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Scarcity or complete lack of single rhamnose residues interspersed within the homogalacturonan regions of citrus pectin

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Abstract

Commercial citrus pectin containing galacturonic acid and rhamnose in a ratio of ~40:1 was saponified and then exhaustively digested with endopolygalacturonase (EPG). The products were separated by ultrafiltration into low-molecular-weight (LMW) and high-molecular-weight (HMW) fractions. The LMW fraction accounted for 80% of the starting material, but for only 10% of the total rhamnose. The molar ratio of galacturonic acid to rhamnose of the LMW fraction was 236, suggesting that very few small Rha-containing oligomers were generated by the EPG digestion. No distinct Rha-containing oligomers were found by various chromatographic analyses of the LMW fraction. The HMW fraction, which only accounted for 10% by weight of the starting pectin, contained more than 85% of the rhamnose. The ratio of GalA to Rha in the HMW fraction was 1.7:1 and partial acid hydrolysis of this fraction produced a series of oligomers consisting of GalA–Rha repeating units, suggesting that it contained rhamnogalacturonan, which has a backbone composed of GalA–Rha disaccharide repeating units. The HMW fraction also contained large amounts of arabinose and galactose, which probably originated from side chains linked to some of the rhamnose residues. We propose that commercial citrus pectin is composed of two regions: the predominant region consists of chains of uninterrupted 1,4-linked α-D-GalA residues with between 60–70% of the residues methyl esterified; and the other region consists of rhamnogalacturonan with a backbone composed of GalA–Rha disaccharide repeating units and neutral sugar side chains.

Keywords: Endopolygalacturonase; Homogalacturonan; Pectin; Rhamnose

1. Introduction

Powell et al. [1] proposed that homogalacturonan (HG) regions of several fruit pectins consist of 1,4-linked α-D-galacturonic acid residues in blocks of ~25 residues separated by single rhamnosyl residues and suggested that this would generate 'kinks' in the otherwise linear structure of the pectins. This conclusion was based on the observation that a partial acid hydrolysis of pectins, presumed to be selective for rhamnosyl linkages, generated material that eluted as a relatively

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homogeneous fraction on a Sephadex G-50 column. The chain length of this material was estimated by end-group analysis to be ~25 residues. This model has become almost universally accepted. However, other experiments in which the linkages of Rha residues in pectins were selectively cleaved, using a lower acid concentration and temperature, have generated HGs that contain less than 1% Rha and have molecular weights such that they must contain up to 200 residues [2].

We reasoned that if there are significant numbers of Rha residues interspersed within the HG region of citrus pectin, then it should be possible to generate short Rha-containing oligomers by endopolygalacturonase (EPG) digestion of saponified pectin. We have previously shown that the active site of a highly purified, commercial EPG of *Aspergillus niger* binds four contiguous, non-esterified GalA residues. The enzyme cleaves the glycosidic bond between the third and fourth residues from the nonreducing end [3,4]. Methyl esterification of any of these four residues prevents hydrolysis. However, esterification of residues outside the four that are bound to the enzyme does not inhibit activity. If a Rha residue is no more inhibitory than a methyl-esterified GalA residue, then we can predict what products are likely to be formed from an HG containing isolated, single Rha residues (see Fig. 1). Complete digestion of pectic acid with this EPG generates monomer, dimer and trimer of GalA in comparable amounts. Thus, we would expect the products of a complete digestion of saponified citrus pectin to be predominantly the monomer, dimer and trimer of GalA, plus a significant amount of oligomers containing four, five, or six GalA residues and one Rha. The expected Rha-containing oligomers were not found.

2. Experimental

Pectin and its saponification.—Commercial citrus pectin (10 g) from Sigma Co., St Louis, MO (No. 9135) was suspended in 30 mL of ethyl alcohol, and 300 mL of water was added to dissolve the pectin. Saponification of the pectin was carried out at room temperature by adjusting the pH of the solution to pH 11 with 1 M NaOH and maintaining it at pH 11 until there was no significant change for 15 min. After saponification, the pH of the solution was adjusted to pH 4 by the addition of glacial acetic acid. Almost complete de-esterification was confirmed by analysis of the degree of esterification as described previously [5].

EPG digestion of pectin.—High-purity *Aspergillus niger* EPG obtained from Megazyme Company, Australia was used without further purification. EPG (100 μL, 2830 nKat) was added to the de-esterified pectin solution, and the mixture was kept at 37 °C for 24 h. The EPG digest was centrifuged in a Sorvall RC2-B centrifuge (12 kg, 10 min) to remove the precipitate. The precipitate was then washed with water (2×50 mL) and centrifuged again. The supernatants were combined and freeze-dried for further fractionation and characterization.

