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Cell wall carbohydrates from fruit pulp of *Argania spinosa*: structural analysis of pectin and xyloglucan polysaccharides

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Abstract—Isolated cell walls of *Argania spinosa* fruit pulp were fractionated into their polysaccharide constituents and the resulting fractions were analysed for monosaccharide composition and chemical structure. The data reveal the presence of homogalacturonan, rhamnogalacturonan-I (RG-I) and rhamnogalacturonan-II (RG-II) in the pectic fraction. RG-I is abundant and contains high amounts of Ara and Gal, indicative of an important branching in this polysaccharide. RG-II is less abundant than RG-I and exists as a dimer. Structural characterisation of xyloglucan using enzymatic hydrolysis, gas chromatography, MALDI-TOF-MS and methylation analysis shows that XXGG, XXXG, XXLG and XLLG are the major subunit oligosaccharides in the ratio of 0.6:1:1.2:1.6. This finding demonstrates that the major neutral hemicellulosic polysaccharide is a galacto-xyloglucan. In addition, *Argania* fruit xyloglucan has no XUFG, a novel xyloglucan motif recently discovered in *Argania* leaf cell walls. Finally, the isolation and analysis of arabinogalactan-proteins showed that *Argania* fruit pulp is rich in these proteoglycans.

Keywords: *Argania spinosa*; Cell wall; Fruit pulp; MALDI-TOF-MS; Pectin; Xyloglucan

1. Introduction

The argan tree (*Argania spinosa* (L.) Skeels) of the family Sapotaceae is endemic to south-western Morocco where it grows over an area of approximately 320,000 square miles.[1,2] This tree has important economic and ecological roles in this part of the world. It protects the soil against both water and wind-induced erosion, and is also used, because of its ability to survive under arid conditions, to arrest desert encroachment. The argan tree also supports indigenous populations economically.[3] It is used to shade crops and agricultural animals necessary for the native economy, but more importantly, different parts of the argan tree serve a number of important uses. The argan seeds are used to produce the oil that is mainly utilised for cooking and is believed to have various medicinal properties (e.g., reducing cholesterol level, improving blood circulation). The argan oil is also widely incorporated into many cosmetic products.[1,4]Much research has been conducted on the oil composition[1] whereas comparatively little information is currently available on the structure of cell wall polysaccharides of argan fruit. Plant cell walls consist of cellulose microfibrils embedded in a matrix of non-cellulosic components such as xyloglucan (XyG) and pectins, as well as arabinogalactan-proteins (AGPs).[5,6] Together, these cell wall polymers form a functional matrix, involved in the control of many aspects of plant growth and development as well as in the interactions of plants with the biotic and abiotic environments.[7,8] They are also widely used in the food industry and as cosmetics or neutraceuticals.[9–11]

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In the course of our studies on the structural and functional properties of cell wall polysaccharides in plants[12–16] particularly in relation to understanding their possible uses in industry, we have undertaken studies aimed at characterising the different classes of cell wall polysaccharides and proteoglycans present in *A. spinosa*. In our recent work on argan leaf cell wall polysaccharides, we have described a novel XyG motif.[16] More recently, Habibi and Vignon[2] reported on the characterisation of xylan polysaccharides of argan seed pericarp. In the present study, we have performed a thorough characterisation of the major cell wall polysaccharides, including XyG, RG-I, RG-II and AGPs, present in *A. spinosa* fruit pulp.

2. Results and discussion

2.1. Monosaccharide composition of *Argania* fruit pulp polysaccharides

In order to determine the nature of different polysaccharides present, the isolated cell wall residue (CWR) was sequentially extracted and the monosaccharide composition of the resulting fractions was determined by gas chromatography (GC) (Table 1). The CWR isolated from fruit pulp contains 58 ± 5% TFA-soluble sugars of which 9% are uronic acids. The main neutral sugars present in the fruit pulp CWR are Ara, Gal and Xyl. After performing Saeman's hydrolysis, Glc was the main sugar released from the CWR, with most of the Glc being of cellulosic origin, whereas only 4% of monosaccharide released after TFA degradation is Glc.

