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Structural characterization of an apple juice arabinogalactan-protein which aggregates following enzymic dearabinosylation

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An arabinogalactan-protein (AGP) was isolated from a cider apple juice and contained arabinose and galactose as the main sugars (Ara/Gal mol ratio 0.67), with some uronic acids (1.4%) and protein (1.7%). Its weight-average molecular weight (M_w) was 80 kDa. Enzymatic dearabinosylation of AGP with a purified α-L-arabinofuranosidase released 95% of arabinose and left a galactan-protein GP_1. Enzymatic degalactosylation of GP_1 with a ß-D-galactosidase released 42% of galactose giving GP_2. Methylation analysis of AGP, GP_1, and GP_2 revealed that AGP is built of inner chains of (1→3)-linked galactosyl residues 6-substituted by (1→6)-linked galactan outer chains 3-substituted by terminal arabinofuranosyl units. After dearabinosylation, ß-D-galactosidase hydrolysed specifically 76% of (1→6)-linked galactan outer chains. Removal of arabinofuranosyl substituents led to partial aggregation of GP_1 upon freeze-drying, and further elimination of (1→6)-linked galactan outer chains by ß-D-galactosidase did not alter the aggregation phenomenon. The formation of aggregates reveals the possibility that enzymatic degradation of AGPs can lead to haze formation in fruit juices. Copyright © 1996 Elsevier Science Limited.

INTRODUCTION

Type II arabinogalactan-proteins (AGPs) are widely distributed in the plant kingdom (Fincher *et al.*, 1983). Aside from their protein moiety (usually < 10%), these proteoglycans consist of a (1→3)-ß-D-galactan core branched at position 6 by 6-linked ß-D-galactan outer chains heavily substituted by terminal α-L-arabinofuranosyl units (Tsumuraya *et al.*, 1984, 1987; Saulnier & Brillouet, 1989; Saulnier *et al.*, 1992; Kikuchi *et al.*, 1993; Pellerin *et al.*, 1993, 1995; Waters *et al.*, 1994). Use of novel enzymes, i.e. endo-(1→6)-ß-D-galactanase and exo-(1→3)-ß-D-galactanases, has allowed elucidation of the fine structure of AGPs with regards to the structure and degree of polymerization (dp) of the outer side-chains (Brillouet *et al.*, 1991; Tsumuraya *et al.*, 1990; Pellerin & Brillouet, 1994). We have characterized a 'neutral' AGP from grape (Saulnier *et al.*, 1992) which yields, after enzymatic dearabinosylation and freeze-drying, a galactan-protein (GP) of lower molecular weight showing in size-exclusion chromatography an additional broad shoulder of an apparent molecular weight higher than that of the starting AGP. We now describe the isolation and characterization of an arabinogalactan-protein from a cider apple juice exhibiting the same behaviour. The formation of high molecular weight GPs upon dehydration is interpreted in terms of aggregation.

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EXPERIMENTAL

Origin and purification of apple juice AGP

The starting material (crude AGP) was obtained as described (Will & Dietrich, 1992) from an apple juice derived from belt pressing combined with water extraction of cider apples without addition of liquefying enzymes. Ultrafiltered and discolourized colloids were treated with purified pectinases (PE, PG), and the remnant polysaccharides were separated by ion-exchange chromatography on DEAE-Sepharose CL-6B, the crude AGP being eluted by 0.05 M NaCl. It was

further purified as follows: crude AGP (200 mg) was dissolved in 10 ml of 0.02 M citric acid buffer (pH 3.0) and loaded at 180 ml/h onto a S-Sepharose Fast Flow column (2.5×6 cm; Pharmacia) equilibrated in the same buffer. The unbound fraction (60 ml) was dialysed against 0.05 M acetate buffer (pH 4.8) and applied at 60 ml/h on a DEAE-Sephacel column (2.5×3 cm; Pharmacia) equilibrated in the same buffer. The unbound fraction (90 ml) was extensively dialysed against distilled water, concentrated under reduced pressure (<40°C), and then freeze-dried.

Enzymatic dearabinosylation of AGP

A solution of AGP (150 mg) in 0.05 M acetate buffer containing 0.02% sodium azide (20 ml, pH 4.2) was incubated at 40°C for 4 days with α-L-arabinofuranosidase from *Aspergillus niger* (Saulnier *et al.*, 1992) (240 μl, activity 623 nKat/ml, i.e. 0.5 nKat/μmol of arabinose contained in the AGP). The degradation was monitored by TLC and the arabinose liberated was determined by the NAD^+–galactose dehydrogenase system (Melrose & Sturgeon, 1983). After boiling the incubation medium for 15 min, the dearabinosylated AGP was freed from arabinose by dialysis against distilled water, and then freeze-dried to give GP_1.

