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Rhamnogalacturonase: a novel enzyme that degrades the hairy regions of pectins*

Henk A. Schols, Carolien C. J. M. Geraeds, Marjo F. Searle-van Leeuwen, Felix J. M. Kormelink, and Alfons G. J. Voragen†
Department of Food Science, Agricultural University, Bomenweg 2, 6703 HD Wageningen (The Netherlands)

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ABSTRACT

A rhamnogalacturonase (RGase), that could degrade the modified hairy regions (MHR) prepared from apple cell walls, was isolated and purified from a technical preparation of *Aspergillus aculeatus*. The RGase cleaved galactopyranosyluronic-rhamnopyranosyl linkages. No activity was observed towards other cell-wall polysaccharides or *p*-nitrophenyl glycosides. The optimal conditions for RGase were pH 3–4 and 40–50°. MHR was degraded by RGase, and methylation analysis and ^{13}C-n.m.r. spectroscopy indicated the products to have a tetrasaccharide backbone of alternating rhamnose and galacturonic acid residues. Some oligomers had a galactose residue 4-linked to rhamnose. The potential value of this new enzyme is considered.

INTRODUCTION

The complex nature of plant cell walls and the structure of the component polysaccharides have been the subject of many publications[1–4]. Pectins have been studied frequently because they are the most important components with respect to the growing, ripening, and processing of fruits and vegetables[5–7]. De Vries *et al.*[8] proposed that pectins consist of highly carboxyl-methylated linear homogalacturonan regions which alternate with "hairy" (ramified) regions that comprise highly branched rhamnogalacturonans. The presence of pectic hairy regions has been reported also for carrot cell walls[9] and the pulp of grape berries[10]. The preparation of apple juice by the liquefaction process involves combinations of pectolytic and cellulolytic enzymes, so that the hairy regions and other pectin fragments which originate from the insoluble protopectin are solubilized[5]. Schols *et al.*[11] isolated these fragments by ultrafiltration and established that their structures were similar to that of the hairy regions described by De Vries *et al.*[8]. The fragments were resistant to degradation by most of the pure and technical pectinase and cellulase preparations, except for a crude preparation obtained from *Aspergillus aculeatus*, which could degrade the rhamnogalacturonan backbone of the hairy regions.

* Hairy (Ramified) Regions of Pectins, Part II. For Part I, see ref. 11.
† Author for correspondence.

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We now report the isolation and characterization of a novel rhamnogalacturonase (RGase) that can split galactopyranosyluronic-rhamnopyranosyl linkages.

EXPERIMENTAL

Substrates. — Beet arabinan and xylan from oat spelts were purchased from Koch–Light, (1→5)-α-L-arabinan (haze arabinan) was isolated from turbid apple-juice concentrate[12,13], CM-cellulose (Akucell AF 0305) was obtained from Akzo (Arnhem), polygalacturonic acid was purchased from ICN (Cleveland), high-methoxyl pectin (degree of methylation, 90%) was prepared in our laboratory[14], and galactan was isolated from potato fibre (obtained from Avebe) according to the modified method of Labavitch *et al.*[15] as described by Rombouts *et al.*[16].

"Modified hairy regions" (MHR) was obtained by ultrafiltration of apple juice produced by the liquefaction process[11]. This polysaccharide has been designated previously as ultrafiltration retentate (UFR) by Voragen *et al.*[5]. MHR was also modified[11] by removal of arabinosyl side chains by treatment with hydrochloric acid (→ MHR-HCl) or with arabinanases (→ MHR-ARA) and by demethylation and deacetylation with sodium hydroxide (→ MHR-S, MHR-HCl-S, and MHR-ARA-S).

Enzyme preparation. — Ultra Sp, an enzyme preparation from *Aspergillus aculeatus,* was kindly provided by Novo Ferment AG (Basel).

Enzyme assays. — The Nelson–Somogyi assay[17] used to measure the increase in reducing end groups did not have the sensitivity needed, and high performance g.p.c.[11] was the only method suitable for measuring the action of the enzyme by monitoring the change in the molecular-weight distribution.

