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ISOLATION AND AN N.M.R. STUDY OF PECTINS FROM FLAX (*Linum usitatissimum* L.)

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ABSTRACT

The EDTA-extracted pectins from flax, which were fractionated by exclusion chromatography according to size, contained galactose, galacturonic acid, rhamnose, glucose, and traces of arabinose. The main components of the major fraction, a high-molecular-weight galactan, were →4)-β-Gal*p*-(1→ chains and β-Gal*p* non-reducing terminal units according to 1D ^{1}H- and ^{13}C-n.m.r. analysis. The low-molecular-weight fractions contained notable amounts of rhamnose and galacturonic acid. On the basis of multipulse n.m.r. (CHORTLE, COSY, and NOESY), a rhamnogalacturonan structure of type I with β-Gal*p* units attached to the HO-4 of rhamnose can be proposed. This polymer probably constitutes the backbone of the high-molecular-weight polysaccharides.

INTRODUCTION

In a whole plant such as flax, the composition of the polysaccharides in the matrix of the cell walls depends on the state of growth and the location in the tissue[1]. For example, the cortex cell walls of flax are enriched in methylated pectins (10% of cell-wall dry weight) and homogalacturonans (5%)[2]. In contrast, the walls of the phloem cells, which are differentiated into fibres, contain mainly cellulose and galactans, and the pectins, which represent <10% of the cell walls, are depleted in arabinose and enriched in galactose, rhamnose, and glucose[3,4]. Primary cell-wall polymers have been classified largely according to their primary structures[5]. Delineation of the primary structures of the pectic cell-wall polysaccharides of flax was the goal of this work. In order to determine the structure of these pectins, they were fractionated and analyzed by ^{1}H- and ^{13}C-n.m.r. spectroscopy.

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RESULTS AND DISCUSSION

Chemical studies. — EDTA-extracted pectins (p_e) from flax represent 11% of the total pectins and 5% of the uronic acids. P_e is weakly charged and its molar mass per unit charge (equiv.) is 1000–1250. The major neutral sugar is galactose and the ratios of Gal, Rha, Glc, and Ara are 1:0.33:0.20:0.04. Exclusion chromatography of p_e gave nine fractions, S400-1/5 and S200-1/4 (in order of decreasing molecular weight[6]) (Table I). For the two major fractions, S400-3 (39%) and S200-2 (16.5%), there was a marked correlation between the neutral sugar/uronic acid ratios (NS) (5.78 and 2.4, respectively) and the galactose contents (81% and 59.4%, respectively). The ratios of Gal, Rha, Glc, and Ara were 1:0.105:0.093:0.04 and 1:0.36:0.20:0.09, respectively, indicating that the GalA/Rha ratios were constant at 2:1. Thus, the decrease in molecular weight may correspond to removal of galactose side-chains from a rhamnogalacturonan backbone which is highly acetylated (20–40%).

N.m.r. studies. — Both ^{1}H- and ^{13}C-n.m.r. spectra were recorded for all of the p_e fractions. The long acquisition times required for the latter spectra, which were obtained with broad-band proton decoupling (full n.O.e.) and with a recycle time of 2.6 s, precluded quantitative spectra. Whilst recommending quantitative ^{13}C-n.m.r. spectra, Gorin[7] pointed out that the 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 T_1 values (0.20–0.52 s with the exception of C-1 of α-GalA, 0.72 s) reported[8] for pectins from *Vigna radiata* Wilczek suggest that the conditions used here were roughly quantitative. An increase in the recycle time (5.2 s) did not alter significantly the relative intensities of the signals. As the width of the peaks varied considerably amongst the various glycosyl units, inter-residue relative intensities were measured both by integration of peak areas and excision of the chart paper and weighing.

