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Mode of action of RG-hydrolase and RG-lyase toward rhamnogalacturonan oligomers. Characterization of degradation products using RG-rhamnohydrolase and RG-galacturonohydrolase[1]

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Abstract

The mode of action of RG-hydrolase and RG-lyase toward purified linear rhamnogalacturonan (RG) oligomers has been studied. Major tools in the characterization of the degradation products were the exo-acting RG-rhamnohydrolase and RG-galacturonohydrolase. They were used to prepare a series of standards of RG oligomers for HPAEC. ^{1}H NMR spectroscopy confirmed the structure assignment made using HPAEC for a selection of isolated degradation products. Identification of degradation products from purified RG oligomers was then performed by comparing retention times of HPAEC peaks with those of standards. RG-hydrolase was able to cleave RG oligomers which contained five Rha units or more, i.e. DP 9 with a Rha unit at both nonreducing and reducing end. Its preferential cleavage site was at four units from the first nonreducing Rha. RG-lyase was active toward oligomers that contained at least six GalA units, i.e. DP 12 with a GalA at the nonreducing and a Rha at the reducing end. The preferential cleavage site was for the smaller oligomers four residues, and for the largest oligomer six residues from the reducing Rha. From the observed cleavage patterns it can be speculated that in hairy regions, the RG stretches have to be at least 13 residues long for RG-hydrolase and 16 residues long for RG-lyase in order to produce one tetramer.

Keywords: Pectin; Structure; Rhamnogalacturonase; Rhamnohydrolase; Galacturonohydrolase; RG-hydrolase; RG-lyase

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1. Introduction

Since Schols et al. [1] described that the enzyme rhamnogalacturonase (RGase) was able to degrade the RG backbone in hairy regions of pectin, several papers have been published dealing with RGase activity [2–9]. Subsequently, a set of enzymes, all with high specificity toward the RG regions of pectin and no activity toward homogalacturonan (HG) regions, has been found in the authors' laboratory, including RG-acetylesterase [10]; RG-rhamnohydrolase [11]; RG-lyase [12], and RG-galacturonohydrolase [13]. RGase as described by Schols et al. [1] is now termed an RG α-D-galactopyranosyluronide-(1,2)-α-L-rhamnopyranosyl hydrolase, abbreviated RG-hydrolase. The discovery of these enzymes enables a better structural characterization of the hairy (ramified) regions of pectin, and also of native plant cell wall pectin [14]. Furthermore, they might become important for production and modification of (potentially) biologically active RG structures. Wound-signal activity has been demonstrated for RG I [15], and hypocotyl elongation for Δ-(4,5)-unsaturated GalA-(1,2)-Rha disaccharides [16].

In this paper we describe the cleavage patterns of RG-hydrolase and RG-lyase toward a series of RG oligomers with different degrees of polymerization (DP's). In the study of the mode of action of enzymes, a time-consuming factor usually is the identification of substrate degradation products, which have to be isolated using preparative chromatography and characterized by e.g. sugar composition analysis, linkage position analysis, NMR spectroscopy, MS. We show how two enzymes, RG-rhamnohydrolase and RG-galacturonohydrolase, able to remove a Rha unit or a GalA unit respectively from the nonreducing end of RG chains, can be used as analytical tools, enabling characterization of enzymic degradation products without tedious isolation procedures. Firstly, the exo-enzymes were used to modify available RG oligomers, in order to have a series of different types of RG oligomers available as standards for high-performance anion-exchange chromatography (HPAEC). RG-hydrolase and RG-lyase degradation products of a mixture of linear RG oligomers were characterized by comparison of retention times of HPAEC peaks with those of the standard oligomers. The validity of this identification was then confirmed by isolating some of these degradation products by size-exclusion chromatography (SEC) and characterizing them by ^{1}H NMR spectroscopy. Finally, RG-hydrolase and RG-lyase were incubated with purified RG oligomers, and the products were characterized by HPAEC. Here the exo-enzymes were used to modify initially formed degradation products into oligomers for which standards were available, enabling confirmation of their identification.

2. Experimental

Preparation of RG oligomers.—Sugar beet pulp was saponified and then hydrolyzed with 0.1 M HCl at 80 °C for 72 h. Linear RG oligomers of DP 4–16, abbreviated **A4**–**A16** (see Table 1) and a mixture of oligomers with a DP > 10, were isolated by ion-exchange chromatography and SEC by Renard et al. [17]. Saponified apple modified hairy regions (MHR-S) was treated with RG-hydrolase, and the RG oligomers produced were isolated using SEC as described [11]. Removal of Gal from these oligomers, and subsequent de-rhamnosylation was carried out as described [11]. A similar isolation procedure was carried out with RG-lyase, and the resulting branched unsaturated RG oligomers were isolated as described [12]. From these unsaturated RG oligomers the Gal was removed using 9.3 μg β-galactosidase mg^{-1} substrate, under the conditions described [11].