Enzyme fractions were screened for contaminating activities by measuring the increase in the reducing end groups[17] after incubation of the enzyme fractions for 1 h at 30° with a 0.1% solution of the substrate in 0.05M sodium acetate buffer (pH 5.0). The digests were analysed by high performance g.p.c.

The activity of the pure enzyme towards various *p*-nitrophenyl glycosides was measured spectrophotometrically[18] at 405 nm, using the extinction coefficient 13 700 $m^{-1}.cm^{-1}$.

Purification of the enzyme. — Fractionation of the crude enzyme preparation included chromatography on Bio-Gel P10 (100–200 mesh), DEAE-Bio-Gel A, and Bio-Gel HTP (Bio-Rad). Gradient elution was performed with an LKB 11 300 Ultrograd Gradient Mixer with the sensor set at an A_{280} value of 0.01.

Other column materials used were obtained by cross-linking[16] alginate with epichlorohydrin (molar ratio of epichlorohydrin to "anhydrouronic acid" of 2.34) and monoQ HR5/5. The latter column was used with a fast protein liquid chromatography (f.p.l.c.) system (Pharmacia).

The protein content of the RGase fraction was determined according to the method of Sedmak[19], slightly modified for using microtiter plates as described by Kormelink *et al.*[18].

SDS–PAGE. — Sodium dodecyl sulphate–polyacrylamide gel electrophoresis was performed with the PhastSystem (Pharmacia) according to Rombouts *et al.*[16]. Bovine serum albumin (M_r 68 000), catalase (M_r 60 000), aldolase (M_r 35 000), trypsin inhibitor (M_r 20 000), and cytochrome C (M_r 12 500) were used as standards. Gels were stained for protein with Coomassie Brilliant Blue 350-R.

Optimum temperature and pH. — The influence of pH and temperature on RGase activity was derived from the change in molecular-weight distribution of MHR-S as measured by high performance g.p.c. after incubation of 0.3% MHR-S with RGase (75 ng of protein) in 0.05M sodium acetate buffers of pH 3.0, 4.0, 5.0, and 6.0 at temperatures of 20, 30, 40, 50, and 60° for 60 min. The enzyme was inactivated by boiling each reaction mixture for 5 min.

Temperature and pH stability. — The stability of the RGase was tested by incubation of RGase (75 ng of protein) in the absence of substrate in 0.05M acetic acid or sodium acetate (pH 3.2, 4.0, 5.0, 6.0, and 7.5) for 90 min at 30°. A second incubation (60 min, 30°) was performed after adjustment of the pH to 5.0 and addition of the substrate (0.3% of MHR-S). The stability to temperature was tested by incubation at temperatures in the range 20–70° in 0.05M NaAc (pH 5.0). The second incubation step was similar to that described above. The enzyme was inactivated by boiling each reaction mixture for 5 min and the samples were analysed by high performance g.p.c.

Product analysis. — RGase was incubated with 3.5% MHR-S or MHR-HCl in 0.05M sodium acetate (pH 5.0) for 20 h at 30°. After inactivation of the enzyme by boiling for 5 min, the digest (~400 mg) was applied on a column (950 × 22 mm) of Sephadex G50 (fine; separation range for dextrans: 200–10 000 Da, Pharmacia) and the products were eluted with 0.05M sodium phosphate (pH 7.0) at 20 mL/h. Fractions (5 mL) were assayed by automated colorometric methods for uronic acids and total neutral sugars[20,21]. The neutral sugar values were corrected for the contribution of the uronic acids in the orcinol assay.

N.m.r. spectroscopy. — The ^{13}C- and ^{1}H-n.m.r. spectra were recorded and interpreted as described[22].

Analytical methods. — Sugar composition and uronic acid content were determined as described[11].