Enzymatic degalactosylation of GP_1

GP_1 (20 mg) was dissolved in 0.5 M NaOH (7 ml), and after neutralization with 2 M HCl, the medium was brought to 0.05 M acetate buffer containing 0.02% sodium azide (final volume 20 ml, pH 4.2). It was then incubated at 40°C for 4 days with ß-D-galactosidase from *Aspergillus niger* (Sigma, G 3522) (75 μl, activity 271 nKat/ml on *p*-nitrophenyl galactopyranoside, i.e. 0.2 nKat/μmol of galactose contained in GP_1). The degradation was monitored by TLC and the galactose liberated was determined by the NAD^+–galactose dehydrogenase system. After boiling the incubation medium for 15 min, the degalactosylated GP_1 was freed from galactose by dialysis against distilled water, and then freeze-dried to give GP_2.

General

Neutral sugars were determined, after hydrolysis with 2 M trifluoroacetic acid (TFA) for 75 min at 120°C (Albersheim *et al.*, 1967), by GLC of the alditol acetate derivatives (Harris *et al.*, 1984) at 210°C on a fused-silica DB-225 capillary column (30×0.32 mm i.d., 0.25 μm film; J&W Scientific) with H_2 as the carrier gas. Uronic acids were measured by the *m*-phenylphenol method (Blumenkrantz & Asboe-Hansen, 1973) using glucuronic acid as a standard. Uronic acids were also identified, after hydrolysis with TFA, by high pH anion-exchange chromatography (HPAEC) on a CarboPac PA-1 (25×0.4 cm; Dionex) with a CarboPac PA-1 guard column (5×0.4 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 with a Dionex DX-300 chromatography system equipped with a PAD detector fitted with a gold working electrode with the following pulse potentials and durations: $E_1 = 0.05$ V, $t_1 = 300$ ms, $E_2 = 0.6$ V, $t_2 = 120$ ms, $E_3 = -0.6$ V, $t_3 = 300$ ms. Protein was determined according to Lowry *et al.* (1951) with ovalbumin as a standard.

Methylation structural analyses of the native, dearabinosylated, and degalactosylated AGPs were performed with sodium methyl sulfinyl carbanion and methyl iodide in dimethyl sulfoxide (Hakomori, 1964); partially methylated alditol acetates were analysed as described (Doco & Brillouet, 1993).

Size-exclusion chromatography and molecular weight determination

The molecular size distribution of the polysaccharides was studied by high-performance size-exclusion chromatography (HPSEC) using 2 serial Shodex OHpak KB-803 and KB-805 columns (30×0.8 cm; Showa Denkko, Japan) with a OHpak KB-800 P guard column (5×0.6 cm), eluted at 30°C with 0.1 M $LiNO_3$ at 1 ml/min from a Waters 510 pump, with online refractive index (Erma-ERC 7512 detector thermostated at 40°C) and UV (Waters 440) detections. Apparent molecular weights (M_r) were obtained by calibration of the system with a narrow pullulan molecular-weight standard [$\ln M_r = 24.04 - 0.804 \times t_R$ (t_R = column retention-time at peak maximum; $r^2 = -0.998$). Weight-average molecular weight (M_w) of the native AGP was determined by low-angle laser-light-scattering (LALLS) as described (Doco & Brillouet, 1993).

Freeze-drying and dehydration procedure

AGP and GP solutions at 2–5 mg/ml in water were frozen at −40°C in a RP2V lyophilisator (CIRP, France). Freeze-drying was then performed under vacuum (0.1 mbar) by slowly heating the samples up to 20°C during 48 h. In order to remove residual water, the samples were finally let at 55°C under reduced pressure for 16 h in a P_2O_5-containing desiccator.

