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STRUCTURAL INVESTIGATION OF A GALACTOGLUCOMANNAN FROM CELL WALLS OF TOBACCO *(Nicotiana tabacum)* MIDRIB

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ABSTRACT

Cell-wall polysaccharides of the midrib of *Nicotiana tabacum* were fractionated into pectin, hemicellulose, and α-cellulose components. From the α-cellulose fraction, a polymer composed of D-galactose, D-glucose, and D-mannose in the molar ratios ~1:2:4 was extracted by aqueous 25% sodium hydroxide–5% boric acid and purified by barium hydroxide precipitation, ion-exchange chromatography, and gel filtration. Methylation analysis, enzymic degradation, and ^{13}C-n.m.r. studies showed that the polysaccharide was built up of (1→4)-linked β-D-glucopyranosyl and β-D-mannopyranosyl residues in the molar ratio ~1:2, and that ~14% of the D-mannosyl residues were substituted at O-6 by α-D-galactopyranosyl or 2-*O*-β-D-galactopyranosyl-α-D-galactopyranosyl side-chains.

INTRODUCTION

In previous studies of the cell-wall polysaccharides of tobacco midrib, pectin[1], galactan[2], arabinoxyloglucan[3–5], 4-*O*-methylglucuronoxylan[6], and acidic polysaccharide (rhamnogalacturonan with such neutral polysaccharides as arabinan and galactan)[7] were isolated and characterised. In a continuation of these investigations, we now report on the structure of a galactoglucomannan (GGM) from the same source.

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RESULTS AND DISCUSSION

The cell-wall material (CWM) obtained from tobacco midrib contained 32.8% of uronic acids, 51.4% of neutral sugars, 4.5% of lignin, and 4.7% of protein. The CWM was fractionated into pectin, hemicellulose, and α-cellulose components, the yields and sugar compositions of which are summarised in Table I. From the hemicellulose fraction, arabinoxyloglucan[3–5], 4-*O*-methylglucuronoxylan[6], and rhamnogalacturonan with arabinan and galactan[7] were isolated and their structures determined. As shown in Table I, almost all of the mannose residues in the CWM were concentrated in the α-cellulose fraction. Therefore, this fraction was extracted with aqueous sodium hydroxide containing boric acid. The polysaccharide fraction obtained from the extract contained mannose together with xylose, galactose, glucose, uronic acids, and small proportions of arabinose and rhamnose (Table I), and was purified by precipitation as the barium complex from the alkaline solution. This method is generally used for the purification of mannose-containing polysaccharides[8]. Further fractionation by ion-exchange chromatography on DEAE-cellulose (AcO^-form) gave a main fraction (crude GGM), which contained mannose, galactose, and glucose together with small proportions of xylose and arabinose, but no uronic acid or rhamnose (Table I). Gel filtration

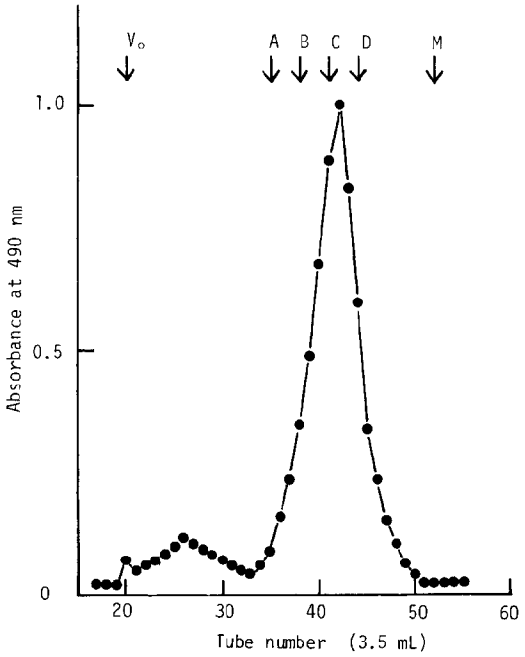

Fig. 1. Elution profile on Sepharose CL-6B of crude GGM. Fractions 19–35 contained PS-H, and 36–50 contained PS-L. Tube numbers at which Blue Dextran (V_o), standard dextrans T-70 (A), T-40 (B), T-20 (C), T-10 (D), and mannose (M) appeared are indicated by arrows.

