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Isolation and characterization of rhamnogalacturonan oligomers, liberated during degradation of pectic hairy regions by rhamnogalacturonase *

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ABSTRACT

Digests of modified hairy regions of apple pectin (MHR) obtained after degradation by rhamnogalacturonase (RGase) were analyzed for oligomer composition using high-performance anion-exchange chromatography and pulsed amperometric detection. A series of oligomers which appear to be characteristic of RGase degradation could be recognized. These oligomers were isolated on a preparative scale by size-exclusion chromatography and preparative anion-exchange chromatography and analyzed for sugar composition. ^{1}H NMR spectroscopy showed that the oligomers consisted of between 4 and 9 sugar units with a backbone of alternating rhamnose and galacturonic acid residues, partly substituted with galactose residues linked to C-4 of the rhamnose moiety. The HPLC elution pattern showed that higher oligomers were also formed during incubation with RGase. These have the same basic structure but may contain other sugar units in addition to those given above. The oligomer composition of RGase digests of MHR isolated from apple, pear, leek, onion, carrot, and potato was very similar. Using anion-exchange chromatography to monitor the degradation of MHR at increasing incubation times, it was found that all the oligomers were present from the initial stages of the enzyme reaction and that the ratio between the different oligomers remained constant with time. Implications of these results for the structure of MHR and the mechanism of RGase action are discussed.

INTRODUCTION

Although the structure of pectic substances has been the subject of many investigations, important questions about their structure remained unanswered.

* Hairy (Ramified) Regions of Pectins, Part V. For Part IV see ref 10.
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Pectin is a hetero-polysaccharide consisting of a linear chain of α-(1 → 4)-linked D-galacturonic acid residues, interrupted at intervals by α-(1 → 2)-linked L-rhamnopyranosyl residues, to which neutral sugar side-chains are connected. Barrett and Northcote[1] first showed that neutral sugars are located in certain areas of the pectin molecule. In enzymic degradation studies of extracted pectins, De Vries et al.[2] found that 95% of the neutral sugars in a pectin were carried by a backbone containing only 5% of the galacturonosyl residues. These regions were named hairy regions. Similar regions were reported for pectins from grapes[3,4], carrots[5], and *Angelica acutiloba* Kitagawa[6]. Characteristic of these hairy regions is the relatively low galacturonic acid content (7–23%) and a high rhamnose content (5–15%), resulting in high ratio of rhamnose to galacturonic acid (0.2–1). The most important accompanying neutral sugars are usually arabinose and galactose; their relative amounts depend on the plant material under investigation.

In previous studies[7], we isolated a polysaccharide fraction from apple juice manufactured by liquefying ground apples with a mixture of pectolytic, hemicellulolytic, and cellulolytic enzymes. In this process, the polysaccharide fraction had been solubilized from the cell walls and was resistant to further degradation by the enzyme mixture. From its sugar and glycosidic linkage composition and from chemical degradation studies, it appeared that the polysaccharide had a structure very similar to the pectic hairy regions as described by De Vries et al.[2]. For obvious reasons, we named this fraction "modified hairy regions" (MHR). Using MHR as a substrate, we discovered and isolated a new enzyme which was able to degrade 4% of the glycosidic linkages in the MHR-backbone and which we named therefore "rhamnogalacturonase" (RGase)[8]. Since it was not possible at that time to separate the various oligosaccharides present in the MHR digest, the low molecular weight products were characterized by NMR spectroscopy of the mixture[9]. The oligomers were found to have a basic structure of α-Rha*p*-(1 → 4)-α-GalA-(1 → 2)-α-Rha*p*-(1 → 4)-GalA. A β-Gal*p* unit was 4-linked to approximately half of the terminal Rha*p* residues and to half of the (1 → 2)-linked Rha*p* residues. With this information, a detailed but still partly hypothetical structure was proposed[7] for MHR.

The accompanying paper[10] describes the characterization of MHR fractions, including their degradability by RGase as followed by changes in the molecular weight distribution of MHR by high-performance size-exclusion chromatography (HPSEC). It was shown that RGase was able to degrade the MHR fractions isolated from various sources. However, to study the low molecular weight degradation products of MHR in more detail, a chromatographic method capable of separating the oligomeric fragments was required.

In this paper, we report an HPLC method using anion-exchange chromatography to separate the oligomers liberated by RGase. Oligomers having a degree of polymerization (dp) between 4 and 9 were isolated on a preparative scale and identified using ^{1}H NMR spectroscopy. Using the HPLC method, the degradation of apple MHR by RGAse was followed with time. Degradation products formed by

RGase during enzymic degradation of MHR from potato fibre, carrot, onion, leek, and pear were compared with those from apple MHR.

EXPERIMENTAL

Isolation of the modified hairy regions and their degradation by RGase.—The MHR were isolated from juices obtained by liquefying the ground raw materials as described[7,10]. The degradation of MHR by RGase was performed after hydrolysis of methyl and acetyl esters by NaOH (resulting in MHR-S) as described by Schols et al.[8].

