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Characterization of two arabinogalactan-proteins from red wine

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Two weakly acidic arabinogalactan-proteins (AGPs) were recovered from a red wine by ethanol precipitation, ion-exchange, affinity and size-exclusion chromatography. They represented, respectively, 4·3 and 5·2% of the total alcohol-precipitable colloids. The more acidic arabinogalactan had higher molecular weight, uronic acid and rhamnose contents and higher arabinose/galactose ratio. Both AGPs had similar amino acid compositions. Linkage studies were performed on native and enzymatically dearabinosylated AGPs. Wine arabinogalactans were built of a 3-linked galactan core cross-linked at position 6, with short 6-linked galactan branches heavily substituted by arabinose at positions 3 and 4. Uronic acids were identified as being mainly galacturonic acid, indicating that wine AGPs might derive from native grape pectins.

INTRODUCTION

Type II arabinogalactan-proteins (AGPs) (Aspinall, 1980) are widely distributed in higher plants (Fincher *et al.*, 1983), including grape (Saulnier & Brillouet, 1989; Saulnier *et al.*, 1992). They have in common a (1 → 3)-D-galactan inner core with (1 → 6)-linked outer chains variably substituted by monomeric (e.g. arabinose) and oligomeric side-chains. Their protein content is usually less than 10%. Degradation of arabinogalactans with purified enzymes arabinofuranosidase (Tsumuraya *et al.*, 1984), β-galactosidase (Sekimata *et al.*, 1989), exo-β-(1 → 3)-D-galactanase (Tsumuraya *et al.*, 1990) and endo-β-(1 → 6)-D-galactanase (Brillouet *et al.*, 1991) is a useful tool in the study of their structural organization.

It has been suggested that AGPs reduce the filterability of wine (Brillouet *et al.*, 1989; Belleville *et al.*, 1990, 1991) and apple juice (Will *et al.*, 1992). A better knowledge of their structures will help to establish new technology(ies) (enzymatic degradation and/or new microfiltration membranes) designed to reduce the negative effect of these polysaccharides on filtration.

An acidic arabinogalactan was formerly isolated from a red wine (Brillouet *et al.*, 1990) and was supposed to be a degradation product of native grape pectins by endogenous pectolytic enzymes. The authors have recently isolated two abundant AGPs from a red wine (Pellerin & Brillouet, 1992) and the present work compares their structure.

EXPERIMENTAL

Wine sample and recovery of colloids by ethanol precipitation

The red wine used as source of AGPs was the same as previously described by Belleville *et al.* (1991), i.e. prepared from mature grapes of 'Carignan noir' harvested in 1989 at the INRA-Pech Rouge/Narbonne Experimental Station. Grapes were destemmed, crushed before fermentation (5 days) and post-macerated (5 days). The wine was concentrated 16 times by ultrafiltration on a Carbosep M5 membrane (MWCO 20 kDa, Tech-sep, France) and depigmented through Sephadex LH-20 (Pharmacia, Sweden); colloids were then precipitated from an aqueous solution by addition of 5 volumes of cold 95% ethanol containing 0·06 M HCl, washed with 80 and 95% ethanol, and dried at ambient temperature.

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Isolation of arabinogalactan-proteins

Ethanol-precipitable colloids (10 g) were loaded on a (5 cm × 28 cm) column of DEAE-Sephacel (Pharmacia) equilibrated in 0·05 M acetate buffer (pH 4·8) and fractionated by applying a NaCl step gradient of 2 column volumes fractions at 0 (elution of neutral polysaccharides), 50, 150 and 250 mM (elution of acidic fractions with increasing uronic acid contents) in starting buffer. The fractionation profile, relative proportions, molar sugar compositions and HPSEC profiles of the different fractions have been recently published (Pellerin & Brillouet, 1992). The 50 and 150 mM fractions contained arabinogalactans and mannans which were eliminated on a (5 cm × 9 cm) Concanavalin A-Ultrogel (Sepracor-IBF, France) column equilibrated in 0·05 M acetate buffer (pH 5·6) containing 0·15 M NaCl, 1 mM $CaCl_2$ and 1 mM $MnCl_2$.

The arabinogalactans were finally separated from low molecular weight components by size-exclusion chromatography on a (5 cm × 70 cm) Sephacryl S-400 HR (Pharmacia) column equilibrated in 0·05 M acetate buffer (pH 4·8) containing 0·15 M NaCl.