RESULTS AND DISCUSSION

Characterization of substrates. — The sugar compositions of MHR and chemical and enzymic modifications are summarized elsewhere (Schols *et al.*[11], Table I). As expected, the sugar compositions of the MHR substrates before and after saponification were identical.

Enzyme purification. — The scheme for the isolation of RGase from Ultra SP is shown in Fig. 1 and the corresponding elution patterns are shown in Fig. 2. Ultra Sp was first desalted on Bio-gel P10 and the fractions which were active against MHR were combined and applied to a column of DEAE Bio-gel A. Elution was performed with the

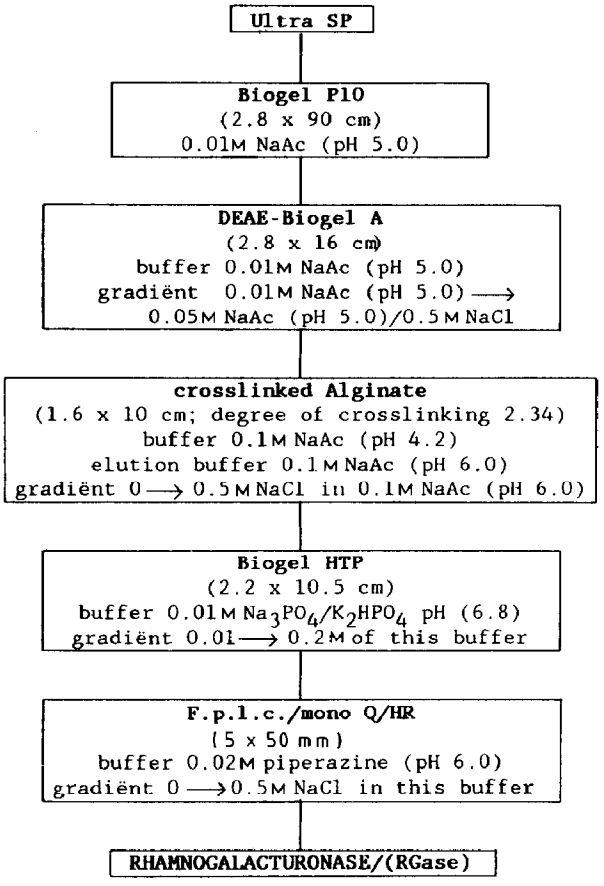


Fig. 1. Scheme for the isolation of RGase from a crude *Aspergillus aculeatus* preparation (Ultra SP); (0.01% NaN_3 was added as buffer preservative in all steps).

gradient mixer controlled by the level sensor (A_{280}) set at 0.01, which made it possible to elute protein peaks with a minimum of contamination. In this step, part of the polygalacturonase activity could be separated from the RGase fraction. The fractions that contained the RGase activity were combined and dialysed against 0.1M sodium acetate (pH 4.2). The remaining polygalacturonase activity was removed by using a column of cross-linked alginate. The combined fractions that contained the MHR-degrading activity were dialysed against sodium phosphate (pH 6.8) and applied to a column of Bio-gel HTP. The first peak was active against MHR when measuring the reducing end groups, but this was due to the presence of arabinanases. The RGase-containing fractions were practically free of other activities. As the RGase was labile in phosphate buffers, the HTP-step was performed without delay in order to avoid inactivation. This sensitivity toward phosphate buffers was described by Karr and Albersheim[23] for their wall-modifying enzyme (WME). For this reason, the fractions from the HTP column were assayed directly for RGase activity and the active fractions were dialysed immediately against 0.1M sodium acetate (pH 5.0).