Enzymes.—RG-hydrolase and RG-lyase from *Aspergillus aculeatus* were purified using the method of Schols et al. [1] and of Kofod et al. [5] respectively. β-Galactosidase from *Aspergillus niger* was purified by Van de Vis [18] and used for degalactosylation of RG oligomers. Procedures for the purification of RG-rhamnohydrolase and RG-galacturonohydrolase from *A. aculeatus* are described in Mutter et al. [11] and in Mutter et al. [13] respectively. All substrates, varying in concentration between 0.018 and 0.05% w/v, unless mentioned otherwise, were incubated in 50 mM NaOAc buffer (pH 5.0), containing 0.01% w/v NaN_3, at 40 °C for 24 h. Type **A** oligomers (see Results) were treated with 2.6 μg RG-galacturonohydrolase mg^{-1} substrate to form type **B** oligomers.

When type **A** oligomers were sequentially treated with the exo-enzymes, RG-galacturonohydrolase and RG-rhamnohydrolase were used in amounts between 2.4 and 2.8 μg and between 9 and 18 μg mg^{-1} substrate respectively.

Type **A** and type **B** oligomers were incubated with 0.18 μg RG-hydrolase and with 0.42 μg RG-lyase mg^{-1} substrate. Subsequent incubation of the RG-hydrolase/RG-lyase digests with the exo-enzymes was carried out with 6 μg of RG-galacturonohydrolase and with 16 μg RG-rhamnohydrolase mg^{-1} substrate.

Digests for preparative isolation of oligomers were produced out of a mixture of type **A** oligomers with a DP > 10, of which 16 mg was incubated for 48 h at 40 °C with 3.1 μg RG-hydrolase in 0.2% w/v solutions in 50 mM NaOAc buffer (pH 4); and of which another 16 mg was incubated for 48 h at 40 °C with 0.88 mg RG-lyase in 0.2% w/v solutions in 50 mM NaOAc buffer (pH 6).

Isolation of oligomeric degradation products formed by RG-hydrolase and RG-lyase.—Concentrated and desalted (using a PD-10 column; Pharmacia Biotech, Uppsala, Sweden) digests were injected on combined Bio-Gel P-4 and P-6 columns mounted in series, eluted with 0.1 M NaOAc buffer (pH 3.6) at 40 °C. Fractions (4–5 mL) were collected and analyzed. The GalA and neutral sugars (using a Rha standard) concentrations were measured by automated meta-hydroxy-diphenyl [19] and orcinol assays [20] respectively. Corrections were made for interference of uronic acids in the neutral sugars assay. Peak-forming fractions were pooled, concentrated on a rotary evaporator, desalted on PD-10 columns, reconcentrated and freeze-dried.

HPAEC analysis.—HPAEC was carried out using a Dionex (Sunnyvale, CA) Bio-LC system equipped with a Dionex CarboPac PA-100 (4×250 mm) column and a Dionex pulsed electrochemical detector in the pulsed amperometric detection (PAD) mode. To minimize errors due to variations in the PAD detector response, HPAEC–PAD was always performed using a freshly polished gold electrode with no difference in level between the gold surface and the surrounding block. A gradient of NaOAc in 100 mM NaOH (1 mL/min) was used as follows: 0–50 min, 0–450 mM; 50–55 min, 450–1000 mM; 55.1–70 min, 0 mM.

^1H NMR spectroscopy.—^{1}H NMR spectra of oligosaccharide solutions in deuterated H_2O were recorded on a Bruker ARX 400 spectrometer at 320 K. Proton chemical shifts were referenced to acetone assigned to 2.225 ppm. Oligosaccharides were deuterium-exchanged twice in 99.9% D_2O before solubilization in 0.5 mL D_2O with a trace of acetone as internal reference.

3. Results

Preparation of a series of standards of RG oligomers.—Five types of linear, strictly alternating RG oligomers were made available, coded **A**–**E**, differing in their reducing and/or nonreducing end sugars. The oligomers are listed in Table 1. The number in the code refers to the DP of the oligomer. The symbols for Rha and GalA and their linkage types in oligomer structures, as explained in Table 1, will be used throughout the paper. The isolation and enzymic modification of RG oligomers types **A**–**E** are described below.

Type **A** oligomers are linear RG oligomers of the type G-(R-G)$_n$-R, with $n = 1$–7, i.e. **A4** to **A16** in Table 1, and have been purified from sugar beet pulp and characterized [17]. Type **A** oligomers were treated with RG-galacturonohydrolase [13], to remove the nonreducing GalA to obtain oligomers of type **B**: (R-G)$_n$-R, with $n = 1$–7, i.e. oligomers **B3** to **B15** in Table 1. **A4** was sequentially treated with RG-galacturonohydrolase and RG-rhamnohydrolase

Table 1
Explanation of codes of RG oligomers: G, α-GalA (1,2)-linked to Rha, or GalA at the reducing end; R, α-Rha (1,4)-linked to GalA, or Rha at the reducing end; uG, α-Δ-(4,5)-unsaturated-GalA (1,2)-linked to Rha. Retention times upon HPAEC under the conditions described in Experimental

