ELSEVIER

Carbohydrate Research 279 (1995) 265–279

CARBOHYDRATE RESEARCH

A xylogalacturonan subunit present in the modified hairy regions of apple pectin ☆

Henk A. Schols [a], Edwin J. Bakx [a], Dick Schipper [b], Alfons G.J. Voragen [a,*]

[a] *Department of Food Science, Wageningen Agricultural University, Bomenweg 2, 6703 HD Wageningen, The Netherlands*

[b] *Royal Gist brocades, P.O.box 1, 2600 MA Delft, The Netherlands*

Received 1 March 1995; accepted 3 August 1995

Abstract

The high molecular weight fraction (fraction A) of the modified hairy regions (MHR) from apple cell walls was treated by rhamnogalacturonase (RGase) after saponification and after deacetylation by rhamnogalacturonan acetylesterase (RGAEase). Three fractions could be recognized by size-exclusion chromatography: rhamnogalacturonan oligomers, residual stubs of the rhamnogalacturonan backbone rich in arabinan side-chains, and a fraction rich in xylose and galacturonic acid. The "xylogalacturonans" obtained after saponification and after enzymic deacetylation had rather similar sugar compositions (xylose:galacturonic acid ratios of 0.4–0.9) and molecular weights. After saponification, the xylogalacturonan was eluted as a single peak on anion-exchange chromatography whereas three peaks were obtained when the MHR was deacetylated by RGAEase, indicating variations in the degrees of methylation. One of the methyl-esterified xylogalacturonan fractions was characterized by NMR spectroscopy: the xylose residues were β-(1 → 3)-linked to some of the galacturonic acid residues within a rather high molecular weight xylo-(1 → 4)-α-galacturonan and a degree of methylation of 39 was calculated. The methyl esters were found to be equally divided between the substituted and unsubstituted galacturonosyl residues.

Keywords: Pectin; Xylogalacturonan; Rhamnogalacturonan; Enzymic degradation

☆ Hairy (Ramified) Regions of Pectins, Part VII. For Part VI, see ref. [39].

* Corresponding author.

SSDI 0008-6215(95)00287-1

1. Introduction

Pectic substances, which represent one of the most important plant cell-wall polysaccharides, have been the subject of many studies but still little is known about their fine structure. De Vries et al. [1] proposed a model in which pectin consists of alternating "smooth" homogalacturonan regions and highly branched "hairy" or ramified regions. Starting from apple juice obtained by an enzymic liquefaction process, we have described [2] the isolation of a pectic fraction having similar characteristics to the hairy regions previously described by De Vries et al. [1]. Since the apple juice hairy regions might have been altered by the action of enzymes present in the technical enzyme preparation, the pectic fraction was designated as modified hairy regions (MHR) [2]. The structure of the MHR could be described in more detail by making use of the enzyme rhamnogalacturonase (RGase) [3] which is able to hydrolyze specifically galactopyranosyluronic–rhamnopyranosyl linkages. MHR consists of the same sugar residues having the same type of sugar linkages as described for rhamnogalacturonan I (RG-I), isolated, for example, from suspension-cultured sycamore cells [4]. However, our modified hairy regions were judged to differ from RG-I since MHR contained a higher proportion of galacturonic acid as compared to rhamnose, whereas RG-I was suggested to consist of strictly alternating rhamnose and galacturonic acid residues [4]. Pectic molecules like the (modified) hairy regions [1,2,5–8] and RG-I [4] have been isolated from many sources and seem to be ubiquitous in plant cell-wall material. A variety of oligomers derived from these types of pectic polymers, consisting of alternating rhamnose and galacturonic acid residues, have been reported [8–14]; side chains of arabinose and/or galactose residues, mainly attached to the rhamnose residues, might be present.

Although many different oligomers have been identified, there is still a lack of information on the sequences of the oligomers within the pectic molecule, since chemical hydrolysis and fragmentation reactions are commonly hampered by poor selectivity. More and more, enzymes are being used as analytical tools in the elucidation of the fine structure of polysaccharides [14–16]. The availability of pure, well-characterized enzymes active toward pectic hairy regions or RG-I type of polysaccharides is increasing and includes rhamnogalacturonases [3,17], rhamnogalacturonan acetylesterase [18], and rhamnogalacturonan rhamnopyranohydrolase [19], in addition to enzymes acting on the side chains of the pectic molecules like arabinanases [20] and galactanases [19,21].

Based on RGase degradation studies, Voragen et al. [15] suggested the existence of three different subunits within ramified pectic molecules extracted by a technical enzyme preparation: a xylogalacturonan unit, residual stubs of the pectic backbone containing arabinan side chains, and rhamnogalacturonan chains of unknown length.

This paper deals with the isolation of the three different subunits and further characterization of the xylogalacturonan subunit. The substitution pattern of xylose residues and methyl esters over the galacturonan backbone could be established by 2D NMR spectroscopy.

2. Experimental

Substrate.—Modified hairy regions (MHR) were isolated from apple and fractionated by size-exclusion chromatography into fractions A, B, C, as described previously [2]. MHR fraction A was used for further studies. Prior to enzymic degradation with RGase, it was first saponified [2]. For this purpose, MHR (25 mg) was treated with 0.1 M NaOH (1 mL; 0°C) for 16 h, followed by neutralization (1 mL of 0.1 M AcOH), and dilution with buffer (9 mL of 0.05 M NaOAc pH 5.0).

Enzymic degradation of MHR fraction A.—Saponified MHR fraction A in NaOAc buffer (1 mL) was incubated with RGase [3] (33 μL; ca. 146 ng of protein), purified from *Aspergillus aculeatus*, for 16 h at 30°C. The enzyme was inactivated by heating (5 min, 100°C) and the digests were analyzed by high-performance size-exclusion chromatography (HPSEC) and high-performance anion-exchange chromatography (HPAEC). For semi-preparative fractionation of the digest, 350 mg of apple MHR fraction A (saponified) (0.4% in 0.05 M NaOAc pH 5.0) was incubated for 20 h using 2 mL of RGase.