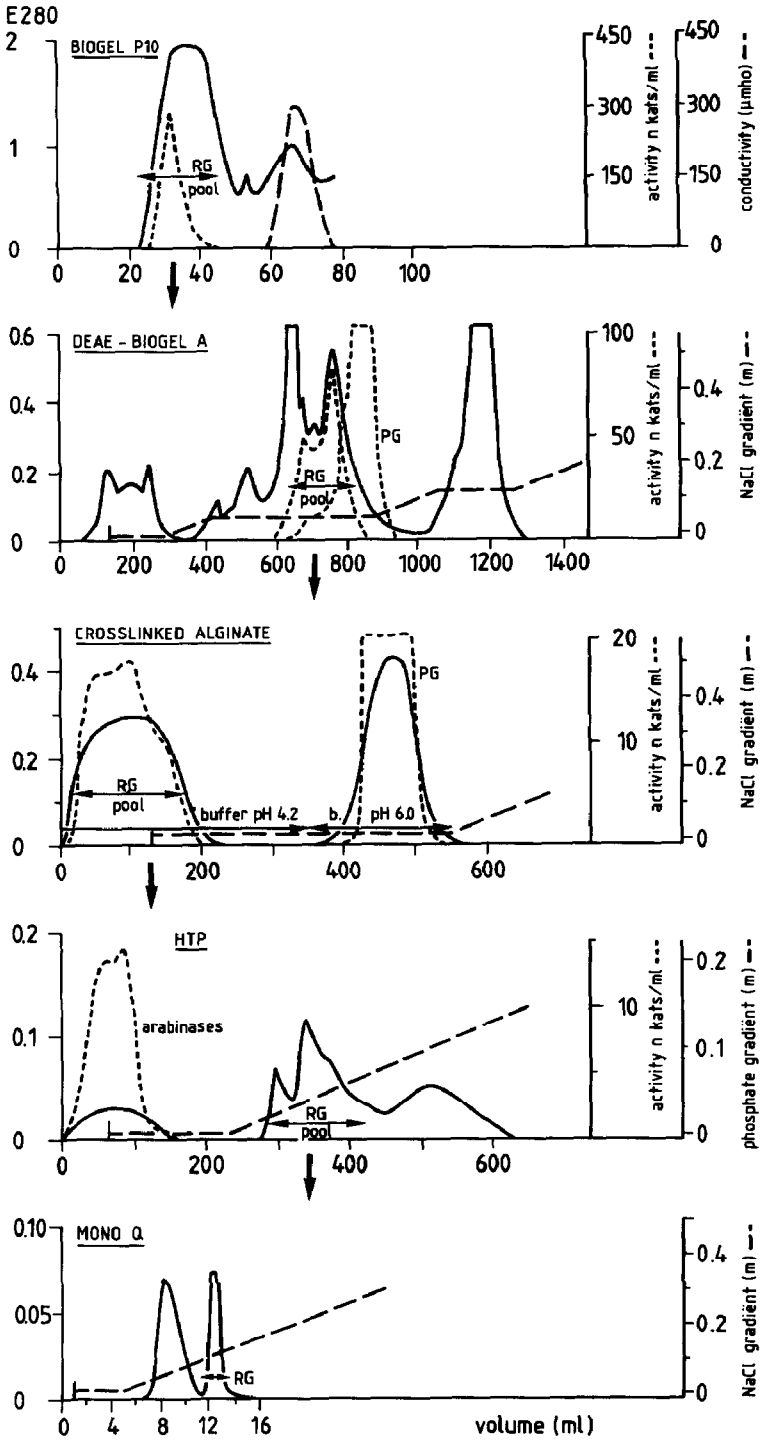


Fig. 2. Elution patterns on various columns used in Fig. 1 for the purification of RGase (RG).

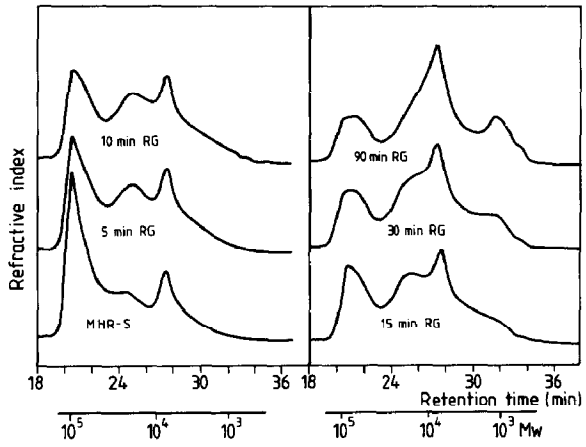


Fig. 3. High performance g.p.c. of saponified MHR (MHR-S) after treatment with RGase (RG) at 30° and pH 5 for various times.

