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Characterization of five type II arabinogalactan-protein fractions from red wine of increasing uronic acid content

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Abstract

Five arabinogalactan-protein conjugates (AGP) were separated from red wine by two successive anion-exchange chromatography steps and further purified to apparent homogeneity by affinity and size-exclusion chromatography. Together they represent more than 40% of total wine polysaccharides, confirming the abundance of AGPs in red wine. The five purified fractions had a common arabinogalactan core with characteristics typical of wine type II AGPs, but differed mainly in their uronic acid content, as evidenced by differences in the strength of their binding to the anion-exchanger. Their uronic acid content and glycosidic linkage composition revealed that the three less acidic AGPs contained from 3 to 7% glucuronic acid, half in terminal non-reducing positions and half in terminal Rha*p*-(1 → 4)-Glc*p*A-(1 → sequences. The two more acidic AGP-containing fractions contained both glucuronic (6.1 and 13.3%, respectively) and galacturonic (1.9 and 2.3%, respectively) acid in association with 2- and 2,4-linked rhamnose, indicating the presence of AG-rhamnogalacturonan fragments.

Keywords: Arabinogalactan; Arabinogalactan-protein; Rhamnogalacturonan I; Wine; Grape; Pectin; Uronic acid

1. Introduction

Type II arabinogalactans (AG) and arabinogalactan-proteins (AGP) form a group of structurally related polysaccharides and proteoglycans widely distributed in the plant

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kingdom [1–3]. They share common structural features based on a ramified (1 → 3)-D-galactan inner core with (1 → 6)-linked D-galactan side chains highly substituted by arabinofuranosyl residues and minor amounts of arabinopyranose, rhamnose, xylose, glucuronic acid, and its 4-*O*-methyl ether.

Typical AGPs commonly contain less than 10% protein. The presence of hydroxyproline as a characteristic amino acid in their protein moiety has led to the inclusion of AGPs in the group of hydroxyproline-rich glycoproteins (HRGPs) [4]. However, the recent characterization of hydroxyproline-deficient AGPs in the medium of suspension-cultured cells of *Daucus carota* [5] or *Acacia senegal* [6] showed that this characteristic is not universal among plant AGPs.

A diversity of AGs and AGPs can be recovered from one plant species, the heterogeneity being attributed either to the size of their protein moiety [7,8] or to their acidic sugar contents and to the identity and mode of linkages of their terminal sugar substituents [9].

AGs and AGPs are present in various plant tissues [3] such as trunks, leaves, seeds, floral organs, in liquid suspension cultures, in plant exudates [10], and in fruits and their derived products [11–14].

In grapes, AGP structures have been found either associated with the hairy regions of water-soluble pectins [11] or as soluble AGPs [12,15]. In fact, wine can be used as a source of type II AGPs and we have already described three different weakly acidic fractions [9,13,16]. The structural organization of grape and wine AGPs has been studied by chemical methods, but also using specific enzymatic degradations with purified α-L-arabinofuranosidase [15], endo-(1 → 6)-β-D-galactanase [17], and exo-(1 → 3)-β-D-galactanase [18]. The 3-linked galactan cores of grape AGPs present high levels of cross-linkage at position 6 giving a characteristic bushy structure, the 6-linked galactan side chains (dp up to 7) being heavily substituted by arabinofuranose linked mainly at C-3 in the grape AGP and C-3,4 in wine acidic AGPs. The polypeptide moieties represent from 3.5 to 13% of the molecules and share typical compositions with hydroxyproline, glycine, serine, and alanine as the main amino acids.

In the present study we report the structural characterization of five AGP fractions from a red wine, separated on the basis of their negative charge density.

2. Experimental

Wine sample and recovery of colloids.—The red wine sample (600 L) was the same as previously used as source of a minor arabinogalactan-protein [16] and was prepared from mature Carignan noir grapes harvested in 1991 at the INRA-Pech Rouge/Narbonne Experimental Station and concentrated to 25 L by ultrafiltration (20 kDa cut-off). Total colloids were precipitated by adding five volumes of cold ethanol to the concentrated wine [19], the precipitate being then dissolved and dialysed against water. The salt-free colloid solution was adjusted to pH 3 with citric acid buffer (final concentration 20 mM) and loaded on a S-Sepharose fast flow cation-exchange column (Pharmacia, Sweden). The almost fully depigmented, unbound fraction represented 97% of total wine polysaccharides (phenol–sulfuric acid basis) and was used as a source of AGPs.