Alternatively, 150 mg of apple MHR fraction A (0.04% in 0.05 M NaOAc pH 5.0) was directly incubated for 18 h at 40°C with 1 mL of RGase in combination with 10 μg of rhamnogalacturonan acetylesterase [18] (RGAEase; kindly provided by Novo-Nordisk, Denmark).

Analytical methods.—The neutral sugar composition of all fractions was determined after hydrolysis with 2 M trifluoroacetic acid (1 h, 121°C) [5] or with 1 M H_2SO_4 (3 h, 100°C) without pre-hydrolysis as described [2]. Uronic acid content was determined colorimetrically using *m*-hydroxybiphenyl as described [2].

Chromatography.—HPSEC was performed on three Bio-Gel TSK columns in series (40XL, 30XL, and 20XL) as described [2]. For HPAEC analysis, a Dionex Bio-LC system which included a quaternary gradient pump, eluent degas (He) module, and pulsed electrochemical detector (PED) in the PAD mode, completed with a Spectra Physics SP8800 autosampler and a Spectra Physics Winner data handling system, was used as described [13]. A CarboPac PA100 column (4 × 250 mm) with guard column (Dionex) was used at a flow rate of 1.0 mL/min. The gradient was obtained by mixing solutions of 0.1 M NaOH and M NaOAc in 0.1 M NaOH. After equilibration for 15 min with 0.1 M NaOH, 20 μL of the sample was injected and a linear gradient to 0.43 M NaOAc in 0.1 M NaOH within 35 min was started. Finally, the column was washed for 5 min with M NaOAc in 0.1 M NaOH.

Size-exclusion chromatography was performed on a column (105 × 26 mm) of Sephacryl S200 (Pharmacia; separation range for dextrans 10^3–8×10^4 Da) or Sephacryl S300 (separation range for dextrans 2×10^3–4×10^5) using a Hiload System (Pharmacia) and 0.1 M NaOAc (pH 5.0) as eluent. Fractions (2.5 mL) were collected and analyzed for neutral sugars and uronides as described [2]; arabinose and galacturonic acid were used as standards, respectively. Pooled fractions were dialyzed and lyophilized before analysis.

Anion-exchange chromatography was performed on a column (250 × 20 mm) of DEAE-Sepharose Fast Flow (Pharmacia). Sample (18–25 mg) was applied on the column at a flow of 1 mL/min (0.005 M NaOAc, pH 5.0); after 60 min the flow was

increased to 5 mL/min and a gradient (in 80 min) to 1 M NaOAc (pH 5.0) was started. Elution with 1 M NaOAc was continued for another 8 min, followed by a gradient up to 2 M NaOAc in 8 min. Fractions (5 mL) were collected and analyzed as described. Pooled fractions were dialyzed and lyophilized before analysis.

NMR spectroscopy.—All of the NMR experiments were performed on a Bruker AMX-600 spectrometer at 350–370 K. Both the homonuclear and heteronuclear experiments were performed using a 5-mm triple resonance probe equipped with gradients. The polysaccharide fraction RGase/RGAEase S300 I-*c* (see Fig. 4; ca. 6 mg) was dissolved in D_2O (0.5 mL). In each experiment except for the HSQC experiment, the residual HDO signal was saturated during the 0.5-s recycle delay. Phase cycling was applied for phase-sensitive detection [22]. The ^1H spectral width in all the homonuclear spectra was 4000 Hz. The TOCSY experiments involved the clean-TOCSY sequence using the MLEV17 sequence [23] for isotropic mixing; the delays during the mixing time were chosen equal to the 90° pulse width for the powerlevel of the spin-lock, which was 20 μs. The total spin-lock mixing time in the TOCSY experiment ranged from 17 to 120 ms. The mixing time in the NOESY and ROESY experiment was 100 ms. For all the homonuclear 2D experiments, a total of 256 1K FIDs, 400 scans each, were collected, zero-filled once in the F2 dimension and twice in the F1 dimension. A squared sine-bell function shifted by $\pi/3$ was applied in both dimensions. In the HSQC experiment, the ^1H spectral width was 18,500 Hz (oversampling) and the ^{13}C spectral width 9300 Hz, and ^{13}C decoupling during acquisition was achieved by the GARP-1 scheme [24]. Residual water signal and artefacts were suppressed by using three gradients as described [25,26]; 256 4K FIDs of 480 scans were collected with a recycle delay of 0.95 s, and the zero filling and digital filtering was identical to the homonuclear experiments. The ^1H chemical shifts were referenced to external sodium 3-trimethylsilylpropionate-d_4 (TSP) via the residual solvent signal, taking into account the temperature dependence of this reference [27]. The ^{13}C chemical shifts refer to dioxane in D_2O at 69.43 ppm, using the spectrometer frequency generating system as a secondary reference.

3. Results

Degradation of MHR fraction A.—MHR was shown [2] to consist of three fractions of polysaccharides differing in molecular weight. Degradation studies of MHR by RGase were hampered by the finding that degradation products of the high molecular weight fraction A were co-eluted with non-degraded molecules of the fractions B and C. For this reason, we only used the most important fraction A in this study. Previous studies also revealed that the enzyme RGase is hindered by the presence of ester groups; therefore MHR fraction A was saponified prior to enzymic degradation. After degradation by RGase, the molecular weight of the saponified fraction A (Fig. 1) shifted completely to lower molecular weight values within 16 h.

