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Cell wall polysaccharides of Chinese Wolfberry (*Lycium barbarum*): Part 2. Characterisation of arabinogalactan-proteins

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ABSTRACT

Arabinogalactan-proteins (AGPs) were isolated from Wolfberry fruit (*Lycium barbarum*) and purified by anion-exchange chromatography and precipitation with Yariv reagent. Linkage and NMR analysis established the structure of Wolfberry AGP as typical of AGP reported from other sources. The data were consistent with a backbone of (1 → 3)-linked β-D-galactopyranosyl residues, many of which were substituted at O-6 with side chains of mainly 5-substituted α-L-arabinofuranosyl residues, terminated with α-(and β-)L-arabinofuranosyl, α-L-rhamnopyranosyl and β-D-glucopyranosyluronic acid residues. An unusual structural feature of the AGP was the occurrence of 4-substituted β-D-glucopyranosyluronic acid residues in the side chains. The AGP, which had a MW average of between 50 and 60 kDa, displayed heterogeneity with regard to both galactose/arabinose ratio and degree of branching. Protein accounted for ~6% of the AGP and was rich in hydroxyproline. Evidence for an interaction between some of the AGP and hydrophobic moieties is presented.

1. Introduction

In a companion paper we reported the structural features of the different types of polysaccharide from both soluble and insoluble cell wall fractions of Wolfberry (Redgwell et al., submitted for publication). Glycoconjugates in the form of arabinogalactan-proteins (AGPs) were identified, confirming several previous reports of these molecules in Wolfberry fruit. In two of these publications (Huang, Tian, & Zheng, 1999; Peng, Huang, Qi, Zhang, & Tian, 2001) it was reported that the glycoconjugates LbGp3 and LbGp4 were AGPs containing a (1 → 4)-linked galactopyranan backbone frequently substituted at O-3 with highly branched side chains terminated predominantly by arabinofuranosyl residues. The authors pointed out the novelty of this structure, as most known plant-derived AGPs carry a (1 → 3)-linked galactopyranan backbone substituted at O-6 with side chains containing additional galactopyranosyl residues as well as arabinofuranosyl residues. However, our investigations identified structural features of Wolfberry AGP that were consistent with those of most plant AGP reported in the literature. That is a backbone of (1 → 3)-linked β-D-galactopyranosyl residues substituted at intervals in the O-6 position with mostly α-L-arabinofuranosyl residues (Redgwell et al., submitted for publication).

Experiments in our laboratory verified the existence of AGP in Wolfberry fruits by the positive reaction of crude polysaccharide preparations to the β-D-glucosyl Yariv reagent, a test specific for AGPs (Van Holst & Clarke, 1985). In the present study we report on a more detailed investigation of Wolfberry AGP fractions isolated and purified by precipitation with the Yariv reagent and subsequently fractionated by anion-exchange chromatography. Their structural features were determined by a combination of linkage analysis and ^{1}H and ^{13}C NMR spectroscopy.

2. Materials and methods

2.1. Plant material

Wolfberries (*Lycium barbarum*) were obtained as dried fruit (water content ~3.3%) from Ningxia Chinese Wolfberry Group Company, Ningxia Province, China.

2.2. Reagents

The β-D-glucosyl Yariv reagent [1,3,5-tris (4-β-D-glucopyranosyloxyphenylazo)-2,4,6-trihydroxy-benzene] was

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purchased from Biosupplies Australia Pty Ltd. Bakerbond Octadecyl (C_{18}) 40 μm Prep LC packing was obtained from J.T. Baker.

2.3. General methods

Compositional, linkage, and total uronic acid analysis, the Yariv plate assay and precipitation with CTAB were all carried out as previously described (Redgwell et al., submitted for publication). Glucuronic and galacturonic acids in polysaccharide hydrolysates were analysed by HPAEC-PAD on a Dionex SLC 3000 instrument according to the SOP 4149-1 procedure using a CarboPac PA-1 (4 mm × 250 mm) column equilibrated with 150 mM NaOH.

2.4. Precipitation of AGP with Yariv reagent

Defatted WSP (30 mg) was dissolved in 7.5 mL of 0.05 M Tris/HCl, pH 8.0. The solution was filtered (Whatman GFA 3 glass fibre) to remove traces of particulate matter and 5 mL of Yariv reagent (1 mg/mL in 1% NaCl) added to 5 mL of the filtrate. The solution was allowed to stand overnight at 4 °C and centrifuged (7000 rpm, 20 min). The supernatant was decanted and the pellet (AGP–Yariv complex) was suspended in 100% methanol by a brief ultrasonication. Following centrifugation the supernatant was discarded and the methanol wash repeated. The pellet was dried in a stream of argon and dissolved in 2 mL of 50% DMSO. Amounts of sodium dithionite (50–75 mg) were added and the solution heated to 50 °C to break the Yariv–AGP complex. The colourless solution was then dialysed for several days and AGP recovered by freeze drying. The supernatant from the Yariv precipitate, containing mostly pectic polysaccharides, was evaporated to dryness and subjected to the same series of steps and recovered after freeze drying.

2.5. High-performance liquid chromatography

Molecular weight analyses were made with an Agilent 1200 series HPLC instrument equipped with a TSKgel G3000PWXL column (7.8 mm × 30 cm) and a GMPWXL column (7.8 mm × 30 cm) in series with a PWXL guard column (6 mm × 4.4 cm) (Tosco Bioscience). The system was fed with 0.1 M sodium nitrate (sodium azide, 0.02%) at 0.5 mL/min. Signals were measured by refractive index. Samples and pullulan standards (Fluka standard set Mp 342–710,000, no. 96351) were dissolved in eluant at 5 mg/mL and 3 mg/mL, respectively. They were filtered on nylon syringe filters (0.13 mm diameter, 0.2 μm), before injection (25 μL). Signals were recorded and treated with ChemStation for LC systems (B.04.01 SP1).