TABLE I

YIELDS AND COMPOSITIONS OF FRACTIONS OBTAINED FROM CWM OF TOBACCO MIDRIB

Fraction	*Yield*[a]	*Uronic acid*[b] *content*	*Neutral sugar*[b] *content*	*Neutral sugar composition (mol %)*						*Protein*[b] *content*
				Rha	*Ara*	*Xyl*	*Man*	*Gal*	*Glc*	
CWM	100	32.8	51.4	2.2	4.0	13.5	4.1	4.7	71.4	4.7
Pectin	29.3	92.6	3.7	11.6	26.4	8.7	—	43.0	10.3	4.8
Hemicellulose	18.3	28.7	40.2	1.7	7.5	60.6	tr	8.3	21.8	4.2
α-Cellulose	39.5	5.2	84.3	0.3	0.9	1.4	4.7	1.6	91.1	0.3
NaOH–borate soluble	3.5	8.6	86.4	2.0	3.5	12.4	36.1	12.7	33.4	n.d.
NaOH–borate insoluble	35.2	2.4	85.6	0.1	0.4	1.4	1.1	0.3	96.7	n.d.
$Ba(OH)_2$ precipitate	3.0	7.7	88.3	1.2	3.0	11.0	38.4	12.0	34.5	n.d.
$Ba(OH)_2$ soluble	0.2	22.4	71.6	13.1	12.1	35.3	5 3	14.4	19.9	n.d.
Crude GGM	1.2	—	92.3	—	1.8	4.6	50.2	12.3	31.1	n.d.
PS-L	1.0	—	95.7	—	0.7	1.5	55.8	14.7	27.4	n.d.
PS-H	0.1	—	94.6	—	7.3	24.4	25.2	6.3	36.2	n.d.

[a]Percentage of dry CWM. [b]Percentage of dry material; n.d., not determined.

(Fig. 1) of the crude GGM on Sepharose CL-6B revealed a major component (PS-L) at the elution volume at 2.2 V_o and a minor component (PS-H) in the region of higher molecular weight, and these were isolated. Their yields and sugar compositions are shown in Table I.

Purified PS-L had $[\alpha]_D^{23}$ −2° (*c* 0.5, 0.5M NaOH), was homogeneous in zone electrophoresis and sedimentation analysis, and gave arabinose, xylose, galactose, glucose, and mannose in the molar ratios 0.7:1.5:14.7:27.4:55.8 on acid hydrolysis. These ratios were not changed by attempted fractionation of PS-L using barium hydroxide. The molecular weight of PS-L was estimated to be ~15,000 by gel filtration on Sepharose CL-6B (Fig. 1).

GGM was methylated by the Hakomori[9] 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, 2,3,6-tri-*O*-methylglucose, 2,3,6-tri-*O*-methylmannose, 2,3-di-*O*-methylglucose, and 2,3-di-*O*-methylmannose as prominent products (Table II). These results suggested that GGM contained a backbone of (1→4)-linked glucopyranosyl and mannopyranosyl residues substituted by terminal galactosyl groups at O-6. By analogy with the galactoglucomannan[10] from wood, it is assumed that the main chain of GGM is β-linked.

GGM was hydrolysed with β-D-mannanase from *Streptomyces* No. 17 and the products were fractionated on Bio Gel P-2. T.l.c. (solvent *A*) of each fraction in Fig. 2 revealed that fraction 1 contained only mannose and that fractions 2–5 contained more than one component: 2, R_{Man} 0.96 and 0.75; 3, R_{Man} 0.66 and 0.47; 4, R_{Man} 0.27, 0.20, and 0.13; 5, R_{Man}0.13 and 0.09. These components were purified by preparative p.c. Fraction 6, eluted at the void volume, contained

TABLE II

METHYLATION ANALYSIS OF GGM

Methylated sugar[a]	T_1[b]	T_2[b]	*Mole percent*	*Mode of linkage*
2,3,5-Ara	0.74	0.73	0.5	T-Ara*f*-(1→
2,3,4-Xyl	0.79	0.80	0.7	T-Xyl*p*-(1→
3,4-Xyl	0.92	1.03	0.6	→2)-Xyl*p*-(1→
2,3,6-Glc	1.13	1.27	29.8	→4)-Glc*p*-(1→
2,3-Glc	1.33	1.67	2.0	→4,6)-Glc*p*-(1→
2,3,4,6-Gal	1.00	1.00	6.4	T-Gal*p*-(1→
2,3,6-Gal	1.11	1.25	4.6	→4)-Gal*p*-(1→
3,4,6-Gal	1.14	1.25	2.8	→2)-Gal*p*-(1→
2,3-Gal	1.34	1.71	0.7	→4,6)-Gal*p*-(1→
2,3,6-Man	1.11	1.21	44.8	→4)-Man*p*-(1→
2,3-Man	1.31	1.58	7.1	→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 10 C 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.

