Carbohydrate Research, 188 (1989) 137–144
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

AN ARABINOGALACTAN–PROTEIN FROM THE PULP OF GRAPE BERRIES*

LUC SAULNIER† AND JEAN-MARC BRILLOUET

Institut National de la Recherche Agronomique, Institut des Produits de la Vigne–Laboratoire des Polymères et des Techniques Physico-chimiques, 9 Place Viala, F-34060 Montpellier (France)

(Received October 1st, 1988; accepted for publication, December 6th, 1988)

ABSTRACT

An arabinogalactan–protein (AGp) was isolated from an aqueous extract of an alcohol-insoluble residue from the pulp of grape berries by ion-exchange and gel-permeation chromatography. AGp contained neutral sugars (89%), mainly arabinose and galactose (mol. ratio, 0.66), uronic acids (3%), and protein (8%), had a weight-average molecular weight ($\bar{M}_w$) of 165,000, and treatment with an α-L-arabinofuranosidase released 85% of the arabinose. Methylation analysis of the native and arabinosidase-degraded AGps revealed inner chains of (1→3)-linked galactosyl residues 6-substituted with (1→6)-linked galactosyl residues 3-substituted by terminal arabinosyl units. Reductive alkaline degradation of AGp showed that the threonine residues were involved in carbohydrate–protein linkages.

INTRODUCTION

Type II arabinogalactans (AGs) and arabinogalactan–proteins (AGps) have been found in most higher plants[1]. We have reported an arabino-3,6-galactan structure for the neutral side-chains associated with the rhamnogalacturonic backbone of water-soluble pectins isolated from the pulp of grape berries[2] and now describe the isolation and characterisation of a water-soluble arabinogalactan–protein.

EXPERIMENTAL

General. — Neutral sugars were determined, after hydrolysis with 2M trifluoroacetic acid (120°, 1.25 h), by g.l.c. of the alditol acetate derivatives[3] 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, 1:10; hydrogen as

*Presented at the XIVth International Carbohydrate Symposium, Stockholm, Sweden, August 14–19, 1988.

†Author for correspondence.

0008-6215/89/$ 03.50

carrier gas at 65 kPa). Uronic acids and proteins were assayed by the *m*-phenylphenol[4] and Lowry[5] methods, respectively. Uronic acids were identified tentatively, after acid hydrolysis of AGp and separation of the acidic and neutral sugars on Dowex-1 resin, by t.l.c. on Kieselgel 60 (Merck), using acetonitrile–water (90:10) and detection (100°, 10 min) with 0.2% naphthoresorcinol in ethanol–sulfuric acid (95:5). Amino-acid composition was determined, after hydrolysis by 6M HCl (110°, 10 h)[6] under N_2, with a Kontron Chromakon 400 auto-analyser.

Extraction and purification of the grape AGp. — An alcohol-insoluble residue (AIR) was prepared from pulp of grape berries (Carignan noir cv.), and extracted thrice with water at room temperature[7]. After extensive dialysis, the water-soluble polysaccharides were separated on DEAE-Sephacel[7] (0.05M acetate buffer, pH 4.8) into an unbound neutral fraction and bound acidic pectic substances. The neutral polysaccharides were further fractionated on a column (V_o 128 mL, V_t 336 mL) of Ultrogel AcA 34 in 0.05M acetate buffer (pH 4.8) containing 0.3M NaCl (35 mL/h) (Fig. 1). The major fraction (I) was the arabinogalactan–protein (AGp).

Determination of molecular weight. — A universal calibration curve[8] [$\mathrm{Ln}([\eta] \times \overline{M}_w)$ *vs.* Kav] was established at 25° with a column (V_o 80 mL, V_t 198 mL) of Sephacryl S400 eluted with 0.2M NaCl (0.02% of NaN_3) at 36 mL/h, using a pullulan calibration kit (Macherey–Nagel) and detection by differential refractometry. The intrinsic viscosities $[\eta]$ of the AGp and pullulan standards were determined at 25° in the same solvent with a Ubbelohde micro-viscosimeter (flow time, 91.27 s).

