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CELL WALL AND EXTRACELLULAR GALACTOGLUCOMANNANS FROM SUSPENSION-CULTURED *RUBUS FRUTICOSUS* CELLS

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Abstract—A galactoglucomannan (GGM) from the primary walls of suspension-cultured *Rubus fruticosus* and its counter part from the extracellular polysaccharides of the culture medium have been isolated. Barium hydroxide purification yielded a homogeneous extracellular polymer which comprised galactose, glucose, and mannose in the ratio 17:24.20. Methylation analysis and ^{13}C NMR data demonstrated that the two galactoglucomannans had the typical structural features of GGM from primary walls in that they consisted of a $\beta(1\rightarrow4)$ linked backbone of interspersed glucosyl and mannosyl residues in which mostly mannose carried substituents at O-6. More than two-thirds of the mannose residues were substituted, whereas glucose had very little substitution.

INTRODUCTION

Higher plant mannans typically represent cell wall storage polysaccharides [1]. They consist of a β-(1→4)-linked linear structural backbone of D-mannose residues in the case of pure mannans. In galactomannans the mannose chain may carry $\alpha(1\rightarrow6)$-linked D-galactosyl substituents. In glucomannans (GM), mannose usually predominates in the main chain in which variable sequences of single or contiguous β-(1→4) glucosyl residues are found. In galactoglucomannans (GGM), single units of α-D-galactopyranose residues may substitute D-mannose and D-glucose at *O*-6, a typical feature of gymnosperm and to a lesser extent angiosperm wood hemicelluloses [2]. In these woods, the usual proportions of glucose to mannose are 1:3 for GM and of galactose, glucose and mannose 1·1.3 for GGM. Different sugar ratios are found in GM from monocotyledonous sources with sometimes equal proportions of glucose to mannose. Also the position of the D-galactosyl substituent may occur at *O*-2 and/or *O*-3 [3, 4].

Mannose-rich cell wall polysaccharides from higher plants are characteristic of secondary walls. However, Simson and Timell [5] suggested the presence of glucomannans in the primary wall of cambial cells from *Populus tremuloïdes* and *Tilia americana*. Mannose usually accounts for a few percent of the sugars of the primary walls of suspension-cultured cells, being of the same order of magnitude as rhamnose. Although the rhamnose-containing galacturonans have been thoroughly investigated, there are only few reports about mannose containing polysaccharides from suspension-cultured cells. However, arabinoglucuronomannans have been isolated both in the cell wall and in the extracellular polysaccharides from suspension-cultured cells of *Nicotiana tabacum* [6, 7]. In these polymers $\alpha(1\rightarrow2)$ D-mannose residues were interspersed in the main chain with $\beta(1\rightarrow4)$-D-glucuronic acid residues both carrying at position three, simple terminal L-arabinofuranose and (1→5) linked α-arabinofuranobiose residues.

Recently, Stevens and Selvendran [8] isolated from cabbage leaves two xyloglucan fractions which contained a certain proportion of mannose. Although they suggested that the mannose residues could be part of an associated glucomannan, they could not separate it from the xyloglucan. Two galactoglucomannans from the extracellular medium and from cell walls of suspension-cultured *N. tabacum* have been isolated and shown to be made up of a backbone of alternating $\beta(1\rightarrow4)$-linked D-glucose and D-mannose residues highly substituted on the mannosyl residue at *O*-6 with α-D-galactopyranosyl or β-D-galactopyranosyl (1→2)-α-D-galactopyranosyl side chains [9, 10].

The study presented herein describes the isolation and characterization of two galactoglucomannans isolated from the primary walls of suspension-cultured *Rubus fruticosus* cells and from the extracellular polysaccharides released into the culture medium. The structure of the two related polymers is presented.

RESULTS AND DISSCUSSION

Carbohydrate analysis of the walls from suspension-cultured cells of *R. fruticosus* between day 10 and day 40 after inoculation of the mixture revealed an overall increase of the net content of neutral sugars in the wall after day 10. Neutral sugars amounted to 58% of the dry wt of the wall at day 10 and increased to 65% at the end of the exponential growth (day 24) to fall to 61% in the stationary phase (day 40). On the other hand, the relative molar proportion of the neutral sugars corresponded to a rather constant deposition of each constituent. The main difference in the relative monosaccharide composition was at day 40 which showed an increase in cellulosic glucose and a decrease in galactose. Mannose represented only 2.5–4% of the total neutral sugars, being present in amounts almost equal to those of rhamnose. The major part of this mannose was recovered in the 2.5 M sodium hydroxide extract (EA) which represent 11% of the total cell wall besides large amounts of xyloglucans. Xyloglucans were purified from EA by extraction with

