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New investigations of the structure of grape arabinogalactan–protein

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ABSTRACT

The structure of an arabinogalactan–protein (AGP) isolated from grape juice was studied by methylation analysis, n.m.r. spectroscopy, and interaction with peanut lectin, after specific degradation with purified enzymes and/or Smith degradation. AGP appeared to be homogeneous with a weight-average molecular weight of 110 000. Treatment of AGP with arabinofuranosidase released 88% of the arabinose and left GP_1. Hydrolysis of GP_1 with an endo-(1→6)-β-D-galactanase removed 50% of the galactose and left GP_2. Smith degradation of GP_1 gave a 3-linked galactan that still contained 3,6-linked residues. Endogalactanase- and Smith-degraded GP_1, but not AGP and GP_1, reacted strongly with peanut lectin. Thus, AGP is a 3-linked galactan cross-linked at positions 6. The core also carries, at positions 6, 6-linked galactan chains heavily 3-substituted with arabinofuranose residues.

INTRODUCTION

Arabinogalactan–proteins (AGPs) are found in most higher plants[1], but their fine structures have not been determined fully. The occurrence of an AGP in the grape has been reported[2], which might be involved in microfiltration membrane plugging[3] and wine-colour stabilisation in wine-making technology. Grape AGP has been characterised partially[2] and further data on the structure are now reported.

EXPERIMENTAL

Extraction and purification of grape AGP. — Grapes (Carignan noir cv.) were harvested at technological maturity in the I.N.R.A. experimental vineyards at Pech Rouge (Gruissan 11300, France) in September 1988. Berries were separated by hand from the stalks and pressed to give a crude must that was centrifuged. The polysaccharides were isolated from the clear juice by precipitation with EtOH (5 vols., 48 h at 4°), and a solution in water was dialysed extensively against distilled water, then concentrated in a vacuum at $<40°$. Proteins were eliminated[4] by shaking with 5:1 $CHCl_3$–1-butanol. Finally, the acidic polysaccharides were separated from the neutral poly-

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saccharides by ion-exchange chromatography on DEAE-Sephacel[2], and AGP was purified from the neutral fraction by size-exclusion chromatography on Ultrogel AcA 34, as described[2].

Enzymes. — An endo-(1→6)-β-D-galactanase was purified to homogeneity from a crude liquid enzyme preparation from *Aspergillus niger* (Gist-Brocades, France)[5]. An α-L-arabinofuranosidase (EC 3.2.1.55) was purified to homogeneity from the same preparation by precipitation with ammonium sulfate to 90%, and successive elutions from Ultrogel AcA 44 (50mM phosphate buffer, pH 7.2), Phenyl-Sepharose CL-6B (M ammonium sulfate), DEAE-Sepharose CL-6B (25mM phosphate buffer, pH 7.2, then a gradient 0→0.5M NaCl), Hydroxyapatite-Ultrogel (mM phosphate buffer, pH 6.8 for adsorption, then a gradient 1→50mM), and two additionnal steps on DEAE Sepharose Cl-6B (25mM phosphate buffer + 0.2M NaCl, pH 7.2, then a gradient 0.2→0.5M NaCl).

The enzyme was homogeneous in SDS-PAGE with an apparent molecular weight of 80 000. It had a specific activity of 663 nkat/mg (1 nkat = 1 nmol of *p*-nitrophenol liberated per s at pH 4.2 and 40° from 2mM *p*-nitrophenyl α-L-arabinofuranoside). The enzyme had no detectable activity on the previously described substrates[6], citrus pectin (Fluka 76 280) and polygalacturonic acid from orange (Sigma P-1879), the optimum pH was 4.5, it was stable in the pH range 4.5–6.5 (16 h, 40°), and the optimum temperature was 60°.

General. — Neutral sugars were determined, after hydrolysis with 2M trifluoroacetic acid (1.25 h, 120°) by g.l.c. of the alditol acetate derivatives, prepared using perchloric acid as the catalyst for acetylation[7], on a fused-silica capillary column (30 m × 0.32 mm i.d.) bonded with OV-225 (0.25-μm film) at 210° (injector and detector temperature, 250°; split ratio, 1:20; hydrogen as carrier gas at 65 kPa). Uronic acids and proteins were assayed by the *m*-phenylphenol[8] and Lowry[9] methods, respectively. Uronic acids were also reduced[10] with $NaBD_4$. T.l.c. was performed on Kieselgel 60 (Merck), using 9:1 acetonitrile–water and detection (10 min, 100°) with 0.2% of naphthoresorcinol in 95:5 ethanol–sulfuric acid. Amino acid composition was determined, after hydrolysis by 6M HCl (10 h, 110°)[11], under N_2, with a Kontron Chromakon 400 auto-analyser.

Enzymic degradation. — A solution of AGP (2.5 g) in 0.1M acetate buffer (0.5 mL, pH 4.2) was incubated for 72 h at 40°, with successive additions of α-L-arabinofuranosidase (6 μL, activity 623 nkat/mL) at 0, 24, and 48 h. The degradation was monitored by t.l.c. and the arabinose liberated was determined by the NAD^+–galactose dehydrogenase system[12]. The degraded AGP was separated from the arabinose by elution from a PD10 column (Pharmacia) with water and the eluate was freeze-dried to give GP_1. A solution of GP_1 (3.5 mg) in 0.1M acetate buffer (1.4 mL, pH 4.2) was incubated at 40°, with successive additions of endo-(1→6)-β-D-galactanase (20 μL) at 0 and 24 h. Aliquots were withdrawn, diluted with EtOH (5 vols.), and centrifuged. Each supernatant solution was air-dried, the residue was hydrolysed by trifluoroacetic acid (2M, 1 h, 100°), and the products were converted into alditol acetates and analysed by g.l.c.. The degraded GP_1 was isolated as described above for degraded AGP, to give GP_2.

For preparative purposes, AGP (300 mg) and GP_1 (60 mg) were each degraded as described above, but with incubation for 24 h with no enzyme supplementation.

