Available online at www.sciencedirect.com
ScienceDirect
Carbohydrate Polymers 70 (2007) 224–235
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

Identification of the connecting linkage between homo- or xylogalacturonan and rhamnogalacturonan type I

G.J. Coenen, E.J. Bakx, R.P. Verhoef, H.A. Schols, A.G.J. Voragen *

Wageningen University, Department of Agrotechnology and Food Sciences, Laboratory of Food Chemistry, P.O. Box 8129, 6700 EV Wageningen, The Netherlands

Received 7 February 2007; received in revised form 2 April 2007; accepted 2 April 2007
Available online 19 April 2007

Abstract

Pectin is of interest both as cell wall component and as food additive. The precise chemical structure of pectin remains under debate, although the structural elements of pectin are rather well described. In order to get more insight in the inter linkage between the various structural elements, apple pectin modified hairy regions were degraded by controlled acid hydrolysis. From the degradation products oligomeric fragments were selected which could represent interconnection points, and these oligomers were characterized using LC–MS and NMR approaches.

It was shown that the oligomers $GalA_3Rha_1$, $GalA_4Rha_2$, and $GalA_5Rha_3$ consisted out of a homogalacturonan and a rhamnogalacturonan type I segment connected via a GalA*p*α-(1 → 2) Rha*p* linkage. In addition, a $GalA_6Rha_3Xyl_1$ oligomer was identified, which consisted out of a xylogalacturonan and a rhamnogalacturonan type I segment. These oligomers indicated that in apple pectin both homogalacturonan and xylogalacturonan were covalently linked to rhamnogalacturonan type I. With these new insights, currently used pectin models were refined.

Keywords: Homogalacturonan; Xylogalacturonan; Rhamnogalacturonan I; Pectin model; Covalent linkage; Pectin structure

1. Introduction

Pectin is an important plant cell wall component, which is thought to influence the porosity and strength of the primary cell wall, and the growth mechanism of the plant cell (Bacic, Harris, & Stone, 1988). Furthermore, it is of great importance to the food industry as it is one of the main natural gelating additives. Next to that, pectin determines to a large extend food processing characteristics of fruits and vegetables e.g. juices, nectars, purees and preserves (de Vries, 2004; Schols & Voragen, 1996). To be able to improve our understanding of pectin, knowledge of its structure is essential in order to comprehend its biological function and to explain its gelling and stabilizing properties (Daas, Boxma, Hopman, Voragen, & Schols, 2001; May, 2000; Voragen, Pilnik, Thibault, Axelos, & Renard, 1995). However, more than 180 years of pectin research (Braconnot, 1825) did not succeed in unambiguously unravel the structure of this plant polysaccharide partly due to the fact that its structure is affected by plant species (de Vries, Voragen, Rombouts, & Pilnik, 1981), plant development stage (Huisman, Schols, & Voragen, 1996), and cell location (Redgwell & Selvendran, 1986).

Pectin may consist out of different associated structural elements, such as homogalacturonan (HG), xylogalacturonan (XGA), rhamnogalacturonan type I (RGI), rhamnogalacturonan type II (RGII), arabinan, and arabinogalactan (Schols & Voragen, 2002). Homogalacturonan has intensively been studied, revealing characteristics such as distribution of methyl esters and acetyl groups over the galacturonan backbone, which contributes to the

* Corresponding author. Tel.: +31 317 482888; fax: +31 317 484893.
E-mail address: fons.voragen@wur.nl (A.G.J. Voragen).

0144-8617/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2007.04.007

understanding of gelling behavior and ripening processes of fruits and vegetables (Albersheim, Darvill, O'Neill, Schols, & Voragen, 1996; Guillotin et al., 2005). Xylogalacturonan also has a HG backbone, but 25–75% of the galacturonic acid (GalA) units are substituted with xylose (Le Goff, Renard, Bonnin, & Thibault, 2001; Schols, Bakx, Schipper, & Voragen, 1995a; Voragen, Beldman, & Schols, 2001). Furthermore the GalA sugar residues comprising the XGA backbone can be methyl-esterified (Schols et al., 1995a). Arabinan and (arabino)galactan (Beldman, Schols, Pitson, Searle-van Leeuwen, & Voragen, 1997; Carpita & Gibeaut, 1993; Mohnen, 1999; Ridley, O'Neill, & Mohnen, 2001; Schols, Posthumus, & Voragen, 1990) are covalently linked to RGI (Albersheim et al., 1996; Lau, McNeil, Darvill, & Albersheim, 1987; McNeil, Darvill, & Albersheim, 1980), were RGII is thought to be covalently linked to HG (O'Neill, Eberhard, Albersheim, & Darvill, 2001, 2004; Ridley et al., 2001; Vidal et al., 2000). The placement of XGA in respect to RGI is indistinct, but regarded exchangeable with HG (Huisman et al., 2001; Schols & Voragen, 1996; Vincken et al., 2003b). When isolating these structural elements, using enzymes, a number of unidentified structures remain to be further characterized (Schols & Voragen, 1994).

Using this information, two types of models for pectin structure have been composed: (1) a model in which the pectin backbone consists out of (alternating) RGI and HG (de Vries et al., 1981; Schols & Voragen, 1996; Voragen et al., 1995); (2) a model in which the backbone consists out of RGI, with HG and XGA as side chains (Vincken et al., 2003a). Arabinan and (arabino)galactan chains are covalently linked to RGI in the different models. Furthermore, both models assume that RGI and HG/XGA are covalently attached, although no linkage has ever been demonstrated (Ridley et al., 2001; Vincken et al., 2003a). The assumption of a covalent linkage between these structures is based upon co-elution (de Vries, Rombouts, Voragen, & Pilnik, 1982), molecular weight shift after endopolygalacturonase or pectate lyase digestion (Schols, Vierhuis, Bakx, & Voragen, 1995b), and the release of RGI, RGII, and oligogalacturonides from endo- and exopolygalacturonase degraded sugar beet pectin (Ishii & Matsunaga, 2001).

It has been shown that HG elements are build up by 81–117 galacturonic acid (GalA) residues (Thibault, Renard, Axelos, Roger, & Crepeau, 1993; Yapo, Lerouge, Thibault, & Ralet, 2007), although no firm evidence has been presented how these HG elements are coupled. Rees and Wight (1971) reported the presence of one single interspersing L-rhamnose unit interspacing HG elements, which would result in a kink in the molecule. Single interspersing rhamnose could, however, not be isolated from an endopolygalacturonase digest of citrus pectin, indicating a scarcity or complete lack of interspersing single rhamnose residues (Zhan, Janssen, & Mort, 1998), indicating that longer Rha-GalA sequences are involved in coupling HG elements.

Identification of the linkage types between two neighboring elements is needed to gain further insight in the structure of pectin. For this reason, we describe in this paper the mild acid hydrolysis of apple pectin hairy region, the enrichment of rhamnose and galacturonic acid and the subsequent isolation and identification of connection points. Finally, using the experimental data described in this paper, currently used pectin models were refined.

2. Experimental

2.1. Modified hairy regions

The starting material was prepared similarly as described by Schols et al. (1990). Homogenized apple tissue was incubated with Rapidase Liq+ (DSM Food Specialities, The Netherlands). The resulting suspension was centrifuged (Pennwalt Sharpless P600 Decanter) and the supernatant was ultra-filtrated (60 kDa). The retentate was freeze dried and named apple MHR (Schols et al., 1990). The apple MHR was saponified in 0.05 M NaOH at 4 °C during 24 h at a concentration of 10 mg/mL. Subsequently the mixture was neutralized with 0.25 M HCl to pH 7 and used for further analysis.

2.2. Controlled acid hydrolysis

Neutralized saponified sample solution (10 mg/mL) was hydrolyzed for 48 h with 0.1 M HCl at 80 °C. Thereafter, the hydrolysate was dialyzed (12 kDa) and lyophilized to remove liberated neutral sugars. A sequential hydrolysis step with 0.05 M trifluoroacetic acid (TFA) at 100 °C for 6 h (10 mg/mL) was conducted to initiate backbone degradation without extensively degrading the polymer. Hereafter, the sample was centrifuged at 18,500*g*. The supernatant was filtered through a P3 glass filter. The filtrate was concentrated with rotational film evaporation (40 °C, 40 mbar) and subsequently freeze dried. The freeze dried hydrolysate (6 g.) was dissolved to a concentration of 400 mg/mL and 3×5 mL was applied onto a column (100×2.6 cm i.d.) of Biogel P2 (Bio-Rad, USA) at 60 °C and eluted with distilled water (30 mL/h). The polymer population was collected and subsequently lyophilized.