TABLE III

YIELDS AND PROPERTIES OF OLIGOSACCHARIDES (**1–9**) DERIVED FROM GGM BY ENZYMIC HYDROLYSIS WITH β-D-MANNANASE

Oligosaccharide	*Yield (mg)*	$R_{Man\text{-}1}$[a]	$R_{Man\text{-}2}$[a]	$[\alpha]_D^{23}$ *(degrees)*	*D.p.*[b]	*Mole percent*		
						Gal	*Glc*	*Man*
1	2	1.00	1.00		1	—	—	100
2	4	0.96	0.84	+8	2	—	48	52
3	13	0.75	0.65	−8	2	—	—	100
4	9	0.66	0.53	−11	3	—	32	68
5	16	0.47	0.41	−20	3	—	—	100
6	12	0.27	0.27	+25	5	18	44	38
7	6	0.20	0.19	+22	6	30	37	34
8	5	0.13	0.14	+6	6	17	35	48
9	5	0.09	0.11	+16	7	29	30	41

[a]Mobilities in t.l.c. relative to D-mannose with solvents *A* ($R_{Man\text{-}1}$) and *B* ($R_{Man\text{-}2}$). [b]Degree of polymerisation.

TABLE IV

METHYLATION ANALYSIS OF OLIGOSACCHARIDES (**1–9**), BEFORE AND AFTER BOROHYDRIDE-REDUCTION, DERIVED FROM GGM BY ENZYMIC HYDROLYSIS WITH β-D-MANNANASE

Methylated sugar[a]	T_1[b]	T_2[b]	*Mole percent*											
			2	**3**	**4**	**5**	**6**	**6-R**[c]	**7**	**7-R**	**8**	**8-R**	**9**	**9-R**
1,2,3,5,6-Man	0.78	0.69	—	—	—	—	—	15	—	11	—	11	—	10
2,3,4,6-Glc	0.97	0.95	53	—	35	—	17	20	19	18	17	18	14	14
2,3,4,6-Man	0.97	0.96	—	55	—	32	—	—	—	—	—	—	—	—
2,3,4,6-Gal	1.00	1.00	—	—	—	—	19	18	17	17	14	16	15	15
2,3,6-Man	1.11	1.21	47	45	65	68	20	—	17	—	35	17	30	14
2,3,6-Glc	1.12	1.27	—	—	—	—	23	23	16	18	19	20	14	17
3,4,6-Gal	1.13	1.25	—	—	—	—	—	—	17	17	—	—	12	13
2,3-Man	1.29	1.58	—	—		—	21	24	15	19	16	18	14	17

[a]1,2,3,5,6-Man = 1,2,3,5,6-penta-*O*-methylmannitol, *etc.* [b]Relative retention times (see Table II). [c]**6-R**, Borohydride-reduced **6**, *etc.*

arabinose, xylose, galactose, and glucose in the molar ratios 7:28:10:56. Since this fraction was considered to be derived from accompanying polysaccharides, such as arabinoxyloglucan[3–5] and galactan[2], it was not studied further. All of the purified oligosaccharides were homogeneous in t.l.c. and Table III shows the yields and properties. The results of methylation analyses of the oligosaccharides before and after reduction with borohydride are shown in Table IV.

Oligosaccharides **2–5** were chromatographically indistinguishable from authentic samples, and, based on the neutral sugar composition, methylation analysis, and ^{13}C-n.m.r. data (Table V), the following structures were determined, β-D-Glc*p*-(1→4)-D-Man, β-D-Man*p*-(1→4)-D-Man, β-D-Glc*p*-(1→4)-β-D-Man*p*-(1→4)-D-Man, and β-D-Man*p*-(1→4)-β-D-Man*p*-(1→4)-D-Man.

