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AN ARABINOGALACTAN FROM THE CULTURE MEDIUM OF *Rubus fruticosus* CELLS IN SUSPENSION

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ABSTRACT

A water-soluble arabinogalactan, isolated from the extracellular medium of suspension-cultured cells of *Rubus fruticosus*, contained arabinose, rhamnose, galactose, and also protein (6.5%) and uronic acid (2.5%). Methylation analysis of the arabinogalactan and the arabinose-free product obtained by mild acid hydrolysis showed that the polysaccharide was a typical arabino-3,6-galactan in which rhamnose and glucuronic acid occupied non-reducing terminal positions. Successive Smith-degradations combined with methylation analysis and ^{13}C-n.m.r. spectroscopy revealed that the arabinogalactan contained a main chain of (1→3)-linked β-D-galactopyranosyl residues with a high degree of branching at positions 6 by (1→6)-linked D-galactopyranosyl side-chains of various lengths, in which several contiguous residues were substituted at positions 3. The polymer is thus an arabinogalactan–protein belonging to the galactans of Type II.

INTRODUCTION

Arabinogalactans have been classified into two main groups which have similar compositions, but in which the D-galactan chains have quite different linkages[1]. Thus, Type I contains a backbone of (1→4)-linked β-D-galactopyranosyl residues to which may be attached single L-arabinofuranosyl units or, in some cases, disaccharide substituents[2]. Type II arabinogalactans are more widespread and occur in abundance in softwoods, cell walls of dicots, and cereals[3], and represent also the main structural feature of certain exudate polysaccharides (for example, gum arabic[4]). The core of Type II galactans shows great structural diversity, but the presence of a main chain of (1→3)-linked β-D-galactopyranosyl residues to which are attached side chains of (1→6)-linked β-D-galactopyranosyl residues seems to be a common feature. The distinction between arabinans associated with galactans and true arabinogalactans has not always been made clearly since degradations may occur during isolation. However, evidence for true arabinans and galactans has been reported[5,6].

Arabinogalactans have been found in the primary walls of suspension-

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cultured cells as side chains attached to the rhamnogalacturonan backbone of the pectic complex. Although pure arabinogalactans have not been isolated from primary cell walls[7], their biological role is suspected to be important[8]. Their close association with pectic polymers[6] or with the hydroxyproline-rich structural wall protein[8] makes them difficult to isolate in a pure form.

The culture medium of cell suspensions seems to contain all the non-cellulosic polysaccharides present in the cell walls[9], and these are often easier to isolate than from the cell walls. Two arabino-3,6-galactans (Type II) were described as extracellular polysaccharides (ECP) of suspension-cultured sycamore cells[10,11], but these appeared to be a continuous spectrum of arabinogalactans which were heterogeneous in size, degree of branching, and degree of arabinosylation[8].

The release of arabinogalactan into the culture medium of suspension cultures of *Vinca rosea* was considered to indicate the turnover of the corresponding cell-wall polymer[12]. The association of the arabinogalactan from *Nicotiana tabacum* with protein has been demonstrated[13], and therefore this polysaccharide exists as an arabinogalactan–protein (AGP).

We now report on the isolation, purification, and structure of the extracellular AGP present in the culture medium of blackberry cells in suspension.

EXPERIMENTAL

General methods. — Uronic acid was determined by the 3-hydroxybiphenyl method[14], protein by the method of Lowry *et al.*[15], and hydroxyproline by the colorimetric procedure described by Kivirikko and Liesmaa[16].

Samples of polysaccharide were hydrolysed in aqueous 72% H_2SO_4 (2 h, 30°) followed by dilution to M acid and hydrolysis for 1 h at 120° in sealed tubes. Neutral sugars were then analysed as their alditol acetates by g.l.c. at 220° on glass columns (2 m × 3 mm) containing 3% of SP-2340 on Chromosorb W-AW DMCS (100–120 mesh); *myo*-inositol hexa-acetate was used as internal standard.

