New rhamnogalacturonan degrading enzymes from *Aspergillus aculeatus*

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Nieuwe rhamnogalacturonaan afbrekende enzymen uit *Aspergillus aculeatus*

Proefschrift
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ABSTRACT

Three new rhamnogalacturonan degrading enzymes were purified from a commercial enzyme preparation, Pectinex Ultra SP, produced by the fungus *Aspergillus aculeatus*. Pectinex Ultra SP is industrially used in the mash treatment of apples and pears in juice production, increasing juice yield. Rhamnogalacturonans are highly branched polysaccharides that are part of the pectin network in the plant cell wall. The purified enzymes were characterized and appeared to be only active toward rhamnogalacturonan and not toward the well-known pectic polysaccharide homogalacturonan. Rhamnogalacturonan rhamnohydrolase is able to remove the terminal nonreducing L-rhamnose residues which are α-(1,4)-linked to D-galacturonic acid residues in rhamnogalacturonans. Rhamnogalacturonan galacturonohydrolase is an enzyme able to remove the terminal nonreducing D-galacturonic acid residues which are α-(1,2)-linked to L-rhamnose residues in rhamnogalacturonans. Both enzymes were essential analytical tools in the study of the mode of action of rhamnogalacturonan hydrolase and a third new enzyme, rhamnogalacturonan lyase, toward linear rhamnogalacturonan oligosaccharides. While rhamnogalacturonan hydrolase cleaves α-D-galacturonic acid-(1,2)-L-rhamnose linkages by hydrolysis, rhamnogalacturonan lyase cleaves the α-L-rhamnose-(1,4)-D-galacturonic acid linkages by β-elimination. Both enzymes act in an endo-fashion, with a degree of multiple attack of 4 and 2.5 respectively toward modified hairy regions of apple. From the degree of multiple attack of these endo-enzymes, combined with information on the mode of action toward linear rhamnogalacturonan oligosaccharides, it could be estimated that the average length of rhamnogalacturonan regions in modified hairy regions of apple is at least 29 sugar residues.

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Stellingen

1. Het gebruik van p-nitrophenylglycosides als substraat voor het opsporen van glycosidase-activiteit is geen garantie dat hiermee alle glycosidases gevonden worden.
   Dit proefschrift, hoofdstuk 2.

2. Omdat er enzymen zijn waarvoor geen geschikte screeningstests bestaan, die essentieel zijn voor de "expression cloning" methode *(H Dalbøge & HP Heldt-Hansen, Mol Gen Genet 243 (1994) 253-260)*, is conventionele enzymzuivering nodig om van dergelijke enzymen de genetische code te kunnen bepalen.
   Dit proefschrift, hoofdstukken 2 en 6.

3. De enorme hoeveelheid werk, nodig voor het opzuiveren van een nieuw enzym, staat in schrille tegenstelling met het beetje tekst dat nodig is om de zuivering te beschrijven in een publicatie.
   Dit proefschrift, hoofdstukken 2 en 6.

4. Uit de structuur van de rhamnogalacturonaan-oligomeren die met behulp van Driselase uit bamboe celwandmateriaal werden verkregen *(T Ishii, Mokuzai Gakkaushi 41 (1995) 561-572)* blijkt dat Driselase, geproduceerd door de schimmel *Irpex lacteus*, zowel een RG-hydrolase als een RG-rhamnohydrolase bevat.

5. Het is volkomen onduidelijk waarom er voor de produktie van Δ-(4,5)-onverzadigd-galacturonzuur-(1,2)-rhamnose disacchariden uit rhamnogalacturonaan twee enzymen, een lyase en een "endorhamnosidase", nodig zouden zijn, zoals gesuggereerd door *SC Fry, S Aldington, PR Hetherington & J Aitken, Plant Physiol 103 (1993) 1-5.*

6. Een zuiver enzym is een relatief begrip zolang de technieken om de zuiverheid te bepalen steeds gevoeliger worden.

7. "Wat de boer niet kent, dat eet hij niet" is soms ook van toepassing op wetenschappers, als ze als referenten een methode moeten beoordelen die ze niet kennen.