Size-exclusion chromatography of RGase digests.—The incubation mixture of MHR fraction A was separated by SEC on a Sephacryl S200 column. Due to the fact that Sephacryl S200 covered a relative narrow range, resulting in a better resolution, three

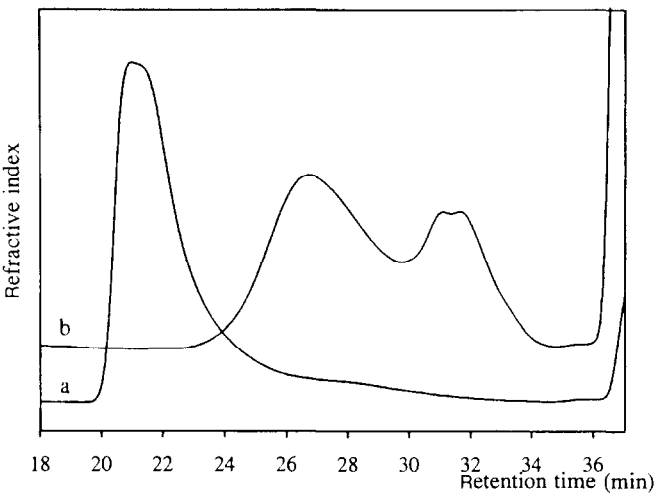

Fig. 1. High-performance size-exclusion chromatography elution pattern of saponified MHR fraction A, before (a) and after (b) treatment with RGase at 30°C for 16 h.

different peaks could be distinguished (Fig. 2), contrary to HPSEC analysis (Fig. 1) which displayed only two fractions. The fractions were pooled and analyzed for their sugar composition (Table 1). Xylose and galacturonic acid were the main sugars in the void fraction; together they accounted for ca. 80% of the sugar residues in this fraction. This is in sharp contrast with the starting material in which arabinose prevails, while xylose and galacturonic acid only make up 35% of the constituent sugars. Minor constituent sugars in fraction I were rhamnose, arabinose, and galactose. The glucose determined in the fractions I–III could not be detected in the starting material. Since

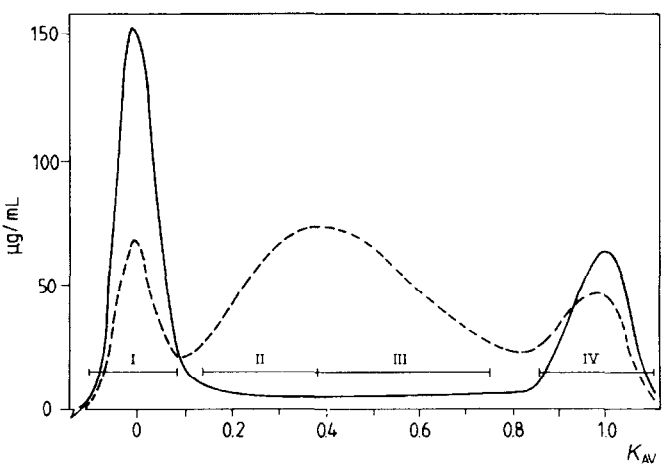

Fig. 2. Size-exclusion chromatography on Sephacryl S200 of MHR fraction A after degradation with RGase: —, uronic acids; ---, neutral sugars.

Table 1
Sugar composition (mol%) of MHR fraction A and fractions I–IV, obtained after Sephacryl S200 size-exclusion chromatography of the digest of MHR fraction A with RGase

Sugar	MHR fr.A	I	II	III	IV
Rha	5	4	1	3	23
Ara	50	8	84	81	11
Xyl	11	38	3	1	0
Man	0	0	0	0	0
Gal	10	4	3	5	29
Glc	0	5	3	3	0
GalA	24	41	6	7	37
dm	28				
da	55				
Xyl:GalA	0.5	0.9	0.5	0.1	0

70% of the xylose residues present in the original MHR fraction were terminally linked, while 20% of all galacturonic acid residues were branched through C-3 [2], it was assumed that this fraction I represents xylogalacturonan-rich molecules.

Fractions II and III covered a broad M_w range and both consisted of arabinose-rich polysaccharides (> 80% arabinose). They contained minor amounts of rhamnose, xylose, galactose, and galacturonic acid with rhamnose to galacturonic acid ratios (varying from 1:6 to 1:2.3) characteristic of a rhamnogalacturonan backbone.

Fraction IV (Fig. 2) represented the oligomeric material eluting at 31–33 min in Fig. 1. These oligomers, analyzed by HPAEC, were similar to the characteristic oligomeric reaction products of RGase [13,28], which consisted of a backbone of alternating rhamnose and galacturonic acid residues in the backbone (4–6 residues) with galactose residues connected to some or all of the rhamnose residues. There were some small differences in the relative amounts of the various oligomers compared to the digest of the whole MHR [13]: in the RGase digest of MHR fraction A, the hexamer $Rha_2GalA_2Gal_2$ and octamer $Rha_3GalA_3Gal_2$ were more evident, while the RGase-oligomers without, or with only one, galactose substitution were present in relatively low concentrations.

To facilitate the isolation of appropriate amounts of the xylogalacturonan fraction, a new and larger batch of saponified fraction A was incubated with RGase and separated on a Sephacryl S300 column. As expected, a better separation between fractions I and II was obtained; however, the resolution between fractions III (arabinose-rich molecules) and IV (the RGase oligomers) decreased (not shown).

Since MHR fraction A is rich in *O*-acetyl groups [2] (55 mol of acetyl per 100 mol of galacturonic acid), while a considerable number of methoxyl groups are present (28 mol per 100 mol of galacturonic acid), experiments were also carried out using RGAEase to remove the *O*-acetyl groups specifically. Prior to use, the RGAEase preparation was examined carefully for side activities on MHR, but no activity other than acetyl release was found. The degradation of MHR fraction A by RGase and RGAEase as monitored by HPSEC was rather slow (presumably caused by the limiting amount of RGAEase) although the expected HPSEC pattern as in Fig. 1 was obtained. Fractionation of the

RGase/RGAEase digest on Sephacryl S300 also gave results similar to those of the RGase digest after saponification.

It should be stated that the elution behaviour of the degraded MHR fraction A (both saponified or RGAEase-treated) was not always consistent. Sometimes, HPSEC analysis of an RGase digest of MHR fraction A revealed that all polymeric material was degraded to smaller fragments, while after inactivation of the enzyme (5 min, 100°C), a high molecular weight fragment (retention time, 20 min) could be observed by HPSEC analysis. We observed that solutions containing degradation products rich in xylose and galacturonic acid residues (i.e., xylogalacturonan) were prone to aggregation (as monitored by HPSEC) and even precipitation sometimes occurred. Although considerable effort was directed to solve these problems, the only way to prevent these undesirable effects was to run the experiments, chromatographic separations, etc., well standardized in a tight time schedule.