RESULTS

Characterization of apple juice AGP

Although the crude apple juice AGP was previously described as 'homogeneous' by size-exclusion chromatography on Superose 12 (apparent molecular weight 50

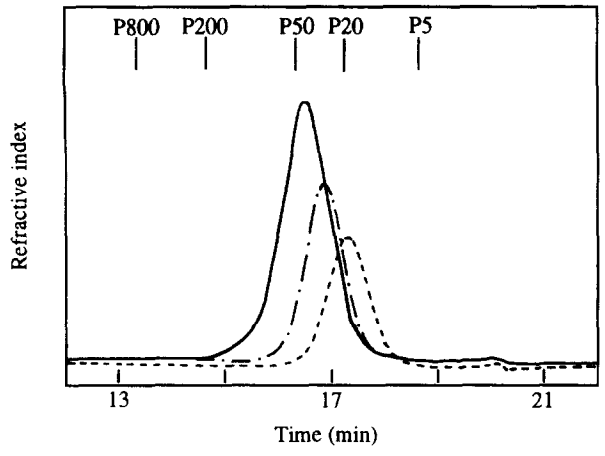

Fig. 1. HPSEC profiles of native apple juice arabinogalactan-protein and of never freeze-dried galactan-proteins. Positions and M_w values in kDa of the pullulan standards are indicated. (——), native AGP; (—•—) GP_1; (-----), GP_2.

kDa) (Will & Dietrich, 1992), two additional ion-exchange chromatographic steps removed minor impurities yielding purified AGP (yield 85%). Further attempts to purify this polymer by other techniques (e.g. SEC) failed to show any heterogeneity. In HPSEC, AGP gave a narrow symmetrical peak (Fig. 1; $t_R = 16.53$ min) with an apparent molecular weight of 46 kDa, a value similar to those previously reported for the crude apple AGP (Will & Dietrich, 1992) and for a radish seed AGP (Tsumuraya *et al.*, 1987). Absolute weight-average molecular weight (M_w) was found equal to 80 kDa by LALLS, a figure lower than determined for a similar grape AGP (Saulnier & Brillouet, 1989; Saulnier *et al.*, 1992).

The composition of this apple juice AGP is given in Table 1. Arabinose and galactose were the main constituents in a molar ratio (Ara/Gal = 0.67) identical to that of a neutral grape AGP (Saulnier & Brillouet, 1989; Saulnier *et al.*, 1992). Traces (< 0.5% each) of rhamnose, fucose, xylose, and glucose were also observed. Uronic acids concentrations were low, glucuronic acid only being detected by HPAEC. Protein was also detected giving a faint UV response co-eluted with the refractometric signal.

Methylation analysis (Table 2) showed an excellent agreement between the relative proportions of the sugars found either from the analyses of the alditol acetates or partially methylated alditol acetates, and the (terminal/branched residue) ratio was equal to 1. Arabinofuranose was present mainly as non-reducing terminal groups (90%), but was also 5-linked (10%). Galactose was mainly 3,6- (62%) and 3-linked (25%), some 6-linked (10%) and terminal (3%) galactose also being present. This distribution is typical of type II AGPs (Tsumuraya *et al.*, 1984; Waters *et al.*, 1994; Cartier *et al.*, 1987) and quite similar to the pattern observed in a neutral AGP from grape (Saulnier & Brillouet, 1989; Saulnier *et al.*, 1992). However, no

Table 1. Composition of AGP, GP_1, and GP_2

	AGP	GP_1	GP_2
Neutral sugars[a]	76.6	70.9	58.8
Uronic acids[a]	1.4	2.2	3.5
Protein[a]	1.7	2.6	3.5
Arabinose[b]	40	4	5
Galactose[b]	60	96	95

[a]Dry weight percent (anhydro sugar basis).
[b]Mol%.

Table 2. Methylation analysis of AGP, GP_1, and GP_2 (Mol%)

Methyl ether	Linkage	AGP	GP_1	GP_2
2,3,5-Ara[a]	Ara*f*-(1→	36.2	2.0	2.0
2,3-Ara	→5)-Ara*f*-(1→	3.9	—	—
2,3,4,6-Gal	Gal*p*-(1→	1.7	8.8	8.1
2,4,6-Gal	→3)-Gal*p*-(1→	15.4	8.6	8.1
2,3,4-Gal	→6)-Gal*p*-(1→	5.8	30.5	7.4
2,4-Gal	→3,6)-Gal*p*-(1→	37.1	12.1	11.0
Free Ara[b]			38.1	38.1
Free Gal[b]				25.2

[a]2,3,5-Ara = 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methyl arabinitol, etc.
[b]Monomeric arabinose or galactose determined in the digestion medium by galactose dehydrogenase-NAD^+ system. Insertion of these data facilitates direct comparisons of proportional amounts of methyl ethers [3,4].

3,4,6-linked was observed contrary to grape and wine AGPs (Saulnier & Brillouet, 1989; Saulnier *et al.*, 1992; Pellerin *et al.*, 1993, 1995; Waters *et al.*, 1994).

