Carbohydrate Research, 182 (1988) 63–78
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

STRUCTURAL STUDIES OF PECTIC SUBSTANCES FROM THE PULP OF GRAPE BERRIES

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(Received December 8th, 1987; accepted for publication, February 24th, 1988)

ABSTRACT

The structure of the neutral sugar side-chains of pectic substances extracted with water (WSP), dilute HCl (HP), and endopectinlyase (AIR/PEL) from an alcohol-insoluble residue (AIR) from the pulp of grape berries was investigated by using chemical methods (methylation, carboxyl-reduction, and periodate oxidation), enzymic degradation with an α-L-arabinofuranosidase, and ^{13}C-n.m.r. spectroscopy. The side chains of WSP are essentially a type II arabinogalactan, whereas those of HP and AIR/PEL are arabinan-like structures associated with minor proportions of type I and II arabinogalactans. The side chains are ~80% degraded by the α-L-arabinofuranosidase, indicating that the α-L-arabinofuranosyl residues are mainly 3-linked to the (1→6)-galactan side-chains of the type II arabinogalactan. The three pectic fractions contain minor amounts of proteins rich in hydroxyproline and serine.

INTRODUCTION

Acidic pectic substances, *e.g.*, pectins, contain a rhamnogalacturonan backbone in which (1→4)-linked α-D-galacturonan chains are interrupted at intervals with α-L-rhamnopyranosyl residues carrying neutral side-chains[1–3]. Pectic substances isolated from the pulp of grape berries[4] contained homogalacturonan (smooth) regions that were degraded by endopolygalacturonase and endopectinlyase, and resistant (hairy) regions that were rich in neutral side-chains[5]. These hairy fragments were purified and fractionated by ion-exchange chromatography[5], and we now report on the application of methylation analysis, periodate oxidation, degradation with an α-L-arabinofuranosidase, and ^{13}C-n.m.r. spectroscopy.

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0008-6215/88/$ 03.50

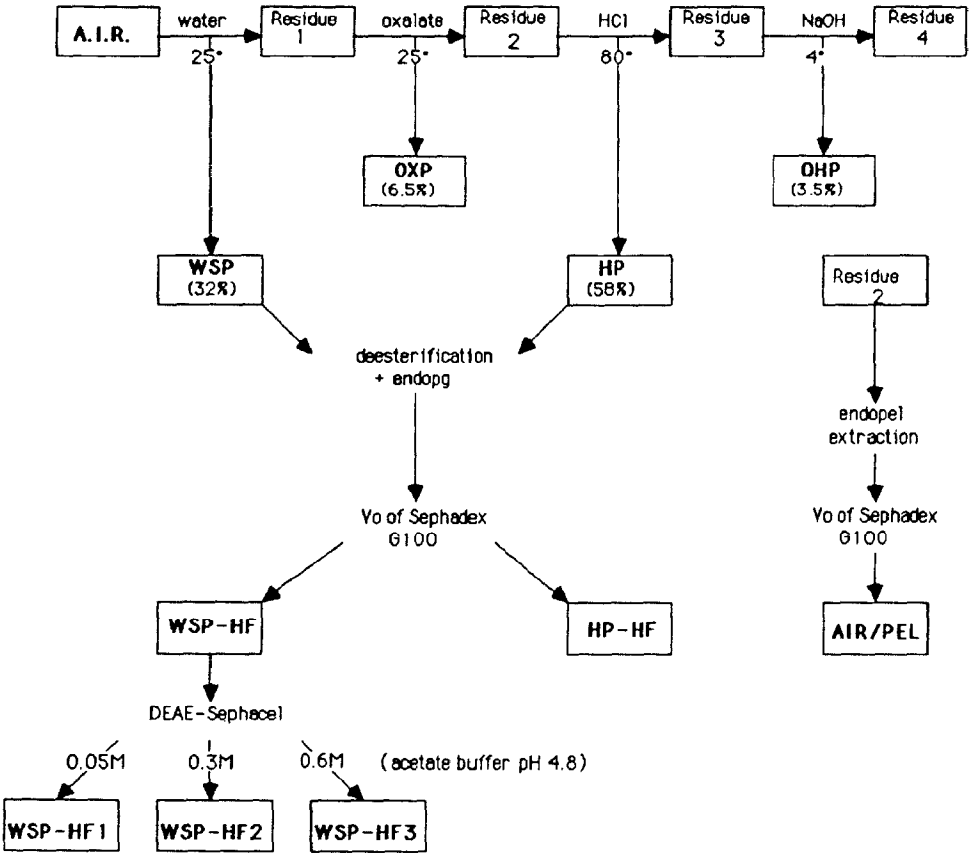

Fig. 1. Isolation of pectic substances from pulp of grape berries[4,5].

EXPERIMENTAL

Pectic substances. — An alcohol-insoluble residue (AIR) was prepared[4] from the pulp of grape berries and extracted sequentially with water, oxalate, hot HCl, and cold NaOH, yielding the fractions WSP, OXP, HP, and OHP, respectively. WSP and HP were further purified by ion-exchange chromatography[4].

Hairy fragments — WSP and HP were degraded, after de-esterification[5], with a purified endopolygalacturonase[6] from *Aspergillus niger*, and the hairy fragments (WSP-HF, HP-HF) containing neutral side-chains were separated from acidic oligomeric products of degradation (Fig. 1) by using Sephadex G100[5]. WSP-HF was fractionated on DEAE-Sephacel[5], yielding an unbound fraction (WSP-HF1) in 0.05M acetate buffer (pH 4.8), and two bound fractions (WSP-HF2/3) eluted by a linear gradient of acetate buffer (0.05→0.8M, pH 4.8.).

AIR was extracted sequentially with water, oxalate, and citrate buffer[5], and then degraded with endopectinlyase[7] to yield a fraction (AIR/PEL), rich in neutral sugars, isolated at the void volume of Sephadex G100 (Fig. 1).

General — α-L-Arabinofuranosidase (426 nkat/mg) was purified to homogeneity from a culture-supernatant solution of the basidiomycetes *Dichomitus squalens*[8]. Pronase was purchased from Boehringer (Mannheim).