2.6. NMR spectroscopy

Resolution-enhanced 1D/2D 500-MHz ^{1}H NMR spectra and 125-MHz ^{13}C NMR spectra were recorded in D_2O on a Bruker DRX-500 spectrometer (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University) at a probe temperature of 292 K. The temperature 292 K was chosen instead of the generally used 300 K, to move the HOD signal upfield. Before analysis, samples were exchanged twice in D_2O (99.9 at.% D, Cambridge Isotope Laboratories, Inc., Andover, MA) with intermediate lyophilization, and then dissolved in 0.6 mL D_2O. Chemical shifts (δ) are expressed in ppm by reference to internal acetone (δ 2.225 for ^{1}H and 31.07 for ^{13}C). Suppression of the HOD signal was achieved by applying a WEFT pulse sequence for 1D experiments and by a pre-saturation of 1 s during the relaxation delay in 2D experiments. 2D TOCSY spectra were recorded using an MLEV-17 mixing sequence with spin-lock times of 10–200 ms. 2D NOESY experiments were performed with a mixing time of 200 ms. Natural abundance 2D ^{13}C–^{1}H HSQC and HMBC experiments were recorded without decoupling during acquisition of the ^{1}H FID. Resolution enhancement of the spectra was performed by a Lorentzian-to-Gaussian transformation for 1D spectra or by multiplication with a squared-bell function phase shifted by π/2.3 for 2D spectra, and when necessary, a fifth order polynomial baseline correction was performed. All NMR data were processed using in-house developed software.

3. Results and discussion

3.1. Isolation of water-soluble polysaccharides

Water-soluble polysaccharides (WSP) were isolated from dried Wolfberries as described by Redgwell et al. (submitted for publication) and consisted predominantly of uronic acid, arabinose and galactose. The polysaccharide content accounted for 50% of the WSP. The protein content was 17.0%.

3.2. Yariv precipitation of AGP

Treatment of WSP with the Yariv reagent yielded a precipitate (henceforth referred to as AGP) that accounted for 20% of the WSP. The composition was consistent with that of an arabinogalactan (Table 1). Arabinose and galactose were present at a ratio of ~1:1. The uronic acid content was 9.4% and rhamnose was also present. The 80% of WSP which remained in the Yariv supernatant contained more than 50% uronic acid. The ratio of arabinose to galactose was 3:1. Glucuronic acid is known to be present as terminal residues in some AGP. Therefore to identify the uronic acid in the AGP and supernatant fractions, hydrolysates of each were examined by high-performance anion-exchange chromatography (HPAEC) on CarboPac PA-1.

Glucuronic acid was the predominant uronic acid in the AGP and galacturonic acid the major uronic acid in the Yariv supernatant fraction, indicating pectic polysaccharides in the latter (Table 1). A Yariv plate assay indicated that some AGP was still present in the supernatant fraction, albeit at much lower levels than in the AGP. Despite the much higher galacturonic acid content of the supernatant fraction, rhamnose (normally associated with the rhamnogalacturonan backbone of pectic polysaccharides) was present at higher levels in the AGP. This suggested that rhamnose may be a structural feature of the AGP and not merely associated with a trace of contaminating pectic polysaccharide in the Yariv precipitate.

The molecular weight profiles of WSP and AGP fractions of the Yariv precipitate (Fig. 1) indicated the polydispersed nature of the WSP which ranged between ~40 and 1000 kDa. The profile of the AGP was dominated by a peak between 50 and 60 kDa. A minor component of higher molecular weight (~200 kDa) was present in the AGP. As this peak coincided with the largest peak in the WSP (i.e. pectic polysaccharides) it is likely to be caused by a small amount of pectic polysaccharide co-precipitating with the AGP during treatment with the Yariv reagent.

3.2.1. Linkage analysis

Analysis of the partially methylated alditol acetates derived from the Yariv purified AGP was done on carboxyl-reduced polysaccharides to allow assignments to be made for the uronic acid components (Table 2). Since no 4-substituted galactopyranosyl residues were detected in the unreduced WSP (data not shown) any 4-substituted galactopyranosyl residues in the carboxyl-reduced WSP were derived from galacturonic acid. The fact that they accounted for more than 40% of the linkage types indicated that pectic polysaccharides accounted for a major part of the soluble polysaccharides in Wolfberry. The presence of 4-substituted

Table 1
Monosaccharide composition of AGP and Yariv supernatant fractions of WSP.

Fraction	Monosaccharide composition (mol%)									Total (μg/mg)
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA	GlcA	
AGP	3.3	–	42.9	0.3	tc	44.3	tc	2.4	7.0	748
Yariv supernatant	1.1	0.2	26.7	3.9	0.6	8.2	6.0	48.8	4.5	643

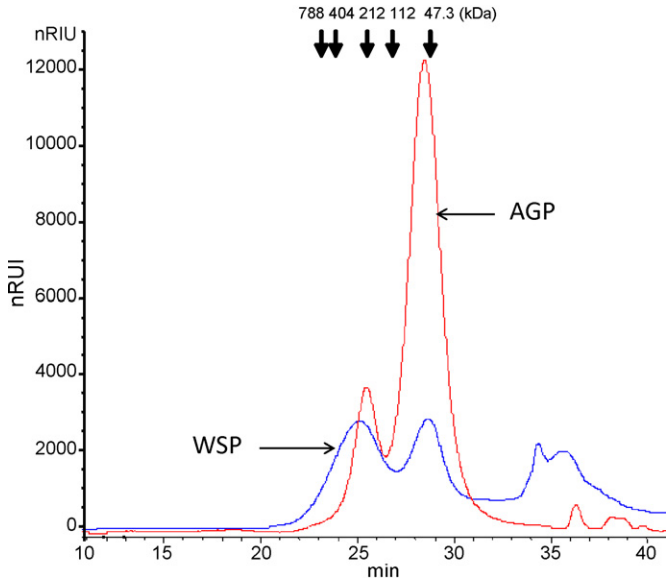

Fig. 1. HPLC profiles of WSP and AGP fractions of Wolfberry.

xylopyranosyl and glucopyranosyl residues indicated the presence of additional types of polysaccharide.

