Carbohydrate Research, 212 (1991) 177–186
Elsevier Science Publishers B.V., Amsterdam

Alfalfa-stem pectins: enzymic degradation and structural characterization of a buffer-soluble fraction

Ronald D. Hatfield
U.S. Dairy Forage Research Center, USDA-Agricultural Research Service and Department of Agronomy, University of Wisconsin, 1925 Linden Drive West, Madison, WI 53706 (U.S.A.)

(Received June 5th, 1990; accepted for publication September 11th, 1990)

ABSTRACT

Carbohydrate material solubilized from alfalfa (*Medicago sativa* L.) stem cell walls with hot 10mM potassium phosphate (pH 7.0) was fractionated into neutral and acidic components by anion-exchange chromatography. Glycosyl-composition and glycosyl-linkage analysis indicated that the neutral fraction contained arabinogalactan type I and that the major acidic fraction was composed of rhamnogalacturonans. The rhamnogalacturonans were resistant to depolymerization by endo-(1→4)-α-D-galacturonanase. Saponification of the rhamnogalacturonans followed by acidification to pH 5 gave a precipitate and a soluble fraction. The precipitate after resolubilization was extensively depolymerized by endo-(1→4)-α-D-galacturonanase. Rhamnogalacturonans in the soluble fraction, which contained a high proportion of neutral sugar residues, were only partially depolymerized by endo-(1→4)-α-D-galacturonanase. Products separated by gel filtration were shown by glycosyl-composition analysis to be composed of different proportions of neutral sugar residues and galactosyluronic residues.

INTRODUCTION

Plant cell walls are composites of polysaccharides, lignin, proteins, water, and ions, differing in composition depending upon the structural or functional role of the cell[1]. Pectic polysaccharides constitute one group of cell-wall components. Studies of pectic fractions have been made on a number of plant species[2,3], including alfalfa[4–6], and have shown these fractions to be complex mixtures of polysaccharides[2–6]. Aspinall *et al.*[4,5,6] previously characterized pectic polysaccharides isolated from alfalfa. They concluded that these polysaccharides were comprised of two types of associated arabinogalactans and rhamnogalacturonans, the latter varying in degree of methyl-esterification. Although they indicated that one of the fractions contained an acidic arabinogalactan[4], its chemical composition was unclear.

Work with pectic materials from other plant species[7–11] has indicated that there are subclasses of pectic polysaccharides containing regions abundantly substituted with neutral sugars, often referred to[8] as "hairy regions". These regions may vary in size and frequency within pectic polysaccharides and are typically composed of arabinose- and galactose-containing segments. Previously we isolated a pectic fraction from alfalfa stems that contained a large amount of neutral sugar[12], similar in composition to the water-soluble fractions identified by Aspinall and Molloy[3]. We were interested in the fine structure and enzymic degradation patterns of the major polysaccharides in this

fraction. This report describes the characteristics of this class of buffer-soluble polysaccharides from alfalfa.

EXPERIMENTAL*

Alfalfa (*Medicago sativa* L.) plants were grown in a greenhouse under high-pressure sodium lamps with a 14 h/10 h day/night regime. Plants were cut 5 cm above the soil line when flower buds had formed but not opened. Harvested plants were frozen immediately, lyophilized, and separated into stems and leaves. Stems were further divided into sections: the apical section (AN) comprising the upper 3–5 cm of main stems and branches; upper sections (UN) comprising the top 7–8 nodes and internodes below the apical section; and the lower section (LN) comprising the 7–8 nodes and internodes at the base of the plant.

Cell wall isolation. — Stem material from each section was cut into pieces 5–10 mm long and subsamples removed for cell wall isolation. The samples were then homogenized in cold (5°) sodium phosphate buffer (10mM + 50mM NaCl, pH 7.0, 50 mL/g dry tissue) in a Waring blendor†. Two drops of octanol were added to reduce foaming. The total homogenate was transferred to a Fleaker filter apparatus (Spectrum) containing two 52 μm Teflon-mesh filters (Spectrum). Cell-wall material was washed with cold 50mM NaCl (2000 mL, 5°), to remove cytoplasmic contaminants, followed by acetone until all residual pigment was removed, and finally 2:1 chloroform–methanol (500 mL). After the final wash, air was drawn through the preparation until it was dry. The cell walls were transferred to a jar mill with 80% ethanol and brought to a final volume of 100 mL. Each sample was milled until the particle size was approximately 500 μm (4–5 h), and collected on a glass fiber filter using vacuum filtration. The milled walls were washed with 80% ethanol, followed by acetone, and air dried.