Size-exclusion chromatography

Molecular weight distribution was studied by high performance size-exclusion chromatography (HPSEC) as reported (Pellerin & Brillouet, 1992) on two serial (0·8 cm × 30 cm) Shodex OHpak KB-803 and KB-805 columns (Showa Denkko, Japan) with a (0·6 cm × 5 cm) OHpak KB-800P guard column, equilibrated at 1 ml min^{-1} with 0·1 M $LiNO_3$ (total volume of the system 23 ml). The elution was monitored with an Erma-ERC 7512 (Erma, Japan) refractive index detector and at 280 nm with a Waters 440 absorbance detector (Millipore, USA) in combination with Waters Baseline 810 software.

The weight average molecular weight (M_w) was estimated using a universal calibration curve (ln ($[\eta] \times M_w$) versus elution time) (Grubisic *et al.*, 1967) established on the above HPSEC system at 25°C with a pullulan calibration kit (Showa Denkko). The intrinsic viscosities were determined at the same temperature in 0·1 M $LiNO_3$ with a AVS-400 capillary viscometer (Schott-Geräte, Germany).

Analytical methods

Protein contents were measured by the method of Lowry *et al.* (1951). Amino acids were determined according to Moore and Stein (1954), after hydrolysis by 6 M HCl, 15 h at 110°C under Ar, with a Kontron Chromakon 400 auto-analyzer (Kontron, Switzerland). Neutral monosaccharides were determined, after hydrolysis with 2 M trifluoroacetic acid (120°C, 75 min) (Albersheim *et al.*, 1967), by GLC of the alditol acetate derivatives (Harris *et al.*, 1984).

Uronic acids were assayed by the *m*-phenylphenol method (Blumenkrantz & Asboe-Hansen, 1973). They were identified, after hydrolysis with 2 M TFA, by thin-layer chromatography on Silica Gel 60 plates (Merck, Germany), using acetonitrile/water (4:1, v/v). The detection was achieved with 0·2% naphtoresorcinol in ethanol/sulfuric acid (95:5, v/v).

Native and degraded arabinogalactans were methylated using sodium methyl sulfinyl carbanion and methyl iodide in dimethyl sulfoxide according to Hakomori (1964). Methylated polysaccharides were extracted with chloroform/methanol (2:1, v/v) and then hydrolyzed in 2 M TFA, hydrolysis products were derivatized into their alditol acetates. Partially methylated alditol acetates were analyzed as described (Brillouet *et al.*, 1989) on two fused-silica capillary columns of DB-1 and DB-225 (J & W Scientific, USA) and areas were corrected by response factors (Sweet *et al.*, 1975).

Enzymatic hydrolysis

α-L-Arabinofuranosidase (EC 3.2.1.55) (623 nkat ml^{-1}) from *Aspergillus niger* was the same as that used by Saulnier *et al.* (1992). AGPs (5 mg) in 0·03 M acetate buffer (pH 4·2) (1 ml) were added with enzyme (10 μl) and incubated for 72 h at 40°C; 3 μl of enzyme was supplemented at 36 h. Arabinose liberated was determined enzymatically with the NAD^+-galactose dehydrogenase system (Melrose & Sturgeon, 1983). The dearabinosylated AGPs were dialyzed and freeze-dried.

RESULTS

Recovery of arabinogalactan-proteins and HPSEC profiles

The fractions eluted by 50 and 150 mM NaCl on DEAE-Sephacel chromatography represented, respectively, 9·8 and 9·1% of the wine colloids. Yeast mannans were eliminated by affinity chromatography on Concanavalin A-Ultrogel. Each unbound fraction eluted as a narrow peak in HPSEC with some assymetry in the low molecular weight range (Pellerin & Brillouet, 1992), which was finally eliminated by chromatography on Sephacryl S-400 HR. Both AGP_1 (50 mM NaCl fraction) and AGP_2 (150 mM NaCl) had close elution times (16·16 and 15·95 min, respectively) and exhibited coeluting UV-absorbing material (Fig. 1). They represented, respectively, 4·3 and 5·2% of the ethanol-precipitable colloids from the wine sample.