Fractionation of total wine polysaccharides.—The unbound polysaccharides (176.5 g by weight) were fractionated by anion-exchange chromatography as follows.

Step 1. The unbound fraction was adjusted to pH 4.6 by adding sodium citrate (final concentration 40 mM) and fractionated in nine aliquots on a DEAE-Macroprep column (5 × 80 cm; Bio-Rad, USA) equilibrated at 20 mL/min in 40 mM sodium citrate buffer pH 4.6. Bound polysaccharides were fractionated by stepwise addition of NaCl (1 L/step; 10, 50, 150, 250 mM in starting buffer).

Step 2. The unbound fraction from DEAE-Macroprep was dialysed against 50 mM sodium acetate buffer (pH 5.4) and loaded again, in three aliquots, on the DEAE-Macroprep column equilibrated at 20 mL/min in 50 mM sodium acetate buffer (pH 5.4). Bound polysaccharides were separated by stepwise addition of NaCl (1 L/step; 30, 60, 100, 200 mM in starting buffer).

Isolation of wine arabinogalactan-proteins.—Five arabinogalactan-containing fractions were recovered, two from step 1 and three from step 2, dialysed against 50 mM sodium acetate buffer (pH 5.6) containing 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 1 mM $MgCl_2$, and loaded on a Concanavalin A-Ultrogel column (5 × 70 cm; Sepracor-IBF, France) equilibrated at 5 mL/min in the same buffer to remove the contaminating yeast mannoproteins. The arabinogalactans were finally purified to apparent homogeneity by size-exclusion chromatography of 1.8-g aliquots on a Sephacryl S-400 HR column (5 × 80 cm; Pharmacia) equilibrated at 7 mL/min in 50 mM sodium acetate buffer (pH 5) containing 50 mM NaCl.

Analytical methods.—Molecular weight distributions were studied by high performance size-exclusion chromatography (HPSEC) [20] by applying 125-μg aliquots of each purified AGP on two serial Shodex OHpak KB-803 and KB-805 columns (0.8 × 30 cm; Showa Denko, Japan) with a OHpak KB-800P guard column (0.6 × 5 cm), equilibrated at 1 mL/min in 0.1 M $LiNO_3$. The weight average molecular weight of each fraction was estimated with a universal calibration curve [ln ($[\eta] \times M_w$) vs. elution time] [21] established with a pullulan calibration kit (Showa Denko), the intrinsic viscosities being determined with a AVS-400 capillary viscosimeter (Schott Geräte, Germany).

Protein contents were measured according to Lowry et al. [22]. Uronic acids were assayed by the *m*-phenylphenol method [23]. For neutral sugar and uronic acid composition analyses, 1 mg polysaccharide fractions were hydrolysed with 2 M trifluoroacetic acid (TFA, 120°C, 75 min) followed by evaporation under a stream of nitrogen. The residues were dissolved in 50 mM acetate buffer (130 μL, pH 5) and incubated with 20 μL (2 nkat) of β-glucuronidase from *Helix pomatia* (Sigma, USA) (37°C for 24 h) to hydrolyse any aldobiouronic acids resistant to acid hydrolysis [24]. The products of successive acid and enzymatic hydrolyses were analysed by GC of their alditol acetate derivatives [15,25] and by high performance anion-exchange chromatography (HPAEC) with a Dionex DX-300 chromatography system equipped with a PAD detector using a CarboPac PA-1 column (0.4 × 25 cm; Dionex, USA) with a CarboPac PA-1 guard column (0.4 × 5 cm) eluted at 1 mL/min with the following gradient of sodium acetate in 100 mM NaOH: 0–5 min, isocratic at 0 mM sodium acetate; 5–20 min, linear gradient up to 150 mM sodium acetate, and final isocratic elution.

Glycosyl-linkage compositions were determined by GC–MS of the partially meth-

ylated alditol acetates. Six mg of sample was methylated using sodium methylsulfinyl carbanion and methyl iodide in dimethyl sulfoxide [26]. Half of each was then carboxyl reduced with lithium triethylborodeuteride (Superdeuteride®, Aldrich, USA) [27]. Half of each carboxyl-reduced sample was methylated again and, finally, all samples [methylated (procedure a); methylated and carboxyl-reduced (procedure b); methylated, carboxyl reduced, and remethylated (procedure c)] were submitted to acid hydrolysis, $NaBH_4$ reduction, and acetylation as described [13]. Partially methylated alditol acetates were analysed as described, their identities being confirmed by GC–MS performed with a HP-5989 MS-Engine (Hewlett–Packard, USA) and areas being corrected by response factors [28].