^{13}C-N.m.r. spectra (75.46 MHz) were recorded with a Bruker AM 300 spectrometer for solutions in $(CD_3)_2SO$. The signal for Me_2SO at 39.6 p.p.m. relative to that of Me_4Si served as the internal reference.

Optical rotations were measured with a Perkin–Elmer polarimeter at 589 nm.

Fractionation of the extracellular polysaccharides (ECP). — The culture medium of suspension-cultured *Rubus fruticosus* cells at day 24 (1000 g, fresh weight) was filtered and then centrifuged at 2000*g* for 15 min at 4°, and aqueous 7% cupric acetate (250 mL for 2 L of filtrate) was added. The precipitated copper salt of the acidic polysaccharide was collected by centrifugation and a solution in aqueous 5% hydrochloric acid was diluted with ethanol (3 vol.). The precipitate was collected by centrifugation, and an aqueous solution was dialysed against water and then freeze-dried to give the carboxyl form of the polysaccharide (3.3 g).

The supernatant solution from the copper acetate precipitation was poured into ethanol (3 vol.), the precipitate was collected by centrifugation, and a solution

in water was dialysed against water and then freeze-dried to give the ethanol-insoluble material (1.7 g). The supernatant solution was poured into acetone (3 vol.), the precipitate was collected by centrifugation, and a solution in water was dialysed against water and then freeze-dried to give the acetone-insoluble material (0.6 g).

A solution of the ethanol-insoluble material (1 g) in water (50 mL) was adsorbed on to a column of cellulose (150 g; previously washed with M NaOH) and eluted with water (330 mL), to give arabinogalactan F-H_2O (0.212 g).

The column was then eluted with 0.5M NaOH (210 mL) followed by M NaOH (185 mL), and the eluates were neutralised, dialysed against water, and freeze-dried to give xyloglucans F-NaOH(0.5M) (0.110 g) and F-NaOH(M) (0.263 g), respectively.

Methylation analysis. — Polysaccharides were methylated according to the methods of Hakomori[17] and Purdie[18].

Methylated polysaccharides were hydrolysed first in aqueous 90% formic acid (1 h, 100°) and then with 2M trifluoroacetic acid (100°, 4 h). The resulting partially methylated sugars were analysed as their acetylated alditol derivatives by g.l.c. on fused-silica capillary columns (25 m × 0.32 mm) containing SP-2340, with a capillary injection-system in the split-mode. Temperature programme: 3 min at 170°, 2°/min to 225°, 225° for 15 min.

G.l.c.–m.s. was performed using a Girdel 3000 apparatus equipped with capillary columns (20 m × 0.3 mm) containing OV-17. Temperature programme: 160° for 4 min, then 2°/min to 210°.

Selective hydrolysis. — Arabinogalactan F-H_2O (200 mg) was hydrolysed with 0.05M oxalic acid for 2 h at 100°. The hydrolysate was dialysed against distilled water and freeze-dried to give F-H_2O-H^+ (120 mg).

Smith degradation. — Fraction F-H_2O-H^+ (100 mg) was oxidised with 0.05M sodium metaperiodate (40 mL) at 20° in the dark for 48 h. Oxidation was stopped by the addition of ethylene glycol, the solution was dialysed against distilled water, and the product in the dialysate was reduced conventionally with $NaBH_4$ for 15 h. The solution was neutralised with acetic acid and then dialysed against distilled water, the non-dialysable material was hydrolysed in M trifluoroacetic acid for 48 h at 20°, the acid was removed by repeated evaporations in H_2O, and the residue was dialysed and freeze-dried, to give the degraded polymer (25 mg). Second and third Smith-degradations were performed under the same conditions, to give 13 and 5 mg, respectively, of degraded polymer.