TABLE I

CHEMICAL DATA FOR FRACTIONS OBTAINED BY EXCLUSION CHROMATOGRAPHY OF EDTA-EXTRACTED PECTINS FROM FLAX

	S400-1	*S400-2*	*S400-3*	*S400-4*	*S400-5*	*S200-1*	*S200-2*	*S200-3*	*S200-4*
Yield	0.02	0.07	0.33	0.08	0.03	0.07	0.14	0.08	0.03
Equiv.	335	525	1120	660	850	940	580	475	350
NS[a]	0.87	2.06	5.78	2.90	4.09	4.65	2.40	1.74	0.96
k_{av}	0.00	0.28	0.54	0.69	1.00	0.13	0.30	0.58	1.00
Mol. wt.	——	——→	100 000	——	——	47 000	21 000	6000	<5000[b]

[a]Ratio of neutral sugars and uronic acids. [b]The S200 column was calibrated[6] by light-scattering measurements on authentic samples of branched flax polymers. Recent calibrations have shown that the mol. wt. is less for linear polymers, *i.e.* <1000 for S200-4 if it was a linear molecule.

The ^{13}C-n.m.r. spectrum of S400-3 (Fig. 1) contained seven strong signals at δ 105.88 (1), 79.18 (0.64), 76.02 (0.98), 74.84 (1.01), 73.38 (0.99), 70.18 (0.28), and 62.28 (1.2), and numerous small signals (5–25% of the intensity of the signal at 105.88 p.p.m.). From comparison of these data with those for β-D-Gal*p*-(1→4)-β-D-Gal-OMe[9] and a β-galactan[10] (Table II), a →4)-Gal*p*-(1→ repeating unit (C-4 79.18 p.p.m.) with a third of the galactosyl residues in non-reducing terminal

