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Carbohydrate Research 337 (2002) 255–263

CARBOHYDRATE RESEARCH

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NMR spectroscopy and chemical studies of an arabinan-rich system from the endosperm of the seed of *Gleditsia triacanthos*

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Received 28 September 2001; accepted 20 November 2001

Abstract

Exhaustive extraction of the endosperm from the seed of *Gleditsia triacanthos* using water at room temperature and 50 °C left a residue, which was further extracted at 95 °C. Precipitation of this extract with 2-propanol yielded major amounts of galactomannan components, while the supernatant was mainly composed of arabinose-rich constituents. Two fractions were obtained by anion-exchange chromatography. The fraction that eluted with water is an arabinan with (1→5) α-L linkages and branching mainly on C-2, accompanied with equal amounts of a low-galactose galactomannan oligosaccharide, and a small proportion of a β-(1→4)-galactan. The fraction eluted with an increased ionic strength consists mainly of a similar arabinan, and lower proportions of a high-galactose galactomannan, galactan, and protein. The arabinan moiety in both fractions was characterized by chemical analysis and 1D and 2D NMR spectroscopic techniques.

Keywords: Arabinan; NMR; legume seeds; β-(1→4)-Galactan; Galactomannan; *Gleditsia triacanthos*

1. Introduction

Arabinans appear in different families of plants, occurring in seeds,[1–6] fruits,[7] roots,[8–10] bark of stems,[11] and trunks.[12–14] They are usually constituted by (1→5)-linked α-L-arabinofuranosyl moieties, variably branched on C-2 and/or C-3 with single or multiple stubs of the same unit.

Another related group of polysaccharides, also distributed widely in the plant kingdom, is constituted by arabinogalactans and arabinogalactan–proteins.[15–18] These polysaccharides can be subdivided in two main types: (a) type I-arabinogalactans, showing a core of 4-linked β-D-galactopyranosyl units, substituted with L-arabinofuranosyl and D-galactopyranosyl stubs on C-3, usually not associated with protein; and (b) type II-arabinogalactans, highly branched polysaccharides consisting of β-D-galactopyranose units joined through C-3 and C-6, the former predominant in the inner part, and the latter on the outer chains, branched with L-arabinofuranosyl, L-arabinopyranosyl and D-galactopyranosyl residues, among other sugars.[18]

The galactomannans of the seed of Leguminosae, constituted by a β-(1→4)-mannan core with variable degrees of substitution in C-6 by α-D-galactopyranosyl single side stubs, have been extensively studied.[19] Particularly, the water-extracted, 85% ethanol-insoluble products from the endosperm of *Gleditsia triacanthos* have been fully characterized,[20] in their three extraction stages, at room temperature, 50 °C and 95 °C. A galactomannan oligosaccharide was also characterized.[21] Following a preliminary account,[22] the study of a system of low-molecular weight, arabinose-rich polysaccharides from the 95 °C water-extracted, 75% 2-propanol-soluble extract from the endosperm of the seed of *G. triacanthos* is reported herein, together with the characterization of its components.

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0008-6215/02/$ - see front matter
PII: S0008-6215(01)00310-X

2. Results

Fraction S_{95} was isolated from the 75% 2-propanol supernatant of the endosperm of *G. triacanthos* extracted exhaustively with water at room temperature and further at 50 and 95 °C. Its weight represented 2.3% of the original endosperm weight. Analytical features of S_{95} are shown on Table 1.

S_{95} was fractionated by anion-exchange chromatography,[21] giving rise to a 'neutral' fraction (N), with 23% yield, and a 'charged' (C) fraction, with 50% yield. The analytical features of both fractions are also shown in Table 1. Arabinose is the main sugar in both fractions, while galactose and mannose show also significant amounts, especially in fraction N. The low-magnitude negative optical rotations are consistent with the presence of major amounts of α-L-arabinofuranans[2,12] and galactomannan oligosaccharides.[21] Neither fraction C nor N react with the Yariv reagent,[23] thus indicating the absence of arabinogalactan–proteins.[17]