Size-exclusion chromatography. — AGP, GP_1, and GP_2 were each fractionated on a FPLC Superose 6 column (Pharmacia, preparative grade.; V_o 23.1 mL, V_t 65.2 mL; plate number per m, 16 000) in 0.2M NaCl at 0.6 mL/h. Solutions (0.2 mL) containing 2 mg/mL of polysaccharide were injected and the eluate was monitored by differential refractometry. AGP (20 mg) was also eluted from a Sephacryl S-400 column (V_o 80 mL, V_t 198 mL) with 0.2M NaCl at 33 mL/h, and 3.3-mL fractions were collected and analysed by g.l.c. Finally, AGP was eluted from an HPSEC system[13] involving 4 Shodex OH-pack columns (B-803–806), using 0.1M $LiNO_3$ at 1 mL/min, with online determination of molecular weight by low-angle laser-light scattering (Chromatix CMX 100, LDC Analytical). The weight-average molecular weight (M_w) was also estimated using a universal calibration curve[14] [$\ln([\eta] \times M_w)$ *vs.* K_{av}] of the Superose 6 column, established at 25° with a pullulan calibration kit (Showa denko). The intrinsic viscosities [η] of the AGP and pullulan standards were determined at 25° in 0.2M NaCl, using an AVS400 viscosimeter (Schott Geräte, Germany) fitted with a Ubbelohde micro-viscosimeter (solvent flow time, 91.27 s).

Periodate oxidation and Smith degradation. — A solution of GP_1 (30 mg) in 30mM $NaIO_4$ (10 mL) was kept at room temperature in the dark. The consumption of $NaIO_4$ was monitored by measuring the absorbance at 225 nm. After 72 h, glycerol (50 μL) was added, and the mixture was dialysed against distilled water (24 h), concentrated to 10 mL, and treated with $NaBH_4$ (0.8M, at room temperature for 48 h). The excess of $NaBH_4$ was destroyed by the addition of glacial acetic acid, the mixture was dialysed against distilled water (24 h), and concentrated to 5 mL, 2M trifluoroacetic acid (5 mL) was added, and the mixture was kept at 20° for 48 h. The extent of degradation was monitored by size-exclusion chromatography on a column of Superose 6. Smith-degraded GP_1 (GP_1S_I) was isolated by freeze-drying and a solution in water was freed from oligomeric products by chromatography on a PD10 column (Pharmacia). GP_1S_I and GP_2 were submitted to periodate oxidation and Smith degradation under similar conditions to give GP_1S_{II} and GP_2S_I, respectively, but size-exclusion monitoring was omitted due to the scarcity of the product.

Controlled acid hydrolysis. — AGP (10 mg) was treated with 0.01M trifluoroacetic acid (2 h, 100°), and then freed from oligomeric products by elution from a PD10 column. The V_o was collected to give a galactan–protein GP_H and the total volume was analysed by t.l.c. GP_H was submitted to Smith degradation to give GP_HS_I.

Methylation analysis. — Native, reduced, and enzyme-, acid-, and Smith-degraded AGP were each methylated according to the Hakomori method[15]. Each product was hydrolysed successively with aqueous 90% formic acid (1 h, 100°) (except for native AGP), then 2M trifluoroacetic acid (1.25 h, 120°). The partially methylated sugars were converted into their alditol acetates[7] and analysed[16] by g.l.c. on fused-silica capillary columns of DB-1 and DB-225. Identification was based on relative retention times and confirmed by g.l.c.–m.s. by coupling the DB-225 column to a Finnigan Mat ITD 400 mass spectrometer (on-column injection; injector temp. 50°→220° at 60°/min; oven

temp. 90°→170° at 10°/min then 5°/min up to 210°; He was the carrier gas). Peak areas were corrected by response factors[17].

Gel-diffusion experiments. — Petri dishes containing 1% of agarose gel in 10mM Tris buffer (pH 7.3) together with 0.9% NaCl and mM $CaCl_2$, were used. Peanut lectin (Sigma; 40 μL, 4 mg/mL)[18] or Yariv reagent (Bio-supplies, Australia; 40 μL, 1 mg/mL)[19] was delivered to a central well and the polysaccharides (40 μL, 1 mg/mL) were put in equidistant peripheral wells. Gels were left at room temperature overnight.

Cleavage of alkali-labile sugar–protein linkages and enzymic degradation of the peptidic moiety. — A solution of AGP (2 mg) in 0.2M NaOH containing 0.3M $NaBH_4$ (1 mL) was incubated[20] at 45° for 6 h, then cooled and treated (10 h, 110°, under N_2) with conc. HCl (1 mL), and the amino acids were analysed. GP_2 (3 mg) was incubated (24 h, 37°) with pronase (50 μg, Boehringer) in 0.1M phosphate buffer (1 mL, pH 7.0) and then injected on the Sephacryl S-400 column as described above.

N.m.r. spectroscopy. — A Bruker AM-400 spectrometer operating in the F.t. mode (^1H 400.13 MHz, ^{13}C 100.57 MHz) was used on solutions in D_2O (10–15 mg in 0.5 mL with 5-mm tubes). Me_2SO was the internal standard (^{13}C, δ 39.5; ^1H, δ 2.72). The spectral window for the ^1H-n.m.r. spectra was 10 p.p.m. for 16 k data points with a pulse width of 8 μs (45°), a 3-s delay between each scan, and an acquisition time of 2 s. ^{13}C-N.m.r. spectra were recorded with complete proton decoupling and a pulse width of 8 μs (60°). The acquisition time was 1.11 s with a 3-s delay between each scan. Partially relaxed ^{13}C-n.m.r. spectra were measured using the inversion–recovery method (180°–τ–90°) with τ values of 150 and 300 ms.

For AGP, a double-quantum-filtered phase-sensitive COSY experiment[21] was performed using a (90°)–(t_1)–(90°)–(90°)–(FID, t_2) sequence. The spectral width in F1 and F2 was 2048 Hz; the number of data points in F2 was 1024, and 512 increments were recorded. The 90° pulse was 21 μs and the total acquisition time was 16 h. Before Fourier transformation, $\pi/2$-shifted squared sine-bell-weighting functions were applied. Magnitude COSY spectra were acquired for GP_1 and GP_2, using a (90°)–(t_1)–(90°)–(FID, t_2) sequence. Acquisition conditions were as above except that 256 increments were recorded. Before Fourier transformation, the data were multiplied with a sine bell. Zero-filling was applied in F1. Phase-sensitive NOESY experiments[22] were performed using a (90°)–(t_1)–(90°)–τ–(90°)–(FID, t_2) sequence and a mixing time (τ) of 100 ms. 512 × 1 K data matrices were obtained and zero-filled to 1 K × 1 K. Prior to Fourier transformation, the first data file was halved to reduce t_1 ridges and $\pi/2$-shifted squared sine-bell-weighting functions were applied[23].

