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A GALACTOGLUCOMANNAN FROM EXTRACELLULAR POLYSACCHARIDES OF SUSPENSION-CULTURED CELLS OF *NICOTIANA TABACUM*

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Abstract—A polysaccharide containing D-xylose, L-arabinose, D-mannose, D-galactose and D-glucose residues in the molar ratio of 0.07:0.16:1.01:1.02:1.0 was isolated from the extracellular polysaccharides of suspension-cultured tobacco cells by ion-exchange chromatography, ammonium sulfate precipitation and gel-filtration. Structural studies were performed by methylation analysis, ^{13}C NMR spectroscopy, partial hydrolysis with acid and cellulase degradation. From these experiments, a structure for the polysaccharide is proposed.

INTRODUCTION

In previous studies on the extracellular polysaccharides (ECP) of suspension-cultured tobacco cells, an arabinoxyloglucan (AXG) was isolated and shown to be structurally similar to the AXG of the midrib of tobacco leaves [1] In continuation of these investigations, we report on the structure of a galactoglucomannan (GGM) from the same source

RESULTS AND DISCUSSION

When a solution of ECP was fractionated using anion-exchange CC, *ca* 50% of the sugars were recovered in a fraction not adsorbed on the column (F-1). High-voltage paper electrophoresis of F-1 suggested the presence of two kinds of polysaccharides, which were separable from each other by ammonium sulfate (AS) precipitation. Thus, the saturated AS-soluble fraction gave a polysaccharide which was further purified by gel-filtration with Sephadex G-75 Table 1 shows the yield and neutral sugar composition of ECP, F-1 and AS-soluble and -insoluble fractions. It was also possible to separate the two polysaccharides of F-1 with barium hydroxide, generally used for purification of mannose-containing polysaccharides [2], instead of AS. In this case, GGM was recovered in the barium hydroxide-insoluble fraction.

The purified polysaccharide had $[\alpha]_D = +39°$ (water, *c* 0.5) and seemed to be a homogeneous preparation from the results of gel-filtration, high-voltage paper electrophoresis and ultracentrifugation. It apparently contained no uronic acid and protein, and gave, on complete acid hydrolysis, D-xylose, L-arabinose, D-mannose, D-glucose and D-galactose in the molar ratio of 0.07.0 16·1.01·1 00.1 02, respectively This ratio was not changed by attempted fractionation of the material with barium hydroxide or ethanol. Its MW was estimated to be *ca* 15 000 by high-performance, gel-permeation chromatography. Therefore, GGM contains *ca* 90 hexoses consisting of equal amounts of galactose, glucose and mannose residues with small amounts of arabinose and xylose residues. The $S_{20,w}$ value was calculated to be 1 13 S.

The ^{13}C NMR spectrum was recorded to determine the anomeric configuration of the sugar residues. GGM showed four anomeric carbon signals at δ 105 2, 103.8, 100.8 and 99.6 The $J_{C\text{-}1,H\text{-}1}$ values for the anomeric carbon signals were also measured and found to be 165, 164, 161 and 171 Hz, respectively. From these and literature data [3–5], the four signals were assigned to the β-D-

Table 1 Yield and neutral sugar composition of fractions from extracellular polysaccharides of tobacco cells

		Neutral sugar composition (mol %)					
Fraction	Yield* (mg)	Xyl	Ara	Rha	Man	Glc	Gal
ECP	—	15	22	1	15	31	16
F-1	1000	22	8	0	13	43	13
AS-insoluble	475	27	13	0	tr	60	tr
AS-soluble, G-75	300	2	5	0	31	31	31

*From 11 of ECP soln.

galactopyranosyl, β-D-glucopyranosyl, β-D-mannopyranosyl and α-D-galactopyranosyl residues, respectively