2.3. Molecular weight distribution

Hydrolyzed polysaccharide was dissolved in distilled water (4 mg/mL) and analyzed by High Performance Size Exclusion Chromatography (HPSEC) performed on a SpectraSystem HPLC (Thermo Separation Products, USA) using three TosoHaas TSK-gel columns in series 4000, 3000, 2500, PWxl (300×7.5 mm; TosoH, Japan) preceded by a TSK PWxl guard column (40×6 mm; TosoH, Japan). The sample (20 μL) was injected and eluted at 30 °C using 0.8 mL/min 0.2 M $NaNO_3$. Detection was performed using a Shodex RI 71 refractive index detector (Showa Denko K.K., Japan).

2.4. Sugar composition

The sugar composition was determined according to De Ruiter, Schols, Voragen, and Rombouts (1992). Samples were dried at 40 °C under vacuum over P_2O_5 and hydrolyzed with 2 M HCl in dry methanol for 16 h at 80 °C followed by 1 h of 2 M TFA at 121 °C. The monomeric sugars were analyzed by high-performance anion-exchange chromatography (HPAEC) using a SpectraSystem HPLC (Thermo Separation Products, USA), equipped with a CarboPac PA-1 column (4 mm ID × 250 mm; Dionex, USA) in combination with a CarboPac PA guard column (4 mm ID × 25 mm) and a ED40 PAD-detector (Dionex, USA) (De Ruiter et al., 1992). A flow rate of 1 mL/min was used with the following gradient of distilled water, 0.1 M NaOH, and 1 M NaAc in 0.1 M NaOH: 0–15 min, 30 mM NaOH; 15–16 min, 30–100 mM NaOH; 16–55 min 0–500 mM NaAc in 0.1 M NaOH. Each run was followed by a 1 M NaAc in 0.1 M NaOH washing step for 14 min and an equilibration step of 100 mM NaOH of 5 min followed by 30 mM NaOH elution for 15 min.

2.5. Fractionation and desalting of oligomers separated by anion-exchange chromatography

Anion-exchange chromatography was performed using a SpectraSystem HPLC (Thermo Separation Products, USA), equipped with a CarboPac PA-1 column (2 mm ID × 250 mm; Dionex, USA) in combination with a CarboPac PA guard column (2 mm ID × 25 mm) and a ED40 PAD-detector (Dionex, USA). A flow rate of 0.2 mL/min was used with the following gradient of NaOAc buffer (pH 5.0) in water: 0–8 min, 10 mM; 8–12 min, 175 mM; 12–108 min 175–860 mM. Each elution was preceded by a 15 min 10 mM NaOAc equilibration step and the run was completed with an 8 min 1 M NaOAc washing step. Oligomer detection was possible after post-column addition of NaOH (1 M; 0.2 mL/min).

The eluate of the anion-exchange chromatography was desalted online by two desalting units, connected in-line after the PAD-detector. First, the Anion Self-Regenerating Suppressor-Ultra 4 mm-unit (ASRS; Dionex, USA) was connected to exchange the sodium ions for hydronium ions (H_3O^+). Next, the Cation Self-Regenerating Suppressor-Ultra 4-mm unit (CSRS; Dionex, USA) was installed to exchange the acetate ions for hydroxide (OH^-). Desalting the eluent was achieved by electrolysis of deionized water (8 mL/min) in both suppressors. Fractions (0.5 min/fraction; 200 μL) were collected in a 96-well Multiscreen-BV plate (Millipore, USA) filled with regenerated Dowex AG 50W-X8 (Bio-Rad Laboratories, USA) in the hydrogen form, using a FC-203B fraction collector (Gilson, USA) to remove the residual salts. After this desalting step, samples were collected in a 96-well plate by centrifugation (1000*g*) of the multiscreen plate. This step was repeated two times with multiscreen plates containing regenerated Dowex AG 50W-X8.

2.6. Annotation of oligomers using MALDI-TOF mass spectrometry

For matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) analysis an Ultraflex workstation (Bruker Daltonics, Germany) equipped with a 337 nm laser was used. The mass spectrometer was operated in the positive mode and calibrated with a mixture of maltodextrins (mass range 300–3000 Da). After a delayed extraction time of 200 ns, the ions were accelerated with a 25 kV voltage. The ions were detected using the reflector mode.

Two microliter of each fraction was automatically transferred from the 96-well plate to the Maldi sample plate and mixed with 2 μl of matrix by using a Symbiot-I robot (PerSeptive Biosystems, USA) and dried under a stream of warm air. The matrix solution was prepared by dissolving 9 mg 2,5-dihydroxy benzoic acid in a ~1 mL acetonitrile:water (300:700 μL) mixture. Mass to charge signals of the sodium adducts were translated in oligomeric structures (*e.g.* 685 $m/z \rightarrow GalA_2Rha_2$), using an in-house software program and HPAEC data. Mass to charge signals, which could not be assigned unambiguously were determined with Post-Source Decay/MALDI-TOF MS, using the same settings as for MALDI-TOF MS. The spectrum was divided into different independently measured segments, which were merged to one PSD spectrum by the FlexAnalysis Software (Bruker Daltonics, Germany) (Verhoef et al., 2005).

2.7. Partial structural elucidation with nanospray mass spectrometry of ^{18}O-labeled oligomers

Detailed structural information was obtained after labeling the reducing end with ^{18}O. The samples were lyophilized and 0.5% formic acid in H_2 ^{18}O (Campro Scientific, the Netherlands) was added, followed by 72-h incubation at 40 °C. Static nanospray MS (NSI–MS) of labeled samples was performed with a LTQ Ion Trap (Thermo Electron, USA). Ten microliter sample was transferred into a capillary needle (New Objective, USA) and MS analysis was carried out in the positive mode using a capillary temperature of 200 °C and a spray voltage of 1.8 kV. The automated tune function generated the remaining settings, using the sample material. Fragmentation was performed using a window of 2 m/z at 25% collision energy.

2.8. Enzymatic degradation of oligomeric fragments with exopolygalacturonase and rhamnogalacturonan galacturonohydrolase

Fractions containing connecting oligomers were dialysed (100 Da; Spectrapore, USA), lyophilized and dissolved in 100 μL distilled water. Thereafter incubated with an overdose of exopolygalacturonase (ExoPG) of *Aspergillus tubingensis* (Kester, Kusters-Van Someren,

Muller, & Visser, 1996). The resulting digest was sequentially digested with rhamnogalacturonan α-D-galacturonohydrolase (RGGH) from *Aspergillus aculeatus* (Mutter, Beldman, Pitson, Schols, & Voragen, 1998a). Incubations were carried out at 37 °C for 24 h, and after each incubation samples were heated for 10 min at 100 °C to inactivate the enzymes. Enzyme activity and overdose were confirmed by incubating polygalacturonic acid and RGI oligomer $GalA_3Rha_3$ standard solutions.

2.9. ^1H NMR spectroscopy of oligomeric fragments

Prior to NMR analysis, samples were reduced to their corresponding alditols. The reduced sugars were sequentially exchanged in 99.96% D_2O (Cambridge Isotope Laboratories, USA) and after freeze-drying dissolved in 99.996% D_2O (Cambridge Isotope Laboratories, USA) and inserted in NMR microtubes (Shigemi, USA). NMR spectra were recorded at a probe temperature of 25 °C on a Bruker AMX-500 spectrometer (Bruker Biospin, Germany) located at the Wageningen NMR Center. Chemical shifts were expressed in parts per million relative to internal acetone: $\delta = 2.225$ ppm for ^1H.

The 1D ^1H proton spectra were recorded at 500.13 MHz using 1024 scans and a sweep width of 3000 Hz. Two-dimensional homonuclear COSY and TOCSY spectra were recorded using standard pulse sequences delivered by Bruker using 512 experiments of 128 scans (Oosterveld, Coenen, Vermeulen, Voragen, & Schols, 2004).

3. Results

3.1. Partial degradation of Apple MHR to generate linkage point fragments

Different glycosidic linkages hydrolyze under different circumstances; therefore it is possible to rather specifically remove sugars. Linkages between two GalA sugars are more stable then aldobiuronic linkages (GalA-Rha) or pseudo-aldobiuronic (Rha-GalA) sugars. Linkages between neutral sugars are the most susceptible to acid hydrolysis; hence controlled acid hydrolysis is frequently used to remove neutral sugars (BeMiller, 1967; Guillon & Thibault, 1990; Thibault et al., 1993). Acetyl and methyl esters were removed by saponification to reduce the number of signals/peaks in spectra and chromatograms. Saponified Apple MHR was partially degraded by controlled acid hydrolysis (hydrochloric acid and trifluoroacetic acid) to breakdown neutral sugar side chains and to release oligomers, involved in the connection between HG and/or XGA to RGI. The degradation was monitored with HPSEC analysis (Fig. 1).

Saponification of the MHR resulted in a similar elution profile, with a small shift to the left of the 30.5 min population, which would be attributed to aggregation of HG chains (Kravtchenko, Berth, Voragen, & Pilnik, 1992). The saponified sample was dialyzed and lyophilized, which explains the absence of low molecular weight peaks. Controlled acid hydrolysis degraded high molecular weight populations to a mixture of oligomers.