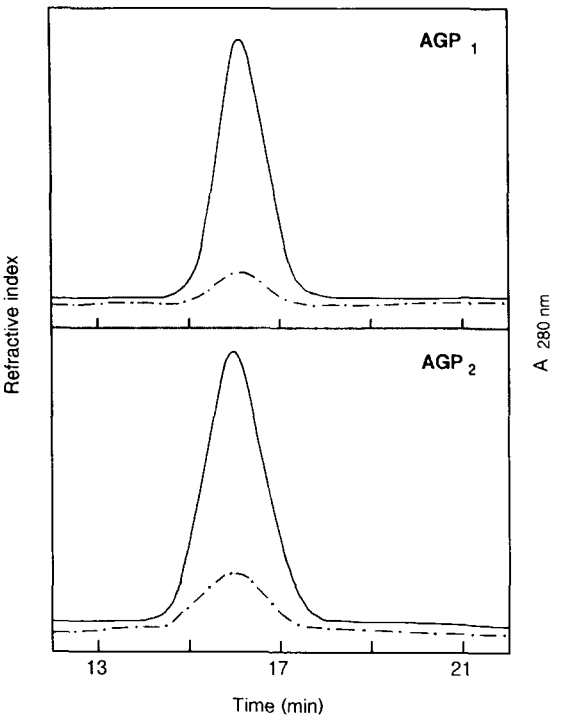

Fig. 1. HPSEC profiles of AGP_1 and AGP_2 on Shodex OHpak-KB columns. Eluates were monitored by differential refractometry (——) and absorbance at 280 nm (– –).

Compositional analyses and physico-chemical characteristics of the AGPs

AGP_1 and AGP_2 were essentially carbohydrate in nature (92·1 and 81·4% sugars), their proteic moiety representing approximately 4% (Table 1). AGP_2 had a higher uronic acid (and rhamnose) content than AGP_1 in accordance with its stronger binding to DEAE-Sephacel.

Arabinose and galactose were largely predominant in both AGPs with respective Ara/Gal ratios of 0·92 (AGP_1) and 0·97 (AGP_2). Glucose and mannose were present as trace amounts. Thin-layer chromatography showed that galacturonic acid was by far the predominant uronic acid, traces of glucuronic acid being also detected in both AGPs.

As reported for grape (Saulnier *et al.*, 1992) and wine (Brillouet *et al.*, 1990) AGPs, intrinsic viscosities were low, and weight average molecular weights (M_w) obtained from the universal calibration curve ($\ln([\eta] \times M_w) = 37{\cdot}042 - 1{\cdot}393 \times$ elution time) were in the range of the previously reported M_w for similar grape AGPs (Table 1).

Amino acid compositions

Amino acid compositions of both AGPs were very similar (Table 2) with high amounts of Ser, Ala, Thr, and Gly, the characteristic arabinogalactan-protein amino acids (Fincher *et al.*, 1983). Hydroxyproline contents were lower than 5%. According to the molecular weights obtained from the universal calibration, to the protein contents and amino acid compositions, the peptidic moieties of AGP_1 and AGP_2 contained, respectively, 60 and 78 amino acid residues per mole (Table 2).

Enzymic degradation and linkage studies of the AGPs

All methylation analyses were in agreement with the corresponding compositional analyses. Methylation analyses of AGP_1 and AGP_2 (Table 3) showed that arabinose and rhamnose were present mainly as terminal non-reducing units, but arabinose was also 5-linked (e.g. 14% of total arabinose in AGP_2), as observed in apple juice AGP (Will & Dietrich, 1992). Galactose was mostly found 3-, 6-, 3,4-, 3,6- and 3,4,6-linked. These data were typical of type II arabinogalactans.

Both wine AGPs were submitted to degradation by arabinofuranosidase yielding GP_1 and GP_2. The extent of dearabinosylation, as measured with the NAD^+-galactose dehydrogenase assay, was 79% for GP_1 and

Table 1. Composition and physico-chemical characteristics of native and dearabinosylated AGPs

Properties and composition	AGP_1	GP_1	AGP_2	GP_2
M_w	176 000	120 000[a]	224 000	168 000[a]
$[\eta]$ (ml g^{-1})	11·64	nd	12·24	nd
Protein[b]	3·7	5·9	3·9	6·7
Uronic acids[b]	4·7	8·0	7·3	8·2
Neutral sugars[b]	87·4	70·3	74·1	64·6
Rhamnose[c]	1·3	1·9	3·5	5·4
Arabinose[c]	46·5	11·4	46·4	16·1
Mannose[c]	1·0	1·8	1·2	2·2
Galactose[c]	50·4	84·1	48·0	75·1
Glucose[c]	0·8	0·8	0·9	1·2

[a]Estimated from extent of dearabinosylation.
[b]Per cent of dry matter.
[c]Mol %.
nd, Not determined.