RESULTS AND DISCUSSION

The pectic polymers in the culture medium of *Rubus fruticosus* cells after 24 days were precipitated as the copper salt[10]. The soluble material was fractionated into ethanol-insoluble and acetone-insoluble components. Analysis of the three products (Table I) confirmed the effectiveness of the fractionation procedure. The

TABLE I

SUGAR ANALYSIS OF THE FRACTIONS OBTAINED FROM THE SUSPENSION-CULTURE FILTRATE OF *Rubus fruticosus* CELLS

Fraction	*Yield*[a]	*Uronic acid*[b]	*Neutral sugars*[b]					
			Rha	*Fuc*	*Ara*	*Xyl*	*Man*	*Gal*	*Glc*
Copper-salt	330	66.5	3.5	tr.[c]	11	1	0.5	15	2.5
Ethanol-insoluble	170	2.5	tr.	3.5	9	26	1	19	39
Acetone-insoluble	60	4.5	—	1.5	3	9.5	15	20	46.5

[a]Mg/100 g of fresh cells. [b]Percentage. [c]Trace.

ethanol-insoluble material contained a mixture of xyloglucans and arabinogalactans, and the acetone-insoluble material contained a mixture enriched in a mannose-containing polymer. The latter fraction could be purified further by barium complexation to give a galactoglucomannan[19].

The ethanol-insoluble material was fractionated by elution from a cellulose column successively with water, 0.5M NaOH, and M NaOH. A hundred-fold excess of cellulose was necessary to adsorb the xyloglucans completely. The fraction (F-H_2O) eluted with water contained only ~3% contamination with other polysaccharides (Table II), together with 2.5% of uronic acid and 6.5% of protein in addition to arabinose and galactose. That the protein moiety was an integral part of the arabinogalactan–protein fraction was indicated by the fact that the overall protein content increased to 12.5% after selective hydrolysis of the arabinose and to 14.2% after Smith degradation. The protein moiety contained only 6.6% of hydroxyproline[20].

The ratio of galactose to arabinose in fraction F-H_2O was 1.5, which reflects the proportions generally found in primary walls[3]. Methylation analysis of the arabinogalactan showed that 90% of the arabinose occupied terminal positions; the remainder was 5-sustituted (Table III), indicating F-H_2O to be an arabinogalactan and not an arabinan–galactan.

TABLE II

FRACTIONATION OF THE ETHANOL-INSOLUBLE FRACTION BY CHROMATOGRAPHY ON CELLULOSE

Fraction	*Yield*[a]	*Neutral sugars (molar ratio)*					
	Rha	*Fuc*	*Ara*	*Xyl*	*Man*	*Gal*	*Glc*
Ethanol-insoluble	100	tr.[b]	4	10	30	0.5	18.5	37
F-H_2O	21.2	4	—	37	1.5	0.5	55.5	1.5
F-NaOH(0.5M)	11	—	3	—	45	—	7	45
F-NaOH(M)	26.3	—	2.5	tr.	30.5	tr.	6.5	60.5

[a]Mg/100 mg of the ethanol-insoluble fraction. [b]Trace.

TABLE III

METHYLATION ANALYSIS DATA

Linkage[a,b]	*Mole per cent of recovered carbohydrate*				
			Smith degradation		
	F-H_2O	*F-H_2O-H^+*	*1*	*2*	*3*
T-Rha*p*	3.5	2.5	—	—	—
T-Ara*f*	43	5	—		
T-Gal*p*	4	10.5	17.5	11	12
5-Ara*f*	5	Trace	—	—	—
3-Gal*p*	5	6.5	35	78	77
6-Gal*p*	4	51.5	32	—	—
3,6-Gal*p*	35.5	24	15.5	11	11

[a]The sugars corresponding to the contaminating xyloglucans and representing <3% of the methylated compounds are not reported. [b]T-Rha*p*, terminal rhamnopyranose; 5-Ara*f*, 5-linked arabinofuranose *etc.*

The main core of the arabinogalactan comprises a (1→3),(1→6)-linked galactan as indicated by the presence of 3- and 6-linked galactose, and the large proportion of 3,6-linked galactose indicated a high degree of branching. The relative amounts of terminal residues suggested that most of the branches were terminated by an arabinosyl group, and only 20% of the galactose residues did not carry a side chain. Thus, the extracellular arabinogalactan from *Rubus fruticosus* is of the same type as that from *Acer pseudoplatanus*[10] or *Nicotiana tabacum*[13].

