Carbohydrate Research, 137 (1985) 173–181
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

A GALACTOGLUCOMANNAN FROM CELL WALLS OF SUSPENSION-CULTURED TOBACCO (*Nicotiana tabacum*) CELLS

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(Received July 3rd, 1984; accepted for publication, August 29th, 1984)

ABSTRACT

Cell-wall polysaccharides of suspension-cultured cells of *Nicotiana tabacum* were fractionated into EDTA-soluble, 5% and 24% potassium hydroxide-soluble, and α-cellulose components. From the 24% potassium hydroxide-soluble fraction, a polysaccharide composed of D-galactose, D-glucose, and D-mannose in approximately equal proportions was purified by ion-exchange chromatography and barium hydroxide precipitation. Methylation analysis, enzymic hydrolysis, and ^{13}C-n.m.r. studies showed that the polysaccharide was built up of (1→4)-linked, alternating β-D-glucopyranosyl and β-D-mannopyranosyl residues, and that ~83% of the mannosyl residues were substituted at O-6 by α-D-galactopyranosyl or 2-*O*-β-D-galactopyranosyl-α-D-galactopyranosyl side-chains.

INTRODUCTION

Galactoglucomannans are one of the major components of cell walls of woody tissues of gymnosperms and angiosperms[1]. Although Simson and Timell[2] suggested the presence of glucomannan in the primary cell-walls of the cambial tissues of trees, no reports have been published concerning the isolation and the structural characterisation of mannose-containing polysaccharide from the primary cell-walls of suspension-cultured cells.

We have surveyed[3] the kinds of polysaccharides present in the cell walls of suspension-cultured tobacco cells. Methylation analysis of the fractions solubilised from the cell-wall materials (CWM) suggested that the 24% potassium hydroxide-soluble fraction contained a galactoglucomannan (GGM) as a hemicellulosic polysaccharide[4]. We now report the isolation and structural investigation of this GGM.

0008-6215/85/$ 03.30

EXPERIMENTAL

The general experimental methods have been reported previously[4,5].

Materials. — β-D-Galactosidase from *Escherichia coli* was purchased from Boehringer Mannheim (West Germany). Cellulase from *Trichoderma viride* (Meicellase, kindly donated by Meiji Seika Ltd.) was partially purified[6] on a gauze column. Mannobiose [β-D-Man*p*-(1→4)-D-Man], β-D-Glc*p*-(1→4)-D-Man, and cellobiosyl-mannobiose [β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→4)-β-D-Man*p*-(1→4)-D-Man] were gifts from Dr. I. Kusakabe. Sepharose CL-6B and standard dextrans were purchased from Pharmacia Fine Chemicals, Bio-Gel P-2 from Bio-Rad Laboratories, and DEAE-cellulose from Seikagaku Kogyo (Japan).

^{13}C-N.m.r. spectra. — For n.m.r. spectroscopy, a Jeol FX-100 spectrometer was used. The ^{13}C-n.m.r. spectra (25.1 MHz) were obtained for solutions in D_2O (oligosaccharides, 1-mm tube) or 0.2M sodium deuteroxide (polysaccharide, 5-mm tube), with internal methanol (δ 49.9 from the signal for Me_4Si). The $J_{C\text{-}1,H\text{-}1}$ value was determined by a gated, ^1H-decoupler sequence to retain the n.O.e.

Mass spectrometry (m.s.). — Field desorption (f.d.)-m.s. was accomplished with a Hitachi M-80 instrument with data processor M-003. Secondary-ion mass spectrometry (s.i.m.s.) was obtained using $[Xe]^-$ as the primary ions, and glycerol matrix (~0.5 μL) was added to the sample on the silver substrate. The acceleration voltages of the primary and secondary ions were 8 and 3 kV, respectively.

Molecular weight. — A solution of polysaccharide (10 mg) in 0.5M NaCl (1 mL) was applied to a column (1.6 × 80 cm) of Sepharose CL-6B pre-equilibrated with 0.5M NaCl containing 5mM disodium ethylenediaminetetra-acetate (EDTA), followed by elution with the same solvent as above (2.6-mL fractions). The carbohydrate content of each fraction was determined by the phenol–sulfuric acid method[7]. The column was calibrated by using standard dextrans[5].