Code	Structure	Retention time (min)
A2[a]	G-R	13.3
A4	G-R-G-R	21.2
A6	G-R-G-R-G-R	27.1
A8	G-R-G-R-G-R-G-R	31.3
A10	G-R-G-R-G-R-G-R-G-R	34.4
A12	G-R-G-R-G-R-G-R-G-R-G-R	36.8
A14	G-R-G-R-G-R-G-R-G-R-G-R-G-R	39.1
A16	G-R-G-R-G-R-G-R-G-R-G-R-G-R-G-R	40.8
B3	R-G-R	12.2
B5	R-G-R-G-R	19.8
B7	R-G-R-G-R-G-R	25.2
B9	R-G-R-G-R-G-R-G-R	29.3
B11	R-G-R-G-R-G-R-G-R-G-R	32.8
B13	R-G-R-G-R-G-R-G-R-G-R-G-R	35.6
B15	R-G-R-G-R-G-R-G-R-G-R-G-R-G-R	37.6
C2	R-G	12.5
C4	R-G-R-G	20.8
C6	R-G-R-G-R-G	26.3
D3	G-R-G	22.5
D5	G-R-G-R-G	28.4
D7	G-R-G-R-G-R-G	32.6
E4	uG-R-G-R	31.0
E6	uG-R-G-R-G-R	35.4
E8	uG-R-G-R-G-R-G-R	38.4
E10	uG-R-G-R-G-R-G-R-G-R	39.3

[a] The number refers to the DP of the oligomer.

[11] to consecutively form oligomers **B3**, **A2**, and monomeric Rha and GalA.

A mixture of RG oligomers, with the majority of the Rha units substituted at C-4 with Gal, was obtained from saponified apple MHR by treatment with RG-hydrolase [1,21]. These oligomers were linearized by treatment with a β-galactosidase from *A. niger*, to obtain type **C** oligomers: $(R\text{-}G)_n\text{-}R\text{-}G$, with $n = 1$–2, with as major products **C4** and **C6** [11]. Sequential degradation of purified **C4** [11] with RG-rhamnohydrolase and RG-galacturonohydrolase finally gave **C2**.

The mixture of linearized type **C** oligomers was de-rhamnosylated with RG-rhamnohydrolase [11] to form oligomers of type **D**: $(G\text{-}R)_n\text{-}G$, with $n = 1$–2, i.e. oligomers **D3** and **D5** in Table 1. However, after this treatment, a third peak was distinctly present, eluting after the former two oligomers upon HPAEC. Plotting the log (k')[2] (capacity factor) of the HPAEC elution versus DP for the three peaks showed the same trend of the curve as observed for types **A** and **B** (see below). This suggested that the third peak was the following in a homologous series and should have the structure G-R-G-R-G-R-G (**D7**). This heptamer was only observed as a distinct peak in the HPAEC chromatogram after de-galactosylation and de-rhamnosylation of the original oligomers.

Unsaturated RG oligomers with all Rha units C-4 substituted with Gal, were purified from saponified apple MHR after treatment with RG-lyase [12]. These oligomers were linearized using a β-galactosidase from *A. niger*, to obtain oligomers of type **E**: $uG\text{-}(R\text{-}G)_n\text{-}R$, with $n = 1$–4. The resulting oligomers **E4** to **E10** were used as standards.

Retention of RG oligomers upon HPAEC.—In Fig. 1 log (k') is plotted versus the DP of the various types of RG oligomers, and for comparison also for HG oligomers. The relationship between log(k') and DP was not linear, as expected for gradient elution [22]. Clearly, the fraction of acidic GalA residues in an oligomer is the most important factor for binding, explaining the increase in capacity factor over the series **B**→**A**/**C**→**D**→HG oligomers. This does not hold for the **E** type of oligomers: the Δ-(4,5)-unsaturated GalA at the nonreducing end causes much stronger retention on HPAEC compared to oligomers of the **A** series. This might be ascribed to the increased acidity of the nonreducing Δ-(4,5)-unsaturated GalA, probably due to the conjugation of the carboxyl group with the double bond between C-4 and C-5, as already observed for HG oligomers [23].

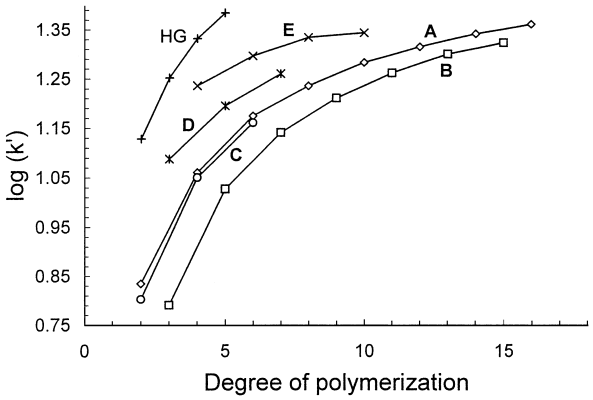

Fig. 1. Log (k') versus DP for HG oligomers (HG), and RG oligomers of type **A, B, C, D**, and **E** (see Table 1).

Preparative isolation and ^1H NMR spectroscopy characterization of enzymically produced RG degradation products.—Large amounts of a mixture of type **A** RG oligomers with a DP > 10, and an average DP of approximately 20 based on HPAEC, were available. Therefore, for preparative isolation of degradation products, this mixture rather than purified RG oligomers was incubated with RG-hydrolase, and a second batch with RG-lyase. The enzyme digests were separated on Bio-Gel P-4 and P-6 columns in series.