Partial hydrolysis of fraction F-H_2O with 0.05M oxalic acid[21] removed ~90% of the arabinose, and methylation analysis (Table III) of the resulting galactan (F-H_2O-H^+) showed that there was an increase in terminal galactose and 6-linked galactose and confirmed that most of the arabinose had been present as terminal units. It was established that many of (1→6)-linked galactosyl residues carried a single arabinofuranosyl substituent at position 3. Thus, fraction F-H_2O-H^+ is essentially a galactan containing mainly 6-linked galactosyl residues, few 3-linked galactosyl residues, and ~25% of disubstituted residues. Smith degradation of fraction F-H_2O-H^+ resulted in a 75% of loss of material, which accorded with the predicted amount based on the presence of ~70% of periodate-vulnerable residues. Methylation analysis of the non-dialysable material (Table III) revealed increased proportions of 3-linked and terminal galactose residues, reduced proportions of 6- and 3,6-linked galactosyl residues, and that the 6-linked galactosyl residues were contiguous and directly linked to the (1→3)-linked galactan main-chain.

The second Smith-degradation was accompanied by ~50% loss of material and gave a (1→3)-linked galactan which still contained a relatively high proportion of short side-chains, and this was confirmed by the results of a third Smith-degrada-

tion. The fact that three consecutive Smith-degradations did not lead to a linear polymer reflects the high degree of branching of the galactan.

The ^{13}C-n.m.r. spectrum (75.46 MHz) of the arabinogalactan contained 16 major signals (Fig. 1). The signals at 108.7 and 103.3 p.p.m. can be assigned to C-1 of α-L-arabinofuranosyl and α-D-galactopyranosyl residues, respectively. These configurations accord with the $[\alpha]_D$ value of −33° (water) of the arabinogalactan[13]. The resonances of the arabinose residues were assigned on the basis of the spectrum of the dearabinosylated galactan (F-H_2O-H^+) and by reference to previous results[22]. Loss of the arabinofuranosyl residues led to the loss of the peak at 78.9 p.p.m. for C-3 of the galactopyranose substituted by terminal arabinose and the appearance of a signal at 73 p.p.m. for C-3 of unsubstituted galactose. Dearabinosylation also enhanced the signals due to the galactan and resolved the signals at 73.0 and at 67.6 p.p.m., to give signals at 73.2 (C-5), 73.0 (C-3), and 68 and 67.8 p.p.m. (C-6 and C-4 of 6-linked Gal) (Table IV). Methylation analysis showed that ~33% of the galactosyl residues in arabinogalactan F-H_2O-H^+ were 3-substituted. Thus, it is surprising that the intensity of the signal of C-3 involved in a glycosidic linkage was not more prominent at 81.7 p.p.m. Similar results have been reported for other highly substituted arabinogalactans, where a broad peak was observed[23,24].

TABLE IV

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

Fraction	*Residue and linkage assignment*	*Chemical shifts (p.p.m.)*					
		C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
Arabinogalactan (F-H_2O)	α-L-Ara*f*-(1→	108.7	81.0	77.4	85.0	61.5	
	→6-β-Gal*p*-(1→	103.3	70.4	72.8 78.9[a]	67.8	73.0	68.1
Dearabinosylated galactan (F-H_2O-H^+)	→6)-β-D-Gal*p*-(1→	103.3	70.3	73.0	67.8	73.2	68.0
Smith-degradation products: 1	→6)-β-D-Gal*p*-(1→	103.3	70.3	73.0	67.8	73.2	68.1
	→3)-β-D-Gal*p*-(1→	103.7 102.8[b]	70.0	81.7	67.3	74.8	60.1
2	→3)-β-D-Gal*p*-(1→	103.7 102.9[b]	70.1	81.9	67.3	74.8	60.2
3	→3)-β-D-Gal*p*-(1→	103.9	70.3	82.0	67.5	75.0	60.3