Isolation of GGM. — CWM isolated from suspension-cultured cells of *Nicotiana tabacum* L., cv. Bright Yellow, was successively treated with 50mM EDTA in 50mM sodium acetate buffer (pH 4.5), and 5% and 24% potassium hydroxide containing 10mM sodium borohydride[3]. The 24% potassium hydroxide-soluble fraction (820 mg) was applied to a column (4.5 × 45 cm) of DEAE-cellulose (AcO^-) which was eluted with water[4]. To a solution of the water-eluted fraction (255 mg) in aqueous 5% sodium hydroxide (200 mL) was added aqueous 5% barium hydroxide (200 mL). The mixture was centrifuged, and a solution of the precipitate in aqueous 5% sodium hydroxide was treated as described above. The material precipitated with barium hydroxide was dissolved in 5% sodium hydroxide, and the solution was neutralised with acetic acid, dialysed against distilled water, and freeze-dried to give a purified polysaccharide (GGM, 125 mg).

Hydrolysis of the polysaccharide with cellulase. — A suspension of GGM (100 mg) in 0.1M sodium acetate buffer (pH 4.5) was incubated with cellulase (20 mg, 7.4 units/mg) for 48 h at 37° with the addition of a few drops of toluene[8]. The mixture was then heated at 100° for 15 min and centrifuged, and the supernatant

solution was treated with Dowex 50W (H^+) resin, concentrated, and freeze-dried (105 mg). A solution of the residue in water (1 mL) was applied to a column (1.6 × 90 cm) of Bio-Gel P-2 and eluted with water. Fractions (2.6 mL) were analysed by the phenol–sulfuric acid method[7]. Some of the oligosaccharide in each fraction was purified by preparative p.c. on Whatman 3MM paper with 6:4:3 1-butanol–pyridine–water. Purities of the oligosaccharides were checked by t.l.c. on silica gel (Merck, 5553) with 3:3:2 1-butanol–ethyl acetate–water[5].

Partial hydrolyses of the oligosaccharides. — *(a) With acid.* Oligosaccharide (~2 mg) was heated with 0.5M trifluoroacetic acid for 1 h at 100°, the acid was evaporated, and the residue was reduced with sodium borohydride and then methylated. The resulting permethylated alditols were subjected to g.l.c. using a glass capillary column (0.28 mm × 50 m) coated with OV-101 at 260°. The retention times of methylated derivatives of disaccharide-alditols were 17.9 (cellobiose), 18.8 (4-*O*-β-D-Glc*p*-D-Man), 19.0 (4-*O*-β-D-Man*p*-D-Glc), and 19.2 (mannobiose), respectively. For the control experiment, cellobiosyl-mannobiose was treated in the same manner as above. G.l.c. revealed the presence of cellobiose, glucosyl-mannose, and mannobiose in the molar ratios 1:1.4:0.7 in the hydrolysate.

(b) With enzyme. Oligosaccharide (~2 mg) in 50 mM sodium acetate buffer (pH 6.0, 1 mL) was incubated with β-D-galactosidase (100 μL, 30 units) for 24 h at 40° with the addition of a few drops of toluene. The mixture was then treated with Dowex 50W (H^+) resin, concentrated, and freeze-dried.

RESULTS AND DISCUSSION

The 24% potassium hydroxide-soluble fraction from the CWM of suspension-cultured tobacco cells was fractionated on a DEAE-cellulose column[4]. One of the major fractions (F-1), which was eluted with water, contained arabinose, xylose, galactose, mannose, and glucose in the molar ratios 7.6:14.0:15.2:17.2:46.0. Zone electrophoresis of F-1 gave two spots, indicating that it contained two kinds of polysaccharide, which were considered to be an arabinoxyloglucan and a galacto-glucomannan (GGM) based on the results of neutral sugar composition and methylation analysis of F-1 compared with those of the neutral fraction of extracellular polysaccharides (ECP) of tobacco cells[9–11]. The two polysaccharides could be separated by precipitation with barium hydroxide, which is generally used for the purification of mannose-containing polysaccharides[12].