Separation of the RG-hydrolase digest resulted in six fractions differing in hydrodynamic volume (Fig. 2a), corresponding to the six major reaction products of the RG-hydrolase digest that were present in the HPAEC elution pattern (not shown). The oligomers present in these six fractions, a-1–a-6,

Table 2
Composition of Bio-Gel fractions a-1–a-6 (from the RG-hydrolase digest)

RG oligomers:	Code	a-1	a-2	a-3	a-4	a-5	a-6
R-G-R-G-R-G-R	**B7**	69	6	5	0	0	0
R-G-R-G-R-G	**C6**	0	53	0	0	0	0
R-G-R-G-R	**B5**	10	11	84	32	18	2
G-R-G-R-G	**D5**	9	0	0	100	42	4
R-G-R-G	**C4**	0	0	17	29	265[a]	30
G-R-G	**D3**	16	7	46	0	0	81[a]

[a] The actual value is higher since the PAD cell was saturated.

Composition is based on area percentages of peaks in the HPAEC elution patterns, relative to the amount of oligomer **D5** in fraction a-4, that was set to 100. Peaks were identified by comparing HPAEC elution behavior with standards as described in the text. Explanation of symbols in Table 1.

[2] k′, capacity factor (comparable with K_{av} in gel filtration), k′ = (elution volume-void volume)/(void volume).

were identified by comparison with standards (see Table 1) using HPAEC, and are shown in Table 2. As the response factors of the various oligomers were not known, the data in Table 2 do not represent the absolute amount of the various oligomers present. In each fraction one oligomer is predominantly present (60–80% of the total peak area). Contaminating oligomers are coming from the neighboring fractions. The exception to this is oligomer **D3** (G-R-G), which is also present in non-neighboring fractions. This trimer **D3** was not found as one of the degradation products of the purified type **A** oligomers (see below). The RG-hydrolase digest was incubated for 48 h, using relatively more enzyme than was used for incubation of only 20 h in case of the purified RG oligomers (see below). Therefore, it is assumed that this trimer is only formed after extensive incubation.

Separation of the RG-lyase digest on SEC resulted in four fractions (Fig. 2b). Fraction b-1, eluting first from the Bio-Gel columns and containing the largest products, was a mixture of

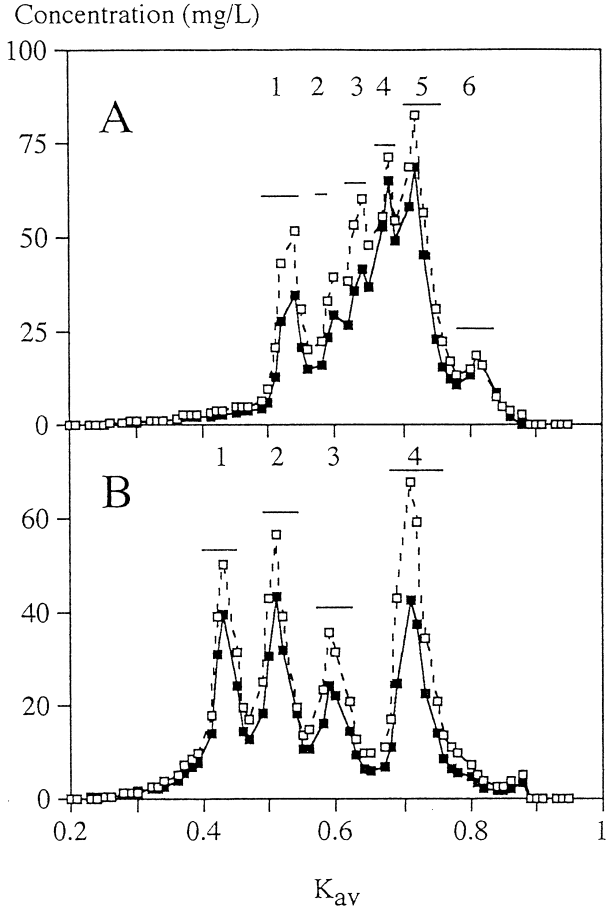

Fig. 2. Bio-Gel P-4 + P-6 fractionation of the digests of the mixture of type **A** oligomers with a DP > 10 made by RG-hydrolase (A) and RG-lyase (B). ■, GalA; □, Rha.

oligomers that were not observed before in the HPAEC elution pattern of the original RG-lyase digest, probably due to the low PAD response factor for large oligomers. Fraction b-2 contained predominantly oligomers **A8** and **E8**, identified by comparison with standards upon HPAEC as described above. This fraction was pooled between the same K_{av} values as fraction a-1, that predominantly contained **B7**. Fraction b-3 contained **E6**, and fraction b-4 contained **E4** as major oligomer. Fraction b-4 was pooled between approximately the same K_{av} values as fraction a-5, which contained predominantly oligomer **C4**. Since except for b-1 the b-fractions contained only one or two different oligomers, composition data were not represented in a table.