Methylation analysis of GGM (Table 2, column 1) revealed terminal arabinofuranosyl (2,3,5-Me_3-Ara), terminal xylopyranosyl (2,3,4-Me_3-Xyl), terminal and 2-substituted galactopyranosyl (2,3,4,6-Me_4- and 3,4,6-Me_3-Gal), 4- and 4,6-substituted glucopyranosyl (2,3,6-Me_3- and 2,3-Me_2-Glc) and 4-, 6- and 4,6-substituted mannopyranosyl (2,3,6-Me_3-, 2,3,4-Me_3- and 2,3-Me_2-Man) residues in the molar ratio of 0.10, 0.11, 2.19, 1.28, 3.00, 0.24, 0.76, 0.44, 2.14, respectively The results of methylation analysis indicated the mode of linkages of each sugar Thus, pentoses are present as non-reducing terminal groups Galactose residues are present as terminal (63%) and 2-linked (37%) Most (93%) of the glucose residues are 4-linked and the rest (7%) are 4,6-linked About two-thirds (64%) of the mannose residues are 4,6-linked and the rest are 4- (23%) and 6-linked (13%)

Since the results of methylation analysis suggested that the non-reducing end groups of GGM were mainly galactosyl residues, the removal of terminal galactosyl residues was tried Thus, the treatment of GGM with oxalic acid yielded the partially hydrolysed GGM (PH-GGM) The PH-GGM had $[\alpha]_D = +25°$ (water, *c* 0.5) and gave, on complete acid hydrolysis, D-galactose, D-glucose and D-mannose residues in the molar ratio of 0.47 1.00 0.78 Methylation analysis of PH-GGM (Table 2, column 2) revealed terminal galactopyranosyl, 4- and 4,6-substituted glucopyranosyl and 4-, 6- and 4,6-substituted mannopyranosyl residues in the molar ratio of 1.35, 3.00, 0.18, 1.29, 0.08, 1.17, respectively. The results indicated that the removal of terminal galactose residue caused the decrease of 2-linked galactose and 4,6-linked mannose residues and the increase of 4-linked mannose residue

These results suggested that GGM has a backbone of β-(1 → 4)-linked glucose and mannose residues and some mannose residues carry side-chains of galactose and *O*-galactosyl-(1 → 2)-galactose residues at C-6.

^{13}C NMR and methylation analysis of GGM indicated the presence of β-(1 → 4)-linked glucose residues, which could be degraded by *T viride* cellulase This cellulase, partially purified with a gauze column, contained no detectable activities of exo-glycosidases GGM and PH-GGM were treated with the cellulase at 37° for 2 days and the products were fractionated on a Bio-Gel P-2 column. For GGM, the elution profile was rather complex and it was not possible to purify each oligosaccharide except for one product (OS-1') On the other hand, PH-GGM gave a relatively simple elution profile and two main oligosaccharides (OS-1 and OS-2), each of which were homogeneous on TLC, were obtained This suggested that the enzyme treatment was not sufficient for GGM degradation, probably because of the presence of various side-chains, and that the removal of the side-chains by mild acid hydrolysis made more complete degradation possible Similar results have been obtained with AXG of the midrib of tobacco leaves, where 2 days of enzyme treatment was insufficient for the degradation of AXG [6] The AXG was completely degraded with the cellulase after 4 days of treatment [4]

Cellulase degradation of GGM and PH GGM yielded three pure oligosaccharides, OS-1', OS-1 and OS-2. Judging from its elution position from a Bio-Gel P-2 column, OS-2 was considered to be a disaccharide, which was confirmed by FD-mass spectrometric analysis The mass spectrum of OS-2 gave a base peak as a cation cluster $[M + Na]^+$ ion at *m/z* 365, indicating the MW of OS-2 to be 342 The results of sugar composition, methylation and NMR analysis (Table 2, column 5 and Table 3) revealed the structure of OS-2 to be *O*-β-D-Man-(1 → 4)-D-Glc

The elution position from a Bio-Gel P-2 column and FD-mass spectrometry (base peak at *m/z* 527) indicated that OS-1 was a trisaccharide Sugar composition, methylation (Table 2, column 4) and NMR (Table 3) analyses, coupled with the fact that the treatment of OS-1 with α-galactosidase from *E. coli* produced OS-2 (checked on TLC) indicated the structure of OS-1 to be *O*-α-D-Gal-(1 → 6)-β-D-Man-(1 → 4)-D-Glc OS-1' was found to be identical to OS-1.

From the above results, the basic structure of GGM could be deduced Thus, it has a main chain of β-(1 → 4)-linked, alternating with D-mannose and D-glucose residues About two-thirds of the D-mannose residues carry α-D-galactose units, and probably β-D-Gal-(1 → 2)-α-D-Gal side-chains, at C-6.

