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Rhamnogalacturonan α-L-Rhamnopyranohydrolase[1]

A Novel Enzyme Specific for the Terminal Nonreducing Rhamnosyl Unit in Rhamnogalacturonan Regions of Pectin

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Two α-L-rhamnohydrolases with different substrate specificities were isolated from a commercial preparation produced by *Aspergillus aculeatus*. The first rhamnohydrolase was active toward *p*-nitrophenyl-α-L-rhamnopyranoside, naringin, and hesperidin and was termed *p*-nitrophenyl-α-L-rhamnopyranohydrolase (pnp-rhamnohydrolase). From the data collected, the enzyme seemed specific for the α-1,2- or α-1,6-linkage to β-D-glucose. The pnp-rhamnohydrolase had a molecular mass of 87 kD (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), a pH optimum of 5.5 to 6, a temperature optimum of 60°C, and a specific activity toward pnp-α-L-rhamnopyranoside (pnp-Rha) of 13 units mg^{-1} protein. The second rhamnohydrolase, on the contrary, was active toward rhamnogalacturonan (RG) fragments, releasing Rha, and was therefore termed RG-rhamnohydrolase. The RG-rhamnohydrolase had a molecular mass of 84 kD, a pH optimum of 4, a temperature optimum of 60°C, and a specific activity toward RG oligomers of 60 units mg^{-1} protein. The RG-rhamnohydrolase liberated Rha from the nonreducing end of the RG chain and appeared specific for the α-1,4-linkage to α-D-galacturonic acid. The enzyme was hindered when this terminal Rha residue was substituted at the 4-position by a β-D-galactose. The results so far obtained did not indicate particular preference of the enzyme for low or high molecular mass RG fragments. From the results it can be concluded that a new enzyme, an RG α-L-rhamnopyranohydrolase, has been isolated with high specificity toward RG regions of pectin.

L-Rhamnosyl residues have been found as constituent sugars in the backbone of pectins in plant cell walls, in which 1,4-linked α-D-galacturonan chains are interrupted at intervals by the insertion of single 1,2-linked α-L-rhamnopyranosyl residues (Barrett and Northcote, 1965; Lau et al., 1985; Colquhoun et al., 1990; O'Neill et al., 1990). In smooth regions of pectin L-Rha residues are reported to occur once on every 25 galacturonic acid residues (Powell et al., 1982), once on every 70 residues (Konno et al., 1986), or once on every 72 to 100 residues (Thibault et al., 1993). In the ramified "hairy" regions of pectin L-Rha is part of the RG backbone, which consists of repeating units of the disaccharide α-(1,2)-L-Rha_p-α-(1,4)-D-Gal_pA (Lau et al., 1985; Colquhoun et al., 1990; Schols et al., 1990a; Schols and Voragen, 1994). The fine structure of hairy regions of pectin is the subject of investigation of several workers (O'Neill et al., 1990; Schols et al., 1990b; Schols and Voragen, 1994; Schols et al., 1994). In addition to chemical methods (Guillon and Thibault, 1989; Mort et al., 1991; Puvanesarajah et al., 1991), enzymes are becoming more important as analytical tools in structural studies because of their high specificity (Guillon et al., 1989; Voragen et al., 1993).

Various pectolytic enzymes, including pectin methylesterases and pectin depolymerases, active toward smooth regions of pectin, have been described (Rombouts and Pilnik, 1980; Pilnik and Voragen, 1993). These enzymes, however, have been shown not to be active toward hairy regions of pectin and in fact most of the studied hairy pectin fragments are released by treatment of cell wall material with these enzymes (O'Neill et al., 1990; Schols et al., 1990b, 1994). Schols et al. (1990a, 1994) described a novel type of enzyme, RGase, that is able to split the RG backbone of hairy regions isolated from the cell walls of different fruit and vegetable sources. RGase was found to liberate specific RG oligomers from the saponified hairy regions, having the basic structure α-L-Rha_p-(1,4)-α-D-Gal_pA-(1, 2)-α-L-Rha_p-(1,4)-α-D-Gal_pA (Colquhoun et al., 1990; Schols et al., 1994). In these oligomers, a β-D-Gal_p unit can be 4-linked to the terminal Rha_p residues or the 1,2-linked Rha_p residues. Searle-van Leeuwen et al. (1992) described a new type of acetylesterase that is specific for hairy regions of pectins. The RGase and acetylesterase were purified from a commercial enzyme mixture derived from *Aspergillus aculeatus*.

We have strong indications that, in analogy to the enzymic degradation of smooth regions, a whole array of enzymes is present in nature, specific for the degradation of hairy or RG

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Abbreviations: HPAEC, high-performance anion-exchange chromatography; HPSEC, high-performance size-exclusion chromatography; HTP, hydroxylapatite; MHR, modified hairy regions; MHR-S, saponified modified hairy regions; PG, polygalacturonase; pnp, *p*-nitrophenyl; pnp-Rha, pnp-α-L-rhamnopyranoside; pnp-rhamnohydrolase, pnp-Rha rhamnohydrolase; RG, rhamnogalacturonan; RGase, rhamnogalacturonase; RGmed, intermediate-sized fragments produced from MHR-S by RGase; RGoligo, low molecular mass fragments produced from MHR-S by RGase, consisting of rhamnogalacturonan oligomers; RGpoly, high molecular mass fragments produced from MHR-S by RGase.

regions of pectin. In the commercial enzyme preparation derived from *A. aculeatus*, we have found several of these specific enzymes. In this report we describe a new type of rhamnohydrolase, specific for the nonreducing terminal rhamnose in RG fragments. This enzyme is compared with a rhamnohydrolase from the same source, active toward other rhamnosides (pnp-Rha, naringin, and hesperidin). The importance of substrate modification for detecting new enzyme activities is demonstrated.

MATERIALS AND METHODS

Substrates

Preparation of MHR

MHR were isolated from apple liquefaction juice and were subsequently saponified (MHR-S) according to the method of Schols et al. (1990b), now using another batch of the experimental preparation Rapidase C600 (Gist-Brocades, Delft, The Netherlands) for liquefaction.