Ultrafiltration of EPG-digested pectin.—The EPG-digested pectin was ultra-filtered using a

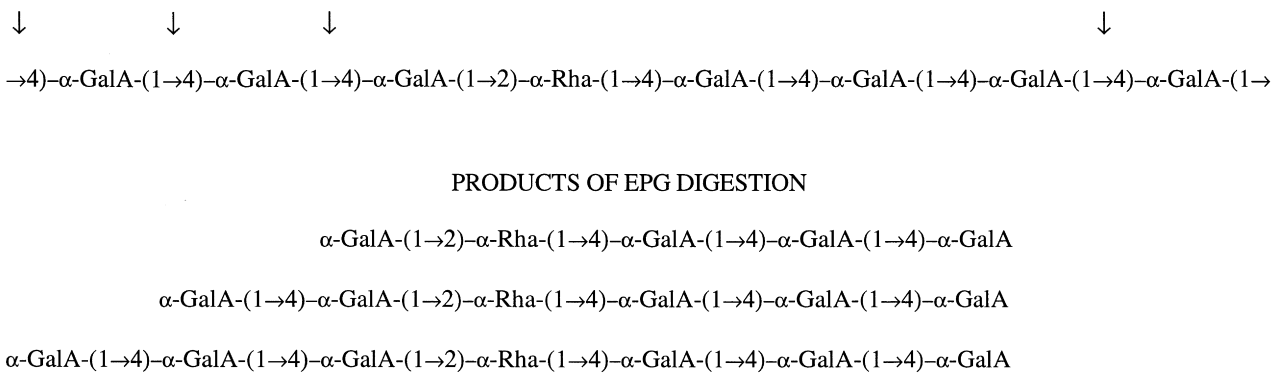

Fig. 1. Schematic diagram of the expected degradation of saponified pectin containing an isolated Rha residue by endopolygalacturonase. Arrows point out all possible cleavage sites for the enzyme. Note that on the nonreducing side of the rhamnose, cleavage at any of the closest four GalA residues would leave too short a GalA segment for further enzyme action. Since the enzyme cleaves between the third and fourth residues of the four residues to which it binds, the linkage between the GalA and Rha residues cannot be cleaved. Toward the reducing side of the Rha, the closest that the enzyme can hydrolyze the polymer would be three residues away. Cleavage four residues away would leave a four-residue stretch of GalA residues that could be cleaved by the enzyme. Thus every oligomer produced should have three GalA residues to the reducing side of the Rha.

YM10 (10 k MW cutoff) membrane (Amicon Inc., Beverly, MA) into two major fractions, a low-molecular-weight fraction (LMW) and a high-molecular-weight fraction (HMW). The LMW fraction was then dialyzed in 1 k MW cutoff tubing (Spectro/Por MWCO1000, Spectrum Medical Industries Inc., Houston, TX) to remove most of the mono- and di-galacturonic acids [6]. The material remaining in the dialysis tubing was designated the medium-molecular-weight fraction (MMW). The fractions obtained by ultrafiltration and dialysis were freeze-dried prior to further characterization.

Anion-exchange chromatography.—Anion-exchange chromatography was performed on a Dionex BioLC system (Dionex Corporation, Sunnyvale, CA) using a 15×0.9 cm column of Poros 50 HQ strong anion-exchange packing (Perseptive Biosystems, Farmington, MA), a 25×2.25 cm column of DEAE-650 S Toyopearl (Supelco Inc., Bellefont, PA), or a semipreparative 25×0.9 cm Carbopac PA1 anion-exchange column (Dionex Corporation, Sunnyvale, CA) and a continuous permanganate post-column detector [7] described previously. Gradient elution was accomplished with ammonium acetate buffer, pH 5.2, at a flow rate of 2 mL/min. The samples were injected with the column equilibrated in 30 mM buffer. After 3 min a linear gradient of from 30 mM to M ammonium acetate over 60 min was used to elute the sample.

Size-exclusion chromatography.—Samples were chromatographed on 50×2.25 cm columns packed with TSK-GEL Toyopearl size-exclusion medium (HW50S or HW55S, Supelco Inc., Bellefonte, PA) in 50 mM ammonium acetate buffer, pH 5.2, and detected with a Shodex RI-71 refractive index detector (Shodex Co., Japan). Pooled fractions were repeatedly freeze-dried to remove ammonium acetate.