Neutral sugars were determined, after hydrolysis with 2M trifluoroacetic acid (1.25 h, 120°), by g.l.c. of their alditol acetate derivatives[9] on a fused-silica capillary column (30 m x 0.32 mm i.d.) bonded with OV-225 (0.25-μm film) at 210° (injector and detector temperature, 250°; split ratio, 1:10; hydrogen as carrier gas at 65 kPa). Arabinose was also determined by the NAD^+/galactose dehydrogenase system[10]. Uronic acids and proteins were assayed by the *m*-phenylphenol[11] and Lowry[12] methods, respectively. Amino acid composition was determined, after hydrolysis by 6M HCl (10 h, 110°)[13], using ninhydrin (440 nm for hydroxyproline) on a Kontron Chromakon 400 auto-analyzer[14,15]. The products of enzymic hydrolysis were separated by t.l.c. on Kieselgel 60 (Merck), using acetonitrile–water (85:15) and detection with 0.5% thymol in ethanol–sulfuric acid (95:5) and heating (10 min, 100°).

Reduction of uronic acids. — Prior to reduction, WSP and HP (2 mg/mL) were de-esterified in 0.05M NaOH for 2 h at 2°, then neutralised to pH 4–5 with M HCl, dialysed against distilled water, and freeze-dried. To a solution of de-esterified polysaccharide (10–15 mL, 1–2 mg/mL) was added 1-cyclohexyl-3-(2-morpholinoethyl)-carbodi-imide metho-*p*-toluenesulfonate[16] [CMC (Fluka); 10 mol of CMC per carboxylic acid equivalent], and the pH was kept at 4.75 with 0.02M HCl for 2 h. M $NaBH_4$ solution (250 mol of $NaBH_4$ per carboxylic acid equivalent) was then added during 30 min, maintaining the pH at 7 with M HCl (1-octanol as anti-foaming agent). After 1.5 h, the mixture was extensively dialysed against distilled water, concentrated, and freeze-dried.

Periodate oxidation. — To an aqueous solution (10 mL) of WSP or HP (20 mg) was added 0.03M sodium periodate (10 mL), and each mixture was kept in the dark at ambient temperature. The consumption of periodate was monitored[17] at 225 nm. After 72 h, each mixture was dialysed extensively against distilled water and then reduced with $NaBH_4$ (5 mg/mL) for 1 day at room temperature. The excess of $NaBH_4$ was destroyed with Amberlite IR-120 (H^+) resin, and each solution was dialysed and then freeze-dried.

Enzymic degradation. — (*a*) A solution of polysaccharide (2–7 mg) in 0.1M acetate buffer (1mL, pH 4.2) was incubated for 24 h with α-L-arabinofuranosidase (1 nkat of enzyme/μmol of arabinose in the polysaccharide). The same amount of enzyme was added and, after 24 h, the arabinose liberated was separated by t.l.c. and determined by the NAD^+/galactose dehydrogenase system. The solution of the degraded polysaccharide was dialysed and freeze-dried prior to methylation analysis.

(*b*) A solution of WSP (10 mg) in 0.1M phosphate buffer (pH 7.5) containing 0.02% of sodium azide was incubated for 24 h at 40° with 50 μL of aqueous pronase

(150 μg/mL). An aliquot was then injected onto a column of DEAE-Sephacel and eluted as described[5]. Polysaccharides were detected by the phenol–sulphuric acid method[18]. Another aliquot was dialysed, freeze-dried, and then analyzed for amino acid composition.

Methylation analysis. — Native, carboxyl-reduced, and arabinosidase-degraded WSP, HP, WSP-HF, HP-HF, and AIR/PEL were each methylated once by the Hakomori method[19] as described by Jansson *et al.*[20], using a time of contact with the methylsulfinylmethanide anion of 2 h. WSP, HP, and their α-L-arabinofuranosidase-degraded products were converted into the H^+ form to ensure dissolution in dimethyl sulphoxide. Each methylated polysaccharide was then extracted with $CHCl_3$–CH_3OH (2:1), and the mixture was washed thrice with water[21] and air-dried at 40° before hydrolysis with aqueous 90% formic acid (1 h, 100°) and 2M trifluoroacetic acid (1.25 h, 120°). The partially methylated sugars were then converted into their alditol acetates and analysed by g.l.c. on (*a*) a fused-silica capillary column (50 m x 0.32 mm i.d.) bonded with OV-1 (0.20-μm film); on-column injection at 35°, 15°/min to 165°, then, after 30 min, to 210° at 15°/min; detector temperature, 250°; hydrogen as carrier gas at 120 kPa; and (*b*) on a fused-silica capillary column (30 m x 0.32 mm i.d.) bonded with OV-225 (0.25-μm film thickness); 170° for 15 min, then 5°/min to 210°; injector and detector temperature, 250°; split ratio, 1:10; hydrogen as carrier gas at 65 kPa. Peak identification was based on retention times using partially methylated alditol acetates from standard polysaccharides[22], and confirmed by g.l.c.-m.s. with the OV-1 column coupled to a Nermag R 1010C mass-spectrometer. Peak areas were corrected by response factors as described by Sweet *et al.*[23].

^{13}C-N.m.r. spectroscopy. — WSP-HF and AIR/PEL (~30 mg) and their arabinosidase-degraded forms (WSP-HF, 12 mg; AIR/PEL, 8 mg) were dissolved in D_2O (1 mL) and, using 5-mm tubes, spectra were recorded at 80°, with complete proton decoupling, using a Bruker WP 100 spectrometer at 25.18 MHz.

RESULTS AND DISCUSSION

Structure of native and carboxyl-reduced pectic substances. — Native pectic substances from grape pulp[4], WSP, HP, and AIR/PEL, and their carboxyl-reduced forms were methylated in one step by the Hakomori procedure[19]. Prior to methylation, native pectins were converted into their H^+ form[24] to ensure solubility in dimethyl sulphoxide. After preliminary saponification with cold dilute sodium hydroxide, 84% and 88% reduction of WSP and HP, respectively, was achieved in one step, whereas it was only 68% for AIR/PEL which had not been de-esterified. In addition, the neutral side-chains from WSP, after fractionation on DEAE-Sephacel[5] (WSP-HF1/3), were subjected to methylation analysis (see Experimental) and the data are reported in Tables I and II. The native and carboxyl-reduced pectins gave 24 partially methylated alditol acetates, which were separated by g.l.c. on OV-1 and OV-225 capillary columns. The methylation was assumed to be

complete since there was a good agreement between the relative percent of each parent sugar as determined by sugar analysis, as their alditol acetates, or methylation; the (terminal/substituted) ratios were also close to unity except for carboxyl-reduced WSP.