Isolation of buffer-soluble polysaccharides. — The milled cell walls (1–2 g) were suspended in 50 mL of potassium phosphate buffer (10mM + 0.02% NaN_3, pH 7.0) and heated for 1 h in a boiling water bath. Samples were cooled to 60–65°, treated with 10 units of α-amylase (Sigma A-3403), and placed in a water bath for 2.5 h at 55°. The α-amylase treatment was imposed to remove possible starch contamination. After incubation the cell walls were pelleted by centrifuging at 2500 × *g* for 10 min. The supernatant containing extracted polysaccharides was decanted into a filtration flask fitted with a glass-fiber filter. The walls were washed four times with 30 mL of deionized water and lyophilized. The amylase treated extract and washes were combined and dialyzed against water (8–10 L) for 48 h and lyophilized. This extract was primarily pectin, thus resembling hot-water extracts. It will be referred to as the HW-extract of the cell wall (Fig. 1).

* Abbreviations: PG, endo-(1→4)-α-D-galacturonanase; TFA, trifluoroacetic acid; THF, tetrahydrofuran.

† Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee of the product by USDA and does not imply its approval to the exclusion of other products that also may be suitable.

DEAE anion-exchange chromatography. — The HW-extract was fractionated on a DEAE Spectra/Gel M column (2.5 × 15 cm). Samples were dissolved in 10 mL (2.5 mg/mL) of 10mM sodium acetate buffer, pH 5.75, and applied to the DEAE column, which was equilibrated with the same buffer. Elution was started with the equilibration buffer, and continued with a linear gradient of NaCl (0–750mM, total volume of 200 mL) in the buffer. Elution profiles were determined by measuring the total neutral sugar and total uronic acid residues in even-numbered fractions (see below for methods). Fractions corresponding to peaks of total neutral sugar and total uronics were pooled, dialyzed against water, and lyophilized. Pooled fraction F4 (Fig. 2) contained the largest amount of uronic acid residues and was comprised of fractions having elution volumes between 210 and 255 mL.

Gel-filtration chromatography. — Uronic acid-rich fractions (F4) pooled from the DEAE column were subjected to gel-filtration chromatography. Samples (5–10 mg) were dissolved in 5 mL of 200mM sodium acetate buffer, pH 3.75, and applied to a Toyopearl HW-55 TSK column (2.3 × 110 cm) equilibrated with the same buffer. Polysaccharides were eluted from the column with 400 mL of the equilibration buffer, at a constant flow of 25 mL/h. Fractions (3 mL) were collected and assayed for total neutral sugars and total uronic acids.

Polysaccharide samples partially degraded with endo-(1→4)-α-D-galacturonanase (EC 3.2.1.15) were fractionated on the same column run under identical conditions. Fractions (3 mL) were collected and assayed for total neutral sugars and total uronic acids. Fractions containing peaks of total carbohydrate and eluting early (F4-S-A, F4-S-B, F4-I-A, and F4-I-B, Fig. 3) were pooled, dialyzed, and lyophilized. Carbohydrate from pooled, later-eluting fractions was recovered by adding ethanol to 80% and collecting the preciptate as a pellet after centrifugation. The pellet was dissolved in water and lyophilized.