All products, present in Bio-Gel fractions a-1–a-6 (Fig. 2a) and b-1–b-4 (Fig. 2b), were derived from strictly alternating RG oligomers by enzymic degradation, and had a strictly alternating RG backbone. Therefore, the aim of structure confirmation by ^{1}H NMR spectroscopy was to establish the nature of the reducing and nonreducing end sugars. Diagnostic signals were the anomeric signals, and the H-4 of nonreducing end GalA (saturated and unsaturated) and of nonreducing end Rha (see Table 3) [12,17,21]. Identification was complicated by variations of the NMR spectra that occurred with varying pH values, especially of the H-5 signal of GalA [24]. Except for fractions a-4 and b-3, which were not pure enough for positive identification of the components, the results (Table 3) confirmed the structure assignments of the main oligomers in the fractions that were made by comparing HPAEC elution behaviour of unknown degradation products with that of RG standards (Table 2). In the following paragraphs, the enzymic degradation products of purified RG oligomers instead of mixtures were characterized by HPAEC, in order to learn more about the mode of action of the RG-hydrolase and RG-lyase.

Degradation of linear RG oligomers by RG-hydrolase.—Oligomers **A4** to **A16** (for explanation of codes see Table 1), and oligomers **B7** to **B15** were incubated with RG-hydrolase. The GalA, released by RG-galacturonohydrolase from type **A** oligomers when type **B** oligomers were formed, was not removed from the solution, since separate experiments showed that GalA did not inhibit RG-hydrolase or RG-lyase. Although minor amounts of reaction products were released from **A8**, RG-hydrolase was only active toward **B9** and larger

Table 3
Diagnostic signals for identification of reducing and non-reducing end Rha and GalA residues of RG oligomers, and their presence in enzyme degradation products

	GalA					Rha			Main oligomer based on ^{1}H NMR
	Int	r.e. (α/β)	n.r.e.	to r.e.Rha (α/β)	U-n.r.e.	Int	r.e. (α/β)	n.r.e.	
H-1	5.00[a,b]	5.28/4.55[b]	5.00[a]	5.08/5.16[a]	5.13[c]	5.27[a,b]	5.22/4.93[a]	5.23[b]	
H-4	4.41[a,b]	4.39/4.32[b]	4.29[a]	4.41[a]	5.81[c]	3.40[a,b]	3.47/3.33[a]	3.35[b]	
Fractions:									
a-6		++	++	+/−	−	++	+/−	+/−	**D3**: G-R-G
a-5	++	++	−	−	−	++	−	++	**C4**: R-G-R-G
a-4	++	?	+	?	−	++	?	+/−	
a-3	+	−	−	+	−	+	+	+	**B5**: R-G-R-G-R
a-2	++	+	−	+/−	−	++	+/-	+	**C6**: R-G-R-G-R-G
a-1	++	−	−	+	−	++	+	+	**B7**: $(R\text{-}G)_3$-R
b-4	−	−	−	++	++	++	++	−	**E4**: uG-R-G-R
b-3	+	?	−	+	+	++	+	−	

[a] [17].
[b] [21,29].
[c] [12].
++: Major signal; +, present; +/−, weak signal; –, absent. Int, internal; r.e., reducing end; n.r.e., non-reducing end; U-n.r.e., Δ-(4,5)-unsaturated GalA non-reducing end. Explanation of symbols in oligomer structure in Table 1.

oligomers. Type **B** oligomers were cleaved at exactly the same positions as the type **A** oligomers they originated from, i.e. **B9** was cleaved as **A10**. Therefore, only the identification of degradation products from type **A** oligomers is described in detail below.

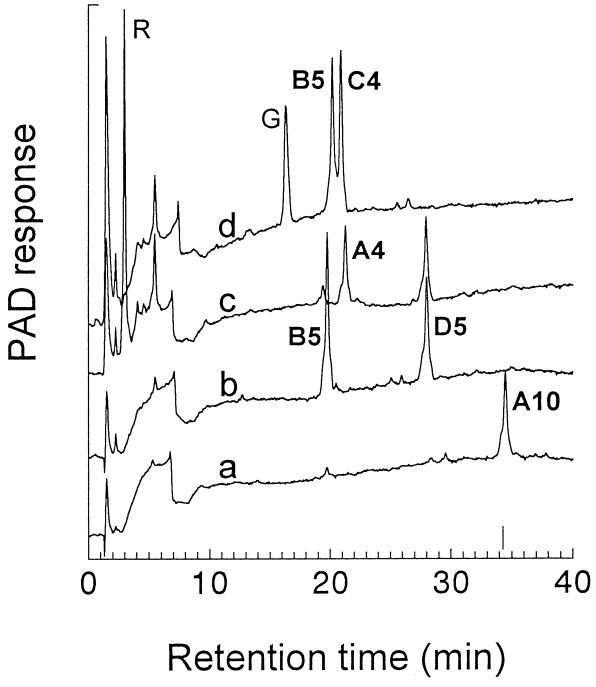

Fig. 3. HPAEC patterns of **A10** (a); after incubation with RG-hydrolase (b); after incubation with RG-hydrolase and subsequently RG-rhamnohydrolase (c); and after incubation with RG-hydrolase and subsequently RG-galacturonohydrolase (d). Explanation of codes in Table 1.