The proportions of arabinose methyl ethers are quite different in the three pectins; WSP contains mainly terminal non-reducing arabinose (68% of total arabinose), whereas HP and AIR/PEL showed descreasing proportions (49% and 27%, respectively). Conversely, the relative proportion of 5-linked arabinose increased from WSP to HP and AIR/PEL (22%, 35%, and 52%, respectively). The proportion of 3,5-linked arabinose indicated a low degree (4%) of branching in WSP, whereas, in HP and AIR/PEL, similar much larger proportions were observed (16% and 19%, respectively). Similar distributions were obtained for the carboxyl-reduced pectins. Thus, the structural features of the arabinose-containing side-chains are closely related in HP and AIR/PEL, and involve arabinan-like structures with a central core of (1→5)-linked arabinofuranosyl residues carrying essentially single substituent on positions 3. Conversely, the WSP arabinose-containing moiety is constituted mainly of terminal units and to a lesser extent of short chains of (1→5)-linked arabinose with few branching points. It is remarkable that the two water-insoluble pectic fractions (HP and AIR/PEL) had similar arabinan-like side-chains, although those of HP may have been degraded somewhat by the acidic conditions used for extraction. Conversely, the water-soluble pectin (WSP), which is thought to be derived from the walls during maturation[25], showed a clearly distinct shape.

There was a complex mixture of galactose methyl ethers, eight derivatives being consistently observed. In WSP, 3,6-linked galactose was the dominant feature (43% of total galactose) followed by 3-linked (17%), 3,4,6-linked (13%), and 6-linked galactose (8%); 4-linked galactose was virtually absent from native WSP. This distribution is typical of type II arabinogalactans[26] which, unlike type I, are not distributed widely in pectins. However, they were detected as side chains in pectins from apple[27], *Panax ginseng*[28], *Hibiscus fulcineus*[29], lemon[30], and lucerne[31], and they were associated with rhamnogalacturonan in walls from suspension-cultured cells of Sycamore[32,33], *Vinca rosea*[34], and *Nicotania tabacum*[35]. Recently, type II arabinogalactans isolated from roots of *Angelica acutiloba*[36] and leaves from *Artemisia princeps*[37] were found to be associated with rhamnogalacturonans. Furthermore, De Vries *et al.*[27] have reported that, in pectic fractions containing relatively high amounts of galactose, which are water-extractable, the galactans were mainly (1→3)/(1→6)-linked. HP and AIR/PEL showed increasing proportions of 4-linked galactose, whereas the type II arabinogalactan features were decreased. The proportion of terminal non-reducing galactose was also increased as compared with WSP. Thus, the galactan moiety of WSP is of type II, having a central core of (1→3)-linked galactopyranosyl residues with side chains of (1→6)-linked galactose with an overall highly branched character as attested by the amounts of 3,6- and 3,4,6-linked galactose. Such higly branched arabinogalactans have been reported in the roots of *Angelica acutiloba*[36], *Cannabis sativa*[38], and *Lolium multiflorum*[39].

TABLE I

METHYLATION ANALYSIS OF NATIVE, CARBOXYL-REDUCED, AND ARABINOSIDASE-DEGRADED WSP, HP, AND AIR/PEL

	WSP			*HP*			*AIR/PEL*		
Methyl ether	*Native*	*Carboxyl-reduced*	*Arabinosidase-degraded*	*Native*	*Carboxyl-reduced*	*Arabinosidase-degraded*	*Native*	*Carboxyl-reduced*	*Arabinosidase-degraded*
234 Rha[c]	[a]3.0	1.1	2.4	2.1	0.5	2.2	1.3	0.5	0.5
34 Rha	5.2	4.8	5.3	22.1	7.3	25.2	9.9	13.0	13.2
3 Rha	1.1	1.0	1.1	6.7	2.2	7.7	4.2	5.0	4.5
Rha	0.6	0.5	0.9	1.3		0.9	0.3	0.3	0.3
Total	9.9(10.5)[b]	7.4(5.0)	9.7	32.2(34.7)	10.0(9.4)	36.0	15.7(21.1)	18.8(19.0)	18.5
234 Fuc	1.3	0.4	1.2	1.3	0.5	0.8	0.6	0.3	0.6
235 Ara	28.0	10.0	8.6	16.2	6.0	3.6	16.5	12.6	1.8
25 Ara	0.6		0.8	1.8	0.6	0.7	1.7	1.0	0.7
35 Ara	0.9	0.5		2.6	1.5	1.3			
23 Ara	8.9	4.8	1.3	11.5	3.5	2.8	31.8	24.3	5.0
2 Ara	1.8	1.4	0.6	5.4	2.0	0.7	11.8	9.9	0.4
Ara	0.8	1.1	0.7	0.4		0.8	0.8		
Total	41.0(40.5)	17.8(13.8)	12.0	37.9(38.0)	13.6(13.6)	9.9	62.6(59.7)	47.8(50.0)	7.9
234 Xyl	1.7	nd	1.2	2.2	0.5	1.8	0.6	0.2	0.4
23 Xyl	3.9	nd	1.7			0.6			
Total	5.6(8.1)	-(3.0)	2.9	2.2(2.0)	0.5(1.0)	2.4	0.6(1.3)	0.2(1.1)	0.4
2346 Gal	4.1	2.8	6.9	6.4	3.6	8.7	4.5	3.5	4.2
246 Gal	6.8	6.0	4.6	3.0	1.3	1.2	1.8	1.6	3.4
234 Gal	3.4	1.6	12.5	1.7	0.5	2.8	0.6	0.4	1.0
236 Gal	0.5	35.6	0.7	2.7	62.3	1.7	3.3	18.1	3.9
26 Gal	2.1	2.6	1.2	2.0	1.6	0.6	2.0	2.3	0.8
23 Gal	0.9		3.7	0.5	0.7	1.1		0.4	0.5
24 Gal	17.5	15.5	11.7	6.0	2.8	4.8	2.0	2.0	1.3
2 Gal	5.5	5.5	1.6	1.0	0.6	0.6	1.8	0.7	0.2
Total	40.8(39.0)	69.6(77.5)	42.9	23.3(22.0)	73.4(75.0)	21.5	16.0(12.6)	29.0(25.5)	15.3