Chromatography.—High-performance anion-exchange chromatography (HPAEC) was performed on a Dionex Bio-LC system (Sunnyvale, CA) which included a quaternary gradient pump, eluent degas (He) module, a (4 × 250 mm) CarboPac PA1 column with matching guard column, and a pulsed electrochemical detector (PED) in the pulsed amperometric detection (PAD) mode. Samples were introduced into the system using a Spectra Physics SP8800 autosampler (San Jose, CA), and chromatograms were recorded with a Spectra Physics Winner system. The effluent was monitored using the PED detector (reference electrode Ag/AgCl) containing a gold electrode. Potentials of E_1 0.1, E_2 0.6, and E_3 −0.6 V were applied for duration times T_1 0.5, T_2 0.1, and T_3 0.1 s. The flow rate was 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 an equilibration step of at least 15 min with 150 mM NaOAc in 100 mM NaOH, 20 μL of the sample was injected, and a linear gradient to 500 mM NaOAc in 100 mM NaOH within 40 min was started. The column was washed for 5 min with M NaOAc in 100 mM NaOH and equilibrated again for 15 min with 150 mM NaOAc in 100 mM NaOH.

Isolation of oligomers from MHR digests.—Size-exclusion chromatography over Sephadex G50[8] was used to separate the oligomers from the high molecular weight fraction in the RGase digest of apple MHR-S.

The oligomeric fraction was further fractionated by preparative HPAEC using the Dionex system as described above. A CarboPac PA1 preparative column (9 × 250 mm) without guard column was used with a flow rate set at 5.0 mL/min. The gradient was slightly modified and 200-μL aliquots were injected. Following detection, the effluent was neutralized by the addition of M AcOH (Peristaltic 2232 pump, Pharmacia/LKB, Uppsala, Sweden) and fractions were collected using a Helirac 2212 fraction collector (Pharmacia/LKB). Pooled fractions were dialysed against distilled water and lyophilized.

Analytical methods.—Samples were hydrolyzed by 2 M CF_3CO_2H acid at 121°C for 1 h and neutral sugars were converted into their alditol acetates as described[7], in order to determine the sugar composition. The uronic acid content was determined colorimetrically using *m*-hydroxybiphenyl as described[7].

NMR spectroscopy.—^1H NMR spectra were recorded for each fraction (1 mg or less in 0.5 mL of D_2O) at 400 MHz with a Jeol GX400 spectrometer. The residual

water signal was suppressed by saturation during the 2-s delay between acquisitions. The number of transients was between 200 and 2000, depending upon the amount of sample available. Acetone was added as internal reference and its chemical shift was set to 2.217 ppm with respect to Me_4Si.

RESULTS

Analysis of oligomeric reaction products of RGase.—Most of the silica gel-based columns used in carbohydrate chromatography, including weak and strong anion-exchangers, were unable to retain the type of oligomer liberated by RGase. Also, chromatography on commercially available polystyrene diphenylbenzene resins was not very successful. On a 2% cross-linked resin (Aminex HPX 22H), successfully used by Hicks and Hotchkiss[11] for the separation of maltodextrins and oligogalacturonides, we could only obtain limited resolution (results not shown). We also applied a newly developed technique which is based on the use of strong anion-exchange resins in combination with pulsed electrochemical detectors and which allows the use of gradient elution for improved separation of oligosaccharides[12–14].

Fig. 1 shows the elution pattern of the RGase digest of apple MHR on a CarboPac PA1 column. Due to the high selectivity of the column under the conditions of the applied gradient, an elution pattern was obtained in which three major peaks and several minor peaks could be distinguished. Although not all components were separated completely, the separation could not be improved by changes in elution conditions. A possible explanation for this observation is the great similarity in structure of the various oligomers (see below). Direct quantification of the different oligomers by comparison of peak areas is not possible due to

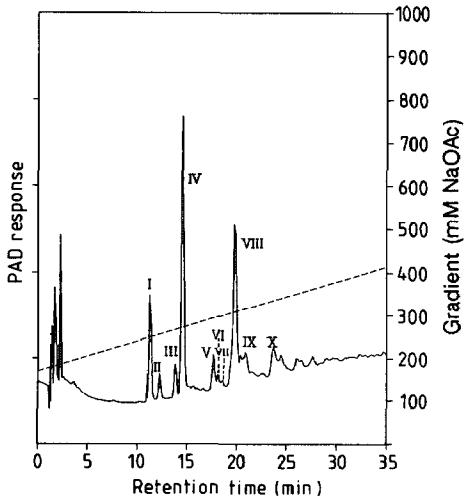

Fig. 1. Elution profile on HPAEC of apple MHR-S after treatment with RGase at 30°C and pH 5.0 for 24 h.

different response factors for different oligosaccharides[12,14–16]. The estimated amounts of unknown oligomers can easily be in error. This is also the case when higher oligomers are quantified using the response factor of some monomeric sugar residues[12,15].