The structural features of type II arabinogalactans found in many AGP consist for the most part of (1→3)-linked β-D-galactopyranosyl residues, some of which are substituted at O-6 with α-L-arabinofuranosyl and β-D-galactopyranosyl residues (Fincher, Stone, & Clarke, 1983; McNeil, Darvill, Fry, & Albersheim, 1984). The linkage analysis of AGP in Table 2 accords with these reports and is consistent with the idea that Wolfberry AGP is made up of a backbone of (1→3)-linked galactopyranosyl residues substituted at intervals in the O-6 position with various combinations of mostly arabinofuranosyl and galactopyranosyl residues. The ratio of 3-substituted to 3,6-disubstituted galactopyranosyl residues (1:2.6) indicated a highly branched molecule. The side chains exist predominantly as 5-substituted arabinofuranosyl residues but the complex nature of the side chains is indicated by the presence of 2-substituted, 3-substituted, and 3,5-disubstituted arabinofuranosyl residues. In addition, 4-substituted glucopyranosyluronic acid residues were present as internal residues in the side chains which is not a commonly reported feature of AGP. Although arabinofuranosyl residues terminate most of the side chains, galactopyranosyl, rhamnopyranosyl and glucopyranosyluronic acid residues also fulfil this function.

Table 2
Linkage analysis of WSP and Yariv precipitated AGP.

Sugar	Linkage	WSP	AGP
Rha*p*	Terminal	1.2	3.1
Ara*f*	Terminal	11.8	22.1
	2-	5.0	1.2
	3-	2.6	3.8
	5-	7.1	16.2
	3,5-	0.6	0.9
Xyl*p*	4-	2.8	–
Gal*p*	Terminal	1.0	1.3
	3-	3.7	11.2
	6-	1.0	3.8
	3,6-	8.9	30.0
Glc*p*	Terminal	1.7	–
	4-	5.2	–
	4,6-	1.7	–
Gal*p*A[a]	4-	43.3	0.4
	3,4-	1.8	–
Glc*p*A[a]	Terminal	0.3	2.0
	4-	0.6	4.0

[a] Glucuronic and galacturonic acids analysed as 6,6-dideuterio-glycosyl residues following carboxyl reduction of the polysaccharides with sodium borodeuteride.

3.2.2. NMR analysis of the AGP fraction

The 1D ^{1}H NMR spectrum of the Yariv-precipitated AGP fraction (Fig. 2) showed considerable overlap of signals with different intensities in the anomeric region (δ 5.3–4.4), and the 2D TOCSY and ^{13}C–^{1}H HSQC spectra revealed the presence of many spin systems. Since this AGP fraction was still mixed with small amounts of other polysaccharides, the assignment of all protons of the different monosaccharide residues was complicated and in some cases not definitive. However, by careful study of the 2D NMR spectra, in particular the TOCSY spectra with different mixing times (10–200 ms), eleven residues, labeled A–K according to decreasing ^{1}H chemical shift values of their anomeric protons (Table 3), could be discriminated, and a single spin system per residue could be assigned.

The broad signal at ~δ 5.26 contained the anomeric protons of two →5)-α-L-Ara*f*-(1→ residues (A, B) and one →3)-α-L-Ara*f*-(1→ residue (C). Although the TOCSY tracks of A H-1 and B H-1 were overlapping, the complete scalar coupling networks H-1,2,3,4,5a,5b could be determined (Bock & Thøgersen, 1982; Cardoso, Ferreira, Mafra, Silva, & Coimbra, 2007; Cardoso, Silva, & Coimbra, 2002; Dourado, Cardoso, Silva, Gama, & Coimbra, 2006; Eriksson, Andersson, Westerlund, Andersson, & Aman, 1996; Nunes, Reis, Silva, Dominques, & Coimbra, 2008). The ^{13}C chemical shifts, deduced from the 2D ^{13}C–^{1}H HSQC spectrum, showed similar data for A and B, except for the H-4/C-4 resonance, most likely due to different sugar substituents at the O-5 position. The presence of the 5-substituted A and B residues was confirmed by the downfield shift of C-5 (δ 69.3, $\Delta\delta$ 5.4 compared to non-substituted α-L-Ara*f* D) (Bock & Pedersen, 1983; Beier & Mundy, 1984). For the overlapping residue C, the TOCSY spectrum showed on the C H-1 track, the cross-peak with C H-2, whereas the remaining signals C H-2,3,4,5a,5b could be found via the C H-2 (δ 4.45) track (Table 3). The HSQC spectrum showed for residue C a downfield shift for C-3 (δ 86.7, $\Delta\delta$ 7.5 compared to non-substituted α-L-Ara*f* D) (Bock & Pedersen, 1983; Beier & Mundy, 1984), in accordance with a 3-substituted α-L-Ara*f*.