DMSO and formation, upon addition of water, of a gel (50% of EA) which could be separated by centrifugation [11]. About 70% of the mannose from EA was recovered in the gel which mainly consisted of a xyloglucan. Precipitation of a mannose-containing polysaccharide with barium hydroxide [12] afforded an insoluble complex NS_3 (46% from the gel) in which 90% of the mannose of the gel was found Even, after three consecutive reprecipitations with 5% barium hydroxide the mannose-linked fraction (NS_3) was still associated with residual xyloglucan as indicated by the high proportion of xylose and glucose (Table 1) Barium precipitation proved to be very efficient for mannose containing polymers as shown by the analysis of the supernatant of the precipitation (S_1) which was enriched in xyloglucan and 1→3 glucan (Tables 1 and 2). Methylation analysis of the insoluble barium complex afforded all the expected methylated derivatives of a xyloglucan plus partially methylated mannose derivatives corresponding to (1→4)-linked mannose residues, nearly one out of two carrying a substituent at *O*-6 (Table 2). It was not possible to distinguish the glucose residues belonging to the glucomannan backbone from those belonging to the xyloglucan, since in both polysaccharides the glucopyranosyl residues have the same type of linkage and substitution. Xyloglucans cannot account for the high proportion of terminal galactose and therefore a part of it must belong to the other polymer which is therefore a galactoglucomannan In xyloglucans, wherever non terminal galactose is found, it is substituted at *O*-2 by a terminal L-fucose Thus, the relative amount of terminal fucosyl residues and of 3,4,6-tri-*O*-methyl galactose suggests that some 2-linked galactosyl units are also present in the structure of the GGM This agrees with the results of Eda *et al* [10]. A part of the preceding analytical and structural results was confirmed by the ^{13}CNMR spectrum of NS_3 The well resolved spectrum showed in the anomeric region the characteristic signals for xyloglucans [11] at δ 105, 102 2 and 98 8 ppm corresponding, respectively, to β-D-galactose, β-D-glucose and α-D-xylose, to which were added signals which can be ascribed to β-D-glucose, β-D-mannose and α-D-galactose residues of the galactoglucomannan at δ 102 9, 100 and 99 7 ppm, respectively The assignment of the latter series of signals could be confirmed by comparison with the spectrum of the pure GGM extracted from the extracellular culture medium

Carbohydrate analysis of the extracellular polysaccharides between day 10 and day 40 showed only discrete variations in the content of the predominant sugars. However, the relative amount of neutral sugars decreased from 50% at day 10 to 35% at day 40. The amounts of uronic acids remained nearly constant Despite the absence of cellulose in the culture medium, the proportion of glucose was still high, representing about one-third of the neutral sugars The galactose content which represented 19% of the neutral sugars was 26% at day 40 This seems to be related to the decrease observed in the cell walls In opposition the proportion of mannose represented from 3% of the total neutral sugars at day 10 to *ca* 6 5% at day 24 to fall to only 2% at day 30 Since most of

Table 1 Changes in sugar composition during the fractionation of EA_{24} from *Rubus*

	Neutral sugars (mol %)					
Fraction	Fuc	Ara	Xyl	Man	Gal	Glc
EA_{24}	3 5	5 5	25 5	10	15 5	40
Gel EA_{24}	3	1 5	23	11	12 5	49
S_1	3 5	2 5	24 5	1	7	61 5
NS_3	3	0 5	14	19 5	18	45

Table 2 Methylation analysis data of *Rubus* galactoglucomannan

	Relative mol %				
Methylated sugar*	Rt†	Gel	S_1‡	NS_3‡	EC-GGM§
2,3,4-Me_3-Fuc	0 7	3 0	3 0	2 5	–
2,3,4-Me_3-Xyl	0 8	15 5	16 5	11 0	–
2,3,4,6-Me_4-Gal	1 2	7 0	2 5	11 5	18 0
3,4-Me_2-Xyl	1 5	5 0	8 5	3 0	–
2,4,6-Me_3-Glc	1 6	3 5	16 5	–	–
2,3,6-Me_3-Man	1 8	4 0	Tr	7 0	15 5
3,4,6-Me_3-Gal	1 9	5 0	3 5	4 5	7 0
2,3,6-Me_3-Glc	2 0	24 5	16 0	31 5	41 5
2,3-Me_2-Man	2 8	5 5	Tr	9 5	16 0
2,3-Me_2-Glc	3 0	27	33 5	19 5	2 0

*2,3,4-Me_3-Fuc = 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-fucitol, etc

†Retention time relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol on SP 2340

‡S_1 Supernatant from barium precipitation, NS_3 barium complex precipitate

§EC-GGM Extracellular galactoglucomannan

the non-cellulosic polysaccharides from the cell wall are also found in the culture medium this suggested that a galactoglucomannan should be present in a substantial quantity amongst the extracellular polysaccharides at day 24.

