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Structure of the repeating units in the rhamnogalacturonic backbone of apple, beet and citrus pectins

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Abstract

After controlled acid hydrolysis of de-esterified apple, beet and citrus pectins, a fraction rich in galacturonic acid and rhamnose was isolated and further fractionated by ion-exchange chromatography. The main peaks, representing for each pectin > 30% of the rhamnose-rich fraction, and 50–78% of its rhamnose, were composed of rhamnose and galacturonic acid in equimolar proportions. Gel-permeation chromatography showed series of homologous oligomers with polymerisation degrees of 6 to 20. NMR data indicated a repetitive structure based on the → 4)-α-D-Gal*p*A-(1 → 2)-α-L-Rha*p*-(1 → unit, where the reducing ends were rhamnose units and the non-reducing ends galacturonic acid residues. The same rhamnogalacturonan oligomers were obtained from apple, beet and citrus pectins.

Keywords: Pectin; Galacturonan; Rhamnogalacturonan; ^{1}H and ^{13}C NMR

1. Introduction

Pectins are a family of polysaccharides defined by the presence of a high proportion of galacturonic acid. One of the main components of the primary cell walls of plants, they are extracted from apple pomace and citrus peels to be used in the food industry as a gelling agent [1]. They are composed of "smooth" homogalacturonic regions alternating with "hairy" regions, the latter being rich in neutral sugars, mainly arabinose,

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galactose, and rhamnose. The homogalacturonic regions are composed of more than 100 consecutive α-(1 → 4)-linked galacturonic acid residues [2], of which the carboxylic group can be methyl-esterified in varying amounts. In addition, pectins from some plants such as beet carry acetyl groups on secondary hydroxyls of the galacturonic acids [3]. In the "hairy" regions, rhamnose residues are present in the main chain, while arabinose and galactose are present as side-chains, connected to the pectic backbone mostly through the C-4 position of rhamnose. The backbone itself is composed of 4-linked α-D-galacturonic acid and 2-linked α-L-rhamnose, mostly in an alternating sequence [4].

However, the size of oligomers presenting such an alternating sequence stays much below the length postulated for the backbone of the "hairy" regions [4,5]. In addition, most of the studies have been carried out using pectins solubilised by enzymes directly from cell walls. It is of interest to study the "hairy" regions, and notably their backbone, in commercial pectins; they add irregularities in the pectin chain that have a major role in the functional properties of these macromolecules, especially in their gelling behaviour.

We have recently used the different sensitivities to acid hydrolysis of the glycosidic linkages in pectins (GalA–GalA > GalA–Rha > Rha–GalA > neutral sugar–neutral sugar) to isolate homogalacturonic regions and estimate their minimum length [2]. The original pectins differed in their molecular weights (citrus > apple ≥ beet) and their rhamnose contents (beet > apple > citrus). We isolated from all three pectins homogalacturonans with weight- and number-average degrees of polymerisations of 72–120, 91–108 and 114–138 for apple, beet and citrus, respectively, and less than 1 mol% of rhamnose. Two more fractions were obtained during this acid hydrolysis: the neutral side-chains were hydrolysed rapidly to form low-molecular-weight oligomers, and a minor fraction, of intermediate molecular weight, rich in galacturonic acid and rhamnose, was observed. The aim of our present work was to isolate and characterise this rhamnogalacturonic fraction, and compare its structure in all three pectins.

2. Experimental

Pectins.—Commercial high methoxyl citrus and apple pectins were obtained from SBI (Beaupte, France) and Obipectin (Bischopsfell, Switzerland) respectively. Beet pectin (lot 64863-0) was obtained from Copenhagen pektinfabrik (Denmark). Oligogalacturonides and maltodextrins were from the laboratory collection.

Purification, de-esterification and acid hydrolysis.—These were carried out as described earlier [2]. After controlled acid hydrolysis (0.1 M HCl at 80°C for 72 h), acid-soluble and acid-insoluble fractions were separated by centrifugation (15 min, 30 000 *g*). The acid-soluble fraction was filtered on Millipore 3 μm membrane, neutralised (to pH 5.5) and dialysed against distilled water (theoretical cut-off of the dialysis membrane: 4–6 kDa) before being freeze-dried. An aliquot of the dialysed hydrolysate from each pectin was redialysed against 0.1 M acetate buffer, pH 4.