Enzyme degradation of isolated fractions. — Pooled fractions, collected from the DEAE column, containing the highest amounts of uronic acid (F4, Fig. 1) were used in enzyme-degradation studies. Endo-(1→4)-α-D-galacturonanase (EC 3.2.1.15) was purified from the commercial enzyme preparation Pectinex (NOVO)[15]. Samples were dissolved in 5 mL of sodium acetate buffer (20mM + 0.01% NaN_3, pH 5.0), and treated with purified PG (3 units, 1 unit = 1 μmol of reducing equivalents released per min). The reaction mixture was incubated for 20 h, with additional enzyme added at 5 h and 15 h. Following incubation the mixture was heated for 2 min at 100° to terminate the reaction, cooled, and applied to the HW-55 TSK column.

Methyl ester analysis of the F4 fraction indicated that approximately 41% of the uronic acid residues were esterified. A portion of the fraction was treated with 0.5M NaOH for 1 h at 25° to saponify the methyl esters. The hydrolyzed samples were cooled on ice and the pH was adjusted to 5.0 with glacial acetic acid. Precipitated material was pelleted and the supernatant carefully removed. Sodium azide was added to the supernatant to a final concentration of 0.01%, then PG was added and incubation was conducted as described above. The precipitate was dissolved in H_2O at 70°, made to 10mM sodium acetate with 200mM acetate buffer, pH 5.0, 10% NaN_3 was added to give a

final concentration of 0.01%, and the sample was treated with PG in the same way.

Partial acid hydrolysis of saponified fraction F4 polysaccharides. — A sample of saponified DEAE-F4 (F4-S, Fig. 1) was dissolved in water, made 0.1M in TFA with 2M TFA, and heated for 1 h at 100°. Polysaccharides remaining in the mixture were precipitated by adding ethanol to 80%. The supernatant was removed and dried under a stream of filtered air. The pellets were dissolved in water and lyophilized. A small amount of the TFA-treated polysaccharides (F4-S-TFA, Fig. 1) was taken up in 20mM sodium acetate buffer, pH 5.0, and treated with PG.

General procedures. — The methyl ester content of polysaccharide samples was determined by the procedure of Wood and Siddiqui[16]. Total sugars were determined by the phenol–sulfuric acid method[13], using arabinose as a standard. Total uronics were determined using the 3-phenylphenol method[14], with galacturonic acid as a standard. Isolated polysaccharides were hydrolyzed with 2M TFA for 1.5 h at 120°. The monosaccharides were analyzed by g.l.c. as their alditol acetates using the procedure of Blakeney *et al.*[17].

Polysaccharides (0.5–2.5 mg) were methylated using *n*-butyllithium and methyl iodide as described by Carpita and Shea[18]. Recovery of the methylated material was accomplished as described by Harris *et al.*[19]. Methylated polysaccharides were hydrolyzed with 2M TFA (1.5 h, 120°) and the monosaccharides converted into partially methylated alditol acetates using the procedure of Blakeney *et al.*[17]. Uronic acids were identified as the corresponding sugar alcohols following reduction with $LiAlD_4$ using the procedure of Lindberg[20], with the following modifications: Each dried, methylated polysaccharide sample was dissolved in 2.5 mL of THF, under argon, and 7.5–10 mg of $LiAlD_4$ was added with mixing. The tubes were immediately capped with Teflon-lined screw caps and heated for 5 h at 72°. After heating, excess $LiAlD_4$ was destroyed by sequentially adding a few drops of ethyl acetate, ethanol, and water. Three mL of chloroform was added, and the organic layer was washed twice with 2 mL of saturated aqueous potassium sodium tartarate. The organic fraction was concentrated under a gentle stream of dry air. The residue was hydrolyzed with 2M TFA (1.5 h, 120°) and converted into partially methylated alditol acetates using the procedure of Blakeney *et al.*[17].

Partially methylated alditol acetates were analyzed by g.l.c. on a 60 m × 0.25 mm DB-1 capillary column (J&W Scientific, 1 μm film thickness). Samples were injected at a column temperature of 150° and separated using a temperature program of 4°/min increase to 250°, with holding at the final temperature for 25 min. The carrier gas was He at a flow rate of 0.7 mL/min. The injector temperature was 275° with a split ratio of 50:1. Peaks were detected with a Hewlett–Packard mass-selective detector, model MSD 5970.