From **A10**[3] (Fig. 3, line a) two major products were formed (Fig. 3, line b). When compared with the available standards, the first peak corresponded to **B5**, and the second peak with **D5**. Together they match the DP 10 from which they originate. The designation of the products was confirmed by subsequent incubation of the reaction mixture with either RG-rhamnohydrolase or RG-galacturonohydrolase. Fig. 3 (line c), shows that after RG-rhamnohydrolase action, **D5** remains, **B5** has disappeared while a new peak is formed and Rha is released. The newly formed peak corresponded to **A4**, as expected from removal of the non-reducing Rha from **B5** by RG-rhamnohydrolase. After RG-galacturonohydrolase action, shown in Fig. 3, line d, **B5** remains, **D5** has disappeared while a new peak is formed and GalA is released. The newly formed peak corresponded to **C4**, as expected from removal of the nonreducing GalA unit from **D5** by RG-galacturonohydrolase. In this manner, using the two exo-enzymes, the original assignments were confirmed.

Since the original oligomer contained GalA at the nonreducing and Rha at the reducing end, **D5** must originate from the nonreducing end and **B5** from the reducing end, and they can therefore be positioned as follows:

[3] Previously, RG-hydrolase was reported to be most active toward DP 12 and higher [25], and RG-lyase toward DP 14 and higher [26]. However, the DP assignment of oligomers was later corrected after characterization by NMR [17] into two units smaller, i.e. DP 10 instead of DP 12 etc.

G-R-G-R-G (**D5**) + R-G-R-G-R (**B5**),

showing that the RG-hydrolase cleaved exactly in the middle of the oligomer, at five units from the nonreducing/reducing end.

Four products were formed from **A12** (not shown). When compared with the available standards, the peaks corresponded (in order of increasing retention on HPAEC) to **B5, B7, D5**, and **D7**. Subsequent degradation of the reaction products by RG-galacturonohydrolase and RG-rhamnohydrolase all gave products for which standards were available, and confirmed the original assignments. The reaction products can be put together to form the original **A12** as follows:

G-R-G-R-G (**D5**) + R-G-R-G-R-G-R (**B7**);

and

G-R-G-R-G-R-G (**D7**) + R-G-R-G-R (**B5**)

showing that the RG-hydrolase cleaved either five or seven units from the nonreducing end (or seven or five units from the reducing end). Since the molar response factor decreased going from **A10** to **A16** (not shown), it could be concluded from the integrals of the products that cleavage at five units from the nonreducing end was preferred above cleavage seven units from the nonreducing end of **A12**.

The assignment of the products released from **A14** was again confirmed using RG-galacturonohydrolase and RG-rhamnohydrolase. The structures identified were, by increased retention on HPAEC: **B5, C4, B7, D5, B9**, and **D7**. With two pairs of these oligomers, **A14** can be formed directly as follows:

G-R-G-R-G (**D5**) + R-G-R-G-R-G-R-G-R (**B9**);

and

G-R-G-R-G-R-G (**D7**) + R-G-R-G-R-G-R (**B7**)

indicating cleavage by RG-hydrolase five or seven units from the nonreducing end, similarly to cleavage of **A12**. After its initial formation, nonamer **B9** was found to decrease in time (not shown), while the amount of **C4** and **B5** increased. Therefore it was assumed that the latter two oligomers resulted from further cleavage of oligomer **B9** by RG-hydrolase. The ratio between oligomers **D5** and **D7** is indicative of which initial cleavage is preferred by RG-hydrolase, since these oligomers could not be degraded further by this enzyme. The ratio between **D5** and **D7** was essentially the same as found in case of degradation of **A12**, and therefore cleavage at five units from the nonreducing end was also preferred by RG-hydrolase in case of **A14**. Traces of the second generation product **C6** were found, but no peaks eluting at retention times of the trimer **B3**, both expected from further cleavage of **B9**.

The structures of the RG-hydrolase reaction products from **A16** could be confirmed using RG-rhamnohydrolase and RG-galacturonohydrolase, and were the following: **B5, C4, B7, C6, D5, B9**, and **D7**. The ratio between the only two products that could be resulting from the nonreducing end, **D5** and **D7**, was again the same as for **A12**, indicating preferred cleavage at five units from the non-reducing end of **A16**. One pair of oligomers formed the original **A16**:

G-R-G-R-G-R-G (**D7**)+R-G-R-G-R-G-R-G-R(**B9**),

where RG-hydrolase had cleaved seven units from the nonreducing end, although only traces of **B9** were found after 20 h incubation. Initially, beside **B11** was also formed (not shown), but both **B9** and **B11** decreased upon prolonged incubation. Therefore, similarly to **A14**, RG-hydrolase most likely further cleaved **B9** into **C4** and **B5**. Separate experiments showed that **B11** could be degraded into:

R-G-R-G (**C4**) + R-G-R-G-R-G-R (**B7**),

and

R-G-R-G-R-G (**C6**) + R-G-R-G-R (**B5**),

which are indeed the other RG-hydrolase products found in the **A16** RG-hydrolase digest.