Final purification involved fractionation using an f.p.l.c. system equipped with a mono Q column.

Characterization of RGase. — RGase was not active against MHR, but was very active towards MHR-S and MHR-HCl. The inhibition of RGase by acetyl groups is discussed below.

RGase was not active against high- or low-esterified pectins, polygalacturonic acid, haze linear arabinan, beet branched arabinan, oat spelts xylan, potato galactan, and carboxymethylcellulose. RGase had weak activity against *p*-nitrophenyl α-L-arabinofuranoside (incubation for 24 h → 70 nkat/mg of protein). There was no activity against the *p*-nitrophenyl α- and β-glycosides of D-Gal*p*, D-Glc*p*, D-Xyl*p*, and those of β-L-Ara*f*, α-L-Ara*p*, α-D-Gal*f*, and α-D-Man*p*.

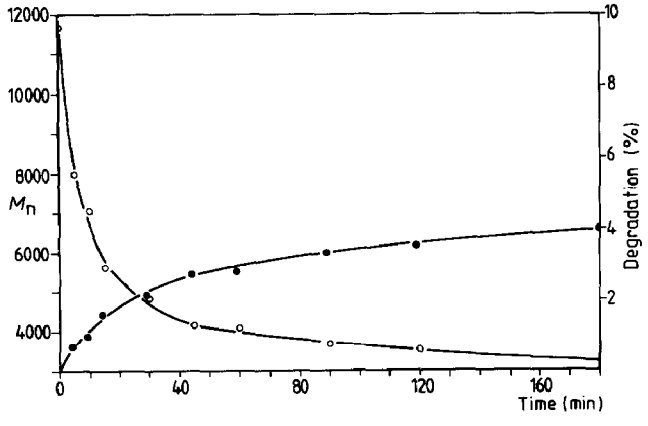


Fig. 4. Decrease of the average M_n (-o-) and the percentage of degradation (-●-) of MHR-S on incubation with RGase at 30° and pH 5 for 3h.

In SDS–polyacrylamide gel electrophoresis, RGase moved as a single band with an M_r value of 51 000.

The specific activity of RGase at pH 5.0 and 30° was calculated from the change in the distribution of mol. wt. as determined by high performance g.p.c. Using commercial g.p.c. software, M_n was calculated from which, together with the substrate concentration, the number of glycosidic linkages cleaved was estimated and expressed in activity units. The value obtained (~ 5 μkat/mg) gives only the order of magnitude, since the content of protein was low, and it applies only to the MHR-S substrate. Moreover, it should be borne in mind that the column system was calibrated with pectin standards.

Figure 3 shows the high performance g.p.c. patterns of MHR-S after incubation with RGase (75 ng of protein). The fraction with the highest M_w in MHR was degraded from the beginning of the reaction and was converted into fractions of lower M_w. The second peak (10 000–30 000) was shifted towards the third peak (7000) which represented a fraction that was resistant to RGase. Low-molecular-weight degradation products ($\sim$ 1500) were formed (see below).

From the high performance g.p.c. data, the average M_n value and percentage of degradation were estimated using commercial g.p.c. software (Fig. 4). The action of the enzyme was limited (<4% degradation after 3 h). With higher concentrations of enzyme and longer times of incubation, the void peak of MHR-S was degraded completely.

RGase was optimally active in the pH range 3–4 (Fig. 5). At pH 5, the activity was reduced to $\sim$50% and, at pH 6, to 20%. In the range 20–60°, the activity of the enzyme was always >75% of the maximal activity which was measured between 40 and 50° (Fig. 5).