Anion-exchange chromatography of the xylogalacturonan fractions.—To check whether the xylogalacturonan fractions of the Sephacryl S300 column were homogeneous according to charge, the pools I were further fractionated on a DEAE Sephacryl Fast Flow column. All carbohydrates were eluted essentially in a single peak (Fig. 3), showing there were no major differences in charge between the various molecules. Pool RGase S300-I appeared therefore to be rather homogeneous, both in molecular weight and charge density, suggesting the presence of only one type of polymer. Compositions of the DEAE-fractions of RGase S300-I were compared to that of the starting material (Table 2). The composition of both pool I-*a* and I-*b* indicates the presence of a xylogalacturonan polymer, although small proportions of rhamnose, arabinose, galactose, and glucose were found to be present. The relatively higher content of galacturonic acid in pool I-*b* might explain the longer retention on the DEAE material.

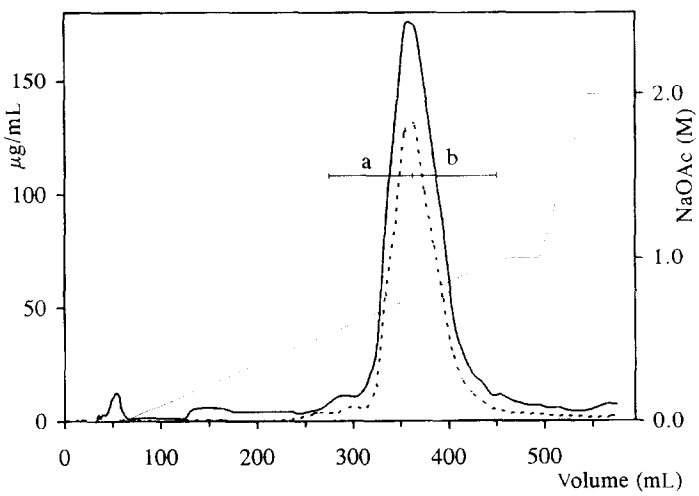

Fig. 3. Anion-exchange chromatography on DEAE Sepharose of fraction I of the Sephacryl S300 fractionation of saponified MHR fraction A after degradation with RGase: —, uronic acid; ---, neutral sugars; thin line, NaOAc gradient.

Table 2
Sugar composition (mol%) of fraction RGase-I obtained after chromatography over Sephacryl S300 of the RGase digest of apple MHR fraction A, and fractions I-*a* and I-*b* obtained after DEAE Sepharose anion-exchange chromatography

Sugar	RGase S300-I	I-*a*	I-*b*
Rha	3	3	2
Ara	6	5	5
Xyl	28	34	25
Man	1	2	3
Gal	4	3	3
Glc	3	1	4
GalA	55	52	58
Xyl:GalA	0.5	0.7	0.4

When the xylogalacturonan fraction, obtained from the RGase/RGAEase-treated MHR fraction A, was chromatographed over a DEAE column, a different elution pattern was obtained (Fig. 4). At least three distinct fractions could be recognised, of which the last fraction, representing 68% of the polysaccharides, was still much less retained on the anion-exchange resin than the xylogalacturonan obtained from the saponified MHR fraction A (eluted at 0.5 M and 0.75 M NaOAc, respectively; Fig. 3 vs. Fig. 4). The sugar composition of the pools (Table 3) confirmed that the same type of material was isolated as before. The starting material (RGase/RGAEase pool I) was rather similar to the corresponding fraction obtained from the saponified material (RGase pool I; Table

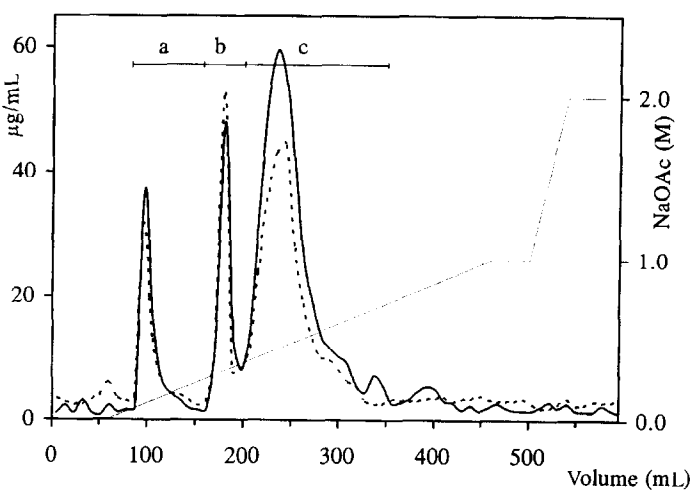

Fig. 4. Anion-exchange chromatography on DEAE Sepharose of fraction I of the Sephacryl S300 fractionation of MHR fraction A after degradation with RGase and RGAEase: —, uronic acid; ---, neutral sugars; thin line, NaOAc gradient.

Table 3
Sugar composition (mol%) of fraction RGase/RGAEase-I obtained after chromatography over Sephacryl S300 of the RGase/RGAEase digest of apple MHR fraction A, and fractions I-*a*, I-*b*, and I-*c* obtained after DEAE Sepharose anion-exchange chromatography

Sugar	RGase/RGAEase I	I-*a*	I-*b*	I-*c*
Rha	4	3	3	5
Ara	11	11	14	6
Xyl	34	26	22	34
Man	1	4	2	1
Gal	5	5	4	4
Glc	2	9	5	3
GalA	44	42	50	47
Xyl:GalA	0.8	0.6	0.4	0.7

2). The most important differences were the relatively higher arabinose and xylose content and the lower galacturonic acid content in pool RGase/RGAEase I, which also resulted in a somewhat higher xylose:galacturonic acid ratio. The three fractions obtained after DEAE-column chromatography of pool RGase/RGAEase I were all rich in xylose and galacturonic acid residues (together responsible for ca. 70% of all sugars), in addition to various amounts of other sugars. The glucose, found in all fractions, was not detected in the starting MHR fraction A and might originate from the column material. The DEAE-pools RGase/RGAEase I-*a* and I-*b* were enriched in arabinose as compared to pool I-*c*, while the xylose content varied slightly. Unfortunately, not enough material was obtained to determine the amount of methyl ester groups and to differentiate further between the three pools. Since pool RGase/RGAEase I-*c* appeared to be the most important pool representing a homogeneous and rather pure xylogalacturonan, which is still methyl-esterified, this fraction was chosen for further characterization by NMR spectroscopy.

