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Different populations of pectic hairy regions occur in apple cell walls ☆

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Abstract

Alcohol insoluble solids from apple were extracted in sequence by buffer at 20°C and at 70°C, EDTA/oxalate, and mild alkali, yielding four populations of pectins. These pectins and the insoluble residue were characterized by their sugar composition, degree of esterification (methyl ester and *O*-acetyl groups), molecular weight distribution, and degradability by the combination of endopolygalacturonase (PG) and pectin esterase (PE) and by rhamnogalacturonase (RGase) after chemical saponification. After PG/PE treatment, the remaining high molecular weight material representing the pectic hairy regions was isolated and characterized. Clear differences were found in the sugar composition of the fractions obtained, while only small variations were observed in the sugar linkage composition. The pectic hairy regions were further degraded by RGase and the digests separated into high molecular weight and oligomeric degradation products. These "RGase oligomers" consisted of between 4 and 9 sugar units with a backbone of alternating rhamnose and galacturonic acid residues, partly substituted with galactose linked to C-4 of the rhamnose moiety. Both the absolute amount of RGase oligosaccharides released as well as the degree of galactose-substitution of the oligomers increased when severer extraction conditions were used. Relatively more RGase oligomers were released from the low molecular weight hairy regions as compared to the high molecular weight fraction. Typical high molecular weight fragments isolated from the RGase digests of various hairy regions included residual segments of the rhamnogalacturonan backbone rich in arabinose and a polymer presumably enriched in xylogalacturonan segments.

Keywords: Apple; Pectin; Extraction; Enzymic degradation; Rhamnogalacturonase

☆ Hairy (Ramified) Regions of Pectins, Part VI. For Part V, see ref. 19.

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1. Introduction

Pectins are important constituents of plant cell walls and are considered to consist partly of homogalacturonan regions (''smooth regions'') which are interrupted by ramified regions [1–3], referred to as hairy regions in this study. The homogalacturonan segments are reported to have a length of 72–100 galacturonic acid residue [3] and part of the galacturonic acid residues are present as methyl esters. The ramified regions, usually obtained after extensive degradation of the homogalacturonan regions by polygalacturonase (PG), may consist completely of alternating rhamnose and galacturonic acid residues as has been demonstrated in Rhamnogalacturonan I (RG-I) isolated from sycamore cells [2]. Hairy or ramified regions have also been isolated from enzyme-treated tissue or pectin fractions from, for example, carrot [4,5], grape berries [6], apple [1,7,8], onion [9], and the roots of *Bupleurum falcatum* L [10]. In most cases the galacturonic acid to rhamnose ratio in these fractions was found to be much higher than a straight alternating RG-I would suggest.

The structure of apple pectins and the hairy regions thereof and their enzymic degradability have been studied extensively [1,7,8,11–15]. The most striking feature of apple pectin is that large amounts of arabinose residues are present in highly branched side-chains [12]. De Vries et al. [16] reported the presence of two types of (arabino)galactans in apple pectic hairy regions: pectins rich in (1 → 3,6)-linked galactan side-chains preferentially extracted by cold buffer and (1 → 4)-linked galactans present in pectins subsequently extracted under harsher conditions.

The structure of the hairy regions, isolated from apple and various other fruits and vegetables using the enzymic liquefaction process, has been studied in detail [17–19]. Since a wide-spectrum enzyme preparation was used, the pectin fraction was designated MHR (modified hairy regions). Rhamnogalacturonase (RGase) has been identified [20] as an enzyme able to cleave galactopyanosyluronic-rhamnopyranosyl linkages within a rhamnogalacturonan segment of the MHR backbone, releasing characteristic oligomers having a tetrameric or hexameric backbone of alternating rhamnose and galacturonic acid residues with a β-Gal*p* unit 4-linked to approximately half of the terminal Rha*p* residues and to half of the (1 → 2)-linked Rha*p* residues. Using RGase, structural subunits present in MHR could be recognized; as well as the characteristic RGase oligomers [19,21], high molecular weight degradation products present in the digest were isolated and characterized. Evidence was obtained [22,23] that apple MHR consisted of a xylogalacturonan subunit, a subunit representing residual stubs of the backbone rich in (long) arabinan side-chains, and a rhamnogalacturonan part of unknown length.

Although studies of the fine-structure of apple MHR, available in high amounts, increased our knowledge on pectin structures present in the cell wall, it should always be realized that structures present in MHR might be the results of enzymic alterations during the cell-wall liquefaction process. The method of isolation also resulted in an accumulation of (modified) hairy regions originating from completely different pectin molecules (varying in extractability or even insoluble pectins) into one single fraction.

In this paper, the distribution of hairy regions over different populations of pectic molecules present in the cell wall is described. After a brief characterization of

differently extracted pectins, attention is paid to the different hairy regions, their degradability by RGase, and the presence and distribution of subunits within these hairy regions.

2. Experimental

Isolation of various pectin fractions.—Golden Delicious apples were extracted with ethanol as described by De Vries et al. [11] to yield alcohol insoluble solids (AIS). The AIS were subsequently extracted using 0.05 M NaOAc buffer (pH 5.2) at room temperature and at 70°C, then 0.05 M EDTA/0.05 M NH_4oxalate in 0.05 M NaOAc (pH 5.2) (70°C) as described [11], followed by an extraction with 0.05 M NaOH (0°C). Fractions obtained were dialysed and lyophilized to yield cold buffer soluble solids (CBSS), hot buffer soluble solids (HBSS), chelating agents soluble solids (ChSS), alkali soluble solids (ASS), and the residue (RES).