Methylation of fractions C and N was carried out first with sodium hydroxide–iodomethane in methyl sulfoxide, and then submitted to two further methylation steps with barium oxide and iodomethane.[14] The molar proportions of arabinose and its methylated derivatives (Table 2) were similar in both fractions and consistent with a (1→5)-linked arabinan, branched mainly on C-2 and less on C-3. Galactose and mannose appear non-methylated in high proportions (especially mannose) in spite of the harsh methylation conditions, a fact that remains only slightly changed throughout the sequence of methylations. Attempts to methylate in chaotropic systems (lithium chloride in methyl sulfoxide) did not improve these results. The presence of 2,3-di-*O*-methylmannose, 2,3,4,6-tetra-*O*-methylgalactose (in similar amounts), and 2,3,6-tri-*O*-methylmannose is in agreement with the known structure of galactomannans. The presence of 2,3,6-tri-*O*-methylgalactose was previously observed in small amounts in the methylation analyses of galactomannans and galactomannan oligosaccharides of the endosperm of legume seeds.[20,21] Small amounts of terminal xylose residues were also detected.[20]

The ^{13}C NMR spectra of fractions C and N are shown on Fig. 1. The assignments of the signals belonging to the arabinan components of C and N (Table 3) were made with the aid of previous work. The backbone arabinose units (B units, Fig. 2) were assigned according to previous work on linear 1→5 arabinans,[24,25] while the assignments of A and C units (Fig. 2) was also aided by previous work.[4,8] The smaller linewidth of the A unit signals facilitated their recognition. It is worth noting that the signal ascribed to the anomeric carbon of A-type arabinose units is shifted upfield with respect to that reported by Capek et al.[8] It is possible that in that work the assignments of C-1 of terminal and 2-substituted units should be exchanged. A recent work on an arabinogalactan, in which the arabinan moiety has also a 5-linked backbone, but with branching on C-3, may have also reversed the assignments of the anomeric signals,[26] as well as those belonging to C-2 and C-4 of B units. Splitting of signals belonging to most of the carbons (but not the anomeric) of B and C units in the 125 MHz spectra may be an indication of the effect from a different environment, mainly originated in vicinal units (diads)[27] or a complexation effect. It was previously shown[2] that branching points tend not to appear in blocks or regularly spaced, but more or less randomly distributed. Methylation analysis indicated the presence of 6–8% of the arabinose units as trisubstituted, and 3% as 3,5-di-

Table 1
Analysis of the 75% 2-propanol-soluble fraction S_{95}, and their subfractions N and C

Fraction	S_{95}	N	C
Carbohydrate [a] (%)	62	79	75
Protein (%)	26	11	20
$[\alpha]_D$ (°)	−49	−28	−40
Average-molecular weight	n.d.	2100	4000
Monosaccharide (mol/100 mol)			
Arabinose	60	48	67
Xylose	3	1	5
Mannose	22	37	14
Galactose	15	14	14

[a] Uronic acids were not detected in any fraction.

Table 2
Molar ratios of methylated sugars after permethylation and hydrolysis of fractions N and C

Methylated sugar [a,b]	N	C
2,3,5-Ara	1.00	1.00
2,3-Ara	1.13	1.09
2-Ara	0.10	0.08
3-Ara	0.69	0.66
Ara	0.18	0.25
2,3,4,6-Gal	0.61	0.18
2,3,6-Gal	0.30	0.16
Mono-Gal	0.17	tr
Gal	0.26	0.31
2,3,6-Man	1.38	0.24
2,3-Man	0.60	0.14
Mono-Man	0.21	tr
Man	0.92	0.61
2,3,4-Xyl		0.19

[a] 2,3,5-Ara = 2,3,5-tri-*O*-methylarabinose, etc.
[b] Trace amounts of 2,3,4,6-Man, 2,6-Man, 2,5-Ara and 3,5-Ara were also detected.