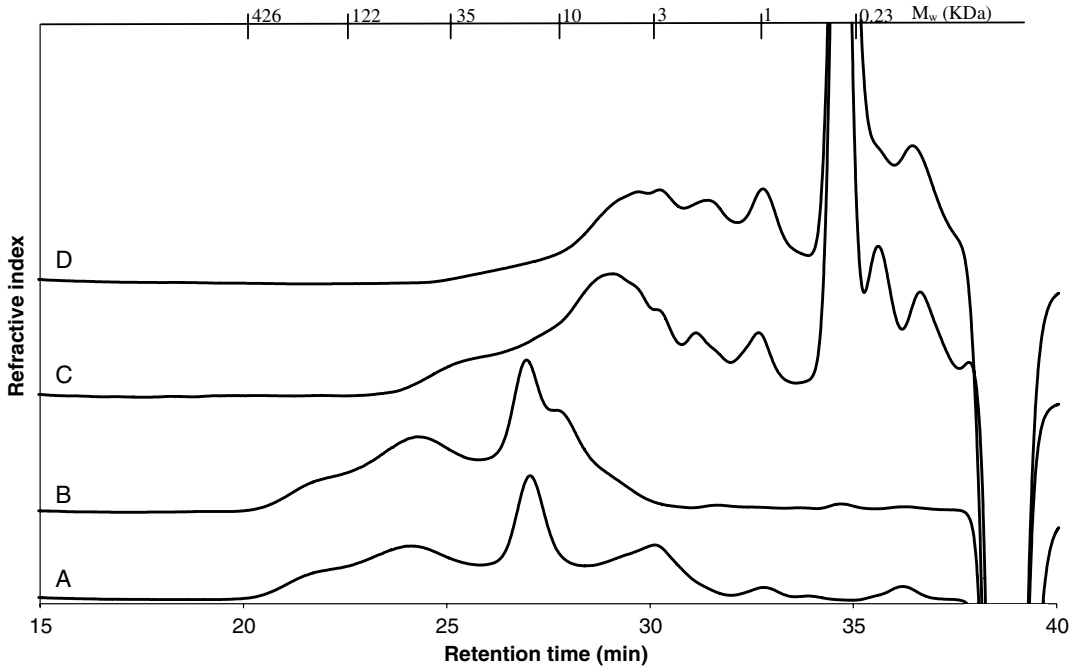

Fig. 1. HPSEC molecular weight distribution profiles of (A) apple MHR, (B) saponified apple MHR, (C) HCl hydrolyzed saponified apple MHR, and (D) TFA and HCl hydrolyzed saponified apple MHR (molecular weight annotations in top *x*-axis are based on pullulan standards).

The first hydrolysis step is performed with HCl, this mild condition almost specifically hydrolyzes neutral sugar linkages. After hydrochloric acid hydrolysis, a peak remains present at 25 min, which is expected to consist primarily out of galacturonic acid residues, since the aldobiuronic galacturonic acid linkage is the most stable pectin linkage next to GalA–GalA (Thibault et al., 1993). TFA was used to partly hydrolyze the aldobiuronic linkages between the molecules constituting the population at 25 min, resulting in a sample containing mainly oligomers and monomers.

3.2. Sugar composition of apple MHR during different controlled acid hydrolysis steps

To monitor the relative enrichment of galacturonic acid and rhamnose in the polymer and oligomer material during controlled acid hydrolysis, the sugar composition was determined (Table 1).

The sugar composition shows that the saponified sample has only minor differences with the original sample, showing that saponification indeed only removed esters. The 48 h 0.1 M HCl 80 °C hydrolysis removed primarily arabinose and galactose. The sequential 6 h, 0.05 M TFA, 100 °C hydrolysis resulted in a further decrease in the neutral sugars as well as the removal of fucose. By removing these neutral sugars the resulting material was relatively enriched in Rha and GalA content and slightly further degraded. Xylose resists hydrolysis better than arabinose and galactose, which is in accordance with the results of Thibault et al. (1993). We hypothesize that the presence of the carboxyl group in close vicinity could provide a protective effect on the β-(1 → 3) xylose linkage stability. Sugar analysis showed that during different hydrolysis steps the mixture was enriched in galacturonic acid and rhamnose moieties as described before (Thibault et al., 1993). From the GalA:Rha ratio 2.2:1 it can be concluded that HG and RGI structures remain present in the sample after TFA and HCl hydrolysis. Enzymes could not be used for enrichment of connection points out of the MHR, since MHR is the enzyme resistant product left after degrading apple pectin with a complex mixture of pectic enzymes.

3.3. Isolation of connecting fragments out of the apple MHR hydrolysate

In order to recognize oligomeric fragments involved in the connection between HG or XGA to RGI, the hydrolysate was fractionated using high-performance anion-exchange chromatography (HPAEC; Fig. 2). The separation was performed at pH 5 to selectively separate galacturonic acid containing oligomers, since neutral oligomers will not bind to the column at these conditions. Galacturonic acid oligomers are annotated using a PGA digest consisting out of mono-, di-, tri- and tetragalacturonic acid. All other peaks were annotated by off line MALDI-TOF MS of desalted fractions (Kabel, Schols, & Voragen, 2001).

The HPAEC elution pattern shows the oligomer composition of the sequential HCl and TFA hydrolyzed sample. Controlled acid hydrolysis of saponified apple MHR resulted in a complex mixture of more than 50 different peaks. The first peak represents neutral sugar oligomers, which do not bind to the column. The gradient is optimized to separate oligomers with at least two galacturonic acid residues; under these conditions mono GalA eluted at 15 min in a broad peak. Peaks are annotated, based on sugar composition of the injected sample, oligomer elution behavior, and apparent MALDI-TOF MS masses. In addition, salt signals, which are usually regarded undesirable because of the negative influence on signal intensity, were used to confirm hypothesized structures. The presence of additional salt signals neighboring the sodium adduct of an oligomer is caused by the exchange of a proton of one or more carboxyl group(s) with a sodium or potassium ion and is therefore indicative for the number of GalA moieties within an oligomer. When more m/z signals are present in one fraction, the annotation is related to the signal intensity. RGI oligomers are identified in the hydrolysate, so controlled acid hydrolysis succeeded in releasing "backbone" oligomers from apple MHR. HG and XGA type oligomers are recognized as well to be present in the hydrolysate.

Depending on their size, oligomers with similar charges and differing in neutral sugars content may co-elute. RGI backbone oligomers with either a single pentose or hexose substitution are identified, which are considered remnants

Table 1
Sugar composition (mol %) of the different MHR fractions

Fraction	Sugar composition (mol %)								
	Fuc	Rha	Ara	Gal	Glc	Man	Xyl	GalA	GlcA
Apple MHR[a]	1	17	8	15	3	0	13	42	1
Saponified apple MHR[a]	2	20	6	18	2	0	11	39	2
HCl-treated saponified apple MHR[a]	1	20	2	14	3	0	8	51	1
TFA- & HCl-treated saponified MHR[b]	0	27	1	4	2	0	6	59	1

[a] Obtained after dialysis (12 kDa).
[b] Obtained by pooling the polymer peak after Biogel P2 separation (distilled water elution).

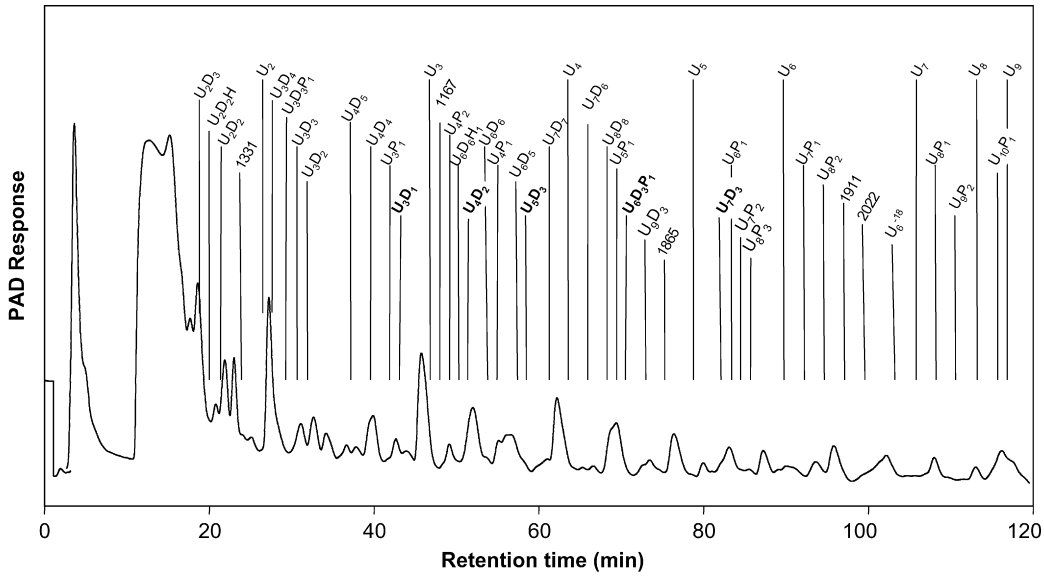

Fig. 2. HPAEC elution profile of an oligosaccharide mixture obtained after mild acid treatment of apple MHR, including peak annotation based on off line MALDI-TOF MS analysis of fractions collected (U = uronic acid; galacturonic acid, D = deoxyhexose; rhamnose and P = pentose; xylose).

of arabinan or galactan side chains (Gur'janov, Gorshkova, Kabel, Schols, & van Dam, 2007; Schols, Voragen, & Colquhoun, 1994). Peaks which could not be annotated were shown by their *m*/*z* value. After fractionation it was possible to identify oligomers using MALDI-TOF MS, which could not be detected in the whole hydrolysate (data not shown). MALDI-TOF MS was used for annotation in combination with a PGA digest, since most other structures are not available as standards.