Gel-diffusion experiments.—Petri dishes containing 1% agarose gel in 10 mM Tris buffer, pH 7.3, containing 0.9% NaCl and 1 mM $CaCl_2$ were used. Yariv reagent antigen (Bio-supplies, Australia; 40 μL, 1 mg/mL) was delivered to a central well, the arabinogalactans (40 μL, 1 mg/mL) were put in equidistant peripheral wells, and the gels were left overnight at 25°C.

3. Results and discussion

Purification of five wine arabinogalactans.—The presence of arabinogalactans was detected in five fractions recovered during the separation of wine polysaccharides on a DEAE-Macroprep column. The other fractions contained negligible amounts of arabinogalactans and were discarded. Contaminating mannoproteins were eliminated by affinity chromatography on Concanavalin A. Five fractions were numbered according to the magnitude of their ionic interactions with the DEAE groups as follows.

Step 1 at pH 4.6: fractions eluted with 10 mM (AGP3) and 50 mM NaCl (AGP4) in citrate buffer.

Step 2 at pH 5.4: fractions unbound to DEAE-Macroprep (AGP0) and eluted with 30 mM (AGP1) and 60 mM NaCl (AGP2) in acetate buffer.

The five AGPs were finally purified to apparent homogeneity by preparative size-exclusion chromatography on Sepharose S-400 HR to give narrow symmetrical peaks in HPSEC (Fig. 1), with respective elution times of 16.20, 15.98, 15.95, 15.86, and 15.53 min for AGP0, 1, 2, 3, and 4. The respective yields of AGP0, 1, 2, 3, and 4 were 53.2, 2.3, 3.7, 9.8, and 3.8 g and they represented, respectively, 30.0, 1.3, 2.1, 5.6, and 2.1% of the total wine polysaccharides (176.5 g) thus confirming the abundance of arabinogalactans in wine.

Composition and physico-chemical characteristics.—Analysis of the five purified fractions (Table 1) revealed that their respective compositions were in accordance with their order of elution from the DEAE-Macroprep column. All fractions were mainly carbohydrate in nature with arabinose and galactose the dominant monosaccharides. However, their uronic acid content increased from 2.7% for AGP0 (unbound to DEAE-Macroprep at pH 5.4) to 20.4% for AGP4 (elution from DEAE-Macroprep at pH 4.6 required 50 mM NaCl concentration). AGP3 and AGP4 were richer in uronic acids than all previously reported grape and wine AGs and AGPs [9,13,15,16].

The protein content decreased inversely with respect to the uronic acid concentra-

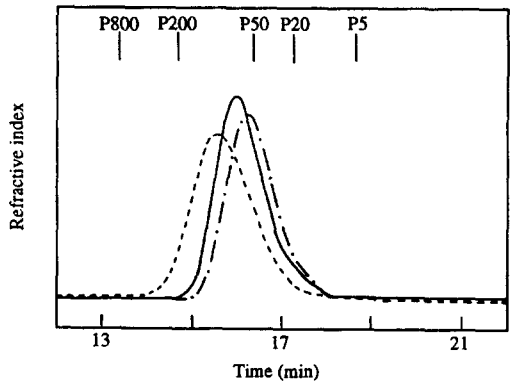

Fig. 1. HPSEC profiles of three purified AGPs from red wine: AGP0 (–·–), AGP2 (———) and AGP4 (— — —) on Shodex OHpak KB columns. Elution times and M_w (kDa) of the pullulan standards are shown.

tions, AGP4 containing less than 1% protein while the other AGPs had a protein content comparable to that previously described for wine AGPs. The five fractions exhibited a strong positive reaction with Yariv antigen, a reagent that binds selectively to plant AGPs [29].

Treatment with β-glucuronidase performed after acid hydrolysis allowed exhaustive hydrolysis of the aldobiouronic acids partially resistant to hydrolysis with TFA. Indeed,

Table 1
Composition and physico-chemical characteristics of wine AGPs

Properties and composition	AGP0	AGP1	AGP2	AGP3	AGP4
% of total wine polysaccharides	30.0	1.3	2.1	5.6	2.1
M_w	184 000	262 000	261 000	236 000	237 000
$[\eta]$ (mL/g)	10.3	9.9	10.4	13.0	20.8
Protein [a]	3.6	2.4	3.0	2.4	0.8
Uronic acids [a]	2.7	6.5	7.4	12.4	20.4
Neutral sugars [a]	79.5 [b]	75.0	76.2	77.0	61.3
	(70.2) [c]	(73.4)	(73.3)	(65.9)	(51.3)
Rhamnose [d]	1.1	2.2	3.1	7.1	10.8
Arabinose [d]	40.5	43.8	39.2	43.2	28.5
Xylose [d]	*				1.3
Mannose [d]	0.5		1.3	1.0	1.9
Galactose [d]	53.8	48.4	49.9	39.8	40.5
Glucose [d]	1.0	0.7	1.1	0.9	1.4
Glucuronic acid [d]	3.1	4.9	5.4	6.1	13.3
Galacturonic acid [d]				1.9	2.3

