Carbohydrate Research, 165 (1987) 31–42
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

DEVELOPMENT OF ARABINANS AND GALACTANS DURING THE MATURATION OF HYPOCOTYL CELLS OF MUNG BEAN (*Vigna radiata* Wilczek)

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(Received October 20th, 1986; accepted for publication, February 21st, 1987)

ABSTRACT

Hypocotyl cell walls contain galactans and arabinans that are soluble in boiling water. During maturation, the Ara/Gal ratio remains unchanged but high-molecular-weight galactans are replaced by smaller polymers. On the basis of the ^{1}H-n.m.r. 2D-COSY(δ–δ, ^{1}H–^{1}H) n.m.r., and ^{13}C-n.m.r. spectra, a (1→5)-α-Ara*f* structure can be proposed for the arabinans in both young and mature cell walls. However, the galactan(s) changed from a probably highly branched to an unbranched (1→4)-β-Gal*p* structure during maturation.

INTRODUCTION

Arabinogalactans closely associated with pectic polymers have been observed in primary walls of suspension-cultured cells[1,2], and the ratios of arabinose to galactose are usually 1.5–2.1. The primary cell walls of Mung-bean hypocotyl contain[3] important amounts of neutral polymers composed mainly of arabinose and galatose (ratio 1.5:1) which can be solubilised together with highly methylated polygalacturonans. However, these preliminary investigations did not determine whether the neutral polymers were arabinogalactans or mixtures of galactans and arabinans, which are known to be present in some primary cell walls[4–9]. Since pectic polysaccharides might play an important role in the development of cell walls, we have fractionated further the pectic material isolated from young and mature hypocotyl cells and now report ^{1}H- and ^{13}C-n.m.r. data on the fractions.

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0008-6215/87/$ 03.50

RESULTS AND DISCUSSION

Chemical studies. — The "water-soluble pectins" (PF_1) were fractionated by anion-exchange chromatography into neutral (NF) and acidic (AF) fractions. The data reported in Table I show that each fraction contained protein, the amount of which decreased notably during cell-wall maturation. In contrast, the ratio of arabinose to galactose, the main constituents of the polymers, remained unchanged and was higher in NF. Xylose, mannose, and glucose were also present. The intrinsic viscosity of NF was particularly high in young cell walls, which is indicative of large macromolecules.

Gel-permeation chromatography of NF on Sepharose CL6B gave three fractions (α, β, and δ) which represented, respectively, 44, 19, and 37% of NF in young cell walls, and 30, 23, and 47% in mature cells. For young walls, the peak α was eluted just after the void volume of the column, indicating a high molecular weight. In contrast, for mature cell walls, the peak α was retained. Therefore, cell-wall maturation is characterised by a decrease in the size of the polysaccharides present in NF. The intrinsic viscosities reported in Table I support this conclusion.

N.m.r. studies. — Both ^1H- and ^{13}C-n.m.r. spectra were recorded for all of the water-soluble pectic polymers. Whilst recommending quantitative ^{13}C-n.m.r. spectra, Gorin[10] pointed out that integrated intensities of the signals of polysaccharides, obtained under the usual operating conditions, are often proportional or quasi-proportional to the number of ^{13}C nuclei present (T_1 values of 0.2 s or less, approximately equal n.O.e. values, at least for methine carbons). The long acquisition times (18–30 h) required for our ^{13}C-n.m.r. spectra, which were obtained with broad-band proton decoupling (full n.O.e.) and with a recycle time of

TABLE I

COMPOSITION[a] OF NEUTRAL (NF) AND ACIDIC (AF) POLYSACCHARIDES EXTRACTED WITH BOILING WATER FROM YOUNG (Y) AND MATURE (M) HYPOCOTYL TISSUES

	Y		*M*	
	NF	*AF*	*NF*	*AF*
Neutral sugars (mol.g^{-1})	274	811	389	354
Rhamnose (molar ratio, %)		3		4
Arabinose	47	41	34	38
Xylose	6	4	5	11
Mannose	7		15	
Galactose	30	47	23	47
Glucose	10	5	23	
Uronic acids (μmol.g^{-1})		931		545
Proteins (mg.g^{-1})	7.1	9.6	1.6	2.2
Intrinsic viscosity	12.5	7.7	2.0	12.5

[a]Sugar and protein contents are expressed with respect to crude-cell-wall dry-matter ratio.