High-performance capillary zone electrophoresis (HPCZE).—Oligosaccharides were labeled with 8-aminonaphthalene-1,3,6-trisulfonate (ANTS) and analyzed on a custom-built capillary electrophoresis instrument as previously described [4].

GLC analysis of sugar composition.—Samples were methanolyzed and trimethylsilylated as described by Chaplin [8] and analyzed on a Varian 3300 gas chromatograph (Varian Associates, Palo Alto, CA) using a DB-1 fused silica capillary column (30 m×0.25 mm, 0.25 μm film thickness: J&W Scientific, Inc., Rancho Cordova, CA) and inositol as internal standard [9].

NMR spectroscopy.—^{1}H NMR spectra of samples in D_2O were recorded at 30 °C on a Varian Unity Inova 400 NMR spectrometer. Chemical shifts are reported in ppm relative to the signal of HOD (4.75 ppm).

Trifluoroacetic acid (TFA) partial hydrolysis of HMW fraction.—The HMW fraction obtained by ultrafiltration was treated with 2 M TFA at 80 °C for 5 h. The hydrolyzed HMW fraction was separately chromatographed on an HW50 size-exclusion column and a PA1 anion-exchange column. Fractions obtained from the PA1 column were analyzed by CZE and NMR spectroscopy.

Matrix-assisted laser desorption time-of-flight mass spectrometry.—Oligosaccharides were dissolved in aqueous 2% acetonitrile containing 0.1% trifluoroacetic acid. This solution was mixed with an equal volume of dihydroxybenzoic acid and dried on the sample plate in air. Spectra were obtained on a Perseptive Biosystems Voyager matrix-assisted laser desorption time-of-flight mass spectrometer in the positive ion mode.

3. Results

Fractionation and quantitation of the pectin.—After EPG digestion of the saponified pectin, 10% of the material was insoluble and was removed by centrifugation. The soluble material was fractionated on a DEAE Toyopearl ion-exchange column, but this column did not give satisfactory resolution of the oligomers. Size-exclusion chromatography of the major peaks eluting from the column showed them to contain a combination of oligomeric and polymeric material. Thus, the soluble portion of the enzyme digest was separated by ultrafiltration with a 10 kD cut-off membrane into relatively high (HMW) and relatively low (LMW) molecular weight fractions. The sugar compositions of the LMW and HMW fractions, the untreated pectin, and the insoluble fraction are given in Table 1. The starting pectin had a similar sugar composition to that reported previously for citrus pectin [5]. Our data confirm the report by Thibault et al. [2] that there is insufficient rhamnose present in the pectin to give one Rha per 25 residues of GalA. The precipitate had a slightly higher ratio of GalA to Rha than the initial pectin sample and contained more noncarbohydrate material. It was not investigated further. The HMW fraction accounted for only 10% by weight

Table 1
Sugar compositions (mol percentage) of commercial citrus pectin and fractions of its EPG digest

Fraction	Weight (g)	Ara	Rha	Xyl	Man	GalA	Gal	Glc	GalA/Rha	Sugars [a] (%)
Intact pectin	10.0	2.0	2.2	0.4	0.2	88.2	6.3	0.8	40.2	54.3
Precipitate	0.9	1.7	1.8	0.4	0.2	92.7	2.5	0.8	52.8	48.4
LMW	8.0	0.6	0.4	0.2	0.1	96.4	1.6	0.6	236.0	79.4
MMW	1.4	2.6	1.4	0.5	0.1	91.2	3.4	0.5	66.9	81.8
HMW	1.0	14.1	18.6	1.4	0.5	32.0	31.8	1.5	1.7	78.5

[a] Percentage of the weight of the sample accounted for by GLC after methanolysis and trimethylsilylation. Polygalacturonic acid is known not to depolymerize completely under these conditions [18]. Thus the GalA content of the intact pectin and precipitate is probably underestimated.

of the pectin but contained more than 85% of total rhamnose. The HMW fraction had a ratio of GalA to Rha of 1.7, indicating most likely that it was predominantly rhamnogalacturonan [10,11], not homogalacturonan. The LMW material, which accounted for 80% by weight of the total pectin but only for 10% of the total rhamnose, had a very high ratio of GalA to Rha. Assuming that all the rhamnose in the LMW fraction was from EPG-digested HG, there could be, at most, only one Rha per 236 GalA residues. We conclude that most of the rhamnose is concentrated in the region giving rise to the HMW fraction rather than evenly distributed throughout the pectin.