RESULTS AND DISCUSSION

Characterisation of AGP. — Purified AGP accounted for ~ 17% of the soluble polysaccharides isolated from grape juice, gave a positive reaction with Yariv antigen which is specific for AGP[24], and contained 90.5% of neutral sugars (Table I), mainly arabinose and galactose with traces of rhamnose, mannose, and glucose. Glucuronic acid accounted for 3.5% of the molecule and was identified when carboxyl reduction

TABLE I

Physical properties and chemical composition of native and enzyme-degraded AGPs

Properties and composition	*AGP*	*GP_1*	*GP_2*
Mol. wt.[a]	110000	80000	50000[d]
$<Rg>$ (Å)[a]	74	68	56
$[\eta]$ (mL/g)	14.0	15.2	n.d.[e]
Proteins[b]	6.0	13.6	17.8
Uronic acid[b]	3.5	4.5	7.9
Neutral sugars[b]	90.5	81.9	74.3
Arabinose[c]	39.0	6.3	5.2
Galactose[c]	61.0	93.7	94.8

[a] Determined by size-exclusion chromatography from the universal calibration curve; $<Rg>$: mean square radii of gyration. [b] Percent dry-weight basis. [c] Mol.%; traces of rhamnose, mannose, and glucose were also detected. [d] Estimated from extent of hydrolysis. [e] Not determined.

gave glucose. Proteins were rich in hydroxyproline (30% of the total amino acids), serine (16%), alanine (14%), threonine (10%), and glutamic acid (10%). These data were similar to previous results[2] on grape AGP, but there were differences in the amino acid composition; in particular, the content of hydroxyproline was higher.

In size-exclusion chromatography, on three different gels, AGP was eluted as a narrow symmetrical peak (Fig. 1) which indicated a narrow distribution of molecular weights, as confirmed by the polydispersity index of 1.12 determined by low-angle laser-light scattering[25]. AGP was eluted between K_{av} 0.37 and 0.71 from a column of Sephacryl S-400. Analysis of the product after each 0.03 K_{av} showed that the arabinose–

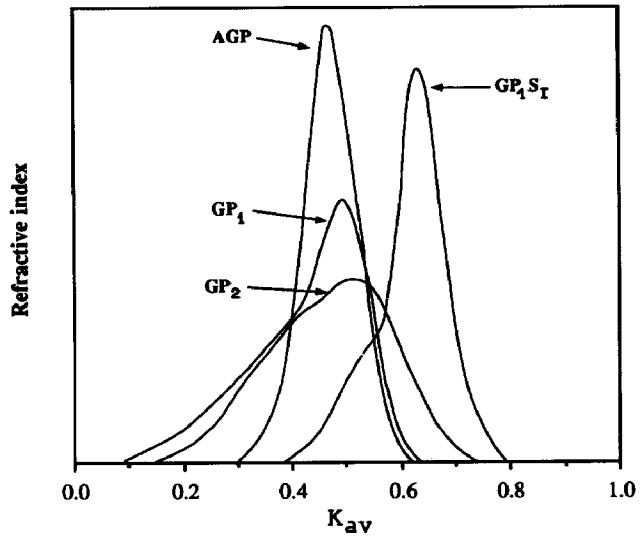

Fig. 1. Size-exclusion chromatography of native and degraded AGPs on Superose 6.

galactose ratio remained constant within experimental error, with a mean value of 0.64.

The intrinsic viscosity [η] of AGP was low (Table I) which suggested a compact structure. The M_w, estimated from universal calibration, was 110 000, whereas a value of 130 000 was determined from the light scattering. The discrepancy may be ascribed to the relative imprecision of the [η] determination due to its low value.

There was good agreement between the composition based on analysis of the alditol acetates and on methylation analysis which indicated complete methylation. Methylation analysis (Table II) showed that terminal arabinose was lost when methylated AGP was subjected to formolysis. Arabinose was found mainly at terminal nonreducing positions, and galactose in 3-, 6-, and 3,6-linked positions, which reflected a typical, highly branched, type II arabinogalactan structure. Reduction of the uronic acid with $NaBD_4$ showed that glucuronic acid was in terminal and 4-linked positions.

The ^{13}C- and ^{1}H-n.m.r. data for AGP are collected in Tables III and IV. α-L-Ara*f* (C-1 at 109.81–111.02 p.p.m.) and β-D-Gal*p* (C-1 at 104.49–105.92 p.p.m.) were detected in the ratio 2:3. The ^{13}C (C-2/5 at 82.89, 78.16, 85.43, and 62.84 p.p.m.) and ^{1}H (see footnotes to Table IV) assignments for the α-L-Ara*f* residue were obtained from 2D heteronuclear [^{1}H–^{13}C] and homonuclear [^{1}H–^{1}H] chemical shift correlation spectra. Similar ^{13}C- and ^{1}H-n.m.r. data have been reported for methyl α-L-arabinofuranoside[26,27] and pectic substances from *Vigna radiata*. At least 4 coupling networks could be attributed to the α-L-Ara*f* residues in the COSY spectrum, which reflected the complexity of the polysaccharide. The ^{13}C signals of the α-L-Ara*f* residue were much sharper than those of the Gal residues which suggested enhanced mobility for these sugars. Polysaccharides have been sequenced according to the ^{13}C T_1 values of the various sugars[28]. For amylopectins[29], the terminal units relax more slowly than those in the main chain.

TABLE II

Methylation analysis of native and degraded AGPs (mol.%)

Methyl ether	*Linkage*	*AGP*	*GP_1*	*GP_2*	*GP_1S_I*	*GP_1S_{II}*	*GP_2S_I*
2,3,4-Rha[a]	Terminal	0.5	0.8	1.3			
2,3,5-Ara	Terminal	34.0	6.3	5.9			
2,5- + 3,5-Ara	1→2,3	0.9	0.9	1.2			
2,3-Ara	1→5	1.1	0.4	0.7			
2,3,4,6-Gal	Terminal	2.6	17.0	25.2	9.3	3.1	4.2
2,4,6-Gal	1→3	16.3	8.0	14.2	78.6	86.6	84.3
2,3,4-Gal	1→6	6.2	35.7	15.2	1.7	0.4	
2,3,6-Gal	1→4	0.3	1.6	1.7			
2,6-Gal	1→3,4	2.1	0.8	0.9			
2,3-Gal	1→4,6	0.5	1.8	2.2		0.3	
2,4-Gal	1→3,6	31.6	25.4	30.6	7.1	7.3	8.7
2-Gal	1→3,4,6	4.0	1.4	1.6	3.3	2.3	2.8

[a] 2,3,4-Rha = 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylrhamnitol, *etc.* 2,3,4,6-, 2,3,6-, and 2,3,4-Glc, and 2,3,4,6- and 3,4,6-Man were observed as traces in AGP, GP_1, and GP_2.