2.6 s, precluded quantitative spectra. An increase in the recycle time (5.2 s) did not alter significantly the relative intensities of the signals (see Experimental) for the most soluble sample, NF_δ, and, thus, the conditions are assumed to be roughly quantitative. The samples were not totally soluble (see Experimental), with the exception of NF_δ and NF_α obtained from mature cell walls. Satisfactory data could not be obtained for AF due to the low solubility. Enzymic hydrolysis of AF gave more soluble material and this study will be reported elsewhere.

The n.m.r. spectra of NF of young cell walls are given in Figs. 1A and B. The region for anomeric proton signals in the ^{1}H-n.m.r. spectrum of NF before chromatography (Fig. 1A-1) contains at least seven signals at 5.41 (b), 5.21 (J ~3 Hz), 5.18 (J ~3 Hz), 5.15 (J ~3 Hz), 5.12 (J ~3 Hz), 4.68 (J ~7 Hz), and 4.67 p.p.m. (J ~7 Hz), with relative intensities of 0.22, 0.08, 0.19, 0.21, 1.00, and 0.46 (for the sum of the last two peaks which overlapped). Similarly, the region for signals of anomeric carbons in the ^{13}C-n.m.r. spectra (Fig. 1B-5) contained at least six signals at 109.0, 108.5, 105.85, 104.0, 103.2, and 101.1 p.p.m., with relative intensities of 1, 0.2, 0.4, 0.1, 0.1, and 0.1, respectively. From the n.m.r. data of the major component ($\delta_{H\text{-}1}$ 5.12, J ~3 Hz and $\delta_{C\text{-}1}$ 109.0), together with the results of g.l.c. analysis and chemical shift data for methyl arabinofuranosides[11–13] (see Table II), an α-arabinofuranoside structure can be proposed tentatively based on the well-known rules[10,14] for the ^{13}C and ^{1}H chemical shifts for O-alkylated glycosides.

Attention was then turned to multi-pulse n.m.r. methods[15]. High-resolution 2D-n.m.r. spectroscopy has been used in the elucidation of the structure of tetrasaccharides[16], homo-oligomers[17], glycospingolipids[18], and polysaccharides[19]. The 2D chemical-shift correlation or COSY experiment is widely used to determine chemical shifts and coupling constants in molecules whose 1D spectra involve severe overlapping of signals. Thus, a phase-sensitive double-quantum-filtered COSY[20] spectrum was recorded for NF (~10–15 mg or 2–3%). The acquisition time was about the same as that required for the ^{13}C-n.m.r. spectra. The proton assignments for the major glycoside were extracted from the COSY spectrum (not given) and the chemical shifts, indicated in Fig. 1A-4, are collected in Table III together with data for methyl α-arabinofuranoside and arabinans which confirm the α-Ara$f$ assignment. Finally, from the results of g.l.c. analysis (see Table I),

TABLE II

^{13}C CHEMICAL SHIFT DATA FOR METHYL ARABINOSIDES IN D_2O[11]

Compound	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*OCH$_3$*
α-D-Araf^a	109.3	81.9	77.5	84.9	62.4	56.1
β-D-Araf^b	103.2	77.5	75.7	83.1	64.2	56.1
α-L- or -D-Ara$p$	105.1	71.8	73.4	69.4	67.3	58.1
β-L- or -D-Ara$p$	101.0	69.4	69.9	69.96	63.8	56.3

$^a J_{H\text{-}1,H\text{-}2}$ 2 Hz[13]. $^b J_{H\text{-}1,H\text{-}2}$ 4.5 Hz[13].