Xylogalacturonan structure from NMR spectra.—In Fig. 5 the ^{1}H (top) and 2D $^{1}H/^{13}C$ HSQC spectra are shown. The broad lines in the region 5.3–2.8 ppm in these complex spectra suggest that there is a high molecular weight carbohydrate polymer present, apart from some low molecular weight impurities. Due to the much smaller line-width of low molecular weight compounds, the contaminating lactic acid (ca. 5–10%; probably introduced during dialysis) seems to dominate the spectra. Despite the high molecular weight of this complex material, the relative good quality of the TOCSY (spectra not shown) and ROESY spectra and the good dispersion in the HSQC spectrum allowed us to assign the ^{1}H and ^{13}C chemical shifts of the main components in this xylogalacturonan fraction. The assignment was much complicated by the presence of methyl esters at part of the galacturonosyl residues, since the $^{1}H/^{13}C$ signals of C-1 and C-5 are split into at least four resonances [29]; the same holds for C-4 [30]. These multiple resonances are caused by the fact that the difference in shielding by a free carboxyl group and an esterified carboxyl group is also felt in preceding and following monomeric units [29]. The degree of methylation (mol of methanol per 100 mol of galacturonic acid residues) was determined by integration of the C-5/H-5 resonances in

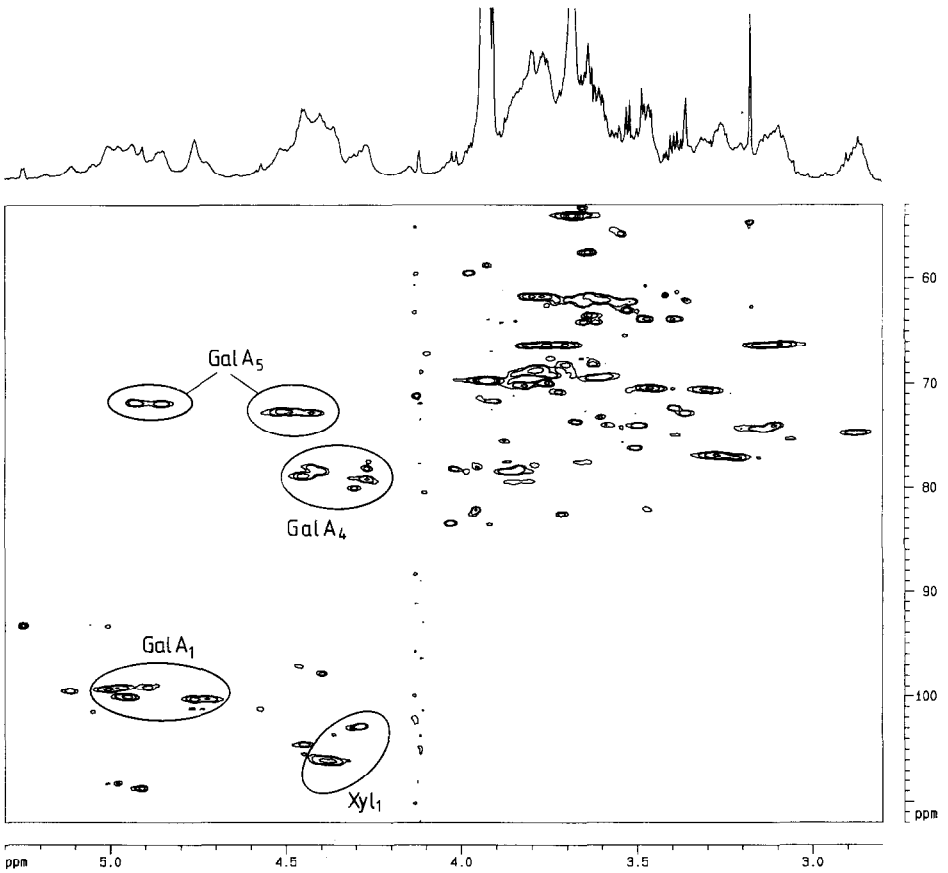

Fig. 5. {^{1}H,^{13}C} HSQC spectrum of a partially methyl-esterified xylogalacturonan fraction (RGase/RGAEase S300-I-*c*) with high-resolution ^{1}H spectrum on top.

the HSQC spectrum and a dm value of 39 was calculated. From the ^{1}H and the HSQC spectra, it could be concluded that no *O*-acetyl groups were present in the fraction investigated.

After assignment of the monomeric units, the type and substitution pattern of the different residues can be deduced from the ^{13}C chemical shifts by comparison with model compounds [31]. The ^{13}C chemical shifts of Xyl^{a} and Xyl^{b} (Table 4) only differ from those of monomeric xylose at C-1, indicating that xylose is mainly terminally linked. The ^{13}C chemical shifts of the galacturonic acid residues differ from the chemical shifts of free galacturonic acid (apart from C-1) at C-4. Galacturonic acid residue GalA^{b} also shows a downfield shift of ca. 8 ppm at C-3, indicating the presence of a substituent at this position. From 2D NOESY (spectrum not shown) and ROESY experiments (Fig. 6), it was established that a β-xylose residue was linked to some of the galacturonosyl residues at O-3. The resonances of the C-2/H-2 of xylose are also

Table 4
^{13}C and ^{1}H chemical shifts (ppm) for monomeric residues [a] of galacturonic acid and xylose as found for the partially methyl-esterified xylogalacturonan fraction (RGase/RGAEase S300-Ic)

Atom no.	GalA[a]		GalA[b]		GalA[monomer]	
	C	H	C	H	C	H
1	99.1;100.0;99.1 [b]	5.10;4.95;4.89	100.2	4.75	92.9	5.21
2	69.4	3.61	70.2	3.82	68.7	3.72
3	68.7	3.79	78.2	3.85	70.1	3.82
4	79	4.4	78.4	4.4	71.5	4.18
5	71.8*;71.8* [d]; 72.7**;72.7**	4.93*;4.85*; 4.52**;4.48**	[c]	[c]	72.2	4.31