Table 1. Yield and monosaccharide composition of the cell wall fractions isolated from defatted fruit pulp of *Argania spinosa* by sequential extraction with inorganic solvents

Glycosyl residues	CWR	*A*FP	*A*F1OH	*A*F4OH
Yield[a]	100	50	8	9
NS[b]	49	45	28	53
UA[b]	9	15	3	3
Rha[c]	5(tr)	6	3	2
Fuc[c]	1(tr)	1	1	3
Ara[c]	33(tr)	40	22	14
Xyl[c]	14(3)	4	39	29
Man[c]	4(3)	2	2	6
Glc[c]	4(88)	2	10	24
Gal[c]	17(tr)	16	13	15
GalA[c]	1(5)	27	6	3
GlcA[c]	11(2)	3	3	1
Protein[b]	nd	tr	8	11

Values between parentheses are data obtained after Saeman's hydrolysis.
CWR, cell wall residue; *A*FP, *Argania* fruit pectin; (*A*F1OH, *A*F4OH) *Argania* fruit alkali-soluble fractions.
[a] Percentage weight of the DMM dry weight.
[b] Percentage weight of fraction dry weight.
[c] Mol percent, NS = neutral sugars, nd = not determined, tr = trace.

2.2. Analysis of pectic polysaccharides

Pectic polysaccharides were isolated from the crude cell wall material. The monosaccharide composition of the *Argania* fruit pectin (*A*FP) extract is presented in Table 1. Approximately 60% of this fraction is carbohydrate in nature (NS + UA). It is mainly composed of Ara, GalA and Gal, suggesting that homogalacturonan (HG) is not the major polysaccharide in this fraction. All other sugars are present in minor amounts. The high quantities of Ara residues and low quantity of Gal in this fraction suggest the occurrence of arabinans and/or arabinogalactans that may be either free or part of RG-I side-chains. However, small galactan chains might also be present. Rha represents 6% of the neutral sugars in the *A*FP fraction, which is very low in relation to the GalA content. According to the classification of pectins by Schols and Voragen[17] that is based in part on the ratio of Rha/GalA, RG-I can be distinguished from HG by its ratio Rha/GalA that ranges from 0.05 to 1. The ratio determined for the *A*FP fraction is 0.22, suggesting a predominance of RG-I polysaccharide. The analysis of purified RG-I and RG-II fractions shows that the RG-I was the main polysaccharide present, whereas RG-II was found to occur as a minor polysaccharide, mostly as a dimer (d-RG-II-B) (Fig. 1). The purified RG-I polysaccharide is rich in Ara and Gal (Table 2). This indicates that Ara and Gal residues detected in the *A*FP fraction are mainly linked to RG-I as side-chains, and that these are richer in arabinans than in galactans. In addition, this finding might reflect a high degree of branching of RG-I in the pulp fruit cell walls of *A. spinosa*. Similarly, Ara is the most abundant sugar

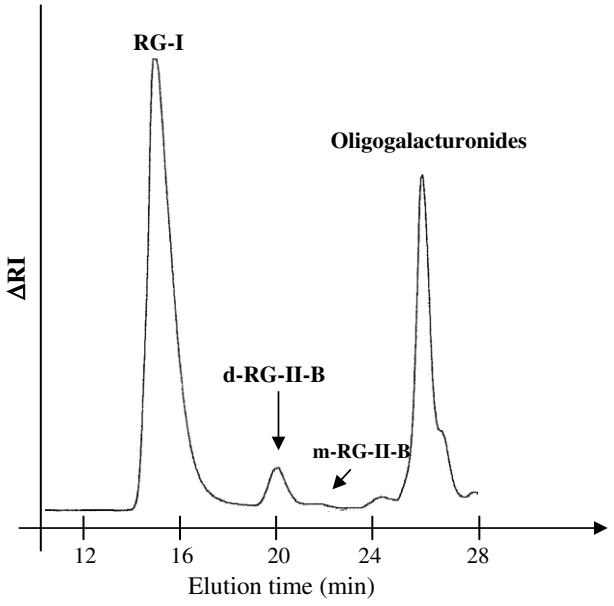

Figure 1. Profiles of RG-I and RG-II isolated from *Argania spinosa* fruit pulp cell walls.