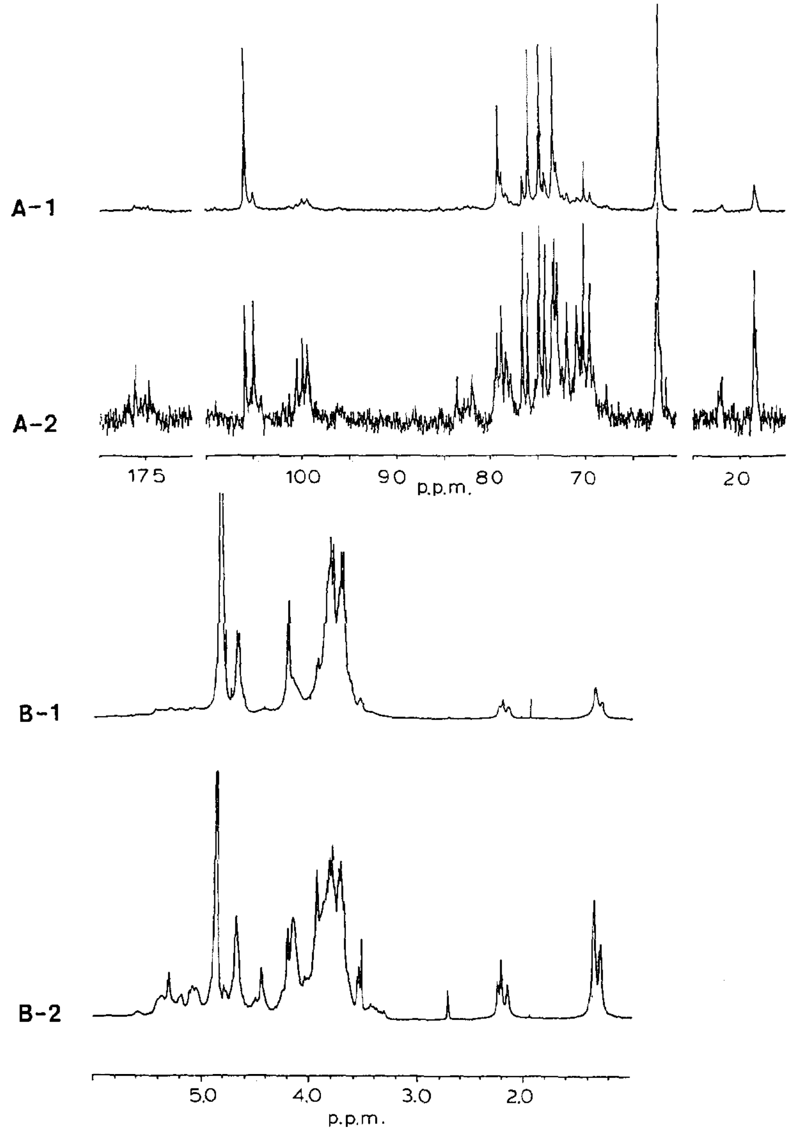

Fig. 1. N.m.r. spectra (D_2O) of the fractions S400-3 (A-1,2) and S200-4 (B-1,2): A, 100-MHz ^{13}C-n.m.r. spectra [internal $(CD_3)_2SO$, δ 39.5]; B, 400-MHz ^{1}H-n.m.r. spectra [internal $(CD_3)_2SO$, δ 2.72].

TABLE II

CHEMICAL SHIFT DATA FOR RELATED GLYCOSIDES

Compound	*Position*						*Ref.*
	1	*2*	*3*	*4*	*5*	*6*	
β-Galactosyl residues							
β-D-Gal*p*-(1→2)-β-D-Gal*p*OMe[a]							
^{13}C	103.2	79.2	73.6	69.6	75.9	61.7	11
β-D-Gal*p*-(1→3)-β-D-Gal*p*OMe[a]							
^{13}C	105.2		84.0				9
β-D-Gal*p*-(1→4)-β-D-Gal*p*OMe[a]							
^{13}C	105.4			78.9			9
-(1→4)-β-Gal*p*-(1→							
^{13}C	105.8	73.3	74.8	79.1	75.95	62.2	10
^{1}H	4.68	3.72	3.81	4.21			10
β-Gal*p*-(1→2)-α-Rha*p*[a]							
^{13}C	105.9	72.2	73.7	69.7	76.2	62.2	12
β-Gal*p*-(1→3)-α-Rha*p*[a]							
^{13}C	105.5	72.4	73.8	69.9	76.3	62.3	12
β-Gal*p*-(1→4)-α-Rha*p*[a]							
^{13}C	104.9	72.9	74.0	69.8	76.4	62.1	12
^{1}H	4.66	3.55	3.67	3.93	3.69		13
β-Glucosyl residues							
β-Glc*p*-(1→4)-α-Rha*p*[a]							
^{13}C	104.4	75.1	77.2	70.8	77.0	61.9	12
α-Rhamnosyl residues							
β-Gal*p*-(1→4)-α-Rha*p*[b]							
^{13}C	95.0	72.0	71.2	82.3	68.1	18.2	12
^{1}H	5.11	3.93	4.02	3.70	3.95	1.35	13
β-Gal*p*-(1→2)-α-Rha*p*[b]							
^{13}C	100.6	78.5	70.9	73.3	70.3	17.8	14
^{1}H	5.03	4.22		3.5		1.26	14
α-Galacturonan							
^{13}C	100.45	69.72	70.44	79.47	72.85		8
^{1}H	5.09	3.80	4.03	4.44	4.80		8

[a] Data for the right-hand sugar residue. [b] Data for the left-hand sugar residue.

positions (C-4, 70.18 p.p.m.) could be proposed for S400-3. Amongst the weaker signals, the peaks at 175, 22, and 18 p.p.m. suggested the presence of small amounts of glycuronic acid, acetyl groups (7%), and deoxy sugars (22%), respectively. The ^{1}H-n.m.r. spectrum (Fig. 1) of S400-3 contained signals for H-1 at 4.67 p.p.m. (d, *J* 8 Hz) and for H-4 at 4.25 p.p.m. (bs) confirming the β-galactan assignment for the major residue. Weak signals at 2.2 (9%) and 1.3 p.p.m. (15%) corroborated the presence of the acetyl groups and deoxy sugars. The n.m.r. spectra of the various S400 and S200 fractions showed a regular increase in the relative proportions of the minor residues as the molecular weight decreased. Thus, S200-4 was selected for study.