Atom no.	Xyl[a]		Xyl[b]		Xyl[c]		Xyl[monomer]	
	C	H	C	H	C	H	C	H
1	105.9	4.40	106.0	4.36	102.8	4.21	97.3	4.54
2	74.3	3.15	74.7	2.88	74.0	3.11	74.9	3.20
3	77.1	3.22	76.9	3.28	76.9	3.28	76.6	3.40
4	70.4	3.47	70.5	3.30	70.4	3.47	70.0	3.60
5	66.2	3.70;3.10	66.2	3.70;3.10	66.2	3.78;3.14	65.9	3.90;3.30

[a] GalA[a], (1 → 4)-linked galacturonosyl residue. GalA[b], (1 → 4)-linked galacturonosyl residue, branched at C-3. Xyl[a], xylose residue, linked to a non-esterified galacturonosyl residue. Xyl[b], xylose residue, linked to an esterified galacturonosyl residue. Xyl[c], xylose residue having deviating C/H-1 chemical shifts for reasons unknown. Xyl[monomer] and GalA[monomer] refer to β-xylose and α-galacturonic acid anomers, respectively.
[b] Due to the partial methyl esterification, it was not possible to assign all resonances to specific galacturonic acid residues.
[c] The chemical shifts of C/H-5 of the substituted galacturonic acid cannot be distinguished from the chemical shifts of the unsubstituted galacturonic acid residues.
[d] *, Methyl-esterified galacturonic acid residue; **, de-esterified galacturonic acid residue.

doubled (Table 4), due to the presence of methyl esters: xylose H-2 protons are more shielded by the methyl-esterified carboxyl group of galacturonic acid. This was confirmed by comparison of the spectra with those from a sample of saponified xylogalacturonan. Thirty-six percent of all xylose residues (as determined using two-dimensional integration as indicated in Fig. 5) were linked to methyl-esterified galacturonosyl residues. The presence of a second (minor) substituent (Xyl[c]) could be detected. The C-1/H-1 chemical shifts differ slightly from the main xylose substituent (Table 4), but the remaining chemical shifts definitely point to a xylose residue. The exact nature of the chemical shift differences is not known, but might have to do with sequence-specific substitution patterns such as the presence of a xylose-substituted galacturonic acid moiety, flanked on both sides by methyl-esterified galacturonic acids. A triad of substituted galacturonosyl residues might also cause this type of difference in shielding. No indications were found for the presence of a substituted xylose residue.

From two-dimensional integration of the C-1 resonances of the galacturonic acid and xylose moieties (Fig. 5), the xylose to galacturonic acid ratio was calculated to be 0.7 which was in good agreement with the overall sugar composition (Table 3). Other residues present and assignable in the HSQC spectrum are α-rhamnose, β-galactose, and α-arabinofuranose. Their chemical shifts agree with those published earlier by

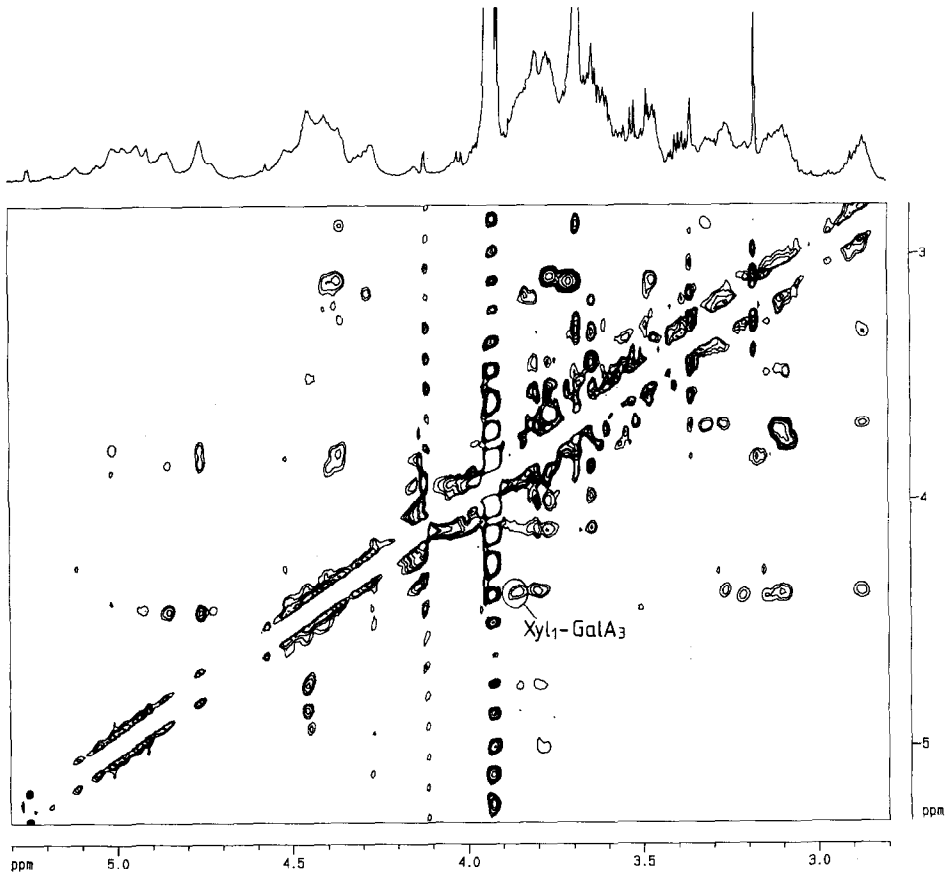

Fig. 6. {^{1}H,^{1}H} ROESY spectrum of a partially methyl-esterified xylogalacturonan fraction (RGase/RGAEase S300-I-*c*) with high-resolution ^{1}H spectrum on top.

Colquhoun et al. [28] on RGase oligomers released from apple MHR. Although not proved, they might originate from residual rhamnogalacturonan fragments flanking the xylogalacturonan.

