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Rhamnogalacturonase B from *Aspergillus aculeatus* Is a Rhamnogalacturonan α-L-Rhamnopyranosyl-(1→4)-α-D-Galactopyranosyluronide Lyase[1]

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The recently described rhamnogalacturonase B, which is able to degrade ramified hairy regions of pectin, was found to be a rhamnogalacturonan α-L-rhamnopyranosyl-(1→4)-α-D-galactopyranosyluronide lyase. The cleavage site and mechanism differ from that of the previously described rhamnogalacturonase A, which is a hydrolase and can now be termed rhamnogalacturonan α-D-galactopyranosyluronide-(1→2)-α-L-rhamnopyranosyl hydrolase.

RGs, which are a part of the backbone of the highly ramified regions of pectin in plant cell walls (Voragen et al., 1993; Schols and Voragen, 1994), are presently the subject of many investigations. These highly branched pectins are not degraded by the classical pectolytic enzymes with activity toward "smooth" homogalacturonan regions of pectin (O'Neill et al., 1990; Schols et al., 1990b). Schols et al. (1990a) were the first to describe an enzyme (RGase) that was able to degrade the ramified regions of pectin. Since then, several papers from other workers have been published dealing with RGase activity (Matsuhashi et al., 1992; Düsterhöft et al., 1993; An et al., 1994; Sakamoto and Sakai, 1994). In addition, two other types of enzyme with high specificity toward hairy regions of pectin have been found, an RG-acetylesterase (Searle-van Leeuwen et al., 1992) and an RG α-L-rhamnopyranohydrolase (Mutter et al., 1994).

Recently, in the authors' laboratory, a new RGase from *Aspergillus aculeatus* was found (referred to by Kofod et al., 1994), named RGase B. Both RGase A and RGase B have been cloned and expressed in *Aspergillus oryzae*. RGase B was shown to be different from RGase A in pI, in pH optimum and stability, and in the reactivity with antibodies raised against RGase A. Furthermore, the oligomers formed by RGase B from MHR differed in elution behavior using HPAEC. Comparison of the primary structures, deduced from the cDNAs encoding the enzymes, indicated that the two RGases were structurally different (Kofod et al., 1994).

In their study, Kofod et al. (1994) could not give evidence that RGase B was indeed an RGase. In the present study we prove that RGase B is an RGase and that the two RGases are indeed different. A more specific nomenclature for the two enzymes is suggested.

MATERIALS AND METHODS

MHR-S were isolated from apple liquefaction juice as described by Mutter et al. (1994). RGase B degradation products of MHR-S were fractionated using Sephadex G-50. Pooled RGase B oligomer fractions were further separated by preparative HPAEC, essentially as described by Schols et al. (1994), using a Dionex (Sunnyvale, CA) PA-100 (22 × 250 mm) at 25 mL min^{-1} with the following gradient of NaOAc in 100 mM NaOH: 0 to 50 min, 200 to 300 mM; 50 to 55 min, 300 to 1000 mM; 55 to 70 min, 200 mM. Fractions were neutralized using acetic acid, pooled, dialyzed, and lyophilized. ^{1}H-NMR spectra of the products (in deuterated H_2O) were obtained at 400 MHz using a JEOL GX400 spectrometer. Two-dimensional NMR experiments (COSY and ROESY) were carried out as described previously (Colquhoun et al., 1990). Highly methoxylated pectin with a degree of methoxylation of 92.3% was prepared at our laboratory according to the procedure of Van Deventer-Schriemer and Pilnik (1976). Polygalacturonic acid was from Fluka. A mixture of linear alternating RG oligomers with a degree of polymerization higher than 18 was kindly provided by Dr. C.M.G.C. Renard (Institut National de la Recherche Agronomique, Nantes, France) and their preparation was essentially as described by Renard et al. (1995).