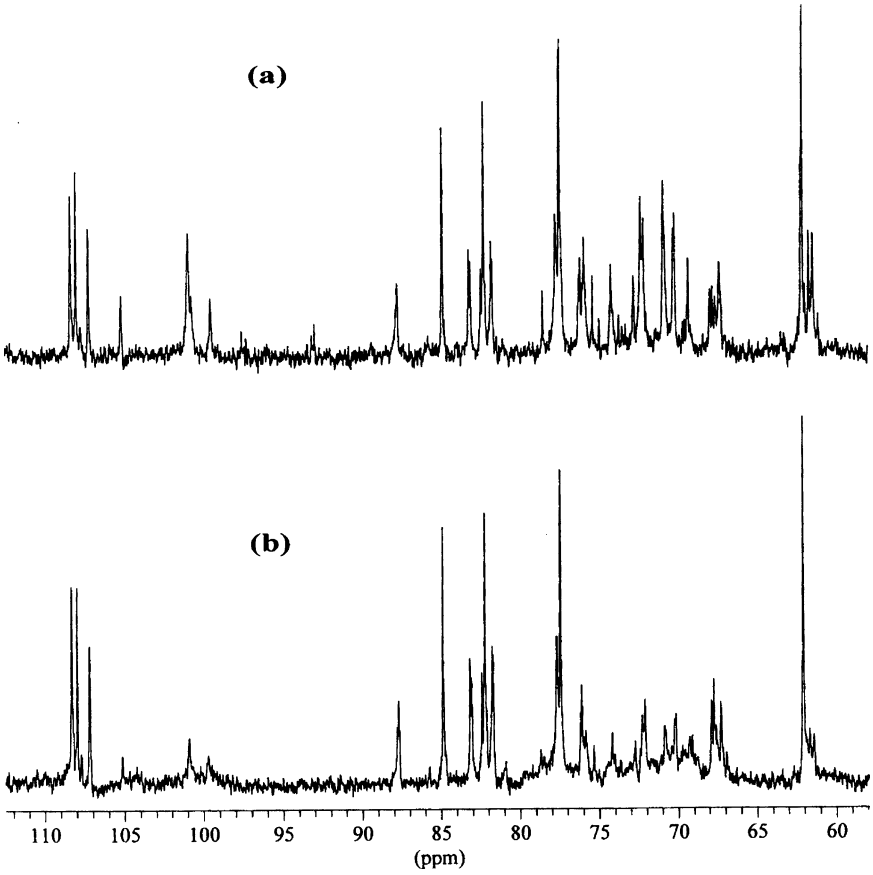

Fig. 1. 125 MHz proton-decoupled ^{13}C NMR spectra of fractions N (a) and C (b).

Table 3
Assignments of the ^{13}C and ^{1}H NMR spectra of fractions N and C [a]

	C-1	C-2	C-3	C-4	C-5	C-6
Arabinan						
Ara-A [b]	108.0	82.2	77.4	84.9	62.0	
	(5.16) [c]	(4.10)	(3.93)	(4.04)	(3.79, 3.70)	
Ara-B [b]	108.3	81.6–81.7	77.6–77.7	83.1–83.2	67.7	
	(5.06)	(4.10)	(3.97)	(4.17)	(3.85, 3.78)	
Ara-C [b]	107.2	87.7–87.8	76.1–76.2	82.4	67.3	
	(5.17)	(4.13)	(4.13)	(4.18)	(n.d.)	
Galactomannan						
Gal	99.6	69.2	70.1	70.1	72.1	62.1
Man-NB [d]	100.9	70.8	72.2	77.4	75.8	61.4
Man-B [d]	100.7	70.8	72.2	77.4	74.1	67.9
Galactan						
Gal	105.1	72.7	74.2	78.5	75.3	61.6

[a] Besides, the following peaks were observed in the ^{13}C NMR spectra: 107.7 and 69.1 ppm (C); 107.7, 97.7, 92.9, 74.9 and 61.8 ppm (N). For the ^{1}H NMR spectra, the assignments (only for the arabinan moiety) for the protons linked to the indicated carbons appear in parentheses.

[b] A, B and C units are represented in Fig. 2.

[c] This signal also correlates with the fourth arabinose unit with ^{13}C NMR absorption at 107.7 ppm (see Fig. 3).

[d] NB, non-branched; B, branched.

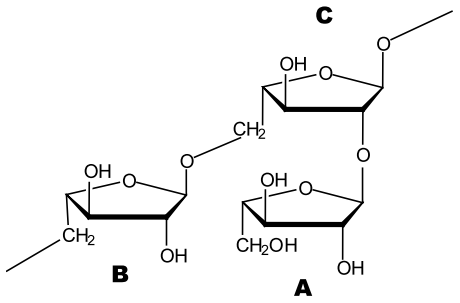

Fig. 2. Schematic structure of the arabinan moiety of fractions N and C.

substituted. The presence of a minor anomeric peak (107.7 ppm) can correspond to such C-3-branched units. Literature has shown diverging values for the anomeric carbon signal of 3,5-disubstituted units: Capek et al.[8] indicated that it should appear at 107.9 ppm, while Dong and Fang[26] located it at 110.2 ppm, in both cases according to our referencing system. On the other hand, for trisubstituted arabinoses, a chemical shift of 107.2 ppm, similar to that of C units (Table 3) is expected.[8]