Table 2 Methylation analyses of GGM, PH-GGM and oligosaccharides

		Relative mol %				
Methylated sugar*	RR_t†	GGM	PH-GGM	OS-1'	OS-1	OS-2
2,3,5-Me_3-Ara	0.68	1	0	0	0	0
2,3,4-Me_3-Xyl	0.76	1	0	0	0	0
2,3,4,6-Me_4-Man	0.97	0	0	0	0	51
2,3,4,6-Me_4-Gal	1.00	21	19	26	37	0
2,3,6-Me_3-Man	1.12	7	18	0	0	0
2,3,6-Me_3-Glc	1.13	29	42	37	33	49
3,4,6-Me_3-Gal	1.14	13	tr	0	0	0
2,3,4-Me_3-Man	1.16	4	1	37	30	0
2,3-Me_2-Man	1.30	21	17	0	0	0
2,3-Me_2-Glc	1.31	2	3	0	0	0

*2,3,5-Me_3-Ara 2,3,5-tri-*O*-methyl arabinose, etc.

†RR_t Retention time relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-galactitol on OV-101

Table 3 ^1H and ^{13}C NMR data for fragment oligosaccharides

Oligosaccharides	Chemical shifts* (coupling constant in Hz)			
	β-D-Man	α-D-Gal	β-D-Glc† (R)	α-D-Glc† (R)
OS-1′	4 74 (1)	5 00 (2 9)	4 64 (6 3)	5 21 (3 9)
	101 1	99 2	96 7	92 8
OS-1	4 73 (1)	5 00 (3 2)	4 65 (8 1)	5 21 (3 9)
	100 8	99.6	96 6	92 6
OS-2	4 73 (1)	—	4 65 (7 8)	5 21 (3 9)
	100.9		96 7	92 7

*Int standard for ^1H NMR was sodium 4,4-dimethyl-4-silapentane-1-sulfonate (δ 0) and that for ^{13}C NMR was methanol (δ 49 8)

†R Reduced end-group

GGM are known to be structural constituent of woody tissues of gymnosperms and angiosperms [7]. They have also been isolated from the stems and leaf tissues of some legumes [8, 9] and the stems of an aquatic moss [10, 11] and a fern [12]. The presence of such polysaccharides has also been demonstrated in the seeds of some species of the Iridaceae and Liliaceae [13]. Recently, this class of polysaccharide has been briefly reviewed [14]. Generally speaking, the GGM so far reported have a main chain of β-(1 → 4)-linked D-glucose and D-mannose residues, to which are attached α-(1 → 6)-linked D-galactose groups as single stubs Moreover, there is no set pattern for the occurrence of the D-hexoses in the main chain and the distribution of the D-galactosyl stubs is also random. Accordingly, tobacco GGM reported here is unique in two respects: firstly, the main chain consists of alternate D-mannose and D-glucose residues and secondly, the stubs occur mainly on the D-mannose residues. A GGM with similar structure was also found in the hemicellulose fraction of the cell-wall material prepared from suspension-cultured tobacco cells [S. Eda *et al.*, unpublished results].

EXPERIMENTAL

General. Concn was performed under red pres at 45° or less Paper electrophoresis was performed on a Whatman GF/A glass microfibre paper at 1500 V for 30 min with 0.1 M sodium tetraborate (pH 9.2) Carbohydrate was detected by heating with H_2SO_4 HPLC was conducted using a constant-flow pump and a differential refractometer for monitoring the column effluent Separations were performed on a column of TOYO SODA TSK-Gel G 3000SW, which was calibrated with T-series dextrans (Pharmacia) using 0.9 % NaCl as eluant. Ultracentrifugation was done using an analytical ultracentrifuge at 60 000 rpm at 20° with samples (5 mg/ml) dissolved in 0 1 M NaCl GC was performed with FID and a column (glass capillary, 0 27 mm × 50 m) coated with OV 101 using N_2 at 1 ml/min. Total carbohydrate was determined by the PhOH–H_2SO_4 method [15] using glucose as standard Neutral sugar analysis was performed by GC of the diethyl dithioacetal TMSi derivatives [16, 3] after hydrolysis in 2 M TFA at 120° for 1 hr Methylation analysis was performed by the method of ref [17] as described in ref [18] GC and GC/MS of the partially methylated alditol acetates were essentially as described in ref. [19] ^{13}C NMR spectrum (25 1 MHz) [20] was obtained using 5 mm tubes (polysaccharide) or 1 mm tubes (oligosaccharide) in D_2O at 70° with MeOH (δ 49.8 from TMS) as int standard The $J_{C\text{-}1,H\text{-}1}$ value was determined by a gated, ^1H-decoupler sequence to retain the NOE ^1H NMR spectra (99 6 MHz) were obtained using 1 mm tubes with deuterium-exchanged (× 3) samples at 70° DSS was used as int standard TLC was performed on Si gel sheets (Eastman 13181) with (a) EtOAc–pyridine–H_2O (4 2 1) or (b) BuOH–*iso*PrOH–H_2O (3 12 4) (both developed twice) and detection with 50 % H_2SO_4