Preparation of RGase Degradation Products of MHR-S: RGpoly, RGmed, and RGoligo

MHR-S was degraded (1% [w/v], 24 h, 40°C, in 50 mM ammonium acetate buffer, pH 4.8) on a large scale by RGase (110 μg protein g^{-1} substrate) from *Aspergillus aculeatus* as purified by Schols et al. (1990a). The degradation products were separated on a column of Sephadex G50, as described by Schols et al. (1990a), using a volatile ammonium acetate buffer (50 mM pH 4.8). Fractions (5 mL) were assayed by automated colorimetric methods for uronic acids (Ahmed and Labavitch, 1977) and total neutral sugars (Tollier and Robin, 1979). The neutral sugar values were corrected for the contribution of the uronic acids in the orcinol assay. Fractions were analyzed by HPSEC and HPAEC as described below. Fractions were pooled as RGpoly (high molecular mass fragments), RGmed (intermediate-sized fragments), and RGoligo (structures of the major products in Table I). Pools were lyophilized several times to remove all buffer. RGpoly, RGmed, and RGoligo represented 51, 12, and 37%, respectively, of the total amount of sugars recovered after lyophilization.

Preparation of the RG Hexamer

A fraction containing the RG hexamer (Table I) was obtained by chromatography of the RGase digest of MHR-S on two Fractogel TSK HW-40 (S) columns (600 × 26 mm) in series using a flow rate of 2.5 mL min^{-1} and 0.1 M sodium acetate, pH 3.0, at 60°C. Fractions were screened on HPAEC as described below, and those containing the RG hexamer were pooled.

Preparation of Degalactosylated Substrates

RG substrates were incubated for 24 h at 40°C in 50 mM sodium acetate, pH 5, with β-galactosidase from *Aspergillus niger* (van de Vis et al., 1991) in amounts sufficient to remove all Gal residues from the substrate in 6 h. This resulted in degalactosylated substrates. The structures of the degalactosylated RG hexamer and the degalactosylated RG octamer are presented in Table I.

Table I. *Structure of the major RG oligomers: the branched hexamer (the RG hexamer) and the mixture of two branched octamers (the RG octamer), produced by RGase out of MHR-S, before and after degalactosylation*

Identification of oligomers was done as described by Schols et al. (1994).

RG hexamer:

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
↗
β-Gal$_p$-(1→4)

RC octamer (mixture of two):

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)-α-Gal$_p$A
↗
β-Gal$_p$-(1→4)

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)-α-Gal$_p$A
↗
β-Gal$_p$-(1→4)

Degalactosylated RG hexamer:

α-Rha$_p$-(1→4)-α-Gal$_p$A-(1→2)-α-Rha$_p$-(1→4)-α-Gal$_p$A

Degalactosylated RG octamer:

α-Rha$_p$-(1→4)-α-Gal$_p$A-(1→2)-α-Rha$_p$-(1→4)-α-Gal$_p$A-(1→2)-α-Rha$_p$-(1→4)-α-Gal$_p$A

Rhamnosides of Non-RG Origin

Other Rha-containing substrates were pnp-Rha, α-solanine, and α-chaconine (Sigma), and naringin and hesperidin (Fluka Chemie AG, Buchs, Switzerland).

Glycans and Glycosides for Side Activity Determination

Substrates used for screening of glycanase activities were CM-cellulose (Akucell AF-2805; Akzo, Arnhem, The Netherlands); xylan ex oat spelts (Sigma); soluble starch (Merck AG, Darmstadt, Germany); potato arabino-β-1,4-galactan (isolated from potato fiber according to the method of Labavitch et al., 1976); larchwood arabino-β-1,3/6-galactan ("stractan"; Meyhall Chemical AG, Kreuzlingen, Switzerland); a linear arabinan kindly provided by British Sugar (Peterborough, UK); high methoxyl pectin (prepared at our laboratory; degree of methylation, 92.3%); and polygalacturonic acid (ICN Biomedicals, Costa Mesa, CA).

The pnp-glycosides used for screening of glycosidase activities were obtained from Koch and Light, Ltd. (Haverhill, UK) and from Sigma: pnp-α-L-Ara_f, pnp-α-L-Ara_p, pnp-α-D-Gal_p, pnp-β-D-Gal_p, pnp-β-D-Gal_f, pnp-α-D-Xyl_p, pnp-β-D-Xyl_p, pnp-β-D-Man_p, pnp-α-D-Man_p, pnp-α-L-Fuc_p, pnp-β-D-Fuc_p, pnp-α-D-Glc_p, pnp-β-D-Glc_p, pnp-β-D-Glc_pA, and pnp-β-D-Gal_pA.

Enzyme Purification

Rhamnohydrolases were purified from the commercial preparation Pectinex Ultra SP-L produced by *A. aculeatus*, kindly provided by Novo Nordisk Ferment, Ltd. (Dittingen, Switzerland). Enzyme purification was carried out at 4°C. All buffers contained 0.01% (w/v) sodium azide to prevent microbial growth. Fractions collected were screened for protein content (A_{280} or the Sedmak method [Sedmak and Grossberg, 1977]), rhamnohydrolase activity toward RG oligomers (RG-rhamnohydrolase), and rhamnohydrolase activity toward pnp-Rha (pnp-rhamnohydrolase). Fractions containing these activities were pooled. Purification steps involved Bio-Gel P10, Bio-Gel HTP hydroxylapatite, and DEAE Bio-Gel A (Bio-Rad Laboratories, Richmond, CA) and MonoS HR 5/5, Superose 12 HR 10/30, and Superdex 75 XK 16/60 prep grade (Pharmacia LKB Biotechnology, Uppsala, Sweden). When using gradient elution, "peak control" was used to elute protein peaks with a minimum amount of contamination. This was done by maintaining the composition of the eluent at a fixed value during elution of the peaks. Concentration by ultrafiltration was done using a YM 30K membrane from Amicon Corp. (Danvers, MA). Further details are given in "Results" (Fig. 2).

Enzyme Assays

Enzyme activities were expressed as units: one unit corresponds to the release of 1 μmol Rha min^{-1} under standard conditions.

These conditions were 50 mM sodium acetate buffer (pH 5) and 40°C. Rhamnohydrolase activity was calculated from the release of Rha as determined by HPAEC. The release of *p*-nitrophenol from pnp-glycosides was measured spectrophotometrically at 405 nm, and activity was calculated using the molar extinction coefficient of 13,700 M^{-1} cm^{-1}.