Sugar composition of the purified oligomers.—Preparative separation of the low molecular weight fraction was performed using a preparative CarboPac PA1 column which showed an equal or sometimes even better resolution between the different oligomers as compared to the analytical CarboPac PA1 column. The combined fractions were analyzed for their sugar composition. Since alditol acetates were prepared from very small quantities (30–100 μg), traces of xylose, mannose, and glucose were found, although these sugars were not detectable in the oligomer mixture before purification. For this reason, only the relative amounts of rhamnose, galactose, and galacturonic acid are given in Table I. Using analytical HPAEC, it was shown that the preparative fractions, I, II, V, VI, VII, and VIII (Table I) were essentially homogeneous. Others were found to be mixtures of closely eluting compounds. The preparative HPAEC isolation and subsequent pooling resulted in only a mixture of fractions III and IV, while fraction IX contained > 70% of fraction VIII. All oligomer fractions except fractions I and V contained galactose, which is in agreement with earlier results[8,9]. However, there was still insufficient information to deduce the complete structure and size of the oligomers, especially in the case of mixtures of fractions III/IV and VIII/IX. Confirmation of structure was also required since the order of elution of the different oligomers is not predictable and was found to depend on sugar residues and linkage types present as well as on the dp[14]. For this reason, further characterization of the oligomers was performed by NMR spectroscopy.

Oligomer structure from ^1H NMR spectra.—In previous work[9], an extensive series of 2D NMR experiments was used to deduce the structures of four rhamnogalacturonan oligosaccharides present as a mixture. To determine the

TABLE I

Sugar composition (mol%) of oligomers, obtained after Sephadex G50 size-exclusion chromatography and preparative HPAEC of the digest of apple MHR-S with RGase

Fraction	Major oligomer [a]	Rha	Gal	GalA
I	**1**	44	1	55
II	**2**	32	18	50
III/IV [b]	**4**	28	36	36
V	**5**	47	3	50
VI	**5, 6**	32	19	49
VII	**6, ?**	26	36	38
VIII	**8a, 8b**	29	35	36
IX	**9**	25	38	37
X	**9, ?**	34	46	21

[a] The numbers of the oligomers correspond with those given in Table II. [b] Components 3 and 4 pooled into one fraction.

linkage positions, it was necessary to know the chemical shifts of protons in the extremely crowded spectral region from 3.5–4.2 ppm and, for this, 2D experiments were essential. With the structural information from the earlier study and the availability of fractions which contain only one major component, it has proved possible to use simple 1D ^{1}H NMR spectra to deduce the structures of further oligomers of this type. Characteristic signals in the less crowded regions of the spectrum provide sufficient information for structure determination, and allow the determination to be made on quantities of material which are insufficient for routine ^{13}C NMR studies. The signals for all the anomeric protons, together with those for H-4 and H-5 of the GalA units are found in the region from 4.3 to 5.4 ppm. Other well-resolved signals are those between 3.3 and 3.5 ppm from H-4 of Rha units (unsubstituted by Gal) and those from H-6 of Rha (substituted and unsubstituted) between 1.22 and 1.31 ppm.

Fig. 2 shows ^{1}H NMR spectra of two of these regions for fractions I, II, and III/IV (mixture of two fractions). Structures of oligomers which have been

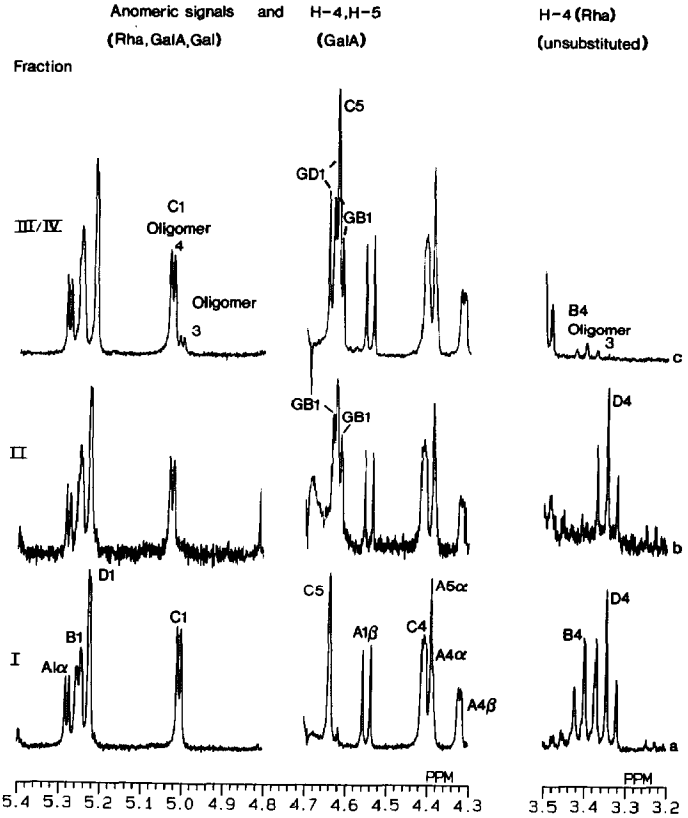

Fig. 2. ^{1}H NMR spectra of fractions containing oligomers based on the $(Rha-GalA)_2$ backbone unit. Major oligomer components of the fractions are a 1; b 2; and c 4. The key to individual assignments is given in Table III. The partly suppressed water signal (4.74 ppm) is not shown.