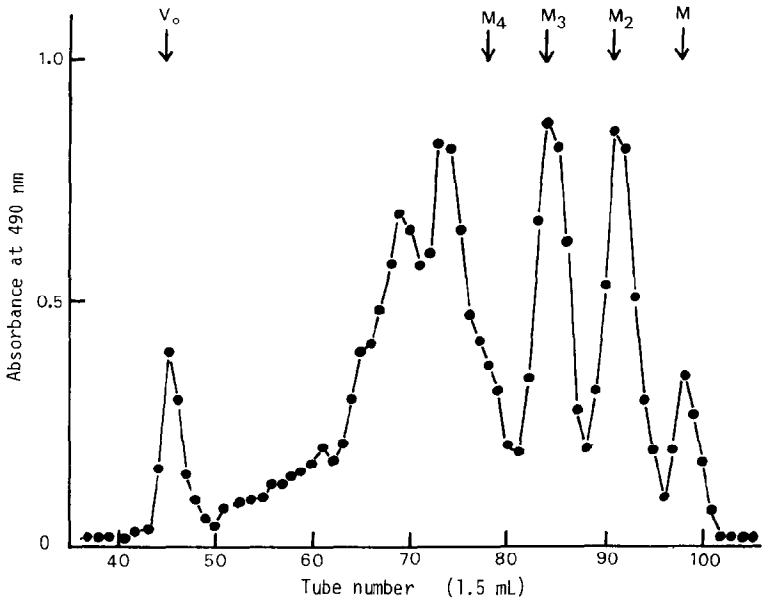

Fig. 2. Gel filtration on Bio-Gel P-2 of the β-D-mannanase hydrolysate of GGM. The fractions and tube numbers were as follows: 1, 97–102, 2, 89–96; 3, 82–88; 4, 72–81; 5, 64–71; and 6, 43–50. Tube numbers at which Blue Dextran (V_0), mannotetraose (M_4), mannotriose (M_3), mannobiose (M_2), and mannose (M) appeared are indicated by arrows.

TABLE V

^{13}C-N M.R. DATA FOR OLIGOSACCHARIDES

Oligosaccharide	*Chemical shift (p.p.m.)*					
	β-D-*Gal*p	α-D-*Gal*p	β-D-*Glc*p	β-D-*Man*p	D-*Man-R*	D-*Glc-R*
2			103.5		94.7	
					94.5	
3				101.1	94.7	
					94.6	
4			103.6	101.0	94.8	
					94.6	
5				101.0	94.7	
				101.2		
6		99.7	103.8	101.0	94.8	
			103.5		94.5	
7	105.6	99.6	103.8	100.9	94.8	
			103.6		94.5	
11				100.9		96.7
						92.7
12		99.3		100.9		96.8
						92.8
13		99.2	103.5	101.0		96.7
				100.8		92.7

Oligosaccharide **6** contained galactose, glucose, and mannose in the molar ratios ~1:2:2 (Table III). Methylation analysis revealed one non-reducing terminal Gal, one non-reducing terminal Glc, one 4-substituted Glc, one 4-substituted Man, and one 4,6-disubstituted Man (Table IV). Methylation analysis of reduced **6** revealed one 4-substituted mannitol residue and the concomitant disappearance of one 4-substituted Man (Table IV), indicating that the reducing terminus of **6** was 4-substituted Man. The ^{13}C-n.m.r. spectrum of **6** contained six signals for anomeric carbons at 103.8, 103.5, 101.0, 99.7, 94.8, and 94.5 p.p.m., which were assigned to β-D-Glc*p* (103.8 and 103.5), β-D-Man*p* (101.0), α-D-Gal*p* (99.7), and reducing D-Man (94.8 and 94.5). Thus, the following structure was proposed for **6**.

β-D-Glc*p*-(1→4)-β-D-Man*p*-(1→4)-β-D-Glc*p*-(1→4)-D-Man
6
↑
1
α-D-Gal*p*

However, the precise sequence of internal D-glucosyl and D-mannosyl residues in **6** remains to be determined.

Oligosaccharide **7** contained galactose, glucose, and mannose in the molar ratios ~1:1:1. Methylation analysis revealed one 2-substituted galactosyl residue in addition to the sugar residues found in **6** (Table IV). The ^{13}C-n.m.r. spectrum of **7** contained seven signals for anomeric carbons at 105.6, 103.8, 103.6, 100.9, 99.6, 94.8, and 94.5 p.p.m., which were assigned to β-D-Gal*p* (105.6), β-D-Glc*p* (103.8 and 103.6), β-D-Man*p* (100.9), α-D-Gal*p* (99.6), and reducing terminal D-Man (94.8 and 94.5). Hence, the following structure was proposed for **7**.

β-D-Glc*p*-(1→4)-β-D-Man*p*-(1→4)-β-D-Glc*p*-(1→4)-D-Man
6
↑
1
β-D-Gal*p*-(1→2)-α-D-Gal*p*

Oligosaccharide **8** contained galactose, glucose, and mannose in the molar ratios ~1:2:3. By comparing the results (Tables III and IV) of sugar composition and methylation analyses of **8** with those of **6**, it was assumed that **8** contained one more Man which was present as an internal 4-substituted residue. Thus, the following structure was proposed for **8**.

β-D-Glc*p*-(1→4)-β-D-Man*p*-(1→4)-β-D-Glc*p*-(1→4)-β-D-Man*p*-(1→4)-D-Man
6
↑
1
α-D-Gal*p*

Oligosaccharide **9** contained galactose, glucose, and mannose in the molar ratios ~2:2:3. By analogy with **8**, **9** contained one more internal 4-substituted Man than **7** (Tables III and IV) and, hence, the following structure was proposed for **9**.