Broadened signals of branch-point residues, due to a reduced rate of segmental motion, suggest that the correlation times of such groups are situated on the other side of the T_1 minimum [$1/T_1 = f(\tau_c)$] with respect to the end groups. Moreover, as ^{13}C relaxation of carbohydrates is dominated by dipolar interactions with attached protons, the T_1 values of ^{13}C in CH_2OH are ~ 50% of those of the ^{13}C in CHOH. The partially relaxed ^{13}C-n.m.r. spectra[30] of AGP are given in Fig. 2. In Fig. 2a, the signals of C-1/4 of α-L-Ara*f* are inverted, the corresponding Gal resonances are almost nulled, and the signals for CH_2OH (62.56–62.84 and 70.50–72.76 p.p.m.) are positive and easily identified. In Fig. 2b, the signals of α-L-Ara*f* are weak, whereas those of β-D-Gal*p* are strong. The ^{13}C assignments for C-3/5 of the Gal residues were based on comparison with data for related galactosyl-containing compounds[31–34]. The signal at 71.43 p.p.m. was identified as due to C-2 of β-D-Gal*p* from a 2D heteronuclear chemical-shift-correlation spectra. All the signals of the β-D-Gal*p* residues were broad which suggested a complex polymer. Only partial ^1H data could be obtained for the Gal residues. The numerous H-1/H-2 correlations in the DQFCOSY[21] spectrum corroborated the ^{13}C data regarding the complexity of the polysaccharide. For all samples, broadened signals (probably of the amino acid residues) were barely visible in the 1D ^1H-n.m.r. spectrum at high field. These peaks did not yield cross-peaks in the 2D spectra and were not assigned.

Degradation of the arabinofuranose substituents. — An α-L-arabinofuranosidase from *Aspergillus niger* selectively removed terminal Ara*f* units from AGP and left a

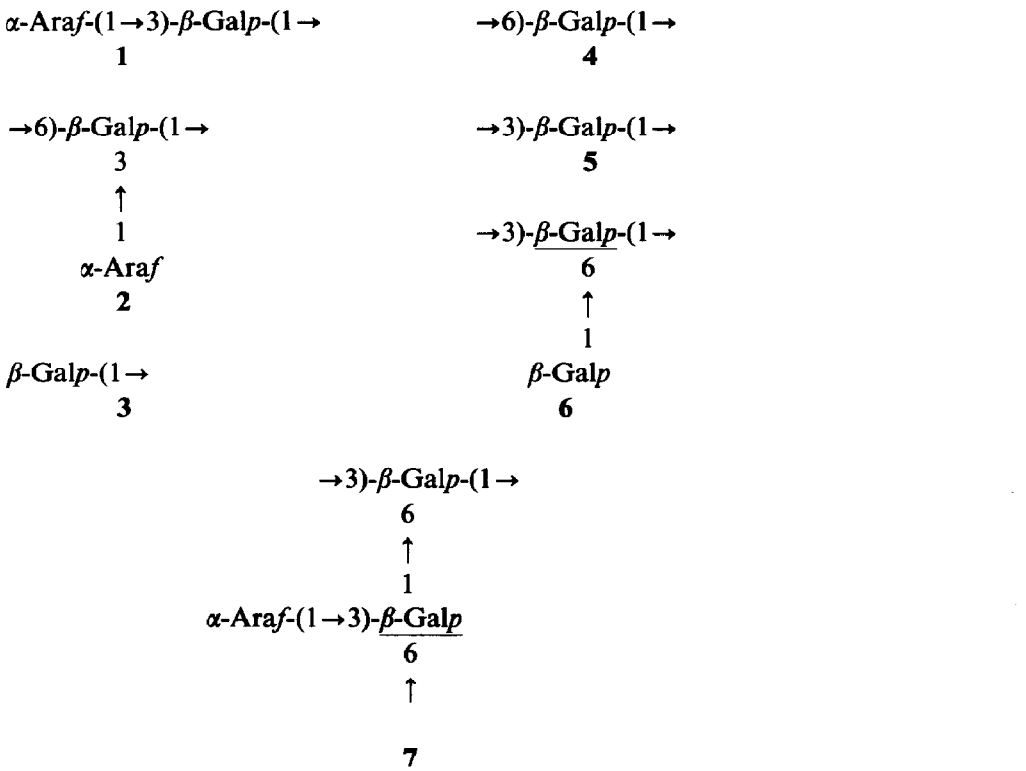

TABLE III

100-MHz ^{13}C-n.m.r. data (D_2O)

Fraction	*Chemical shifts*	*Integral*[a] *(%)*[b]		*Assignment*[c]	*Fraction*	*Chemical shifts*	*Integral*[a] *(%)*[b]	*Assignment*[c]
AGP	111.02	0.15			GP_2	104.37–106.40	1.00	C-1 β-Gal*p* of **3–6**
	110.78	0.70	(41)	C-1 α-Ara*f* of **1** and **2**		85.83	0.03	
	109.95	0.15				82.83–84.31	0.38	C-3 β-Gal*p* of **5** and **6**
	109.81					77.07–78.60	0.15	
	104.49–105.92	1.00	(59)	C-1 β-Gal*p* of **2–6**		76.84	0.35	C-5 β-Gal*p* of **3**
	85.43	1.00		C-4 α-Ara*f* of **1** and **2**		75.86–76.74	0.23	
	85.18		(7.5)			74.64–75.68	0.50	C-5 β-Gal*p* of **4–6**
	82.89	1.00		C-2 α-Ara*f* of **1** and **2**		74.55	0.24	C-3 β-Gal*p* of **3**
		0.45		C-3 β-Gal*p* of **5** and **6**		73.32–74.48	0.49	C-3 β-Gal*p* of **4**
	81.45–82.10	0.52		C-3 β-Gal*p* of **1** and **2**		72.52	0.35	C-2 β-Gal*p* of **3** and **4**
	78.16	1.00		C-3 α-Ara*f* of **1** and **2**		70.60–72.36	1.10	C-2 β-Gal*p* of **5** and **6**
	77.41							C-6 β-Gal*p* of **5** and **6**
	76.43–76.65	0.56		C-2 β-Gal*p* of **1**, **2**, and **4–6**		70.40	0.40	C-4 β-Gal*p* of **3–6**
	74.09–75.86	1.00		C-5 β-Gal*p* of **1**, **2**, and **4–6**		70.14	0.35	C-4 β-Gal*p* of **5** and **6**
				C-3 β-Gal*p* of **3** and **4**		62.46–63.21	0.79	C-6 β-Gal*p* of **3** and **5**
	70.50–72.76	1.75		C-2 β-Gal*p* of **1–6**				
				C-6 β-Gal*p* of **2** and **4–6**				
	70.16	0.30		C-4 β-Gal*p* of **3–6**				
	70.01	0.60		C-4 β-Gal*p* of **1** and **2**				
	62.84	1.00		C-5 α-Ara*f* of **1** and **2**				
	62.56	0.65		C-6 β-Gal*p* of **1**, **3**, and **5**				

