occur in plants other than as insoluble calcium oxalate crystals, the mole ratio of oxalate to calcium is found to vary from 7 to <1 (Fassett, 1973). In tropical root crops and particularly in the aroids, viz. taro *Colocasia*, taro *Xanthosoma*, giant taro, giant swamp taro, and elephant foot yam, calcium oxalate is present as fine needlelike crystals or raphides (Sakai and Hanson, 1974; Sunell and Healey, 1979; Nixon, 1987; Bradbury and Holloway, 1988). The occurrence of these crystals has been considered as either the cause or a contributing cause of the acridity of some species of taro, giant taro, and giant swamp taro (Sakai and Hanson, 1974; Tang and Sakai, 1983), which causes irritation to the skin and swelling of the mouth and throat.

The two main toxic effects of oxalate poisoning are (1) acute poisoning, resulting in hypocalcaemia after ingestion of high levels of soluble oxalates, and (2) (more commonly) chronic poisoning in which calcium oxalate crystals are deposited in the kidneys, resulting in renal damage (Connor, 1977). The presence of oxalate in foods has also been implicated in reducing the bioavailability of essential minerals such as calcium (Kelsay, 1985). We have developed a method for the determination of calcium oxalate as well as water-soluble oxalate and other organic acids. The methods were applied to the tropical root crops of the South Pacific.

MATERIALS AND METHODS

Root crops were harvested in Papua New Guinea (PNG), Solomon Islands, Fiji, Western Samoa, Kiribati, Tonga, and Ponape State of the Federated States of Micronesia and cleaned, and the fresh weight was recorded. They were sent by air freight to Canberra and were stored at 15 °C

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0021-8561/89/1437-0337$01.50/0