Figure 5 shows that RGase was most stable at pH 4–5; at pH 3, only 43% degradation of MHR-S occurred. After incubation at pH 7.5, 50% of the activity remained. Figure 5 also shows that RGase was stable in the range 20–50° and there was a marked decrease in activity at 60°.

Thus, RGase is active over a broad range of conditions (pH 3–5, 20–60°).

Characterization of products of RGase action. — RGase was active only on MHR substrates. Figure 6 shows the degradation before and after saponification. MHR and

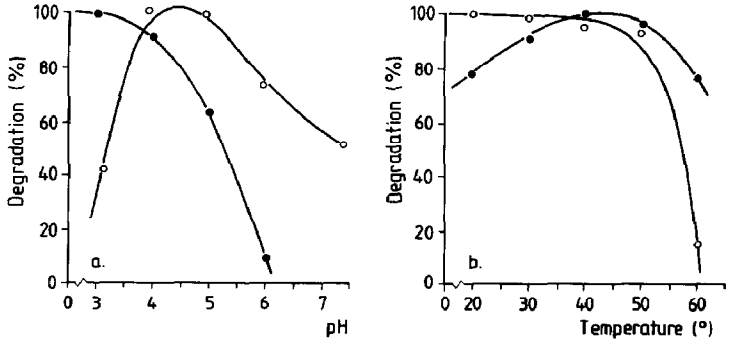


Fig. 5. The pH and temperature curve (-●-) and pH and temperature stability curve (-o-) for RGase.

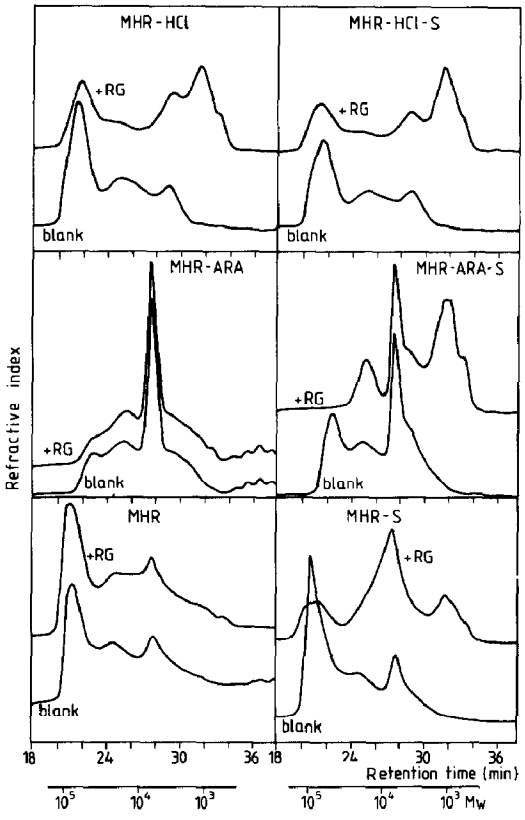


Fig. 6. High performance g.p.c. of MHR substrates, before and after incubation with RGase at 30° and pH 5 for 3 h.

MHR-ARA underwent little or no degradation. After removal of the methoxyl and acetyl groups, however, the resulting MHR-S and MHR-ARA-S were degraded readily to resistant polymeric fractions and oligomers. The difference in the patterns of elution of MHR-ARA and MHR-ARA-S must be ascribed to their preparation from different batches. MHR-HCl with degrees of methylation and acetylation of 33 and 10%, respectively, was degraded in about the same way as saponified MHR-HCl, indicating that the methoxyl groups did not hinder the RGase or that they are located in the regions which were not degraded by the enzyme.