Recombinant RGase B from *Aspergillus aculeatus* was purified starting from lyophilized crude culture supernatant of an *Aspergillus oryzae* transformant (A 1560) producing recombinant RGase B, kindly provided by Novo Nordisk

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Abbreviations: COSY, correlation spectroscopy; GalA, α-D-galacturonic acid; HPAEC, high-performance anion-exchange chromatography; MHR, modified hairy regions from apple pectin; MHR-S, saponified MHR; NaOAc, sodium acetate; RG, rhamnogalacturonan; RGase, rhamnogalacturonase; RG-hydrolase, RG α-D-galactopyranosyluronide-(1→2)-α-L-rhamnopyranosyl hydrolase; RG-lyase, RG α-L-rhamnopyranosyl-(1→4)-α-D-galactopyranosyluronide lyase; Rha, rhamnose; ROESY, rotating frame Overhauser effect spectroscopy; us-GalA, unsaturated galacturonic acid.

A/S (Copenhagen, Denmark), essentially as described by Kofod et al. (1994). Native RGase A from *A. aculeatus* was purified using the method of Schols et al. (1990a). SDS-PAGE and IEF were performed as described by Mutter et al. (1994).

All incubation mixtures contained 0.65 mL of 0.1% (w/v) substrate solution (except for the linear RG oligomers: 0.02% [w/v]) and 0.05 mL of RGase B solution (2.92 μg mL^{-1} for recombinant RGase B; 1.23 μg mL^{-1} for RGase A). Incubations for determination of the specificity of RGase B toward various substrates were carried out at 30°C. Activities were calculated from the increase in A_{235}, as measured every 60 s using a Beckman DU-62 spectrophotometer equipped with a Soft-Pac Kinetics module. For further details see "Results." The number of linkages cleaved was expressed in activity units (one unit of enzyme producing 1 μmol unsaturated products min^{-1}) using a molar extinction coefficient of 4800 M^{-1} cm^{-1} (MacMillan et al., 1964).

High-performance size-exclusion chromatography was performed using three Bio-Gel TSK columns in series (40XL, 30XL, and 20XL) as described by Schols et al. (1990b) and calibrated using pectin standards (in the range of 196 to 100,000 D).

HPAEC was carried out using a Dionex Bio-LC system equipped with a Dionex CarboPac PA-100 (4 × 250 mm) column and a Dionex PED detector in the pulsed amperometric detection mode. A gradient of NaOAc in 100 mM NaOH (1 mL min^{-1}) was used as follows: 0 to 45 min, 100 to 380 mM; 45 to 55 min, 380 to 500 mM; 55 to 60 min, 500 to 1000 mM; 60 to 80 min, 100 mM.

RESULTS

Recombinant RGase B was purified from the culture supernatant of *A. oryzae*. The purified enzyme moved as a single band on SDS-PAGE and IEF. As already mentioned by Kofod et al. (1994), the HPAEC elution behavior of the RGase B oligomers as produced from MHR-S is very different from that of the RGase A oligomers (Fig. 1).

To determine the structure of the oligomeric RGase B reaction products and to gain more information about what part of the MHR-S is attacked by the enzyme, the degradation products of MHR-S as produced by RGase B were fractionated using a Sephadex G-50 size-exclusion column. Fractions containing RGase B oligomers were pooled and further purified using preparative HPAEC.

One- and two-dimensional NMR experiments (COSY and ROESY) were used to determine the structure of the oligomers. Figure 2 shows the ^{1}H-NMR spectrum of the smallest oligosaccharide, which elutes at 22.5 min in Figure 1. (^{1}H-NMR spectra were recorded at 27 and 50°C to shift the residual water resonance and reveal all signals in its locality.) The spectrum differed in important respects from spectra of RGs released by RGase A action (Colquhoun et al., 1990; Schols et al., 1994). The doublet at δ 5.81 (J = 3.4 Hz) was not present in the spectra of RGs reported earlier (Colquhoun et al., 1990), and the absence of any signals at δ 5.28 and 4.55 indicated that GalA could not be the reducing end residue. Comparison with the spectra of the linear