Isolation of the hairy regions (HR).—Solutions of the various pectin fractions (0.2%) in 0.05 M NaOAc (pH 5.0) were incubated (20 h, 40°C) with the following purified enzymes, free from relevant side-activities: RGase from *Aspergillus aculeatus* [17,20], PG from *Kluveromyces fragiles* [17], and pectin esterase (PE) from *Aspergillus niger* [17]. The pectins present in CBSS, HBSS, and ChSS still contained methyl esters which limited the action of PG (not shown). For this reason, purified fungal PE was added in sufficient amounts to de-esterify all pectins within 5 h. After incubation, enzymes were inactivated (5 min, 100°C) and the digests were analyzed by HPSEC (high-performance size-exclusion chromatography) and HPAEC (high-performance anion-exchange chromatography). Fractions were also incubated on a semi-preparative scale using ~ 1 g of substrate in 200 mL of 0.05 M NaOAc (pH 5.0). Since the ASS fraction was almost insoluble in the buffer, 2 mM EDTA was added to the buffer and the mixture was heated (5 min, 100°C) to enhance solubilization. The digests were concentrated and applied to a Sephacryl S300 column as described below, resulting in the fractions HR-I and HR-II.

To check whether the homogalacturonan regions in CBSS, HBSS, and ChSS were completely removed by the mixture of PG and PE, the HR-fractions were chemically saponified as described below and subjected to an additional incubation with PG. All digests were analyzed by HPSEC and HPAEC.

Saponification of the hairy regions.—The methyl ester and *O*-acetyl groups in the HR were removed by treatment with 0.05 M NaOH as described [17,18], followed by neutralization. After adjustment of the solutions to the desired buffer pH and molarity, they were directly used in enzymic degradation studies.

Analytical methods.—Samples were hydrolyzed by M H_2SO_4 (100°C, 3 h) and neutral sugars were converted into their alditol acetates [24] to determine the sugar composition. The insoluble AIS and RES fractions were first pre-hydrolyzed using aqueous 72% H_2SO_4 (30°C, 1 h) to include cellulosic material [24]. The uronic acid content was determined colorimetrically using 3-hydroxybiphenyl [25]. Methylation analysis was performed as described previously [17].

Chromatography.—HPSEC was performed on three Bio-Gel TSK columns in series (40XL, 30XL, and 20XL) as described [17]. 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 pulsed amperometric mode (PAD), completed with a Spectra Physics SP8800 autosampler and a Spectra Physics Winner data handling system was used as described [19]. A CarboPac PA100 column (4 × 250 mm) with matching 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 an equilibration step of 15 min with 0.1 M NaOAc in 0.1 M NaOH, 20 μL of the sample was injected and a linear gradient to 0.35 M NaOAc in 0.1 M NaOH within 40 min was started. The column was washed for 5 min with M NaOAc in 0.1 M NaOH and equilibrated again for the next injection.

Size-exclusion chromatography (on a preparative scale) of CBSS-HR-II, HBSS-HR-I, and ChSS-HR-II was performed on a column (100 × 26 mm) of Sephacryl S300 (separation range for dextrans 2×10^3–4×10^5) using a Hiload System (Pharmacia) and 0.05 M NaOAc (pH 5.0) as eluent (2.5 mL/min). Fractions (2.5 mL) were collected and analyzed for neutral sugars and uronides as described; arabinose and galacturonic acid were used as standards, respectively. Pooled fractions were dialysed and lyophilized before analysis.

3. Results

Characterization of the various pectin fractions.—Starting from the alcohol insoluble solids isolated from Golden Delicious apples, four different pectin fractions were obtained: cold buffer soluble solids (CBSS), hot buffer soluble solids (HBSS), chelating agents soluble solids (ChSS), and alkali soluble solids (ASS), along with the remaining insoluble residue (RES). All fractions were analyzed for their sugar composition and degrees of esterification (Table 1). It can be seen that the proportion of galacturonic acid residues varied between 45 and 80 mol% in the pectin fractions, while the RES fraction is relatively poor in this sugar (10 mol%). The degree of methylation (dm) is rather high

Table 1
Sugar composition (mol%) of pectins, extracted from apple AIS

Sugar	Pectin fraction					
	AIS	CBSS	HBSS	ChSS	ASS	RES
Rha	2(1.19) [a]	1(0.10)	2(0.09)	3(0.23)	3(0.13)	1(0.52)
Fuc	1(0.87)	tr(0.01)	tr(0.01)	tr(0.01)	tr(0.01)	2(0.79)
Ara	12(8.05)	10(0.70)	15(0.69)	28(1.92)	33(1.23)	9(3.96)
Xyl	8(5.13)	1(0.07)	2(0.09)	4(0.26)	5(0.17)	11(4.95)
Man	2(2.00)	1(0.08)	2(0.09)	1(0.06)	1(0.04)	3(1.60)
Gal	7(5.71)	5(0.41)	6(0.35)	8(0.65)	11(0.53)	6(3.45)
Glc	38(31.2)	2(0.16)	2(0.13)	1(0.12)	2(0.08)	58(31.5)
GalA	30(26.8)	80(7.71)	71(4.37)	55(4.99)	45(2.31)	10(5.82)
OMe	83(4.0)	73(1.02)	72(0.58)	82(0.74)	0	0
OAc	19(1.7)	3(0.07)	5(0.08)	13(0.23)	0	0
Total sugars (% w/w)	81	93	83	63	45	88

[a] Gram quantities per residue in the fractions originating from 100 g of AIS.