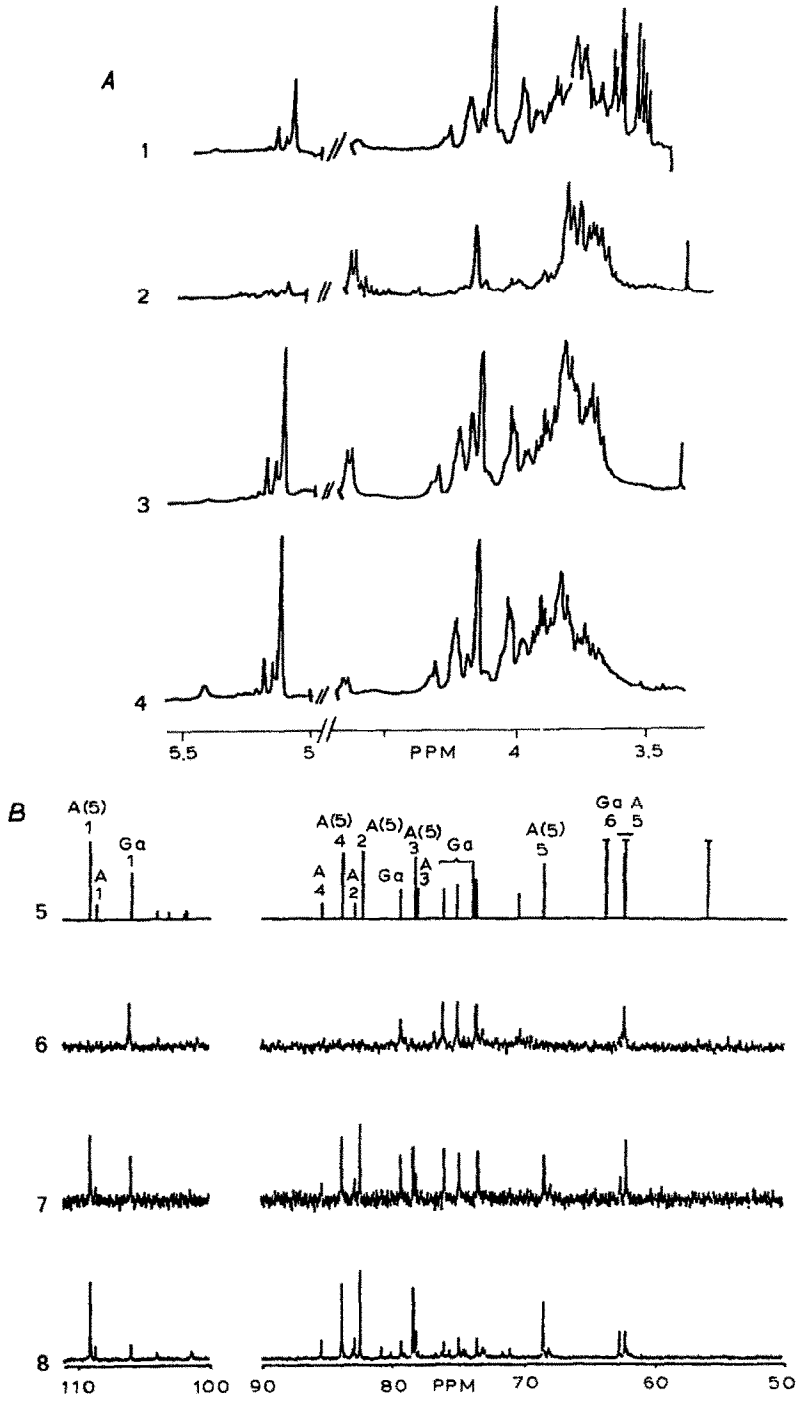

Fig. 1. N.m.r. spectra (D_2O) of the neutral polysaccharides isolated from young hypocotyl tissues. Spectra were performed on NF and the α, β, and δ fractions obtained after gel permeation. *A*, 400-MHz ^{1}H-n.m.r. spectra (internal Me_2SO, δ 2.72); *B*, 100-MHz ^{13}C-n.m.r. spectra (internal Me_2SO, δ 39.5): NF 1 and 5; NF_α, 2 and 6; NF_β, 3 and 7; NF_δ, 4 and 8; A, α-Araf; Ga, β-Galp.

n.m.r. data for the second most-abundant glycoside [signals for H-1 at δ 4.67 (J ~7 Hz), H-2 at δ 3.70 (COSY), and C-1 at δ 105.85], and the chemical shift data for methyl galactopyranosides (see Table IV), a β-galactopyranose structure can be proposed.

Most of the information concerning the nature of the glycosidic linkages was obtained from the purified fractions. The ^{13}C-n.m.r. spectrum of the fastest-eluted fraction (NF_α, Fig. 1B-6) was that of a galactan (6 strong signals at 105.8, 79.1, 75.95, 74.8, 73.3, and 62.2 p.p.m., with intensities of 1, 0.6, 1, 1, 0.9, and 1.3, respectively). For purposes of comparison, the chemical shift data for various substituted methyl β-galactopyranosides are collected in Table IV. None of the major ^{13}C signals could be attributed to C-4 of β-Gal*p* with HO-4 unsubstituted, whereas the signal at 62.2 p.p.m. could be assigned to C-6 of β-Gal*p* with HO-6 unsubstituted. Moreover, the absence of signals down-field of 80 p.p.m. indicates that HO-3 is not involved in a glycosidic linkage. These data are compatible with a 4-*O*-β-Gal*p* structure, possibly branched, for the major component. For β-Gal*p*, an up-field shift (β-effect) is expected for the carbon atoms β to hydroxyl groups involved in glycoside linkages[10,27]. The down-field shift observed for the signal of C-3 of 4-*O*-β-Gal*p* is therefore surprising, although similar shifts have been reported[10,28]. Both the existence of several weak signals and the relative intensity of the peak at 79.1 p.p.m. suggest that other galactosyl residues are also present, but the poor signal-