Characterization of LMW and MMW fractions.—We have indicated that EPG treatment of HG should generate oligosaccharides containing from four to six GalA residues and one Rha residue, if the HG contains the putative isolated Rha residues. Such oligosaccharides would be expected to pass through a 10 kD cut-off ultrafiltration membrane, since GalA oligomers of up to at least 15 residues can, to some extent, pass through this membrane (see Fig. 2a). Capillary electrophoresis of the ANTS-labeled LMW fraction showed that mono-, di-, and tri-GalA were the most abundant oligosaccharides and only small amounts of longer oligomers were detected (see Fig. 2b). Since only the reducing ends of the oligosaccharides are labeled, the ratios of peak areas give an estimate of the relative molar proportions of each species detected. The $GalA_n$ oligomers (Fig. 2b) were tentatively identified by comparison of their migration rates to $GalA_n$ oligomers (Fig. 2a) prepared by autoclave hydrolysis of pectic acid [12], followed by passage of the acid-soluble fraction through the same 10 kD cut-off ultrafiltration membrane used to fractionate the EPG-digested pectin. Because the electrophoresis was carried out at pH 2.5, the charge on each oligomer was contributed predominantly by the ANTS label. Thus the rate of migration was inversely proportional to the size of the oligomer.

To enrich for oligomers over three residues in length, the LMW fraction was dialyzed against water in 1000 MW cut-off tubing. The sugar composition (see Table 1) of the retained material (MMW) indicated a significant enrichment for Rha-containing oligomers, and there was a considerable decrease in the amount of material recovered. The MMW fraction accounted for only ~17.5% by weight of the LMW fraction. The MMW fraction still contained an abundance of dimer and trimer of GalA (Fig. 2c). There were also oligomers migrating in the region anticipated for five- to seven-residue oligomers (see Fig. 2c). We estimate that these oligomers represent

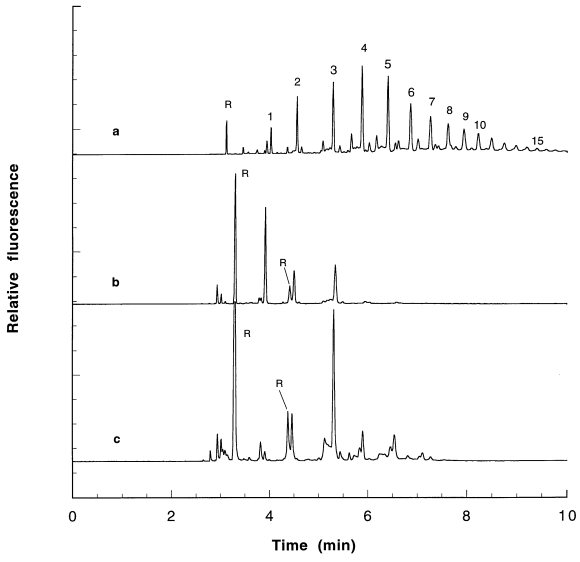

Fig. 2. Capillary zone electropherograms of ANTS-labeled (a) autoclave hydrolysate of pectic acid which passed through a 10 kD cut-off ultrafiltration membrane, (b) LMW fraction, and (c) MMW fraction. The numbers indicate how many residues of GalA were in the labeled oligomer. R indicates peaks from the reagent.

~10 mol% of the MMW fraction. The MMW fraction was chromatographed on a Dionex PA1 column as shown in Fig. 3b. Individual peaks were collected (see Fig. 3b) and tentatively identified by a combination of sugar compositional analysis, ^{1}H NMR spectroscopy, mass spectrometry, and by comparison of their elution times with standards on ion-exchange chromatography (Fig. 3a) and CZE. Table 2 summarizes the tentative identifications for the peaks in Fig. 3b. There was insufficient Rha in proportion to GalA in any of the fractions to indicate more than a trace of the Rha-containing oligosaccharides we expected. Some of the peaks were shown to contain methyl or ethyl esterified oligogalacturonides (Table 2). The ethyl esters were probably formed during the saponification of the pectin, which was performed in the presence of ethanol to aid in the suspension of the pectin during its dissolution. The presence of methyl esters showed that saponification was not complete. The PA1 chromatogram was considerably simplified after the MMW fraction was itself saponified (see Fig. 3c). As anticipated, peak 4 (the methyl and ethyl esterified tetramer) disappeared, and a significant peak for the tetramer of GalA appeared, but in addition, a peak for the pentamer of GalA became prominant. Peaks 10, 11 and 12 remained unchanged.