The polysaccharide purified by precipitation with barium hydroxide was homogeneous in zone electrophoresis and gel filtration on Sepharose CL-6B. It had $[\alpha]_D^{23}$ +55° (*c* 0.5, 0.1M sodium hydroxide), and gave arabinose, xylose, galactose, mannose, and glucose in the molar ratios 1.3:1.4:31.7:34.1:31.5 on acid hydrolysis. These ratios were not changed by attempted fractionation of this polysaccharide using barium hydroxide. Optical rotation data indicated the galactose, glucose, and mannose to be D. The molecular weight was estimated to be 20,000 by gel filtration on Sepharose CL-6B.

TABLE I

METHYLATION ANALYSIS OF GGM

Methylated sugars[a]	T_1[b]	T_2[b]	*Mole percent*	*Mode of linkage*
2,3,5-Ara	0.74	0.73	1	T-Ara*f*-(1→
2,3,4-Xyl	0.79	0.80	1	T-Xyl*p*-(1→
3,4-Xyl	0.92	1.03	1	→2)-Xyl*p*-(1→
2,3,4,6-Gal	1.00	1.00	19	T-Gal*p*-(1→
3,4,6-Gal	1.14	1.25	7	→2)-Gal*p*-(1→
2,3,6-Glc	1.13	1.27	33	→4)-Glc*p*-(1→
2,3-Glc	1.33	1.67	5	→4,6)-Glc*p*-(1→
2,3,6-Man	1.11	1.21	5	→4)-Man*p*-(1→
2,3-Man	1.31	1.58	24	→4,6)-Man*p*-(1→

[a]2,3,5-Ara = 2,3,5-tri-*O*-methylarabinose, *etc.* [b]Retention time of the derived alditol acetate on an OV-101 column programmed at 2°/min from 150 to 220° (T_1) and on a Silar 10C column programmed at 2°/min from 150 to 220° (T_2), respectively, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol.

The ^{13}C-n.m.r. spectrum of GGM contained four signals for anomeric carbons at 105.3, 103.3, 101.0, and 99.7 p.p.m. with $J_{C\text{-}1,H\text{-}1}$ values of 165, 164, 162, and 172 Hz, respectively. From these and literature data[11,13,14], the four signals were assigned to β-D-Gal*p*, β-D-Glc*p*, β-D-Man*p*, and α-D-Gal*p*, respectively.

GGM was methylated by the Hakomori[15] method, the methylated product (which showed no i.r. absorption for hydroxyl groups) was hydrolysed, and the products were converted into the alditol acetates. G.l.c.–m.s. then revealed derivatives of 2,3,4,6-tetra-*O*-methylgalactose, 3,4,6-tri-*O*-methylgalactose, 2,3,6-tri-*O*-methylglucose, 2,3-di-*O*-methylglucose, 2,3,6-tri-*O*-methylmannose, and 2,3-di-*O*-methylmannose as prominent products (Table I).

These results suggested that GGM contained a backbone of (1→4)-linked β-D-glucopyranosyl and β-D-mannopyranosyl residues substituted by terminal D-galactopyranosyl groups at O-6. Therefore, GGM was hydrolysed with a partially purified cellulase from *T. viride* and the hydrolysates were fractionated on Bio-Gel P-2 (Fig. 1). Each fraction was rechromatographed on the same column. Fractions 1 and 2 (oligosaccharides **1** and **2**, respectively) appeared to be homogeneous in t.l.c. Although fractions 3 and 6 each contained several components (t.l.c.), the yields were too low to permit further investigation. Fractions 4, 5, and 7 also contained several components (t.l.c.), and the major component of each fraction was purified by preparative p.c. (to give oligosaccharides **3**, **4**, and **5**, respectively). Fraction 8 contained galactose, glucose, and mannose in the molar ratios of 32:34:33. Since components of this fraction were not mobile in t.l.c. and p.c., it was not studied further. The oligosaccharides obtained after the purification were homogeneous in t.l.c., and the yields and properties of them are summarised in Table II. The results of methylation analyses of these oligosaccharides before and after borohydride reduction are shown in Table III.