The presence of galactomannan oligosaccharides with a high Man/Gal ratio (ca. 3.5) in N and with a lower ratio (ca. 1.8) in C was evidenced in the spectra by their characteristic signals (Fig. 1, Tables 3 and 4), coincident to those reported earlier,[21,27] and by the methylation analysis (Table 2). It is noteworthy that the signals of both units are broader than those coming from the arabinan moiety. This is consistent with a lower degree of freedom, even for the relatively mobile, (1→6)-linked galactopyranosyl units. Other minor signals were observed. As explained earlier, the resonance at 107.7 ppm indicates a variant of arabinose, possibly branched on C-3. A peak at 105.1 ppm, which represents around 7% (Table 4) of the total area of the anomeric peaks for fraction N, may be ascribed to a β-D-galactopyranosyl unit. The appearance of the other five (C-2–C-6) peaks agrees with the presence of a 4-linked β-homogalactan[24] (Table 3), in concordance with the appearance of 2,3,6-tri-*O*-methylgalactose in its methylation analysis (Table 2). Similar signals appear in the spectrum of C, but with much lower intensity, together with others that indicate the anomeric carbons of β-galactose or β-xylose units. The linewidth of the signals of the galactan moiety are comparable to those of the arabinan backbone. The appearance of signals at 92.9 and 97.1 ppm in the spectrum of N suggests the presence of reducing galactoses, indicating the low degree of polymerization of the homogalactan. An attempt to integrate the anomeric signals of the ^{13}C NMR spectra gives the values shown in Table 4. Although the integrals of these spectra are not proportional to the relative proportions of the carbons that gave rise to them due to their different T_1's and NOEs, a semiquantitative calculation, at least for the arabinan moiety, can be considered possible[8] in the present recording conditions. However, the galactomannan moiety is considerably less mobile according to the broader signals observed, and thus may be partly underestimated in this calculation. When observing the ^1H NMR integrated spectra, the actual proportions of mannose are very close to those encountered by chemical analysis of C and N (Table 1). The proportion of terminal arabinose and branching points (A and C units, Fig. 2) are about the same in both cases, as expected, while the amount of unbranched arabinoses is higher in both N and C. The counterbalance of galactoses appearing in the compositional analysis of Table 1 is explained by the presence of a partially branched β-(1→4)-galactan (see above).

Table 4
Integrated areas for the anomeric regions of the ^{13}C NMR spectra of fractions N and C

	N	C
Ara*f* A (108.0)	15	23
Ara*f* B (108.3)	21	35
Ara*f* C (107.2)	16	23
Other Ara*f* (107.7)	4	5
β-Gal*p* (105.1)	7	4
Non-branched Man in galactomannan (100.9)	20	3
Branched Man in galactomannan (100.7)	9	3
Gal*p* stubs in galactomannan (99.6)	8	3

The HETCOR spectrum of C is shown in Fig. 3, while the COSY experiment is depicted in Fig. 4. N gave similar 2D experiments. The assignments of the peaks in the ^1H NMR spectra are shown in Table 3. The signals were assigned with the aid of previously determined spectra of similar products,[4,17,24,26,28] as well as the help of 2D NMR techniques. In a recent paper,[26] the signals corresponding to the anomeric protons of A-like and B units appear reversed, as happens with the ^{13}C NMR spectrum. This fact is predictable, as proton assignments were originated in C–H correlations. The agreement of our data with previous reports[4,24] may be an indication that the assignments of the signals at 5.11 and 5.18 ppm in the paper of Dong and Fang[26] should be exchanged. In the ^1H NMR spectra minor signals in the anomeric zone are observed indicating various arabinose variants, while the signals for the galactose in the galactomannan are shown more evidently in N than in C. Signals belonging to the anomeric protons of β-linked sugars are superseded by the HOD signal.