Preparation of GGM and PH–GGM Suspension cultures of tobacco cells were prepared as described in ref [3] After filtration and centrifugation to remove cells, the ECP soln was dialysed against 10 mM Tris–HCl buffer (pH 8) for 24 hr The clear soln was then applied to a column (2 5 × 15 cm) of DEAE-cellulose equilibrated in the same buffer The buffer washing fraction was collected, dialysed against H_2O and freeze-dried (F-1). F-1 (1 g) was dissolved in H_2O (200 ml) and $(NH_4)_2SO_4$ added to satn After the removal of ppt by centrifugation, the supernatant was dialysed, concd and heated on a column (2 6 × 85 cm) of Sephadex G-75 using 0 1 M NaCl as eluant After dialysis and freeze-drying, a purified polysaccharide (GGM) was obtained (300 mg)

Partial acid hydrolysis of GGM was done according to ref [9] GGM (200 mg) was treated with 25 mM oxalic acid for 6 hr at 100° and the product was eluted from a column (1 6 × 90 cm) of Bio-Gel P-2 (minus 400 mesh) The material recovered in the void vol fraction contained D-galactose, D-glucose and D-mannose residues in the molar ratio of 0 47.1 00 0 78 (PH–GGM, 130 mg), whereas the material in the monosaccharide fraction (50 mg) consisted of D-xylose, L-arabinose, D-mannose, D-glucose and D-galactose residues in the molar ratio of 0 02 0 07 0.06 0 08 1 0

Cellulase degradation Cellulase from *Trichoderma viride* (Meicellase, kindly provided by Meiji Seika Ltd) was partially purified by fractionation on a gauze column [21] The GGM or PH–GGM (100 mg) suspended in 0 1 M NaOAc buffer (pH 4 5) was incubated with the partially purified cellulase (20 mg, 68 units) at 37° for 2 days under a few drops of toluene After a digestion period, the reaction was stopped by heating at 100° for 30 min After the insoluble material was removed by centrifugation, the supernatant was deionized by passing through a Dowex 50W (H^+) column and freeze-dried The cellulase-degraded products were dissolved in 1 ml H_2O and fractionated by gel-filtration on a column (1.6 × 90 cm) of Bio-Gel P-2 From GGM, one oligosaccharide (OS-1′) was obtained in a homogeneous state, whereas from PH–GGM two oligosaccharides (OS-1 and OS-2) were obtained

OS-1′ (yield, 10 mg), eluted at 1 88 V_0, $[\alpha]_D$ + 62° (H_2O, *c* 0.2), RR_f glc = 0 29 (a), 0 38 (b) It gave, on complete acid hydrolysis, D-mannose, D-glucose and D-galactose residues in the molar ratio of 1 29 1 0 1 14

OS-1 (yield, 28.5 mg), eluted at 1.84 V_0, $[\alpha]_D$ + 75° (H_2O;

c 0 15), RR_f glc = 0 29 (a), 0 38 (b) It gave, on complete acid hydrolysis, D-mannose, D-glucose and D-galactose in the molar ratio of 1 16 1 0 1 06

OS-2 (yield, 33 mg), eluted at 2 08 V_0, $[\alpha]_D$ + 21° (H_2O; c 0 21), RR_f glc = 0 61 (a), 0 66 (b) It gave, on complete acid hydrolysis, D-mannose and D-glucose residues in the molar ratio of 1 13 1 0

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