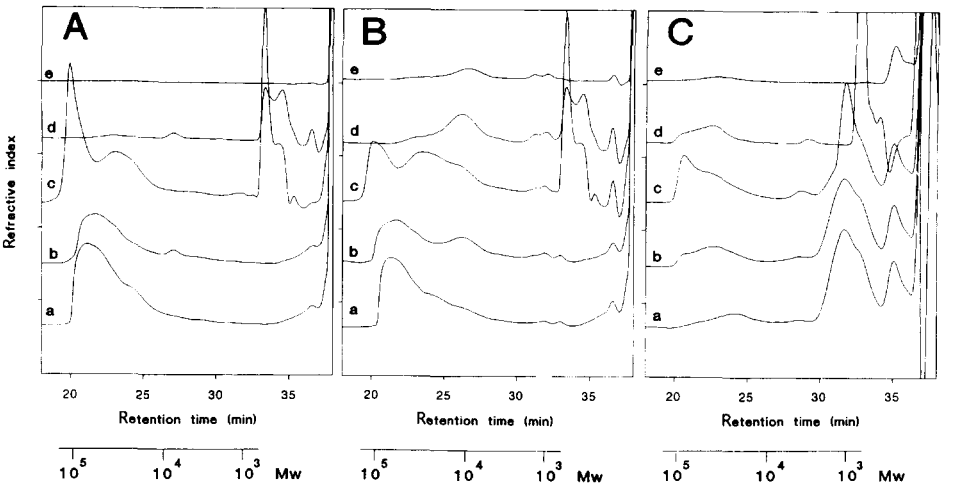

Fig. 1. High-performance size-exclusion chromatography elution patterns of various chemically saponified extracts from apple AIS before degradation (A), after treatment by RGase (B), and after treatment with PG and PE (C): a, CBSS; b, HBSS; c, ChSS; d, ASS; e, RES.

for CBSS, HBSS, and ChSS, while no ester groups were present in ASS and RES as a result of saponification. The ratio of neutral sugars to galacturonic acid is low for the easily extractable pectins (CBSS and HBSS) and higher for the pectins extracted with chelating agents (ChSS) and alkali (ASS). This higher ratio for ChSS and ASS is mainly due to the increased amounts of arabinose and galactose in these extracts.

Since most arabinose and galactose side-chains are reported [1,22] to be arranged in blocks (the so called hairy regions), the location of these blocks in the pectic backbone was studied by the use of RGase. Fig. 1A shows the HPSEC elution patterns of the saponified pectins without any enzyme treatment. Peaks appearing at 34 min did not represent carbohydrates and were caused by some residual amounts of EDTA (ChSS fraction) or other salts present in the sample. It can be seen that CBSS, HBSS, and ChSS consisted of high molecular weight pectins, although a broad molecular weight range is covered. No ASS and RES material was eluted from the column. This was expected for the RES fraction (insoluble in buffer and dilute alkali), but according to the isolation scheme and the characteristics shown in Table 1, ASS should contain soluble pectic material as well. Although ASS pectins were almost insoluble in the incubation buffer, the addition of a small amount of chelating agent (2 mM EDTA) solubilized most material, but these pectins were still not eluted from the HPSEC column material. The non-saponified pectins were eluted at somewhat longer retention times (i.e., lower molecular weights), probably due to ionic effects of the free carboxyl groups (results not shown).

Enzymic degradation of various pectin fractions.—The HPSEC elution patterns of the various pectins after treatment by RGase are shown in Fig. 1B. Small amounts of oligomers were liberated and appeared at 32–33 min. Using HPAEC, it was shown that these oligomers represented the characteristic RGase oligomers as reported before [19].

The release of RGase oligomers will be discussed in detail below. Next to the formation of oligomers, changes in the elution patterns can be observed in the polymeric range of the various pectin fractions. Some material is apparently resistant to further degradation by RGase, while another part shifts to lower molecular weight ranges. In general, RGase action resulted in the release of small amounts of the typical RGase oligomers; CBSS, however, showed almost the same HPSEC elution behaviour before and after enzyme treatment. In the HBSS and ChSS fraction, RGase action resulted in the conversion of part of the high molecular weight material into material of lower molecular weights. In the ASS and the RES fraction, RGase was able to solubilize material which appeared near 26 min.

The HPSEC elution patterns of the different pectins after PE/PG treatment are shown in Fig. 1C. As expected for CBSS, HBSS, ChSS, and ASS pectin, this enzymic degradation resulted in a drastic shift in retention time for a major part of the material. Degradation products were mainly galacturonic acid and some galacturonic acid oligomers in the range of dp 2–7 (HPAEC, results not shown) which were eluted under HPSEC conditions at retention times of 30 min or more. Smaller amounts of galacturonosyl oligomers were found in the digest of ASS and RES. Residual high molecular weight material, resistant to the combination of PG and PE, was recognized to be present in all digests, although relative amounts varied. From the HPSEC elution patterns, it was calculated that 14, 27, and 53% of the polymers present in the original material were eluted at retention times below 30 min (> 5000 Da) for the PE/PG digests of CBSS, HBSS, and ChSS pectins, respectively. The PE/PG-resistant polymeric material was found to be present in reasonable amounts in the digest of ASS and in minor amounts in the digest of RES.

Isolation and characterization of pectic hairy regions.—The polymeric material remaining after PE/PG treatment is believed to represent the so called pectic hairy regions. To study these pectin segments, they were produced and isolated in larger

Table 2
Sugar composition (mol%) of pectic hairy regions population I and II, obtained after treatment of different pectin fractions of apple AIS with pectin esterase and polygalacturonase, and size-exclusion chromatography

Sugar	CBSS-HR		HBSS-HR		ChSS-HR		ASS-HR		RES-HR	
	I	II	I	II	I	II	I	II	I	II
Rha	4	4	5	5	4	4	3	4	3	5
Fuc	2	1	tr [a]	tr	tr	tr	tr	tr	tr	tr
Ara	49	44	52	49	47	66	52	55	52	54
Xyl	5	2	5	3	10	4	5	6	4	6
Man	4	1	1	1	tr	0	1	2	0	0
Gal	18	29	19	21	14	9	20	17	29	16
Glc	4	1	1	1	tr	0	2	3	0	0
GalA	14	19	18	20	25	16	18	14	12	19
Rha/GalA	0.29	0.36	0.28	0.25	0.16	0.25	0.17	0.29	0.25	0.26
Ara/Gal	2.72	1.62	2.74	2.33	3.36	7.33	2.60	3.24	1.79	3.38
Relative amount of HR [b]	1.0	4.8	5.2	4.9	10.6	2.5	3.2	2.2	0.7	0.6