Chromatographic methods.—A Sephadex G-50 (Pharmacia, Uppsala) column (2.5 × 85 cm) was eluted ascendingly with 0.1 M sodium acetate buffer pH 4, and a Bio-Gel P-4 (Biorad, St Louis) column (2.5 × 90 cm) with 0.1 M sodium acetate buffer pH 3.6

[6] at 40°C with a flow rate of 24.5 mL/h. A Biorad AG 1X8 (Biorad, St Louis) column (1.6 × 54 cm) was percolated by ammonium acetate buffer pH 6 at a flow rate of 1.33 mL/min. The column was first washed with 200 mL of 0.05 M buffer, then with a gradient up to 1 M in 600 mL (beet) or 1.2 M in 730 mL (apple and citrus), followed by 200 mL at the final ionic strength. The fractions collected were assayed for uronic acid and neutral sugars using galacturonic acid and galactose (Sephadex G-50) or rhamnose standards. Peak-forming fractions were pooled and desalted either by dialysis or by filtration on a Sephadex G-10 (Pharmacia, Uppsala) column (1.3 × 80 cm) washed by water.

HPSEC was carried out on a Shodex OH-pak SB-802.5 column (8 × 300 mm) (Showa Denko K.K., Tokyo) equipped with a Shodex OH-pak SB-800-P guard column, percolated with 50 mM sodium acetate buffer pH 4 at 0.5 mL/min at room temp with refractive index detection.

Analytical methods.—All analyses were carried out as described earlier [2]. Hydrolysis for 3 h with 2 M trifluoroacetic acid at 120°C [7] was used for neutral sugar determination.

^{13}C and ^{1}H NMR spectroscopy.—^{13}C and ^{1}H NMR spectra of solutions of oligosaccharides in D_2O (15–30 mg/mL) were recorded at 80°C with a Bruker AM 500 spectrometer operating at 125.76 and 500.14 MHz, respectively. Spectral width of 25 kHz and recycling time of 1.05 s were used for ^{13}C NMR and chemical shifts were measured in ppm from the signal of external dimethyl sulfoxide and converted to values related to tetramethylsilane. A spectral width of 12 kHz and a recycling time of 1.4 s

Table 1
Balance of the repartition of the sugars during the 72-h hydrolysis of the pectic acids and dialysis of their soluble fraction [a]

	GalA	Rha	Fuc	Ara	Xyl	Man	Gal	Glc
Apple								
Pectic acid	64.1	3.1	0 [b]	1.8	2.1	0.4	9.0	13.1
Insoluble fraction	61.5	0.4	0	0	0.6	0.1	0.3	1.4
Soluble fraction								
before dialysis	7.5	2.2	0.2	1.0	1.0	0.2	6.5	10.1
after dialysis	6.0	1.1	0	0	0.2	0	0.2	0.5
Beet								
Pectic acid	60.4	6.9	0	12.3	0.4	0	15.3	0.4
Insoluble fraction	50.4	1.0	0	0.1	0.5	0	1.2	0.2
Soluble fraction								
before dialysis	12.0	6.2	0.3	8.2	0.2	0	12.1	0.3
after dialysis	9.3	2.7	0	0	0.1	0	0.4	0.1
Citrus								
Pectic acid	70.8	2.6	0.4	5.2	0.4	0.4	9.4	0.9
Insoluble fraction	63.1	0.9	0	0.1	0.1	0.1	0.3	0.1
Soluble fraction								
before dialysis	7.2	1.8	0.2	2.6	0.1	0.1	6.9	0.6
after dialysis	5.4	0.8	0	0	0	0	0.1	0

[a] All data are expressed as weight% of the original pectic acid.
[b] 0: < 0.05%.

were used for ^{1}H NMR, and chemical shifts were measured in ppm from the signal of external 4,4-dimethyl-4-silapentane-1-sulfonate.