[a] Percent of dry matter.
[b] After acid hydrolysis followed by treatment with β-glucuronidase.
[c] After acid hydrolysis only.
[d] Mol%.
* No entry indicates < 0.5% of the total.

no disaccharides could be detected by HPAEC after treatment with β-glucuronidase. Glucuronic and also galacturonic acid release increased after the enzyme treatment indicating that the preparation from *Helix pomatia* probably also contains an α-galacturonidase. The use of such an enzyme preparation is very useful since it allows a complete quantification of not only the uronic acids but also the neutral sugars (Table 1). For each AGP, the increase in neutral sugar yield (mainly galactose for AGP0, 1, and 2, galactose and rhamnose for AGP3 and 4) was at least half of the total uronic acid amount. However, the sum (galacturonic + glucuronic acids) remained, in most cases, smaller than the uronic acid amount as evaluated by colorimetric analysis with *m*-phenylphenol.

Glucuronic acid was the only uronic acid detected in AGP0, 1, and 2, and was associated with galacturonic acid in the more acidic AGP3 and 4. no. 4-*O*-methylglucuronic acid could be detected by HPAEC. Although the most abundant neutral monosaccharides were, in all cases, arabinose and galactose, the rhamnose content increased in proportion to the uronic acids from AGP0 to AGP4. Traces of glucose and mannose remained associated in the five purified fractions and some xylose was also found in AGP4.

As previously reported, intrinsic viscosities of the purified wine AGPs were low in accordance with their compact structure [9,15,16]. However AGP4 was two times more viscous than AGP0, 1, and 2, suggesting that its structural organization could be somewhat different. The weight average molecular weights (M_w) were obtained from the universal calibration curve [ln $([\eta] \times M_w) = 37.477 - 1.4209 \times$ elution time] and ranged from 180 to 260 kDa.

Glycosyl-linkage analysis.—The five arabinogalactan fractions were permethylated according to Hakomori [26] followed by hydrolysis, reduction, and acetylation of the partially methylated alditols (procedure a). Good agreement was observed between the

Table 2
Methylation analysis of wine AGPs (relative mol%)

Methyl ether	Linkage	AGP0	AGP1	AGP2	AGP3	AGP4
2,3,4-Rha [a]	Terminal	0.9	2.0	2.4	3.6	9.3
3,4-Rha	1 → 2	*			0.6	1.0
3-Rha	1 → 2,4				1.6	2.2
2,3,5-Ara	Terminal	37.2	40.4	36.0	35.1	24.4
2,3-Ara	1 → 5	1.2	1.1	1.2	5.7	4.6
2-Ara	1 → 3,5				3.0	2.5
2,3,4,6-Gal	Terminal	3.1	2.2	2.2	2.9	3.5
2,4,6-Gal	1 → 3	9.7	8.5	8.4	6.2	8.1
2,3,4-Gal	1 → 6	5.4	2.4	2.7	2.5	4.2
2,3,6-Gal	1 → 4				1.1	1.6
2,6-Gal	1 → 3,4	3.3	3.6	4.0	3.9	1.8
2,3-Gal	1 → 4,6	1.5	1.0	1.4	0.7	
2,4-Gal	1 → 3,6	26.9	26.0	28.3	23.3	32.9
2-Gal	1 → 3,4,6	10.8	12.8	13.4	9.6	3.9

[a] 2,3,4-Rha denotes 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylrhamnitol, etc.
* No entry indicates < 0.5% of the total.

relative mole ratios obtained from compositional (alditol acetates) and structural (partially methylated alditol acetates) analyses. The five purified fractions presented all the characteristics of grape and wine type II AGPs (Table 2), arabinose being mainly in terminal non-reducing positions while galactose was mostly 3-, 6-, 3,6-, and 3,4,6-linked. Some arabinose was 5- and 3,5-linked, especially in AGP3 and 4. Rhamnose was in a terminal non-reducing position in AGP0, 1, and 2, but also 2- and 2,4-linked in AGP3 and 4. These data indicate that the five purified fractions contained typical wine arabinogalactan structural features based on a 3-linked bushy galactan core with 6-linked galactan side chains heavily substituted at positions 3 and 4 by arabinofuranose [9,13]. However, the galacturonic acid-containing AGPs were characterized by the simultaneous presence of 2- and 2,4-linked rhamnopyranose, two residues commonly found in rhamnogalacturonan I chains [27,30].