[a] tr = Trace. [b] The amount of HR-I was arbitrarily set to 1.0.

amounts. Digests having identical elution behaviour during HPSEC analysis, as described above, were obtained and were fractionated preparatively using Sephacryl S300 (results not shown). Also on Sephacryl S300 the digest showed two different high molecular weight populations, next to large amounts of uronide-oligomers eluting in the "included" volume of the column. These two different high molecular weight populations were pooled separately, dialysed, and lyophilized to yield Hairy Regions I and II (HR-I and HR-II). As can be seen from Table 2, the relative amounts of the various HR-fractions which have been isolated differed significantly and are in agreement with the relative amounts per fraction as shown in Fig. 1C. From the sugar composition of the fractions (Table 2), it can be seen that the relative amounts of galacturonic acid in all HR-I and HR-II fractions are significantly lower than in the original pectin fraction. The hairy regions are especially rich in arabinose (44–66 mol% compared with 12 mol% in

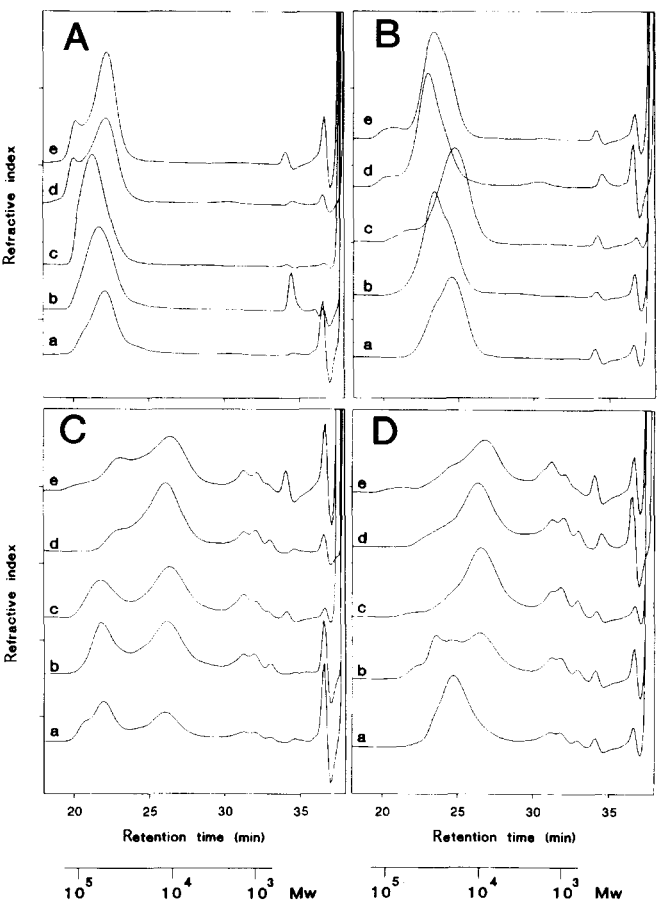

Fig. 2. High-performance size-exclusion chromatography elution patterns of HR-I and HR-II fractions isolated from various extracts from apple AIS before degradation (A and B, respectively) and after treatment with RGase (respectively C and D): a, CBSS; b, HBSS; c, ChSS; d, ASS; e, RES.

AIS) and galactose residues (9–29 mol% compared with 7 mol% in AIS). Rhamnose and xylose residues were present in minor amounts (3–5 and 2–10 mol%, respectively), while glucose, mannose, and fucose residues were present only in some of the fractions. Although small differences in the sugar composition between HR-I and HR-II for each pectin extract were found, no specific trends in the sugar composition of either HR-I or HR-II could be observed. Unfortunately, not enough material was obtained to allow examination of the amount of methyl esters resistant to PE actions and the amount of *O*-acetyl groups in the HR-I and HR-II fractions.

Figs 2A,B show the HPSEC elution patterns of the HR-I and HR-II fractions. Clear differences in retention times can be seen between the corresponding fractions I and II. Sometimes a broader molecular weight distribution could be observed within one group of hairy regions (Fig. 2A, lines d and e). It should be mentioned that the first distinct peak for ASS-HR-I and RES-HR-I is eluted in the "void volume" of the column set used and might not represent a distinct population. No differences in elution pattern were observed when HR-fractions were saponified prior to analysis by HPSEC.

It was confirmed that PE/PG had degraded all the homogalacturonan regions by chemical saponification of the HR-fractions prior to re-incubation by PG. HPSEC analysis showed little change (results not shown), while only very limited amounts of monomeric galacturonic acid could be detected by HPAEC.

Methylation analysis was carried out without reducing the galacturonosyl residues. The results obtained were in good agreement with those published [12,16,17,23]. They showed that, in the HR-fractions isolated from CBSS, HBSS, and ChSS, half of the rhamnose residues were 2-substituted while the other half were also branched through C-4. In ASS-HR, a higher proportion of rhamnose was branched. The glycosidic linkage composition found for arabinose is typical of a highly branched arabinan chain, while no principal differences were observed for the various HR-fractions. In general, half of the 3- and 5-substituted arabinose residues carried one or two terminally linked arabinose residues, with the exception of ChSS-HR-II which is more linear.

Galactose residues were found to be involved in many different types of linkages. In CBSS-HR, they were mainly 3-, 6-, and 3,6-substituted; in the other HR-fractions, 4-substituted galactose residues were also present in about equal amounts. A relatively high proportion of all galactose residues were terminally linked. Xylose was mainly present as terminally linked residues, although the proportion of terminally linked xylose decreased for the HR-fractions from ASS and RES.