3. Discussion

Galactomannans are the main polysaccharide constituents from the endosperm of the seed of *G. triacanthos*.[20] Within the alcohol-soluble products, extracted with water at room temperature from the

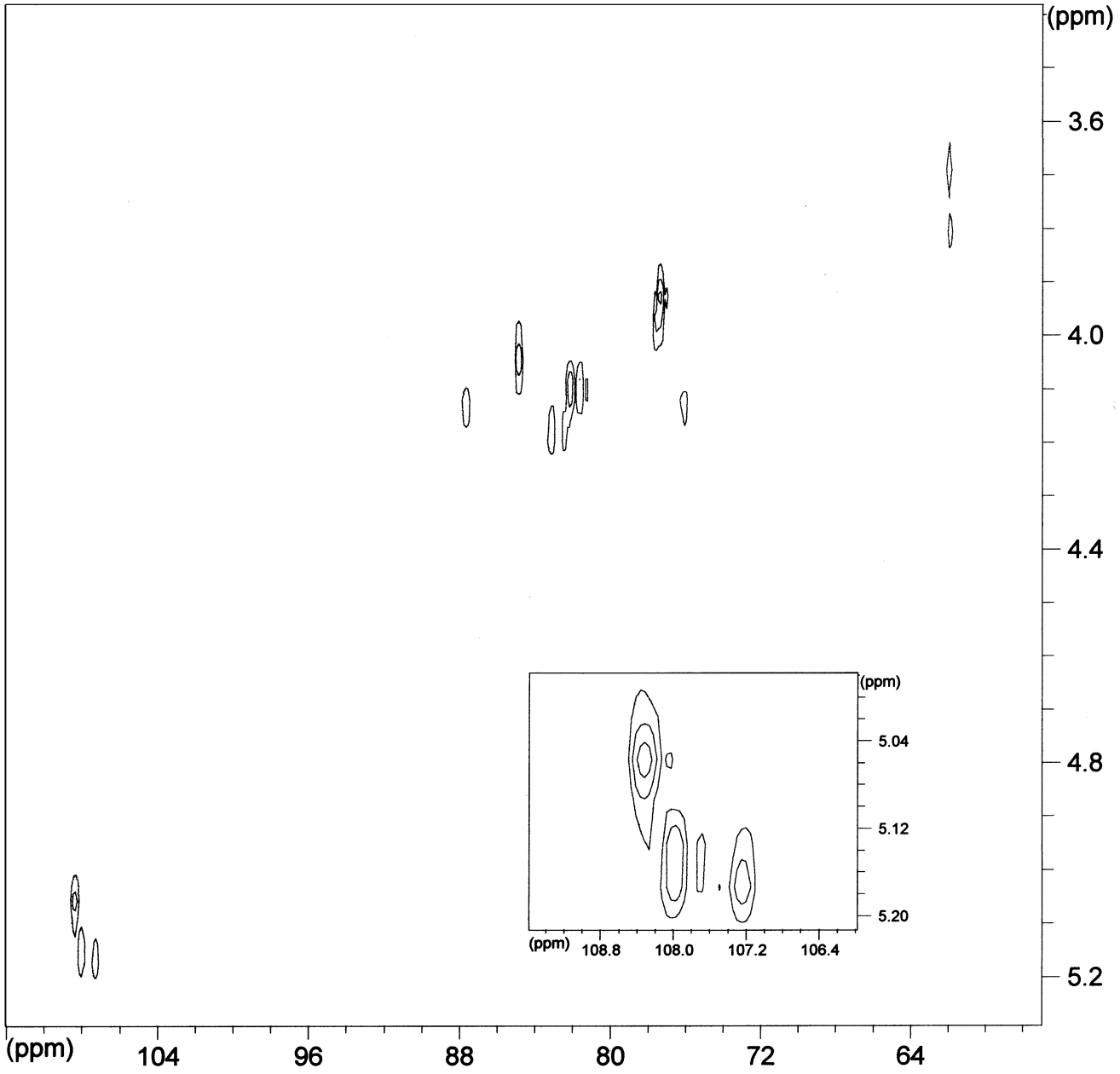

Fig. 3. HETCOR spectrum of fraction C. The inset represents an enlargement of the anomeric region.

endosperm, galactomannan oligosaccharides scarcely substituted with terminal arabinofuranose or fucopyranose stubs and, possibly, with lateral chains having more than one galactose unit (the additional one carrying a $1\rightarrow4$ linkage) have been encountered.[21] The galactomannan oligosaccharides, as well as their high-molecular weight counterparts, exist in aqueous solution in ordered forms producing lower free-energy aggregates, an interaction which also exists with the non-galactomannan components of these fractions.[21]