RESULTS

DEAE column chromatography. — Treatment of alfalfa cell walls with hot 10mM phosphate buffer extracted approximately 33% of the total pectic materials of the wall matrices of the different stem sections. The polysaccharides contained in these stem

extracts were similar in their content of uronic acids and their neutral sugar composition (Table I). Therefore, for a description of their characterization these polysaccharides will be discussed as a single group, and will be referred to as the HW-extract.

Polysaccharides in the HW-extract were analyzed following the scheme outlined in Fig. 1. The extract was resolved into neutral and charged fractions using DEAE anion-exchange chromatography. Although small amounts of carbohydrate did not bind to the column, the majority of the polysaccharides were retarded, and eluted as one peak (F4, Fig. 2). Smaller amounts of retarded material eluted in fractions F2 and F3, just ahead of F4.

TABLE I

Total uronic acid and neutral sugar composition of polysaccharides solubilized with hot phosphate buffer from different alfalfa stem sections

Stem section[a]	*Uronic acid (mol %)*	*Neutral sugars (mol %)*[b]						
		Rha	*Fuc*	*Ara*	*Gal*	*Xyl*	*Man*	*Glc*
AN	46.7	11.6	3.9	37.2	25.9	9.2	2.8	9.3
UN	42.5	11.2	2.6	44.2	30.6	8.0	2.8	9.6
LN	43.8	10.4	2.8	32.3	27.2	7.4	4.7	15.3

[a] AN, apical nodes and internodes; UN, upper nodes and internodes; LN; lower nodes and internodes. [b] Of the total neutral sugar.

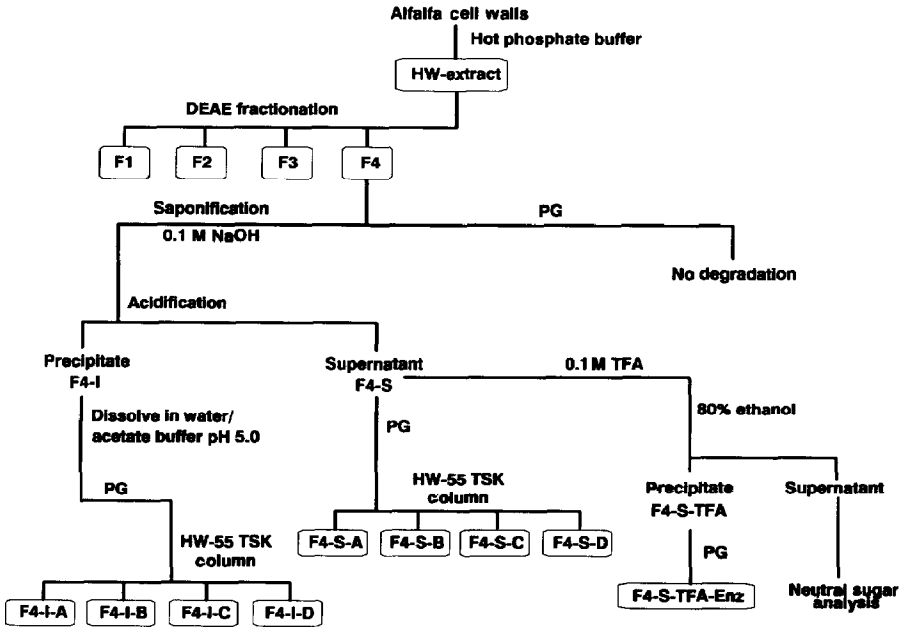

Fig. 1. Flow chart for the fractionation and analysis of polysaccharides in hot-buffer extracts of alfalfa stems. Abbreviations as described in the text.