Purification by complexation of the acidic polymers with copper acetate [13] led to a supernatant which was successively precipitated with ethanol and acetone. Most of the mannose was recovered in the acetone-insoluble fraction. Barium hydroxide purification afforded fairly pure galactoglucomannan (Table 3) in which there was less than 6% of a contaminant xyloglucan. The extracellular GGM (EC-GGM) had $[\alpha]_D = +23°$ (DMSO ; *c* 0.48). Methylation analysis corresponded to a (1→4)linked glucosyl and mannosyl backbone in which mostly mannose carries substituents at *O*-6 (Table 2). More than two thirds of the mannose residues are substituted whereas glucose is essentially unsubstituted The substituents are galactosyl residues mainly as single terminal units (72%) but also as short (1→2)-linked stubs.

The ^{13}C NMR spectrum of EC-GGM (Fig 1) was very well resolved showing *inter alia* five individualized signals in the region of the anomeric carbon atoms [7]. The resonance at lowest field at 105.3 ppm was characteristic of a β-D-galactopyranose. The C-1 at δ 103.3 and 100.9 ppm were respectively assigned to the β(1→4) linked glucopyranosyl and mannopyranosyl units. The two close signals at δ 99.8 and 99 7 ppm corresponded to the C-1 of α-linked galactopyranosyl residues. The signal at 99.8 ppm was assigned to terminal α-D-galactose. This resonance is moved slightly upfield due to the substitution at C-2 of a few α-D-galactose residues. It seems that about one third of the galactosyl units have a β-configuration and two thirds an α-configuration Confirmation of the structural analysis could be found with the signals of C-4 substituted glucose and mannose at 79.8 and 79.5 ppm, respectively, and with the C-6 substituted carbon atoms of glucose and mannose at 69.4 and 69.5 ppm.

From the above results it is concluded that similar galactoglucomannans exist in the cell wall of *Rubus fruticosus* and in the culture medium. In both cases the GGM represents only a small percentage of the cell wall or the extracellular polysaccharides This might not be the only mannose containing polymer since complex glucuronomannans have been characterized in primary cell walls [7,14]. However, apart from the GGM from tobacco cells, no other galactoglucomannans from suspension cells have been described. It confirms that in primary walls of dicotyledons these polymers of the family of glucomannans exhibit characteristic structural

Table 3 Sugar analysis of the fractions obtained during purification of extracellular galactoglucomannan (EC-GGM) from *Rubus*

	Neutral sugars (mol %)					
Fraction	Fuc	Ara	Xyl	Man	Gal	Glc
NS acetone	1 5	4	11 5	15	20 5	47 5
NS barium hydroxide	–	–	3	32	27	38
S barium hydroxide	2	7	19 5	10.5	20	41