2346 Glc	1.4	2.3	0.4	1.0	1.0	0.6	2.1	1.1	0.5
236 Glc		2.5	1.6	1.1	1.1	1.1	0.4	0.8	1.7
234 Glc			0.6	1.0			2.0	2.0	0.6
Total	1.4(1.9)	4.8(0.7)	2.6	3.1(3.3)	2.1(0.9)	1.7	4.5 (5.3)	3.9 (4.4)	2.8
Ara free[d]			28.7			27.7			54.5
Terminal/substituted ratio	1.1	0.5	0.8	1.1	1.1	0.9	1.0	0.8	1.0

[a]Relative mole ratio. [b]Values in brackets are from direct analysis. [c]234 Rha = 1-*O*-acetyl-2,3,4-tri-*O*-methylrhamnitol, etc. [d]Determined by using the galactose dehydrogenase/NAD^+ system.

TABLE II

METHYLATION ANALYSIS OF WSP-HF AFTER FRACTIONATION ON DEAE-SEPHACEL

Methyl ether	*WSP-HF1*	*WSP-HF2*	*WSP-HF3*
234 Rha[c]	0.3[a]	0.8	1.4
34 Rha	0.8	0.4	16.0
3 Rha	0.2	tr.	4.6
Rha	tr.	tr.	0.3
Total	1.3(1.6)[b]	1.2(1.6)	22.3(16.2)
234 Fuc	0.2	0.2	0.7
Total			
235 Ara	30.9	30.0	14.4
25 Ara	0.6	0.7	0.8
35 Ara			0.2
23 Ara	1.6	3.9	25.7
2 Ara	0.3	0.3	6.8
Ara			0.3
Total	33.4(30.9)	34.0(51.6)	48.2(54.3)
234 Xyl	0.4	0.8	0.6
23 Xyl			3.8
Total	0.4	0.8(1.6)	4.4(4.2)
2346 Gal	3.8	3.6	2.6
246 Gal	13.4	9.8	3.9
234 Gal	4.6	2.5	1.2
236 Gal	0.5	0.4	1.8
26 Gal	2.3	4.5	2.0
23 Gal	0.7	0.8	
24 Gal	28.8	25.7	8.3
2 Gal	6.8	15.5	2.0
Total	60.9(60.6)	62.8(43.8)	21.8(20.1)
2346 Glc	1.6	0.3	0.9
236 Glc	1.4	0.4	1.7
234 Glc	0.8	0.3	
Total	3.8(6.9)	1.0(1.2)	2.6(5.2)
Terminal/substituted ratio	0.8	0.6	0.7

[a]Relative mole ratio. [b]Values in brackets are from direct analysis. [c]234 Rha = 1-*O*-acetyl-2,3,4-tri-*O*-methylrhamnitol, etc.

Although the similarities of HP and AIR/PEL are less obvious than for the arabinan moiety, the presence of (1→4)-galactan, which is absent from WSP, is a common feature.

After carboxyl-reduction, 4-linked galactose became the preponderant glycosidic linkage arising from the rhamnogalacturonic backbone of each of the three pectic fractions. Comparison of the relative proportions of the galactose methyl ethers with those from the unreduced material showed an increase in 3,4-linked galactose, indicating branching through position 3 of galacturonic acid. This alkali-stable linkage has already been reported[1], xylose or glucuronic acid being the substituting sugars. Terminal and 4-linked glucose increased after carboxyl-reduc-

tion, arising from glucuronic acid as end groups or substituted at position 4, possibly by terminal rhamnose[40].

Rhamnose occurred in terminal positions mainly in WSP, which is a minor component of type II arabinogalactans[41]. The 2- and 2,4-linked rhamnoses are typical constituents of the rhamnogalacturonan backbone[1,2], commonly half of the rhamnosyl residues being 2,4-linked[2]. In our pectins, the 2,4-linked rhamnose accounted for 11% (WSP), 21% (HP), and 27% (AIR/PEL) of the total rhamnose, which is close to values (30%) reported for the pectins from *Nicotania tabacum*[42,43].

Terminal fucose and xylose were minor components in the three pectins, whereas 4-linked xylose was found only in WSP. Terminal and 2-linked mannose were detected as traces.

After extensive degradation of the rhamnogalacturonic backbone of WSP by an endopolygalacturonase[5], hairy fragments (WSP-HF), corresponding to neutral polymers covalently linked to resistant acidic moieties, were fractionated further on DEAE-Sephacel[5] into WSP-HF1 (unbound fraction) and WSP-HF2 and WSP-HF3 (eluted at low and high ionic strength, respectively). Each fraction was methylated and the results are shown in Table II. WSP-HF1 and WSP-HF2 were similar in their content of neutral sugar and had similar (neutral/acidic sugar) ratios (19 and 14 for WSP-HF1 and WSP-HF2, respectively)[5]; they accounted for ~80% of the total neutral sugars (~40% each) and ~20% of the galacturonic acid of WSP-HF. Although some discrepancies were noted in the proportions of neutral sugar as determined by direct analysis or methylation, their structures were almost identical and similar to that of the parent WSP, a type II arabinogalactan heavily substituted by terminal arabinose being the prominent feature. On the other hand, WSP-HF3 (~20% of neutral sugars and ~80% of galacturonic acid of the inital WSP-HF)[5] exhibited a structure markedly similar to that of the side chains of HP and AIR/PEL, a (1→5)-linked arabinan being the major feature, associated with minor proportions of 3,6-linked and 4-linked galactans. 4-Linked galactopyranosyl residues and 4-linked xylose are found only in WSP-HF3, as in HP and AIR/PEL. According to the content of rhamnose, if it is assumed that neutral side-chains are attached mainly to positions 4 of rhamnose kinks[2,3], then the 3,6-arabinogalactans in WSP-HF1 and WSP-HF2 must be of high d.p., wheras the arabinans of WSP-HF3 could be of low molecular weight which is general for neutral arabinans[1]. Therefore, a further fractionation of the bulk of side chains of WSP clearly indicated some degree of heterogeneity[5], type II arabinogalactan and arabinan side-chains being carried by different portions of the rhamnogalacturonan core.