The structures $GalA_3Rha_1$, $GalA_4Rha_2$, $GalA_5Rha_3$ and $GalA_6Rha_3Xyl_1$ (represented in bold in Figure 2) are of hybrid nature. Their galacturonic acid to rhamnose ratio, indicates the presence of a RGI segment and a HG segment within one oligomer. Since the *m*/*z* ratios were annotated based on sugar composition of the injected sample and elution behavior, these annotations were confirmed with Post-Source Decay (PSD)/MALDI-TOF MS (data not shown). The first three oligomers are expected to be involved in the HG to RGI linkage, the $GalA_6Rha_3Xyl_1$ oligomer indicates a XGA to RGI linkage. The xylose annotation for the pentose rather than arabinose (which has an equal mass) in $GalA_6Rha_3Xyl_1$, was based upon the fact that almost no arabinose was present in the sample.

3.4. A GalA trimer is covalently linked to an alternating Rha-GalA segment

More detailed structural information was obtained by the analysis of connecting oligomers with nanospray ionization–mass spectrometry (NSI–MS) after labeling their reducing end with ^{18}O. Labeling was performed to establish if generated fragments included the reducing end of the oligomer. The fragments are annotated according to the systematic nomenclature of Domon and Costello (1988). When analyzing the MS^1 spectrum of labeled oligomer fractions, signals next to the labeled mother ion appeared to be present having an additional +2 Da increase (data not shown). Galacturonic acid oligomers and maltodextrin standards were analyzed to explain this phenomenon (data not shown). The mass increase of 2 Da occurred only with GalA oligomers, which contain carboxylic acid groups. Furthermore, the number of peaks with an increment of 2 Da depends on the number of uronic acid residues within the oligomer. No increment of 2 Da was found for the maltodextrin standard. The relation with the uronic acid leads us to the "multiple ^{18}O label" hypothesis that there is an exchange between the carboxylic oxygen and ^{18}O.

It becomes evident from the signals at 569 and 551 *m*/*z* (a), that the oligomer with 717 *m*/*z* consists out of a galacturonic acid trimer connected to rhamnose. The position of the rhamnose is determined with the MS^3 spectrum of the 365 *m*/*z* (^{18}O-labeled GalA-Rha) fragment (b), which indicates that rhamnose contains the reducing end (189 *m*/*z*). Both spectra represented in Fig. 3 show labeled uronic acids moieties, but this was recognized as the "multiple ^{18}O label" artifact. Additionally, primarily B and Y fragments are formed, which also annotate rhamnose as the reducing end, since in the positive mode the glycosidic oxygen is principally retained on the fragment including the reducing end (van Alebeek, Zabotina, Beldman, Schols, & Voragen, 2000). Because the galacturonic trimer fragment does *not* contain the glycosidic oxygen, it is likely positioned on the non reducing end, leaving the reducing end as only possible location for the rhamnose. To corroborate the annotation of rhamnose as the reducing end, $GalA_5Rha_3$ was labeled by reduction through $NaBH_4$, which gives a specific mass label of +2 Da on the reducing end. This experiment (data not shown) unambiguously annotates rhamnose at the reducing end.

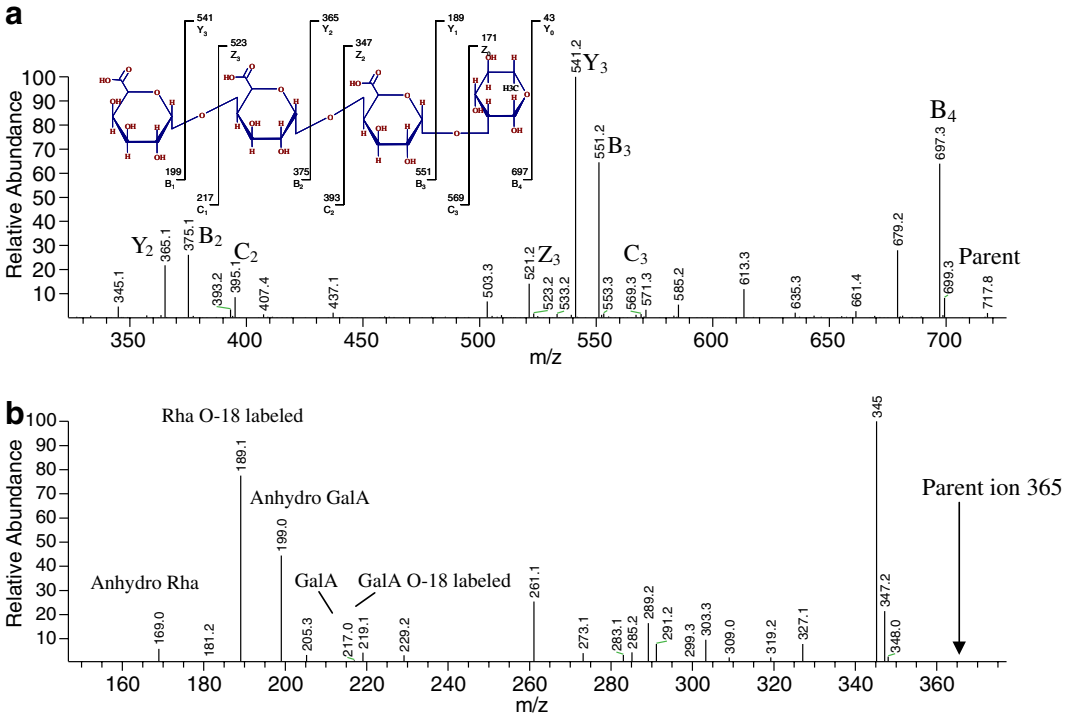

Fig. 3. MS2 fragmentation pattern of ^{18}O-labeled connecting oligomer $GalA_3Rha$ (717 *m/z*) (a), and MS3 fragmentation pattern of 365 fragment (b) analyzed in the positive mode, A schematic representation of the oligomer is given, with assumed linkages between the sugars.

The spectrum indicates the presence of one dominant oligomer with lower amounts of impurities. These impurities consist out the potassium adduct of $GalA_3Xyl$, like the 585 *m/z* ($galA_3$-K^+ adduct) oligomer (Fig. 3a). Other interference like the 571, 553, 395, and 219 *m/z* signals come from $GalA_3Rha$ oligomers, which have ^{18}O-labeled (+2 *m/z*) galacturonic acid instead of a labeled reducing end. Combining these data results in the schematic structure represented in the inlay in Fig. 3a. The linkages are still hypothetical, but the corresponding fragment weights will be applicable for all possible glycosidic cleavages.

Next to the $GalA_3Rha$ oligomer, $GalA_4Rha_2$ and $GalA_5Rha_3$ oligomers were as well collected during the HPAEC run of the MHR hydrolysate (retention times, respectively, 51 and 58 min) and sequentially ^{18}O-labeled. The spectra of these labeled oligomers are shown in Fig. 4.

Both spectra (Fig. 4a and b) show a similar fragmentation pattern. The 551 *m/z* signal indicates a galacturonic acid trimer within these oligomers. There is a loss of galacturonic acid–rhamnose blocks resulting in mainly B type fragments. This indicates that the galacturonic acid trimer fragment is coming from the non-reducing end (van Alebeek et al., 2000). This is confirmed by ^{18}O-labeling, which assigns rhamnose as the reducing end. In these spectra signals are as well present coming form "multiple ^{18}O-labeling", as described for the $GalA_3Rha_1$ oligomer.

Both spectra contain the B fragment 697 *m/z*, which is as well an apparent signal in Fig. 3. MS^3 experiments with the 697 *m/z* fragment for the different oligomers $GalA_3Rha_1$, $GalA_4Rha_2$, $GalA_5Rha_3$, resulted in similar spectra (data not shown), indicating structural resemblance. In both MS^2 spectra (Fig. 4a and b) fragments are observed like $GalA_2Rha$, which can only be generated by double cleavages in the oligomer, which is as well-observed before (Hilz, de Jong, Kabel, Schols, & Voragen, 2006).