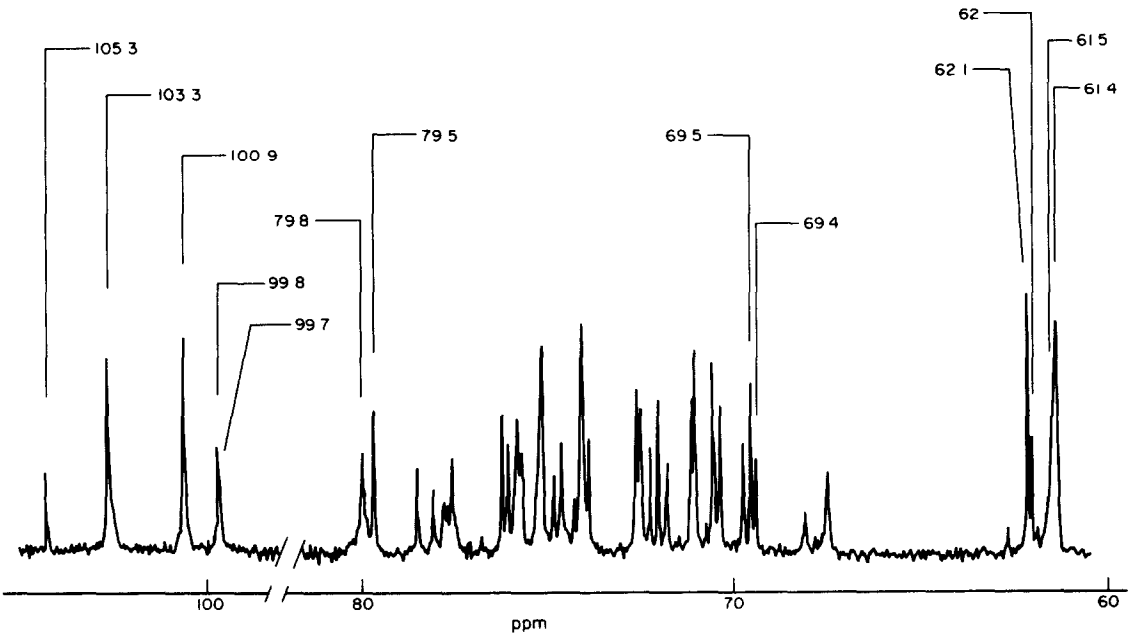

Fig. 1 ^{13}C NMR spectrum of the extracellular galactoglucomannan from *Rubus*.

features with nearly all of the substitution on mannose at *O*-6 and with both α- and β-D-galactopyranosyl side chain residues, some of them substituted at *O*-2 with a terminal galactose

EXPERIMENTAL

Analytical procedures Uronic acid was determined by the 3-hydroxydiphenyl method [15], protein by the method of ref [16] Samples of polysaccharide were hydrolysed with 1 M H_2SO_4 acid, for 6 hr at 100° in sealed tubes After neutralization with $BaCO_3$ the neutral sugars were analysed as acetylated alditol derivatives by FID-GC on glass columns (2 m × 3 mm) containing 3% of SP 2340, isothermal 220°, with N_2 (30 ml/min) as carrier gas *meso*-Inositol hexa-acetate was used as int st ^{13}C NMR (75 46 MHz) were obtained using 5 mm tubes, at 80°, in DMSO-d_6 (δ 39 6 from TMS) for cell wall GGM and in D_2O with acetone (δ 31 7 from TMS) as int st for EC-GGM Polysaccharides were methylated according to the methods of refs [17,18] Methylated polysaccharides were hydrolysed first in aqueous 90% HCO_2H (1 hr, 100°) then with 2 M TFA (4 hr, 100°) The partially methylated sugars were analysed by GC as their acetylated alditol derivatives using a fused-silica SP-2340, WCOT column (25 m × 0.32 mm) programmed 170°/3 min to 225°/15 min at 2°/min with He as carrier and the inj system in the split mode was GC/MS analysis performed with an apparatus equipped with WCOT OVI7 column (20 m × 0 3 mm) prog 160°/4 min to 210° at 2°/min.

Origin of blackberry cell walls Cells were derived from callus of *R fruticosus* and subcultured on Heller's medium supplemented with vitamin B1 (1 mg/1) and glucose (20 g/l) The cells were harvested at 10, 18, 24, 30 and 40 days after transfer by filtration of the culture medium through a sintered glass funnel and washing with H_2O Cell walls were isolated by the method of ref [19]

Sequential extraction of walls The various steps of the extn procedure were as described in ref [19] except that DMSO treatment was omitted and alkaline extn performed only with 2 5 M NaOH soln (20°, 18 hr under N_2) to give the EA fraction

Fractionation of extracellular polysaccharides (ECP) Pectic polymers in the culture medium of *Rubus* cells at day 24 (1 kg fr wt) were pptd as Cu salts (250 ml of 7% cupric acetate per 2 l of filtrate) The sol material was fractionated into EtOH insol (1 7 g) and Me_2CO-insol (0 6 g) components [13] The latter fraction could be further purified to give extracellular galactoglucomannan (EC-GGM)

Preparation of cell wall GGM and EC-GGM Cell wall GGM was obtained from EA as previously described [19] Gel formation was carried out as described in ref [11] To a soln of S(EA-DMSO) in DMSO (500 mg/100ml), 30 ml of H_2O was added in portions of 10 ml The gel formed was collected by centrifugation, dialysed and lyophilized to give 242 mg of gel EA_{24} This fraction (215 mg) was dissolved in 1 25 M NaOH (150 ml) and addition of 5% $Ba(OH)_2$ soln (150 ml) provided an insol fraction (NS_1) which was treated twice to obtain NS_3 (100 mg) composed of xyloglucan and GGM Similarly, EC-GGM (130 mg) was obtained from the Me_2CO insol fraction (900 mg)

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