Analytical data (Tables II and III) for oligosaccharides **1** and **2** were in good

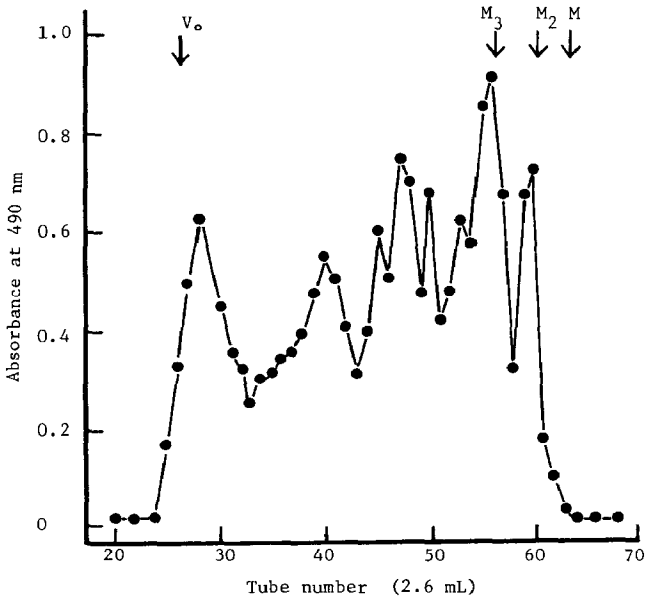


Fig. 1. Gel filtration on Bio-Gel P-2 of the cellulase hydrolysate of GGM. The fractions and tube numbers were as follows: 1, 59–61; 2, 55–58; 3, 52–54; 4, 49–51; 5, 46–48; 6, 44–45; 7, 38–43; 8, 25–31. The tube numbers at which Blue Dextran (V_0), mannotriose (M_3), mannobiose (M_2), and mannose (M) appeared are indicated by arrows.

agreement with those for disaccharide (4-*O*-β-D-Man*p*-D-Glc) and trisaccharide [α-D-Gal*p*-(1→6)-β-D-Man*p*-(1→4)-D-Glc], respectively, as previously reported[5]. These structures were further confirmed by m.s. of the permethylated alditols, the fragmentation patterns of which are shown in Scheme 1.

Oligosaccharide **3** contained galactose, glucose, and mannose in the molar ratios of ~1:2:2. The f.d.-mass spectrum of **3** contained an ion at *m/z* 851 for $[M + Na]^+$ as its base peak, indicating that **3** is a pentasaccharide. Although methylation analysis of a pentasaccharide was expected to give five kinds of methylated

TABLE II

YIELDS AND PROPERTIES OF OLIGOSACCHARIDES (**1–5**) DERIVED FROM GGM BY ENZYMIC HYDROLYSIS WITH CELLULASE

Oligosaccharide	*Yield (mg)*	R_{Man}[a]	*Mol. wt.*[b]	*Mole percent*		
				Gal	*Glc*	*Man*
1	7	0.65	342	—	48	52
2	13	0.43	504	31	34	35
3	6	0.20	828	18	38	45
4	8	0.15	990	31	33	36
5	9	0.05	n.d.[c]	31	35	34

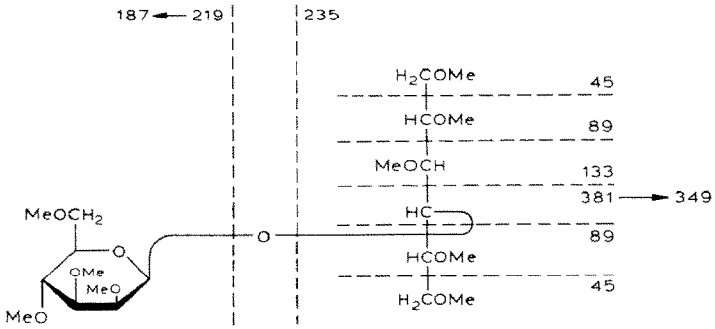
[a]Mobilities in t.l.c. relative to D-mannose. [b]Determined by f.d.-m.s. or s.i.m.s. [c]Not determined.