Table 2. Amino acid composition of wine AGPs

Amino acid	AGP_1		AGP_2	
	Relative molar ratio	Molar composition[a]	Relative molar ratio	Molar composition[a]
Asx	8·0	5	8·1	6
Hyp	4·6	3	4·2	3
Thr	13·8	8	13·6	11
Ser	21·8	13	20·7	16
Glx	7·4	4	7·1	5
Gly	10·3	6	12·7	10
Ala	13·5	8	14·8	11
Val	4·9	3	4·8	4
Ile	2·0	1	2·1	3
Leu	5·4	3	4·0	2
Tyr	1·1	1	1·7	3
Phe	1·4	1	1·3	1
Lys	0·9	1	1·7	1
His	2·9	2	1·9	1
Arg	1·4	1	0·9	1
Orn	0·6		0·4	
Total		60		78

[a]Proximate number of amino acid residues per molecule of AGP, calculated from M_w and protein content (see Table 1).

72% for GP_2. The weight recoveries were in the range 90–100% for all other constituents and were of 12 and 20% for residual arabinose in GP_1 and GP_2, respectively. HPSEC of dearabinosylated samples showed a shift of elution time to 16·35 min for GP_1 (Fig. 2) and to 16·12 min for GP_2. M_w determinations based on the loss of arabinose (Table 1) showed, by inserting elution times in the universal calibration curve, that intrinsic viscosities were little affected by dearabinosylation.

The loss of arabinose induced an increase of 6-linked galactose at the correlative expense of 3,6- and 3,4,6-linked galactose (Table 3), confirming that arabinose is 3- (Saulnier & Brillouet, 1989; Saulnier *et al.*, 1992) and 3,4-linked (Brillouet *et al.*, 1990) to (1 → 6)-galactan chains. The decrease in 3- and 3,4-linked galactose and the increase of terminal galactose indicated that chains might be terminated by Ara (1 → 3)-Gal and Ara (1 → 3,4)-Gal moieties.

Periodate oxidation and Smith degradation performed on dearabinosylated products as previously described (Saulnier *et al.*, 1992) yielded undegraded 3-linked cores with mutual interlinkages at position 6 (data not shown).

DISCUSSION

The authors' results confirm that type II arabinogalactan-proteins are abundant in red wines and point to the slight variability of their structure. In comparison to a previous study (Brillouet *et al.*, 1990), in which a homogenous acidic AGP was released from DEAE-Sephacel by low NaCl concentration, two fractions with different uronic acid contents were recovered in the present study. The weakly acidic AGP_1 described here showed strong identity with the previously reported AGP for its structure, protein and uronic acid contents, and M_w. However, the more acidic AGP_2 was not found in the previous study. The wine used in the earlier study was prepared from crushed grapes rapidly heated at 70°C, a process denaturating endogenous pectolytic enzymes, then pressed before fermentation, thus removing insoluble cell wall material. Conversely, the present wine was obtained after fermentation and post-maceration in the presence of insoluble cell wall material. This wine-making procedure enhanced the enzymatic release of cell wall pectic fragments, resulting in a wine which was twice richer in polysaccharides than the previous one (Belleville *et al.*, 1990). Thus, wines prepared from the same cultivar may exhibit variations in their polysaccharidic content, depending on the year of vintage and the type of wine-making involved.

The two AGPs described here showed differences in their sugar composition. As compared with AGP_1, AGP_2 had a higher content of uronic acid and rhamnose and a higher Ara/Gal ratio. The uronic acids were identified as being mostly galacturonic acid. The AGPs studied might be fragments of grape pectins among which these structures are abundant (Brillouet, 1987; Saulnier *et al.*, 1988). The molecular weight of AGP_2 was the highest amongst grape and wine AGPs, which could be due to the presence of longer rhamnogalacturonic chains.