Enzymatic dearabinosylation of AGP

Treatment of AGP with an α-L-arabinofuranosidase from *Aspergillus niger* resulted in the release of ~95% of arabinose as the sole sugar. GP_1 was obtained in a 72% dry matter yield which corresponds well to the losses in arabinose. GP_1 was correlatively enriched in uronic acids and protein (Table 1).

Methylation analysis of GP_1 (Table 2) revealed that residual arabinose was essentially in the terminal non-reducing position. As formerly observed for grape AGP (Saulnier & Brillouet, 1989; Saulnier *et al.*, 1992), the action of the arabinosidase markedly increased the proportional amount of 6-linked galactose at the corresponding expense of 3,6-linked galactose showing that Ara*f* was 3-linked to 6-linked galactan chains. Emergence of terminal galactose in an equal proportional amount to the disappearance of 3-linked galactose suggests that most chains must be terminated by Ara*f*-(1→3)-Gal*p*-(1→ moieties.

Enzymatic degalactosylation of GP_1

Treatment of GP_1 with a ß-D-galactosidase from *Aspergillus niger* resulted in the release of ~42% of

galactose as the sole sugar. GP_2 was obtained in a 70% dry matter yield (i.e. 50% of the starting AGP).

Methylation analysis of GP_2 (Table 2) revealed that the ß-D-galactosidase eliminated specifically 76% of 6-linked galactose, other linkages being virtually untouched. This enzyme had no action on the native AGP. Similar results were obtained after degalactosylation of a dearabinosylated grape AGP with an endo-(1→6)-ß-D-galactanase (Saulnier *et al.*, 1992). Thus dearabinosylation of external 6-linked galactan chains made them susceptible to the action of that ß-D-galactosidase (Hirano *et al.*, 1994).

Behaviour of GP_1 and GP_2 in HPSEC

When an aliquot of a solution of GP_1 which has never been freeze-dried (i.e. just after elimination of arabinose by dialysis) was injected in the HPSEC system, a narrow symmetrical peak was observed at $t_R = 16.89$ min (Fig. 1) giving an apparent molecular weight of 35 kDa. Taking into account the extent of dearabinosylation (or loss of dry matter) and the molecular weight of AGP (46 kDa), an apparent molecular weight of 35 kDa can also be calculated for GP_1. GP_2, when injected in the HPSEC system after elimination of galactose by dialysis, gave a narrow symmetrical peak eluted at $t_R = 17.29$ min (Fig. 1). Its apparent molecular weight of 25 kDa was in accordance with the extent of degalactosylation (or loss of dry matter).

Freeze-dried dearabinosylated AGP, i.e. GP_1, was dissolved at 1 mg/ml in 0.1 M $LiNO_3$ (or 0.05 M acetate buffer, pH 4.2) to give a clear solution (no haze) which was injected in the HPSEC system. In both cases, GP_1 eluted as a main peak ($t_R = 16.65$ min) with a broad shoulder at $t_R = 15.60$ min (Fig. 2). The observed pattern of aggregation did not depend on the concentration of the GP_1 solution in the range 1→4 mg/ml before freeze-drying. Preliminary experiments have shown that when solutions of freeze-dried GP_1 in 0.05 M acetate buffer (pH 4.2) were heated (15 min, 100°C), the shoulder became less important. Furthermore, long-term storage of a frozen solution of GP_1 that has never been freeze-dried alters slightly its HPSEC profile: the peak becomes skewed, sloping more gently at its leading edge. An apparent molecular weight of 98 kDa was estimated for the shoulder population. Hence, dearabinosylation of AGP and subsequent freeze-drying resulted in a reaction product of apparent molecular weight higher than that of starting AGP and ~3 times higher than that of GP_1. Absolute weight-average molecular weight (M_w) determination by LALLS gave 165 kDa, an aberrant value if compared to the 80 kDa found for the weight-average molecular weight of native AGP.