Both $GalA_4Rha_2$ and $GalA_5Rha_3$ oligomers contain a GalA trimer (remaining HG segment) on the non-reducing end and a rhamnose (remaining RGI segment) at the reducing end. The absence of connection points with the HG segment on the reducing end is remarkable, since in the Voragen model (Voragen et al., 1995), the pectin backbone consists out of alternating HG and RGI structural elements, which would yield oligomers, with HG on the reducing end as well. The weaker linkage stability of pseudo-aldobiuronic linkage is a possible explanation for the specific formation of these oligomers during hydrolysis (Thibault et al., 1993), but since RGI segments (up to $GalA_8Rha_8$) are isolated as well this explanation is not unequivocal.

3.5. A xylose substituted GalA trimer is covalently linked to an alternating Rha-GalA segment

The $GalA_6Rha_3Xyl_1$ oligomer (Fig. 5; retention time 71 min) was analyzed with nanospray ionization–mass spectrometry (NSI–MS) after ^{18}O-labeling to obtain detailed structural information (Fig. 5).

The spectrum shows a dominant $GalA_6Rha_3Xyl_1$ oligomer and a small $GalA_7Xyl_3$ signal. The impurity was attributed to the higher content of $GalA_7Xyl_3$(XGA

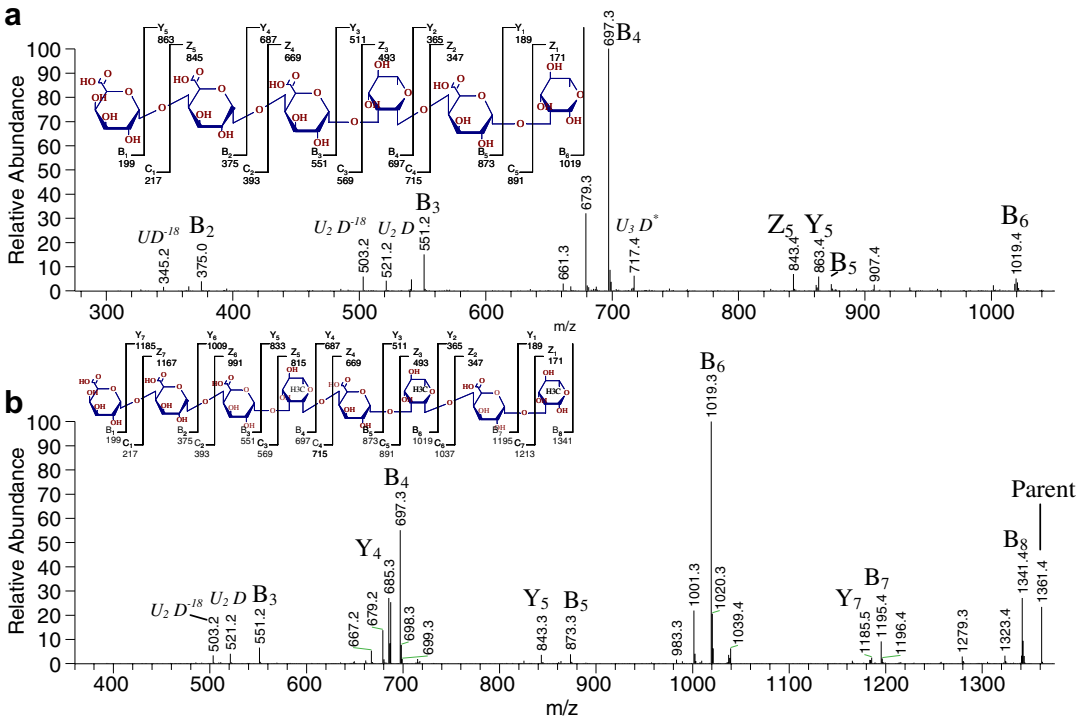

Fig. 4. MS2 fragmentation patterns of ^{18}O-labeled key oligomeric fragments $GalA_4Rha_2$ [1039 m/z] (a), and of $GalA_5Rha_3$ [1361 m/z] (b) analyzed in the positive mode. Asterisk represents ^{18}O label, $^{-18}$ represents −18 m/z mass loss, presumably due to anhydro sugar or structural rearrangement. In both spectra a schematic representation of the oligomer is given, with assumed linkages between the sugars.

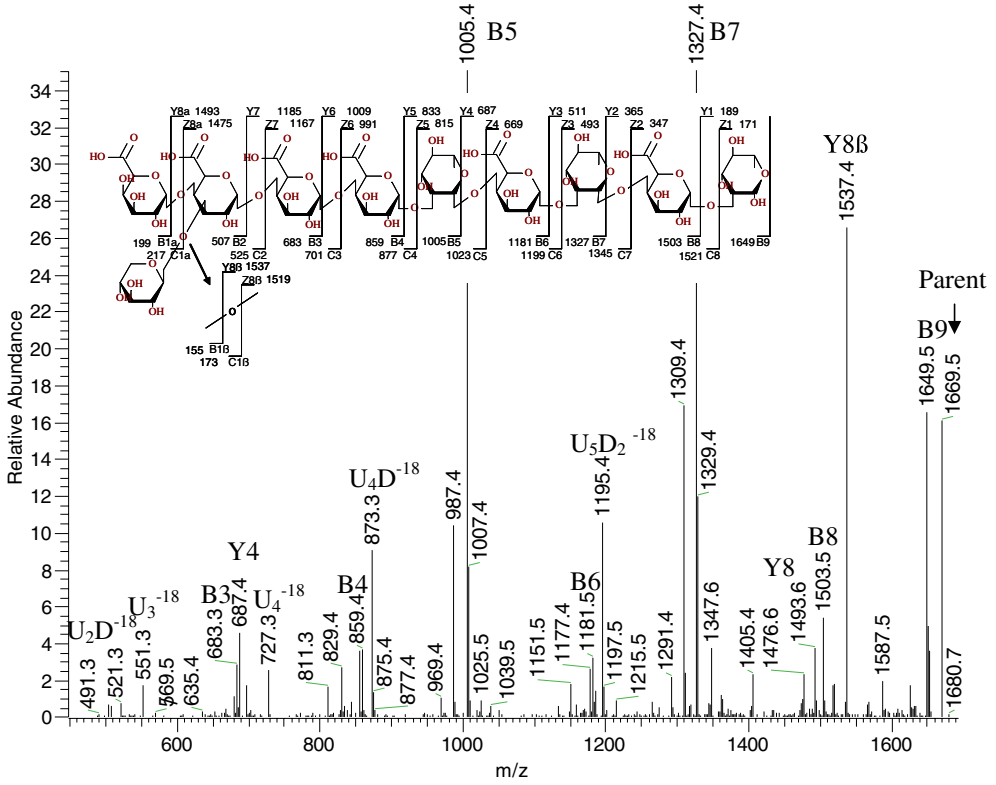

Fig. 5. MS2 fragmentation pattern of $H_2{}^{18}O$-labeled oligomer $GalA_6Rha_3Xyl_1$ [1669 m/z]. In the spectra a schematic representation of the oligomer is given, with assumed linkages between the sugars.

fragment; recognized by the loss of a second consecutive xylose [1405 *m/z*]) in the hydrolysate in relation to $GalA_6Rha_3Xyl_1$ (connection point), which resulted in co-elution of the connecting oligomer and the end of the $GalA_7Xyl_3$ peak. In analogy to the structures discussed before, rhamnose is located at the reducing end of this oligomer. The 727 *m/z* fragment shows that a galacturonic acid tetramer is part of the molecule. Xylose is substituted to one of the uronic acids located at the non-reducing end, which can be concluded from the 507 *m/z* fragment ($GalA_2Xyl$). The release of a single GalA fragment, indicated by the 1493 *m/z* signal, indicated that the second GalA sugar from the end terminus is substituted with this xylose. Combination of these results leads to an oligomer consisting out of a XGA element, attached to a RGI element, as represented in the inlay. It was not possible to further characterize the structures on a linkage level with NSI–MS, nor by investigation of ring fragmentations as described by Quemener, Ordaz-Ortiz, and Saulnier (2006) and Zaia (2004). On an abundantly present $GalA_3Rha_3$ oligomer, no recognizable cross ring cleavages could be detected, which led to discontinuation of this approach.