Enzymic degradation. — A solution of AGp (2 mg) in 0.1M acetate buffer (1 mL, pH 4.2) was incubated at 40° for 72 h with successive additions of α-L-arabinofuranosidase from *Dichomitus squalens*[9] (426 nkat.mg^{-1}) at 0, 24, and 48 h (1 nkat/μmol of arabinose in the polysaccharide). The arabinose liberated was determined by the NAD^+–galactose dehydrogenase system[10]. The degraded AGp was freed from arabinose by elution from a column (10 × 1 cm) of Sephadex G25 with water, and then freeze-dried.

Methylation analysis. — Native and arabinosidase-degraded AGps were methylated[11] once, and the products were hydrolysed successively with aqueous 90% formic acid (100°, 1 h) and then 2M trifluoroacetic acid (120°, 1.25 h). The partially methylated sugars were converted into their alditol acetates and analysed[2] by g.l.c. on fused-silica capillary columns bonded with OV-1 and OV-225. Peak areas were corrected[12] by response factors.

Cleavage of alkali-labile sugar–protein linkages. — AGp (2 mg) was incubated[13] with 0.2M NaOH containing 0.3M $NaBH_4$ (1 mL) for 6 h at 45°. After cooling, 1 mL of conc. hydrochloric acid was added for the hydrolysis (110°, 10 h under N_2) and the amino acids were analysed.

RESULTS AND DISCUSSION

Composition and physico-chemical properties of grape AGp. — Water-soluble polysaccharides from grape pulp have been separated[7] into a neutral (25%)

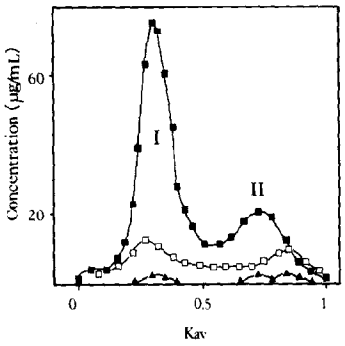

Fig. 1. Gel-permeation chromatography on Ultrogel AcA 34 of water-soluble neutral polysaccharides from the pulp of grape berries: —■—, neutral sugars; —▲—, uronic acids; and —□—, proteins.

and an acidic fraction (75%). The latter has been studied elsewhere[2,7,14], and the former was fractionated (Fig. 1) on Ultrogel AcA 34 into two components I (Kav 0.3, 70%) and II (Kav 0.7, 30%). The fraction I (AGp) exhibited co-elution of Lowry- and *m*-phenylphenol-positive materials together with neutral sugars. Arabinose and galactose (mol. ratio, 0.66) were the major sugar constituents associated with minor amounts of mannose and glucose, and traces of rhamnose, fucose, and xylose (Table I). AGp also contained unidentified uronic acid (3% as galacturonic acid) that migrated in t.l.c. beyond galacturonic, glucuronic, and 4-*O*-methylglucuronic acid, and could be 4-*O*-methylgalacturonic acid which has been reported[1] in some AGps. Protein (8%) was also detected.

The intrinsic viscosity $[\eta]$ of AGp was low and comparable to those of similar AGps[15,16]. AGp gave a symmetrical peak (Kav 0.54) after re-chromatography on Sephacryl S400 (Fig. 2), showing low polydispersity and apparent homogeneity. Using the equation $\mathrm{Ln}([\eta] \times \overline{M}_w) = 18.997 - 8.586 \times \mathrm{Kav}$ (R 1.00), established

TABLE I

PHYSICAL PROPERTIES AND CHEMICAL COMPOSITION OF GRAPE AGP

Mol. wt.	165,000
$[\eta]$ (mL/g)	10.8
Proteins (%)	8
Uronic acids (%)	3
Neutral sugars (%)	89
Neutral sugar composition (mol %)[a]	
Arabinose	38.7
Mannose	2.8
Galactose	57.6
Glucose	0.9

[a]Traces of rhamnose, fucose, and xylose were also detected.