3. Results

Initial pectic acids.—Commercial pectins were purified from low-molecular-weight contaminants by ethanol precipitation and de-esterified prior to acid hydrolysis [2]. Variations in the sensitivity to acid hydrolysis of galacturonosyl–galacturonosyl linkages were removed by ensuring that all galacturonosyl residues were de-esterified prior to hydrolysis. The de-esterification process was efficient, giving dm and da values of 1.2 and 0.1 for apple, 2 and 0.1 for citrus but still of 7.5 and 5.2 for beet (representing in that case 9 and 12 mg/g of pectic acid). The galacturonic acid contents of the pectic acids (Table 1) were 604 (beet), 641 (apple) and 708 mg/g (citrus). The high glucose content in apple pectin is due to starch, present in apples at maturation and up to 3–4 weeks after harvest [8].

Hydrolysis.—As previously, after 72 h of hydrolysis we obtained soluble and insoluble material in the reaction vials. These two fractions were separated by centrifugation, with yields of insoluble fractions of 64, 53 and 65% of the pectic acids for apple, beet and citrus, respectively, and yields of soluble fractions of 29, 39 and 20%. Some loss of sugars occurred during hydrolysis, particularly arabinose and galactose. The insoluble fractions have been characterised previously [2].

Chromatography on Sephadex G-50 of the 72-h soluble fractions (Fig. 1) showed for all three pectins two distinct fractions: a neutral fraction, eluting at the total volume, and

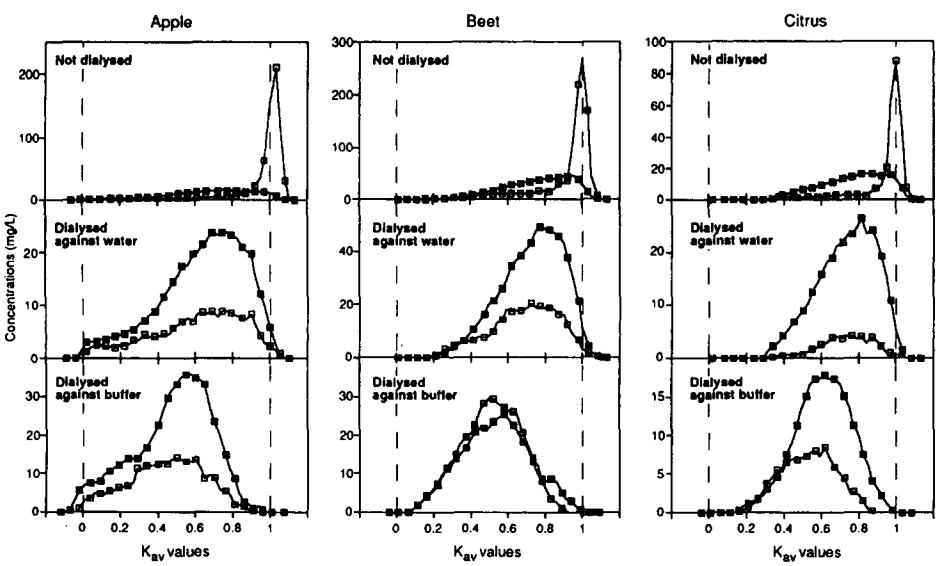

Fig. 1. Chromatography of the soluble fraction of hydrolysates (72 h, 0.1 M HCl, 80°C) from pectic acids of apple, beet and citrus, before dialysis, after dialysis against distilled water and after dialysis against acetate buffer, on Sephadex G50 eluted by 0.1 M acetate buffer, pH 4.5. □: neutral sugars; ■: uronic acids.