TABLE II

Structures of identified oligomers, obtained after degradation of apple MHR-S by RGase

1	α-Rha*p*-(1 → 4)-α-Gal*p*A-(1 → 2)-α-Rha*p*-(1 → 4)-Gal*p*A
2	α-Rha*p*-(1 → 4)-α-Gal*p*A-(1 → 2)-α-Rha*p*-(1 → 4)-Gal*p*A β-Gal*p*-(1 → 4)
3	β-Gal*p*-(1 → 4)-α-Rha*p*-(1 → 4)-α-Gal*p*A-(1 → 2)-α-Rha*p*-(1 → 4)-Gal*p*A
4	β-Gal*p*-(1 → 4)-α-Rha*p*-(1 → 4)-α-Gal*p*A-(1 → 2)-α-Rha*p*-(1 → 4)-Gal*p*A β-Gal*p*-(1 → 4)
5	α-Rha*p*-(1 → 4)-α-Gal*p*A-(1 → 2)-α-Rha*p*-(1 → 4)-α-Gal*p*A-(1 → 2)-α-Rha*p*-(1 → 4)-α-Gal*p*A-(1 → 2)-α-Rha*p*-(1 → 4)-Gal*p*A
6	α-Rha*p*-(1 → 4)-α-Gal*p*A-(1 → 2)-α-Rha*p*-(1 → 4)-α-Gal*p*A-(1 → 2)-α-Rha*p*-(1 → 4)-Gal*p*A β-Gal*p*-(1 → 4)
8a	β-Gal*p*-(1 → 4)-α-Rha*p*-(1 → 4)-α-Gal*p*A-(1 → 2)-α-Rha*p*-(1 → 4)-α-Gal*p*A-(1 → 2)-α-Rha*p*-(1 → 4)-Gal*p*A β-Gal*p*-(1 → 4)
8b	β-Gal*p*-(1 → 4)-α-Rha*p*-(1 → 4)-α-Gal*p*A-(1 → 2)-α-Rha*p*-(1 → 4)-α-Gal*p*A-(1 → 2)-α-Rha*p*-(1 → 4)-Gal*p*A β-Gal*p*-(1 → 4)
9	β-Gal*p*-(1 → 4)-α-Rha*p*-(1 → 4)-α-Gal*p*A-(1 → 2)-α-Rha*p*-(1 → 4)-α-Gal*p*A-(1 → 2)-α-Rha*p*-(1 → 4)-Gal*p*A β-Gal*p*-(1 → 4) β-Gal*p*-(1 → 4)

identified are given in Table II and the lettering scheme for individual sugar units is given in Table III, together with selected chemical shifts. Fraction I contained only the tetramer **1** which is unsubstituted by β-Gal. Assignments (Fig. 2a) were obtained from earlier work[9] and the chemical shifts were in good agreement with those reported previously. The dp of **1** was established by integration of the anomeric signals of the nonreducing sugar rings with respect to the total integral ($\alpha + \beta$ forms) of the A1 resonances of the reducing end GalA unit. It may be noted that the chemical shift of the B1 resonance is affected by the anomeric form of the neighbouring A ring, and that there is apparent broadening of the B4 resonance (in comparison with D4) for the same reason.

Fraction II consisted entirely of the pentamer **2**, in which ring B is substituted with a β-Gal unit. The anomeric proton of this unit gave a doublet (J 7.6 Hz) at 4.625 ppm which overlapped with the C5 resonance of the GalA ring (Fig. 2b). It was found previously[9] that linkage of a β-Gal unit at O-4 of the Rha ring produced a downfield shift of 0.27 ppm for the H-4(Rha) resonance. The location of Gal on ring B, rather than ring D, was shown by the disappearance of the B4 triplet in Fig. 2b (shifted downfield from 3.399 ppm in Fig. 1a), whereas the D4 triplet was unaffected. Other chemical shift changes which accompany the linkage of the β-Gal unit to ring B are those of C1 and B6 (moved 0.02 and 0.06 ppm downfield, respectively) and C5 (moved 0.01 ppm upfield), but the shift of B1 is hardly changed. Similar changes occur with the higher dp oligomers discussed below. It is also known[9] that the chemical shifts of B3 and B5 are appreciably affected by Gal substitution, but these shifts are not readily measurable from the 1D spectra used here.