GP_1	105.21	0.50	C-1 β-Gal*p* of **5** and **6**	GP_2S_I	105.93	0.10	C-1 β-Gal*p* of **3**
	104.98	0.50	C-1 β-Gal*p* of **3** and **4**		105.60	0.90	C-1 β-Gal*p* of **5**
	82.64–84.02	0.30	C-3 β-Gal*p* of **5** and **6**		83.60	0.90	C-3 β-Gal*p* of **5**
	76.72	0.30	C-5 β-Gal*p* of **3**		76.69	0.10	C-5 β-Gal*p* of **3**
	75.31	0.30	C-5 β-Gal*p* of **4**		76.32	0.90	C-5 β Gal*p* of **5**
	75.20	0.30	C-5 β-Gal*p* of **5** and **6**		74.14	0.10	C-3 β-Gal*p* of **3**
	74.35	0.30	C-3 β-Gal*p* of **3**		72.65	0.20	C-2 β-Gal*p* of **3**
	74.20	0.30	C-3 β-Gal*p* of **4**		71.84	1.00	C-2 β-Gal*p* of **5**
	72.32	0.80	C-2 β-Gal*p* of **3** and **4**		70.18	1.00	C-4 β-Gal*p* of **3**
	70.43–72.73	1.20	C-2 β-Gal*p* of **5** and **6**		70.00		C-4 β-Gal*p* of **5**
			C-6 β-Gal*p* of **4** and **6**		64.09		
	70.22	1.00	C-4 β-Gal*p* of **3–6**		62.52	1.2	C-6 β-Gal*p* of **3** and **5**
	62.63	0.60	C-6 β-Gal*p* of **3** and **5**				

[a] Fraction of the corresponding glycosyl (Ara*f* or Gal*p*) C-1 integral. [b] Fraction of the total signals for C-1. [c] Underlined residue in formula where appropriate.

TABLE IV

400-MHz ^{1}H-n.m.r. data for native and enzyme-degraded AGPs in D_2O

Fraction	*Chemical shifts*		*Relative values*		*H-1 Integral (%)*[a]	*Compatible structures*
	H-1	*H-2*	$J_{1,2}$	$J_{2,3}$		
AGP	5.47	4.22[b]	s[h]	s	6	
	5.30	4.25[c]	s	s		
	5.27	4.25[c]	s	s	35	α-Ara*f*
		4.22[b]	s	s		
	5.11	4.16	s		1	
	4.72	3.80	l		16	
	4.51–4.56	3.67–3.75	l	l	42	β-Gal*p*
	4.48	3.53[d]	l	l		
GP_1	4.74	3.82	l		31	
	4.45–4.56	3.58	l	l	69	
GP_2	4.72	3.80[e]	l		44	
	4.43–4.56	3.65–3.71	l	l	56	
GP_2S_1	4.68	3.77[f]	l	l	90	**5**
	4.61	3.60[g]	l	l	10	

[a] Percent of total H-1 signals. [b] H-3 at 4.00 and H-4 at 4.16 p.p.m. [c] H-3 at 3.98 and H-4 at 4.16 p.p.m. [d] H-3 at 3.69 p.p.m., 22% of total Gal*p*. [e] H-3 at 3.85 and H-4 at 4.19–4.32 p.p.m. [f] H-3 at 3.85 and H-4 at 3.91 p.p.m. [g] H-3 at 3.66 and H-4 at 3.91 p.p.m. [h] Key: s, small; l, large.

galactan–protein GP_1. As observed[2] with a similar enzyme from *Dichomitus squalens*, only ~ 88% of arabinose was released from the AGP. Enzyme supplementation did not increase the extent of hydrolysis, and only arabinose was released (t.l.c.). Amino acid composition of the protein and uronic acids remained unaltered after the treatment with the enzyme (Table I), and GP_1 gave a positive reaction with Yariv antigen.

GP_1 was eluted from a column of Superose 6 (Fig. 1) as a main peak at K_{av} 0.5, which reflected a molecular weight lower than that of AGP (K_{av} 0.48). However, there was a shoulder in the higher K_{av} range which remains unexplained. Although this heterogeneity resulted from the action of arabinofuranosidase, such side reactions as transglycosylation or reversion[35,36] are unlikely to produce such an increase in hydrodynamic volume. The intrinsic viscosity of GP_1 was low (Table I) and close to the value for AGP, which suggested that treatment with the enzyme had not modified the overall conformation of the macromolecule. The value of M_w, estimated by universal calibration and assuming a K_{av} of 0.5 for GP_1, was 80 000, which agreed well with that (79 000) calculated from the M_w of AGP and the extent of hydrolysis.

The methylation analysis data for GP1 are reported in Table II. The residual Ara was mainly in terminal nonreducing positions which suggested that the resistance to arabinosidase may have been due to steric hindrance. The effects of the enzyme on the structure were similar to those reported[2], *e.g.*, an increase in 6-linked and terminal Gal and a decrease in 3,6-linked and 3-linked Gal. Therefore, most of the Ara*f* was 3-linked to 6-linked galactan chains.