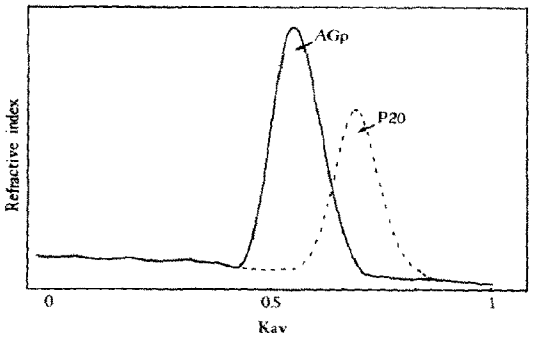

Fig. 2. Gel-permeation chromatography on Sephacryl S400 of grape AGp (P20, standard pullulan, $\overline{M}_w$ = 23,700, $\overline{M}_w/\overline{M}_n$ 1.07).

from the universal calibration curve (Fig. 3), a weight-average molecular weight ($\overline{M}_w$) of 165,000 was obtained, giving an average d.p. of ~1,000 for the carbohydrate moiety of the AGp. This molecular weight is far higher than that (52,000) reported[15] for radish seed AGp, but which was obtained from a calibration with dextrans which is valid only for polymers having similar structures. Otherwise, the hydrodynamic volume[8] of macromolecules ($[\eta] \times \overline{M}_w$) has to be taken into account.

Glycosidic linkages and degradation by arabinofuranosidase. — Native and arabinofuranosidase-degraded AGps were methylated fully in one step by the Hakomori procedure, and the products were converted conventionally into the partially methylated alditol acetates which were analysed by g.l.c. (Table II). In AGp, 95% of arabinose was present mainly as non-reducing terminal groups, but minor amounts of 5-, 3-, and 2-linked arabinose residues were also present. The main features of the galactan moiety were 3,6- (51%) and 3-linked (29%) galactose, although terminal, 3,4,6-, 3,4-, and 6-linked galactose residues were also detected in minor proportions. This distribution is typical[1] of type II AGps and reflects extensive branching already observed in neutral sugar side-chains of grape water-

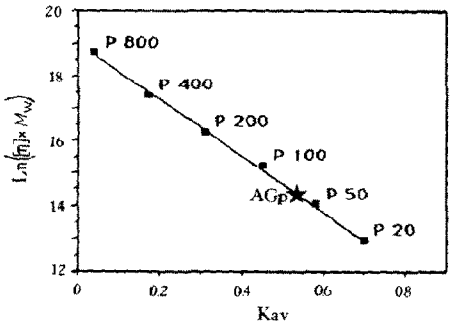

Fig. 3. Universal calibration curve for the column of Sephacryl S-400 with pullulans P-800 ($\overline{M}_w$ 853,000), P-400 (380,000), P-200 (186,000), P-100 (100,000), P-50 (48,000), P-20 (23,700).

TABLE II

METHYLATION ANALYSIS OF NATIVE AND ARABINOSIDASE-DEGRADED AGPS

Methyl ether	*Linkage*	*Native AGp*	*Arabinosidase-degraded AGp*
234 Rha[a]	L-Rha*p*-(1→	0.4[b]	0.3
234 Fuc	L-Fuc*p*-(1→	tr.[c]	tr.
235 Ara	L-Ara*f*-(1→	34.8	5.7
25 Ara	→5)-L-Ara*f*-(1→	0.3	0.3
35 Ara	→3)-L-Ara*f*-(1→	0.2	0.0
23 Ara	→2)-L-Ara*f*-(1→	1.2	0.0
Total		36.6	6.0
2346 Gal	D-Gal*p*-(1→	2.8	9.8
246 Gal	→3)-D-Gal*p*-(1→	18.0	10.7
234 Gal	→6)-D-Gal*p*-(1→	4.0	19.8
236 Gal	→4)-D-Gal*p*-(1→	1.1	0.7
26 Gal	→3,4)-D-Gal*p*-(1→	1.8	0.0
23 Gal	→4,6)-D-Gal*p*-(1→	0.0	1.1
24 Gal	→3,6)-D-Gal*p*-(1→	31.7	17.8
2 Gal	→3,4,6)-D-Gal*p*-(1→	2.3	0.6
Total		61.7	60.6
236 Glc	→4)-D-Glc*p*-(1→	0.4	0.2
2346 Man	D-Man*p*-(1→	0.3	0.2
346 Man	→2)-D-Man*p*-(1→	0.5	0.4
Total		0.8	0.6
Free Ara[d]			32.8
Terminal/substituted ratio		1	0.8

[a]234 Rha = 1-*O*-acetyl-2,3,4-tri-*O*-methylrhamnitol, *etc.* [b]Relative molar ratio. [c]Trace. [d]Determined by galactose dehydrogenase/NAD^+ system.

soluble pectins[2]. Similar AGs have been found in *Angelica acutiloba*[17], *Cannabis sativa*[18], *Carica papaya*[19], and *Lolium multiflorum*[20].