The hydrolysis of the aqueous-extracted alcohol-soluble S_{95} yielded mannose and galactose, as could be expected from galactomannan oligosaccharides, but also major amounts of arabinose. The extract could not be fractionated by gel-permeation chromatography in a chaotropic solvent like 7 M urea.[29] 'Fractionation' of S_{95} was achieved by means of anion-exchange chromatography and gave rise to a 'neutral' product, eluted with water (N), and a 'charged' product that was eluted when the ionic strength was raised (C). Both products have the same qualitative composition, with arabinose as the main sugar, carrying similar amounts of galactomannans in the first case, and lesser amounts in the second. High amounts of protein remain soluble in the hydroalcoholic supernatants and accompany fractions C and N through the fractionation process (Table 1),

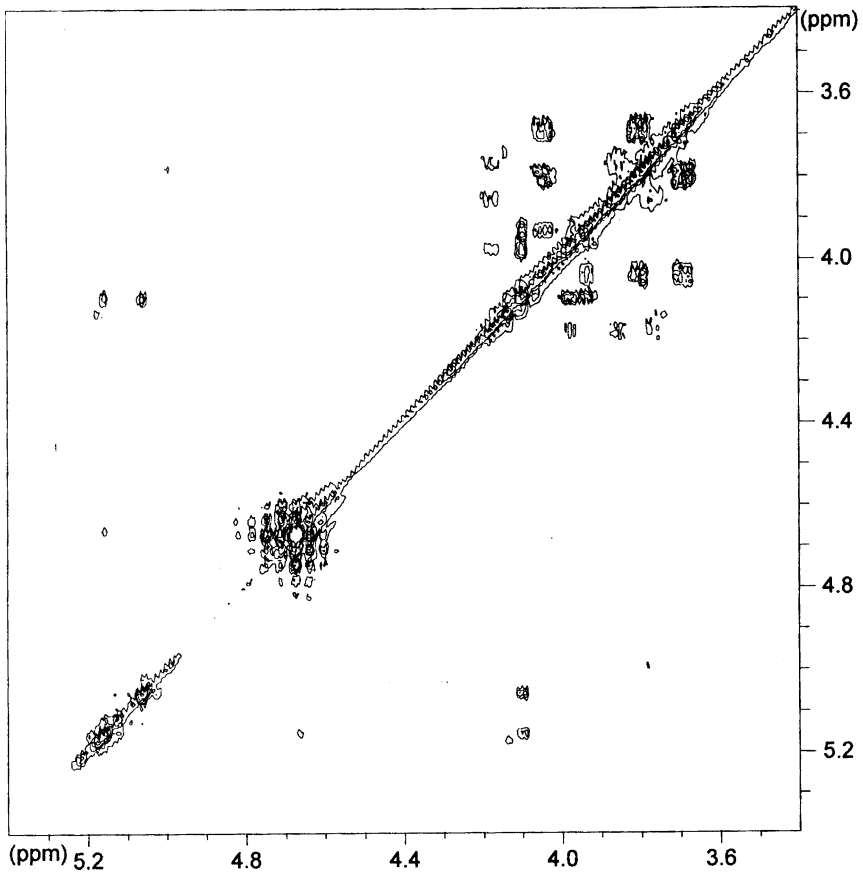

Fig. 4. 500 MHz COSY spectrum of fraction C. The large signal around 4.6 ppm corresponds to the HOD peak.

although the protein is probably only associated to the carbohydrates.[21,29] The separation of S_{95} into a 'neutral' and a 'charged' fraction is hard to explain in terms of a true ion-exchange reaction, as no uronic acid or any other acid group were detected in the original or in the final products. Considering that no clear-cut separations were obtained, the fractionation can be originated in the protein accompanying the carbohydrates or in the interaction of the dextran gel with some components of the mixture.[9] In either case, the interactions of the polysaccharide components within themselves might be responsible for the presence of the mixture in both fractions.