Enzyme fractions were screened for contaminating activities by incubation for 30 min and 24 h with 0.25% (w/v) solutions of selected substrates for glycanase activities and 0.02% (w/v) solutions for glycosidase activities. Protein concentrations used in these experiments are described in "Results" (Table IV). The digests from the glycanase assays were analyzed by HPSEC and HPAEC.

Gel Electrophoresis

Electrophoresis was carried out with a PhastSystem (Pharmacia) according to the instructions of the supplier. The molecular mass was estimated by SDS-PAGE on a 10 to 15% polyacrylamide gel (Pharmacia). A low molecular mass kit (Pharmacia) from 14.4 to 94.0 kD was used for calibration. The isoelectric points were deduced from a pH 3 to 9 IEF gel using the standards from the broad isoelectric point calibration kit (Pharmacia). The gels were stained with Coomassie brilliant blue R-250.

Influence of pH, Temperature, and Buffer Salt Concentration

General substrate concentrations were 0.1% (w/v) degalactosylated RGoligo for the RG-rhamnohydrolase and 0.02% (w/v) pnp-Rha for the pnp-rhamnohydrolase. Incubations took place for 30 min, and preincubation (in stability experiments) occurred for 1 h at 40°C in 50 mM sodium acetate buffer (pH 5) unless mentioned otherwise.

The optimum pH for the rhamnohydrolases was determined using McIlvaine buffers (mixtures of 0.1 M citric acid and 0.2 M sodium hydrogenphosphate) in the pH range 3 to 8. Final protein concentrations were 0.16 μg mL^{-1} for the RG-rhamnohydrolase and 0.70 μg mL^{-1} for the pnp-rhamnohydrolase.

The stability of the enzymes at different pH values was measured using the same McIlvaine buffers as described above in which enzymes were preincubated. Final protein concentrations were 4.42 μg mL^{-1} for the RG-rhamnohydrolase and 4.86 μg mL^{-1} for the pnp-rhamnohydrolase. After preincubation an aliquot of the preincubated solution was added to substrate solution in sodium acetate (pH 5), resulting in a total buffer salt concentration of approximately 0.15 M for the RG-rhamnohydrolase and 0.2 M for the pnp-rhamnohydrolase. Final protein concentrations in incubation mixtures were 0.29 μg mL^{-1} for the RG-rhamnohydrolase and 1.87 μg mL^{-1} for the pnp-rhamnohydrolase.

The optimum temperature for the rhamnohydrolases was determined at different temperatures in the range 2 to 70°C. Final protein concentrations were 0.16 μg mL^{-1} for the RG-rhamnohydrolase and 0.35 μg mL^{-1} for the pnp-rhamnohydrolase. The stability of the enzymes at different temperatures was measured by preincubation of the enzymes in 50 mM sodium acetate (pH 5) at different temperatures. After cooling, substrate was added and incubation took place with final protein concentrations of 0.32 μg mL^{-1} for the RG-rhamnohydrolase and 0.70 μg mL^{-1} for the pnp-rhamnohydrolase.

The influence of buffer salt concentration on the activity

of the enzymes was determined by incubating enzymes in sodium acetate buffer (pH 5) with molarities in the range 50 mM to 1.2 M. Final protein concentrations were 0.18 $\mu g\ mL^{-1}$ for the RG-rhamnohydrolase and 2.81 $\mu g\ mL^{-1}$ for the pnp-rhamnohydrolase. The higher protein content for the pnp-rhamnohydrolase compared to the RG-rhamnohydrolase was necessary since the pnp-rhamnohydrolase was slowly inactivated during storage.

Analytical Methods

Sugar composition of MHR and other RG substrates was determined after methanolysis and subsequent hydrolysis with TFA as described by de Ruiter et al. (1992). The molecular mass distribution of substrates before and after enzyme treatment was determined by HPSEC as described by Schols et al. (1990b).

HPAEC was performed using a Dionex Bio-LC system (Sunnyvale, CA) equipped with a Dionex CarboPac PA-100 (4 × 250 mm) and a Dionex pulsed electrochemical detection detector in the pulsed amperometric detection mode. Rha was determined isocratically using 100 mM NaOH at a flow rate of 1 mL min^{-1}. RG oligomers, degradation products thereof, and digests of glycanase assays were analyzed with the same system using a gradient of sodium acetate in 100 mM NaOH as follows: 0 to 5 min, 0 mM; 5 to 35 min, 0 to 430 mM; 35 to 40 min, 430 to 1000 mM; 40 to 45 min, 1000 mM; 45 to 60 min, 0 mM.

RESULTS

Preparation and Characterization of RG Substrates

The freshly prepared apple MHR, used as a model for RG substrates, contained the same populations on HPSEC as the original MHR. The RGase degradation products had identical retention times on HPAEC as those obtained from the previously described MHR. The sugar compositions of MHR-S and RGase degradation products thereof (RGpoly, RGoligo, and the RG hexamer) are given in Table II. The only difference in MHR compared with the MHR isolated by Schols et al. (1990b) was a lower Ara content (20 compared to 55 mol%) and a higher Rha:GalA ratio (0.50 compared to 0.29). RGpoly contained the higher molecular mass degradation products and consisted of relatively low amounts of Rha and high amounts of Xyl and GalA. RGoligo and the RG hexamer consisted mainly of Rha, Gal, and GalA. The structure of the major RGase products, the (branched) RG hexamer and the (branched) RG octamer (mixture of two), is shown in Table I.

Table II. *Sugar composition (in mol%) of RG substrates: MHR-S and RGase degradation products thereof (RGpoly, RGoligo, and the RG hexamer)*

Sugar	MHR-S	RGpoly	RGoligo	RG hexamer
Rha	16	9	29	31
Ara	20	23	10	5
Xyl	11	19	2	3
Gal	18	13	27	24
Glc	2	3	3	4
Uronic acid	33	34	29	34

Table III. *Removal of Gal and GalA from RG substrates by the β-galactosidase from A. niger*

The data represent the percentages of sugar removed as compared with the amount of the sugar originally present in the substrate. This was determined using quantitative HPAEC.