Table 2. Monosaccharide composition (mol %) of RG-I and RG-II pectic polysaccharides isolated from the cell wall of *Argania spinosa* fruit pulp

Glycosyl residues	*A*FRG-I	*A*FRG-II
Rha	12	20
Fuc	0	5
Ara	60	34
Xyl	1	6
Man	0	1
Glc	1	7
Gal	21	17
GalA	3	11
GlcA	3	8
Api	nd	5
2-O-Me-Fuc	1	10
2-O-Me-Xyl	1	7
Kdo	nd	nd
Dha	nd	nd
Aceric A	nd	nd

nd = not detected; (RG-I = rhamnogalacturonan-I; RG-II = rhamnogalacturonan-II).

in RG-II representing more than one third (34%) of all sugars present in this polysaccharide. Interestingly, the Ara content of *Argania* RG-II is much higher than in most other species including *Arabidopsis thaliana* (14%),[18,19] sycamore (13%)[20] and pumpkin (9%).[21]

2.3. Analysis of xyloglucan in the alkali-soluble fraction

Monosaccharide compositions of both alkali-soluble fractions (*A*F1OH and *A*F4OH) is given in Table 1. The major sugars present in both extracts are Xyl, Glc, Ara and Gal. The abundance of Xyl and Glc is indicative of the presence of XyG. However, the ratio of Xyl/Glc is 3.9 and 1.2 for the *A*F1OH and *A*F4OH fractions, respectively. Thus, it is very likely that these extracts also contain other polysaccharides such as xylans and arabinoxylans. Gal is known to be part of XyG side-chains,[22,13] and Ara on arabinoxylans,[23] and sometimes on XyG. The content of Fuc is very low in both alkali-soluble fractions suggesting that XyG side-chains might be underfucosylated. In addition, residual pectic compounds may also be present in the alkali solubilised fractions as indicated by the presence of GalA and Ara residues. To further analyse XyG structure, *A*F1OH and *A*F4OH fractions were then submitted to hydrolysis with *endo*-(1→4)-β-D-glucanase, which cleaves β-(1→4)-glucosidic linkages of the XyG backbone that occur adjacent to unbranched glucose residues. Endoglucanase generated XyG oligosaccharide (XyGO) containing fractions, namely *A*F1XyGO and *A*F4XyGO. As shown in Table 3, sugar composition of both fractions *A*F1XyGO and *A*F4XyGO is similar. Xyl and Glc are the major neutral sugars and the ratio of Xyl/Glc is 1.6 and 1.3 for the *A*F1XyGO and *A*F4XyGO fractions, respectively. Generally, the ratio of Xyl/Glc in most XyG that occurs in higher plants is about 0.75.[22,13] The low quantity of Fuc in both XyG-containing fractions (1–2%) indicate that, like in *Argania* leaf XyG,[16] this sugar is almost absent from fruit pulp XyG.

Table 3. Monosaccharide composition (mol %) of the fractions, *A*F1XyGO and *A*F4XyGO, derived from the alkali-soluble fractions of *Argania spinosa* fruit pulp by hydrolysis with *endo*-glucanase

Glycosyl residues	*A*F1XyGO	*A*F4XyGO
Rha	nd	nd
Fuc	1	2
Ara	8	6
Xyl	47	41
Man	3	7
Gal	12	13
Glc	29	31

nd = not detected.

Methylation analysis performed on fraction *A*F4XyGO shows the presence of 1,6-Glc*p* and 1,4,6-Glc*p* residues, typical for the substituted cellulose-like backbone of XyG. T-Xyl*p*, T-Gal*p* (terminal galactopyranose) as well as 1,2-Xyl*p* and 1,2-Gal*p* are also present (Table 4). The proportion of T-Fuc*p* is very low confirming the underfucosylation of XyG of the *Argania* fruit pulp cell wall. Together, the methylation analysis data of the *A*F4XyGO fractions are consistent with the presence of XyG, most likely in the form of galactoxyloglucan. Similar data were found for the *A*F1XyGO fraction (not shown). To determine the structure of the XyG subunits, we performed MALDI-TOF-MS analysis on the *A*F4XyGO fraction. Taking into consideration the specificity and mode of action of the endoglucanase, sugar composition and molecular masses of the known XyGOs,[13,16,24] we were able to identify different XyGOs present (Table 5). The signal *m*/*z* 1555 that corresponds to fucosylated fragments XLFG and/or XFLG is very low, which is consistent with the low level of Fuc