The arabinofuranosidase released 85% of the arabinose of AGp, indicating this sugar to be the α-L-furanose form. Methylation analysis of the arabinosidase-degraded AGp revealed that the residual arabinose was mainly in terminal non-reducing positions, and the resistance to arabinosidase may have been due to steric hindrance. The action of the enzyme markedly increased the proportion of 6-linked galactose at the expense of 3,6-linked galactose, suggesting that arabinose is mainly 3-linked to (1→6)-linked galactan chains. The reduction in the proportion of 3-linked galactose residues with a concomitant increase in terminal non-reducing galactose residues suggests that numerous chains must be terminated by Ara*f*-(1→3)-Gal*p*-(1→ moieties. Similar effects have been observed after treatment of a radish seed AGp[15] by a yeast arabinofuranosidase. A ^{13}C-n.m.r. study of similar

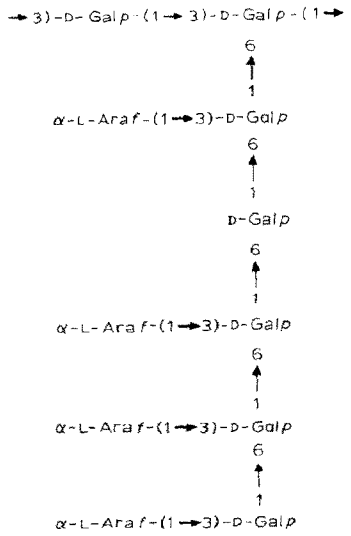

Fig. 4. A possible partial structure of grape AGp (arbitrary chain-length).

arabino-3,6-galactans as neutral side-chains of grape pectins[2] showed that, after degradation by the same arabinosidase, the only new signal was for the unsubstituted C-3 of galactopyranose, whereas the signal for C-6 remained unaltered. Cartier *et al.*[21] observed the same effect on a chemically de-arabinosylated AG from *Rubus fruticosus*. Thus, the main point of substitution of 6-linked galactose is position 3. Some variations were also noted, such as the disappearance of 3,4,6- and 3,4-linked galactose and the appearance of 4,6-linked galactose.

The equivalence of 3,6- and 6-linked galactose in the arabinosidase-degraded AGp does not accord with a comb-like structure where (1→6)-galactan chains are branched at positions 6 of a (1→3)-galactan main chain, but more likely indicate a bushy structure where (1→6)-galactan outer chains are branched at position 6 of short (1→3)-galactan chains mutually interlinked[22]. The structure of a (1→3)-galactan obtained after two Smith degradations of an AG from *Rubus fruticosus*[21] supports this hypothesis.

Thus, the main structural feature of AGp (Fig. 4) is consistent with (1→3)-galactan inner chains carrying, on positions 6, (1→6)-galactan chains heavily 3-substituted by single non-reducing arabinose residues as suggested for radish AGps[15,23]. Minor structural features, such as 6-substitution of (1→3)-linked galactose by arabinose, cannot be excluded on the basis of methylation data of arabinosidase-degraded AGp.

Amino-acid composition and carbohydrate–protein linkages. — Amino-acid analysis of the protein fraction of AGp revealed high proportions of hydroxyproline, serine, glycine, and alanine (53% of the total amino acids; Table III),

TABLE III

AMINO-ACID COMPOSITION OF GRAPE AGP

Amino acid		*Amino acid*	
Aspartic acid	4.3[a]	Valine	3.9
Hydroxyproline	10.7	Isoleucine	2.5
Threonine	8.4	Leucine	3.3
Serine	17.7	Tyrosine	0.8
Glutamic acid	9.8	Phenylalanine	1.2
Proline	4.9	Aminobutyrate	2.1
Glycine	11.0	Lysine	3.2
Alanine	13.8	Histidine	1.1
Methionine	0.2	Arginine	1.1

[a]Relative molar ratio.