Substrate	Gal Removed	GalA Removed
	%	%
MHR-S	40	1
RGpoly	12	3
RGoligo	84	4
RG hexamer	95	6

Degalactosylation of RG Substrates

The rhamnohydrolase with activity toward RG substrates appeared to be hindered by Gal side chains attached via a β-linkage to the 4-position of Rha. Therefore, RG substrates were treated with a β-galactosidase from *A. niger*. Effectivity of the treatment is presented in Table III for various substrates. A substantial amount (40%) of the Gal could be removed from MHR-S. This is in contrast with RGpoly, from which only 12% could be removed in this manner. Almost all Gal could be removed from RGoligo and from the RG hexamer. The removal of Gal from RGpoly and from RGoligo together accounted for 92% of the Gal removed from MHR-S (removal of Gal from RGmed was not investigated). Apparently the Gal residues in RGoligo were a suitable substrate for the β-galactosidase, in contrast with the polymeric fragments. Gal residues were released from substrates without degradation of the backbone, as indicated by HPSEC of incubation mixtures (results not shown).

Figure 1 shows HPAEC elution patterns of RGoligo and the RG hexamer before and after treatment with β-galactosidase. When another gradient was used, the peak eluting at 5 min in Figure 1 could be identified as Gal (results not shown). Furthermore, a small peak at 18 min, resulting from GalA, could be seen. Apparently the β-galactosidase contained traces of a GalA-releasing enzyme. The GalA signal was rather high compared with those of RGoligo. However, it is known that pulsed amperometric detection does not provide a uniform response to the same functional groups (Lee, 1990). Therefore, the peak areas do not give information about the molar ratio of monomers and oligomers. In Table III the percentage of GalA that was liberated is presented. The reaction products after GalA removal could not be recognized on HPAEC, probably because of the minor amounts of GalA released. The retention times of the newly formed RG oligomers without Gal side chains were reduced (from 23 min for the RG hexamer to 21 min for the degalactosylated RG hexamer; from 25 min for the RG octamer to 24 min for the degalactosylated RG octamer). The structures of the newly formed degalactosylated oligomers, previously described as oligomer 1 and 5, respectively, by Schols et al. (1994), is presented in Table I.

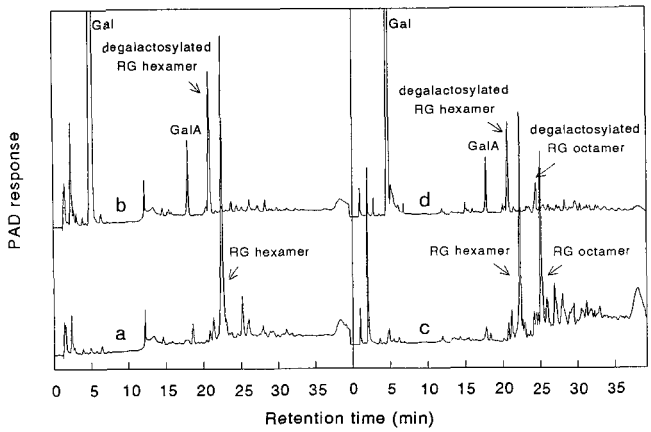

Figure 1. HPAEC of the RG hexamer (a), the degalactosylated RG hexamer (b), RGoligo (c), and degalactosylated RGoligo (d). Solutions of the RG hexamer were 0.03% (w/v) and of RGoligo 0.1% (w/v) (on a total sugar basis) in 50 mM sodium acetate buffer (pH 5.0). The structures of the RG oligomers after Schols et al. (1994) are presented in Table I. PAD, Pulsed amperometric detection.

Purification of Rhamnohydrolases

During purification of rhamnohydrolases from *A. aculeatus*, column fractions were screened toward pnp-Rha, which was intended to be the model substrate for rhamnohydrolase activity, and toward degalactosylated RGoligo, which was the actual substrate of interest. In early stages of purification, enzyme activities toward pnp-Rha and toward degalactosylated RGoligo were co-eluting (results not shown).

The purification scheme is given in Figure 2. Purification was commenced by desalting 60 mL of Pectinex Ultra SP-L on a Bio-Gel P10 column. Desalted protein (774 mg by Sedmak) was applied onto a DEAE Bio-Gel A column. After the column was washed, a sodium chloride gradient was used. Of the desalted material most of the protein was bound to the anion exchanger at pH 5. Both bound and unbound fractions contained rhamnohydrolase activity toward pnp-Rha as well as toward degalactosylated RGoligo.

Fractionation was continued with the unbound protein fractions (84 mg by Sedmak), which were pooled, concentrated by ultrafiltration, and applied onto the cation exchanger MonoS HR 5/5. After the column was washed, the protein was eluted with a sodium chloride gradient. Screening of column fractions revealed that the hydrolytic activities toward pnp-Rha and degalactosylated RGoligo resulted from different enzymes (Fig. 3). These two enzymes were given the preliminary names pnp-rhamnohydrolase and RG-rhamnohydrolase because of their apparent substrate specificity (as shown below). Enzyme fractions were pooled, and the purification of both enzymes was continued (see Fig. 2) and will be discussed separately below.

Purification of pnp-Rhamnohydrolase

Further purification of the pnp-rhamnohydrolase involved Superdex 75 prep grade to remove some low molecular mass protein material. The larger part of the protein did not bind

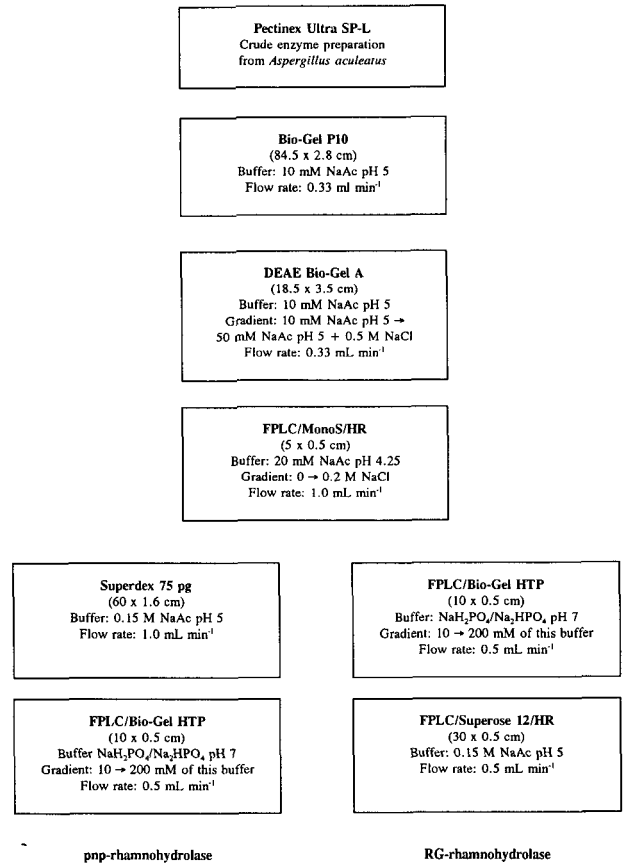

Figure 2. Scheme of the purification of the pnp-rhamnohydrolase and the RG-rhamnohydrolase from a crude preparation of *A. aculeatus*. FPLC, Fast protein liquid chromatography; NaAc, sodium acetate.