[a] →6)-β-D-Gal*p*-(1→
3
↑
1
α-L-Ara*f*

[b] →3)-β-D-Gal*p*-(1→
6
↑
1
β-D-Gal*p*

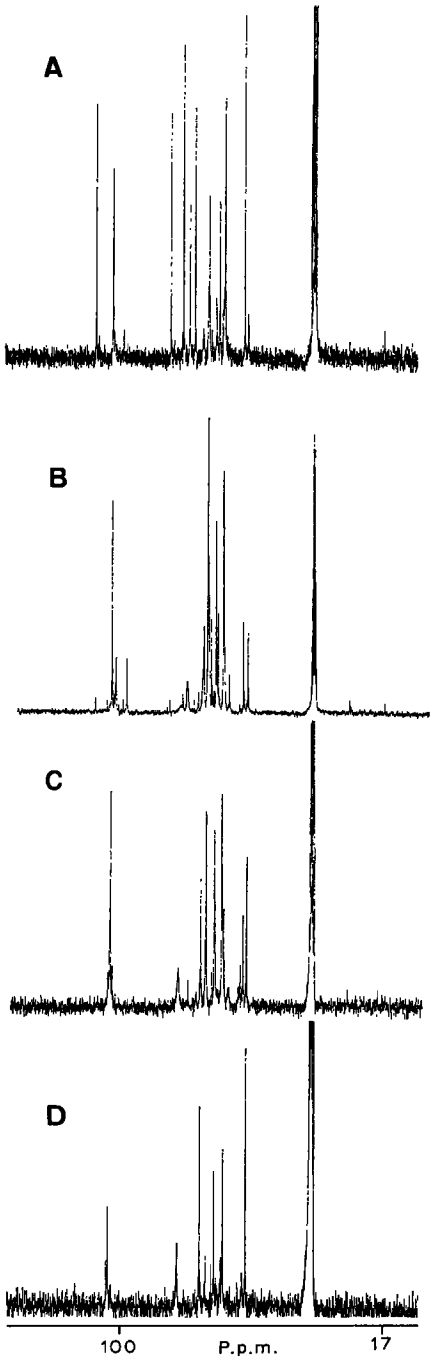

Fig. 1. ^{13}C-N.m.r. spectra: A, arabinogalactan (F-H_2O); B, dearabinosylated galactan (F-H_2O-H^+); products of 1st (C) and 2nd (D) Smith degradations.

The complexity of the C-3 resonances, due to the differently substituted 3-linked D-galactopyranosyl residues, was reduced only slightly in the spectra of the non-dialysable products of the three Smith-degradations. This could reflect conformational irregularity within the galactan chain.

The progressive modifications in the substitution pattern of the products of the Smith degradations allowed several of the signals to be assigned to the 3- and the 6-linked D-galactopyranosyl residues (Table IV). In particular, the complex signals for C-1 of the β-galactosyl residues could be assigned, since the (1→3)-galactan obtained after the third Smith-degradation showed only one signal at 103.9 p.p.m. The signal (103.3 p.p.m.) at higher field can be assigned therefore to (1→6) linkages. Homopolymers provide better references for signal assignment in polysaccharides than glycosides[24].

The foregoing data indicate that the extracellular arabinogalactan produced by suspension-cultured cells of *Rubus fruticosus* is an arabinogalactan and not an arabinan–galactan.

The polymer is similar to that from *Nicotiana tabacum*[25], but the proportion of hydroxyproline in the protein moiety is low compared to data in the literature[3,25,26] and, by analogy with other arabinogalactan–proteins[25,27], it is likely that the uronic acid is glucuronic acid. The correlation between this extracellular AGP and the arabinogalactans of Types I and II identified in the cell walls of *Rubus fruticosus* is being studied.

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