Fraction III/IV consisted mainly of the hexamer **4** plus a small amount (~ 10%) of pentamer **3**. The presence of a second β-Gal unit was indicated by the

TABLE III

Selected ^{1}H chemical shifts [a] (ppm) for rhamnogalacturonan oligomers

Oligomer	Unit	Gal	Rha	GalA	Gal	Rha	GalA	Gal	Rha	GalA (r.e.) [b]
		GF	F	E	GD	D	C	GB	B	A
1	H-1					5.227	5.006		5.257(α) [c]	5.280(α)
									5.248(β)	4.548(β)
	H-4					3.346	4.407		3.399	4.391(α)
										4.320(β)
	H-5						4.638			4.391(α)
	H-6					1.227			1.242	
2	H-1					5.228	5.028	4.625	5.251	[d]
	H-4					3.346	4.412		[e]	
	H-5						4.627			
	H-6					1.228			1.301	
4	H-1				4.636	5.216	5.030	4.625	5.251	[d]
	H-4					[e]	4.409		[e]	
	H-5						4.626			
	H-6					1.291			1.301	
5	H-1		5.228	5.003		5.264	5.014		5.257	[d]
	H-4		3.345	4.407		3.394	4.407		3.403	
	H-5			4.642			4.656			
	H-6		1.227			1.243			1.243	
6	H-1		5.227	5.000		5.261	5.032	4.629	5.261	[d]
	H-4		3.343			3.392			[e]	
	H-6		1.227			1.243			1.303	
8a	H-1	4.637	5.216	5.044	4.624	5.262	5.014		5.262	[d]
	H-4		[e]			[e]			3.403	
	H-6		1.289			1.302			1.243	
8b	H-1	4.637	5.216	5.004		5.262	5.030	4.624	5.262	[d]
	H-4		[e]			3.394			[e]	
	H-6		1.289			1.243			1.302	
9	H-1	4.637	5.216	5.038	4.624	5.261	5.030	4.624	5.261	[d]
	H-4		[e]			[e]			[e]	
	H-6		1.289			1.303			1.303	

[a] ±0.003 ppm. [b] r.e. = reducing end. [c] Effect of neighbouring r.e. [d] Ring A parameters same as **1**. [e] Ring substituted by Gal, H-4 shifted to ~3.67 ppm.

addition of a second doublet at 4.636 ppm (Fig. 2c). In this region, the two H-1 doublets of β-Gal overlap with the C5 resonance (4.626 ppm). Fig. 2c shows the disappearance of the D4 resonance, corresponding to complete Gal substitution at the O-4 position of ring D. However, small residual signals from B4 (3.399 ppm) and C1 (5.006 ppm) are assigned to the pentamer **3**. Gal substitution of the terminal Rha residue shifts D1 upfield by 0.01 ppm, and D6 downfield by 0.06 ppm, but leaves other readily measurable resonances unchanged. Again, there are similar observations with higher dp oligomers. The oligosaccharides **1–4** were previously identified[9] in a mixture which contained all four species, but it was not possible to be very specific about the relative amounts of each component. Separation of individual components has allowed finer structural details (such as

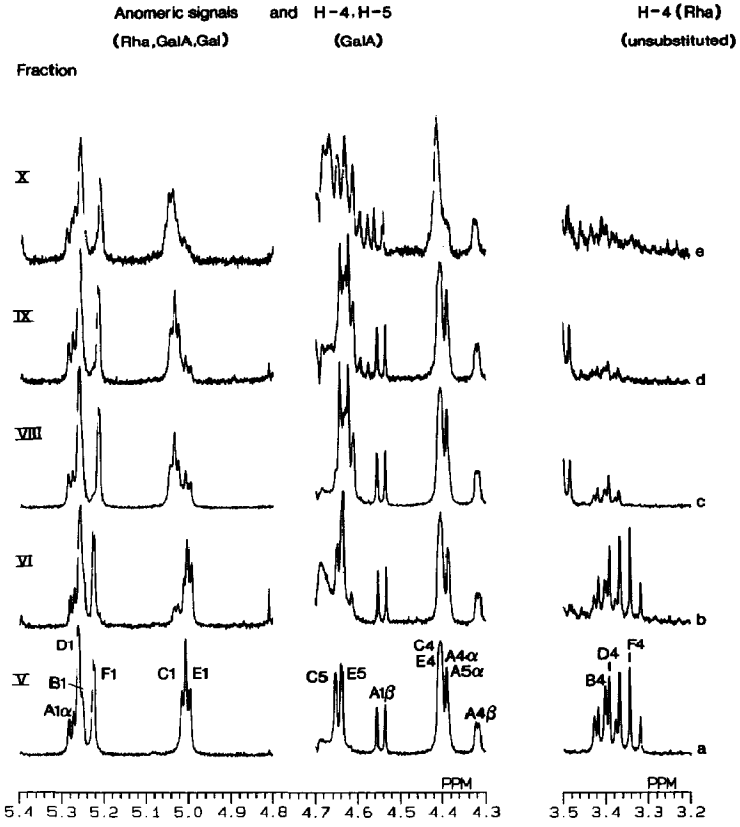

Fig. 3. ^{1}H NMR spectra of fractions containing oligomers based on the $(Rha-GalA)_3$ backbone unit. Major oligomer components of the fractions are a **5**; b **5**, **6**; c **8a**, **8b**; d **9**; and e **9**+?

the exact position of Gal substitution) to be determined and has shown that the major constituents are the tetramer and hexamer.