Partial characterization of HMW.—Since we were unable to detect oligomers containing Rha in the LMW fraction, we partially characterized the HMW fraction to determine the nature of the Rha-containing polymeric material. This fraction is unlikely to contain small Rha-containing oligomers since GalA oligomers of $\leq$15 residues pass through the 10 kD ultrafiltration membrane (see Fig. 2a). Anion-exchange chromatography of the HMW fraction on a PA1 column showed no evidence of any discrete oligomers, except for traces of the trimer and unsaturated tetramer of GalA. Most of the material eluted as a very broad peak across the region where the dimer to decamer of GalA would elute. The material also eluted from an HW-55 size-exclusion column as a broad peak in the region corresponding to pullulan standards of 180–23 kD.

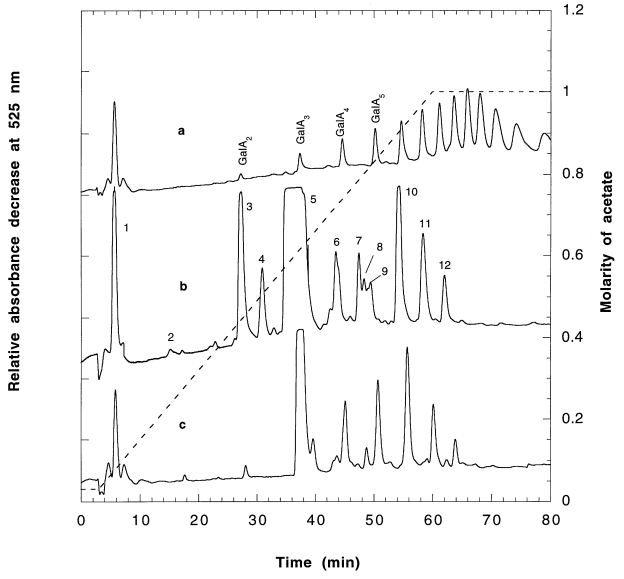

Fig. 3. HPAEC chromatograms (PA1 column) of (a) autoclave hydrolysate of pectic acid as described in Fig. 2, (b) MMW fraction, and (c) resaponified MMW fraction.

The HMW fraction was treated for 5 h at 80 °C with 2 M TFA, and the products were fractionated on an HW-50 size-exclusion column. The arabinose and galactose eluted in the included volume, indicating that the neutral sugar side chains of the HMW fraction had been hydrolyzed. Material from the center of the broad peak eluting just after the void volume was labeled with ANTS and analyzed by CZE. Two different series of peaks were detected (Fig. 4b). The faster migrating series co-migrated with the homologous series prepared from pectic acid by autoclave hydrolysis (Fig. 4a), indicating that it was composed of homogalacturonan fragments that had not been digested by EPG. The slower migrating homologous series had much wider spacings between peaks (see Fig. 4b). When the entire partial acid hydrolysate was fractionated on a PA1 anion-exchange column, two series were again apparent (Fig. 5b). The later eluting series co-chromatographed with the $GalA_n$ oligomers (Fig. 5a). However, the other series of peaks eluted much earlier from the column (see Fig. 5b). The second numbered peak of the earlier eluting series was shown by ^{1}H NMR spectroscopy (see Fig. 6), and by comparison to published spectra [13–16], to be $[GalA–Rha]_2$. The third numbered peak appeared to be $[GalA–Rha]_3$ because of the similarity of its ^{1}H NMR spectrum to that of the previous peak with the exception of the lesser intensities of the signals from the reducing Rha residue and of the two H-1 signals of the adjacent GalA residue corresponding to the two anomeric forms of the reducing Rha. We presume that the other peaks in the series contain increasing numbers of the repeating disaccharide. In the CZE experiments (e.g., Fig. 4), the oligomer migration rate was dependent on both the charge and radius of gyration of the labeled oligomer. With neutral