RGase degradation of HR.—Using HPSEC, the HR-I and HR-II fractions were also examined for their degradability by RGase (Figs 2C,D). RGase treatment resulted in partial degradation of the high molecular weight material, along with the formation of oligomers appearing at 32–33 min. The HR-fractions originating from pectins extracted under more severe conditions seemed to be degraded more readily. HR-I fractions of CBSS, HBSS, and ChSS were incompletely degraded and, along with the oligomers, a new population also appeared at 26 min. About half of the material was eluted at the same time as the non-treated material. The HR-I fractions of ASS and RES were converted almost completely into lower molecular weight fragments. Also most of the HR-II fractions shifted readily to longer elution times. RGase acted differently on CBSS HR-II as compared to the other HR-II fractions. Although RGase was also able to

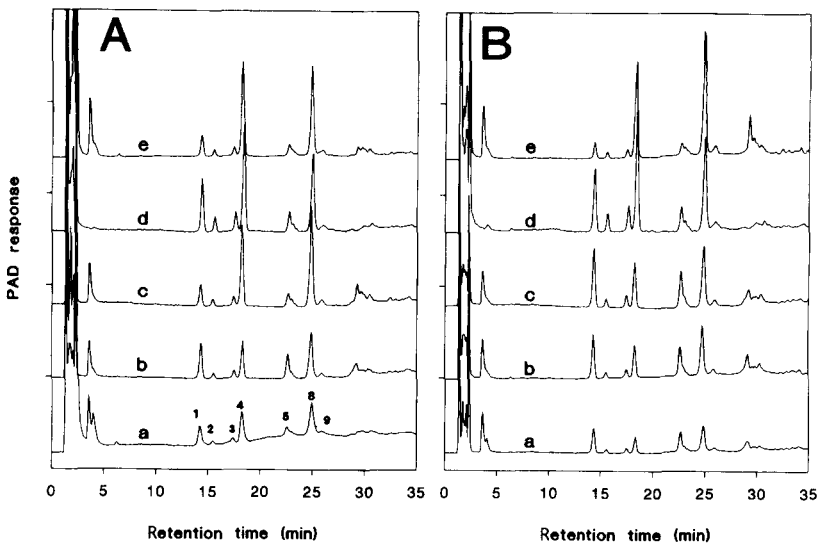

Fig. 3. High-performance anion-exchange chromatography elution patterns of HR-I (A) and HR-II (B) isolated from various extracts from apple AIS after treatment with RGase: a, CBSS; b, HBSS; c, ChSS; d, ASS; e, RES.

liberate oligomers from CBSS HR-II, the bulk of the material was eluted at about the same retention time as the non-treated material.

Characterization of oligomeric degradation products.—RGase digests of HR-I and HR-II were also analyzed by HPAEC and, as can be seen in Figs 3A,B, characteristic RGase-oligomer profiles were obtained. In addition to peaks already identified [19] (Table 3), minor quantities of unknown oligomers eluted at higher buffer concentrations could be observed. It can be seen that relatively low amounts of oligomers could be released from CBSS HR-I and HR-II, while these amounts increased for HR-digests obtained from the pectins which were more difficult to extract. Another clear observation is that RGase liberated relatively high amounts of oligomers **1** and **5** (without galactose substitution) from the HR-I and HR-II fractions of CBSS and HBSS and from the HR-II fraction of ChSS pectin. RGase treatment of the other HR-fractions resulted in the release of higher amounts of oligomers containing two rhamnose residues carrying a galactose branch (oligomers **4** and **8a / b**). Except for the HR-I and HR-II fraction of the ChSS pectin, only minor differences could be observed in the HPAEC elution profile of the RGase digests of HR-I and HR-II of the same pectin fraction. To be able to draw conclusions from data instead of judging only the chromatograms, the RGase oligomers were quantified using galacturonic acid as standard (Table 4). It can be seen that, on a sugar weight basis, the relative proportion of each oligomer increased from the CBSS fraction to the ASS fraction, while for the HR-fractions originating from the RES fraction this proportion was somewhat lower. It also can be concluded that more oligomers were formed from the HR-II fractions as compared to the higher molecular weight material of the HR-I fractions.

Table 3
Structures of identified oligomers, obtained after degradation of apple MHR-S by RGase [19]

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)-Gal *p*A
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) β-Gal *p*-(1 → 4) α-Rha *p*-(1 → 4)-Gal *p*A β-Gal *p*-(1 → 4)

Table 4
Relative amounts [a] of the various oligomers liberated during the incubation of different apple hairy regions by rhamnogalacturonase

Pool		Oligomer							
		1	**2**	**3**	**4**	**5**	**8a/b**	**9**	Total
CBSS	I	4	1	1	7	2	9	1	25
	II	6	1	1	4	7	9	1	29
HBSS	I	8	1	2	7	7	12	1	38
	II	12	2	3	9	12	16	2	56
ChSS	I	7	2	3	20	7	28	3	70
	II	18	2	4	13	16	24	3	80
ASS	I	15	4	6	31	9	25	3	93
	II	19	5	8	37	12	33	6	120
RES	I	5	1	2	23	5	24	3	63
	II	4	1	2	26	6	37	3	79

[a] Expressed in μg of GalA per mg of sugars in the hairy regions.

Characterization of polymeric degradation products.—Although not enough material has been isolated yet to enable study of all polymeric fragments which remained after digestion of the HR-fractions by RGase, some digests were fractionated on Sephacryl S300. We have chosen for this examination: CBSS-HR-II which hardly shifted towards lower molecular weights after RGase treatment; HBSS-HR-I which was divided into two high molecular weight populations by RGase; and ChSS-HR-II, shifting as one peak to lower molecular weights after degradation by RGase (Figs 2C, 2D). Elution patterns as determined from neutral sugar and uronide content in eluent fractions after chromatography on Sephacryl S300 are shown in Fig. 4. As expected, great resemblance was noticed between these elution patterns and the HPSEC patterns as shown in Figs 2C, 2D. From the elution patterns on Sephacryl S300, it can be seen that the polysaccharide fraction eluting in the column's void volume contained quite a few galacturonic acid residues. The same is true for the RGase-oligomers eluting at the included volume ($K_{av} = 1.0$).