Spectra of the fractions with a longer retention time on the HPAEC column are shown in Fig. 3. Fraction V (Fig. 3a) contained only the linear hexamer **5**, unsubstituted by Gal. The dp of the oligomer was obtained by integration of the anomeric resonances (as for **1**), but a comparison of Fig. 3a with Fig. 2a also shows clearly the new resonances assigned to the additional GalA and Rha units. Resonances associated with the terminal reducing and nonreducing units have the same chemical shifts as in **1**. Thus, F1, F4, and F6 have chemical shifts similar to those of the corresponding resonances from the terminal Rha residues of **1**. The B and D rings of **5** may be distinguished using the signals in the H-4(Rha) region since the B4 chemical shift is slightly affected by the anomeric form of the neighbouring A ring (cf. **1**). This gives a slight broadening of the B4 resonance, which can therefore be assigned as the highest frequency triplet of the three H-4(Rha) multiplets.

The partial loss of B4 signal intensity in the spectrum of fraction VI (Fig. 3b) showed that VI did not consist of a single oligomer but was an approximately equimolar mixture of **5** and **6**. Substitution of ring B with a β-Gal changed the C1 chemical shift to 5.032 ppm and the loss of intensity of the high-frequency H-1(GalA) doublet (5.014 ppm) suggested that the assignment of C1 and E1 in **5** was as indicated in Fig. 3a. Fraction VII (not shown) was rather similar to VI but contained a higher proportion of **6**, mixed with the other mono-Gal isomers.

Fraction VIII (Fig. 3c) consisted mainly of a mixture of the two isomers **8a** and **8b**. Disappearance of the F4 signal showed that the terminal Rha was completely substituted by β-Gal and this was confirmed by the change of the F1 shift to 5.216 ppm (as in **4**). Anomeric signals of the β-Gal units are assigned as the doublet centred on 4.637 ppm (Gal linked to terminal Rha; this doublet was not found in the spectrum of **6**) and the two doublets centred on 4.624 ppm (Gal linked to units B and D). Resonances C1 and E1 were shifted downfield by Gal substitution of rings B and D, respectively. If fraction VIII had consisted only of **8a** and **8b**, the two downfield C1/E1 doublets would be expected to have the same intensity as the upfield pair. Integration shows a ratio of 2:1 rather than 1:1, so VIII must also contain some of the nonamer **9**. In fraction IX (Fig. 3d), this ratio has increased to 4:1 and from this it is estimated that the proportion of **9** is ca. 30% in fraction VIII and 60% in fraction IX.

It was thought that fraction X might contain oligomers based on a $(Rha–GalA)_4$ backbone unit. However, the spectrum (Fig. 3e) was similar to that of **9**, and although H-1(Rha) and H-1(GalA) regions appeared more complex, integrations showed that the molecule was still based on the $(Rha–GalA)_3$ unit. Absence of signals from the 3.4 ppm H-4(Rha) region indicated a high level of Gal substitution (confirming the results of Table I). A doublet (J 7.2 Hz) on 4.59 ppm, which was just evident in Fig. 3d, had become more prominent in Fig. 3e. This signal could arise from the anomeric proton of β-Gal units which form part of longer galactan side chains. Shortage of material prevented further investigations of this fraction.

NMR studies of fractions V–IX have therefore shown the existence of a series of oligomers based on a hexameric rhamnogalacturonan unit, with progressively increasing amounts of galactose. Clean separation of the individual oligomers becomes more difficult as the molecular size increases. NMR provides a convenient and rapid alternative to conventional analytical methods when only small quantities of sample are available, even if the samples contain a mixture of closely related compounds.

Time curves of the degradation of apple MHR by RGase.—Now that the oligomers have been separated by HPAEC and their structures have been established by NMR spectroscopy, the HPAEC method can be used to investigate the formation of these reaction products during enzymic degradation of apple MHR. The degradation was monitored by both HPSEC and HPAEC. The HPSEC elution patterns are similar to the chromatograms previously published by Schols et al. (ref. 8, Fig. 3). The elution patterns obtained on the anion-exchange column are