Table 2
Identification of PA1 column fractionated MMW

Peak	Digested fragment	GalA (mol%) [a]	GalA/Rha (molar ratio)	Identified by			
				NMR [b]	CZE	HPLC	MS
1	Neutral sugars	2.7	–	✓		✓	
2	GalA	98.0	–	✓	✓	✓	
3	$GalA_2$	99.4	–	✓	✓	✓	✓
4	Esterified $GalA_4$ [c]	100.0	–	✓			✓
5	$GalA_3$	99.6	541.0	✓	✓	✓	✓
6	Unknown [c,d]	85.7	27.1	✓			
7	Unsatd. $GalA_3$ [e]	92.6	83.3	✓			✓
8	Unknown [c,d]	93.2	31.2	✓			
9	$GalA_5$	89.7	33.5	✓			
10	Unsatd. $GalA_4$ [d,e]	96.8	92.0	✓			✓
11	Unknown [d]	98.5	104.1	✓			
12	Unknown [d]	99.0	158.8	✓			

[a] Mol percent of identified sugars.
[b] Comparison to ^{1}H NMR spectra of well characterized $GalA_n$ oligomers [19] [20].
[c] Esterification indicated by the presence of ^{1}H NMR signals at 4.95 and 4.93 ppm [20]. For peak 4, ethyl ester was indicated by a triplet at 1.32 ppm correlated only to a quartet at 4.26 ppm in the TOCSY spectrum. Methyl ester was indicated by extra signal intensity at 3.84 ppm.
[d] These appear by sugar composition and ^{1}H NMR to be oligomers of GalA. Peak 6 contains small amounts of fucose, xylose and other neutral sugars and must be a mixture of neutral sugar-containing GalA oligomers. Peaks 10, 11 and 12 give the ^{1}H NMR expected for $GalA_n$ oligomers but also have signals at 4.16, 4.19, 4.81 and 4.93 ppm indicative of the presence of an as yet unidentified component.
[e] Both ^{1}H NMR signal at 5.8 ppm [20] and the appropriate masses appearing on the mass spectra confirmed the presence of unsaturated tri- and tetra-GalAs.

sugar-derived oligomers, the charge comes only from the triply charged ANTS label. GalA oligomers, even at the pH of 2.5 used for the electrophoresis, contribute to the overall charge of the ANTS derivatives. Thus $GalA_n$ oligomers migrate faster than neutral sugar oligomers. The $[GalA–Rha]_n$ series of oligomers has a wider spacing between peaks because of the increase of two sugar residues between members, and a migration rate between that expected for a GalA oligomer and a neutral sugar oligomer because of the presence of both noncharged and charged sugar residues.

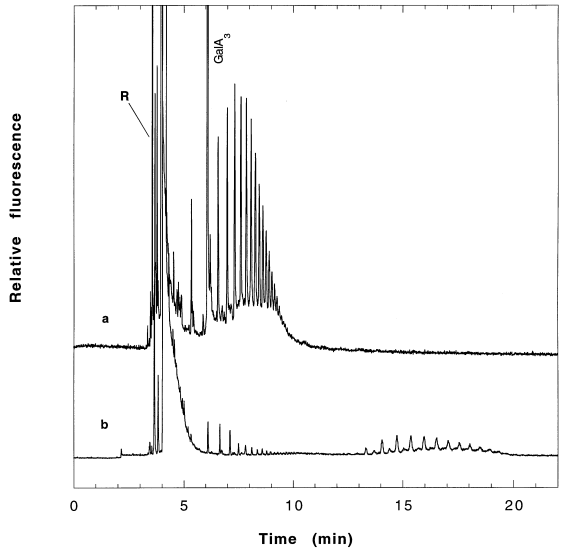

Fig. 4. CZE electropherograms of (a) autoclave hydrolysate of pectic acids soluble at pH 2 and (b) polymeric portion of partial TFA hydrolysate of the HMW fraction.

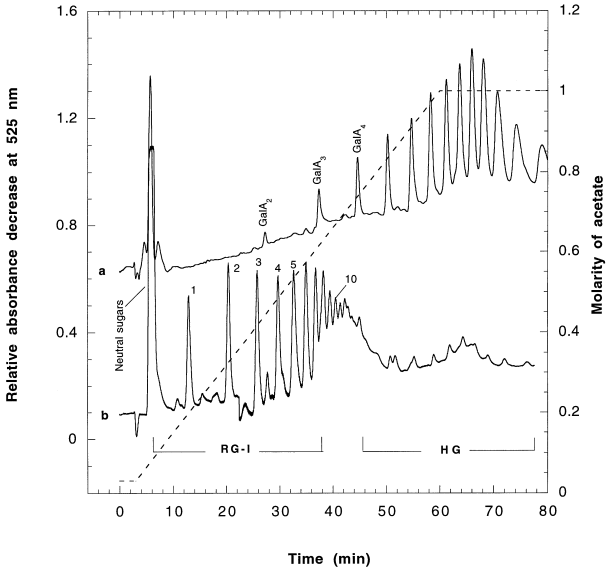

Fig. 5. HPAEC chromatograms (PA1 column) of an aliquot of (a) autoclave hydrolysate of pectic acid (b) the entire TFA partial hydrolysate of HMW. The numbers indicate the number of disaccharide repeating units in the rhamnogalacturonan fragments.