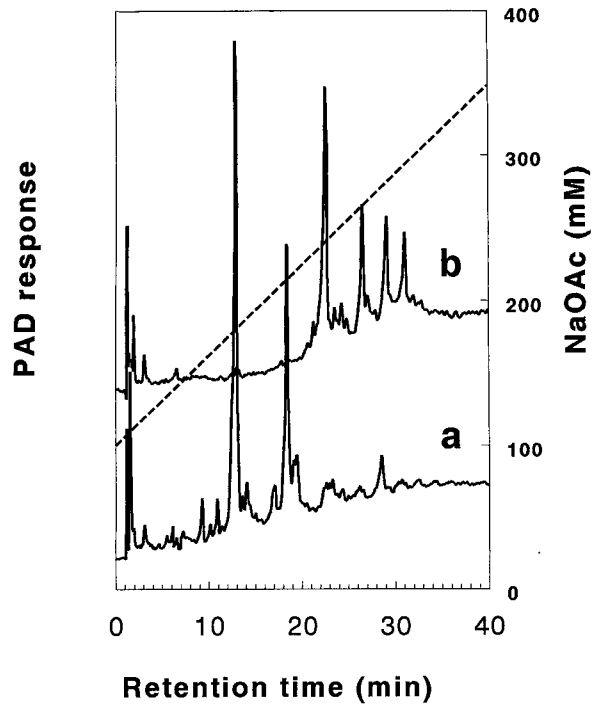
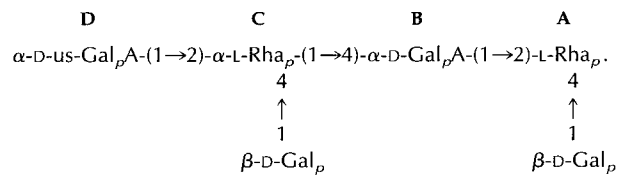


Figure 1. Typical HPAEC chromatograms of MHR-S after degradation by RGase A (a) and MHR-S after degradation by RGase B (b); – – – – –, NaOAc gradient; PAD, pulsed amperometric detection.

RG oligomers that were produced using acid hydrolysis (C.M.G.C. Renard, personal communication), suggested that, as in those oligomers, Rha was the reducing end unit with H-1 signals at δ 5.22 (α) and 4.94 (β). From the COSY experiment the doublet at δ 5.81 was found to belong to a four-proton spin-coupling network that had chemical shifts and coupling constants characteristic of an α-linked Δ-4,5-us-GalA residue at the nonreducing terminus (Tjan et al., 1974). For this residue the anomeric signal was at δ 5.13, and the doublet at δ 5.81 was assigned to the olefinic proton. Further assignments (via COSY) and integration of the anomeric region showed that, in addition to the terminal units, the oligosaccharide had one α-GalA, one α-Rha, and two β-Gal residues. The structure deduced for the oligosaccharide is:



Assignments in the down-field region are given in Figure 2, and the chemical shifts are summarized in Table I. The linkage positions were established in the same way as before (Colquhoun et al., 1990) by the occurrence of ROESY cross-peaks, which correlated with protons D1/C2, C1/B4, and B1/A2. The anomeric pairs D1/C1 and B1/A1 were also correlated in the ROESY spectrum, a feature that appears to be characteristic for (1→2) linkages. The chemical shifts of protons A4 and C4 (Table I) showed that both Rha