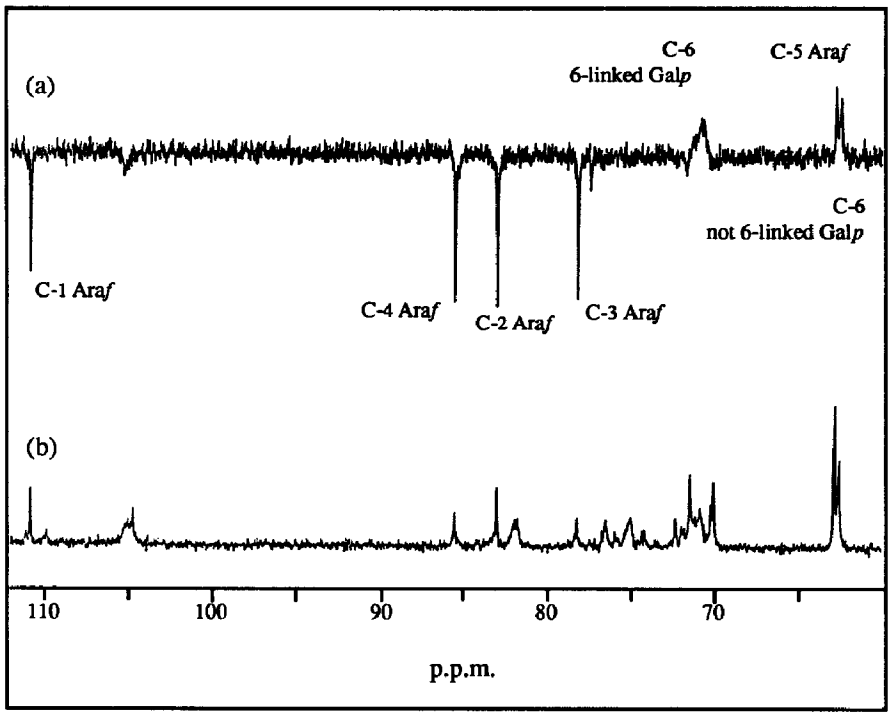

Fig. 2. Partially relaxed ^{13}C-n.m.r. spectra of AGP: (a) τ 0.15 s and (b) τ 0.30 s, where τ is the delay in the inversion–recovery pulse sequence.

N.m.r. experiments confirmed these observations (Tables III and IV). Comparison of the ^{13}C-n.m.r. spectra of GP1 and AGP showed that the signals for α-L-Ara*f* residues and 3-linked β-D-Gal*p* with the C-3 signal between 81.45–82.10 p.p.m. had disappeared and there were sharp signals for two new residues, namely, terminal β-D-Gal*p* (C-1/6 at 104.98, 72.32, 74.35, 70.22, 76.72, and 62.63 p.p.m.) and 6-linked-β-D-Gal*p* (C-1/5 at 104.98, 72.32, 74.20, 70.22, and 75.31) with intensities about one-third of the total for C-1. These units were identified by comparison with ^{13}C data for related galactosyl-containing compounds. The intensities of the signals of the terminal β-D-Gal*p* residues were roughly twice those expected from chemical analysis, but it is known[29] that the carbons of terminal units experience stronger n.O.e.'s than those of internal residues. In a similar way, a partially relaxed ^{13}C-n.m.r. spectrum confirmed the ^{13}C signals of the CH_2OH groups of the various β-D-Gal*p* residues. Two strong signals of equal intensities at 70.84 and 71.06 p.p.m. for C-6 of the 6-linked β-D-Gal*p* residues were observed. Broad signals were observed for C-3 of 3-linked-β-D-Gal*p* residues at 82.64–84.02 p.p.m. (about one-third of the total for C-1) and the broadened base of the C-6 signal at 62.63 p.p.m. suggested that a significant proportion of these sugars was not branched at position 6. The ^{1}H-n.m.r. spectrum of GP_1 reflected essentially two groups of coupling networks. The H-1 and H-2 peaks of the major components (69%) resonated at 4.45–4.56 and 3.58 p.p.m., respectively, and showed the fine structure

expected for β-D-Gal*p* units [several d in the H-1 region (*J* 8 Hz), and a dd(t) in the H-2 region]. Signals of the minor component were markedly broadened (H-1 at 4.72 p.p.m. and H-2 at 3.82 p.p.m.) (31%).

In gel-diffusion with peanut lectin, which is specific for the β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→moiety[37], GP_1 gave a weak precipitation band, whereas AGP gave no reaction. As postulated[2], this result confirmed that most of the terminal nonreducing Gal*p* residues belong to 6-linked chains, which hampered interaction with terminal Gal*p* of 3-linked chains.

Controlled acid hydrolysis of AGP resulted in incomplete removal of terminal Ara*f* and traces of Gal were also removed (t.l.c.). Nevertheless, the product GP_H was eluted as a single peak from a column of Superose 6 (K_{av} 0.54), and methylation analysis gave results close to those obtained for GP_1 (Table II).

Degradation of (1→6)-linked β-D-galactose chains. — GP_1 was degraded by endo-(1→6)-β-D-galactanase to give GP_2, whereas AGP was resistant to the enzyme. Of the galactose content of GP_1, ~ 50% was removed by this treatment, leaving uronic acids and protein unchanged. GP_2 gave a positive reaction with the Yariv antigen. Chromatography of GP_2 on Superose 6 clearly showed that a product of higher molecular weight was eluted as a shoulder of the main peak (K_{av} 0.56; Fig. 1). It is assumed that these two components reflect the heterogeneity of GP_1 and were not induced by the treatment with the endogalactanase. Although the enzyme was highly purified, traces of contaminant arabinofuranosidase had partially hydrolysed (~ 50%) the remaining Ara of GP_1; (1→6)-β-D-galactobiose and D-galactose were the final products.

The methylation analysis data for GP_2 are reported in Table II. Calculations, based on methylation analysis of GP_1 and GP_2, and taking into account that only Gal (extent of hydrolysis 50%) was released from GP_1, showed that 80% of 6-linked Gal residues inititally present in GP_1 were removed by the enzyme, 3-linked Gal chains being untouched. Therefore GP_2 was enriched in 3-linked Gal chains as compared to GP_1, but still contained 6-linked Gal residues (proportion similar to that of 3-linked residues). Enzyme supplementation failed to split these residual (1→6) linkages. The galactan backbone of GP_2 was still highy substituted, as attested by the high proportion of 3,6-linked Gal residues. Therefore, the endogalactanase had shortened the 6-linked Gal chains, which exhibited a d.p. of at least 7, as suggested by the detection of galacto-oligosaccharides up to d.p. 7 in the early stage of enzyme action[4].