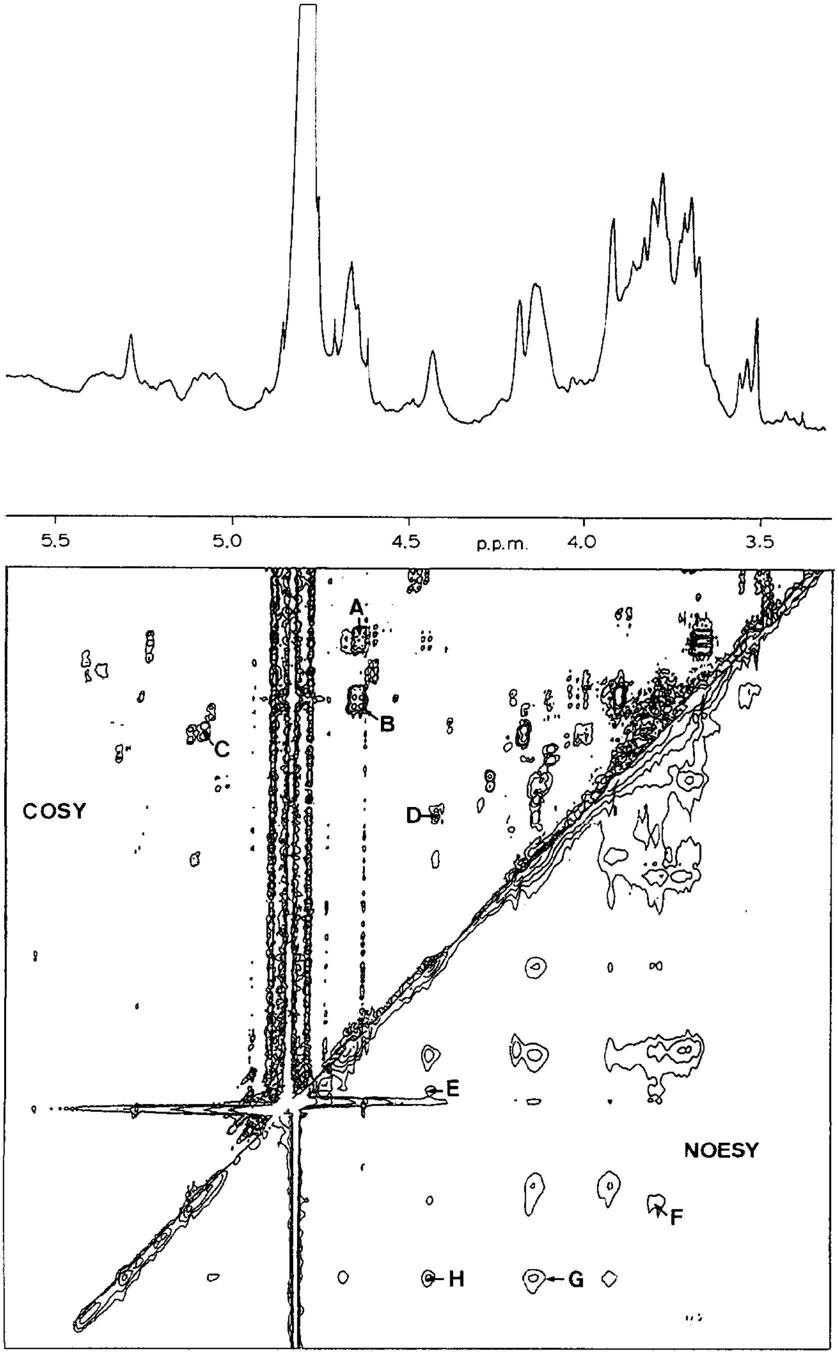

Fig. 2. 400-MHz 2D-n.m.r. spectra of S200-4 in D_2O (20 mg/0.5 mL): upper left, COSY; lower right, phase-sensitive NOESY. The corresponding 1D spectrum is given above along the F2 axis. The following intra-residue cross-peaks (C COSY, N NOESY, ′ for the right-hand residue) are indicated: **A** (C) for H-1/H-2 of β-Gal*p*-(1→4)-α-Rha*p*, **B** (C) for H-1/H-2 of →4)-β-Gal*p*-(1→, **C** (C) for H-1/H-2 of →4)-α-GalA-(1→, **D** (C) for H-3/H-4 of →4)-α-GalA-(1→, **E** (N) for H-5/H-4 of →4)-α-GalA-(1→, **F** (N) for H-1/H-2 of →4)-α-GalA-(1→, **G** (N) for H-1′/H-2′ of β-Gal*p*-(1→4)-α-Rha*p*. The inter-residue cross-peak **H** (N) for H-1/H-4′ of α-Rha*p*-(1→4)-α-GalA is also labelled.

As the resolution of the ^{13}C- and ^{1}H-n.m.r. spectra of S200-4 (Fig. 1) was poor, multi-pulse methods were used. The 1D (δ–δ, ^{1}H–^{13}C) correlation method[15], CHORTLE, which has been applied to small molecules[16], has been useful in the study of polysaccharides[17]. This technique, which extracts proton chemical shifts from the intensities of $^{1}J_{C,H}$-related carbons, theoretically requires only the acquisition of two ^{13}C-n.m.r. spectra. The resolution of the 1D ^{13}C-n.m.r. spectrum is retained and the resolution in the proton dimension is inversely proportional to $2(S/N^{*}\tau)$, where S/N is the conventional signal-to-noise ratio and τ is the proton evolution time. Therefore, CHORTLE, DQFCOSY, COSY, and NOESY spectra were recorded for S200-4.