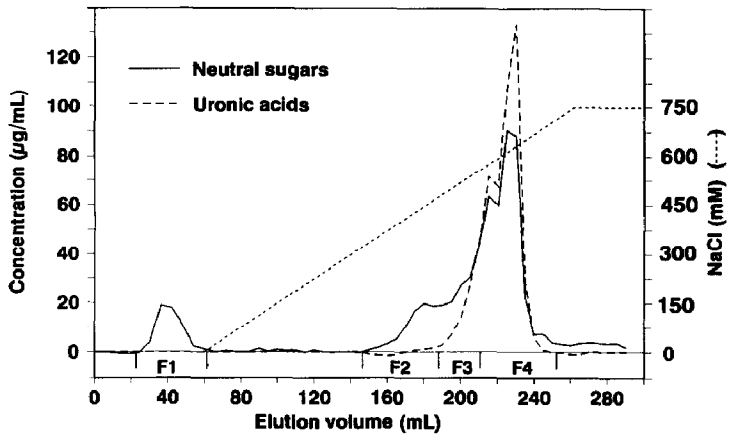

Fig. 2. Elution profile of alfalfa-stem HW-extract (10 mg carbohydrate) chromatographed on a DEAE-Spectra/Gel column. Contents of individual tubes were pooled into four fractions, F1 (22–58 mL), F2 (142–186 mL), F3 (187–210 mL), and F4 (211–240 mL).

TABLE II

Partially methylated alditol acetates derived from polysaccharides of alfalfa-stem cell walls, fractionated by anion-exchange chromatography

Glycosyl residue	*Methylated alditol*	*Linkage position(s)*	*Proportions (mol fraction)*			
			F1[a]	*F2*	*F3*	*F4*
Ara	2, 3, 5	T	0.184	0.145	0.147	0.126
	2, 5 & 3, 5	3 & 2	0.037	0.032	0.052	0.011
	2, 3	5	0.307	0.156	0.065	0.326
	2	3, 5	0.113	0.026	0.065	0.069
	3	2, 5	0.034	0.018	0.030	0.042
Gal	2, 3, 4, 6	T	0.010	0.022	0.029	0.046
	2, 3, 6	4	0.132	0.091	0.085	0.067
	2, 4, 6	3	0.023	0.038	0.030	0.033
	2, 3, 4	6	0.017	0.053	0.056	0.035
	2, 4	3, 6	0.071	0.170	0.110	0.007
	2	3, 4, 6	0.002	0.008	0.011	0.049
	3	2, 4, 6		0.003		0.010
Rha	3, 4	2		0.003	0.004	0.071
	3	2, 4		0.018	0.045	0.080
Xyl	2, 3, 4	T	0.008	0.017	0.014	0.025
	3, 4 & 2, 3	2 & 4	0.014	0.174	0.05	0.004
	3	2, 4		0.021	0.005	
Fuc	2, 3, 4	T		0.005	0.002	trace
Glc	2, 3, 4, 6	T				
	2, 3, 6	4	0.042			trace
	2, 6	3, 4		0.003		
	2, 3	4, 6				trace

[a] Column fractions as shown in Fig. 2.

DEAE fractions F1, F2, F3, and F4 varied in their content of uronic acid residues, giving values of 6.3%, 14.9%, 42.3%, and 44%, respectively. By methylation analysis and reduction, the uronic acid residues in F1 were identified as (1→4)-linked galactosyluronic acid; in F2 as (1→4)-linked glucosyluronic (22%), (1→2,4)-linked galactosyluronic (8%), and (1→4)-linked galactosyluronic (70%) acid; in F3 as (1→2,4)-linked galactosyluronic (4%) and (1→4)-linked galactosyluronic (96%) acid; and in F4 as (1→2,4)-linked galactosyluronic (2%) and (1→4)-linked galactosyluronic (98%) acid. The four fractions were similar in neutral sugar composition, consisting primarily of Ara, Gal, Xyl, and Rha. Linkage analysis of the neutral sugar components of fractions F1, F2, F3, and F4 indicated the presence of both arabinans and arabinogalactans (Table II).

Fraction F4 contained the major portion of the total carbohydrate in the HW-extract. In neutral sugar somposition it was similar to the other fractions, containing mainly arabinose (57%), galactose (25%), and rhamnose (15%). Rhamnosyl residues comprised a more significant portion of the total carbohydrate in this group of polysaccharides, with nearly equal percentages being (1→2)- and (1→2,4)-linked. The linkage pattern of arabinose was similar to that of the other fractions. However, F4 contained larger amounts of (1→3,4,6)-linked galactose.