8. Wie een niet gebaande weg inslaat mag wel een reservetank meenemen.

9. In de publieke discussie over zorgverdeling wordt de optie dat de vader de hoofdzorg voor kinderen en huishouden op zich neemt ten onrechte buiten beschouwing gelaten.

10. Een maatschappij die geen plaats heeft voor minder begaafden of minder validen heeft geestelijk niets te bieden.
"Unnatural selection", Time, 22 september 1997

11. Het invoeren van een korte siësta op een werkdag zou de productiviteit van een werknemer die veel denkwerk verricht enorm verhogen.

12. In Afghanistan, waar vrouwen van de fundamentalistische heersers steeds minder rechten krijgen, is sprake van ernstige schending van de rechten van de mens.
"Taliban weren vrouwen Kabul uit ziekenhuizen", De Volkskrant, 25 oktober 1997.

13. Het krijgen en opvoeden van kinderen in combinatie met het doen van promotie-onderzoek is een waardevolle managementservaring.

Stellingen behorende bij het proefschrift

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Margien Mutter
Wageningen, 15 december 1997

Voorwoord

Welkom, lezer van dit proefschrift, op - zeer waarschijnlijk - de allereerste pagina van het binnenwerk dat u daadwerkelijk leest.
Promoveren is een pittig project waar veel mensen bij betrokken zijn. Mijn collega's hebben er door hun aanwezigheid, hulp, interesse, steun en humor, op het werkvlak zowel als op het privé-vlak, voor gezorgd dat ik een goede tijd heb gehad en heel wat wijzer ben geworden. Naar de volgende mensen gaat mijn welgemeende dank met name uit.
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Ik heb van mijn promotie-periode een behoorlijk avontuur gemaakt, door naast de bevalling die het schrijven van een proefschrift vormt, nog drie "echte" bevallingen

door te maken, met alle levende consequenties van dien natuurlijk. Dit was een spannende, prachtige, en onvermijdelijk ook vermoeiende combinatie. Toen ik aankondigde dat er een tweede op komst was, toevallig in de tijd dat ik met mijn tweede artikel bezig was, rees bij sommigen het vermoeden dat ik voor elk geschreven artikel nu ook een kind zou krijgen! Hoewel het daar wel even op leek, toen er ook nog een derde kind achteraan kwam, kan ik u verder geruststellen.

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Aan mijn ouders

Aan Roland, Myrthe, Linde en Tijmen

Contents

Chapter 1

General Introduction

FRUIT AND VEGETABLE PROCESSING

First introduced in the 1930's, exogenous enzymes are now applied worldwide in industrial fruit and vegetable processing (Omran et al., 1986). The action of enzymes provides a natural, low cost processing aid, as alternative or improvement for traditional methods. Moreover, the use of enzymes enabled the development of completely new processes (e.g. liquefaction and maceration) and therewith new products. The enzymes used are commonly mixtures as present in culture liquids derived from nonpathogenic fungi like *Aspergillus*, and therefore generally regarded as safe (GRAS).

Fruit and vegetables also contain endogenous enzymes, and, depending on the process, their action can have desirable and undesirable consequences. Endogenous pectin methylesterases for instance play an important role in citrus processing, where they cause cloud loss due to the precipitation of enzymically de-esterified pectin with calcium ions (Voragen and Pilnik, 1989). Although desirable for lemon and lime juices, this is undesirable in the production of orange juice. Heat inactivation of pectin methylesterase causes flavor loss, and therefore *cloud stabilization* of orange juice can for instance be achieved by adding exogenous polygalacturonase which degrades the low ester pectin formed, before it can coagulate with calcium.

The oldest and still the largest use of pectinases is fruit juice *clarification*, applied mainly to deciduous juices (e.g. apple) and grape juice (Pilnik and Voragen, 1993). Traditionally the fruit was crushed and the pulp pressed. The resulting raw press juice is thought to contain a persistent cloud of cell wall fragments and complexes, consisting of positively charged cytoplasmatic protein cores with an outer layer of negatively charged pectin chains. Electrostatic repulsion stabilizes the cloud. When pectinases are added as processing aids, e.g. polygalacturonase + pectin methylesterase or pectin lyase alone (depending on the degree of esterification of the pectin), the pectin chains are degraded and the thereby exposed positively charged protein cores can then aggregate with oppositely charged particles to form larger aggregates. Filtration or centrifugation then yields a clear juice.