The n.m.r. data for the anomeric protons and carbons of this fraction are collected in Table III. The region for C-1 resonances, which contained signals integrating for 47% of the total C-1 resonances, contained strong signals at 105.87 and 104.85 p.p.m., whereas the corresponding region for H-1 signals showed heavy overlapping. At least 10 H-1/H-2 correlations were visible in the COSY spectrum of S200-4 (Fig. 2) but the proton coupling network could be extracted only for the two major units, namely, cross-peaks **A** and **B**. The numerous cross-peaks strongly suggest that this fraction, which was obtained in the inclusion volume of the column, contained a mixture of oligomers. For the low-molecular-weight range (1000–5000), reducing units would be detected if the S200-4 fraction contained a single type of molecule. This information has been collected in Table IV along with the corresponding ^{13}C-n.m.r. data.

TABLE III

N.M.R. DATA FOR THE C-1 AND H-1,2 OF S200-4

C-1 (p.p.m.)	*Strength*[a]	*Chemical shift of $^{1}J_{C,H}$-related proton*[b] *(p.p.m.)*	*H-1 (p.p.m.)*	*Integral*	$J_{1,2}$[c] *(Hz)*	*H-2*[c] *(p.p.m.)*	$J_{2,3}$[c] *(Hz)*
α-Glycosyl region							
99.00	w	5.07 (0.04)	5.58	0.32			
99.31	m	5.14 (0.02)	5.32–5.39			3.65–3.53	
99.83	m	5.31 (0.03)	5.29		x	3.82	
100.40	m	5.09 (0.03)	5.26				
101.18	w	5.42 (0.13)	5.09	0.16	x	3.77	y
101.87	w	5.23 (0.15)	5.07		x	4.11	x
			5.05		x	3.76	y
			5.03		x	3.70	y
β-Glycosyl region							
104.85	s	4.66 (0.01)	4.66-	0.51	y	3.55	y
105.87	s	4.66 (0.01)	4.68		y	3.71	y

[a] Key: w, weak; m, medium; s, strong; the α-glycosyl region represents 53% of the total for C-1. [b] From the CHORTLE spectrum; standard error in parentheses. [c] From the COSY spectrum. Key: x 2–4 Hz, y ~8 Hz.