4. Discussion

The use of a pure and defined enzyme like RGase has the advantage that information will be obtained on oligomeric degradation products as well as on higher molecular weight fragments, without the formation of potential artefacts. HPSEC analysis of the RGase/RGAEase digest of the MHR fraction A showed that the degradation was comparable with the degradation of the saponified substrate with RGase only. This indicates either that the sequences of alternating rhamnose and galacturonic acid residues of MHR fraction A where RGase acts are not methyl-esterified or that RGase is

not hindered by these methyl ester groups. Three subunits could be distinguished in MHR fraction A after digestion by RGase. Oligomeric fragments were obtained from the strictly alternating rhamnogalacturonan fraction of the pectin backbone. All oligomeric fragments released by RGase from saponified MHR fraction A have been identified before [13,28], although the relative amounts of the various oligomers differed slightly from the digest of the whole MHR [13]. Since more galactose-substituted oligomers were present in the digest of MHR fraction A and since fraction C was hardly degraded by RGase, we can conclude that more linear RGase oligomers can be liberated from MHR fraction B.

Almost all of the arabinose residues from MHR fraction A were recovered as intermediate molecular weight fragments (fractions II and III), very rich in arabinose and representing residual stubs of the pectic backbone carrying arabinan side-chains. These fragments could not be further degraded by RGase, implying that either the rhamnose and galacturonosyl residues are not present in the alternating order needed by the enzyme or are too highly branched for RGase to split in the backbone.

The third subunit, which was also eluted on an anion-exchange column as a single peak, was a xylogalacturonan fragment, which consisted mainly of galacturonic acid and xylose residues, but still contained some other sugar residues. After saponification of MHR fraction A and degradation by RGase, the xylogalacturonan was homogeneous, both for molecular weight and charge distribution. When the MHR fraction A was only deacetylated (RGase/RGAEase digest), at least three distinct fractions were obtained on anion-exchange chromatography and all fractions were eluted much earlier than the saponified xylogalacturonan. As these fractions had only slight differences in sugar composition, their elution behaviour strongly suggests variations in the proportions of galacturonic acid residues carrying methyl esters, with pools RGase/RGAEase I-*a* and I-*b* being more esterified than pool I-*c*. The presence of methyl esters at the carboxyl groups of the galacturonic acid residues was confirmed by NMR: for pool I-*c*, a degree of methylation of ca. 40 could be calculated from integration of the NMR signals. The NMR data suggest that the methoxyl groups are equally distributed among substituted and unsubstituted galacturonic acid residues. Matsuura [32,33] mentioned that the xylogalacturonan isolated from kidney bean cotyledons was highly esterified, although no values were given and no suggestions were made concerning the distribution of the ester groups over the backbone. Unfortunately, not enough material was available to determine the degree of methylation of fractions I-*a* and I-*b*. However, the presence of these fractions indicates that there is not an equal distribution of methoxyl groups over the various xylogalacturonan molecules and that distinct groups of esterified xylogalacturonan molecules exist.

The galacturonic acid residues present in the fractions RGase/RGAEase I-*c* were (1 → 4)-linked; two-thirds of them carried a terminal β-linked xylose residue attached to the O-3. The ratio of galacturonic acid residues to xylose residues as estimated by NMR is in excellent agreement with the sugar composition and indicates that a certain variation in xylose substitution within various xylogalacturonan molecules may occur. The structural features of the xylogalacturonan are in good agreement with the structure proposed before [15] and also with data on the sugar linkage composition of the parental MHR fraction [2] which showed that 70% of the xylose residues in apple MHR are

terminally linked. Since NMR spectroscopy revealed that xylose was found as terminal residues only, this may suggest that apple MHR fractions B and C [2] contained most of the (1 → 3)- and (1 → 2)-linked xylose residues found for the complete MHR fractions although the relative amount of xylose in fractions B and C is much lower as compared to fraction A [2].

A xylogalacturonan polymer has not previously been described in full detail to be present in apple pectic substances, although Barrett and Northcote [34] described in 1965 the isolation from apple tissue of an aldobiouronic acid consisting of a xylose and a galacturonic acid residue. Other researchers also reported the presence of a terminally linked xylose in pectic substances from apple [2,35], probably linked directly to the galacturonosyl residues as was concluded from base-catalyzed β-elimination reactions. Xylogalacturonans polymers having xylose present as single-unit side chains have been reported earlier [32,33,36,37]. Bouveng [36] described a rather pure xylogalacturonan, originating from the pollen of the mountain pine, with a xylose:galacturonic acid ratio of 0.5 in which the xylose was also linked to O-3 of the galacturonosyl residues in the backbone. Matsuura [32,33] described the isolation and characterization of a xylogalacturonan from kidney bean cotyledons having a xylose to galacturonic acid ratio of 0.5. Kikuchi and Sugimoto [37] described a xylogalacturonan fragment (dp 21), isolated after mild acid hydrolysis of an acidic polysaccharide in soy sauce. The xylose to galacturonic acid ratio was reported to be 0.2. The same authors [37] established that the xylose residue was in the β form in their fraction by using a pure β-xylosidase from *Aspergillus sojae*. Recently, the occurrence of a xylose-rich pectic fraction isolated from pea hulls has been described [38] in which xylose and galacturonic acid together represented 55–72% of all sugar residues.

It can be concluded that the modified hairy regions of apple pectin are more regular in structure than was reported before. The presence of a xylogalacturonan subunit replaces the xylose substitution of solitary galacturonosyl residues as presumed before [2]. The elution behaviour of the xylogalacturonan fractions on HPSEC suggested a rather high molecular weight (20,000–30,000), while such a high molecular weight is confirmed by the broad lines in the NMR spectra. Besides the xylogalacturonan originating from apple MHR which was isolated from apple tissue using the enzymic liquefaction process, we recently isolated polymeric xylogalacturonan-rich fractions from the hairy regions of apples which had been treated by polygalacturonase and pectinesterase [39] without unknown side activities.

Many similarities have been reported for MHR fractions originating from various plant materials [5] and this might also be the case for the presence of the different subunits described here. However, the relative amounts of the subunits might vary considerably as illustrated by the low xylose content in, for example, carrot and leek MHR, and the relatively low arabinose content in MHR from onions [5].