Enzyme degradation of DEAE fraction F4. — Degradation of DEAE F4 with purified PG was monitored by evaluating the elution profile from an HW-55 TSK gel column. Undegraded F4 polysaccharides eluted rapidly from the column with K_{av} (Fig. 3A) ranging from 0.09 to 0.25, indicative of a polydisperse group. After treatment with PG, the elution profile remained unchanged. Saponification of the polysaccharides with 0.5M NaOH followed by acidification to pH 5.0 resulted in the formation of supernatant (F4-S) and precipitate (F4-I) fractions. Treatment of the soluble fraction (F4-S) with PG resulted in a shift to lower molecular weights (Fig. 3B). However, a significant portion of the neutral sugars remained as polymeric fragments (K_{av} = 0.19 to 0.43) containing small amounts of uronic acid. Uronic acid-enriched fractions were retained on the column and eluted with K_{av} = 0.82 to 0.97.

The four degradation fractions (Fig. 3B) differed in molecular size, neutral sugar to uronic acid ratio, and galacturonic acid to rhamnose ratio, and showed slight differences in the linkage patterns of their neutral sugar components (Table III). Three of the fractions, F4-S-A, F4-S-B, and F4-S-C, contained more neutral sugar than uronic acid residues, giving neutral sugar to uronic acid ratios of 7.6, 6.2, and 2.7, respectively. The galactosyluronic to rhamnosyl ratio for F4-S-A, F4-S-B, and F4-S-C increased with decreasing size of the degradation products, with values of 0.9, 1.7, and 3.3, respectively. Fraction F4-S-D contained the smallest fragments, with a neutral sugar to uronic acid ratio of 0.7 and a galactosyluronic acid to rhamnosyl ratio of 18.4.

Treatment of fraction F4-I (Fig. 1) with PG resulted in an elution profile (Fig. 3C) that was similar to the profile from DEAE F4 supernatant (F4-S, Fig. 3B). The high molecular weight material (K_{av} 0.09 to 0.42) was a smaller portion of the total than the uronic acid-rich fragments (K_{av} 0.69 to 1.0). The neutral sugar compositions of the early-eluting fractions were the same as those found for the corresponding degradation products from the soluble fraction (F4-S).

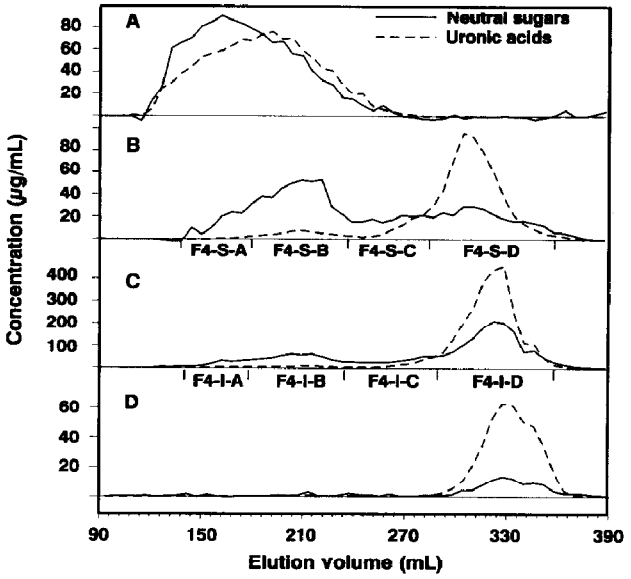

Fig. 3. Elution profiles of DEAE-F4 and its deesterified, enzyme-degraded derivatives, chromatographed on a HW-55 TSK gel column of void volume 120 mL, included volume 350 mL. A, DEAE-F4, untreated; B, DEAE-F4-S after degradation by PG; C, DEAE-F4-I after degradation by PG; D, DEAE-F4-S pretreated with 0.1M TFA (1 h at 100°), then degraded by PG.