3.6. Indications for a α-(1 → 2) linkage between GalA trimer and RGI moiety within the oligomers

The oligomers $GalA_5Rha_3$ (1359 Da) and $GalA_6Rha_3Xyl_1$ (1667 Da) are enzymatically degraded with ExoPG and RGGH (Fig. 6) to confirm the given structure and to get more information about the linkage between the HG or XGA element to the RGI element. ExoPG attacks the substrate from the non-reducing end and is able to remove terminally (1→)-linked galacturonic acid residue from HG chains, while it is also known that ExoPG is tolerant for a xylose substation, removing a GalA-Xyl dimer (Beldman et al., 1996). RGGH is able to release a galacturonic acid connected to a rhamnose from the non-reducing side of RGI chains and unable to liberate galacturonic acid from HG (Mutter et al., 1998a).

Both oligomers $GalA_5Rha_3$ and $GalA_6Rha_3Xyl_1$ are stepwise enzymatically degraded to the RGI end-product with rhamnose on both sides, which indicates that HG is indeed linked to RGI, most probably α-(1 → 2). It was not possible to determine if the enzymes were absolutely specific for α-(1 → 4) (ExoPG) and α-(1 → 2) (RGGH), since side-activities for these enzymes were not investigated so far, due to the unavailability of suitable substrates (Beldman et al., 1996; Benen, Vinken, & Alebeek, 2002; Kester et al., 1996; Mutter et al., 1998a).

Table 2
^{1}H chemical shifts (ppm) of the reduced $GalA_5Rha_3$ oligomer determined at 500 MHz

Residue	H-1	H-2	H-3	H-4	H-5	H-6
α-L-Rha_{red}	5.22	4.06	3.96	3.46	3.85	1.23
α-L-Rha_{int}	5.26	4.12	3.88	3.41	3.78	1.25
α-D-$GalA_{RG}$	5.01	3.91	4.11	4.42	4.72–4.65	
α-D-$GalA_{HG}$	5.05[a]	3.95	4.06	4.41	4.80–4.65	

[a] Tentative assignment (weak signal).

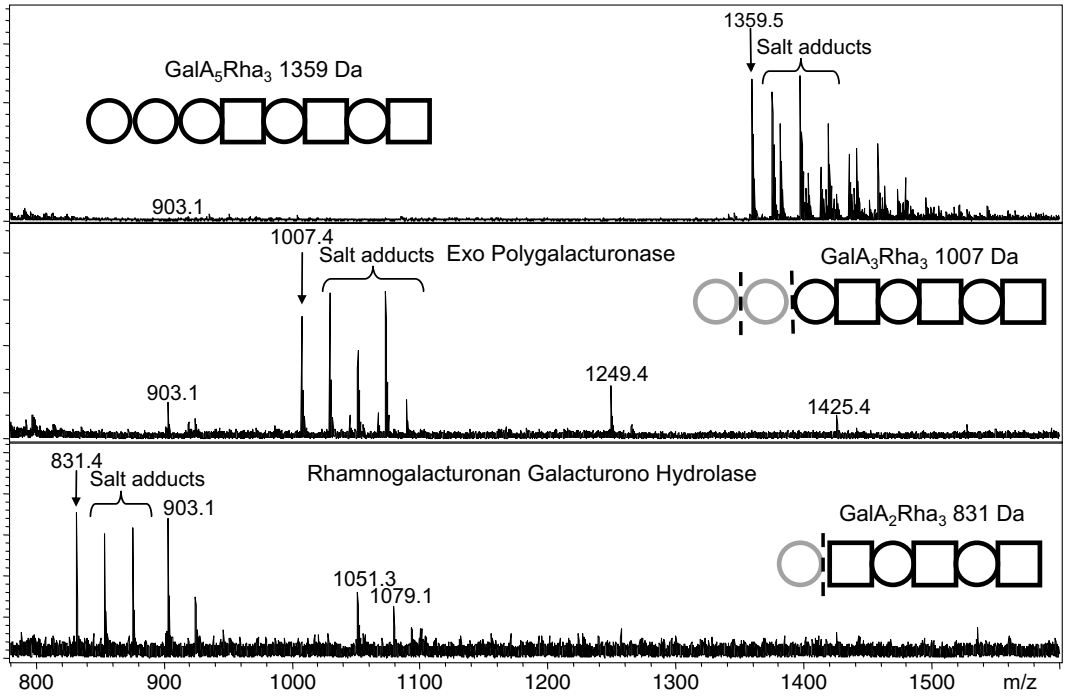

Fig. 6. MALDI-TOF Mass spectra of the $GalA_5Rha_3$ oligomer, before and after treatment with ExoPG and RGGH, with schematic representation in the right side (○, galacturonic acid; □, rhamnose).

3.7. GalA trimer is α-(1 → 2) linked to RGI moiety within connecting oligomer $GalA_5Rha_3$

To substantiate the structural information about the linkages within the connecting oligomers provided by the enzymatic digestions, ^{1}H chemical shifts were determined by NMR spectroscopy of the reduced $GalA_5Rha_3$ oligomer (Table 2).

The presence of only one anomeric signal for *internal* rhamnose points out that both rhamnose sugars present in the oligomer are linked in the same way. The ^{1}H chemical shifts are in good agreement with those reported previously (Colquhoun, de Ruiter, Schols, & Voragen, 1990; Mutter et al., 1998b), being specific for α-(1 → 2) linked rhamnose, within a RGI backbone. The α-D-$GalA_{HG}$ spin system belongs to HG (ppm anomeric signal) (Bushneva, Ovodova, Shashkov, & Ovodov, 2002; Habibi, Mahrouz, & Vignon, 2005; Kardosova et al., 2004; Schols et al., 1995b). The H^1 chemical shifts differ slightly from the literature value, but the remaining chemical shifts definitely point toward an internal α-D-$GalA_{HG}$. The α-D-$GalA_{RG}$ signal is coming from the alternating Rha-GalA unit (ppm anomeric signal) (Habibi et al., 2005; Mutter et al., 1998b; Renard, Lahaye, Mutter, Voragen, & Thibault, 1998; Schols et al., 1990). The ^{1}H signal of the reduced rhamnose is outside the anomeric region, where it is overlapped by the other signals in the bulk region. This position was, however, already demonstrated by MS results. An α-L-Rha was identified as well, indicating incomplete reduction of the reducing end (Schols et al., 1990). However, the β-L-Rha signal could not be detected, meaning that only a small proportion was not reduced. Chemical shifts distinctive for other structures were not present.

The →(4)-α-D-Gal*p*A-(1 → 4)-α-D-Gal*p*A-(1 → 2)-α-L-Rha linkage has been suggested previously for an acid-soluble pectin fraction of *Opuntia ficus-indica* (Habibi et al., 2005). This suggestion was based on relative amounts of different linkages determined by NMR and GC–MS techniques. The → (4)-α-D-Gal*p*A-(1 → 4)-α-D-Gal*p*A-(1 → 2)-α-L-Rha structure itself was, however, not identified. Since other isomer structures could be drawn up as well, the resulting structure remained ambiguous.

For the $GalA_6Rha_3Xyl_1$ oligomer it was not possible to obtain a good NMR signal, possibly due to the low quantity. But looking to the homology with the $GalA_5Rha_3$ oligomer in the results described above, we postulate that the linkage between XGA and RGI will as well be α-(1 → 2).

Furthermore, results were in agreement with the enzymatic degradation patterns. Combining these findings, the structure for connection between RGI and HG is proposed (Fig. 7).

4. Conclusion

By controlled acid hydrolysis of apple MHR a mixture of oligomers was obtained. This mixture was enriched in galacturonic acid and rhamnose content during various hydrolysis and purification steps, as shown by sugar composition analysis. HPAEC at pH 5 in combination with MALDI-TOF MS revealed that this MHR hydrolysate consisted out of RGI, HG, and XGA type oligomers next to neutral sugars. Furthermore, oligomer structures with features of HG as well as XGA and RGI were present which indicates that these are the linkage between HG/XGA and RGI structural elements. MS^n experiments with these labeled structures ($GalA_3Rha$, $GalA_4Rha_2$, $GalA_5Rha_3$, $GalA_6Rha_3$, and $GalA_6Rha_3Xyl_1$ oligomers) showed that the position of the RGI block was at the reducing end of the oligomer and the HG/XGA block was at the non-reducing end. Both blocks were α-(1 → 2) linked, as was indicated by enzymatic degradation with ExoPG and RGGH and NMR spectroscopy. To our knowledge this is the first time that the covalent linkage of a HG or a XGA structural element to RGI was demonstrated at oligomer level. These results partly correspond with the model of Voragen et al. (1995), were the backbone consisted out of consecutive HG and RGI structural elements. Since it could not be ruled out that we only identified part of the connecting points present in the pectin and not all unknown oligomers could be identified so far, the model where HG is positioned as a RGI side chain (Vincken et al., 2003b) could not be excluded irrevocably. The absence of oligomers which contain HG structures at the reducing end could indicate that the backbone consisted out of only out one HG and one RGI structural element. This hypothesis does, however, not account for the Mw of pectins (50–100 kDa), considering that the RGI element is 12 kDa and the HG element is 17.5 kDa (Prade, Zhan, Ayoubi, & Mort, 1999; Yapo et al., 2007; Zhan et al., 1998). The ratio Rha:GalA in apple (1:21), citrus peel (1:31), soy (1:3.5), black currant (1:20), bilberries (1: 21), and sugar beet (1:8) pectins (Hilz, Bakx, Schols, & Voragen, 2005; Voragen et al., 2001; Yapo et al., 2007) would advocate for more, or longer, HG chains per RGI unit. Additional research is needed to determine the size of various pectin structural elements, in order to elucidate the macromolecular build up of this polymer in a more detailed manner.