Table 5
Sugar composition (mol%) of degradation products obtained, after treatment of different hairy regions fractions of apple AIS with RGase, by chromatography on Sephacryl S300 (Fig. 4)

Sugar	CBSS-HR-II			HBSS-HR-I				ChSS-HR-II	
	b	*c*	*d*	*a*	*b*	*c*	*d*	*c*	*d*
Rha	5	2	3	5	4	2	2	2	3
Fuc	1	tr [a]	tr	1	tr	tr	tr	tr	tr
Ara	35	49	70	23	32	70	71	62	73
Xyl	4	2	2	14	9	2	2	8	1
Man	2	1	2	2	2	1	1	2	1
Gal	37	34	7	24	23	13	13	7	7
Glc	2	1	3	3	3	2	2	1	2
GalA	15	9	14	28	26	10	10	19	12

[a] tr = Trace.

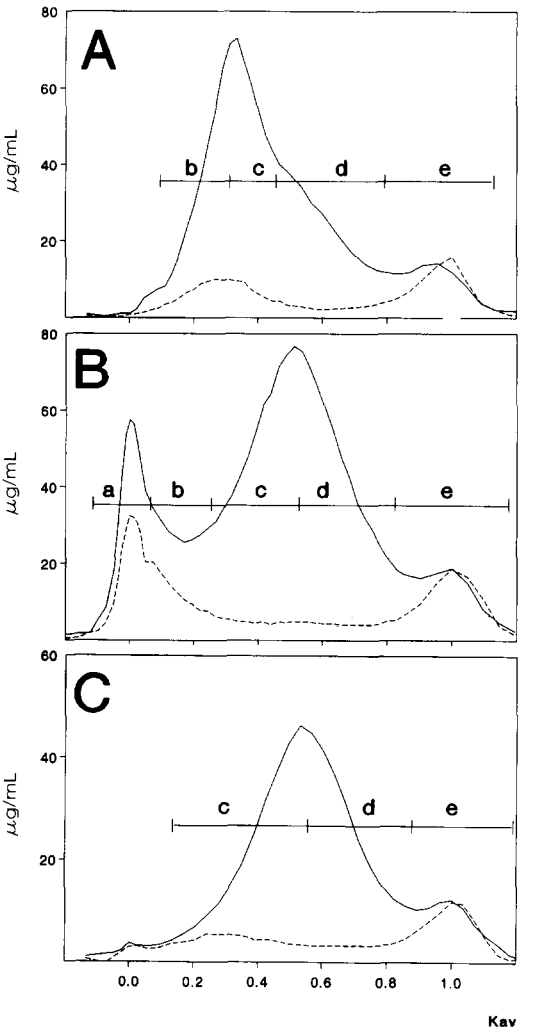

Fig. 4. Size-exclusion chromatography on Sephacryl S300 of the RGase digest of CBSS-HR-II (A), HBSS-HR-I (B), and ChSS-HR-II (C): (———), neutral sugars; (----), uronic acids.

Fractions were pooled as indicated. Pool *e* contained the oligomers as analyzed by HPAEC and discussed before. Other pools were examined for their sugar composition after a dialysis step (Table 5). From both Fig. 4A and Table 5, it appeared that pool *c* of the degraded CBSS-HR-II is (at least partly) an intermediate of pool *b* and *d*. The same trends can be observed even more clearly for the sugar composition of the pools obtained after degradation of HBSS-HR-I by RGase. The highest molecular weight pools (*a* and *b*) consisted of arabinose, xylose, galactose, and galacuronic acid, along with rhamnose and some other minor sugar residues. The ratio arabinose:galactose is ca. 1:1 as was found for CBSS-HR-II-b. The pools *c* and *d*, representing the population eluting at 26 min under HPSEC conditions (Fig. 2D), consisted mainly of arabinose

residues (70 mol%), along with some galacturonic acid, galactose, and rhamnose residues. Both pools *c* and *d* of the digest of ChSS-HR-II were rich in arabinose and the composition of pool *d* resembled that of the other pools *d*. The first half of the peak on Sephacryl S300 (pool *c*) contained somewhat more xylose residues as compared to pool *d*.

4. Discussion

Extracts.—Using rather mild extraction conditions, around 75% of all galacturonic acids present in the AIS of Golden Delicious apples were recovered in four extracts. These fractions, CBSS, HBSS, ChSS, and ASS, represent 10, 7, 13, and 10%, respectively, of AIS on a weight basis. Although the ASS fraction was obtained by alkali extraction at low temperature, it is believed that by this treatment only ester linkages are saponified and hydrogen bonds detached without changing the polysaccharide structures. Fractions CBSS, HBSS, and ChSS comprised 64% of the galacturonic acid present in the AIS. Renard and Thibaul [27] extracted 66% of the galacturonic acids in a one-step process using buffer with or without CDTA or EDTA at higher temperature. Aspinall and Fanous [12] recovered 62% of the galacturonic acid present in apple CWM (cell wall material) in their hot water extract and 25% after extraction by oxalate. The total yield of polysaccharides extracted using buffer and chelating agents (CBSS + HBSS + ChSS) was 30% and this value is comparable with data reported before: 32% [27] and 27% [12]. Although CDTA is recommended more and more in the literature to replace EDTA in complexing calcium ions to extract pectins [28], we still used EDTA since we preferred to compare our results with those of De Vries et al. [11]. It has been demonstrated [27] that EDTA did not differ much in complexation behaviour from CDTA (at pH 4.5) and only slightly higher concentrations of EDTA are needed to complex the same amounts of calcium ions [27]. For this reason, in our experiments the extractions were carried out three times after which no more material could be extracted.