The complete scalar coupling networks H-1,2,3,4,5a,5b of both residues D and E, starting at the anomeric signals at δ 5.09, could be determined from the TOCSY spectrum, in connection with the HSQC spectrum and by connectivities found in HMBC experiments. There is also another residue present in minor amounts with H-1 at δ 5.09, but this could not be identified. The set of chemical shifts of residues D and E are characteristic for a terminal α-L-Ara*f* residue and a terminal β-L-Ara*f* residue, respectively (Bock & Pedersen, 1983; Capek, Matulova, Navarini, & Suggi-Liverani, 2010; Cardoso et al., 2007; Dourado et al., 2006; Eriksson et al., 1996; Tan, Qui, Lamport, &

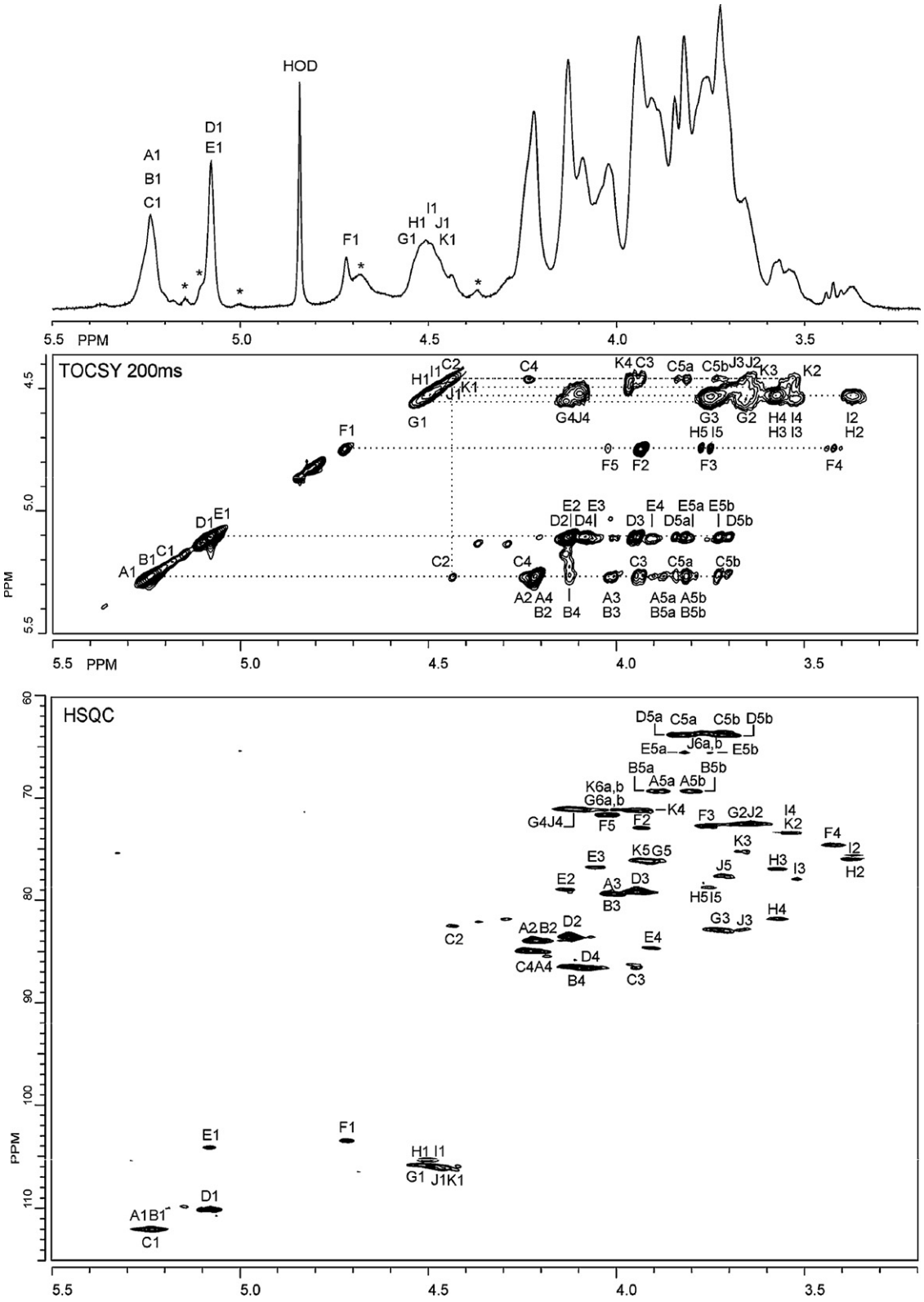

Fig. 2. The 1D ^{1}H NMR spectrum, the TOCSY spectrum (200 ms) and the HSQC spectrum of AGP fraction, recorded in D_2O at 292 K. *Unidentified carbohydrate contamination.

Kieliszewski, 2004). The α- and β-anomeric configurations of D and E, respectively, are supported by their C-1 values deduced from the HSQC spectrum (residue D, α-L-Ara*f*, C-1 at δ 110.2; residue E, β-L-Ara*f*, C-1 at δ 104.3). Also the E C-5 signal at δ 65.7 is indicative of terminal β-L-Ara*f* (Bock & Pedersen, 1983; Cardoso et al., 2007). Finally, the C-4 chemical shifts of A–E at ~δ 85 confirmed furanose ring forms (Beier & Mundy, 1984; Bock & Thøgersen, 1982).

The anomeric signal at δ 4.72 could be attributed to a terminal α-L-Rha*p* residue (F) (Gutiérrez, Martinéz, Sanabria, de Pinto, & Igatuburu, 2005; Nunes et al., 2008; Tan et al., 2004) and the

Table 3
^{1}H and ^{13}C chemical shift assignments (δ, ppm) of monosaccharide residues present in the AGP fraction.