Table 3. Methylation analysis of native and dearabinosylated AGPs (mol %)

Methyl ether	Linkage	AGP_1	GP_1	AGP_2	GP_2
2,3,4-Rha	Terminal	1·0	1·4	2·4	2·9
3-Rha	1->2,4	0·2	0·7	0·6	1·0
Total[a]		1·2 (1·3)	2·1 (1·3)	3·0 (3·5)	3·9 (3·7)
2,3,5-Ara	Terminal	38·5	6·5	33·4	8·4
2,5-Ara	1->3	0·4	0·3	0·5	0·3
3,5-Ara	1->2	0·6	0·3	0·6	0·4
2,3-Ara	1->5	2·9	1·1	5·6	2·2
Total[a]		42·4 (46·5)	8·2 (7·6)	40·1 (46·4)	11·3 (11·1)
2,3,4,6-Gal	Terminal	1·0	3·4	0·9	3·2
2,4,6-Gal	1->3	7·9	4·9	6·6	4·0
2,3,4-Gal	1->6	3·2	26·4	3·9	22·1
2,3,6-Gal	1->4		0·5		1·0
2,6-Gal	1->3,4	4·1	0·6	3·6	0·8
2,3-Gal	1->4,6	1·7	3·2	1·8	3·7
2,4-Gal	1->3,6	25·9	16·0	25·4	15·9
2-Gal	1->3,4,6	11·4	1·6	11·6	2·3
Total[a]		55·2 (50·4)	56·6 (56·4)	53·8 (48·0)	53·0 (52·1)
2,3,6-Glc	1->4			0·4	
2,3-Glc	1->4,6	0·6		0·6	0·4
Total[a]		0·6 (0·8)		1·0 (0·9)	0·4 (0·8)
2,3,4,6-Man	Terminal			0·2	
2,3,6-Man	1->4	0·6	0·2	0·9	0·3
2,4-Man	1->3,6			1·0	0·3
Total[a]		0·6 (1·0)	0·2 (1·2)	2·1 (1·2)	0·6 (1·5)
Free Ara[b]			32·9		30·8

[a]Values in parentheses correspond to monosaccharide analyses (Table 1); for dearabinosylated products GP_1 and GP_2, mol % has been calculated by including the liberated arabinose as a distinct monosaccharide.
[b]Determined by NAD^+-galactose dehydrogenase system.

Although having similar uronic acid contents, the glucuronic acid containing grape AGP (Saulnier & Brillouet, 1989; Saulnier *et al.*, 1992) did not bind to DEAE-Sephacel, while the authors' wine AGP_1, having galacturonic acid as its major uronic acid, did. While isolated glucuronosyl moieties are terminal and/or 4-linked (Tsumuraya *et al.*, 1987; Saulnier *et al.*, 1992), contiguous galacturonosyl residues must form long enough chains with sufficient density of charge to bind to the exchanger.

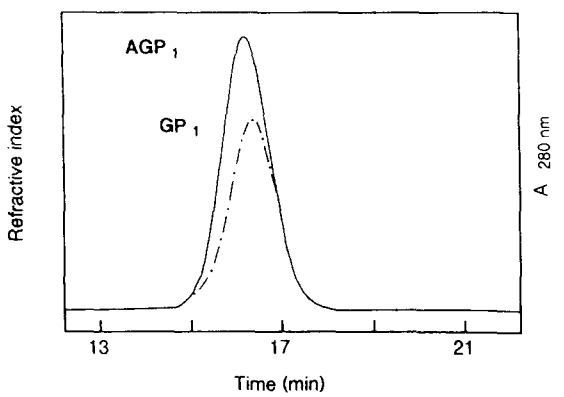

Fig. 2. Compared HPSEC profiles of native AGP_1 (——) and dearabinosylated GP_1 (–·–).

The methylation data obtained for native and degraded AGPs confirmed the structure previously proposed for one wine AGP (Brillouet *et al.*, 1990). Although arabinose was present mainly as terminal non-reducing units, AGP_2 had a significantly higher amount of 5-linked arabinose than AGP_1, a structural feature found in AGPs from ryegrass (Bacic *et al.*, 1987), radish seed (Tsumuraya *et al.*, 1987) and apple (Will & Dietrich, 1992). The (1 → 6)-galactan chains were heavily substituted at positions 3 and 4 by arabinofuranose. The 3-linked galactan core had a bushy structure with high level of cross-linkages at positions 6.

The use of new specific enzymes seems to be now necessary to study further the fine structure and molecular organization of type II AGPs.

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