TFA treatment before enzyme degradation. — A subsample of F4-S (Fig. 1) was hydrolyzed with mild acid (0.1M TFA) to remove arabinosyl substitutions before degradation with PG, and designated F4-S-TFA. The elution profile of the enzyme-degraded de-arabinosylated material (F4-S-TFA-Enz) on the HW-55 column (Fig. 3D) indicated a more complete hydrolysis had been achieved. The higher molecular weight fractions were no longer present. Neutral sugar analysis of the TFA hydroylzates indicated arabinose and galactose as the major sugars, with rhamnose, xylose, and glucose also present as minor components.

DISCUSSION

Carbohydrate material solubilized from the cell walls of alfalfa stems with hot 10mM phosphate buffer was a complex mixture of polysaccharides typical of the pectic components of plant cell walls[2,3]. The neutral fraction (F1, see Fig. 2) contained type I arabinogalactans. It is unlikely that this fraction represents a mixture of galactan and arabinan, because homopolysaccharides are rare in plant cell walls[2,3]. The charged fractions (F2, F3, and F4) from the DEAE column all had similar neutral-sugar compositions and glycosyl-linkage patterns. The predominance of (1→3,6)-linked galactosyl residues was indicative of type II arabinogalactans[3]. These fractions also contained (1→2)- and (1→2,4)-linked rhamnose along with lesser amounts of xylose and glucose.

The majority of the carbohydrate (F4) was composed of rhamnogalacturonan-

TABLE III

Partially methylated alditol acetates derived from the enzyme-resistant residues of the deesterified, PG-digested polysaccharides of fraction F4-S

Glycosyl residue	*Methylated alditol*	*Linkage position(s)*	*Proportions (mol fraction)*			
			F4-S-A[a]	*F4-S-B*	*F4-S-C*	*F4-S-D*
Ara	2, 3, 5	T	0.164	0.158	0.087	0.097
	2, 5 & 3, 5	3 & 2		0.024	0.021	0.029
	2, 3	5	0.332	0.252	0.100	0.108
	2	3, 5	0.049	0.063	0.069	0.035
	3	2, 5	0.029	0.037	0.013	0.017
Gal	2, 3, 4, 6	T	0.045	0.032	0.018	0.022
	2, 3, 6	4	0.119	0.085	0.165	0.182
	2, 4, 6	3	0.027	0.029	0.015	
	2, 3, 4	6	0.046	0.055		
	2, 4	3, 6	0.021	0.103	0.067	0.059
	2	3, 4, 6		0.005	0.012	0.011
	3	2, 4, 6				
Rha	3, 4	2	0.045	0.029	0.063	0.032
	3	2, 4	0.093	0.056	0.012	0.016
Xyl	2, 3, 4	T		0.008	0.029	0.030
	3, 4 & 2, 3	2 & 4		0.076	0.138	0.142
	3	2, 4		0.007	0.019	0.014
Fuc	2, 3, 4	T		0.005	0.025	0.028
Glc	2, 3, 4, 6	T	0.022			
	2, 3, 6	4	0.017	0.003	0.124	0.142
	2, 6	3, 4		0.002		0.009
	2, 3	4, 6			0.021	0.026

[a] Column fractions as shown in Fig. 3B.

type polysaccharides that were resistant to PG degradation. PG requires a completely demethylated galacturonan for maximum hydrolytic action[15,21], producing tri-, di-, and mono-galactopyranosyluronic acids as limit products. The lack of activity against alfalfa rhamnogalacturonans before saponification was due to a combination of methyl-ester and neutral-sugar substitutions. The two fractions formed after saponification of F4 clearly had different structural compositions, as indicated by their solubilities and PG-degradation patterns. Both fractions (F4-S and F4-I) contained highly substituted regions that resembled structures characterized in pectins from other plants and referred to as[8,11] "hairy regions".