Residue	H-1 C-1	H-2 C-2	H-3 C-3	H-4 C-4	H-5a; H-5b C-5	H-6a; H-6b C-6
A →5)-α-L-Ara*f*-(1→						
^{1}H	5.27	~4.23	4.01	~4.22	~3.89; 3.80	–
^{13}C	112.0	84.0	79.4	85.0	69.3	
B →5)-α-L-Ara*f*-(1→						
^{13}C	~5.26	~4.22	4.01	4.12	~3.89; 3.80	–
^{1}H	112.0	84.0	79.4	86.5	69.3	
C →3)-α-L-Ara*f*-(1→						
^{1}H	5.25	4.45	3.94	4.23	~3.83; 3.73	–
^{13}C	112.1	82.6	~86.7	85.1	~64.0	
D α-L-Ara*f*-(1→						
^{1}H	5.09	~4.12	3.94	~4.09	~3.83; 3.71	–
^{13}C	110.2	83.5	79.2	~86.7	63.9	
E β-L-Ara*f*-(1→						
^{1}H	5.09	4.14	4.05	3.90	3.82; 3.74	–
^{13}C	104.3	79.0	76.8	84.7	65.7	
F α-L-Rha*p*-(1→						
^{1}H	4.72	3.93	3.77	3.43	4.03	1.25 (CH_3)
^{13}C	103.6	72.7	72.4	74.7	71.8	
G →3,6)-β-D-Gal*p*-(1→						
^{1}H	~4.53	~3.66	~3.74	~4.13	~3.92	~4.10; 3.97
^{13}C	106.3	72.5	82.9	71.2	76.1	71.2
H →4)-β-D-Glc*p*A-(1→						
^{1}H	~4.52	3.38	3.57	3.58	~3.76	–
^{13}C	105.5	76.1	77.1	81.9	78.7	
I β-D-Glc*p*A-(1→						
^{1}H	~4.52	3.38	3.53	3.54	~3.76	–
^{13}C	106.0	75.7	78.2	73.5	78.7	
J →3)-β-D-Gal*p*-(1→						
^{1}H	~4.45	~3.73	3.68	~4.10	3.72	~3.80; 3.76
^{13}C	106.4	72.5	82.9	71.2	77.5	63.8
K →6)-β-D-Gal*p*-(1→						
^{1}H	~4.45	3.53	3.67	~3.95	~3.93	~4.10; 3.97
^{13}C	106.4	73.5	75.1	71.2	76.1	71.2

~ means average value of overlapping signal.

complete spin system was deduced from the TOCSY spectrum (Table 3). The methyl group of residue F is reflected by the high-field signal at δ 1.25 (not shown).

The broad NMR peak at ~δ 4.50 contained at least five anomeric signals, belonging to a →3,6)-β-D-Gal*p*-(1→ residue (G), a →4)-β-D-Glc*p*A-(1→ residue (H), a terminal β-D-Glc*p*A residue (I), a →3)-β-D-Gal*p*-(1→ residue (J), and a →6)-β-D-Gal*p*-(1→ residue (K). By means of 2D TOCSY (10–200 ms), ^{13}C–^{1}H HSQC and HMBC experiments (spectra not shown), most of the proton and carbon atoms could be assigned (Table 3).

The assignment of the G, J, and K Gal*p* residues was complicated due to a less intensive magnetization transfer (H-4) and extensive overlap of H-2 and H-3. The TOCSY G, J, and K H-1 tracks are typical for β-D-Gal*p* residues, showing only three cross peaks H-2, H-3, H-4. The values for H-5 and H-6a,b followed from the HSQC spectrum. The spin systems of G, J, and K, including the downfield positions of H-4, are in agreement with the *galacto*-configuration (Bock & Thøgersen, 1982; Capek et al., 2010; Nunes et al., 2008). Taking into account the ^{13}C data of G, J, and K, the downfield shifts of G C-6 (δ 71.2, $\Delta\delta$ 9.2; Me-β-D-Gal*p*, $\delta_{C\text{-}6}$ 62.0), and G C-3 (δ 82.9, $\Delta\delta$ 9.1; Me-β-D-Gal*p*, $\delta_{C\text{-}3}$ 73.8), demonstrated a 3,6-disubstituted residue, the downfield shift of J C-3 (δ 82.9, $\Delta\delta$ 9.1) a 3-substituted residue, and the downfield shift of K C-6 (δ 71.2, $\Delta\delta$ 9.2) a 6-substituted residue (Bock & Pedersen, 1983).

Residues H and I were identified as an internal →4)-β-D-Glc*p*A-(1→ residue and a terminal β-D-Glc*p*A residue, respectively, by the typical chemical shifts of H-2,3,4,5, and the absence of H-6 (Dobruchowska, Gerwig, Babuchowski, & Kamerling, 2008). The downfield shift of H C-4 (δ 81.9, $\Delta\delta$ 8.4) is in accordance with a 4-substituted H residue.

Comparison of the substitution patterns revealed by the NMR data with those obtained in the methylation analysis (Table 2) showed a good overlap of data. Because of the low amounts of some substitution patterns, it is understandable that not all substitution patterns seen in the methylation analysis are picked up in the complicated 2D NMR spectra.

In the 2D NOESY spectrum (not shown), the strong inter-residual connectivities (NOEs) G(1 → 3)G, G(1 → 3)J, J(1 → 3)G, and J(1 → 3)J suggest a (1 → 3)-linked β-D-galactopyranose backbone, heavily substituted at O-6. Furthermore, other observed inter-residual NOEs, A(1 → 6)G, B(1 → 6)G, C(1 → 6)G, G(1 → 6)G, and K(1 → 6)G, indicate the high diversity of substitution of the G residues in the backbone. But G residues are also present in the side-chains, because of the inter-residual NOEs A(1 → 3)G and B(1 → 3)G. This is also the case for the J residues, because of the presence of the NOEs A(1 → 3)J and C(1 → 3)J. The different substitutions of A and B are indicated by the NOEs E(1 → 5)B and D(1 → 5)A. Other observed inter-residual NOEs, like A(1 → 3)C and B(1 → 3)C, further demonstrate the complexity of the side-chains. According to the NOEs E(1 → 5)B and I(1 → 6)K, the terminal residues E and I are mainly linked to B and K, respectively. The terminal residue F is linked to H, which is linked to K, according to the NOEs F(1 → 4)H and H(1 → 6)K, respectively.