Periodate oxidation. — WSP and HP consumed 0.58 and 0.77 mol of periodate per mol of "anhydrosugar", respectively. The theoretical consumption, calculated on the basis of methylation experiments of unreduced samples and assuming that galacturonic acid was only (1→4)-linked, gave 0.83 and 0.94 mol/mol, respectively. These differences could be explained by under-oxidation or the presence of 3,4-substituted galacturonic acid which would be resistant to periodate. For the latter, 28% and 19% of galacturonic acid would be substituted on position 3, for

WSP and HP, respectively. Analysis of oxidised product revealed that 31 mol % of galacturonic acid was present, both for WSP and HP, but galactose was the main sugar (56 and 50 mol %, for WSP and HP, respectively). The recovery of galactose was higher than expected from calculation and it was concluded that some methyl-esterified galacturonic acid residues were reduced during treatment of the oxidised products with $NaBH_4$. Methylation analysis of the oxidised and reduced WSP gave mainly 4- (18%), 3- (13%), and 3,6-linked galactose (35%), whereas HP gave 2,4-linked rhamnose (7%), 3,5-linked arabinose (6%), and 4-linked galactose (48%), the last component arising from reduced galacturonic acid, probably carrying alkali-labile substituents (*e.g.*, acetyl groups) which were lost during the methylation.

Hydrolysis of pectic substances by α-L-arabinofuranosidase. — When native WSP, HP, and AIR/PEL were treated with an α-L-arabinofuranosidase, arabinose only was liberated (t.l.c.). The extent of hydrolysis (expressed as the percentage of the total arabinose), as determined by the NAD^+/galactose dehydrogenase system, was 70% for WSP, 73% for HP, and 87% for AIR/PEL. The degraded polysaccharides were then extensively dialysed and methylated. The data are shown in Table I. All arabinofuranosidic linkages were affected by the enzyme treatment. Terminal non-reducing arabinosyl units are the best substrate for α-L-arabinofuranosidases but they also liberate arabinose at a lower rate from (1→5)- and (1→3)-arabinofuranosidic linkages[44]. The enzyme from *Rhodotorula flava* liberated 56% of the arabinose from the arabinogalactan of *Angelica acutiloba*[36], with corresponding values of 31% for *Artemisia princeps*[37], 83–96% for radish seeds[45], and 80% for radish leaves[46], leaving some resistant terminal, 5-linked[36,37,45], or 2-linked[46] arabinosyl units, as with the enzyme from *Dichomitus squalens*[8]. The different limits of hydrolysis could reflect various degrees of steric hindrance due to the highly branched character of the galactan backbone. Variations of the proportions of galactose methyl ethers obtained from arabinosidase-degraded and native pectins (Table I) indicate the site of attachment of the arabinosyl units. Since the proportions of 3-, 3,6- and 3,4,6-linked galactose decreased, whereas those of terminal, 6-, and 4,6-linked galactose increased after treatment with the enzyme in both WSP and HP, the outer chains of (1→6)-linked galactose must carry, on positions 3, terminal and/or short chains of 5-linked arabinose, and the inner (1→3)-linked core could be substituted on positions 6. Some chains must also be terminated by the (Ara*f*→Gal*p*→) moiety because of the enhancement of terminal galactose after the action of the enzyme. The sensitivity of the arabinogalactans of WSP and HP to the arabinosidase is similar to those from *Gladolius*[47] or *Artemisia princeps*[37]. AIR/PEL behaved differently, 3- and 4-linked galactose being increased by the action of the α-L-arabinofuranosidase, whereas 3,6- and 3,4,6-linked galactose were decreased and terminal galactose was unchanged, which is consistent with branching of arabinose on position 6 of (1→3)- and position 3 of (1→6)-linked galactose residues. AIR/PEL could be compared on the basis of enzyme sensitivity to arabinogalactans from *Angelica acutiloba*[36] and radish seeds[45].