Trends in the sugar composition of the extracts showed some correlation with the severity of extraction. Galacturonic acid was the main sugar residue in all extracts, while increasing amounts of rhamnose, arabinose, xylose, and galactose were found in the sequence CBSS, HBSS, ChSS, and ASS. However, the relative proportion of the neutral sugars differed in each extract. The ratio of relative amounts of individual sugars present in CBSS compared to ASS was 1:3 for rhamnose; 1:3.3 for arabinose; 1:5 for xylose, and 1:2.2 for galactose. In general, it can be concluded the pectins contained more rhamnose and more neutral sugars when severer extraction conditions were used for the extraction. The sugar composition showed good agreement with published data [11–13], although sometimes different extraction procedures were followed.

From Table 1, it was calculated that the pectins in CBSS, HBSS, and ChSS, containing 64% of the galacturonic acid residues, contained 58% of the methyl ester groups but only 22% of the acetyl ester groups present in AIS. From the data it can be concluded that galacturonic acid residues in the pectic material in ASS and RES had an average dm value of 96 and a degree of acetylation (da) value of 40, assuming that all

the acetyl groups were present on galacturonic acid residues. It can be concluded that the pectins which were more difficult to extract (ChSS, ASS) were more branched with neutral sugars and were more methylated and acetylated than the more easily extractable material. Some galacturonic acid residues (22%) could not be extracted at all and remained in the residue, together with most of the fucose, xylose, and glucose residues.

Distribution of hairy regions over pectin molecules.—HPSEC analysis revealed that the extracted pectins were all of high molecular weight, since they were eluted near the void of the columns used. Although the ASS fraction was soluble in EDTA/buffer, no peaks appeared after analysis by HPSEC, indicating that the pectins precipitated completely on the column material. The hairy regions are considered to be present in blocks along the pectin molecules. To verify this for our fractions, RGase was used to hydrolyze linkages between rhamnose and galacturonic acid residues in the hairy regions. After treatment of the chemically saponified pectin extracts by RGase, it was demonstrated that RGase was able to release the characteristic RGase-oligomers from all intact pectins. Since RGase is active only within the hairy regions, the different effects of RGase on the elution patterns of the various pectins indicate differences in the proportion and distribution of hairy regions in these pectins. If RGase is active on the hairy regions present in the middle of a pectin molecule, a drastic decrease in the molecular weight of the molecules should be noticed. Since this is not the case for CBSS pectin, it may be suggested that the pectins in CBSS consist of long homogalacturonan chains having hairy regions at the end of these chains. For HBSS and ChSS pectin, a more complex structure may be present.

Hairy regions.—All extracts and the residue were treated by PG, or for those pectins which still contained methyl ester groups by the combination PE and PG, to degrade the homogalacturonan segments. The major part of the pectins was degraded to low molecular weight material while some high molecular weight material remained. It has been reported that PE is able to saponify only part of the methyl esters present [29]. However, this proved to be no limitation for PG to degrade all homogalacturonan segments present since re-incubation of the chemically saponified HR-fractions with PG did not result in further degradation as monitored both by HPSEC and HPAEC. The observations described were in full agreement with the study of De Vries et al. [1] who used pectinlyase instead of PG or PE/PG. As expected, for CBSS, HBSS, and ChSS, a correlation was found between the amount of the PE/PG-resistant material (hairy regions) and the amount of neutral sugars in the intact pectins. As a result of the higher rhamnose content in the pectins extracted under severe conditions, it can be assumed that more rhamnogalacturonan-type regions are present in these pectins.

By HPSEC as well as by chromatography on Sephacryl S300, the hairy regions could be roughly separated into two fractions. The relative amounts of these fractions varied for the different pectin extracts. PG did not liberate large quantities of hairy regions from the insoluble residue, probably indicating that some neutral sugar chains present in the hairy regions are linked to the hemicellulosic or cellulosic matrix in an unknown way. It is less clear why only limited amounts of HR were released from the ASS fraction.

The sugar compositions of the fractions were representative of hairy regions of apple pectins as described [1]. No logical regularity in the relative amounts of the sugars

present in HR-I and HR-II was recognized. Sometimes a higher arabinose, galactose or galacturonic acid content was found for HR-I, but in other cases the opposite was true. The ratio of arabinose to galactose residues varied markedly for the various HR-fractions and fluctuated from 1.6:1 for CBSS-HR-II to 7.3:1 for ChSS-HR-II. Xylose was usually present in higher concentrations in the HR-I fractions. The rhamnose to galacturonic acid ratio varied from 0.16 for ChSS-HR-I to 0.36 for CBSS-HR-II. These values are in the same range as reported before for hairy [1] (0.22) and modified hairy regions [17] (0.29) of apple pectins. Great resemblance is also observed when mol% values of the sugars are compared with those given by De Vries et al. [1] (hairy regions of type E pectin: arabinose 50%, xylose 5%, galactose 13%, rhamnose 5%, and galacturonic acid 23%), although we found slightly lower amounts of galacturonic acid residues and slightly higher amounts of galactose. Comparison of our HR-fractions with MHR [17] show that MHR has a lower galactose content and is relatively richer in xylose residues. This might be due to enzyme treatment during the isolation of MHR.