The linkage sequence of some of the monosaccharide residues of the AGP fraction could be confirmed by unravelling the heteronuclear HMBC spectrum (not shown). Strong correlations were observed for G H-1–G C-3, J H-1–J C-3, and J H-1–G C-3, supporting a (1 → 3)-linked β-D-galactopyranose backbone. The 6-substitution of G was supported by strong correlations for G H-1–G C-6, A/B H-1–G C-6 and K H-1–G C-6. The linkages of the terminal D and E residues were confirmed by the correlations D/E H-1–A/B C-5. Furthermore, the correlation F H-1–H C-4 was clearly visible, as well as H H-1–K C-6.

Table 4
Amino acid composition of protein content of AGP (Yariv precipitatable) and supernatant (non-Yariv precipitatable) fractions from WSP.

Amino acid	AGP		Supernatant	
	mol%	μg/mg	mol%	μg/mg
Aspartic acid	7.5	5.1	8.8	17.5
Glutamic acid	8.9	6.7	9.7	21.9
Hydroxyproline	9.3	6.1	13.4	26.6
Serine	10.5	5.3	10.3	15.8
Alanine	10.1	4.2	6.1	7.5
Glycine	9.5	3.1	9.4	9.4
Histidine	2.9	2.4	2.3	5.7
Arginine	2.2	2.1	2.9	7.8
Threonine	4.9	2.9	5.6	10.1
Proline	4.4	2.5	7.0	12.1
Tyrosine	1.5	1.4	2.1	6.0
Valine	3.7	2.2	3.3	5.8
Methionine	2.1	1.6	1.6	3.5
Cystine	1.2	1.5	2.4	9.4
Isoleucine	3.5	2.3	2.8	5.4
Leucine	4.0	2.7	3.7	7.3
Phenylalanine	2.2	2.0	2.2	5.7
Tryptophan	3.4	3.1	1.4	4.1
Ornithine	4.1	2.9	0.9	1.9
Lysine	4.1	3.0	4.1	9.2
Total	100	63.1	100	192.7

In Fig. 3 a hypothetical model, containing all the structural features as determined by NMR analysis, is depicted for the arabinogalactan present in the AGP fraction. The Wolfberry AGP is made up of a backbone of (1 → 3)-linked β-D-galactopyranosyl residues. On average, 5 of 7 galactosyl residues in the backbone are substituted at O-6 with complex side-chains consisting of various combinations of mostly α-L-arabinofuranosyl and β-D-galactopyranosyl residues, terminated mostly with α-L-arabinofuranosyl residues. However, other terminal residues, like β-L-arabinofuranosyl, α-L-rhamnopyranosyl, and β-D-glucopyranosyluronic acid residues are present. The terminal α-L-rhamnopyranosyl residues are always (1 → 4)-linked to β-D-glucopyranuronic acid and the β-D-glucuronic acids are always (1 → 6)-linked to β-D-galactopyranosyl residues.

3.2.3. Protein content and amino acid composition

Based on a summation of the individual amino acids the Yariv precipitatable AGP and the supernatant fraction contained 6.3% and 19.3% protein, respectively (Table 4). The protein backbones of AGP are often rich in hydroxyproline, serine and alanine (Fincher et al., 1983) and this pattern is reflected in the composition of Wolfberry AGP. However, the composition of the supernatant fraction was also high in these amino acids and was similar but not identical to that of the AGP. The Yariv plate assay revealed that small amounts of AGP remained in the supernatant fraction. Either this fraction contains a non-Yariv precipitatable AGP which is more highly proteinated than the AGP precipitated by the Yariv reagent, or it contains a hydroxyproline-rich protein.

3.2.4. Fractionation of AGP

The health benefits of Wolfberry AGP or glycoconjugates have been attributed to specific structural forms of the molecule. Glycoconjugates have been reported that have differed in terms of their individual monosaccharide composition, molecular weight and galactose to arabinose ratios (Huang, Lin, Tian, & Ji, 1998). The WSP was therefore depectinated by precipitation with CTAB and the AGP-enriched supernatant from the WSP (Redgwell et al., submitted for publication) then eluted on to a DEAE-Sepharose anion-exchange column and polysaccharide fractions recovered by sequential elution with 0.05, 0.1, 0.2, 0.4 and 0.8 M NH_4HCO_3. The monosaccharide composition of each of these fractions was given in Redgwell et al. (submitted for publication). In the present study we subjected the 0.05, 0.1, 0.2 and 0.4 M fractions from the DEAE-Sepharose column to additional purification by treatment with the Yariv reagent. The precipitated polymers (AGPs) and the supernatants from each fraction were recovered and subjected to compositional and linkage analysis.

The ratio of arabinose to galactose in the 0.05, 0.1, 0.2, 0.4 and 0.8 M AGP fractions was 1:1.3, 1:1.4, 1:0.9, 1:0.7 and 1:0.7, respectively (Table 5). Significantly there is a marked increase in the arabinose to galactose ratio in 0.05, 0.4 and 0.8 M supernatant fractions from the Yariv treatment. Compositional analysis of 0.05, 0.4 and 0.8 M supernatant fractions showed them to have an arabinose to galactose ratio of ~5:1. It suggests that there is an additional type of arabinan present in WSP which can be separated from the AGP in the WSP on the basis of its non-interaction with the Yariv reagent. Linkage analysis showed that the 0.05 M supernatant fraction contained 2-substituted arabinofuranosyl residues as the predominant structural feature of the polysaccharide (Table 6). This contrasted with the AGP fractions where terminal and 5-substituted arabinofuranosyl residues were the dominant structural forms in the polysaccharide. It is concluded that Wolfberry contains, in addition to AGP, a neutral arabinan polysaccharide the major structural feature of which is a backbone of (1 → 2)-linked arabinofuranosyl residues.