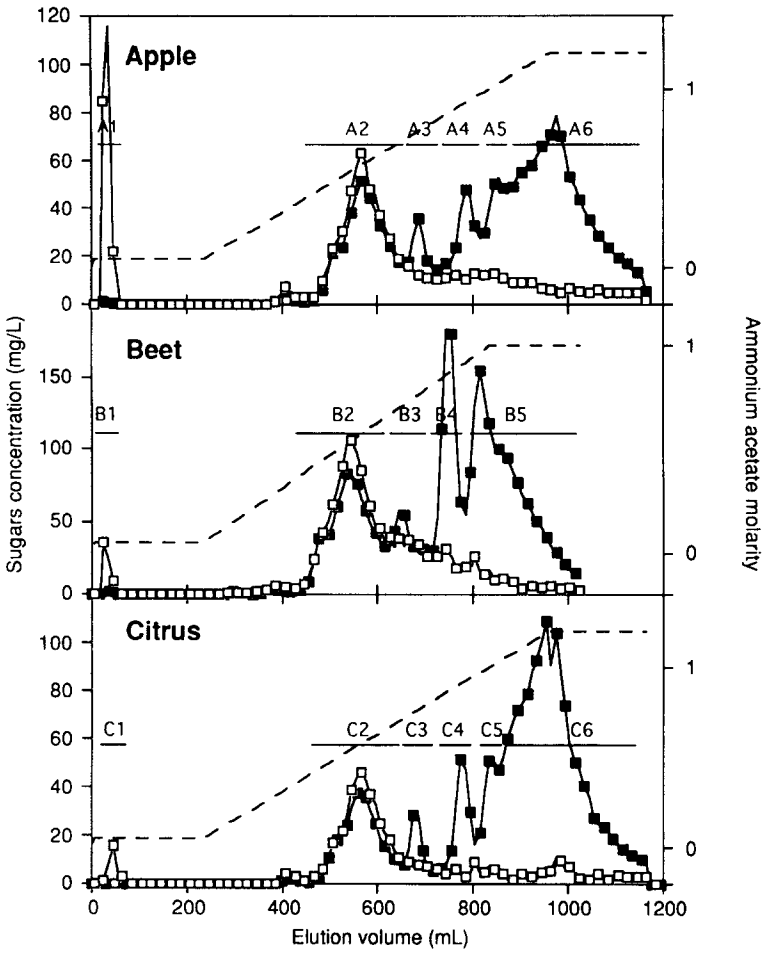

Fig. 2. Chromatography of the soluble fraction (after dialysis against water) of hydrolysates (72 h, 10.1 M HCl, 80°C) from pectic acids of apple, beet and citrus on Biorad AG 1X8 column percolated with ammonium acetate buffer, pH 6. □: neutral sugars; ■: uronic acids; --: buffer molarity.

an acidic fraction, of higher molecular weight. There was some high-molecular-weight material for apple and (in a lower proportion) for beet, while for citrus nothing was present for K_{av} values lower than 0.2. The proportion of neutral sugars in the 72-h soluble acidic fraction was lowest for citrus and highest for beet.

Dialysis against distilled water led to loss of the neutral peak at the total volume (Fig. 1). The composition of the dialysed fractions showed complete elimination of arabinose and almost total of galactose and glucose (Table 1). There was also a loss of rhamnose and galacturonic acid, probably mainly present in very small molecules (Table 1). However, the chromatograms show retention (partial?) of acidic products with low molecular weight after dialysis against water. Difficulties in dialysing oligogalacturonides against water have been reported by Mort et al. [9]: with membranes with a

Table 2
Yields and compositions of Biorad AG 1X8 fractions. For coding of the fractions see Fig. 2

Fractions	Percentage of injected material	Composition (mol%) [c]					
		GalA	Rha	Xyl	Man	Gal	Glc
Apple							
A1	10.3 [a] (2.1) [b]	0	0	0	15	14	71
A2	31.1 (31.8)	53	41	2	1	1	2
A3	7.8 (3.8)	60	31	5	1	1	1
A4	10.0 (8.8)	70	17	10	1	1	1
A5	9.4 (10.0)	81	9	8	0	1	1
A6	25.6 (25.4)	91	4	3	1	1	1
Beet							
B1	1.3 (0.7)	0	0	0	8	62	27
B2	35.5 (36.6)	56	41	0	0	2	1
B3	11.2 (12.1)	59	37	1	0	2	1
B4	16.8 (11.3)	77	20	1	0	1	1
B5	36.1 (23.4)	84	13	1	0	1	1
Citrus							
C1	1.5 (0.3)	0	0	12	34	34	20
C2	30.0 (16.0)	45	49	0	1	0	5
C3	6.1 (2.7)	44	44	4	3	1	2
C4	9.6 (4.3)	62	23	5	3	2	2
C5	9.3 (3.5)	85	9	3	0	1	1
C6	47.1 (43.9)	95	4	1	0	1	0

[a] Calculated from the colorimetric measurements of galacturonic acid and total neutral sugars.
[b] Calculated from the galacturonic acid (colorimetry) and neutral sugars (measured as alditol acetates) after desalting.
[c] Measured on desalted fractions. Fractions A1 to A4, B1 to B3 and C1 to C4 were dialysed and all other fractions were desalted on Sephadex G-10.

theoretical cut-off of 12–14 kDa only oligogalacturonides of a degree of polymerisation < 7 were more than 50% removed by dialysis after 24 h against distilled water.