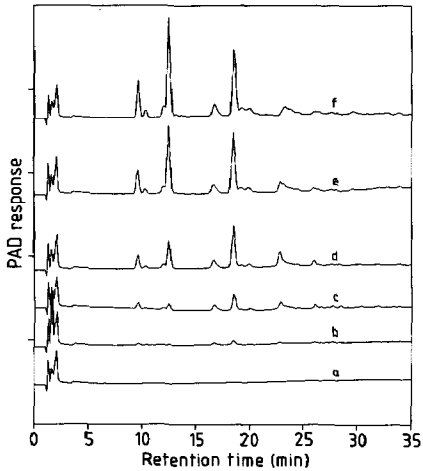

Fig. 4. Elution profile on HPAEC of apple MHR-S after treatment with RGase at 30°C and pH 5.0 for various incubation times: a, blank; b, 5 min; c, 15 min; d, 60 min; e, 300 min; f, 20 h.

shown in Fig. 4. The separation shown in Fig. 1 is significantly better than that shown in Fig. 4. The first separation was obtained on a new CarboPac PA1 column and could not be reproduced for large series of digests. However, the separation shown in Fig. 4 could be reproduced numerous times (more than 500 injections), even after using the column intensively for 1 year. From Fig. 4, it can be seen that almost all of the oligomers were present from the initial stage of the RGase action and that they increased together during the incubation time. The preponderant oligomers in the initial stage were also the most important oligomers after prolonged incubation times (Fig. 4; 5 min vs. 20 h of incubation). It can be seen

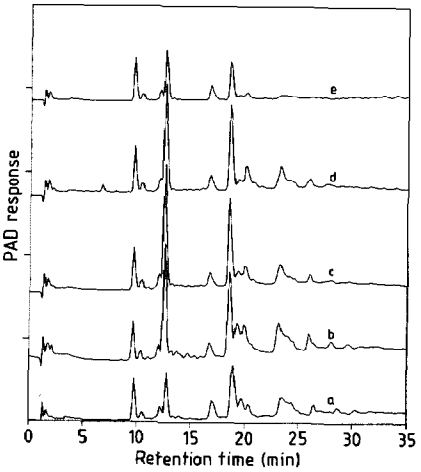

Fig. 5. Elution profile on HPAEC of MHR-S, isolated from various sources after treatment with RGase at 30°C and pH 5.0 for 24 h: a, carrot; b, onion; c, leek; d, potato fiber; e, pear.

from Fig. 4 that the proportion of oligomer **4** is increasing slightly during incubation when compared to the proportion of the other oligomers.

Degradation of MHR from other sources by RGase.—Using the liquefaction process, hairy regions could be isolated from a variety of fruit and vegetable tissues[10]. Striking similarities were observed with respect to sugar composition, rhamnose–galacturonic acid ratio, degree of acetylation, and degradability by RGase as monitored by HPSEC[10]. The RGase digests of MHR originating from potato fibre, carrot, onion, leek, and pear tissue were examined by HPAEC and the elution patterns were compared with those obtained for apple MHR digests. Fig. 5 shows the elution patterns after 20 h of incubation. After this incubation time, the degradation can be considered as complete[8,10]. The elution pattern of pear MHR resembled that of apple MHR, and indicated a similar structure for both MHR preparations. This is also indicated by the similarity in sugar composition[10]. In the digest of onion MHR, some oligomeric compounds were found which elute between hexamer **4** and octamer **8**, as well as significant quantities of higher oligomers. These higher oligomers seemed to be resistant to further degradation.

DISCUSSION

It can be concluded that the newly developed HPAEC method is of great value in the study of the fine structure of modified hairy regions, and the pattern of action of RGase. Complete resolution of the oligomers eluting at higher concentrations of sodium acetate could not be achieved, although Hotchkiss and Hicks[13] and Koizuma et al.[12] reported separations with high resolution for oligogalacturonides and oligoglucosides up to a dp of 40–70. The poor resolution found for some of the RGase oligomers is probably caused by the fact that these oligomers have a repeating unit which differs in substitution. In contrast, the galacturonic acid oligomers and maltodextrins have simple linear structures. Structural diversity of the rhamnogalacturonans will result in (higher) oligomers having the same dp but with galactose residues linked at different positions in the molecule. They may not be readily separable.

Using the HPAEC method, we were able to isolate pure oligomers which could be partly characterized by their sugar composition. The different ratios of constituent rhamnose, galacturonic acid, and galactose residues indicated the presence of oligomers with different dp and different degrees of galactose substitution as found previously[9]. This was confirmed using NMR spectroscopy to identify components (**1–4**) based on the $(Rha–GalA)_2$ unit, whilst additional oligomers with a basic $(Rha–GalA)_3$ unit were also recognized (**5**, **6**, **8**, and **9**). The latter series has not been reported before in the literature. It should be mentioned that characterization of various oligomers from rhamnogalacturonan I, obtained after chemical degradation, has been reported[17,18] previously. The use of the specific rhamnogalacturonase, however, resulted only in the oligomers discussed, while the less specific chemical methods (e.g., β-elimination) resulted also in oligomers with side

chains containing one or more arabinose and two galactose residues. In these studies[17,18], separation of the differently prepared oligomers was usually performed after reduction of the carboxyl group followed by permethylation of the free hydroxyl groups. This explains why the structure of the oligomers isolated differs enormously. However, the structure of the original rhamnogalacturonan as deduced from the information reported is quite similar. It can be concluded that isolation of the native oligomers and characterization by NMR as described here is more direct, and the samples remain available for further experiments.