Table 4. Methylation analysis of *A*F4XyGO fraction generated from *Argania spinosa* fruit pulp

Methylation product[a]	*A*F4XyGO[b]
T-Xyl*p*	11.4
T-Fuc*p*	0.5
1,4-Xyl*p*	13.2
1,2-Xyl*p*	5.6
T-Man*p*	1.2
1,4-Man*p*	10.6
T-Gal*p*	15.4
1,2-Gal*p*	tr
1,4-Glc*p*	8.3
1,6-Glc*p*	12.5
1,4,6-Glc*p*	21.4

[a] Linkage of monosaccharides. T-Gal denotes 1-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, etc.

[b] Percentage of total area of the identified peaks. tr: trace.

Table 5. Positive-ion mode MALDI-TOF-MS of the oligosaccharides generated from *Argania spinosa* fruit XyG

m/z	Relative intensities	Assignment	Compound
953	13	$[M+Na]^+$	XXGG
1085	21	$[M+Na]^+$	XXXG
1115	7	$[M+Na]^+$	XXGGG and/or GXXGG
1247	24	$[M+Na]^+$	XXLG and/or XLXG
1409	31	$[M+Na]^+$	XLLG
1555	4	$[M+Na]^+$	XLFG or XFLG
	Total = 100		

content previously observed (see Table 3). The mass spectrum for the *A*F1XyGO was similar to that obtained for the *A*F4XyGO fraction and was therefore not shown. Interestingly, the novel XyG motifs XUFG previously found in argan leaves,[16] as well as the octasaccharides XXFG and XLFG, which are present in *Argania* leaves, were not found in the cell walls of fruit pulp.

2.4. Analysis of arabinogalactan-proteins

Total AGPs were isolated from argan fruit pulp and quantified using rocket gel electrophoresis as shown in Figure 2. The data indicate that *Argania* fruits are highly rich in AGPs (0.325 mg/g of fresh weight). Monosaccharide compositions of the total population of AGPs isolated from the fruit pulp cell wall is shown in Table 6. Gal and Ara are the main monosaccharides present in the carbohydrate moiety accounting for about 70% of all sugars. Other sugars including Xyl, Fuc, Man, Rha and uronic acids are also present although in very low quantity.

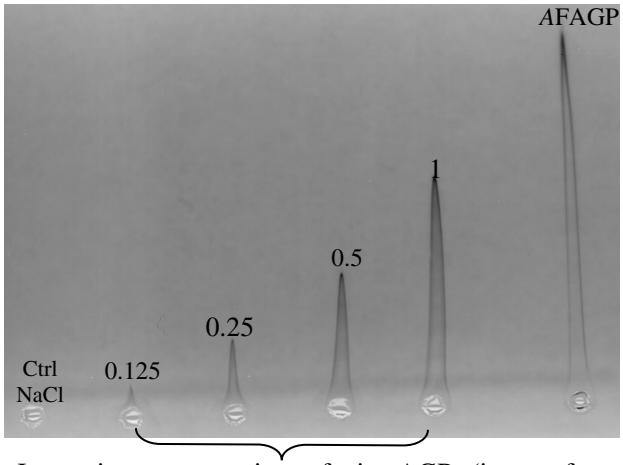

Figure 2. Quantification of total arabinogalactan-proteins (*A*FAGPs) from *Argania* fruit pulp by rocket electrophoresis. Wine AGPs[27] are used as standard.

Table 6. Monosaccharide composition (mol %) of purified fraction of AGPs extracted from *Argania spinosa* fruit pulp

Glycosyl residues	*A*FAGP
Rha	8.6
Fuc	4.5
Ara	28.5
Xyl	7.2
Man	1.6
Gal	47.0
UA	2.5

UA = uronic acid.

3. Materials and methods

3.1. Plant materials and preliminary treatment

A. spinosa fruits were collected from the southern Morocco. Fruit pulp (52 g) was frozen with liquid nitrogen and ground into a fine powder. The powder (6 g) was treated sequentially with hexane, chloroform, MeOH and 80% EtOH to remove hydrocarbons, lipids, flavonoids and oligomers before being treated with the α-amylase (Megazyme) to remove starch. The cell wall residue (CWR; yield 2 g) was then freeze-dried.