β-D-Glc*p*-(1→4)-β-D-Man*p*-(1→4)-β-D-Glc*p*-(1→4)-β-D-Man*p*-(1→4)-D-Man
6
↑
1
β-D-Gal*p*-(1→2)-α-D-Gal*p*

GGM was also hydrolysed with a partially purified cellulase from *Trichoderma viride* and the products were fractionated on Bio-Gel P-2. Each fraction in Fig. 3 was rechromatographed on the same column. Fractions 1 and 2 appeared to be homogeneous in t.l.c. Although fraction 3 contained three components (t.l.c.), the yield of this fraction was too low to permit further study. Fractions 4–6 each contained several components. The major component of each fraction was purified by preparative p.c. Fraction 7 contained galactose, glucose, and mannose in the molar ratios 23:28:44. Since components of this fraction were not mobile in t.l.c. and p.c., it was not studied further. Fraction 8, eluted at the void volume, contained galactose only, indicating it to be derived from an accompanying

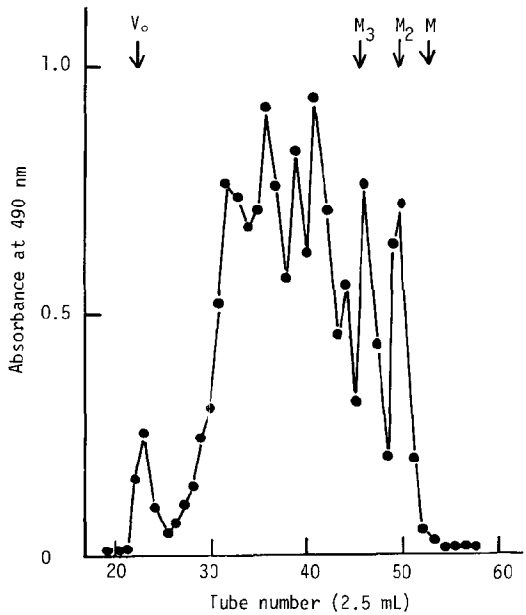

Fig. 3. Gel filtration on Bio-Gel P-2 of the cellulase hydrolysate of GGM. Fractions and tube numbers were as follows: 1, 49–53; 2, 46–48; 3, 44–45; 4, 41–43; 5, 39–40; 6, 33–38; 7, 26–34; and 8, 22–25. Tube numbers at which Blue Dextran (V_o), mannotriose (M_3), mannobiose (M_2), and mannose (M) appeared are indicated by arrows.

TABLE VI

YIELDS AND PROPERTIES OF OLIGOSACCHARIDES (**11–15**) DERIVED FROM GGM BY ENZYMIC HYDROLYSIS WITH CELLULASE

Oligosaccharide	*Yield (mg)*	$R_{Man\text{-}1}$[a]	$R_{Man\text{-}2}$[a]	$[\alpha]_D^{23}$ *(degrees)*	*D.p.*[b]	*Mole percent*		
						Gal	*Glc*	*Man*
11	14	0.64	0.65	+12	2	—	51	49
12	12	0.38	0.42	+33	3	31	34	35
13	10	0.15	0.19	+23	5	18	38	45
14	8	0.11	0.16	+25	6	33	33	34
15	12	0.04	0.05	+15	9	21	33	46

[a]Relative mobilities in t.l.c. (see Table III). [b]Degree of polymerisation.

TABLE VII

METHYLATION ANALYSES OF OLIGOSACCHARIDES (**11–15**), BEFORE AND AFTER BOROHYDRIDE-REDUCTION, DERIVED FROM GGM BY ENZYMIC HYDROLYSIS WITH CELLULASE

Methylated sugar[a]	T_1[b]	T_2[b]	*Mole percent*							
			11	**12**	**13**	**13**-R[c]	**14**	**14**-R	**15**	**15**-R
1,2,3,5,6-Glc	0.79	0.71	—	—	—	15	—	13	—	7
2,3,4,6-Man	0.97	0.96	52	—	20	22	17	19	12	13
2,3,4,6-Gal	1.00	1.00	—	31	17	20	16	17	13	13
2,3,6-Man	1.11	1.21	—	—	—	—	—	—	21	24
2,3,6-Glc	1.12	1.27	48	35	39	21	35	21	33	23
3,4,6-Gal	1.13	1.25	—	—	—	—	15	15	8	8
2,3,4-Man	1.15		—	34	—	—	—	—	—	—
2,3-Man	1.29	1.58	—	—	20	22	17	16	11	12

[a]1,2,3,5,6-Glc = 1,2,3,5,6-penta-*O*-methylglucitol. [b]Relative retention times (see Table II). [c]**13**-R, Borohydride-reduced **13**, *etc.*

polysaccharide, galactan. The purified oligosaccharides were homogeneous in t.l.c., and yields and properties of the oligosaccharides are shown in Table VI. The results of methylation analyses of the oligosaccharides before and after borohydride reduction are shown in Table VII.