TABLE III

METHYLATION ANALYSIS OF OLIGOSACCHARIDES **1–5** BEFORE AND AFTER REDUCTION WITH BOROHYDRIDE

Methylated sugars[a]	T_1[b]	T_2[b]	*Mole percent*									
			1	**2**	**2-R**[c]	**3**	**3-R**	**4**	**4-R**	**5**	**5-R**	**5-PD**[d]
1,2,3,5,6-Glc	0.79	0.71	—	—	27	—	11	—	9	—	6	7
2,3,4,6-Man	0.97	0.96	47	—	—	14	15	—	—	10	9	12
2,3,4,6-Gal	1.00	1.00	—	34	38	17	19	32	35	25	26	28
2,3,6-Man	1.11	1.21	—	—	—	11	12	—	—	—	—	—
2,3,6-Glc	1.12	1.27	53	30	—	39	23	37	20	30	24	27
3,4,6-Gal	1.13	1.25	—	—	—	—	—	—	—	11	12	—
2,3,4-Man	1.15		—	36	35	10	11	18	19	—	—	—
2,3-Man	1.29	1.58	—	—	—	9	10	14	17	23	23	26

[a]1,2,3,5,6-Glc = 1,2,3,5,6-penta-*O*-methylglucitol, *etc.* [b]Relative retention times (see Table I) [c]**2**-R, Borohydride-reduced **2**, *etc.* [d]**5**-PD, **5**-R partially degraded with β-D-galactosidase.



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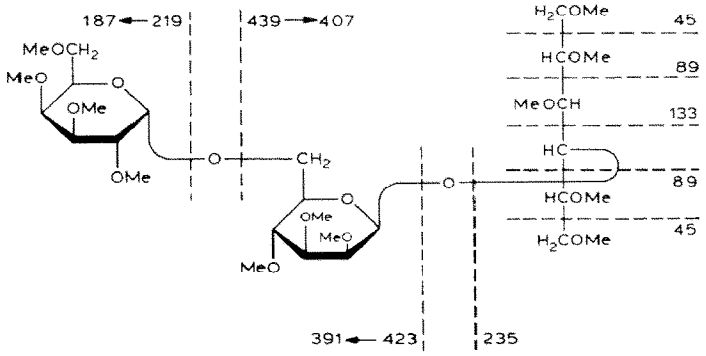

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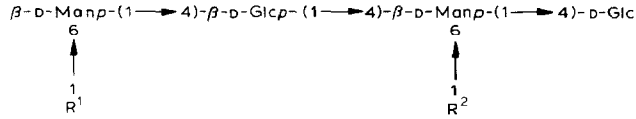
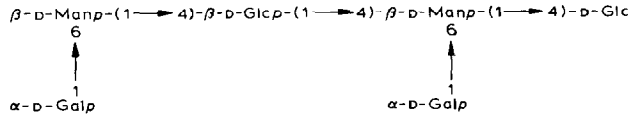
Scheme 1. Mass fragmentation patterns of the permethylated alditols of **1** and **2**.



3-1 R^1 = α-D-Galp, R^2 =
3-2 R^1 = H, R^2 = α-D-Galp

sugars at most, **3** gave six kinds of methylated sugars (Table III). This result suggests that **3** is not homogeneous, but contained at least two oligosaccharides whose structures may be **3**-1 and **3**-2.

Oligosaccharide **4** contained galactose, glucose, and mannose in approximately equal proportions. S.i.m.s. of **4** gave an ion at *m/z* 1014 due to $[M + Na + H]^+$ as its base peak, indicating **4** to be a hexasaccharide. Methylation analysis revealed two non-reducing terminal Gal, two 4-substituted Glc, one 6-substituted Man, and one 4,6-disubstituted Man (Table III). Methylation analysis of reduced **4** revealed one 4-substituted glucitol residue and the concomitant disappearance of one 4-substituted Glc, indicating that the reducing terminus of **4** was 4-substituted Glc. The ^{13}C-n.m.r. spectrum of **4** contained seven signals for anomeric carbon atoms at 103.6, 101.3, 101.0, 99.7, 99.3, 96.8, and 93.8 p.p.m., which were assigned to β-D-Glc*p* (103.6), β-D-Man*p* (101.3 and 101.0), α-D-Gal*p* (99.7 and 99.3), and reducing terminal D-Glc (96.8 and 93.8). Partial hydrolysis of **4** with acid gave **1** and 4-*O*-β-D-glucopyranosyl-D-mannose (GM) which were identified as the methylated alditol derivatives. Thus, the structure of **4** depicted was confirmed.