In order to characterize the oligomers, enzyme digests of MHR-S and MHR-HCl were eluted from Sephadex G50 and the fractions were assayed for neutral sugars and uronic acids. The elution pattern of the MHR-S digest is shown in Fig. 7. A similar pattern was obtained for the MHR-HCl digest and the sugar compositions of the fractions are presented in Table I. Almost all of the xylose was present in the void fraction A, the xylose/galacturonic acid ratio of which was high. The arabinose residues were also recovered from the MHR-S digest in fraction A, and galactose was present in all fractions. Rhamnose, galactose, and galacturonic acid preponderated in the oligom-

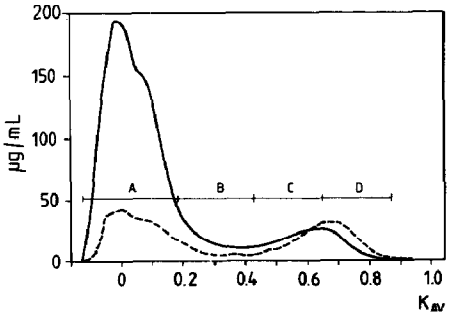


Fig. 7. Chromatography on Sephadex G50 of MHR-S after degradation with RGase: ——, neutral sugars; ----, uronic acid.

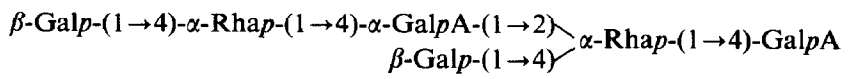
ers with d.p. 4–8. The sugar compositions of the fractions D were similar and illustrated that the patterns of action of RGase on the two substrates were similar. A slight difference was observed in the arabinose contents of fraction C. In fractions C and D, the rhamnose:galacturonic acid ratio was 1. This ratio was also found[24–26] for rhamnogalacturonan I, isolated from tissue-cultured sycamore cells.

The structures of the oligomers present in fraction D have been elucidated by methylation analysis and n.m.r. studies in the following paper[22]. Fraction D appeared to be a mixture of a tetramer, two pentamers, and a hexamer. These oligomers had a tetramer backbone of alternating rhamnose and galacturonic acid with rhamnose at the non-reducing end. The pentamer had a galactose residue linked to the terminal rhamnose *or* to the (1→2)-linked rhamnose and the hexamer (1) had a galactose residue linked to each rhamnose residue. This type of oligomer has not been isolated hitherto from apple cell walls or liberated from pectic hairy regions using a pure enzyme.

TABLE I

Sugar composition (mol%) of the fractions of degraded MHR-S and MHR-HCl after separation on Sephadex G50 (see Fig. 7)

	MHR-S				*MHR-HCl*			
	A	*B*	*C*	*D*	*A*	*B*	*C*	*D*
Rha	3	9	29	40	4	7	25	35
Ara	71	60	12	3	3	5	6	3
Xyl	7	2	1	0	37	19	1	0
Man	0	0	1	0	0	0	0	0
Gal	7	14	29	19	13	19	30	26
Glc	0	1	1	1	1	1	1	0
GalA	13	15	28	38	42	50	37	35
Rha:GalA	0.23	0.60	1.04	1.05	0.10	0.14	0.68	1.00



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The above-mentioned structures of the oligomers, together with chemical and structural analysis, led to a possible model of MHR (Schols *et al.*[11], Fig. 4).

The wall-modifying enzyme (WME) described by Karr *et al.*[23] might be similar to RGase. WME was active against the primary cell walls of suspension-cultured sycamore cells and was necessary for the degradation of the cell walls catalyzed by the commercial enzyme preparation. Purified WME was inactive against numerous polysaccharides, glycosides, and peptides.

Ultra SP from *Aspergillus aculeatus* has been described[27] as an enzyme which can degrade a polysaccharide fraction (SPS) present in soy-protein isolates. The reported sugar composition of SPS is similar to those of MHR-ARA and MHR-HCl (rhamnose:galacturonic acid ratio of 0.44). However, RGase showed no activity towards SPS.

RGase may be useful in studies of the structures of complex pectic polysaccharides. The formation of oligomers by the action of RGase indicates that the complex hairy regions from apple pectin are more regular in structure than has been assumed hitherto. It is proposed that the hairy regions of apple pectin are composed in part of different repeating units as suggested by De Vries[28]. Work on the fine structure of the MHR is in progress.

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