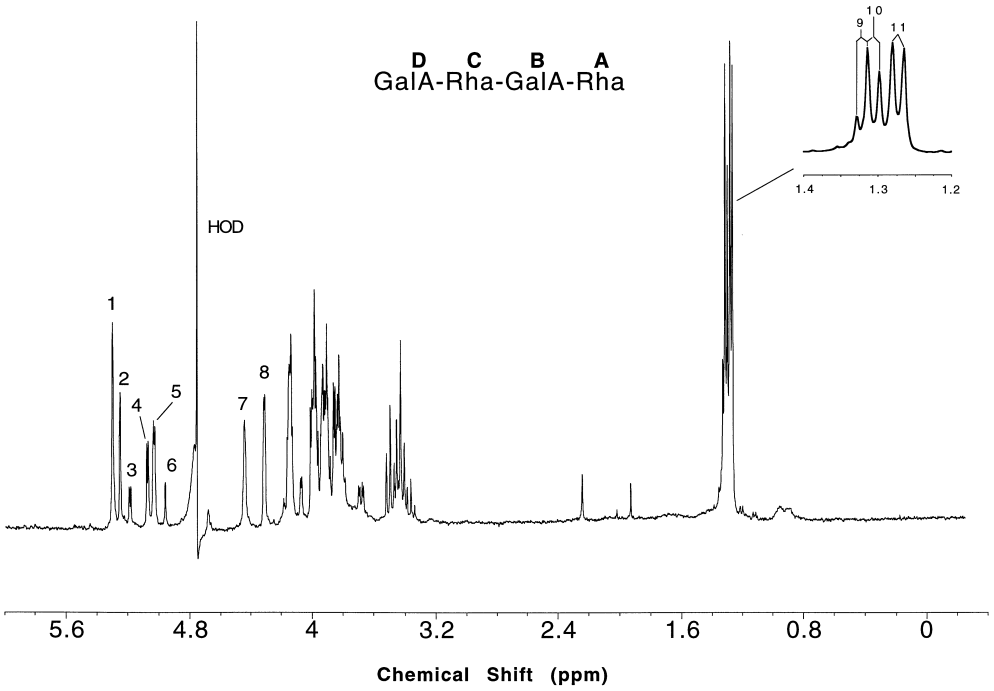

Fig. 6. ^{1}H NMR spectrum of the material in the peak numbered '2' in Fig. 5b. The numbered peaks in Fig. 6 are assigned as 1: **C** H-1, 2: **A** H-1a, 3: **B** H-1 (on Rha β), 4: **B** H-1 (on Rha α), 5: **D** H-1, 6: **A** H-1b, 7: **B** H-4, 8: **D** H-4, 9: **A** H-6b, 10: **A** H-6a, 11: **C** H-6. Bold letters refer to the individual sugar residues as indicated in the figure.

From the above results we conclude that the HMW is predominantly composed of the strictly repeating $[GalA–Rha]_n$ rhamnogalacturonan with neutral sugar side chains in combination with some HG that was not digested by the EPG.

4. Discussion

Despite the continued acceptance in the literature of the presence of isolated Rha residues within the HG regions of pectins, e.g. [17], we were unable to isolate oligogalacturonides containing single Rha residues from an EPG digest of saponified citrus pectin. We could predict which oligosaccharides should be produced and where they should elute upon high-resolution ion-exchange chromatography or CZE, but could not detect them. The sugar composition of the starting pectin indicated that there was one Rha residue for every 40 GalA residues. However, 85% of the Rha in citrus pectin was found to be present in a strictly repeating $[GalA–Rha]_n$ rhamnogalacturonan. Thus, there is only one Rha residue per 200–300 GalA residues in the HG regions. Renard et al. [15] also found that citrus pectins contain rhamnogalacturonan. It is furthermore possible that small fragments of the RG region of pectin are generated by hot acid extraction of the pectin from citrus pulp, and that these are the source of the trace amounts of Rha in the LMW fraction.