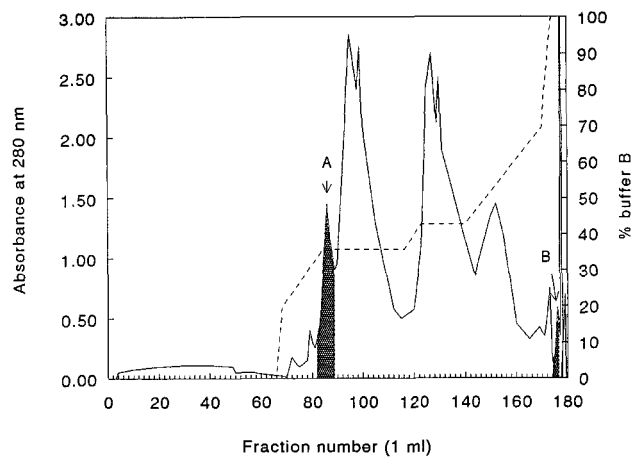

Figure 3. Chromatography of the unbound fraction from the DEAE Bio-Gel A column on a MonoS HR cation-exchange column, using a sodium chloride gradient from 0 to 0.2 M in 20 mM sodium acetate (pH 4.25; see Fig. 2). ——, A_{280}; - - - -, sodium chloride gradient (% buffer B); A, activity toward pnp-Rha:pnp-rhamnohydrolase; B, activity toward RGoligo:RG-rhamnohydrolase.

to the subsequent Bio-Gel HTP column, in contrast with the pnp-rhamnohydrolase. The pnp-rhamnohydrolase eluted at 60 mM sodium phosphate buffer. The protein in the HTP-bound fraction accounted for less than 1‰ in weight of the originally desalted protein (774 mg). Characterization of the pnp-rhamnohydrolase was performed using this HTP-bound fraction.

Purification of RG-Rhamnohydrolase

Further purification of the RG-rhamnohydrolase involved Bio-Gel HTP. The major part of the protein did not bind onto this column, in contrast with the RG-rhamnohydrolase. The RG-rhamnohydrolase eluted at 160 mM sodium phosphate buffer. Characterization of the RG-rhamnohydrolase was performed using this HTP-bound fraction, which also accounted for less than 1‰ of the originally desalted protein (774 mg).

An endo-PG was still present in the HTP-bound fraction of the RG-rhamnohydrolase. This endo-PG could be separated from the RG-rhamnohydrolase using gel filtration chromatography (Superose 12, Fig. 2). Endo-PGs of various sources were not active toward hairy regions or RG oligomers (Schols et al., 1990b; Mutter et al., 1993).

A disturbing factor in the purification and characterization of the RG-rhamnohydrolase was the presence of a co-eluting GalA-releasing enzyme with activity toward RGoligo. No (satisfactory) separation of RG-rhamnohydrolase and the GalA-releasing enzyme could be obtained using ion-exchange chromatography at different pH values (using a salt gradient) or using a pH gradient. Gel filtration chromatography, chromatofocusing, and several types of affinity chromatography were also not effective. The separation of the two enzymes, with apparently similar isoelectric points and molecular masses, is currently under investigation. Although the GalA-releasing enzyme was still co-eluting with the RG-rhamnohydrolase on Bio-Gel HTP, the former was inactivated by the sodium phosphate buffer at pH 7 during separation and storage of the enzyme fractions afterward. For the reasons described above the HTP-bound RG-rhamnohydrolase could be used for characterization experiments.

Characterization of pnp-Rhamnohydrolase

The molecular mass of the purified pnp-rhamnohydrolase was estimated using a Superose 12 column. A calibration curve was made with standard proteins in the range 45 to 450 kD. The pnp-rhamnohydrolase eluted in the range 80 to 90 kD. The major protein band using SDS-PAGE was found at 87 kD (Fig. 4), presumably originating from the pnp-rhamnohydrolase. Two other bands were found at 67 and 49 kD. The isoelectric point of the major enzyme band in IEF was 4.8 (Fig. 4). The enzyme was optimally active at pH 5.5 to 6 in McIlvaine buffers and at 60°C in sodium acetate buffer. Sodium acetate concentrations (at pH 5) above 0.3 M strongly inhibited the enzyme: at 0.44 M only 15% of the activity at 50 mM was left. The pnp-rhamnohydrolase was stable for 1 h in 50 mM sodium acetate buffer (pH 5) up to temperatures of 50°C. Incubation for 30 min at 40°C in McIlvaine buffer (pH 5) caused a decrease in activity of about 50%. The conductivity of the McIlvaine buffer at pH 5 was equal to that of a 0.3 M sodium acetate buffer (pH 5). This suggests that the inactivation was caused primarily by the difference in ionic species and not in ionic strength. When the enzyme was also preincubated for 1 h in this McIlvaine buffer, no significant further inactivation was observed. No influence of the pH on stability was observed: the enzyme appeared equally stable in the pH range 3 to 8. The pnp-rhamnohydrolase was tested on several substrates for other activities (Table IV). Next to an endo-PG, only traces of a few other activities were present. At the optimum temperature of 60°C in 50 mM sodium acetate buffer (pH 5) an activity of 13 units mg^{-1} protein toward pnp-Rha was found for the pnp-rhamnohydrolase.