Dialysis of the 72-h hydrolysates against buffer led to loss of the fraction at high K_{av} values compared to dialysis against water: the actual cut-off was then closer to the theoretical cut-off, as observed by Mort et al. [9]. There was an increase in the neutral sugars to galacturonic acid ratio but, as the loss was considerable ($> 60\%$ of the samples), we chose to continue with the samples dialysed against water.

Ion-exchange fractionation.—The three samples had similar chromatography patterns on Biorad AG 1X8 resin, with one fraction (A2, B2 or C2) containing similar amounts of neutral and acidic sugars, eluting at an ammonium acetate molarity of ~ 0.6 mol/L. This fraction represented more than 30% of the fractionated material (Table 2) and was composed of approximately equimolar amounts of galacturonic acid and rhamnose, plus minor amounts of other neutral sugars. It contained most of the rhamnose from the fractionated material: 78% for apple, 62% for beet and 50% for citrus (calculated from yields and compositions of the dialysed fractions). At higher buffer molarities, the chromatograms showed the superposition of sharp peaks for the uronic acids and a gradual decrease of the neutral sugars. These fractions contained increasing proportions

of galacturonic acid and decreasing proportions of rhamnose. For apple, xylose represented a notable part of the neutral sugars in fraction A3 to A6; for beet, rhamnose was almost the only neutral sugar. In citrus these fractions contained increased amounts of xylose but also mannose, galactose and glucose. The presence of xylose as single residues or short side-chains linked to galacturonic acid has already been detected in apple [10,11] and 3-xylopyranosylgalacturonic acid was reported for citrus pectins [12]. At maximal buffer molarity a trailing peak, almost exclusively acidic, was obtained. Only apple showed a notable non-retained fraction, mostly composed of glucose, probably from starch contamination in the original pectin. In all three cases yields (calculated from the colorimetric data) were between 97 and 108%, showing that all the injected material eluted from the column.

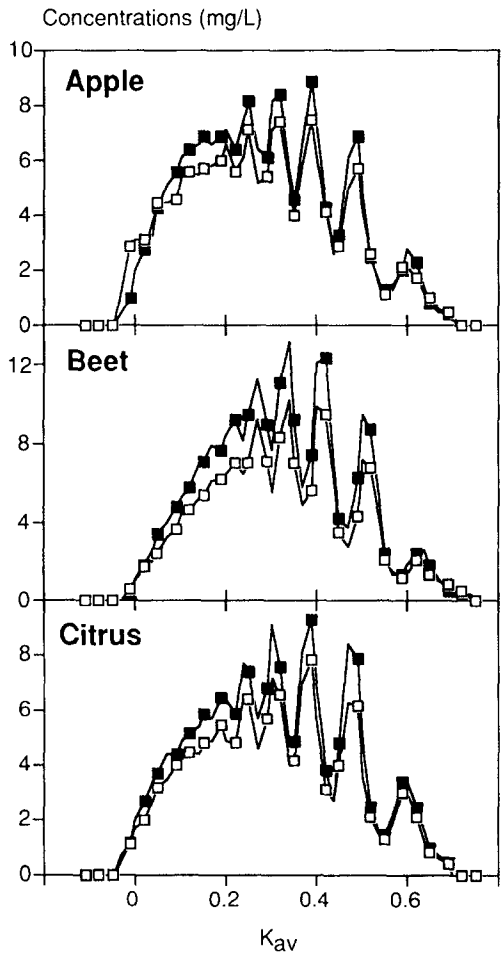

Fig. 3. Chromatography of the rhamnogalacturonic fractions (fractions A2, B2 and C2 from Fig. 2) of the soluble fraction of hydrolysates (72 h, 0.1 M HCl, 80°C) from pectic acids of apple, beet and citrus, on a Bio-Gel P-4 column eluted with 0.1 M sodium acetate buffer pH 3.6 at 40°C. □: neutral sugars; ■: uronic acids.