TABLE IV

N.M.R. DATA (D_2O) OF THE ASSIGNED GLYCOSYL RESIDUES FOR THE ACIDIC FRACTION (S200-4) OF EDTA-EXTRACTED FLAX

Atom	^{13}C (p.p.m.)	*Relative* intensity	*Chemical shift of* $^1J_{C,H}$*-related proton*[a] *(p.p.m.)*	^1H*-X* *(p.p.m.)*[b]	$J_{X,X-1}$ *(Hz)*	$J_{X,X+1}$ *(Hz)*
-(1→4)-β-Gal*p*-(1→						
1	105.87	1	4.65 (0.01)	4.68		y
2	73.34	0.7	3.72 (0.02)	3.71	y[c]	y
3	74.80	1.6	3.89 (0.02)	3.81	y	x
4	79.17	0.7	4.20 (0.08)	4.20	x	
5	75.95	1.2	3.80 (0.04)			
6	62.22	1.6	3.84 (0.02)			
Gal of β-Gal*p*-(1→4)-α-Rha*p*-(1→						
1	104.86	1	4.65 (0.01)	4.66		y
2	73.18	1.6	3.59 (0.03)	3.55	y	y
3	74.18	1.5	3.70 (0.02)	3.71	y	x
4	70.12	1.7	4.03 (0.03)	3.95	x	
5	76.55	1.7	3.73 (0.02)			
6	62.37	1.9	3.80 (0.01)			
-(1→4)-α-GalA-(1→						
1	na[d]		na	5.05	nr[e]	
2	69.42	1	3.95 (0.01)	3.82	nr	y
3	70.82	0.9	4.04 (0.06)	4.04	y	nr
4	78.20	0.9	4.42[f]	4.44	nr	
5	72.83	1.2	4.59 (0.09)	4.78	nr	
6	174.5–176.8					

[a]From the CHORTLE spectrum; standard error in parentheses. [b]From the COSY and COSYDQF spectra. [c]x 2–4, y ~8 Hz. [d]Not assigned. [e]Not resolved. [f]4.38 p.p.m. for 0.25-ms delay and 4.47 p.p.m. for 0.75-ms delay: least-squares fit could not be obtained.

The ^{13}C signal (105.87 p.p.m.) noted above for the →4)-β-Gal*p*-(1→ repeating unit of S400-3 represented ~25% of the glycosyl units for S200-4. There was an almost perfect fit between the ^1H- and ^{13}C-n.m.r. data of this unit and the data given for the β-galactan extracted from cell walls of mung bean hypocotyls[10]. The relative intensities of the ^{13}C signals varied considerably, suggesting that several oligomers were present. Again, about two-thirds of the β-galactose residues are 4-linked. With the exception of the chemical shift of the H-3 resonance ($\delta_{CHORTLE} - \delta_{COSY}$ = 0.08 p.p.m.), the CHORTLE ^1H shifts correlated well with those for the COSY spectrum (only the cross-peak for H-1/H-2, **B**, is labelled).

The other major β-glycosyl residue gave ^{13}C signals (intensity) at 104.86 (1), 76.55 (1.7), 74.18 (1.5), 73.18 (1.6), 70.12 (1.7), and 62.37 p.p.m. (1.9). A β-Gal*p*-(1→4)-α-Rha*p*-(1→ structure can be proposed for this residue based on comparison of the ^{13}C-n.m.r. data, particularly the chemical shifts of the resonances for C-1 and C-2, with those of various β-galactosylrhamnopyranoses[12] (Tables II and IV).

Again, the variations in the relative intensities suggested that several units contributed to these peaks. The signals at 4.66, 3.55, 3.71, and 3.95 p.p.m. for H-1,2,3 and 4, respectively, and the fine structure (from the COSY cross-peaks; only the cross-peak for H-1/H-2, **A**, is labelled) are almost identical to the data[13] for the Gal unit of β-Gal*p*-(1→4)-α-Rha*p*.