Traces of Rha have often been reported in purified GalA oligomers. These oligomers are usually prepared by partial acid hydrolysis, or by enzyme digestion of pectin, followed by ion-exchange chromatography, with no prior size fractionation. Thus, it is likely that the Rha originates from rhamnogalacturonan in the pectin. RG is heterogeneous with respect to molecular weight and side-chain composition and it elutes from anion-exchange columns over a very wide range of ionic strengths. Thus, all fractions from the column are likely to contain some RG.

The absence of isolated Rha residues in pectins has relevance to the study of the biosynthesis of pectins. It would be unusual for one glycosyl transferase to be capable of transferring a GalA to the nonreducing end of both a pre-existing chain of GalA residues and to a Rha residue. A different GalA transferase would likely be required to start the continuation of the growing HG chain after insertion of a single Rha. If a GalA transferase that were only capable of adding GalA to Rha continued the chain elongation after addition of a Rha

to the end of an HG chain, then there would be a switch to synthesis of rhamnogalacturonan. This would result in a block co-polymer with the HG region toward the reducing end and the RG region toward the nonreducing end. Alternatively, pectin chain synthesis may start as a rhamnogalacturonan, but after some time a second galacturonosyl tranferase takes over that can only transfer GalA residues to GalA residues, leading to blocks in the opposite order to the case above. In either case, this would explain the almost universal co-extraction of RG and HG regions of pectins.

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References

[1] D.A. Powell, E.R. Morris, M.J. Gidley, and D.A. Rees, *J. Mol. Biol.*, 155 (1982) 517–531
[2] J.-F. Thibault, C.M.G.C Renard, M.A.V. Axelos, P. Roger, and M.-J. Crépeau, *Carbohydr. Res.*, 283 (1993) 271–286
[3] A.J. Mort and E.M.W. Chen, *Electrophoresis*, 17 (1996) 379–383
[4] Z. Zhang, M.L. Pierce, and A.J. Mort, *Electrophoresis*, 17 (1996) 372–378
[5] N.O. Maness, J.D Ryan, and A.J. Mort, *Anal. Biochem.*, 185 (1990) 346–352
[6] A.J. Mort, B.M. Moerschbacher, M.L. Pierce, and N.O. Maness, *Carbohydr. Res.*, 215 (1991) 219–227
[7] J. Thomas and A.J. Mort, *Anal. Biochem.*, 223 (1994) 99–104
[8] M.F. Chaplin, *Anal. Biochem.*, 123 (1982) 334–341
[9] P. Komalavilas and A.J. Mort, *Carbohydr. Res.*, 189 (1989) 261–272
[10] M. McNeil, A.G. Darvill, S.C. Fry, and P. Albersheim, *Ann. Rev. Biochem.*, 53 (1984) 625–663
[11] H.A. Schols, M.A. Posthumus, and A.G.J. Voragen, *Carbohydr. Res.*, 206 (1990) 117–129
[12] B. Robertsen, *Physiol. Plant Pathol.*, 28 (1986) 137–148
[13] J. An, L. Zhang, M.A. O'Neill, P. Albersheim, and A.G. Darvill, *Carbohydr. Res.*, 264 (1994) 83–96
[14] M. Mutter, I.J. Colquhoun, H. Schols, G. Beldman, and A.G.J. Voragen, *Plant Physiol.*, 110 (1996) 73–77
[15] C.M.G.C. Renard, M. Crépeau, and J.-F. Thibault, *Carbohydr. Res.*, 275 (1995) 155–165
[16] H.A. Schols, A.G.J. Voragen, and I.J. Colquhoun, *Carbohydr. Res.*, 256 (1994) 97–111
[17] J.S. Grant Reid, *Carbohydrate Metabolism: Structural Carbohydrates*, in P.M. Dey and J.B. Harborne (Eds.), *Plant Biochemistry*, Academic Press, San Diego, 1997, pp 205–236.
[18] B. Quemener, J.-F. Thibault, C. Brunet, and M.-J. Crépeau, *Carbohydr. Res.*, 190 (1989) c7–c10
[19] V.-M. Lo, M.G. Hahn, and H. van Halbeek, *Carbohydr. Res.*, 255 (1994) 271–284
[20] S.B. Tjan, A.G.J Voragen, and W. Pilnik, *Carbohydr. Res.*, 34 (1974) 15–23