The structure of the identified oligomers confirmed earlier findings[8,9] that RGase is able to hydrolyze galacturonic acid–rhamnose linkages in the backbone in an endo-fashion. However, the variety, sequence, and relative amounts in which the oligomers are formed in the course of the reaction are not typical of an endo-attack. All the oligomers identified are formed from the beginning and none of them appears to be degraded after prolonged incubation times. Even after an incubation time of 24 h, the hexamer **5** is still present (Fig. 1) and is apparently not degraded further. The relative amounts of **1** and **4** increased slightly compared with **5** and **8**. Since there is no increase of small RGase oligomers at the expense of larger RGase oligomers, the increase in relative amounts of **1** and **4** must originate from polymeric material. The observed pattern of formation of the oligomers with time may be related to the structure of the rhamnogalacturonan regions in MHR. It is suggested that RGase is only able to hydrolyze linkages in the backbone after recognition of sequences of alternating rhamnose and galacturonic acid residues of a certain length. Depending on the structure of the backbone of MHR, this will result in the liberation of an oligomer with a tetrameric backbone, a hexameric backbone, or possibly even higher oligomeric fragments.

Although the oligomers identified consisted only of galacturonic acid, rhamnose, and galactose, it is likely that higher oligomers might exist containing other sugar residues. Size-exclusion chromatography over Sephadex G50 showed[8] that a fraction containing higher oligomers (dp 10–20) with an arabinose content of 12 mol% was present in the RGase digest of apple MHR. So far it is not known whether the arabinose is present as a single unit or as longer side chains (linear or branched).

The identified oligomers were also found in RGase digests of MHR from potato fibre, carrot, onion, leek, and pear. In spite of slight differences, there is a striking similarity in the elution patterns. Again, the ratio between the various oligomers formed in the course of the reaction remained rather constant, as described above for the RGase degradation products of apple MHR. Since all incubations were performed using the same substrate concentration, small differences in the total amount of liberated oligomers (Fig. 5) can be explained by the different sugar composition of the substrates. Depending on the source, some MHR fractions (apple and pear) contained considerably more arabinose, present as arabinan side chains of the rhamnogalacturonan. As a result of this, the actual substrate for RGase is present in lower concentrations. When HPSEC chromatograms of the

various MHR preparations after RGase degradation were compared[10], the differences in amounts of oligomers formed were even more distinct. In contrast to HPSEC with refractive index detection, it should be realized that examination of the HPAEC elution patterns is not an appropriate method for absolute quantification of oligomers formed, because the response factor for the oligomers might be different. For this reason, HPSEC is a more valid method for judging the amount of oligomers formed after degradation with RGase.

These findings confirm that the structures of MHR isolated from a variety of sources are rather similar. As well as the similarity in sugar composition, degree of esterification, and degradability by RGase, the newly obtained elution patterns of the RGase digests can be seen as further evidence for the statement[7,10,19] that MHR is more regular in structure than generally assumed. In addition to the smaller oligomers such as **1**, **4**, and **8**, a similar profile of higher oligomers is liberated from the various MHR substrates, suggesting a high degree of structural homology between substrates. We believe that the breakdown pattern indicates that the rhamnogalacturonan degradation products do not originate from a long "homogeneous" backbone of rhamnose and galacturonic acid residues as suggested by O'Neill et al.[20] for rhamnogalacturonan I. Since a variety of RG oligomers are liberated initially, and not degraded further, a more complex distribution of short rhamnogalacturonan units over the whole "hairy region" of the molecule is suggested. An adapted model of the Modified Hairy Regions of apple pectin has been proposed[21] in which MHR consists of three subunits: a xylogalacturonan subunit interrupted by a subunit consisting of an arabinan-rich stub of the rhamnogalacturonan backbone and a subunit in which the identified rhamnogalacturonan oligomers are dominantly present. From this point of view, the various MHR under investigation are different from the RG-I as described by the Albersheim group[20], although interruption of the rhamnogalacturonan chain with two or more galacturonic acid (or rhamnose) residues is certainly possible and may explain the observed action of rhamnogalacturonase. In addition, RGase was found to be inactive on RG-I supplied by Albersheim's group (unpublished results). Since RGase incubation of the MHR fractions from diverse plant tissues resulted in comparable HPAEC elution patterns, the technique can be used to screen plant cell wall material for the presence of this type of pectic molecule. Now that the smaller oligomers liberated from MHR are well characterized, future research will be directed towards the high molecular weight RGase degradation products of MHR, the segments of the rhamnogalacturonan backbone having various ratios of rhamnose to galacturonic acid, and the liberated "higher" oligomers with dp > 10.

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