TABLE III

^{1}H-N.M.R. CHEMICAL SHIFT DATA FOR α-ARABINOSYL COMPOUNDS

Compound	*Ref.*	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-5'*
Me α-Ara*f*	12	4.91[a]	4.04	3.93	4.02	3.80	3.69
Arabinan	21	5–5.4[b]					
Arabinan	22	5.1[c]					
NF_Y		5.12[d]	4.16	4.04	4.24	3.91	3.81

[a]J ~2 Hz[13]. [b]Coupling constant not measurable. [c]J ~1 Hz. [d]J ~3 Hz.

TABLE IV

^{13}C-N.M.R. CHEMICAL SHIFT DATA FOR β-GALACTOPYRANOSIDES IN D_2O

Compound	*Ref.*	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
Me β-Gal*p*	11	104.9	71.8	73.9	69.8	76.2	62.1
Me 3-Me-β-Gal*p*	23	103.9	69.8	82.0	64.2	75.1	61.2
3,4-Me_2-β-Gal*p*	24	97.7	72.4	84.0	76.5	75.9	61.9
Me 2,3,4,6-Me_4-β-Gal*p*	23	105.3	81.5	84.6	76.1	73.9	72.0
β-D-Gal*p*-(1→2)-(Me β-D-Gal*p*)[a,b]	25	103.2	79.3	73.6	69.6	75.9	61.7
β-D-Gal*p*-(1→3)-(Me β-D-Gal*p*)[a,b]	26	105.2		84.0			
β-D-Gal*p*-(1→4)-(Me β-D-Gal*p*)[a,b]	26	105.4			78.9		
β-Gal*p*-(1→4)-	NF_α	105.8	73.3	74.8	79.1	75.95	62.2

[a]Data for the right-hand sugar residue. [b]At 70°.

to-noise ratio in the spectrum of this barely soluble fraction precluded their assignments.

The ^{13}C-n.m.r. spectrum (Fig. 2B-6) of NF_α, obtained from mature cell walls, contained six equally intense signals which indicated a homogalactan composed of a unique glycosyl residue. The ^{1}H chemical shift data for NF_α (obtained from the COSY spectrum), shown in Table V, are almost identical (taking into account the up-field solvent shift of −0.4 p.p.m.) to those reported for the 4-*O*-β-Gal*p* unit of globotetraosylceramide[30]. Hence, an unbranched structure can be proposed for the galactan in NF_α obtained from mature cell walls. However, the type of branching in the galactan(s) in NF_α obtained from young cell walls could not be determined from the n.m.r. data which are compatible with a mixture of unbranched and branched galactans.

From the ^{13}C-n.m.r. spectra in Figs. 1B(6-8) and 2B(6-8), the intermediate fraction NF_β obtained from either young or mature cell walls appeared to contain a mixture of the polymers in the corresponding NF_α and NF_δ fractions. No changes in the ^{13}C-n.m.r. spectra of NF_δ were observed during maturation. The ^{13}C chemical shifts of the signals of the major component of NF_δ are collected in Table VI along with data for methyl α-arabinofuranoside and some arabinans. Clearly, most of the arabinosyl residues are 5-linked (C-5 signal at 68.4 p.p.m.).