Linkage analysis of the AGP fractions confirmed the structural features that were discussed in Redgwell et al. (submitted for publication) (Table 6). They are those reported for the arabinogalactan moiety of many plant AGP. Differences among the fractions

Table 5
Monosaccharide composition of Yariv-precipitated AGP and Yariv supernatant polysaccharides from DEAE-Sepharose fractions of WSP1.

Fraction	Monosaccharide composition (mol%)								Total (μg/mg)
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	HexA	
AGP									
0.05 M	0.7	–	40.6	0.5	0.4	52.6	0.9	4.3	619
0.1 M	0.7	–	37.6	0.6	0.3	52.5	0.5	7.9	625
0.2 M	3.1	–	41.9	0.2	3.1	39.5	0.4	11.9	660
0.4 M	2.8	–	48.4	0.2	0.4	33.8	0.6	13.8	628
0.8 M	2.7	0.2	53.7	1.3	2.0	37.7	2.3	ND	351
Supernatant									
0.05 M	0.3	0.3	66.2	6.1	1.9	12.6	9.8	2.8	466
0.1 M	2.0	0.1	41.7	9.4	0.5	33.6	1.5	11.3	619
0.2 M	3.4	0.1	48.2	2.4	0.5	29.7	2.0	13.6	582
0.4 M	5.0	0.5	44.6	2.7	1.2	8.9	11.2	26.0	466
0.8 M	3.6	0.5	48.4	4.6	2.7	10.8	29.4	ND	155

ND: not determined.

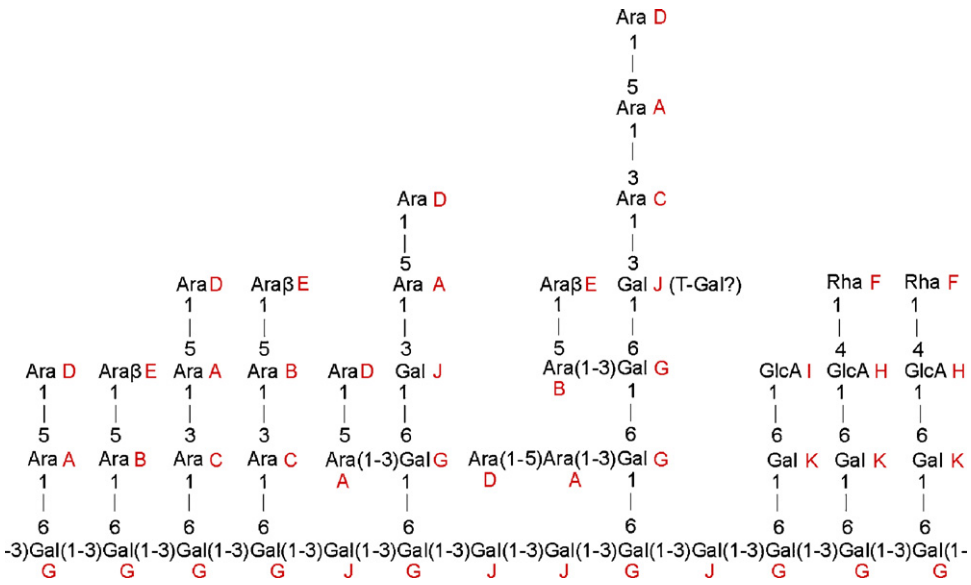

Fig. 3. Hypothetical model of the structural features of the AGP glycan.

occurred mostly in the ratio of 3-substituted to 3,6-disubstituted galactopyranosyl residues which reflected a variation in the degree of branching of the AGP. Thus, for the 0.05, 0.1, 0.2 and 0.4 M AGP fractions the 3,6-disubstituted galactopyranosyl residues were 1.1, 1.3, 2.5 and 4.2 times that of the 3-substituted residues, respectively. It seems that the more branched AGP were more strongly bound to the anion-exchange column. This would make sense, since the affinity for DEAE-Sepharose is based primarily on the acidity of the polysaccharides. The more branch point side chains allow for potentially more glucuronic acid residues per unit length of the AGP.

3.3. Polysaccharide interaction with other cellular components

Moderate amounts of protein and unidentified material co-purified with many of the polysaccharide fractions during the work up procedures. It was thought that some of the unknown material could be polyphenolic in nature as the fruit is known to be rich in flavanoids, etc. An attempt was made to remove this material by passing an aqueous solution of WSP through a column of silica bound Octadecyl C_{18} reverse phase material.

As expected, the majority of brown phenolic material bound to the column. However, surprisingly, so did a significant proportion of the WSP. The fraction which passed straight through the column accounted for 54.1% of WSP and was rich in pectic polysaccharides (Table 7). The remainder was retained on the C_{18} matrix. Of this, 32% was recovered following elution with 50% methanol. Both the water and 50% methanol fractions gave a strong positive test for AGP when subjected to the Yariv plate assay. Under normal circumstances all water-soluble polysaccharides are strongly hydrophilic and would not therefore be expected to be retained on a matrix designed to bind hydrophobic molecules, hence, the presence of a high concentration of pectic polysaccharides and AGP in the water fraction. However, the fact that a major part of the AGP did not pass through the column, indicated that they had formed an association with a hydrophobic molecule. To a degree this association appeared to be selective, as almost all the glucan and xylan content of WSP was contained in the 50% methanol fraction and not the water elu-

Table 6
Linkage analysis of Yariv precipitated AGP and Yariv supernatant fractions from DEAE-Sepharose fractions of WSP1.