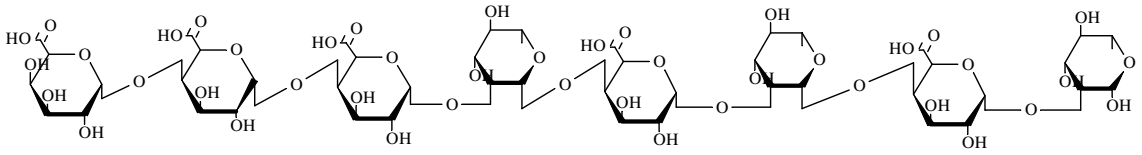

Fig. 7. Representation of a connection between rhamonogalacturonan type I and homogalacturonan.

Acknowledgment

This research was conducted within the framework of the Carbohydrate Research Centre Wageningen.

References

Albersheim, P., Darvill, A. G., O'Neill, M. A., Schols, H. A., & Voragen, A. G. J. (1996). An hypothesis: The same six polysaccharides are components of the primary cell wall of all higher plants. In J. Visser & A. G. J. Voragen (Eds.), *Pectins and pectinases* (pp. 47–53). Amsterdam: Elsevier.

Bacic, A., Harris, P., & Stone, B. A. (1988). Structure and function of plant cell walls. In J. Preiss (Ed.). *The biochemistry of plants, carbohydrates* (Vol. 14, pp. 297–369). London: Academic Press.

Beldman, G., Mutter, M., Searle-Van Leeuwen, M. J. F., Van den Broek, L. A. M., Schols, H. A., & Voragen, A. G. J. (1996). New enzymes active towards pectic structures. In J. Visser & A. G. J. Voragen (Eds.). *Pectins and pectinases* (Vol. 14, pp. 231–245). New York: Elsevier Science.

Beldman, G., Schols, H. A., Pitson, S. M., Searle-van Leeuwen, M. J. F., & Voragen, A. G. J. (1997). Arabinans and arabinan degrading enzymes. In R. J. Sturgeon (Ed.). *Advances macromolecular in carbohydrate research* (Vol. 1, pp. 1–64). London: JAI Press INC.

BeMiller, J. N. (1967). Acid-catalized hydrolisis of glycosides. *Advances in carbohydrate chemistry, 22*, 25–108.

Benen, J. A. E., Vinken, J.-P., & Alebeek, G. W. M. (2002). Microbial pectinases. In G. B. Seymour & J. P. Knox (Eds.), *Pectins and their manipulation*. Oxford: Blackwell Publishing Ltd.

Braconnot, H. (1825). Recherches sur un nouvel Acide universellement répadu dans tous les végétaux. *Annales de chimie et de physique, 28*, 173–178.

Bushneva, O. A., Ovodova, R. G., Shashkov, A. S., & Ovodov, Y. S. (2002). Structural studies on hairy region of pectic polysaccharide from campion *Silene vulgaris* (*Oberna behen*). *Carbohydrate Polymers, 49*(4), 471.

Carpita, N. C., & Gibeaut, D. M. (1993). Structural models of primary cell walls in flowering plants: Consistency of molecular structure with the physical properties of the walls during growth. *The Plant Journal, 3*(1), 1–30.

Colquhoun, I. J., de Ruiter, G. A., Schols, H. A., & Voragen, A. G. J. (1990). Identification by n.m.r. spectroscopy of oligosaccharides obtained by treatment of the hairy regions of apple pectin with rhamnogalacturonase. *Carbohydrate Research, 206*(1), 131–144.

Daas, P. J. H., Boxma, B., Hopman, A. M. C. P., Voragen, A. G. J., & Schols, H. A. (2001). Nonesterified galacturonic acid sequence homology of pectins. *Biopolymers, 58*, 1–8.

De Ruiter, G. A., Schols, H. A., Voragen, A. G. J., & Rombouts, F. M. (1992). Carbohydrate analysis of water-soluble uronic acid-containing polysaccharides with high-performance anion-exchange chromatography using methanolysis combined with TFA hydrolysis is superior to four other methods. *Analytical Biochemistry, 207*(1), 176.

de Vries, J. (2004). Hydrocolloid gelling agents and their applications. In P. A. Williams & G. O. Philips (Eds.). *Gums and stabilisers for the food industry* (Vol. 12, pp. 23–31). Cambridge: The Royal Society of Chemistry.

de Vries, J. A., Rombouts, F. M., Voragen, A. G. J., & Pilnik, W. (1982). Enzymic degradation of apple pectins. *Carbohydrate Polymers, 2*, 25–33.

de Vries, J. A., Voragen, A. G. J., Rombouts, F. M., & Pilnik, W. (1981). Extraction and purification of pectins from alcohol insoluble solids from ripe and unripe apples. *Carbohydrate Polymers, 1*, 117–127.

Domon, B., & Costello, C. E. (1988). A systematic nomenclature for carbohydrate fragmentations in fab-ms/ms spectra of glycoconjugates. *Glycoconjugate Journal, 5*, 397–409.

Guillon, F., & Thibault, J.-F. (1990). Oxidative cross-linking of chemically and enzymatically modified sugar-beet pectin. *Carbohydrate Polymers, 12*(4), 353.

Guillotin, S. E., Bakx, E. J., Boulenguer, P., Mazoyer, J., Schols, H. A., & Voragen, A. G. J. (2005). Populations having different GalA blocks characteristics are present in commercial pectins which are chemically similar but have different functionalities. *Carbohydrate Polymers, 60*(3), 391.

Gur'janov, O. P., Gorshkova, T. A., Kabel, M., Schols, H. A., & van Dam, J. E. G. (2007). MALDI-TOF MS evidence for the linking of flax bast fibre galactan to rhamnogalacturonan backbone. *Carbohydrate Polymers, 67*(1), 86.

Habibi, Y., Mahrouz, M., & Vignon, M. R. (2005). Isolation and structural characterization of protopectin from the skin of *Opuntia ficus-indica* prickly pear fruits. *Carbohydrate Polymers, 60*(2), 205.

Hilz, H., Bakx, E. J., Schols, H. A., & Voragen, A. G. J. (2005). Cell wall polysaccharides in black currants and bilberries – characterisation in berries, juice, and press cake. *Carbohydrate Polymers, 59*(4), 477.

Hilz, H., de Jong, L. E., Kabel, M. A., Schols, H. A., & Voragen, A. G. J. (2006). A comparison of liquid chromatography, capillary electrophoresis, and mass spectrometry methods to determine xyloglucan structures in black currants. *Journal of Chromatography A, 1133*(1-2), 275.

Huisman, M. M. H., Fransen, C. T. M., Kamerling, J. P., Vliegenthart, J. F. G., Schols, H. A., & Voragen, A. G. J. (2001). The CDTA-soluble pectic substances from soybean meal are composed of rhamnogalacturonan and xylogalacturonan but not homogalacturonan. *Biopolymers, 58*(3), 279–294.

Huisman, M. M. H., Schols, H. A., & Voragen, A. G. J. (1996). Changes in cell wall polysaccharides from ripening olive fruits. *Carbohydrate Polymers, 31*(3), 123.

Ishii, T., & Matsunaga, T. (2001). Pectic polysaccharide rhamnogalacturonan II is covalently linked to homogalacturonan. *Phytochemistry, 57*, 969–974.

Kabel, M. A., Schols, H. A., & Voragen, A. G. J. (2001). Mass determination of oligosaccharides by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry following HPLC, assisted by on-line desalting and automated sample handling. *Carbohydrate Polymers, 44*(2), 161.

Kardosova, A., Ebringerova, A., Alfoldi, J., Nosal'ova, G., Matakova, T., & Hribalova, V. (2004). Structural features and biological activity of an acidic polysaccharide complex from *Mahonia aquifolium* (Pursh) Nutt. *Carbohydrate Polymers, 57*(2), 165.

Kester, H. C. M., Kusters-Van Someren, M. A., Muller, Y., & Visser, J. (1996). Primary structure and characterization of an exopolygalacturonase from *Aspergillus Tubingensis*. *European Journal of Biochemistry, 240*, 738.

Kravtchenko, T. P., Berth, G., Voragen, A. G. J., & Pilnik, W. (1992). Studies on the intermolecular distribution of industrial pectins by means of preparative size exclusion chromatography. *Carbohydrate Polymers, 18*(4), 253.