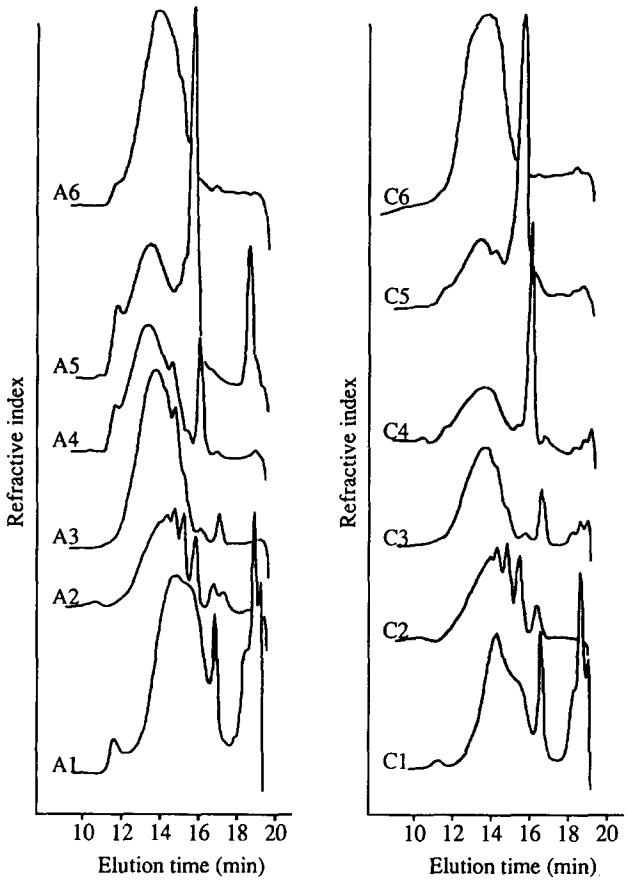

Fig. 4. HPSEC patterns of the fractions from Biorad AG 1X8 ion-exchange chromatography of apple and citrus 72-h hydrolysates on a Shodex OH-pak SB-802.5 column (eluted with 50 mM sodium acetate buffer, pH 4). The void volume of the column was at 11.7 min and its total volume at 20 min. For coding of the fractions see Fig. 2.

Molecular weight distribution in the fractions from Biorad AG 1X8.—Bio-Gel P-4 patterns of fractions A2, B2 and C2 (Fig. 3) indicated that they were composed of oligomers with constant rhamnose to galacturonic acid ratios, constituting a homologous series. The column was calibrated with oligogalacturonides and maltodextrins; molecular weights of ~ 980, 1325 and 1630 could be calculated for the first three peaks, in close agreement with molecular weights of 984, 1306 and 1628 calculated for trimers, tetramers and pentamers, respectively, of the GalA–Rha unit. Extrapolating the calibration to lower K_{av} values, rhamnogalacturonan oligomers with up to eight repeats of the GalA–Rha units gave distinct peaks.

HPSEC traces of Biorad AG 1X8 fractions from apple and citrus (Fig. 4) differed in the presence in apple fractions of material with higher molecular weight, eluting here at the void volume (elution time of 11.7 min). Fractions A2 and C2 showed peaks at 16.6, 15.7, 15.1 and 14.6 min, and the higher oligosaccharides were not separated, coalescing