Recent structural analyses[31] of mannosyl glycopeptides and oligosaccharides relied heavily on correlation of the chemical shift data for H-1 and H-2. The additivity rules, for the correlation of the chemical shifts of similar hydrogen atoms in different oligosaccharides, were useful in the conformational analysis of these compounds when the differential chemical shifts were >0.1 p.p.m.[29]. Dabrowski *et al.*[30] also showed that the chemical shifts of the signals for H-1 and H-2 in glycosphingolipids present regularities related to the type, anomeric configuration, site of glycosidic linkage, and sequence of the sugar components. A computerised approach to the analysis of oligosaccharides by high-resolution ^{1}H-n.m.r. spectroscopy has appeared recently[32]. The g.l.c. analysis of NF obtained from young hypocotyl tissues, given in Table VII, indicate that this polymer contains arabinose, galactose, and glucose as well as small proportions of xylosyl and mannosyl residues. The ^{13}C-n.m.r. spectrum (Fig. 1B-8) contains 5 groups of signals (intensity ±10%) at 109.0 (66%), 108.5 (11%), 105.8 (12%), 103.9 (5%), and 101.3 p.p.m.

TABLE V

^{1}H-N.M.R. CHEMICAL SHIFT DATA FOR β-GALACTOPYRANOSYL COMPOUNDS

Compound	*Ref.*	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*	*H-6'*
Me β-Gal*p*[a]	29	4.20	3.39	3.53	3.81	3.57	3.69	3.64
-(1→4)-β-Gal*p*-(1→3)-[b]	30	4.26	3.31	3.41	3.82	—	—	—
NF_α (from mature cell walls)[a]		4.68	3.72	3.81	4.21	—	—	—

[a]In D_2O. [b]Globotetraosylceramide in $(CD_3)_2SO$.

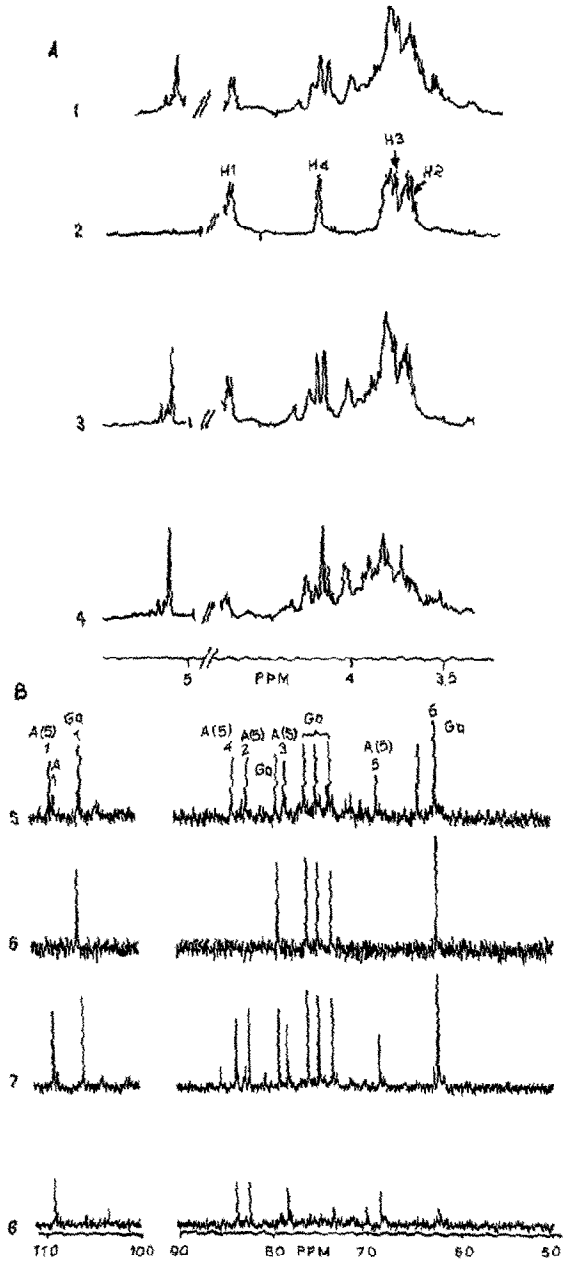

Fig. 2. N.m.r. spectra (D_2O) of the neutral polysaccharides isolated from mature hypocotyl tissues. Symbols as in Fig. 1.

TABLE VI

^{13}C-N.M.R. CHEMICAL SHIFT DATA[a] FOR α-ARABINOFURANOSIDES

Compound	*Ref.*	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*
Me α-Ara*f*	11	109.3	81.9	77.5	84.9	62.4
Arabinan unbranched	6	108.7	82.5	77.8	85.2	62.3
5-linked	6	108.15	82.2	77.8	83.4	67.3 / 68.0 [b]
	5	108.1	81.5	77.5	82.9	67.6
	22	108.8	82.1	78.1	83.5	68.2
NF_δ 5-linked		109.0	82.3	78.2	83.7	68.4

[a]In D_2O. [b]Unassigned.