Lau, J. M., McNeil, M., Darvill, A. G., & Albersheim, P. (1987). Treatment of rhamnogalacturonan I with lithium in ethylenediamine. *Carbohydrate Research, 168*(2), 245.

Le Goff, A., Renard, C. M. G. C., Bonnin, E., & Thibault, J. F. (2001). Extraction, purification and chemical characterisation of xylogalacturonans from pea hulls. *Carbohydrate Polymers, 45*(4), 325.

May, C. D. (2000). Pectins. In G. O. Phillips & P. A. Williams (Eds.), *Handbook of hydrocolloids* (pp. 169–188). Cambridge: Woodhead Publishing.

McNeil, M., Darvill, A., & Albersheim, P. (1980). Structure of plant cell walls 10. rhamno galacturonan I a structurally complex pectic polysaccharide in the walls of suspension cultured sycamore cells acer-pseudoplatanus cells. *Plant Physiology, 66*(6), 1128.

Mohnen, D. (1999). Biosynthesis of pectins and galactomannans. In D. Barton, K. Nakanishi, & O. Meth-Cohn (Eds.). *Comprehensive natural products chemistry* (Vol. 3, pp. 497–527). Elsevier.

Mutter, M., Beldman, G., Pitson, S. M., Schols, H. A., & Voragen, A. G. J. (1998a). Rhamnogalacturonan alpha-D-galactopyranosyluronohydrolase. An enzyme that specifically removes the terminal nonreducing galacturonosyl residue in rhamnogalacturonan regions of pectin. *Plant Physiology, 117*(1), 153–163.

Mutter, M., Colquhoun, I. J., Beldman, G., Schols, H. A., Bakx, E. J., & Voragen, A. G. J. (1998b). Characterization of recombinant rhamnogalacturonan alpha-L-rhamnopyranosyl-(1,4)-alpha-D-galactopyranosyluronide lyase from *Aspergillus aculeatus*. An enzyme that fragments rhamnogalacturonan I regions of pectin. *Plant Physiology, 117*(1), 141–152.

O'Neill, M. A., Eberhard, S., Albersheim, P., & Darvill, A. G. (2001). Requirement of borate cross-linking of cell wall rhamnogalacturonan ii for arabidopsis growth. *Science, 294*, 846–849.

O'Neill, M. A., Ishii, T., Albersheim, P., & Darvill, A. G. (2004). Rhamnogalacturonan II: Structure and function of a borate cross-linked cell wall pectic polysaccharide. *Annual Review of Plant Biology, 55*(1), 109–139.

Oosterveld, A., Coenen, G. J., Vermeulen, N. C. B., Voragen, A. G. J., & Schols, H. A. (2004). Structural features of acetylated galactomannans from green Coffea arabica beans. *Carbohydrate Polymers, 58*(4), 427–434.

Prade, R., Zhan, D., Ayoubi, P., & Mort, A. (1999). Pectins, pectinases and plant–microbe interactions. *Biotechnology and Genetic Engineering Reviews, 16*, 361–391.

Quemener, B., Ordaz-Ortiz, J. J., & Saulnier, L. (2006). Structural characterization of underivatized arabino-xylo-oligosaccharides by negative-ion electrospray mass spectrometry. *Carbohydrate Research, 341*(11), 1834.

Redgwell, R. J., & Selvendran, R. R. (1986). Structural features of cell-wall polysaccharides of onion *Allium cepa*. *Carbohydrate Research, 157*, 183–199.

Rees, D. A., & Wight, A. W. (1971). Polysaccharide conformation. Part VII. Model building computations for 1,4-galacturonan and the kinking function of L-rhamnose residues in pectic substances. *Journal of Chemical Society (B)*, 1366–1372.

Renard, C. M. G. C., Lahaye, M., Mutter, M., Voragen, A. G. J., & Thibault, J.-F. (1998). Isolation and structural characterisation of rhamnogalacturonan oligomers generated by controlled acid hydrolysis of sugar beet pulp. *Carbohydrate Research, 305*, 271–280.

Ridley, B. L., O'Neill, M. A., & Mohnen, D. (2001). Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochemistry, 57*, 929–967.

Schols, H. A., Bakx, E. J., Schipper, D., & Voragen, A. G. J. (1995a). A xylogalacturonan subunit present in the modified hairy regions of apple pectin. *Carbohydrate Research, 279*, 265.

Schols, H. A., Posthumus, M. A., & Voragen, A. G. J. (1990). Structural features of hairy regions of pectins isolated from apple juice produced by the liquefaction process. *Carbohydrate Research, 206*(1), 117.

Schols, H. A., Vierhuis, E., Bakx, E. J., & Voragen, A. G. J. (1995b). Different populations of pectic hairy regions occur in apple cell walls. *Carbohydrate Research, 275*(2), 343.

Schols, H. A., & Voragen, A. G. J. (1994). Occurrence of pectic hairy regions in various plant cell wall materials and their degradability by rhamnogalacturonase. *Carbohydrate Research, 256*(1), 83.

Schols, H. A., & Voragen, A. G. J. (1996). Complex pectins: Structure elucidation using enzymes. In J. visser & A. G. J. voragen (Eds.). *Pectins and pectinases* (Vol. 14, pp. 3–19). Amsterdam: Elsevier Science B.V..

Schols, H. A., & Voragen, A. G. J. (2002). The chemical structure of pectins. In G. B. Seymour & J. P. Knox (Eds.), *Pectins and their manipulation*. Oxford: Blackwell Publishing Ltd.

Schols, H. A., Voragen, A. G. J., & Colquhoun, I. J. (1994). Isolation and characterization of rhamnogalacturonan oligomers, liberated during degradation of pectic hairy regions by rhamnogalacturonase. *Carbohydrate Research, 256*(1), 97.

Thibault, J.-F., Renard, C. M. G. C., Axelos, M. A. V., Roger, P., & Crepeau, M.-J. (1993). Studies of the length of homogalacturonic regions in pectins by acid hydrolysis. *Carbohydrate Research, 238*, 271.

van Alebeek, G., Zabotina, O., Beldman, G., Schols, H. A., & Voragen, A. G. J. (2000). Structural analysis of (methyl-esterified) oligogalacturonides using post-source decay matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Journal of Mass Spectrometry, 35*(7), 831–840.

Verhoef, R., Beldman, G., Schols, H. A., Siika-aho, M., Ratto, M., Buchert, J., et al. (2005). Characterisation of a 1,4-[beta]-fucoside hydrolase degrading colanic acid. *Carbohydrate Research, 340*(11), 1780.

Vidal, S., Doco, T., Williams, P., Pellerin, P., York, W. S., O'Neill, M. A., et al. (2000). Structural characterization of the pectic polysaccharide rhamnogalacturonan II: Evidence for the backbone location of the aceric acid-containing oligoglycosyl side chain. *Carbohydrate Research, 326*(4), 277.

Vincken, J.-P., Schols, H. A., Oomen, R. J. F. J., Beldman, G., Visser, R. G. F., & Voragen, A. G. J. (2003a). Pectin- the hairy thing: Evidence that homogalacturonan is a side chain of rhamnogalacturonan I. In A. G. J. Voragen, H. A. Schols, & R. G. F. Visser (Eds.), *Advances in pectin and pectinase research* (pp. 47–60). New York: Springer.

Vincken, J.-P., Schols, H. A., Oomen, R. J. F. J., McCann, M. C., Ulvskov, P., Voragen, A. G. J., et al. (2003b). If homogalacturonan were a side chain of rhamnogalacturonan I. Implications for cell wall architecture. *Plant Physiology, 132*(4), 1781–1789.

Voragen, A. G. J., Beldman, G., & Schols, H. (2001). Chemistry and enzymology of pectins. In B. V. McCleary & L. Prosky (Eds.), *Advanced dietary fibre technology* (pp. 379–397). Oxford: Blackwell Publishing Ltd.

Voragen, A. G. J., Pilnik, W., Thibault, J. F., Axelos, M. A. V., & Renard, C. M. G. C. (1995). Pectins. In A. M. Stephen (Ed.), *Food polysaccharides and their applications* (pp. 287–339). New York: Marcel Dekker.

Yapo, B. M., Lerouge, P., Thibault, J.-F., & Ralet, M.-C. (2007). Pectins from citrus peel cell walls contain homogalacturonans homogenous with respect to molar mass, rhamnogalacturonan I and rhamnogalacturonan II. *Carbohydrate Polymers, 69*(3), 426–435.

Zaia, J. (2004). Mass spectrometry of oligosaccharides. *Mass Spectrometry Reviews, 23*(3), 161–227.

Zhan, D., Janssen, P., & Mort, A. J. (1998). Scarcity or complete lack of single rhamnose residues interspersed within the homogalacturonan regions of citrus pectin. *Carbohydrate Research, 308*, 373–380.