^{13}C-N.m.r. spectroscopy. — The spectra of WSP-HF, its arabinosidase-

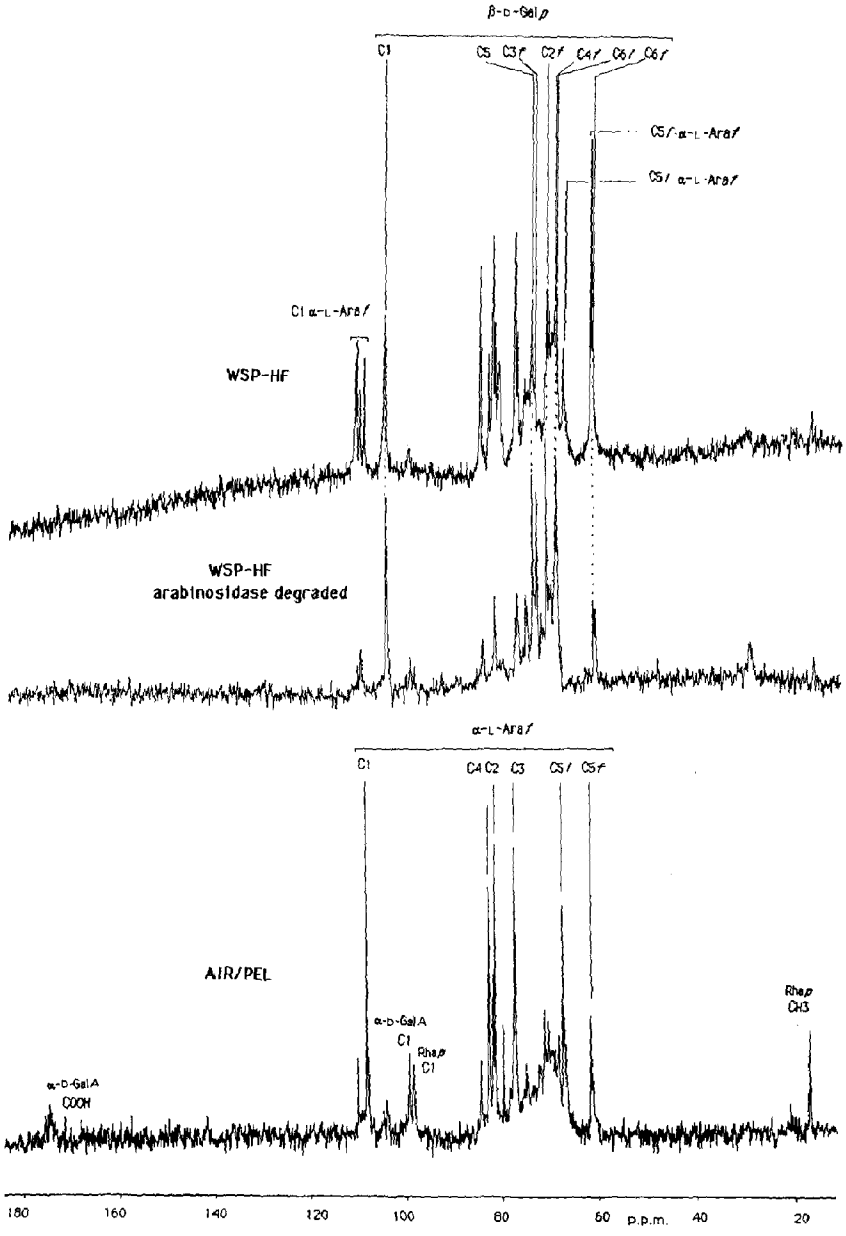

Fig. 2. ^{13}C-N.m.r. (25.18 MHz) spectra of native and arabinosidase-degraded WSP-HF and AIR/PEL (*l* and *f* indicate carbon position involved or not in glycosidic linkage, respectively).

degraded form, and AIR/PEL were interpreted on the basis of methylation and literature data[48,49] (Fig. 2, Table III). The spectrum of WSP-HF exhibits all the features of a type II arabinogalactan characterised by the typical sets of signals at low field given by α-L-arabinofuranosyl moieties and at the higher field by the β-D-galactopyranosyl residues. Terminal arabinosyl units preponderate highly in WSP-HF as shown by methylation analysis, thus an intense signal is expected for C-1 of this residue. In fact, four peaks were observed at 110.3, 109.95, 109.2, and 108.5 p.p.m., which suggests that the chemical shifts of the C-1 resonance from arabinofuranosyl units are not related simply to their patterns of substitution but also to their location in the galactan core. These signals disappear after the treatment with arabinosidase, confirming the above interpretation. Signals are also observed at 62.3 and 67.9 p.p.m. for C-5 of terminal and 5-linked arabinofuranosyl residues, the latter peak disappearing after treatment with arabinosidase. Some terminal arabinosidase-resistant α-L-arabinofuranosyl residues are still detected, as indicated by the remaining signals at 62.3 and 109.9 p.p.m. for C-5 and C-1, respectively. The galactan core gives only one intense broad peak at 104.2 p.p.m. for C-1 of

TABLE III

MAIN FEATURES OF THE ^{13}C-N.M.R. SPECTRA[a] OF WSP-HF AND AIR/PEL

WSP-HF	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
α-L-Arabinofuranosyl						
terminal	109.9	82.3	77.8	84.9	62.3	
5-linked	108.5	81.8	77.2	83.2	67.9	
β-D-Galactopyranosyl						
3-linked	104.2	71.8	81.1	70.3	74.7	61.8
6-linked	104.2	71.8	73.7	70.3	74.7	69.3
3,6-linked	104.2	71.8	81.1	70.3	74.7	69.3
3,4-linked	104.2	71.8	81.1		74.7	61.8
3,4,6-linked	104.2	71.8	81.1		74.7	69.3
α-Rhamnopyranosyl						
2-linked	98.9					17.3
AIR-PEL	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
α-L-Arabinofuranosyl						
terminal	109.9	81.8	77.7	84.1	61.8	
5-linked	108.1	81.4	77.4	82.8	67.5	
3,5-linked	107.6	81.4	79.8	82.8	61.8	
α-Rhamnopyranosyl						
2-linked	98.9					17.3
α-D-Galacturonopyranosyl						
4-linked	99.8					173.8

[a]Chemical shift (δ, p.p.m.).

β-D-galactopyranosyl residues. Other galactopyranosyl signals were attributed on the basis of methylation analysis of the arabinosidase-degraded form. The action of arabinosidase gives rise to a well resolved signal at 73.7 p.p.m., which is typical[48] for unsubstituted C-3 of β-D-galactopyranosyl residues. This result confirms that the main point of linkage of arabinofuranosyl residues in on positions 3 of 6-linked galactopyranosyl chains. The good resolution observed for the spectrum of the arabinosidase-degraded WSP-HF suggests a structural regularity of the galactan core.

The spectrum of AIR/PEL is dominated by signals of α-L-arabinofuranosyl moieties. According to methylation data and previous assignments[49], the intense peaks at 108.1, 82.8, 81.4, 77.4, and 67.5 p.p.m. are to be ascribed, respectively, to C-1, C-4, C-2, C-3 and C-5 of 5-linked arabinofuranosyl residues, confirming the pre-eminence of an arabinan-like structure. Unfortunately, the spectrum (not shown) of its arabinosidase-degraded form was of low quality due to the small amount of sample available. Nevertheless, the disappearance of arabinofuranosyl signals was observed with concomitant reinforcement of C-1 signals at 104.2 p.p.m. for β-D-galactopyranosyl residues, 99.8 p.p.m. for 4-linked α-D-galacturonic acid residues, and 98.9 p.p.m. for 2-linked α-L-rhamnosyl residues. Typical signals were also observed at high field (173.8 p.p.m.) for the carboxyl group of galacturonic acid and at low field (17.3 p.p.m.) for the methyl group of rhamnose.