(6%), respectively. The phase-sensitive double-quantum-filtered COSY spectrum of NF_δ is given in Fig. 3 as a contour plot. In the corresponding 1D ^{1}H-n.m.r. spectrum, traced above the contour plot, the anomeric protons have been labelled A-I. Similarly, the cross-correlation peaks, arising from coupling between H-1 and H-2, have also been labelled. The chemical shifts for the H-2 signal extracted from this COSY spectrum are collected in Table VIII. The splitting patterns of the H-1 and H-2 multiplets can often be extracted from the phase-sensitive double-quantum-filtered COSY spectrum[33]. This was achieved in a second experiment in which the resolution in F2 was 2.4 Hz/pt. and that in F1 was 4.8 Hz/pt. (see expanded areas in Fig. 3). Thus "large" coupling constants (J ~8 Hz) are readily distinguished from "smaller" ones (J ~2 Hz). For example, the cross-peaks labelled G have the correct fine structure for a β-Glc*p* residue in both F1 (H-1, d, J ~8 Hz) and F2 (H-2, dd ~t, J ~9 Hz). The exact values of $J_{1,2}$ and $J_{2,3}$ can be measured in the 1D ^{1}H-n.m.r. spectrum (see expanded region in Fig. 3) since the signal for H-2 of β-Glc*p* is at the high-field limit for sugar protons[30]. Only ~10% of the glucosyl residues have the β-Glc*p* structure. The relative values of $J_{1,2}$ and $J_{2,3}$ (the exact values in brackets were taken from the 1D ^{1}H-n.m.r. spectrum) and H-1 integrals are also given in Table VIII. Finally, the structures compatible with all the experimental data are indicated.

TABLE VII

SUGAR COMPOSITION[a] OF THE α, β, AND δ FRACTIONS OBTAINED AFTER GEL PERMEATION OF NF_γ

Compound	NF_α (%)	NF_β (%)	NF_δ (%)
Arabinose	24.0	45.9	60.4
Xylose	0.9	1.2	1.6
Mannose	0.7	1.9	2.6
Galactose	72.4	46.8	23.7
Glucose	2.0	4.2	11.7

[a]The values represent molar ratios (%) with respect to the total neutral sugars released after acid hydrolysis of each fraction.

TABLE VIII

H-1 AND H-2 CHEMICAL SHIFT DATA OBTAINED FROM 400-MHz ^1H–^1H DOUBLE-QUANTUM-FILTERED PHASE-SENSITIVE COSY (δ–δ, ^1H–^1H) SPECTRUM OF NF_δ IN D_2O

^1H 1D Spectrum label	Chemical shifts		Relative values[a]		H-1 integrals (%)[b]	Compatible structures
	H-1	H-2	$J_{1,2}$	$J_{2,3}$		
A	5.43	3.63	s	c or l	8	
B	5.41	3.63	s	c or l		
C	5.21	4.15	s	s	1	α-Man*p*[c]
D	5.18	4.15	s(1.5)	s	7	β-Glc*f*[d]
E	5.15	4.29	s(1.2)	s	7	β-Gal*f*[d]
F	5.12	4.15	s(1.5)	s(<1)	58	5-*O*-α-Ara*f*[d]
G	4.68	3.29	l(7.9)	l(9.5)	1.5[e]	β-Glc*p*[f or g]
H	4.67	3.71	l(7.3)	l	12.5[e]	β-Gal*p*[g]
I	4.54	3.38	c or l	c or l	5	

[a]Key: c, overlapping signals; s, "small" (J ~2–4 Hz); l, "large" (J ~8 Hz) from 2D spectrum (2.4 Hz/pt.); bracketed values from the resolution-enhanced 1D spectrum (0.5 Hz/pt.). [b]% of the sum of the H-1 integrals. [c]C-1 amongst peaks at 101.3 p.p.m. [d]C-1 amongst peaks at 108.5–109.0 p.p.m. $J_{1,2} + J_{2,3}$ = 2–3 Hz for β-Gal*f* and β-Glc*f*, whereas $J_{1,2} + J_{2,3}$ = 8–9 Hz for α-Gal*f* and α-Glc*f*[13]. [e] From 1D spectrum. [f]C-1 amongst peaks at 103.9 p.p.m. [g]C-1 at 105.8 p.p.m.