Characterization of RG-Rhamnohydrolase

By gel filtration using a Superose 12 column the RG-rhamnohydrolase activity eluted in the range 80 to 90 kD. In Figure 4 SDS-PAGE of the MonoS-bound and the HTP-bound RG-rhamnohydrolase is shown. The purified RG-rhamnohydrolase showed a major band on SDS-PAGE with a molecular mass of 84 kD, presumably originating from the RG-rhamnohydrolase, and a minor band of 71 kD. The endo-PG that was present in the HTP-bound RG-rhamnohydrolase (as mentioned above) showed a longer retention time using gel filtration chromatography. This enzyme was presumably responsible for the protein band occurring at 71 kD upon SDS-PAGE. Molecular masses for endo-PGs near this value are reported in the literature (Rombouts and Pilnik, 1980). Because of a shortage of enzyme fraction, this gel filtration experiment could not be repeated on a larger scale. With IEF (Fig. 4) three bands in the isoelectric point range of 4.9 to 5.4 were found for the HTP-bound RG-rhamnohydrolase.

The RG-rhamnohydrolase was optimally active at pH 4 in McIlvaine buffers and at 60°C in sodium acetate buffer. Sodium acetate concentrations above 0.1 M slowly inactivated the enzyme: at 0.5 M only 33% and at 1.1 M only 10% of the activity at 50 mM was left. The enzyme was stable for 1 h up to temperatures of 60°C in 50 mM sodium acetate buffer (pH 5). Similarly to the pnp-rhamnohydrolase, measuring the activity in McIlvaine buffer (pH 5) for 30 min caused a decrease in activity of about 50%. This was probably partly due to the higher conductivity of the mixture compared with 50 mM sodium acetate buffer (pH 5). Preincubation in McIlvaine buffers for 1 h caused a decrease in activity of 15 to 20%. No influence of the pH was observed: the enzyme seemed equally stable in the pH range 3 to 8. At the optimum temperature of 60°C in 50 mM sodium acetate buffer (pH 5) an activity of 60 units mg^{-1} protein toward degalactosylated RGoligo was found for the RG-rhamnohydrolase. Side activities next to the two enzymes mentioned above were very few, as shown in Table IV.

Comparison of pnp-Rhamnohydrolase and RG-Rhamnohydrolase

For further characterization the pnp-rhamnohydrolase and RG-rhamnohydrolase were incubated with several Rha-con-

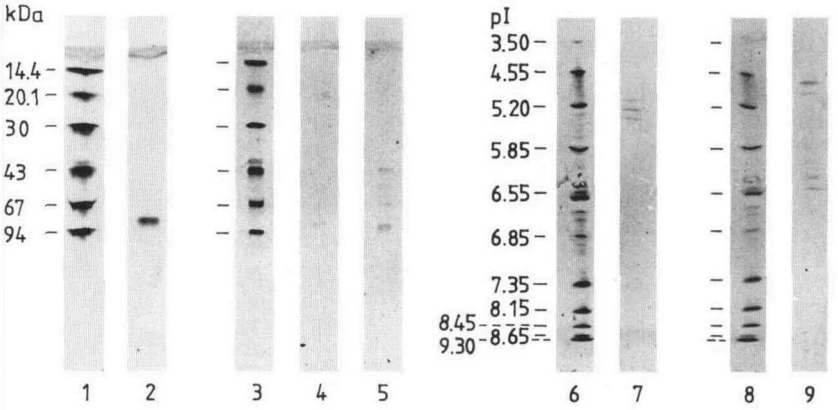

Figure 4. SDS-PAGE, Coomassie staining. Lane 1, Molecular mass standards; lane 2, MonoS-bound RG-rhamnohydrolase; lane 3, molecular mass standards; lane 4, HTP-bound RG-rhamnohydrolase; lane 5, HTP-bound pnp-rhamnohydrolase. IEF, Coomassie staining. Lane 6, Broad isoelectric point standards; lane 7, HTP-bound RG-rhamnohydrolase; lane 8, broad isoelectric point standards; lane 9, HTP-bound pnp-rhamnohydrolase.

taining substrates (Table V). Preliminary experiments showed that a concentration of 300 μM terminal, nonreducing, unbranched Rha was sufficient for the enzymes (results not shown) to have first-order kinetics under the conditions described in Table V. From Table V it can be seen that the pnp-rhamnohydrolase was also active toward naringin and hesperidin. It was not active toward α-solanine or α-chaconine or any of the RG substrates. From the data collected, the pnp-rhamnohydrolase seemed specific for the α-1,2- or α-1,6-linkage to β-D-Glc. One of the two Rha units in α-chaconine is also α-1,2-linked to β-D-Glc, but the other α-linked rhamnosyl residue in α-chaconine is linked to position-4 of the same glucosyl residue (Schreiber, 1968). This might be the reason the enzyme is not active. The activity of the pnp-rhamnohydrolase toward pnp-Rha was lower than found in previous experiments (e.g. 13 units mg^{-1} as mentioned above). The enzyme was found to be slowly inactivated during storage in 0.1 M sodium acetate buffer (pH 5). No reasonable explanation could be given for this observation, since the stability experiments in sodium acetate buffer did not indicate inactivation.

The RG-rhamnohydrolase, on the other hand, was only active toward the RG substrates. Therefore, the enzyme seemed to be specific for the terminal, nonreducing Rha α-1,4-linked to GalA. The RG-rhamnohydrolase appeared to be hindered by the Gal side chains attached via a β-1,4-linkage to Rha. The activity of the enzyme toward degalactosylated substrates is shown in Table V. This table shows very clearly that removal of Gal increased the amount of available substrate for the RG-rhamnohydrolase: activity increased almost 5-fold for MHR-S and 8- to 10-fold for RGpoly, RGoligo, and the RG hexamer. The amount of Rha released in 24 h confirmed that more Rha could be released from the substrate after treatment with β-galactosidase.

The RG-rhamnohydrolase activity toward the various RG substrates (oligomers versus polymers) was of the same order of magnitude: 20 to 60 units mg^{-1} (Table V). No degradation of the backbone occurred upon Rha release, as indicated by HPSEC of incubation mixtures (results not shown). It is interesting to note that the activities toward the degalactosylated RG hexamer and degalactosylated MHR-S were both high (52–58 units mg^{-1}), although the difference in molecular mass is substantial. The same applied for degalactosylated RGoligo and degalactosylated RGpoly (24–33 units mg^{-1}). These results suggest that the RG-rhamnohydrolase had no preference for high or low molecular mass substrates.