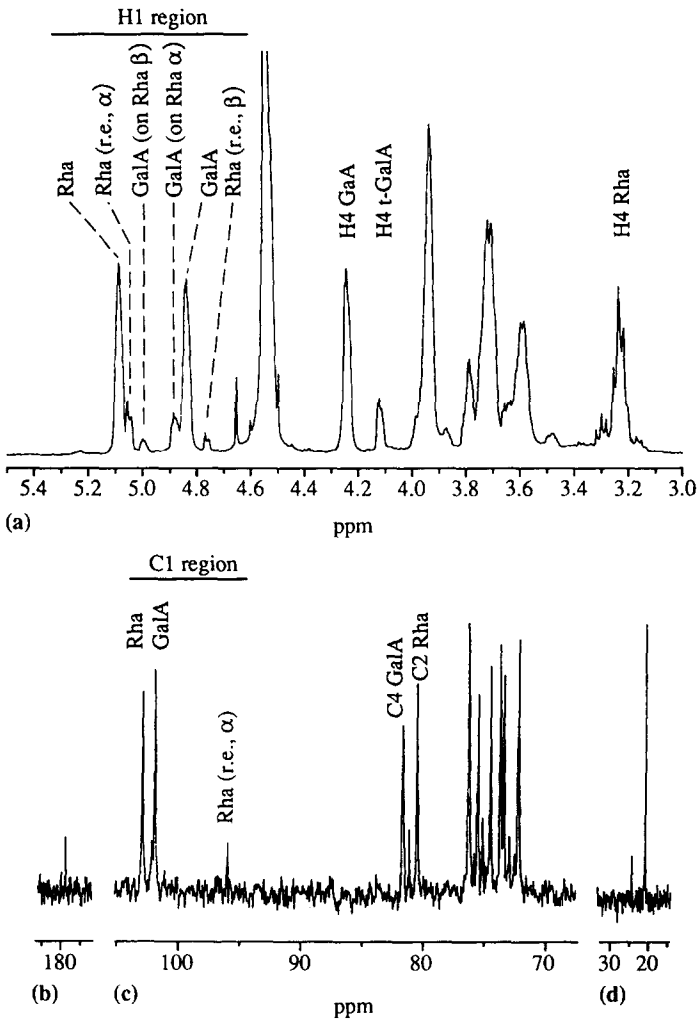

Fig. 5. NMR spectra of fraction B2: (a) ^{1}H NMR spectrum (400 mHz) H1/5 region; (b) ^{13}C NMR (100 mHZ), C-6 (GalA) region; (c) C-1/5 region; (d) C-6 (Rha) region.

in a wide peak. Fractions A6 and C6 showed only one wide peak (elution time 14 min). Fractions A3 to A5 and C3 to C5 were characterised by the superposition of a wide peak and a sharp peak, at 16.9, 16.1 and 15.6 min in fractions 3, 4 and 5, respectively (i.e. at elution times of oligogalacturonides of DP 2, 3 and 4). This reinforced the impression that those fractions contained two types of material: pure oligogalacturonides (sharp peaks observed in the ion-exchange and HPSEC chromatograms), and neutral-sugars-containing oligomers (tailing peak on Biorad AG 1X8 and wide peaks on HPSEC).

NMR spectroscopy.—The anomeric region of the ^{1}H NMR spectrum of B2 (Fig. 5) contained six signals: two main signals at 5.08 and 4.83 ppm, corresponding to the H-1 of α-rhamnose and α-galacturonic acid residues in a →4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→ chain [13,14], and four minor signals at 5.05, 4.99, 4.88 and 4.76 ppm,

attributed as shown on Fig. 5 [13,15]. Peaks at 5.05 and 4.76 were typical signals for reducing rhamnose residues, and signals for reducing galacturonic acid residues were absent [13,14]. The H-4 signals of rhamnose (3.2–3.3 ppm) confirmed the exclusive presence of unsubstituted rhamnose residues. Two signals were present for the H-4 of galacturonic acid, indicating the presence of non-reducing terminal galacturonic acid residues. Fractions A2 and C2 gave ^{1}H NMR spectra identical to that of B2. The ^{13}C spectrum of B2 comprised 12 well resolved signals, confirming the presence of a repetitive galacturonic acid–rhamnose dimer. The two main signals in the anomeric regions, at 102.8 and 101.8 ppm, were due to C-1 of α-L-rhamnose and α-D-galacturonic acid, respectively, and the minor signal at 95.9 ppm to the C-1 of a reducing-end rhamnose [16]. The other signals were similar to these reported by Coulqhoun et al. [13,14] for "B" rhamnose and "C" galacturonic acid, i.e. the internal, non substituted residues of their oligomers.