Thus, hypocotyl cell walls contain galactans and arabinans, which are both soluble in boiling water. Although, during maturation, the ratio of Ara–Gal in the pectic fraction solubilised by boiling water remains nearly unchanged, high-molecular-weight, probably highly branched, galactans were replaced by small unbranched polymers.

EXPERIMENTAL

General methods. — Polysaccharide fractions were hydrolysed with 0.5M H_2SO_4 (100°, 1 h) in sealed tubes and the hydrolysates were neutralised with $SrCO_3$. Monosaccharides were converted into their alditol acetates[34] and analysed by g.l.c. on a column (200 × 0.05 cm) of 3% of ENCSS.M coated on Gas-Chrom Q (100–120 mesh) at 170° (N_2, 30 mL/min) with *myo*-inositol as the internal standard.

Isolation of the pectic polymer. — Seeds of *Vigna radiata* (L) Wilczek were soaked in tap water for 2 h, then placed on moist vermiculite, and covered with a wet cloth. After storage for 3 days at 26° in the dark, the seedlings with hypocotyls 45 mm (±5 mm) long were selected and segments were excised[3] from 2 parts of the hypocotyl. The cell walls were isolated and the pectic fractions were extracted as described previously[3]. The pectic fraction extracted by boiling water was concentrated to a small volume and submitted to ion-exchange chromatography on columns (20 × 1.8 cm) of DEAE-Sepharose CL-6B (Pharmacia) equilibrated with 0.05M sodium acetate buffer (pH 4.7) (ref. 35). Neutral polysaccharides were not bound and acidic polysaccharides were eluted with M buffer. Both neutral and