Arabinans have been found in primary cell walls of different parts of plants of many families.[1–14] They usually carry a backbone of α-(1→5)-linked L-arabinofuranosyl units, more or less branched by single or multiple stubs of the same kind on positions 2 and/or 3. Various degrees of branching have been found: a linear (1→5)-arabinan has been found in apple juice[7] and in the seed coat of several legume seeds,[6,30] while an almost linear one was found in the hypocotyl cells of *Vigna radiata*.[24] In the early papers, most of the branching was encountered on C-3,[1,5,12] while in the seeds of the horsebean, an arabinan with a low degree of branching mostly on C-2, has been found.[9] It is worth noting that for rapeseed, early papers suggested structures with branching on C-3,[1,2] while a more recent one showed most of the substitution on C-2.[4] In this case, a highly branched structure was proposed, in which the side stubs are also branched. For *Althaea officinalis*, large amounts of branches on both C-2 and C-3 have been found,[8] while in the white willow, some non-branched 3-linked units were encountered.[14] Methylation and NMR analyses of C and N show that the arabinose derivatives follow the usual (1→5) backbone of α-L-arabinofuranoses partially branched on C-2 (Fig. 2), and in much lower amounts on C-3. About 55% of the arabinoses in the main chain are not branched.

Methylation, compositional, and NMR spectra analyses show a considerable degree of galactomannan presence in N (ca. 45%) and a minor presence in C (ca. 22%). Their Gal/Man ratio is sharply different, a fact which was considered of taxonomical importance.[30] Heavy undermethylation is observed for the galactomannan components in spite of the harsh methylation conditions. Non-methylated arabinose is usually found in the methylation analysis of arabinans, indicating the existence of double substituted branching

points, but the presence of non-methylated galactose and mannose is in disagreement with the expected structure of galactomannans, homogalactans, or even the galactan core of a type I arabinogalactan.[15] However, the methylation pattern of the galactomannan component is not the usual for undermethylated products, as only few monomethylated derivatives were found (Table 2). Thus, the presence of permethylated and non-methylated polysaccharides (but not undermethylated ones) is suggested, due to the blocking of some hydroxyl groups because of the association of the galactomannan oligosaccharides previously observed in similar products.[21]

The presence of 4-linked galactopyranose units in small amounts was previously assigned to double stubs in the galactomannan molecules, being the one attached to the mannose with the α configuration and carrying on C-4 a second D-galactose unit.[20,21] However, NMR data show that, at least in this case, this methylated unit is more compatible with a (1→4)-β-D-galactan core of an arabinogalactan of type I[15] with a few, undetectable arabinan substitutions, similar to the homogalactans encountered in the mature hypocotyl cells of *V. radiata*,[24] *Lupinus albus*,[31] *Solanum tuberosum*,[32] and other sources.[15] In those examples, the galactans also appeared associated with arabinans or other pectic substances.[15] The appearance of reducing-end galactose units in the ^{13}C NMR spectrum of N is consistent with the separate existence of those galactan oligosaccharides. In C, the presence of small signals at 104.7 and 104.2 ppm might be also indicative of a similar structure, but branched on C-3, as other type I arabinogalactans.[15] Although 2,6-di-*O*-methylgalactose (indicative of a 3,4-disubstituted unit) was not detected in the methylation analysis of C, a low degree of branching is possible, given the known difficulty for methylating such polysaccharides.[32] On the other hand, these signals can also correspond to β-D-xylopyranosyl terminal units (Tables 1 and 2).

Arabinans usually appear associated with pectic materials, i.e., galactans, arabinogalactans, and galacturonate-containing pectins, in various parts of primary cells.[4,15,18] Linear arabinans were found together with other polysaccharides in the seed coats of *Schizolobium parahybum*,[6] *S. amazonicum*[30] and *Cassia fastuosa*.[30] In *S. parahybum* the accompanying polysaccharides are possibly galactomannans, while the galactomannan-rich endosperm also carried arabinose-containing polysaccharides.[6] The presence of galactomannan polysaccharides, together with small amounts of oligosaccharides of the same type in the endosperm of albuminated seeds, is well known.[19–21] Arabinose-rich carbohydrates were also found in some cases.[6,29,33] However, to the best of our knowledge, this work reports for the first time a system of associated low-molecular-weight galactomannans, galactans/arabinogalactans and arabinans in the endosperm of this kind of seeds. Their biological role is not completely understood, but it was suggested that low-molecular-weight carbohydrates might contribute to stabilize enzymes (e.g., α-galactosidases) in the endosperm by preventing dehydration.[34–36] These carbohydrates might also contribute to the formation of weak bonds in the cell wall, which is needed for the swelling properties of the galactomannan-containing endosperm.[37]