To study the uronic acid linkages, the permethylated AGPs were submitted to reduction of their methyl-esterified carboxyl groups followed either by hydrolysis, reduction, and acetylation (procedure b) and remethylated before hydrolysis, reduction, and acetylation (procedure c). Comparison of the methyl ethers obtained by these different procedures allows the unambiguous identification of the galactose arising from galacturonic acid. For example, 4-linked galacturonic acid is obtained as 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methylgalactitol in procedure b and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylgalactitol in procedure c. With the five purified AGPs, the increase in glucose and

Table 3
Glycosyl linkage composition (in mg/g) of two wine AGPs: methylated (a), methylated and carboxyl reduced (b), methylated, carboxyl reduced, and remethylated (c)

Methyl ether	Linkage	AGP2			AGP4		
		a	b	c	a	b	c
2,3,4-Rha [a]	Terminal	18	14	19	50	42	43
3,4-Rha	1 → 2	*			6	8	11
3-Rha	1 → 2,4				12	23	28
2,3,5-Ara	Terminal	263	227	297	132	83	106
2,3-Ara	1 → 5	9	9	11	25	23	29
2-Ara	1 → 3,5				14	14	16
2,3,4,6-Gal	Terminal	16	14	16	19	12	16
2,4,6-Gal	1 → 3	62	59	71	44	45	55
2,3,4-Gal	1 → 6	20	18	22	23	24	28
2,3,6-Gal	1 → 4	3	3	4	9	8	37
2,6-Gal	1 → 3,4	30	28	32	10	11	16
2,3-Gal	1 → 4,6	10	11	11		23	
2,4-Gal	1 → 3,6	208	202	228	179	193	217
2-Gal	1 → 3,4,6	99	100	113	21	25	24
2,3,4,6-Glc	Terminal			25			37
2,3,6-Glc	1 → 4			28			70
2,3,4-Glc	1 → 6		20			34	
2,3-Glc	1 → 4,6		26	7		57	
	Total	738	731	884	544	625	733

[a] 2,3,4-Rha denotes 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylrhamnitol, etc.
* No entry indicates < 0.5% of the total.

galactose yields corresponded to the respective amounts of glucuronic and galacturonic acids estimated by HPAEC analysis (Table 1) indicating that reduction of the carboxyl groups was quantitative.

The results obtained for AGP2 and AGP4 with the three successive procedures are given in Table 3. Glucuronic acid is found as terminal non-reducing and 4-linked residues and the (terminal/4-linked Glc*p*A) molar ratios decreased from AGP0 to AGP4 with respective values of 1.2, 1, 0.9, 0.64, and 0.52 for AGP0, 1, 2, 3, and 4. The (terminal Rha*p*/4-linked Glc*p*A) molar ratio was close to 0.75 in the five AGPs, suggesting that most terminal Rha*p* is found in terminal Rha*p*-(1 → 4)-Glc*p*A-(1 → sequences, a disaccharide commonly found in the side chains of acidic AGPs [2,6].

Among galactosyl methyl ethers of the galacturonic acid-containing AGP3 and 4, an equivalent increase of 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methylgalactitol in procedure b and of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylgalactitol in procedure c clearly indicated that galacturonic acid was essentially 4-linked. The carboxyl-reduction also led to an increase in 2- and 2,4-linked rhamnose, thus confirming that these residues were linked to Gal*p*A. The molar ratios of 4-linked Gal*p*A, 2-linked and 2,4-linked Rha*p*, and terminal non-reducing Gal*p* were 1:0.3:1:0.85 and 1:0.33:1:0.87 for AGP3 and 4, respectively. All these data lead to the conclusion that AGP3 and AGP4 fractions contained rhamnogalacturonan I fragments, the rhamnosyl residues of which are heavily substituted at position 4 by known substituents of pectin hairy regions [27,30,31], e.g. terminal Gal*p* or short arabinan side chains as indicated by the specific occurrence of 5- and 3,5-linked Ara*f* in both fractions. These fractions should originate from the hairy regions of native grape pectins [11], although the mechanism of their release during winemaking is not known.

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