Since the nonreducing end in RG oligomers, consists of Rha, the RG-rhamnohydrolase acted from the nonreducing end. In Figure 5 the degalactosylated RG hexamer and degalactosylated RGoligo are shown before and after incubations (for 0.5 and 12 h) with the RG-rhamnohydrolase. The released Rha eluted at 3 min. The peak at 18 min resulted

Table IV. *Side activities of pnp-rhamnohydrolase and RG-rhamnohydrolase*

++, Activity visible after 30 min of incubation; +, activity visible after 24 h of incubation; −, activity not visible after 24 h of incubation. Reaction mixture: 0.25 % (w/v) substrate solution for polymeric substrates and 0.02 % (w/v) substrate solution for pnp-glycosides in 50 mM sodium acetate buffer (pH 5.0) at 40°C; final protein concentrations for 30-min incubation: 0.19 μg mL^{-1} for pnp-rhamnohydrolase and 0.18 μg mL^{-1} for RG-rhamnohydrolase; for 24-h incubation 1.87 and 1.77 μg mL^{-1}, respectively.

Substrate	pnp-Rhamnohydrolase	RG-Rhamnohydrolase
CM-cellulose	−	−
Xylan	−	+
Starch	−	−
Galactan	−	−
Stractan	−	−
Arabinan	−	−
High-methoxyl pectin	+	+
Polygalacturonic acid	++	++
pnp-α-L-Ara_f	−	−
pnp-α-L-Ara_p	−	−
pnp-α-D-Gal_p	+	+
pnp-β-D-Gal_p	+	−
pnp-β-D-Gal_f	−	−
pnp-α-D-Xyl_p	+	−
pnp-β-D-Xyl_p	−	−
pnp-β-D-Man_p	−	−
pnp-α-D-Man_p	−	−
pnp-α-L-Fuc_p	−	−
pnp-β-D-Fuc_p	−	−
pnp-α-D-Glc_p	−	−
pnp-β-D-Glc_p	+	−
pnp-β-D-Glc_pA	+	−
pnp-β-D-Gal_pA	−	−

Table V. *Action of pnp-rhamnohydrolase and RG-rhamnohydrolase on various Rha-containing substrates (Rha release determined by HPAEC)*

Reaction mixture: substrate concentration adjusted to be at least 300 μM terminal, nonreducing, unbranched Rha for pnp-Rha, naringin, hesperidin, α-solanin, α-chaconin, and degalactosylated substrates in 50 mM sodium acetate buffer (pH 5.0) at 40°C. Final protein concentrations for 30-min incubation: 0.19 μg mL^{-1} for pnp-rhamnohydrolase and 0.18 μg mL^{-1} for RG-rhamnohydrolase; for 24 h incubation: 1.87 and 1.77 μg mL^{-1}, respectively (24 h was not end point in the incubation, results not shown).

Substrate	Type of Linkage Involving Rha	pnp-Rhamnohydrolase		RG-Rhamnohydrolase	
		Activity	Rha released from total in 24 h	Activity	Rha released from total in 24 h
		units mg^{-1}	%	*units* mg^{-1}	%
pnp-Rha	α-1	2.3	88.0	0	0
Naringin	α-1,2 to β-D-Glc_p	2.2	68.9	0	0
Hesperidin	α-1,6 to β-D-Glc_p	2.2	62.9	0	0
α-Solanin	α-1,2 to β-D-Gal_p	0	0	0	0
α-Chaconine	α-1,2 and α-1,4 to β-D-Glc_p	0	0	0	0
RGoligo	α-1,4 to α-D-Gal_pA	0	0	(3.2)[a]	1.3
degal.[b] RGoligo	α-1,4 to α-D-Gal_pA	0	0	32.9	21.0
RG hexamer	α-1,4 to α-D-Gal_pA	0	0	(6.1)	3.0
degal. RG hexamer	α-1,4 to α-D-Gal_pA	0	0	52.6	31.1
MHR	α-1,4 to α-D-Gal_pA	0	0	(2.5)	0.1
MHR-S	α-1,4 to α-D-Gal_pA	0	0	(12.9)	0.3
degal. MHR-S	α-1,4 to α-D-Gal_pA	0	0	57.6	1.3
RGpoly	α-1,4 to α-D-Gal_pA	0	0	(3.0)	0.1
degal. RGpoly	α-1,4 to α-D-Gal_pA	0	0	24.3	4.4

[a] For RG substrates that were not degalactosylated the calculated activities might be underestimated because of insufficient substrate; therefore, data are presented in parentheses. [b] degal., Degalactosylated.

from GalA, which was released by the β-galactosidase treatment as mentioned before. Since Rha was the only monomer released, the apparent structures of the newly formed RG oligomers, a trimer and pentamer, are: α-Gal_pA-(1→2)-α-Rha_p-(1→4)-α-Gal_pA and α-Gal_pA-(1→2)-α-Rha_p-(1→4)-α-Gal_pA-(1→2)-α-Rha_p-(1→4)-α-Gal_pA, respectively. These newly formed oligomers were retarded more on the CarboPac PA-100 column than the tetramer and hexamer from which they originated. This behavior with regard to the degree of polymerization of the molecules was also observed for the dimer α-(1,2)-L-Rha_p-α-(1,4)-D-Gal_pA, which eluted earlier from the column than monoGalA (Schols et al., 1994). Again, it must be noted that the difference in response factor between GalA and the tetramer and pentamer gives rise to misinterpretation of the molar ratios of monomers and oligomers.