References

[1] J.A. De Vries, F.M. Rombouts, A.G.J. Voragen, and W. Pilnik, *Carbohydr. Polym.*, 2 (1982) 25–33.
[2] H.A. Schols, M.A. Posthumus, and A.G.J. Voragen, *Carbohydr. Res.*, 206 (1990) 117–129.

[3] H.A. Schols, C.C.J.M. Geraeds, M.J.F. Searle-van Leeuwen, F.J.M. Kormeling, and A.G.J. Voragen, *Carbohydr. Res.*, 206 (1990) 105–115.
[4] M. O'Neill, P. Albersheim, and A.G. Darvill, in P.M. Dey (Ed.), *Methods in Plant Biochemistry*, Vol. 2, *Carbohydrates*, Academic Press, London, 1990, pp 415–441.
[5] H.A. Schols and A.G.J. Voragen, *Carbohydr. Res.*, 256 (1994) 83–95.
[6] P. Odonmazig, D. Badga, A. Ebringerová, and J. Alföldi, *Carbohydr. Res.*, 226 (1992) 353–358.
[7] H. Kiyohara, J.-C. Cyong, and H. Yamada, *Carbohydr. Res.*, 182 (1988) 259–275.
[8] H. Kiyohara, J.-C. Cyong, and H. Yamada, *Carbohydr. Res.*, 193 (1989) 201–214.
[9] M. McNeil, A.G. Darvill, and P. Albersheim, *Plant Physiol.*, 70 (1982) 1586–1591.
[10] H. Kiyohara, H. Yamada, and Y. Otsuka, *Carbohydr. Res.*, 167 (1987) 221–237.
[11] H. Yamada, H. Kiyohara, and Y. Otsuka, *Carbohydr. Res.*, 170 (1987) 181–191.
[12] J.R. Thomas, A.G. Darvill, and P. Albersheim, *Carbohydr. Res.*, 185 (1989) 279–305.
[13] H.A. Schols, A.G.J. Voragen, and I.J. Colquhoun, *Carbohydr. Res.*, 256 (1994) 97–111.
[14] J. An, L. Zhang, M.A. O'Neill, P. Albersheim, and A.G. Darvill, *Carbohydr. Res.*, 264 (1994) 83–96.
[15] A.G.J. Voragen, H.A. Schols, and H. Gruppen, in F. Meuser, D.J. Manners, and W. Seibel (Eds.), *Plant Polymeric Carbohydrates*, Royal Society of Chemistry, Cambridge, UK, 1993, pp 3–15.
[16] N.K. Matheson and B.V. McCleary, *Adv. Carbohydr. Chem. Biochem.*, (1986) 147–176.
[17] L.V. Kofod, S. Kauppinen, S. Christgau, L.N. Anderson, H.P. Heldt-Hansen, K. Dörreich, and H. Dalboge, *J. Biol. Chem.*, 269 (1994) 29182–29189.
[18] M.J.F. Searle-van Leeuwen, L.A.M. van den Broek, H.A. Schols, G. Beldman, and A.G.J. Voragen, *Appl. Microbiol. Biotechnol.*, 38 (1992) 347–349.
[19] M. Mutter, G. Beldman, H.A. Schols, and A.G.J. Voragen, *Plant Physiol.*, 106 (1994) 241–250.
[20] P. Lerouge, M.A. O'Neill, A.G. Darvill, and P. Albersheim, *Carbohydr. Res.*, 243 (1993) 359–371.
[21] C.M.G.C. Renard, H.A. Schols, A.G.J. Voragen, J.-F. Thibault, and W. Pilnik, *Carbohydr. Polym.*, 15 (1991) 13–32.
[22] D.J. States, R.A. Haberkorn, and D.J. Ruben, *J. Magn. Reson.*, 48 (1982) 286–292.
[23] C. Griesinger, G. Otting, K. Wuthrich, and R.R. Ernst, *J. Am. Chem. Soc.*, 110 (1988) 7870–7872.
[24] A.J. Shaka, P.B. Baker, and R. Freeman, *J. Magn. Reson.*, 64 (1985) 547–552.
[25] A. Bax and S.S. Pochapsky, *J. Magn. Reson.*, 99 (1992) 638–643.
[26] S. Medvedeva, J.-P. Simorre, B. Brutscher, F. Guerlesquin, and D. Marion, *FEBS Lett.*, 333 (1993) 251–256.
[27] R.E. Hoffmann and D.B. Davies, *Magn. Reson. Chem.*, 26 (1988) 523–525.
[28] I.J. Colquhoun, G.A. de Ruiter, H.A. Schols, and A.G.J. Voragen, *Carbohydr. Res.*, 206 (1990) 131–144.
[29] H. Grasdalen, O.E. Bakøy, and B. Larsen, *Carbohydr. Res.*, 184 (1988) 183–191.
[30] D. Schipper, unpublished results.
[31] J.H. Bradbury and G.A. Jenkins, *Carbohydr. Res.*, 126 (1984) 125–156.
[32] Y. Matsuura, *Nippon Nôgeikagaku Kaishi*, 58 (1984) 253–259.
[33] Y. Matsuura, *Nippon Nôgeikagaku Kaishi*, 58 (1984) 1111–1115.
[34] A.J. Barrett and D.H. Northcote, *Biochem. J.*, 94 (1965) 617–627.
[35] J.A. De Vries, F.M. Rombouts, A.G.J. Voragen, and W. Pilnik, *Carbohydr. Polym.*, 3 (1983) 193–205.
[36] O. Bouveng, *Acta Chem. Scand.*, 19 (1965) 953–963.
[37] T. Kikuchi and H. Sugimoto, *Agr. Biol. Chem.*, 40 (1976) 87–92.
[38] R.M. Weightman, C.M.G.C. Renard, and J.-F. Thibault, *Carbohydr. Polym.*, 24 (1994) 139–148.
[39] H.A. Schols, E. Vierhuis, E.J. Bakx, and A.G.J. Voragen, *Carbohydr. Res.*, 275 (1995) 343–360.