Protein content and amino acid composition. — The data for AIR, WSP,

TABLE IV

AMINO ACID COMPOSITION OF AIR, WSP, PRONASE-TREATED WSP, WSP-HF, HP, HP-HF, AND AIR/PEL

Amino acid	*AIR*	*WSP*	*Pronase-treated WSP*	*WSP-HF*	*HP*	*HP-HF*	*AIR/PEL*
Aspartic acid	10.1[a]	6.6	8.0	5.5	6.1	4.6	7.0
Hydroxyproline	0.5	17.1	11.5	2.9	12.9	18.6	6.3
Threonine	6.7	8.8	8.3	5.3	7.0	6.1	4.8
Serine	7.5	15.5	15.3	21.4	6.0	12.4	14.4
Glutamic acid	11.6	7.2	9.9	13.0	7.5	9.3	11.0
Proline	2.2	1.9	0.0	2.5	11.2	8.5	4.6
Glycine	10.7	9.6	11.9	16.5	10.2	9.6	13.5
Alanine	8.9	15.3	12.0	9.7	5.8	6.9	8.7
Methionine	1.3						
Valine	5.8	4.3	4.3	3.5	5.3	5.4	4.2
Isoleucine	4.5	2.2	2.5	1.9	2.8	1.6	2.5
Leucine	7.2	4.6	5.2	2.9	4.5	3.1	4.1
Tyrosine	3.1	1.0	1.9	0.6	2.8	1.6	2.3
Phenylalanine	5.2	1.6	2.3	1.4	3.3	1.1	2.3
Ornithine	0.1	0.9		8.0	0.6	2.8	4.4
Lysine	7.5	2.5	3.4	2.2	6.0	6.9	5.4
Histidine	3.3	0.9	1.4	2.1	4.6	1.5	2.5
Arginine	3.8	0.0	2.1	0.6	3.4	0.0	2.0

[a]Relative mole ratio.

WSP-HF, HP, HP-HF, and AIR/PEL are reported in Table IV. Sulfur-containing amino acids and tryptophan were lost under the conditions used. The pectolytic enzymes (endoPG and endoPEL), used for preparation of hairy fragments, did not interfere since, according to their molecular weights[6,7], they were well separated on Sephadex G100 from polysaccharides eluted at the void volume.

The protein contents (% by weight) were significantly higher in HP (~10%) and AIR/PEL (~11%), than in WSP (~2%). Furthermore, WSP-HF was enriched in proteins as compared to WSP, which indicates that protein material could be linked to the neutral side-chains. The presence of proteins associated with purified pectins has been reported[50,51], and treatment of potato tissue with endoPEL and/or endoPG released polysaccharidic material associated with proteins[52].

WSP was rich in hydroxyproline, serine, alanine, and glycine (58% of the total amino acids), which is a typical feature of arabinogalactan-proteins[41] (AGps). Extensive treatment with pronase did not affect significantly the amino acid composition of the protein moiety of WSP or its elution behaviour on DEAE-Sephacel. The resistance of AGps to proteolytic enzymes has been reported[38] and might be due to the glycosylation of the peptide fragments or the steric hindrance of the enzyme. WSP-HF is depleted in hydroxyproline compared to the parent pectin and is rich in serine (21%) which may be implicated in carbohydrate-protein linkages in AGps[38,46].

HP exhibited a slightly different distribution, hydroxyproline, proline, and glycine being the preponderant amino acids. HP-HF was similar in composition to HP, although it was enriched in hydroxyproline and serine. AIR/PEL was similar in composition to HP-HF, except for the content of hydroxyproline which was lower.

The AIR had a high content of protein (~33% by weight) because of the co-precipitation of soluble proteins with polysaccharides during treatment[4] of the grape pulp with alcohol. The amino acid composition of the AIR is very different from that of the pectins which are extracted, since hydroxyproline represents only 0.5% of the total amino acids. Thus, the high level of hydroxyproline, which is the main constituent of wall proteins[2], is a specific feature of proteins associated with pectic polysaccharide in grape pulp.

Thus, it is concluded that the neutral side-chains of acidic pectic substances from pulp of grape berries have very complex structures. The water-soluble pectin (WSP) is a type II arabinogalactan, and the results are consistent with a (1→3)-galactan core carrying side-chains of (1→6)-galactan heavily substituted on positions 6 by single arabinosyl units. Conversely, water-insoluble pectic substances extracted from cell walls, either by dilute acid (HP) or endopectinlyase (AIR/PEL), show arabinan-like structures associated with minor proportions of arabinogalactans of both types I and II. The three pectic fractions contain minor amounts of proteins rich in hydroxyproline, serine, and glycine, which relates them to AGps. The occurrence of AGPs has been suggested in grape juice[53], and further investigations are now in progress in order to identify these protein-carbohydrate linkages and the role of these arabinogalactans in wine making.

ACKNOWLEDGMENTS

We thank Mrs. P. Williams for technical assistance, Mr. J. Sarris (INRA-IPV, Laboratoire de Biochimie Métabolique, Montpellier, France) for the amino acid analyses, Mr. C. Bosso (CERMAV-CNRS, St Martin d'Hères, France) for the g.l.c.-m.s. analyses, and Professor F. M. Rombouts (Agricultural University of Wageningen, The Netherlands) for a generous gift of endopectinlyase.