4. Conclusion

After controlled acid hydrolysis of pectic acids from apple, beet and citrus, rhamnogalacturonan fractions were obtained, which were composed of a series of linear homologous oligomers presenting a strictly alternating sequence [4)-α-D-GalA-(1 → 2)-α-L-Rha-(1], with rhamnose at the reducing end. In contrast, oligomers generated by rhamnogalacturonase [13,14] present galacturonic acid at the reducing end and their rhamnose residues are partly substituted by galactose. Oligomers with degrees of polymerisation up to about 20 (10 repeats of the GalA–Rha unit) were obtained. They represented up to 78% (apple) of the rhamnose present. We obtained similar fractions from all three pectins, confirming the fundamental common blueprints of pectins [2].

We do not know at this point whether the tailing of the neutral sugar concentration during ion-exchange chromatography corresponds to presence of oligomers with the same repetitive structure and increasing molecular weights, or to oligomers with increasing proportions of galacturonic acid. Some rhamnose was lost during dialysis: was it present as rhamnose itself, as small galacturonic acid-rhamnose oligomers, or as oligomers composed exclusively of rhamnose, that might have been generated by cleavage of regions with consecutive rhamnose residues? After 72 h of hydrolysis, it seems likely that extensive cleavage occurred in the rhamnogalacturonic areas of the pectic backbone. Though the molecular weight calculated by McNeil et al. [5] for the rhamnogalacturonan regions of pectins is likely to be overestimated, as they used globular proteins and dextran standards, the longest oligomers we generated are still very far from a length that would account for the hydrodynamic volumes of the "hairy regions" of pectins. Shorter hydrolysis times might give more indications on the length of the rhamnogalacturonic areas.

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References

[1] C.D. May, *Carbohydr. Polym.*, 12 (1990) 79–100.
[2] J.-F. Thibault, C.M.G.C. Renard, M.A.V. Axelos, P. Roger, and M.-J. Crépeau, *Carbohydr. Res.*, 238 (1993) 271–286.
[3] F.M. Rombouts and J.-F. Thibault, *Carbohydr. Res.*, 154 (1986) 189–203.
[4] A.G.J. Voragen, W. Pilnik, J.-F. Thibault, M.A.V. Axelos, and C.M.G.C. Renard, in A.M. Stephen and I. Dea (Eds.), *Food Polysaccharides,* Marcel Dekker, 1995, pp 287–339.
[5] M. Mcneil, A.G. Darvill, and P. Albersheim, *Plant Physiol.*, 66 (1980) 1128–1134.
[6] J.-F. Thibault, *J. Chromatogr.*, 194 (1980) 315–322.
[7] B. Quemener and J.-F. Thibault, *Carbohydr. Res.*, 206 (1990) 277–287.
[8] N. Gorin, F. Bonisolli, F.T. Heidema, W. Klop, and A.A.Z. Williams, *Lebensm. Unters. -Forsch.*, 166 (1978) 157–161.
[9] A.J. Mort, B. Moerschacher, M.L. Pierce, and N.O. Maness, *Carbohydr. Res.*, 215 (1991) 219–227.
[10] C.M.G.C. Renard, A.G.J. Voragen, J.-F. Thibault, and W. Pilnik, *Carbohydr. Polym.*, 16 (1991) 137–154.
[11] H.A. Schols and A.G.J. Voragen, *Carbohydr. Res.*, 256 (1994) 83–95.
[12] G.O. Aspinall, J.W.T. Craig, and J.L. Whyte, *Carbohydr. Res.*, 7 (1968) 442–452.
[13] H.A. Schols, A.G.J. Voragen, and I.J. Colquhoun, *Carbohydr. Res.*, 256 (1994) 97–111.
[14] I.J. Colquhoun, G.A. de Ruiter, H.A. Schols, and A.G.J. Voragen, *Carbohydr. Res*, 206 (1990) 131–144.
[15] P. Komalavilas and A.J. Mort, *Carbohydr. Res.*, 189 (1989) 261–272.
[16] S. Eda, K. Miyabe, Y. Akiyama, A. Onishi, and K. Kato, *Carbohydr. Res.*, 158 (1986) 205–216.