Oligosaccharide **11** contained glucose and mannose in the molar ratio ~1:1, and methylation analysis revealed one non-reducing terminal Man and one 4-substituted Glc. The ^{13}C-n.m.r. spectrum of **11** contained three signals for anomeric carbons at 100.9, 96.7, and 92.7 p.p.m., which were assigned to β-D-Man*p* (100.9) and reducing terminal D-Glc (96.7 and 92.7). Thus, **11** was 4-*O*-β-D-mannopyranosyl-D-glucose.

Oligosaccharide **12** contained galactose, glucose, and mannose in the molar ratios ~1:1:1, and methylation analysis revealed one non-reducing terminal Gal,

one 4-substituted Glc, and one 6-substituted Man (Table VII). The ^{13}C-n.m.r. spectrum of **12** contained four signals for anomeric carbons at 100.9, 99.3, 96.8, and 92.8 p.p.m., which were assigned to β-D-Man*p* (100.9), α-D-Gal*p* (99.3), and reducing terminal D-Glc (96.8 and 92.8). Hence, **12** was *O*-α-D-galactopyranosyl-(1→6)-*O*-β-D-mannopyranosyl-(1→4)-D-glucose.

Oligosaccharide **13** contained galactose, glucose, and mannose in the molar ratios ~1:2:2, and methylation analysis revealed one non-reducing terminal Gal, one non-reducing terminal Man, two 4-substituted Glc, and one 4,6-disubstituted Man (Table VII). Methylation analysis of the reduced **13** indicated a reducing terminal Glc in **13**. The ^{13}C-n.m.r. spectrum of **13** contained six signals for anomeric carbons at 103.5, 101.0, 100.8, 99.2, 96.7, and 92.7 p.p.m., which were assigned to β-D-Glc*p* (103.5), two β-D-Man*p* (101.0 and 100.8), α-D-Gal*p* (99.2), and reducing terminal D-Glc (96.7 and 92.9). Hence, the following structure was proposed for **13**.

β-D-Man*p*-(1→4)-β-D-Glc*p*-(1→4)-β-D-Man*p*-(1→4)-D-Glc*p*
6
↑
1
α-D-Gal*p*

Oligosaccharide **14** contained galactose, glucose, and mannose in the molar ratios ~1:1:1, and methylation analysis before and after reduction with borohydride showed that **14** contained one more 2-substituted Gal than **13**, indicating that a β-D-galactopyranosyl-(1→2)-α-D-galactopyranosyl side-chain was linked to O-6 of the internal mannosyl residue of the main chain of **13**.

Oligosaccharide **15** contained galactose, glucose, and mannose in the molar ratios ~2:3:4, and methylation analysis revealed one non-reducing terminal Gal, one non-reducing terminal Man, two 4-substituted Man, three 4-substituted Glc, one 2-substituted Gal, and one 4,6-disubstituted Man (Table VII). Methylation analysis of reduced **15** showed that **15** contained a 4-substituted Glc at the reducing terminus. Thus, **15** was considered to have a backbone of (1→4)-linked β-D-Glc and β-D-Man residues in the molar ratio 3:4, to which a β-D-Gal*p*-(1→2)-α-D-Gal*p* side-chain was attached at O-6 of the internal 4-substituted Man.

The above results indicate tobacco GGM to contain a main chain consisting of (1→4)-linked β-D-mannopyranosyl and β-D-glucopyranosyl residues in the molar ratio of ~2:1, with ~14% of the mannosyl residues carrying α-D-galactopyranosyl or probably β-D-galactopyranosyl-(1→2)-α-D-galactopyranosyl side-chains at O-6. Moreover, GGM gave three groups of oligosaccharides on hydrolysis with β-D-mannanase and cellulase, consisting of (*1*) D-mannose residues only (**3** and **5**), (*2*) D-mannose and D-glucose residues (**2, 4,** and **11**), and (*3*) D-mannose, D-glucose, and D-galactose residues (**6–9, 12–15**). In the last group, the D-galactose residues were linked at O-6 of the D-mannosyl residues. These results suggest that

the GGM comprises an unsubstituted (1→4)-β-D-mannan region and a region consisting of (1→4)-linked β-D-mannopyranosyl and β-D-glucopyranosyl residues in the molar ratio ~1:1 with α-D-galactopyranosyl or β-D-galactopyranosyl-(1→2)-α-D-galactopyranosyl side-chains at O-6 of the mannosyl residues.