The regions of the ^{1}H- and ^{13}C-n.m.r. spectra of S200-4, which contain mainly the signals for anomeric protons and carbons of α-glycosyl residues, 5–5.6 (48% of the total for H-1) and 98–102 p.p.m. (53% of the total for C-1), respectively, present >8 and 6 different peaks, respectively. In the COSY spectrum (Fig. 2), several of the H-1's gave H-2 cross-peaks in the region 3.70–3.82 p.p.m. Most of the cross-peaks showed a small $J_{1,2}$ value in F2, and a large $J_{2,3}$ value in F1 as expected for α-GalA. The chemical shift data for the coupling network presenting the most intense cross-peaks (the cross-peaks for H-1/H-2, **C**, and H-3/H-4, **D**, are labelled), which are collected in Table IV, were as follows: 5.05, 3.82, 4.04, 4.44, and 4.78 p.p.m. for H-1/5 respectively. These shifts and the fine structure of the correlation peaks are almost identical to the data reported for various →4)-α-GalA-(1→ homogalacturonan structures[8,18,19]. The NOESY spectrum of S200-4 (Fig. 2) contained most of the expected intra-residue cross-peaks for α-GalA: H-5/H-4 and H-1/H-2, **E** and **F**. The H-4 signal integrated for ~0.25 of the total for H-1. Again, both the numerous H-1/H-2 cross-peaks in the COSY spectrum and a minor H-4 cross-peak with a proton at 4.2 p.p.m. suggest that S200-4 contains a mixture of oligomers. Comparison of the chemical shifts for the resonances for H-2/5 and the CHORTLE data led to the assignments for C-2/5 of 69.42, 70.82, 78.20, and 72.83 p.p.m., respectively, which were almost identical to those of an α-galacturonan isolated from mung bean hypocotyl[8] (Table II). The ^{13}C signals were broader and there was a poorer fit between the COSY and CHORTLE data. A least-squares-fit could not be obtained from the data for C-4 (the mean value of the proton chemical shift obtained using 2 delay values is given in Table IV).

The total coupling networks for the rhamnosyl residues could not be established from the available data. The relative intensities of the Me-5 signals at 1.28 and 1.34 p.p.m. (I 0.81) per proton (0.81/3) as compared to that of the total H-1 was 0.27, suggesting a GalA/Rha ratio of 1:1. The corresponding chemical shifts of the resonance for the H-5 of Rha, which were extracted from the COSY spectrum, were 3.76 and 3.83 p.p.m. respectively. This indicated the rhamnosyl residues to be α, as it is known that the H-5 of β-rhamnosyl residues resonates at higher field (generally <3.50 p.p.m.)[20]. Low-field shifts, which have been described for the Me-5 of 4-linked α-rhamnosyl residues in both ^{1}H- and ^{13}C-n.m.r. spectra[21], were observed for the major rhamnosyl residue (1.35 and 18.34 p.p.m.) of S200-4 as compared to the minor rhamnosyl residue (1.27 and 18.13 p.p.m.). The envelope at 4.1–4.2 p.p.m. in the ^{1}H-n.m.r. spectrum of S200-4 was noticeably greater than in that of S400-3 (Fig. 1). Although H-2 of 2-linked α-Rha was expected to resonate in this region[14], only one set of cross-peaks, H-1 at 5.07 p.p.m. and H-2 at 4.11 p.p.m., was observed in the COSY spectrum. This result could be due to a small