TABLE IV

EFFECT OF REDUCTIVE ALKALI TREATMENT ON AGP AMINO-ACIDS

Amino acid	*Before treatment*	*After treatment*
Serine	72[a]	70
Threonine	34.3	22.8
Alanine	56	50.8

[a]Nmol/mg of AGp.

which is a typical feature of arabinogalactan–proteins[1]. Treatment of AGp with alkaline borohydride decreased the proportion of threonine, whereas those of serine and alanine were affected little (Table IV). These results suggest that the AGp is *O*-glycosylically linked to threonine residues in the polypeptide chain, whereas serine is not involved. However, an alkali-resistant linkage of the AGp to hydroxyproline cannot be ruled out[20].

ACKNOWLEDGMENTS

We thank Mrs. M. Nicol (INRA-IPV, Laboratoire de Biochimie Métabolique, Montpellier) and Mr. Risterucci (CIRAD, Montpellier) for the amino acid analyses.

REFERENCES

1 G. B. FINCHER, B. A. STONE, AND A. E. CLARKE, *Annu. Rev. Plant Physiol.*, 34 (1983) 47–70.
2 L. SAULNIER, J.-M. BRILLOUET, AND J.-P. JOSELEAU, *Carbohydr. Res.*, 182 (1988) 63–78.
3 P. ALBERSHEIM, D. J. NEVINS, P. D. ENGLISH, AND A. KARR, *Carbohydr. Res.*, 5 (1967) 340–345.
4 N. BLUMENKRANTZ AND G. ASBOE-HANSEN, *Anal. Biochem.*, 54 (1973) 484–489.
5 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265–275.

6 R. R. SELVENDRAN, *Phytochemistry*, 14 (1975) 1011–1017.
7 L. SAULNIER AND J.-F. THIBAULT, *Carbohydr. Polym.*, 7 (1987) 329–343.
8 Z. GRUBISIC, P. REMPP, AND H. BENOIT, *Polym. Lett.*, 5 (1967) 753–759.
9 J.-M. BRILLOUET, J.-C. MOULIN, AND E. AGOSIN, *Carbohydr. Res.*, 144 (1985) 113–126.
10 J. MELROSE AND R. J. STURGEON, *Carbohydr. Res.*, 76 (1983) 247–253.
11 S. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
12 D. P. SWEET, R. H. SHAPIRO, AND P. ALBERSHEIM, *Carbohydr. Res.*, 40 (1975) 217–225.
13 F. DOWNS AND W. PIGMAN, *Methods Carbohydr. Chem.*, 7 (1976) 200–204.
14 L. SAULNIER AND J.-F. THIBAULT, *Carbohydr. Polym.*, 7 (1987) 345–360.
15 Y. TSUMURAYA, Y. HASHIMOTO, AND S. YAMAMOTO, *Carbohydr. Res.*, 161 (1987) 113–126.
16 G. B. FINCHER AND B. A. STONE, *Aust. J. Biol. Sci.*, 27 (1974) 117–132.
17 H. YAMADA, Y. OTSUKA, AND S. OMURA, *Planta Med.*, (1986) 311–314.
18 A. HILLESTAD AND J. K. WOLD, *Phytochemistry*, 16 (1977) 1947–1951.
19 J. N. BEMILLER AND S. DIKKO, *Carbohydr. Res.*, 158 (1986) 173–181.
20 A. BASIC, S. C. CHURMS, A. M. STEPHEN, P. COHEN, AND G. B. FINCHER, *Carbohydr. Res.*, 162 (1987) 85–93.
21 N. CARTIER, G. CHAMBAT, AND J.-P. JOSELEAU, *Carbohydr. Res.*, 168 (1987) 275–283.
22 G. O. ASPINALL, in J. PREISS (Ed.), *The Biochemistry of Plants*, Vol. 3, Academic Press, New York, 1980, pp. 473–500.
23 Y. TSUMURAYA, Y. HASHIMOTO, S. YAMAMOTO, AND N. SHIBUYA, *Carbohydr. Res.*, 134 (1984) 215–228.