The cleavage patterns are summarized in Fig. 4. Since initially **B9** was formed in case of **A14**, and **B11** in case of **A16**, first cleavage apparently occurred close to the non-reducing end. When the DP increased, the cleavage possibilities also increased (Fig. 4): beside cleaving four units from the first nonreducing Rha, RG-hydrolase also cleaved six units from the first non-reducing Rha.

However, except for **B11**, cleavage at four units from the non-reducing Rha was preferred. Going from **B9** to larger oligomers in Fig. 4, cleavage options only increased when beside an additional GalA also an additional Rha was present on the nonreducing end, i.e. in **B11, B13** and subsequently **B15**. This suggests that the number of Rha units, or perhaps the number of the structural units R-G, determines whether or not the oligomer can be cleaved by RG-hydrolase. Based on the current results, the size of the RG-hydrolase subsite can be estimated to be at least nine sugar units. If the number of Rha units is indeed determinant, then an additional GalA on the reducing end (type **D** oligomers) would have no influence on the cleavage.

Degradation of linear RG oligomers by RG-lyase.—When RG-lyase was incubated with **A4** to **A16**, and **B9** to **B15**, the smallest oligomer that could be degraded was **A12**. From **A12** (Fig. 5, line a) two peaks were formed (Fig. 5, line b), eluting at almost the same retention times. These peaks (30.94 and 31.38 min) corresponded to **E4** and **A8**, together matching with **A12**. The identification of **A8** was confirmed by subsequent incubation of the reaction mixture with RG-galacturonohydrolase, that resulted in release of GalA and the formation of a new peak that corresponded to **B7** (Fig. 5, line c). No confirmation of the unsaturated product could be given, since no enzyme was available, nor reported in the literature, able to remove the Δ-(4,5)-unsaturated GalA from the non-reducing end of RG fragments. As expected, RG-rhamnohydrolase was not able to remove Rha from the oligomers in the digest. The non-reducing Δ-(4,5)-unsaturated GalA unit must result from the cleavage by RG-lyase, since the original **A12** contains a saturated GalA at the non-reducing end. The structures therefore can be positioned as follows to form the original **A12**:

G-R-G-R-G-R-G-R (**A8**) + uG-R-G-R (**E4**),

showing that RG-lyase cleaved at four units from the reducing end (or eight units from the non-reducing end).

Similar characterization was carried out for the larger oligomers, and the results are summarized in Fig. 6. Since initially **A12** was formed from **A16** (not shown), first cleavage apparently was close to the reducing end. **A16** was the only oligomer that was preferentially cleaved at six units from the reducing end of the RG chain. Similarly to what was observed for RG-hydrolase, the cleavage

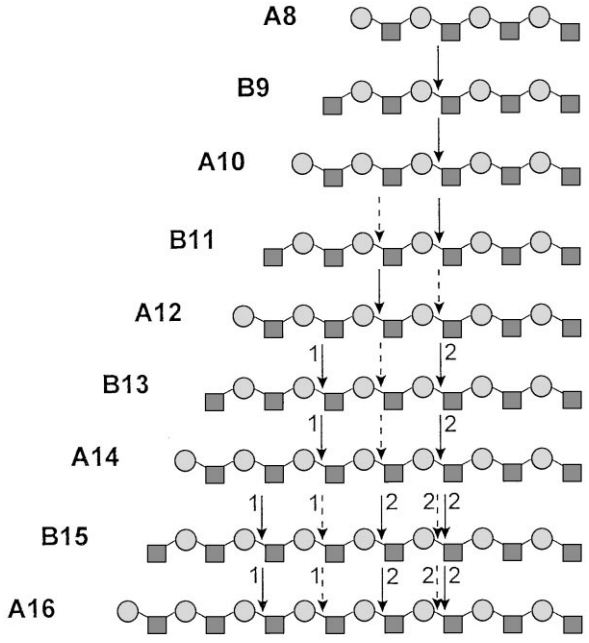

Fig. 4. Cleavage patterns of RG oligomers by RG-hydrolase. Symbols: ●, α-GalA (1,2)-linked to Rha; ■, α-Rha (1,4)-linked to GalA, or Rha at the reducing end. The solid arrows indicate preferential cleavage, the dotted arrows indicate least preferred cleavage. The numbers refer to 1, first cleavage; and 2, second cleavage.