REFERENCES

1 G. O. Aspinall, in J. Preiss (Ed.), *The Biochemistry of Plants,* Vol. 3, Academic Press, 1980, pp. 473–500.
2 A. G. Darvill, P. Albersheim, M. McNeil, J. M. Lau, W. S. York, T. Stevenson, J. Thomas, S. Doares, D. J. Gollin, P. Chelf, and K. Davis, *J. Cell Sci. Suppl.*,2 (1985) 203–217.
3 P. M. Dey and K. Brinson, *Adv. Carbohydr. Chem. Biochem.*, 42 (1984) 265–382.
4 L. Saulnier and J.-F. Thibault, *Carbohydr. Polym.*, 7 (1987) 329–343.
5 L. Saulnier and J.-F. Thibault, *Carbohydr. Polym.*, 7 (1987) 345–360.
6 J.-F. Thibault and C. Mercier, *J. Solid-Phase Biochem.*, 2 (1978) 295–304.
7 F. E. A. van Houdenhoven, *Doctoral Thesis, Agricultural University of Wageningen, The Netherlands,* 1975.
8 J.-M. Brillouet, J.-C. Moulin, and E. Agosin, *Carbohydr. Res.*, 144 (1985) 113–126.
9 P. Albersheim, D. J. Nevins, P. D. English, and A. Karr, *Carbohydr. Res.*, 5 (1967) 340–345.
10 J. Melrose and R. J. Sturgeon, *Carbohydr. Res.*, 76 (1983) 247–253.
11 N. Blumenkrantz G. Asboe-Hansen, *Anal. Biochem.*, 54 (1973) 484–489.
12 O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265–275.
13 R. R. Selvendran, *Phytochemistry,* 14 (1975) 1011–1017.
14 J. V. Benson, Jr., M. J. Gordon, and J. A. Patterson, *Anal. Biochem.*, 18 (1967) 228–240.
15 J. V. Benson, Jr., *Anal. Biochem.*, 50 (1972) 477–493.
16 R. L. Taylor and H. E. Conrad, *Biochemistry,* 11 (1972) 1383–1388.
17 G. O. Aspinall and R. J. Ferrier, *Chem. Ind. (London),* (1957) 1216.
18 M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350–356.
19 S. I. Hakomori, *J. Biochem. (Tokyo),* 55 (1964) 205–208.
20 P.-E. Jansson, L. Kenne, H. Liedgren, B. Lindberg, and J. Lönngren, *Chem. Commun. Univ. Stockholm,* 8 (1976) 1–20.
21 P. J. Harris, R. J. Henry, A. B. Blakeney, and B. A. Stone, *Carbohydr. Res.*, 127 (1984) 59–73.
22 J.-M. Brillouet, *Biochimie,* 69 (1987) 713–721.
23 D. P. Sweet, R. H. Shapiro, and P. Albersheim, *Carbohydr. Res.*, 40 (1975) 217–225.
24 T. J. Waeghe, A. G. Darvill, M. McNeil, and P. Albersheim, *Carbohydr. Res.*, 123 (1983) 281–304.
25 J. M. Labavitch, *Annu. Rev. Plant Physiol.*, 32 (1981) 385–406.
26 A. E. Clarke, R. L. Anderson, and B. A. Stone, *Phytochemistry,* 18 (1979) 521–540.
27 J. A. De Vries, C. H. Den Uijl, A. G. J. Voragen, F. M. Rombouts, and W. Pilnik, *Carbohydr. Polym.*, 3 (1983) 193–205.
28 T. F. Solov'eva, L. V. Arsenyuk, and S. Ovodov, *Carbohydr. Res.*, 10 (1968) 13–18.
29 K. S. Bajpai and S. Mukherjee, *Indian J. Chem.*, 9 (1971) 33–35.
30 G. O. Aspinall and I. W. Cotrell, *Can. J. Chem.*, 48 (1970) 1283–1289.
31 G. O. Aspinall and J. A. Molloy, *J. Chem. Soc., C,* (1968) 2994–2999.
32 G. O. Aspinall, J. A. Molloy, and J. W. T. Craig, *Can. J. Biochem.*, 47 (1969) 1063–1070.
33 K. W. Talmadge, K. Keegstra, W. D. Bauer, and P. Albersheim, *Plant Physiol.*, 51 (1973) 158–173.

34 Y. TAKEUCHI AND A. KOMAMINE, *Physiol. Plant.*, 50 (1980) 113–118.
35 S. EDA, Y. AKIYAMA, K. KATO, A. ISHIZU, AND J. NAKANO, *Agric. Biol. Chem.*, 47 (1983) 1783–1789.
36 H. YAMADA, H. KIYOHARA, J.-C. CYONG, AND Y. OTSUKA, *Carbohydr. Res.*, 159 (1987) 275–291.
37 H. YAMADA, Y. OTSUKA, AND S. OMURA, *Planta Med.*, (1986) 311–314.
38 A. HILLESTAD AND J. K. WOLD, *Phytochemistry*, 16 (1977) 1947–1951.
39 A. BASIC, S. C. CHURMS, A. M. STEPHEN, P. COHEN, AND G. B. FINCHER, *Carbohydr. Res.*, 162 (1987) 85–93.
40 G. O. ASPINALL AND K. G. ROSELL, *Carbohydr. Res.*, 57 (1977) c23–c26.
41 G. B. FINCHER, B. A. STONE AND A.E. CLARKE, *Annu. Rev. Plant Physiol.*, 34 (1983) 47–70.
42 S. EDA AND K. KATO, *Agric. Biol. Chem.*, 44 (1980) 2793–2801.
43 S. EDA, K. MIYABE, Y. AKIYAMA, A. OHNISHI, AND K. KATO, *Carbohydr. Res*, 158 (1986) 205–216.
44 A. KAJI, *Adv. Carbohydr. Chem. Biochem.*, 42 (1984) 383–394.
45 Y. TSUMURAYA AND Y. HASHIMOTO, *Carbohydr. Res.*, 161 (1987) 113–126.
46 Y. TSUMURAYA, Y. HASHIMOTO, S. YAMAMOTO, AND N. SHIBUYA, *Carbohydr. Res.*, 134 (1984) 215–228.
47 P. A. GLEESON AND A. E. CLARKE, *Biochem. J.*, 181 (1979) 607–621.
48 C. HERVE DU PENHOAT, V. MICHON, AND R. GOLDBERG, *Carbohydr. Res.*, 165 (1987) 31–42.
49 N. CARTIER, G. CHAMBAT, AND J.-P. JOSELEAU, *Carbohydr. Res.*, 168 (1987) 275–283.
50 F. M. ROMBOUTS AND J.-F. THIBAULT, *Carbohydr. Res.*, 154 (1986) 177–187.
51 M. BARBIER AND J.-F. THIBAULT, *Phytochemistry*, 21 (1982) 111–115.
52 S. ISHII, *Plant Physiol.*, 62 (1978) 586–589.
53 J.-C. VILLETTAZ AND R. AMADO, *Lebensm.-Wiss. Techno.*, 14 (1981) 176–181.