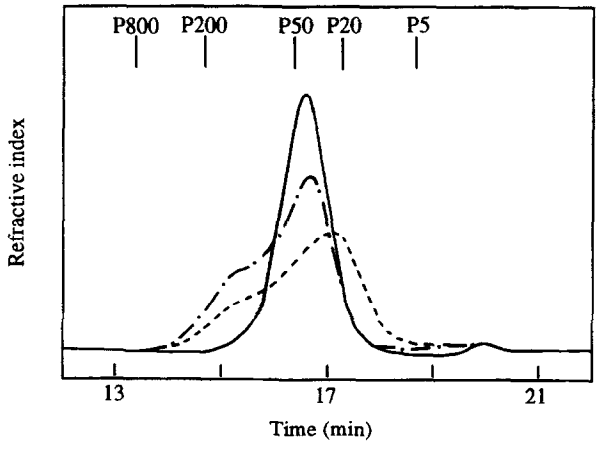

Fig. 2. HPSEC profiles of freeze-dried native apple juice arabinogalactan-protein and galactan-proteins. Positions and M_w values in kDa of the pullulan standards are indicated. (——), native AGP; (—•—) GP_1; (-----), GP_2.

This behaviour led us to think that GP_1 might form aggregates upon freeze-drying. Indeed, after dissolution of freeze-dried GP_1 in 0.5 M NaOH followed by neutralization with 2 M HCl, a narrow symmetrical peak ($t_R = 16.89$ min) was observed in HPSEC as in the case of a solution of never freeze-dried GP_1 (Fig. 1). A lower concentration of NaOH (0.1 M) was insufficient to restore symmetry. Thus, preliminary dissolution in 0.5 M sodium hydroxide was achieved prior to treatment with ß-D-galactosidase. Upon freeze-drying, GP_2 exhibited a similar shoulder aside the main product of hydrolysis (Fig. 2).

DISCUSSION

This paper describes the fine structure of a type II arabinogalactan-protein from apple juice very similar, with regards to its arabinogalactan moiety, to a 'neutral' grape AGP and to a previously reported apple juice AGP (Will & Dietrich, 1992). Apple juice AGP is built on a central 3-linked galactan core carrying, at position 6, 6-linked galactan side-chains substituted at C-3 by terminal arabinofuranose, which could be almost quantitatively removed through the action of a purified α-L-arabinofuranosidase. Exhaustive dearabinosylation rendered the 6-linked galactan side-chains sensitive to the action of a ß-D-galactosidase.

The dearabinosylated molecule, i.e. GP_1, exhibited the ability to form aggregates after freeze-drying and gave an anomolous M_w value in LALLS when compared to that obtained for the native AGP. This aggregation phenomenon was reversible and could be avoided when the product was dissolved in NaOH solutions. Linearization of (1→6)-linked galactan outer chains by α-L-arabinofuranosidase was first thought to be responsible for aggregation by bringing these chains together. However, after ß-D-galactosidase had removed most of these chains, aggregation

was still evident from the HPSEC chromatogram so that linearization of (1→3)-linked galactan inner-chains could also be involved. Thus, the cause of this phenomenon is still unknown. Finally, the chromatographic aggregation profile, i.e. the main reaction product being still present aside the shouldering fraction, suggests a certain heterogeneity which remains to be elucidated. This phenomenon has already been observed for freeze-dried dearabinosylated, and also degalactosylated highly purified grape AGP (Saulnier *et al.*, 1992), but was not understood at the time. Saulnier *et al.* (1992) showed that structures responsible for the main peak and the shoulder fraction were identical, which reinforces the possibility of formation of aggregates upon freeze-drying. Aggregation was also observed for an enzymatically-debranched linear arabinan from apple juice concentrates (Churms *et al.*, 1983) and for a linear arabinan from red wine (Belleville *et al.*, 1993).

Thus, our results indicate the possibility that enzymatic degradation (especially dearabinosylation) of neutral pectic polysaccharides commonly found in fruit juices and their derived-products, including wine, can lead to the formation of high molecular weight aggregates which in turn can induce reduction of clarity through the formation of haze (Belleville *et al.*, 1993) or plug filtration membranes (Will *et al.*, 1992). Although the aggregation phenomenon did not appear if the dearabinosylated apple AGP remained in solution, it may arise upon concentration of juice or cooling.

Frequent use of enzymes as technological tools in juice processing might, since they contain arabinofuranosidase activities, induce or increase these aggregation phenomena. Haze formation in apple juice which is due to linearization of arabinans can be prevented by adding enzyme mixtures containing arabinanases (Beldman *et al.*, 1993). In a similar manner, fungal enzyme preparations would have to be enriched in type II galactanases and galactosidases to depolymerize type II galactans. Endo-(1→6)-ß-D-galactanases (Brillouet *et al.*, 1991) and exo-(1→3)-ß-D-galactanases (Tsumuraya *et al.*, 1990; Pellerin & Brillouet, 1994) are good candidates for such a purpose.

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