GP_2 gave a strong precipitation line in a gel-diffusion experiment with peanut lectin, which showed that endogalactanase had eliminated external 6-linked Gal chains, thereby allowing the interaction of the β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→moiety of the internal 3-linked Gal backbone with the lectin.

The n.m.r. data of GP_2 are reported in Tables III and IV, and in Fig. 3. The ^{13}C signals corresponding to the 6-linked Gal*p* residues (*i.e.* C-5 and C-3 at 75.31 and 74.20 p.p.m., respectively) were much weaker (< 15% of the total for C-1), whereas those of terminal and 3-linked Gal residues were enhanced (50 and 38%, respectively, of the total for C-1). The peaks appeared to be multiple resonances with the exception of those of

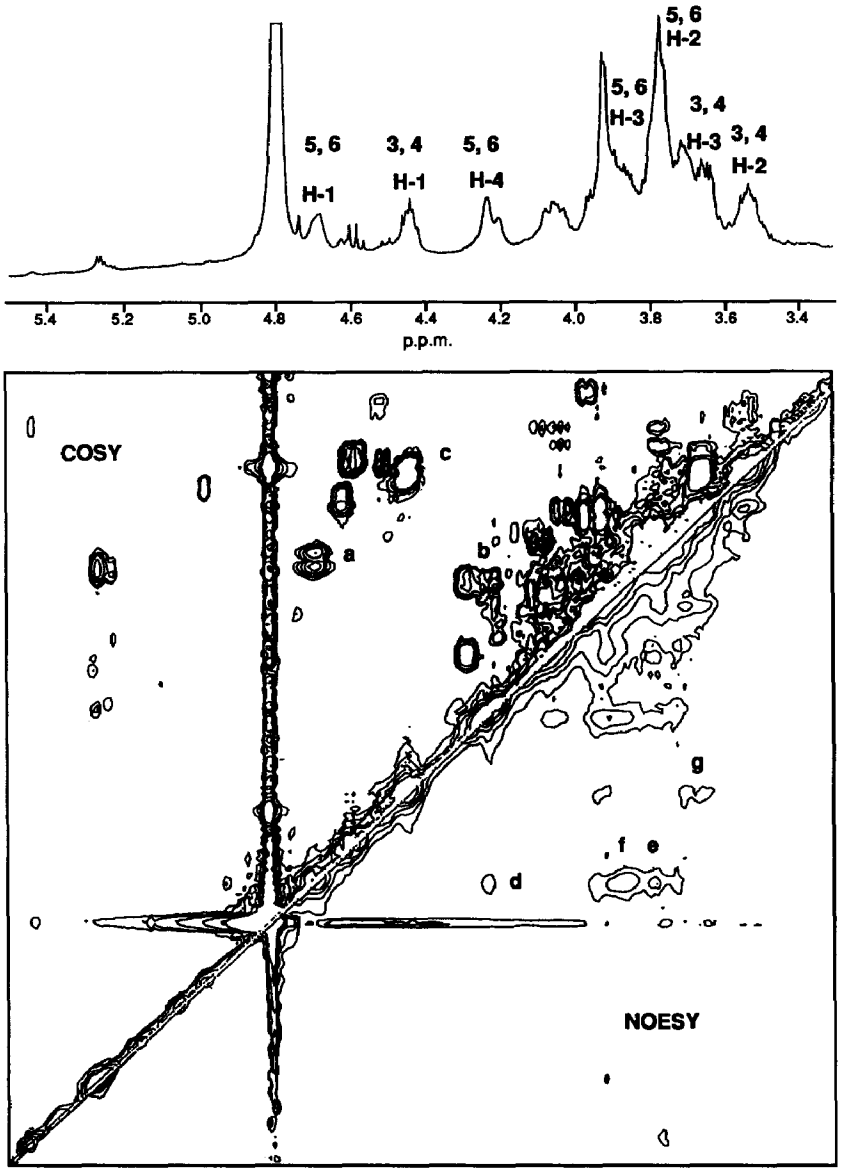

Fig. 3. 400-MHz 2D-n.m.r. spectra of GP_2 in D_2O (2.4 mg/0.5 mL): the corresponding 1D spectrum is given above along the F2 axis. The following cross-peaks are indicated: COSY, **a** H-1/H-2 of **5** and **6**, **b** H-4/H-3 of **5** and **6**, **c** H-1/H-2 of **3** and **4**; NOESY, **d** H-1/H-4 of **5** and **6**, **e** H-1/H-2 of **5** and **6**, **f** H-1/H-3,5 of **5** and **6**, **g** H-1/H-3,5 of **3** and **4**.

the terminal units. The complex, highly branched nature of the polysaccharide was also evident from the multiple ^1H peaks. Sharp signals for reducing α- and β-Gal residues were present in both the ^1H- (<5%) and ^{13}C-n.m.r. spectra, undoubtedly due to incomplete destruction of the enzyme at the end of the hydrolysis reaction. Both the COSY and the phase-sensitive NOESY[22] spectra of GP2 are given in Fig. 3. The latter

spectrum was acquired with a mixing time of 100 ms and, under these conditions, H-1/H-3,5 cross-peaks labelled g were just visible for the Gal residues with H-1 resonating at 4.5 p.p.m. In contrast, the coupling network for the H-1/4 (labelled d–f) of the Gal residues with H-1 resonating at 4.72 p.p.m. could be extracted from the cross-peaks, taking into account the assignments available from the COSY experiment. The n.O.e. between H-1′ of the glycosylating residue and H-4 of the aglycon in 3-linked Gal chains is expected[37] to be an order of magnitude less than that between H-1′ and H-3 (*i.e.*, 0.7% as compared to 7%). Judging from the intensity of the H-1/H-4 cross-peaks (about a third of the H-1/H-3,5 cross-peaks), it seems likely that, for this mixing time, spin diffusion dominates the cross relaxation of the protons of this residue. This finding suggests that these residues are located in the main chain of the polysaccharide where movement is the most restricted. It is known[39] that, when a direct path for inter-residue propagation of spin diffusion exists, cross-peaks between the corresponding anomeric protons are present in the NOESY spectrum. For the (1→3,1→4)-linked β-D-glucan from barley, cross-peaks between the anomeric protons of the (1→3)- and (1→4)-linked β-Glc residues are indeed observed[40] in the NOESY spectrum acquired with a mixing time of 100 ms. Cross-peaks between the anomeric protons resonating at 4.72 and 4.5 p.p.m. are not observed, suggesting that these two types of residues are not located in the same chain. The latter signals correspond either to side- chain residues attached to HO-6 or to residues belonging to a distinct region of the macromolecule.