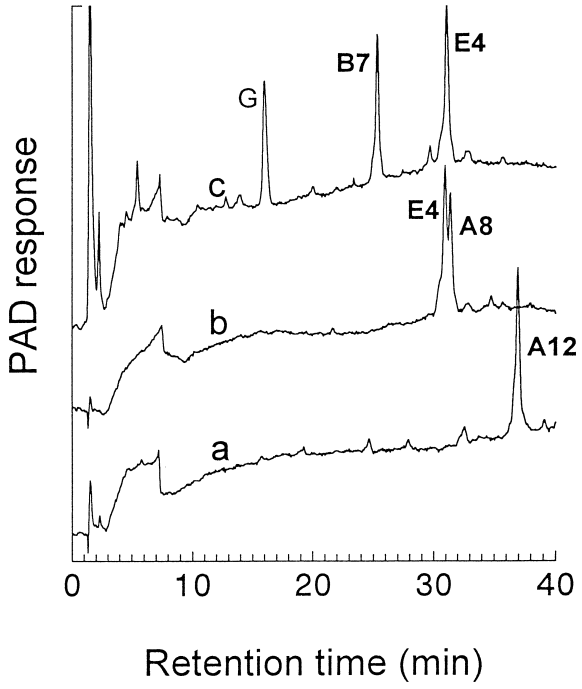

Fig. 5. HPAEC patterns of **A12** (a); after incubation with RG-lyase (b); and after incubation with RG-hydrolase and subsequently RG-galacturonohydrolase (c). Explanation of codes in Table 1.

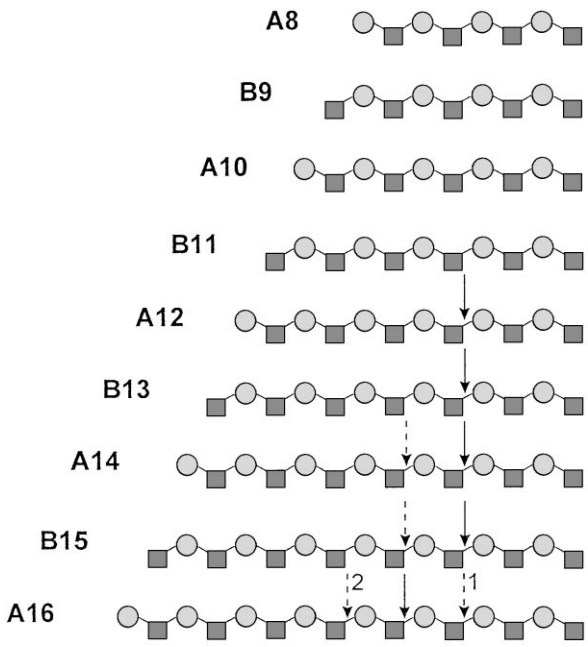

Fig. 6. Cleavage patterns of RG oligomers by RG-lyase. Symbols: ●, α-GalA (1,2)-linked to Rha; ■, α-Rha (1,4)-linked to GalA, or Rha at the reducing end. The solid arrows indicate preferential cleavage, the dotted arrows indicate least preferred cleavage. The numbers refer to 1, first cleavage; and 2, second cleavage.

options increased with increasing DP of RG oligomers. In this case when going from **A12** to larger oligomers in Fig. 6, the cleavage options increased when besides an additional non-reducing Rha unit also an additional GalA was present, in contrast with the cleavage by RG-hydrolase. This suggests that the number of GalA units, or perhaps the number of G-R units, determines if and how the oligomer can be cleaved by RG-lyase. The present results suggest that the size of the RG-lyase subsite will be 12 sugar units. However, if the number of GalA units is indeed determinant, then removal of the reducing Rha can be expected to make no difference for RG-lyase, and the size of the subsite might be 11 residues.

4. Discussion

As shown in this paper, both RG-hydrolase and RG-lyase are able to degrade linear RG oligomers. However, from apple MHR-S predominantly galactosylated oligomers are released by RG-hydrolase [11] and RG-lyase [12,28]. This means that in apple MHR-S the RG stretches must predominantly be galactosylated, and if linear or partly galactosylated regions are present, they are too short for the enzymes to release oligomers from. However, Schols et al. [28] showed that in native apple pectin RG regions without Gal substitution do exist, and also that RG-hydrolase is able to release oligomers therefrom.

In apple MHR-S, the strictly alternating RG regions degradable by RG-hydrolase and RG-lyase are interspersed with the other structural units: the xylogalacturonan and the arabinan side chain-rich RG stubs [13]. Suppose that the enzymes would act on these RG regions in MHR-S in the same way as they do on the oligomers in this study, then these RG regions would have to be at least 9 units (Rha at both ends) long for RG-hydrolase and 12 units (GalA at non-reducing, Rha at reducing end) for RG-lyase to cleave. For a second cleavage resulting in the release of the oligomers **C4** or **C6** from MHR-S [11] the RG stretch would have to be 13 or 15 units long, respectively, for RG-hydrolase. For RG-lyase the length would have to be 16 units to release the tetramer **E4**. Previously [27] RG-hydrolase was found to act toward MHR-S with a degree of multiple attack of 4, and RG-lyase of 2.5. This means that during the lifetime of an individual enzyme-substrate complex, on average 4 respectively 2.5 linkages are cleaved following the first. Therefore, after the first cleavage, RG-hydrolase would need an RG region of 29 residues to release two **C4** and two **C6** oligomers (9+4+4+6+6). RG-lyase for instance would need a stretch of 22 residues in order to be able to release one tetramer **E4** and one hexamer **E6** (12+4+6) (in this case a degree of multiple attack of 2). This implies that the RG regions in MHR-S must be rather longer than suggested by Schols and Voragen [14], i.e. around 13 units.

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