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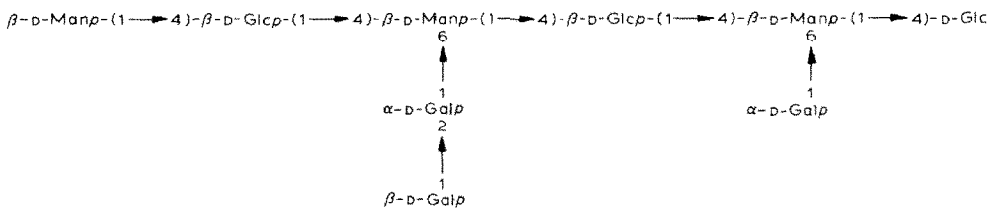
Oligosaccharide **5** contained galactose, glucose, and mannose in approximately equal proportions. The differences of neutral sugar contents between **5** and reduced **5** indicated that **5** was a nonasaccharide. Methylation analysis revealed two non-reducing terminal Gal, one non-reducing terminal Man, one 2-substituted Gal, three 4-substituted Glc, and two 4,6-disubstituted Man (Table III). Methylation analysis of reduced **5** indicated a reducing terminal Glc in **5**. The ^{13}C-n.m.r. spectrum of **5** contained five signals for anomeric carbon atoms at 105.4, 103.4, 101.0, 99.8, and 99.6 p.p.m., which were assigned to β-D-Gal*p* (105.4), β-D-Glc*p* (103.4), β-D-Man*p* (101.0), and α-D-Gal*p* (99.8 and 99.6). Partial hydrolysis of **5** with β-D-galactosidase removed only 2-substituted galactose (Table III), indicating that a terminal galactosyl group was β-(1→2)-linked to a galactosyl residue. Furthermore, partial hydrolysis of **5** with acid gave **1** and GM. Hence, **5** was considered to have a backbone consisting of six alternating (1→4)-linked residues of β-D-Glc*p* and β-D-Man*p*, to which α-D-Gal*p* and β-D-Gal*p*-(1→2)-α-D-Gal*p* side-chains are attached at O-6 of the internal Man.



5

The foregoing results indicate the tobacco GGM to contain a backbone consisting of (1→4)-linked, alternating β-D-glucopyranosyl and β-D-mannopyranosyl residues, with ~83% of the mannosyl residues carrying α-D-galactopyranosyl or β-D-galactopyranosyl-(1→2)-α-D-galactopyranosyl side-chains at O-6.

The GGMs are structural constituents of woody tissues of gymnosperms and angiosperms. They have also been isolated from the stems and leaf tissues of some legumes[16,17], the stems of an aquatic moss[18], and a fern[19]. The presence of such polysaccharides has also been demonstrated in the seeds of some species of the Iridaceae and Liliaceae[20]. Generally, they consist of (1→4)-linked β-D-mannopyranosyl and β-D-glucopyranosyl residues to which α-D-galactopyranosyl groups are attached as single stubs. However, there is no set pattern for the occurrence of the D-hexose residues in the main chain, and the distribution of the D-galactopyranosyl stubs is also random. Therefore, it is concluded that the structural features of our GGM are unique in two respects: firstly, the backbone consists of alternate D-glucosyl and D-mannosyl residues; secondly, the stubs occur mainly on the D-mannosyl residues. A GGM with a similar structure was also isolated from the ECP of suspension-cultured tobacco cells.

ACKNOWLEDGMENTS

The authors thank Dr. I. Kusakabe for supplying authentic oligosaccharides, and Dr. A. Ohnishi for valuable discussions and comments.

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