The ^{13}C signals of GP_2 are very broad for the 3-linked sugars and sharper for the terminal and 6-linked residues. This finding suggests that the sugars whose anomeric protons resonate at 4.72 p.p.m. correspond to structures **5** and **6**, whereas the residues whose anomeric protons resonate at 4.5 p.p.m. correspond to structures **3**, **4**, and possibly **7**, as this fraction contains ~ 5% of terminal Ara*f* residues, according to the results of methylation analysis. Based on these tentative assignments, it can be estimated that most of the 3,6-linked Gal residues determined by methylation analysis correspond to fragment **6**.

Periodate oxidation and Smith degradation of GP_I gave GP_IS_I. After 48 h, the consumption of $NaIO_4$ was constant (5.70 μmol per mg of GP_I), which was in good agreement with the value (5.65 μmol/mg) estimated from composition and methylation analysis. Therefore, it was estimated that all of the Ara and 56% of the Gal were removed from GP_I after Smith degradation. The oxidised and reduced GP_I was submitted to controlled hydrolysis with M trifluoroacetic acid at 20° and the hydrolysis was monitored by gel-permeation chromatography[41] on Superose 6. The initial K_{av} of oxidised GP_I was 0.55 and increased to a constant value of 0.62 after hydrolysis for 18 h. The product GP_IS_I was eluted as a main peak that presented a shoulder at high K_{av} (Fig. 2), which was attributed to initial heterogeneity of GP_I. GP_IS_I was devoid of Ara and exhibited 3-linked Gal as the main constituent (Table II), although some of the 6-linked Gal chains substituted by Ara in GP_I were still present in GP_IS_I. Methylation analyses of the main peak and the shoulder fraction revealed identical structures. A second Smith-degradation of GP_IS_I gave GP_IS_{II} with elimination of the residual 6-linked Gal (Table II). Smith degradation of GP_H gave a galactan which exhibited a structure very similar

to GP_1S_I (Table II). Significant proportions of 3,6-linked Gal residues were present in GP_1S_I and GP_1S_{II} (Table II), indicating that 3-linked chains are probably interlinked through positions 6. Both GP_1S_I and GP_1S_{II} gave positive reactions with peanut lectin and Yariv antigen.

Smith degradation of GP_2 gave GP_2S_I, methylation analysis of which indicated a structure similar to that of GP_1S_{II} (Table II). The ^{13}C- and ^1H-n.m.r. data of a sample (2.5 mg/0.5mL) of GP_2S_I are collected in Tables III and IV. Signals for two types of β-D-Gal*p* residues were observed. On the basis of the ^{13}C chemical shifts, the signals of the major (90%) and the minor (10%) components were assigned to **5** (signals for linked C-3 and unlinked C-6 at 83.60 and 65.52 p.p.m., respectively) and **3**, respectively. The signal-to-noise ratio in the ^{13}C-n.m.r. spectrum was poor, due to the paucity of sample, and signals for **6** were not detected. In the ^1H-n.m.r. spectrum, signals at 4.68 p.p.m. (<5% of the total for H-1) were observed. The partial proton-coupling networks of H-1/4 were extracted from the COSY spectrum and the chemical shifts for the signals corresponding to **5** are analogous to those of **5** and **6** in GP_2. At this point, it was possible to assign tentatively the broad singles at 4.19 and 4.23 p.p.m. in the spectrum of GP_2 (Fig. 4) to E and F, respectively. The 28:72 ratio of these signals is in agreement with the 5:6 ratio (32:68) determined by methylation analysis.

Degradation of the peptide moiety. — AGP was treated with alkaline borohydride in order to catalyse β-elimination of glycosidically linked amino acids, *e.g.*, threonine and serine. The treatment did not modify the proportion of amino acids, in contrast to the previous report[2], where threonine was involved in a carbohydrate–protein linkage. Although the grapes in the two studies were harvested at technological maturity, the harvest for the first report was at the end of October (1984), whereas the harvest in the present study was at the beginning of September (1988) due to different weather conditions between 1984 and 1988. Therefore, variations observed in amino acid composition and protein–carbohydrate linkage of AGP may have been induced by the different physiological conditions undergone by the grape in 1984 and 1988. Digestion of GP_2 with pronase did not modify the elution pattern on Sephacryl S-400 or the amino acid composition. The resistance of AGPs to proteolytic enzymes has been reported[42]. Although steric hindrance should have been reduced by digestion of AGP with arabinofuranosidase and endogalactanase, the glycosylation and/or the presence of a high proportion of hydroxyproline may still explain the inefficiency of the action of proteolytic enzymes on GP_2.

It is concluded that grape AGP is composed of a central 3-linked core surrounded by external 6-linked galactan chains highly substituted by terminal Ara*f* units. The broadened signals in the ^{13}C-n.m.r. spectra for 3-linked Gal, as compared to those of terminal Ara*f* and 6-linked Gal, could reflect a lower rate of segmental motion compatible with a central position of the 3-linked chains. A significant proportion of the 3,6-linked residues remained in the periodate-resistant backbone of AGP. Similar results were observed after Smith degradations of arabinogalactan from *Rubus fruticosus*[43]. Moreover, the presence of periodate-resistant galactan blocks interrupted by vulnerable 6-linked Gal residues, as described in some arabinogalactans[41,44], was not in

accord with the results of gel-permeation chromatography, where the K_{av} observed for Smith-degraded GP_1 was in good agreement with the loss of weight due to Smith degradation. Indeed, on the assumption of a nearly constant intrinsic viscosity of 15 mL/g for GPS_1 and GP_1, a molecular weight of 28 000 was calculated from the universal calibration curve, whereas a value of 29 000 was calculated from the loss of carbohydrate residue and proteins[41] during Smith degradation.

However, from the present results, it is not possible to give an average d.p. of the 3- and 6-linked galactan chains. The possibility of several carbohydrate sub-units branched through hydroxyproline residues on a peptide backbone has been proposed[1] as the general organisation of carbohydrate and peptide moieties of AGP. For grape AGP, hydroxyproline is the best candidate for the carbohydrate–protein linkage, but the presence of carbohydrate sub-units is not probable according to the results of pronase digestion and periodate oxidation.

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