The GGMs are the structural constituents of woody tissues of gymnosperms and angiosperms[10]. They have also been isolated from the stems and leaf tissues of some legumes[11,12] and from stems of an aquatic moss[13] and a fern[14]. Generally speaking, 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, we conclude that the structural feature of our GGM is similar to those obtained from wood, with the major differences being the presence of some repeating blocks of both D-mannose and D-glucose residues and the absence of any linkages between D-galactose and D-glucose residues.

EXPERIMENTAL

Materials. — β-D-Mannanase from *Streptomyces* No. 17 was purified as previously reported[15]. Cellulase from *T. viride* (Meicellase, kindly donated by Meiji Seika Ltd.) was partially purified[16] on a gauze column. Mannobiose [β-D-Man*p*-(1→4)-D-Man], mannotriose [β-D-Man*p*-(1→4)-β-D-Man*p*-(1→4)-D-Man], mannotetraose [β-D-Man*p*-(1→4)-β-D-Man*p*-(1→4)-β-D-Man*p*-(1→4)-D-Man], β-D-Glc*p*-(1→4)-D-Man, β-D-Glc*p*-(1→4)-β-D-Man*p*-(1→4)-D-Man, and β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→4)-D-Man were prepared from the hydrolysates of copra mannan[17] and konjak mannan[18] by β-D-mannanase. Sepharose CL-6B, Sephadex LH-20, and standard dextrans (Blue Dextran, dextrans T-70, T-40, T-20, and T-10) were purchased from Pharmacia Fine Chemicals, Bio-Gel P-2 from Bio-Rad Laboratories, and DEAE-cellulose from Seikagaku Kogyo (Tokyo, Japan).

General methods. — All evaporations were performed under reduced pressure at <45° (bath). Optical rotations were measured, unless stated otherwise, for aqueous solutions at 23° with a JASCO Model DIP-181 polarimeter and a 100-mm micro-cell. Zone electrophoresis was performed on Whatman GF/A glass-fibre paper (47 × 5 cm) at 1600 V for 40 min. Ultracentrifugation analysis was conducted at 25° with a Hitachi 282 analytical centrifuge apparatus.

T.l.c. was conducted on silica gel (Merck, 5553) with *A*, 1-butanol–2-propanol–water (3:12:4); *B*, 1-butanol–ethyl acetate–water (3:3:2); and detection with 50% sulfuric acid containing 1% of orcinol. Preparative p.c. was performed on Whatman 3MM paper by multiple irrigations with 1-butanol–pyridine–water (6:4:3). Reducing sugars were detected with alkaline silver nitrate.

G.l.c. was performed with a Shimadzu Model GC-7A instrument, fitted with a flame-ionisation detector, on *A*, a glass column (200 × 0.3 cm) containing 0.2% of poly(ethyleneglycol adipate), 0.2% of poly(ethyleneglycol succinate), and 0.4%

of silicone XF-1150 on Gas Chrom P (100–120 mesh); *B*, a glass-capillary column (50 m × 0.28 mm) coated with OV-101; *C*, a glass-capillary column (50 m × 0.28 mm) coated with Silar 10C. Peak areas were measured with a Hewlett–Packard Model 3380A digital integrator. G.l.c.–m.s. was accomplished with a Hitachi M-80 instrument (20 eV) with data processor M-003, using columns *B* and *C*.

For n.m.r. spectroscopy, a JEOL FX-100 spectrometer was used. The ^{13}C-n.m.r. spectra (25.1 MHz) were obtained, using a 1-mm tube, for solutions in D_2O at 70° (internal methanol, δ 49.9 from the signal for Me_4Si).

Analyses. — *(a) Sugar.* Polysaccharide material (5–10 mg) was hydrolysed[19] with aqueous 3% sulfuric acid in a sealed tube at 120° for 1 h. The neutral sugars were determined as the alditol acetates by g.l.c. (column *A*). For quantitative analysis, methyl β-D-glucopyranoside was added to the hydrolysates as an internal standard before neutralisation with barium carbonate[20]. The contents of uronic acids in the hydrolysates were determined by the *m*-hydroxybiphenyl method[21] with D-galacturonic acid as a standard. Oligosaccharides (0.2–2 mg) were hydrolysed in a sealed tube with 2M trifluoroacetic acid at 120° for 1 h.

(b) Methylation. Solutions of polysaccharides (5–10 mg) or oligosaccharides (1–2 mg) in methyl sulfoxide (1–2 mL) were methylated[9] with sodium methylsulfinylmethanide (0.5–1 mL) and methyl iodide (1–2 mL). The methylated products were extracted with chloroform, purified by elution from a column (1.2 × 90 cm) of Sephadex LH-20 with chloroform–methanol (1:1), and hydrolysed[22] with 90% formic acid–0.25M sulfuric acid. Each hydrolysate was neutralised with barium carbonate, deionised with Dowex 50W (H^+) resin, and concentrated. The residual methylated sugars were analysed by g.l.c. (columns *B* and *C*) as their alditol acetates, and identified on the basis of comparison of retention times and m.s. data with those of standards when available[3–5,23].