4. Experimental

Plant material.—The seeds of *G. triacanthos* were collected from ripe pods at the Ciudad Universitaria (Buenos Aires, Argentina). The separation of the endosperm from the embryo and testa was carried out by hand after swelling the seeds by soaking them in boiling water for 10 min, as reported earlier.[33] Negligible solubilization of material occurred.[20]

Extraction and fractionation.—The endosperm (77.5 g) was exhaustively extracted with water as already described.[20,21] Product S_{95} was obtained from the extraction at 95 °C, after precipitation of the galactomannans with 2-propanol up to a concentration of 75%, removal of the 2-propanol from the supernatant in a rotatory evaporator, and final freeze-drying. S_{95} was dissolved in water, cleared by centrifugation (the precipitate was mostly composed by proteins) and fractionated with an anion-exchange column (2.5 × 30 cm) of DEAE-Sephadex A-50 (CO_3^{2-}) equilibrated with water. Elution with water yielded fraction N; elution of the bound products with a solution of 0.2 M ammonium carbonate gave fraction C, after evaporation and freeze-drying. No product eluted at higher ionic strengths.

Analytical methods.—Total carbohydrates were assayed by the phenol–H_2SO_4 method,[38] using arabinose as standard. Protein was quantitated by the method of Lowry et al.,[39] using bovine serum albumin as standard. Molecular weights were calculated from the reducing power, which was determined by the method of Park and Johnson.[40] Uronic acids were determined by the method of Filisetti-Cozzi and Carpita.[41] Optical rotations were measured using 0.4–0.6% (w/v) solutions in water. The proportions of monosaccharides constituting the polysaccharides were determined by gas chromatography (GLC) of the hydrolyzates (2 M CF_3COOH, 90 min, 120 °C), using aldononitrile acetates. These derivatives were analyzed by GLC using a capillary column (30 m × 0.25 mm) coated with SP-2330 (0.20 μm), using nitrogen as the carrier with a flow rate of 1 mL/min, in the split mode (split ratio 1:100). Runs were carried out isothermally at 220 °C. Injector and detector (FID) were kept at 230 °C.

Methylation analyses.—Methylation of C and N was carried out by the method of Ciucanu and Kerek.[42] The methylated products were isolated after dialysis and lyophilization, and then re-methylated with barium oxide and methyl iodide (2×16 h, with intermediate dialysis), as reported by Karácsonyi et al.[14] The permethylated sugars were hydrolyzed (2 M CF_3COOH, 90 min, 120 °C) and derivatized to the alditol acetates. They were identified and quantitated by GLC and GLC–MS, using the same GLC column indicated above, and the oven temperature programmed as reported elsewhere.[43]

NMR spectroscopy.—The spectra were obtained on a Bruker AM 500 spectrometer provided with a 5-mm probe, at rt. The samples were dissolved in 1:1 D_2O–water (for ^{13}C NMR spectra) or D_2O (for HETCOR and COSY experiments). Acetone was used as an internal standard, and chemical shifts were referred to Me_4Si by calibrating the acetone Me peak as 31.1 ppm (^{13}C) and its proton resonance at 2.20 ppm. Pulse sequences for 2D techniques were supplied by the spectrometer manufacturers.

Synthesis of Yariv reagent.—The synthesis was carried out by the method of Yariv et al.[44] as modified by Nothnagel (personal communication). In an ice bath, 27.1 mg of *p*-aminophenyl-β-D-glucopyranoside (0.10 mmol) were dissolved in 0.5 mL of 0.6 M HCl solution. Then 0.1 mL of 1.5 M $NaNO_2$ (0.15 mmol) were added slowly with continuous stirring. After 1 h, 1.25 mL of a phloroglucinol solution (0.02 M, titrated to pH 9 by adding 0.5 M NaOH solution) were added on an ice bath. The reaction was kept at pH 9 using 0.5 M NaOH solution. After 2 h, the reaction mixture was dialyzed and lyophilized. The fractions were treated with the Yariv reagent as described by Gane et al.[17] in their modification of the procedure of Jermyn and Yeow.[23]

Acknowledgements

The authors are indebted to Drs M.N. Mazzini and A.E. Manzi, for their fruitful work on this seed, opening the field for further work like the present one. Dr E. Nothnagel is also acknowledged for kindly supplying a modified procedure for the synthesis of the Yariv reagent. This work was supported by grants from CONICET and UBA.

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