Methylation analysis revealed that the sugar linkage composition of all HR-fractions was very similar to that reported earlier for neutral sugars present in apple pectic substances [12,16,17,26] and is not repeated here. Rhamnose residues were branched via C-4 as described [16,17]. In contradiction to De Vries et al. [16] no 3-substituted rhamnose residues were found in our HR-fractions. Arabinose was present in highly branched chains, as has been reported before [12,16,26]. Schols et al. [17] found more linear arabinan side-chains, probably due to enzymic modification during the isolation of their modified hairy regions. Xylose residues were mainly terminally linked, again in agreement with the literature [16,17]. The glycosidic linkages of galactose residues in the CBSS-HR-fractions indicated the presence of (1 → 3,6)-galactans (type II arabinogalactan) as described by others [16,30]. Type I (arabino)galactans [(1 → 4)-linked] predominated in the other fractions. Relatively high proportions of terminally linked galactose were observed to be present in all HR-fractions and this is in full agreement with earlier findings [1,17,19,21].

Enzymic degradation of HR by RGase.—The action of RGases isolated from *Aspergillus aculeatus* and *Trametes sanguinea* IFO 6490 towards native pectins from apple and beet has been reported before [26,31]. Since these experiments were performed on insoluble cell-wall material [26,31] or on material solubilized after extensive heat treatment under alkaline conditions [31], it was hardly possible to use the data obtained to reveal the structure of individual pectins. As illustrated above in our experiments, RGase was active towards extracted pectins, liberating typical RGase oligomers as deduced from their HPSEC and HPAEC elution behaviour.

From the HPSEC elution patterns of the RGase-digested HR-fractions, which were isolated in a defined way, it was concluded that differences exist between the various HR-fractions and thus between the various pectins. Sometimes, the HR-population shifted completely to smaller molecular weight material, but in the case of CBSS-HR-II, incubation by RGase resulted in the release of RGase-oligomers while the remaining polysaccharide was eluted in a very similar fashion to the starting material. This might suggest that (as argued before for CBSS-pectin) CBSS-HR-II contained some alternating rhamnose and galacturonic acid residues in its backbone, not in the middle of the hairy region segment, but more likely at the ends of it. Another possibility might be the

presence of a two populations of pectins, co-eluting with each other, of which only the minor one was degraded by RGase.

Differences are also observed when the elution patterns of the oligomers obtained by HPSEC are compared. First of all, it should be stated that the same types of oligomers were formed from the non-modified HR-fractions as from MHR [19,20]. This indicated that the single-unit side chains of galactose are not a result of the action of enzymes during the liquefaction process, but that these are present in the original pectins and hairy regions. However, the relative amount of the oligomers carrying galactose residues varied for the type of pectin from which the hairy regions originated. The relative amount of each oligomer was estimated using galacturonic acid as standard. Although PAD response depends on size and the sugar residues and linkage types present [19], quantification, even without having the proper standard compounds, enables us to draw conclusions more readily than by only judging the elution pattern. It should be remarked that especially the concentrations of the higher oligomers will be underestimated. Parallel to the increasing amount of HR obtained when severer extraction conditions were used, the amount of oligomers released for the same quantity of HR also increased significantly. Relatively more linear oligomers having a tetrameric or hexameric backbone of alternating rhamnose and galacturonic acid residues were found for the easily extractable pectins (CBSS and HBSS). The ratio of oligomers having a tetrameric rhamnogalacturonan unit to oligomers having a hexameric unit is similar for all HR-digests, while dissimilarities were observed for the ratio of singly branched tetrameric rhamnogalacturonan unit to doubly branched tetramer unit: the presence of the latter was more pronounced in the digests of HR-I fractions. Estimation of similar ratios for the hexameric backbone unit was not carried out since separation and quantification of the oligomers consisting of one galactose residue is difficult under the conditions used. From structural studies of apple MHR, Voragen et al. [22] proposed that subunits consisting of alternating rhamnose and galacturonic acid residues (with part of the rhamnose residues being branched by a galactose residue at C-4) are present in apple MHR (subunit III). These subunits were partly degraded by RGase into the characteristic RGase-oligomers [20,22]. It can be concluded that these rhamnogalacturonan segments are present in all pectic molecules investigated in this study, but variations in the relative amounts of the various RGase-oligomers within these segments are present.

Using chromatography on Sephacryl S300, some regularities were observed for the HR-fractions. In the three digests investigated, an arabinose-rich polymer was recognized, resembling the subunit II as described [22] for apple MHR population A. This type of arabinan-rich remnant of the backbone was also reported by Renard et al. [26] after incubation of CDTA-insoluble solids of apple with RGase (1% rhamnose, 73% arabinose, 2% xylose, 12% galactose, and 7% galacturonic acid). The *a* and *b* pools obtained after chromatography over S300 were relatively enriched in xylose residues and had a relatively low rhamnose to galacturonic acid ratio. Although the relative amount of xylose was at a maximum 14 mol%, it suggests the presence of a xylogalacturonan. Barrett and Northcote [32] had reported in 1965 the occurrence of an aldobiouronic acid of xylose and galacturonic acid, isolated after partial hydrolysis of apple pectin. The existence of a xylogalacturonan polymer in apple MHR population A has been demonstrated [23]. More detailed information on the characteristics and relative

amounts of the xylogalacturonan subunit, rhamnogalacturonan subunits rich in arabinan side-chains, and the RGase oligomers as found for the population of apple MHR having the highest molecular weight will be discussed elswhere [23].

It can be concluded that pure enzymes can be used as tools to study small differences in the fine structure of pectins. This was illustrated by differences in the PE/PG- and RGase-degradability of pectin extracted by cold buffer when compared to that extracted by hot buffer. Many researchers have combined these pectins into one single fraction. Another example of the complexity of the pectins is that RGase incubation of HR-fractions having a rather similar sugar composition resulted in clear differences in the relative amounts of the RGase oligomers obtained (both relatively to each other and in absolute amounts).

More research is being carried out to characterize the proposed subunits present in (other) HR-fractions in more detail, to establish their distribution over the rhamnogalacturonan backbone, and to study the distribution of the hairy regions over the pectic molecules.

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