Monosaccharide	Linkage	mol%							
		0.05 M		0.1 M		0.2 M		0.4 M	
		AGP	SUP	AGP	SUP	AGP	SUP	AGP	SUP
Rha*p*	Terminal			1.2	1.7	2.3	1.9	2.8	1.9
	3,4-						0.7		2.1
Ara*f*	Terminal	19.9	20.7	20.8	25.5	23.8	23.5	26.7	15.4
	2-	2.2	30.4	1.2	5.3	1.0	3.1	1.4	14.7
	3-	3.3	6.7	2.7	3.4	3.5	4.6	4.5	4.8
	5-	11.7	7.5	12.1	14.2	16.7	18.8	19.2	24.3
	3,5-					0.8	2.0	1.4	7.0
Gal*p*	Terminal	3.0	8.1	2.8	1.7	1.9	1.8	1.2	3.0
	3-	23.9	1.9	22.2	8.2	13.1	8.0	7.4	2.1
	6-	8.0	1.4	7.6	3.1	3.5	2.8	3.3	1.4
	3,6-	26.0	3.7	28.1	23.9	32.6	29.0	31.2	6.3
	4-								2.1
Glc*p*	Terminal				0.6		1.2		7.0
	4-	1.4	8.5	1.1	0.9	0.7	1.1	0.9	5.8
	4,6-		4.7						1.2
Xyl*p*	Terminal		1.7		0.8				
	4-		3.1		10.4		1.8		1.6

Table 7
Yield and monosaccharide composition of WSP fractions recovered from C_{18}-reverse phase column.

Fraction	Yield (%)	Monosaccharide composition (mol%)								Total (μg/mg)
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	HexA	
WSP	100	1.4	0.1	27.7	3.0	0.6	12.6	7.8	46.8	607
Water	54.1	1.1	0.1	14.8	1.2	0.3	8.5	0.7	73.4	754
50% CH_3OH	32.0	1.4	0.2	39.9	7.2	1.5	15.0	20.3	14.5	492
80% CH_3OH	3.0	1.6	0.3	35.3	2.0	0.9	9.7	41.8	8.5	334

ate. A small part of WSP fraction was even more strongly bound to the C_{18} and was only recovered in 80% methanol. This fraction was particularly rich in glucan (Table 7).

The fact that much of the brown phenolic material accompanied the polysaccharides in the methanol fractions leads to the speculation that part of the polysaccharide content in Wolfberry is attached to polyphenolic compounds. Whether this association occurs naturally in vivo, or is a consequence of inadvertent interactions during the extraction process has yet to be determined.

4. Concluding discussion

The structural features of the AGP in Wolfberry were those commonly documented for plant AGP from a wide spectrum of plant sources. That is a backbone of (1 → 3)-linked β-D-galactopyranosyl residues, substituted at intervals in the O-6 position with various combinations of α-L-arabinofuranosyl and β-D-galactopyranosyl residues. Up to ∼10% of the molecule may exist as β-D-glucopyranosyluronic acid residues which occurred as terminal and 4-substituted residues on some side chains. α-L-Rhamnopyranosyl residues also existed as terminal sugars. The structural features reported here contradict earlier reports that Wolfberry AGPs carry a backbone of (1 → 4)-linked β-D-galactopyranosyl residues (Huang et al., 1999; Peng, Huang, et al., 2001) and confirm the assignment of Zhang and Zhang (2007) that the backbone is a (1 → 3)-β-D-galactan.

The total AGP fraction obtained by the use of the Yariv reagent possessed an arabinose to galactose ratio of ∼1:1. However, the fraction could be separated into several structural forms in which this ratio varied. This variation comes from the polydispersed structure of the AGP. The number of different structural forms identified will depend on the type of technique used and the degree to which it can distinguish subtle differences in the molecular forms of the AGP. It is believed that these structural forms represent the different glycoconjugates reported in the literature.

The reported LbGp3 (Huang et al., 1999) was composed of arabinose and galactose in a 1:1 ratio. Carbohydrate accounted for 93.6% of the fraction. This appears very close to the composition of the total AGP reported in this paper which contains 6.3% protein. However, LbGp4 and LbGp5 were reported to possess markedly different compositions which included rhamnose, glucose, mannose and xylose as significant components of the glycoconjugates (Huang et al., 1998; Peng, Qi, Tian, & Zhang, 2001). Although all these sugars were originally present in WSP of the present study, only rhamnose was consistently present in the AGP fraction through the whole purification procedure. It is therefore probable that the additional monosaccharides were derived from low amounts of contaminating polysaccharides of a different type which were not completely removed by the work up procedures used in these publications.

In the light of the results of the present paper and the growing publicity on the health benefits of Wolfberries, the role that the glycoconjugates or AGP play in these benefits needs to be reassessed. For one thing their structural features appear to be closely related to AGP found widely in the plant kingdom. Logically therefore it would be expected that similar effects would result from the consumption of AGP from other sources. To an extent claims to this effect have already occurred as the reporting of larch arabinogalactan as an immune enhancer testifies (Kelly, 1999). However, more than one type of polysaccharide has been demonstrated to possess immune enhancing properties (Schepetkin & Quinn, 2006). More rigorous clinical studies need to be done with purified AGP if specific health benefits attributable to them are to be validated.

If AGP in general prove to have specific health benefits then Wolfberry does not appear to be that rich a source of the compounds. Coffee beans, an everyday food item, are a much richer source of AGP (Redgwell, Curti, Fischer, Nicola, & Fay, 2002) and larch plants are already processed as a commercial source of purified arabinogalactan. This is not to say that the publicised benefits associated with the consumption of Wolfberry fruit are invalid. It may mean, however, that they are not specifically related to the AGP, but could result from the collective impact of a mixture of polysaccharides and phytochemicals unique to Wolfberry fruit.

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