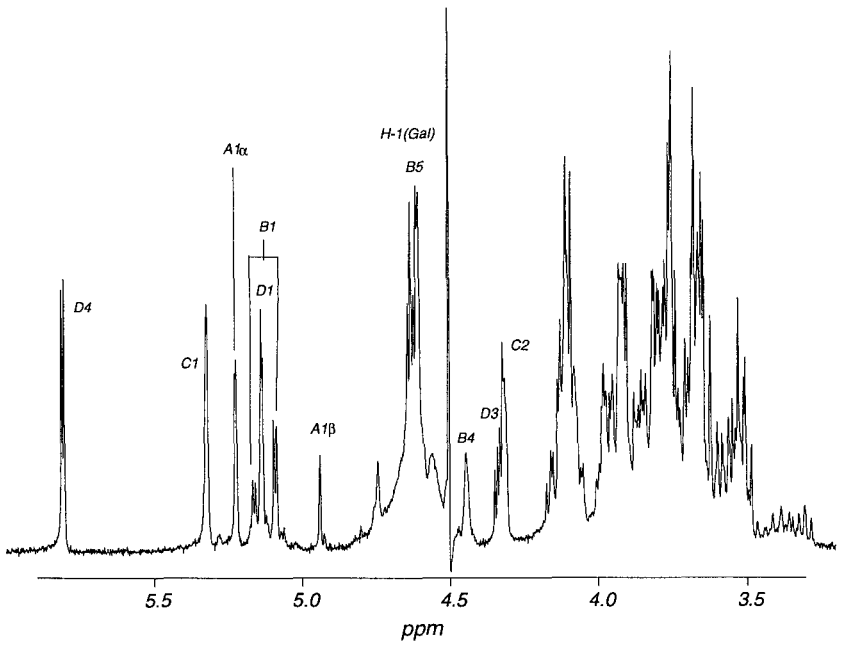


Figure 2. The 400-MHz ^{1}H-NMR spectrum (50°C) of the smallest RGase B oligosaccharide. Residues are coded as in the text. The two B1 doublets are for GalA linked to α- and β-Rha-reducing end groups. ppm, Parts per million.

residues were 4-substituted by β-Gal (Colquhoun et al., 1990). Weak signals below 3.5 parts per million were associated with a small amount of unidentified impurity and did not arise from H-4 of unsubstituted Rha units.

In addition to the features described here, the larger oligomers in the series (eluting at 26.5, 29, and 31 min in Fig. 1) had new anomeric signals at δ 5.28 (Rha) and 5.07 (GalA). These arose from additional internal residues in an extended RG backbone. All of the Rha residues appeared to be (1,2,4) linked. Further details of these spectra will be published elsewhere.

Apparently, RGase B cleaved the RG backbone by β elimination, leaving Rha at the reducing end and an us-GalA at the nonreducing end, indicating the action of a lyase. It is well known that a pectin lyase also cleaves the backbone by β elimination (Albersheim et al., 1960; Rexová-Benková and Markovic, 1976) and introduces a double bond between C-4 and C-5 of the GalA residue at the nonreducing end. Conjugation of the double bond with the carboxyl group at C-5 gives a characteristic absorption maximum at 235 nm. When the action of RGase B toward MHR-S was followed at 235 nm, an increase in the A was indeed observed, confirming lyase activity. For comparison, the action of RGase A from *A. aculeatus* toward MHR-S was measured in the same way and, as expected for a hydrolase, no increase of the A_{235} was observed.

Lyase activity of RGase B toward various substrates was measured (Table II). The optimum pH, measured in McIlvaine buffers, was confirmed to be 6, as reported before (Kofod et al., 1994). The lyase activity toward MHR-S was 20% higher in 20 mM Tris-HCl buffer, pH 8, than in 50 mM NaOAc buffer, pH 6. Different buffers have been found to significantly influence the activity of pectin lyases (Voragen, 1972). The different ionic species used, however, might also influence the molar extinction coefficient (not further investigated). The enzyme had no absolute requirement for calcium ions, although a positive effect was observed. RGase B was also active toward the linear, alternating RG fragments. These fragments consist of an alternating RG backbone with Rha at the reducing end and GalA at the nonreducing terminus (Renard et al., 1995). Since NMR revealed that a Δ-4,5-us-GalA was present at the nonreducing end of the products, the lyase must cleave the linkage between an Rha and a GalA in the backbone. No activity was found toward polygalacturonic acid at pH 6 or pH 8 either with or without 1 mM Ca^{2+} in the reaction

Table I. *^{1}H Chemical shifts for the smallest RGase B oligosaccharide*

n.d., Not determined. –, Not present.