(c) Protein. The content of protein was calculated by multiplication of the nitrogen content by 6.25. Total nitrogen was determined by conventional elemental analysis.

Molecular weights. — 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[24]. The column was calibrated by using standard dextrans.

D.p. of oligosaccharides. — D.p. values of <4 were determined by gel filtration of oligosaccharides on Bio-Gel P-2. D.p. values of >5 were calculated from the differences in the contents of neutral sugars before and after borohydride reduction of the oligosaccharides. The neutral sugar content was determined by the phenol–sulfuric acid method[24].

Preparation of CWM from tobacco midrib. — *Nicotiana tabacum* L., cv. Bright Yellow, was grown in a greenhouse and harvested at an early stage of flower breeding. Midribs were cut from the leaves with a razor brade and stored in a freezer until use.

CWM from tobacco midrib was prepared according to the method of Ring and Selvendran[25]. The frozen midribs (fresh weight, 4.0 kg) were ground to a powder and then blended with aqueous 1% sodium deoxycholate (SDC). The suspension was passed through a French pressure cell at 200–300 kg/cm^2 instead of ball-milling. The resulting slurry was centrifuged, and the insoluble residue was washed with cold aqueous 1% SDC and then with cold water. The residue was sequentially extracted with phenol–acetic acid–water (2:1:1, w/w/v), aqueous 90% methyl sulfoxide, and chloroform–methanol (1:1) overnight at 20°, washed with chloroform–methanol, acetone, and ether, and dried *in vacuo*, to give CWM (95.6 g).

Sequential extraction of CWM. — CWM (2 g) was extracted with 50mM EDTA in 50mM sodium acetate buffer (pH 4.5, 3 × 200 mL) at 100° for 1 h. The combined extracts were dialysed and freeze-dried. The depectinated material was delignified[26] with acidified sodium chlorite solution at 70° for 4 h. The resulting holocellulose was successively extracted with 200 mL each of aqueous 5% and 24% KOH containing 10mM $NaBH_4$ under nitrogen at 20° for 20 h. The combined extracts were adjusted to pH 5 with acetic acid, dialysed, and freeze-dried, and the residue was washed with water and acetone, and dried *in vacuo*, to give an α-cellulose fraction.

Isolation of GGM. — The α-cellulose fraction (25.5 g) was extracted with 100 vol. of aqueous 25% NaOH containing 5% of boric acid and 1% of $NaBH_4$ at 25° for 48 h under nitrogen. The extract (2.3 g) was dissolved in 200 vol. of aqueous 5% NaOH, and 200 vol. of aqueous 5% barium hydroxide was added[8]. The mixture was centrifuged, and a solution of the precipitate in 100 vol. of aqueous 5% NaOH was neutralised with acetic acid, dialysed against distilled water, and applied to a column (4.5 × 45 cm) of DEAE-cellulose (AcO^- form). The fraction eluted with water was dialysed against distilled water, and freeze-dried to give crude GGM (800 mg). A solution of crude GGM in aqueous 5% NaOH was neutralised with acetic acid, and dialysed against 0.5M NaCl containing 5mM EDTA. The clear solution was eluted from a column (2.6 × 90 cm) of Sepharose CL-6B with 0.5M NaCl containing 5mM EDTA. The eluate was dialysed and freeze-dried, to give a purified polysaccharide (GGM).

Hydrolyses of the polysaccharide. — *(a) β-D-Mannanase.* A suspension of the polysaccharide (150 mg) in 0.1M McIlvaine buffer (pH 6.8) was incubated with β-D-mannanase (3 mL, 60 units/mL) at 45° with the addition of a few drops of toluene[15]. After 24 h, the mixture was heated in a boiling water bath for 15 min and then centrifuged, and the supernatant solution was concentrated to 2 mL, applied to a column (1.6 × 90 cm) of Bio-Gel P-2, and eluted with water. Fractions (1.5 mL) were analysed by the phenol–sulfuric acid method[24].

(b) Cellulase. A suspension of the polysaccharide (150 mg) in 0.1M sodium acetate buffer (pH 4.5) was incubated with cellulase (30 mg, 7.4 unit/mg) for 48 h at 37° with the addition of a few drops of toluene[3–5]. After incubation, the mixture was heated in a boiling water bath for 15 min and then centrifuged, and the supernatant solution was treated with Dowex 50W (H^+) resin, concentrated, and freeze-

dried. A solution of the residue (130 mg) in water (2 mL) was applied to a column of Bio-Gel P-2 as in (*a*).

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