$J_{1,2}$ value. The glycosyl residue with H-1 resonating at 5.30 p.p.m. (tentatively assigned as α-Rha*p*; no cross-peaks were observed in the COSY spectrum) gives cross-peaks both with the protons resonating at 4.44 (**H**, H-4 of α-GalA) and 4.2 p.p.m. (**G**, tentatively assigned as H-2 of α-Rha*p*), suggesting that -α-Rha*p*-(1→4)-α-GalA- linkages are present. Me-5 of α-Rha*p* resonating at 1.35 p.p.m. presents cross-peaks with protons resonating at both 4.66 (H-1 of Gal*p*) and 3.7 p.p.m. (possibly H-4 of α-Rha*p*). The characteristic ^{13}C chemical shifts expected for the resonances of a 2,4-disubstituted α-rhamnosyl residue [78 (C-2)[14], 82 (C-4)[12], and <70 p.p.m. (C-5)[22]] were all present in the spectrum of S200-4 (Fig. 1). The ^{13}C resonances were broad and the corresponding ^1H shifts could not be extracted from the CHORTLE spectra.

Thus, of the two types of polymer, isolated from the EDTA-extracted pectins from flax, the low molecular-weight component (probably a mixture of oligomers) had a rhamnogalacturonan I type structure with a →2)-α-Rha*p*-(1→4)-α-GalA-(1→ repeating unit with β-galactan side-chains attached to HO-4 of Rha, about two-thirds of which were 2,4-linked. The average length of the β-galactosyl side-chains was ~3 units. A rhamnogalacturonan with similar branching has been described for suspension-cultured sycamore cell walls[23–25]. The high-molecular-weight component consists essentially of a 4-linked β-galactan, a polymer which has already been isolated from primary cell walls[26–29]. The minor rhamnogalacturonan component was probably the backbone. These polymers were acetylated (10–20%).

EXPERIMENTAL

Isolation of the pectic polymer. — Pectins were extracted successively with cold water (→ p_f), boiling water (→ p_c), aq. 5% ammonium oxalate (→ p_o), and disodium EDTA (→ p_e) from flax which had been presoaked in the open for 17 d[5]. The isolation of p_e (84% yield) was achieved by size-exclusion chromatography in three successive series on columns (2.5 × 70 cm) of Sephacryl (Pharmacia) S200 and S400 with 0.2M NaCl at 120 mL/h. The separation on S200 yielded 63% of high- and 37% of low-molecular-weight polysaccharides. The latter fraction was then purified twice on S400 and the former fraction was purified twice on S200, furnishing a total of nine fractions.

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 (10–30 mg in 0.5 mL with 5-mm tubes). The pH of the S200-4 fraction was 5.3 and $(CD_3)_2SO$ was the internal reference (δ_C 39.5, δ_H 2.72). The spectral window for the ^1H-n.m.r. spectra in Figs. 1 and 2 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 μs (90°). The acquisition time was 1.11 s with a 1.5-s delay between each scan, and 20 000–50 000 transients of 64k data points were accumulated for a total acquisition time of 18–30 h.

When the ^{13}C-n.m.r. spectrum of S200-4 was recorded with a recycle time of 5.2 s, the mean variation compared to the same spectrum acquired with a typical (2.6 s) recycle time was 3% for 31 peaks. Two CHORTLE spectra using τ 0.25 and 0.75 ms were acquired in one experiment (total acquisition time of 72 h) and one CHORTLE spectrum using τ 3.2 ms was acquired in a second experiment.

Double-quantum-filtered phase-sensitive COSY and NOESY experiments were performed using (90°) – (t_1) – (90°) – (90°) – (FID, t_2) and (90°) – (t_1) – (90°) – (τ) – (90°) – (FID, t_2) sequences respectively. The mixing time (τ) in this latter experiment was 0.10 s. The spectral width in F1 and F2 was 2049 Hz; the number of data points in F2 was 2048, and 512 increments were recorded. The 90° pulse was 9 μs and the total acquisition time was 38 h. Before Fourier transformation, the data were multiplied with a $\pi/2$ shifted squared sine bell. Zero filling was applied in F1. The COSY experiment was performed using a (90°) – (t_1) – (90°) – (FID, t_2) sequence and the same spectral widths were used; the number of data points in F2 was 1024, and 512 increments were recorded. Before Fourier transformation, the data were multiplied with a $\pi/2$ sine bell, and zero filling was applied in F1.

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