Unit	Proton	Chemical Shift (δ)					
		H-1	H-2	H-3	H-4	H-5	H-6
Rha	A_α	5.22	3.97	4.09	3.71	3.95	1.34
	A_β	4.94	4.06	n.d.	n.d.	n.d.	n.d.
GalA	B	5.08, 5.16[a]	3.94, 3.98[a]	4.13, 4.15[a]	4.43	4.63	–
Rha	C	5.32	4.32	4.08	3.62	3.85	1.29
us-GalA	D	5.13	3.80	4.34	5.81	–	–
Gal[b]		4.63	3.50	3.66	3.90	n.d.	n.d.

[a] Two values are for unit B linked to α and β forms of the reducing end unit, respectively. [b] Two residues, δ values differ by < 0.01 ppm.

Table II. *Lyase activity of RGase B toward various substrates (units mg^{-1}), determined from the increase in A_{235} using an extinction coefficient of 4800 M^{-1} cm^{-1}*

n.d., Not determined.

Substrate	pH 6[a]		pH 8[b]	
	−Ca	+Ca[c]	−Ca	+Ca[c]
MHR-S	8.8	9.8	10.6	11.8
Linear RG oligomers	3.9	n.d.	n.d.	n.d.
Pectin degree of carboxy-methoxylation 92.3%	0	0	0	0
Polygalacturonic acid	0	0	0	0

[a] 50 mM NaOAc, pH 6. [b] 20 mM Tris-HCl, pH 8. [c] 1 mM $CaCl_2$.

mixture or toward highly methoxylated pectin (the optimal substrate for pectin lyase; Voragen and Pilnik, 1989).

DISCUSSION

Homogalacturonan-cleaving enzymes comprise both hydrolases and lyases (Rombouts and Pilnik, 1980). Recently, a hydrolase specific for RG regions was described (Schols et al., 1990a). Here, we show that a lyase type of enzyme, with the same substrate specificity, also exists. The results show that the recently discovered RGase B from *A. aculeatus* is indeed an RGase and, moreover, that it is a lyase, specific for RGs, cleaving the linkage between Rha and GalA in the backbone and leaving an us-GalA at the nonreducing end and an Rha at the reducing end of the product. This is in contrast with RGase A (Schols et al., 1990a), which cleaves the linkage between a GalA and Rha in the backbone. A more specific nomenclature for the two RGases, RGase A and RGase B, is now necessary. Based on the linkage split and the cleavage mechanism, the name RG-lyase is suggested for RGase B. RGase A should then be named RG-hydrolase.

To our knowledge, no lyases with activity toward RG or hairy regions of pectin have been described in the literature. Filamentous fungi such as *A. aculeatus* more frequently produce pectin lyases (Rombouts and Pilnik, 1980) than pectate lyases. Okai and Gierschner (1991) reported the presence of five major isoenzymes of endo-polygalacturonase, as well as endo-pectin lyase and pectin-esterase in the commercial mixture Pectinex Ultra SP-L (Novo Nordisk Ferment, Dittingen, Switzerland), produced by *A. aculeatus*. The optimal pH (6) for RG-lyase is in the range reported for pectin lyases (between 4.9 and 6.5), whereas the optimum pH for pectate lyases is between 8.0 and 9.0 (Burns, 1991). RG-lyase has no absolute requirement for calcium ions as pectate lyases do, but calcium ions have a positive effect. The pI for RG-lyase (5.1) is in the range reported for pectin lyases (3.5–8.9), whereas most pectate lyases are basic proteins (Rombouts and Pilnik, 1980).

The discovery of new pectolytic enzymes like RG-lyase and, previously, RG-hydrolase (Schols et al., 1990a), RG-acetylesterase (Searle-van Leeuwen et al., 1992), and RG-rhamnohydrolase (Mutter et al., 1994) is very important with respect to the increasingly widely recognized function of polysaccharides as generators of signaling molecules, "oligosaccharins." There are indications that, in addition to homogalacturonic fragments, RG fragments are involved in plant processes, such as phytoalexin elicitation, wound signaling, hypersensitive response, morphogenesis, lignification, and ethylene synthesis (Aldington et al., 1991). The availability of well-characterized RG oligosaccharides, produced or modified by specific enzymes, will enable a more detailed investigation of the structure-activity relationships of these biologically active oligosaccharides.

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