3.2. Chemical isolation of cell wall polysaccharides

CWR (1.6 g) was sequentially fractionated to give a pectic fraction and an alkali-soluble polysaccharide fraction as previously described by Ray et al.[16] Briefly, CWR was treated sequentially with 0.05 M CDTA (*trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid), 0.1 M sodium carbonate, 1 M and 4 M KOH containing 0.4% $NaBH_4$ to isolate non-cellulosic polysaccharides. All extracts were acidified to pH 6 with acetic acid, concentrated, dialysed and finally freeze-dried. CDTA and sodium carbonate extracts were mixed together in one fraction, identified as the argan fruit pectin fraction (*A*FP 770 mg). The polysaccharide fractions soluble in 1 M and 4 M KOH were termed *A*F1OH (yield 112 mg) and *A*F4OH (yield 132 mg), respectively. The cellulose rich residue was washed thoroughly with water containing acetic acid, then with deionised water, and finally dried by solvent exchange to yield α-cellulose (fraction named *A*FINS, 402 mg).

3.3. Enzymatic digestion of alkali-soluble fractions with β-1,4-endoglucanase

Fraction *A*F4OH (42 mg) was dissolved in 9 mL of 50 mM NaOAc (pH 5) and the mixture was incubated with an *endo*-β-(1→4)glucanase (Megazyme International, Ireland) for 24 h at 37 °C with constant shaking. The resulting digest was heated for 15 min at 100 °C to inactivate the enzyme, and the product precipitated in 80% EtOH (v/v). The precipitate was removed by

centrifugation and the 80% ethanol-soluble xyloglucan oligosaccharides (named *A*F4XyGO) were isolated by removing excess ethanol and then freeze-dried. In a similar manner, an *A*F1XyGO fraction was generated from the *A*F1OH fraction (46 mg).

3.4. Digestion and isolation of RG-I and RG-II

To investigate the structure of the pectic polysaccharides RG-I and RG-II, the CWR (200 mg) was treated with endopolygalacturonase exactly as described previously in Ishii et al.[25] The presence of m-RG-II and d-RG-II-B in the endopolygalacturonase digests was determined by comparing their retention times with those of the authentic mRG-II and d-RG-II-B from sugar beet and red wine.

3.5. Arabinogalactan-proteins—purification and quantification

AGPs were isolated from *A. spinosa* fruits according to the procedure of Shultz et al.[26] The AGPs were quantified by rocket electrophoresis as described by Ding and Zhu[27] and the monosaccharide composition was determined by GC. Red wine AGPs[28] were used as standard.

3.6. Monosaccharide analysis

Total sugars, total uronic acids and the monosaccharide composition of different cell wall fractions (CWR, *A*FP, *A*F1OH, *A*F4OH, *A*FAGP, *A*F1XyGO, *A*F4XyGO, *A*FRG-I and *A*FRG-II) were determined by GC.[16,17] Alternatively, neutral sugar composition was determined after hydrolysis with 1 M H_2SO_4 for 1 h at 100 °C. Reduction and acetylation were carried out by the method of Blakeney and Stone[29] and the alditol acetates were analysed by GC and GC–MS.

3.7. MALDI-TOF mass spectrometry

MALDI-TOF mass spectrometry was performed on a micromass (Manchester, UK) TOF spec E mass spectrometer equipped with a nitrogen laser operating at 337 nm. Mass spectra were recorded in the reflection mode with positive ion detection using 2,5-dihydroxybenzoic acid (10 mg/mL) as the matrix.[16]

3.8. Methylation analysis

Polysaccharides were methylated using sodium hydroxide and methyl iodide in dry DMSO according to the method of Blakeney and Stone[29] The permethylated polysaccharides were hydrolysed with 2.5 M TFA at 100 °C for 1 h, reduced with 1 M $NaBD_4$ in 2 M NH_4OH for 1 h at 60 °C and acetylated using acetic anhydride and pyridine as a catalyst. The partially methylated alditol acetates (PMAA) were analysed by GC–MS using DB-225 JW columns. The PMAA was identified as previously described by Ray and Lahaye.[30]

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