DISCUSSION

Two α-L-rhamnohydrolases with entirely different substrate specificities were isolated. The first one, the pnp-rhamnohydrolase, is an α-L-rhamnopyranohydrolase active toward pnp-Rha, naringin, and hesperidin. Pnp-Rha is used as a model substrate for rhamnohydrolase activity (Romero et al., 1985). Naringin is a bitter flavanone glycoside present in citrus juices, and hesperidin is a nonbitter flavanone glycoside in citrus (Chase, 1974). No reports could be found in which one enzyme was mentioned to be active toward all three substrates. More than one band was found on SDS-PAGE and IEF for the pnp-rhamnohydrolase. Therefore, it is possible that the observed activities are the result of more than one enzyme. Rhamnohydrolases that were active toward both naringin and pnp-Rha were described from *Penicillium* spp. (Romero et al., 1985; Hsieh and Tsen, 1991), from *Corticium rolfsii* (Chase, 1974), and from *A. niger* (Hsieh and Tsen, 1991; molecular mass 90 kD, optimum pH 4.5, optimum temperature 55–65°C, broad pH stability range). Gunata et al. (1988) mentioned an α-L-rhamnopyranosidase from *Penicillium* spp. that was active toward both pnp substrates and grape monoterpenyl disaccharide-glycosides (Rha α-1,6-linked to β-D-Glc). Rhamnohydrolases from *A. niger* active toward naringin were described by Ono et al. (1977) (optimum pH 4.5, optimum temperature 50°C) and Park and Chang (1979) (optimum pH 5, optimum temperature 40°C). Naringinases were reported to be isolated from celery seeds and leaves of the shaddock (Chase, 1974). Nothing was mentioned in the latter reports about activity of the enzymes toward pnp-Rha or hesperidin. Separate enzymes hydrolyzing naringin and hesperidin were reported from *A. niger* (Chase, 1974; pH and temperature optima of pH 4.5, 50°C and pH 3.5, 60°C, respectively). A hesperidinase from *A. niger* was reported (Sanchez et al., 1987), but again activity toward pnp-Rha or naringin was not mentioned. Bushway et al. (1988, 1990) described rhamnohydrolase activities that were able to liberate the Rha units from both α-solanine and α-chaconine. Both α-solanine and α-chaconine are glycoal-

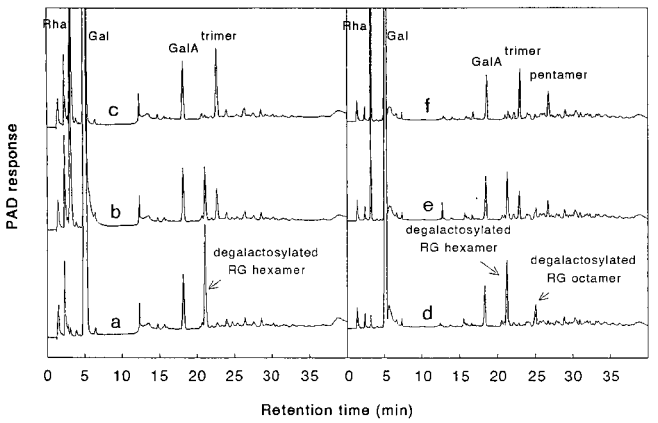

Figure 5. HPAEC of the degalactosylated RG hexamer (a), the degalactosylated RG hexamer after 0.5 h of incubation with RG-rhamnohydrolase (b), the degalactosylated RG hexamer after 12 h of incubation with RG-rhamnohydrolase (c), degalactosylated RGoligo (d), degalactosylated RGoligo after 0.5 h of incubation with RG-rhamnohydrolase (e), and degalactosylated RGoligo after 12 h of incubation with RG-rhamnohydrolase (f). Solutions of the RG hexamer were 0.03% (w/v) and of RGoligo 0.1% (w/v) (on total sugar basis) in 50 mM sodium acetate buffer (pH 5.0). The structures of the RG hexamer, the RG octamer, the degalactosylated RG hexamer, and degalactosylated RG octamer are presented in Table I. The suggested structures of the newly formed oligomers from the degalactosylated RG hexamer and octamer, a trimer and pentamer, respectively, are: α-Gal_pA-(1→2)-α-Rha_p-(1→4)-α-Gal_pA and α-Gal_pA-(1→2)-α-Rha_p-(1→4)-α-Gal_pA-(1→2)-α-Rha_p-(1→4)-α-Gal_pA. PAD, Pulsed amperometric detection.

kaloids present in potato (*Solanum tuberosum*) and work synergistically in their antifungal activity (Fewell and Roddick, 1993). For α-chaconine they observed a difference in hydrolysis of the different Rha units in α-chaconine, depending on the incubation temperature (Bushway et al., 1988). The enzyme preparation used, however, was not purified and nothing was mentioned about activity toward other Rha-containing substrates.

From the results it can be concluded that the second enzyme that was identified is a new enzyme, which is highly specific for RG regions of pectin. So far, the RG-rhamnohydrolase seems equally active toward low and high molecular mass RG fragments. The systematical name for the enzyme should be RG α-L-rhamnopyranohydrolase, abbreviated RG-rhamnohydrolase. The results show that the enzyme splits off the terminal, nonreducing Rha from RG fragments. The galactosyl residue β-1,4-linked to the terminal nonreducing Rha prevents the action of the enzyme on this rhamnosyl residue. The degalactosylation of the RG substrates was, therefore, important for the recognition and characterization of the enzyme. Furthermore, the RG-rhamnohydrolase was only able to continue its action after subsequent removal of GalA (results not shown). Therefore, the enzyme can be classified as an exo-enzyme, in contrast with the endo-action of an enzyme like RGase (Schols et al., 1990a). The purified RG-rhamnohydrolase represented only minor amounts of the total enzyme preparation. It is very likely that this type of enzyme activity is limiting in the total degradation of pectic hairy regions to monomers (e.g. for the synthesis of sequestrants out of GalA). Furthermore, a set of RG glycohydrolases can be of great value in the structural characterization of RG fragments.

Few publications deal with the enzymatic degradation of hairy regions of pectin. The degradation of the backbone of the hairy regions of fruit and vegetables by RGase was described by Schols et al. (1990a, 1990b, 1994) and Schols and Voragen (1994). Matsuhashi et al. (1992) and Düsterhöft et al. (1993) found indications that similar enzymes able to degrade the RG backbone are present in the enzyme preparation Driselase from *Irpex lacteus*. Guillon et al. (1989) used enzymes able to degrade the "hairs" of the hairy regions of beet pectins: the arabinan and galactan side chains attached to Rha.

In further research we will focus on purification of RG-rhamnohydrolases from *A. aculeatus* in larger quantities and on a more detailed characterization of the pattern of action of the enzymes. Synergism with other enzymes like the GalA-releasing enzyme or backbone-degrading enzymes active toward hairy regions will also be studied.

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