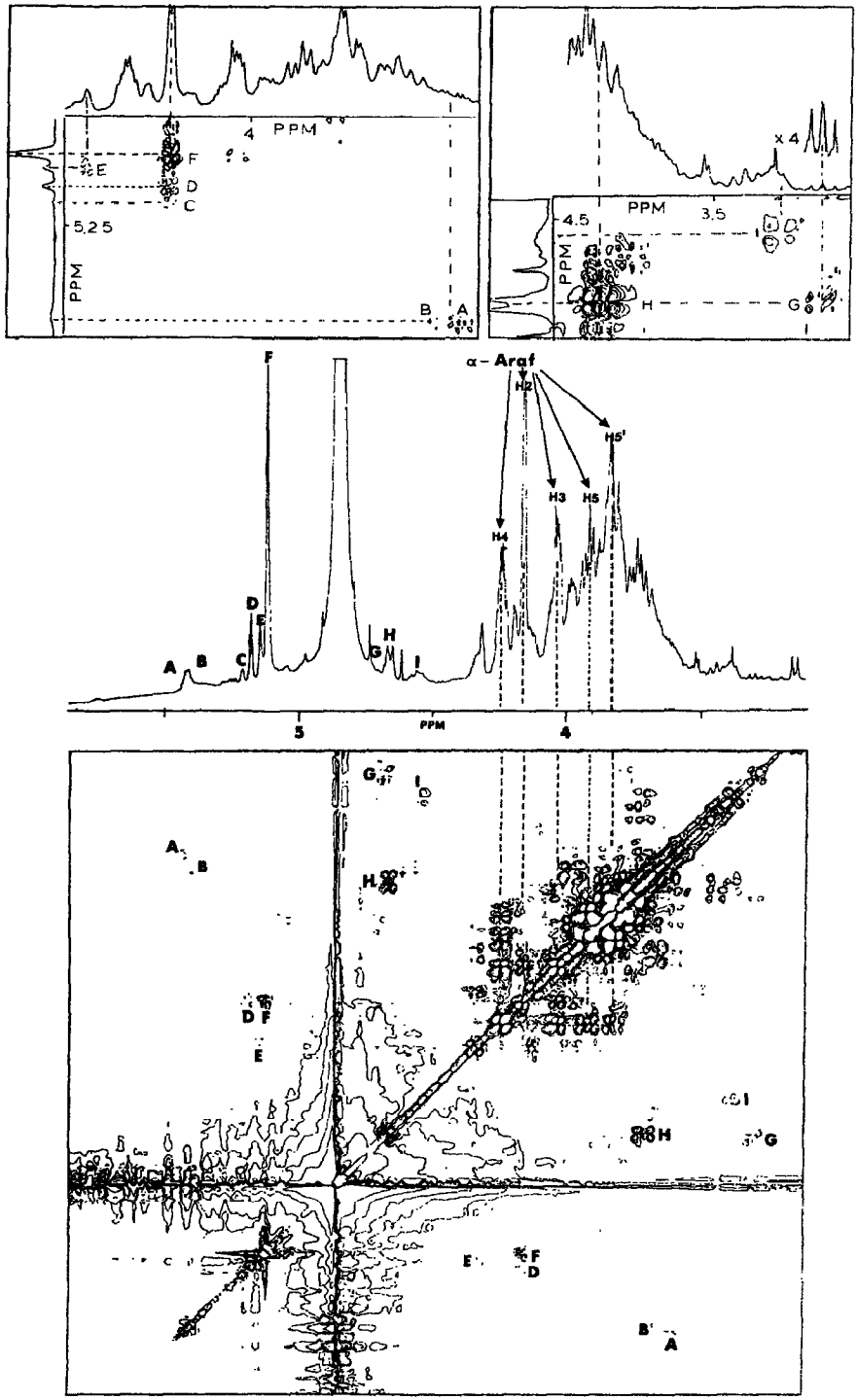

Fig. 3. Symmetrised 400-MHz n.m.r. DQF phase-sensitive COSY (δ–δ, ^1H–^1H) spectrum of ~15 mg of NF_δ in D_2O. Cross peaks corresponding to coupling between H-1 and H-2 of the sugar residues are labelled (A–I) both in the 1D and COSY spectra. Expanded regions with the corresponding 1D spectra along both the F1 and F2 axes are given above (same scale).

acidic fractions were dialysed against H_2O and concentrated to a small volume. The neutral fraction, in 0.05M acetate buffer, was applied to a column (90.0 × 1.8 cm) of Sepharose 6B (Pharmacia) and eluted at 45 mL/h with the same buffer. Fractions (4 mL) were collected, and analysed for their total sugar content using the anthrone reagent[36]. Appropriate fractions were combined, dialysed against H_2O, concentrated to a small volume, and analysed. Uronic acids were estimated by the *m*-biphenyl method[37] (using polygalacturonic as standard) and proteins by the method of Lowry *et al.*[38]. The relative viscosities of the polysaccharides were measured with a Beckman low-shear rotary viscosimeter on 0.01–0.109% solutions in 0.1M NaCl. The specific viscosities were used to determine the intrinsic viscosities[39].

N.m.r. spectroscopy. — A Bruker AM-400 spectrometer operating in the F.t. mode at 400.13 MHz for ^1H and 100.57 MHz for ^{13}C was used. Samples were dissolved in D_2O at 70° [10–15 mg in 0.5 (2) mL with 5(10)-mm tubes]. Dissolution was incomplete except for NF_δ and NF_α obtained from mature cell walls. $(CD_3)_2SO$ was the internal reference (δ_C 39.5, δ_H 2.72). The spectral window for the ^1H-n.m.r. spectra in Figs. 1A and 2A, which were acquired with solvent presaturation (HOD at 4.82 p.p.m.), was 10 p.p.m. for 16k data points with a pulse width of 8 μs (45°) and an acquisition time of 1.02 s. ^{13}C-N.m.r. spectra were recorded with complete proton decoupling and a pulse width of 8 (17 for the 10-mm probe) μs (90°). The acquisition time was 1.11 s with a 1.5-s delay between each scan. The ^{13}C-n.m.r. spectrum of NF_δ was recorded twice under identical conditions, leading to a ±8% mean variation of the integral intensities for 21 peaks. When this spectrum was recorded with a recycle time of 5.2 s, the mean variation in integral intensities for the same 21 peaks was ±10%; 30,000–50,000 transients of 64k data points were accumulated for a total acquisition time of 18–30 h. The intense signals at 182, 56, and 25 p.p.m. present in the ^{13}C-n.m.r. spectrum of NF (Y) [182 and 25 p.p.m. for NF (M)] could not be assigned and were probably due to impurities.

Double-quantum-filtered phase-sensitive COSY experiments[20] were performed using a (90°) – (t_1) – (90°) – (90°) – (FID, t_2) sequence. The spectral width in F1 and F2 was 1220 or 2304 Hz; the number of data points in F2 was 1024, and 512 increments were recorded. The 90° pulse was 7.6 μs and the total acquisition time was 16 h (38 for NF_α from mature cell walls). Before Fourier transformation, the data were multiplied with a $\pi/2$ shifted squared sine bell. Zero filling was applied in F1.

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