The glycosyl composition of the alfalfa hairy regions isolated after PG degradation was similar to what has been described in the literature[7-11]. However, these regions from alfalfa pectins were richer in neutral sugars and had lower ratios of galactosyluronic to rhamnosyl residues. Fractions F4-S-A and F4-S-B were of high molecular weight, indicative of polymeric material attached to a relatively short galacturonan backbone. Fraction F4-S-C was smaller and contained less than half as much neutral sugar as the preceding fractions. The lower frequency of (1→2,4)-linked rhamnose in this fraction suggests that there may be more frequent substitutions on the galacturonan backbone

as opposed to the rhamnose units. These substitutions are likely monomeric in nature, as judged from the elution profile and the ratio of neutral sugar to galacturonic acid residues. This may explain the additional terminal groups found on linkage analysis of these fractions. Mild acid hydrolysis followed by PG treatment resulted in an elimination of the higher molecular weight fractions F4-S-A and F4-S-B (Fig. 3B, D). The more extensive degradation of acid-treated F4-S suggests that substitutions were probably through arabinose units on rhamnose and galacturonic acid residues.

The second group of rhamnogalacturonans (F4-I) exhibited a much lower degree of neutral-sugar substitution. Degradation of this fraction by PG after saponification of the methyl ester groups resulted in a smaller proportion of higher molecular weight fragments (Fig. 3C). Because initial degradation was prevented, a higher degree of methyl esterification was indicated. The composition of the material eluting in the higher molecular weight range resembled that of the "hairy region" of F4-S. Therefore, the rhamnogalacturonans from alfalfa stems could be divided into two classes based upon the frequency and extent of neutral-sugar substitutions.

REFERENCES

1 E. L. Hirst, D. J. MacKenzie, and C. B. Wylam, *J. Sci. Food Agric.*, 10 (1959) 19–26.
2 A. Darvill, M. McNeil, P. Albersheim, and D. P. Delmer, in N. E. Tolbert (Ed.), *The Biochemistry of Plants, A Comprehensive Treatise*, Vol. 1, Academic Press, New York, 1980, pp. 91–162.
3 A. M. Stephen, in G. O. Aspinall (Ed.), *The Polysaccharides*, Vol. 2, Academic Press, New York, 1983, pp. 98–193.
4 G. O. Aspinall and R. S. Fanshawe, *J. Chem. Soc.*, (1961) 4215–4225.
5 G. O. Aspinall, B. Gestetner, J. A. Molloy, and M. Uddin, *J. Chem. Soc.*, (1968) 2554–2559.
6 G. O. Aspinall and J. A. Molloy, *J. Chem. Soc.*, (1968) 2994–2999.
7 A. J. Barrett and D. H. Northcote, *Biochem. J.*, 94 (1965) 617–627.
8 J. A. DeVries, F. M. Rombouts, A. G. J. Voragen, and W. Pilnik, *Carbohydr. Polym.*, 2 (1982) 25–33.
9 J. F. Thibault, *Phytochemistry*, 22 (1983) 1567–1571.
10 B. J. H. Stevens and R. R. Selvendran, *Phytochemistry*, 23 (1984) 107–115.
11 F. M. Rombouts and J. F. Thibault, *Carbohydr. Res.*, 154 (1986) 189–203.
12 R. Hatfield, *Plant Physiol.*, 83S (1987) 245.
13 M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350–356.
14 N. Blumenkrantz and G. Asboe-Hansen, *Anal. Biochem.*, 54 (1973) 484–489.
15 E. M. Shea and R. D. Hatfield, *Plant Physiol.*, 89S (1989) 850.
16 P. J. Wood and I. R. Siddiqui, *Anal. Biochem.*, 39 (1971) 418–428.
17 A. B. Blakeney, P. J. Harris, R. J. Henry, and B. A. Stone, *Carbohydr. Res.*, 113 (1983) 291–299.
18 N. S. Carpita and E. M. Shea, in C. J. Biermann and G. D. McGinnis (Eds.), *Analysis of Carbohydrates by GLC and MS*, CRC Press, Inc., Boca Raton, Florida, 1989, pp. 157–216.
19 P. J. Harris, R. J. Henry, A. B. Blakeney, and B. A. Stone, *Carbohydr. Res.*, 127 (1984) 59–73.
20 B. Lindberg, *Methods Enzymol.*, 28B (1972) 178–195.
21 J. F. Thibault and C. Mercier, *J. Food Biochem.*, 2 (1978) 379–393.