A further development was the introduction of a process called *pulp enzyming*, also referred to as *Maische Fermentierung* and *optimized mash enzymation*. Similar pectinases as used for clarification were in this case added to the pulp of soft fruits e.g. black currants, cherries, strawberries, raspberries, and

bananas (Voragen et al., 1992). Traditional pulping of these fruits, that contain much soluble pectin, results in a semi-gelled mass that is difficult to press. Addition of pectolytic enzymes to the pulp results in degradation of the pectins responsible for gelling, and therewith enables better pressing and higher juice yields. This procedure was also successfully used for grapes and overripe, stored apples, where the soluble pectin content has increased and the same problem exist as with the soft fruits. Enzyme treatment of the pulp of olives, palm fruit and coconut, to increase the oil yield, has also been described (Pilnik and Voragen, 1993).

A process named *maceration* was developed for the production of cloud stable, viscous, and pulpy drinks called nectars, prepared from e.g. pears, peaches, apricots, berries, guava, papaya and passion fruit (Rombouts and Pilnik, 1986). By using the enzyme polygalacturonase, pectin lyase, or pectate lyase (the latter enzyme in case of vegetables, that have a higher pH than fruits), restricted degradation solubilizes the middle lamella pectin, resulting in a suspension of loose cells, which are rather well intact. Vegetable purees with high contents in soluble solids, pigments, and vitamins (e.g. β-carotene in carrot) can be obtained, which are applied in baby foods and as base material for cloudy vegetable juices. Endogenous pectin methylesterase has to be inactivated to prevent further degradation of the pectin which would turn the process into to pulp enzyming technology (Pilnik and Voragen, 1993).

Through the combined action of pectolytic and cellulolytic enzymes, plant cell walls can be degraded thus far, that they cannot withstand the osmotic pressure from inside anymore, resulting in cell wall collapse and juice release without pressing. This process is called *liquefaction*, and results in very high yields (Pilnik and Voragen, 1993). Liquefaction is a very good alternative for processing of vegetables and fruits that yield no juice on pressing, or for which no processes have been developed yet, like mango, guava, and bananas. However, in Europe no specific cellulase preparations are allowed in the process, so enzyme preparations with high cellulase side activities have to be used (Stutz, 1996). Furthermore the increased acidity of the juice, due to the release of GalA by the liquefying enzymes, can be a problem, since it usually is higher than the legally allowed acidity. Several technical problems were also hard to overcome. However, the liquefaction process is currently successfully used by apple juice producing companies in North and South America, and by companies in Europe that produce juice for export to those countries (Stutz, 1996). In Europe, a process called *pomace liquefaction* is preferred. This process involves traditional pressing, resulting in a juice A and pomace. Subsequently water is added to the pomace, which is then liquefied, resulting in juice B. Beside the advantage of allowing the producer to use his traditional presses, yields of 100% and more are possible with this method, and the availability of two different juices allows the producer to target specific markets. Pomace liquefaction is currently used for instance in the production of apple juice (Grassin and Fauquembergue, 1993) and pineapple juice (Sreenath et al., 1994).

The introduction of this new enzyme technology obviously has introduced new problems. In concentrates of clear apple juice, made using the liquefaction process, haze appeared, which was shown to consist of crystallized linear arabinan chains

(Pilnik and Voragen, 1993). The enzyme preparation used in the process originally released branched arabinans, then debranched them (by arabinofuranosidases), but did not contain enzymes able to further degrade the linear arabinans. The solution to this problem evidently was supplementation of the used enzyme preparation with endo-arabinanases that could degrade the linear haze-forming arabinans. This is a typical result from the major drawback of the current enzyme applications, i.e. the use of ill-defined enzyme mixtures. By selecting particular microorganisms and growth conditions, several major enzyme activities are usually obtained, beside many unknown side activities. However, progress has been made with the expression cloning of fungal enzyme genes (Dalbøge and Heldt-Hansen, 1994), enabling the large-scale production of single enzymes by genetically manipulated microorganisms. These single enzymes can be used to formulate *tailor-made* enzyme mixtures. Then, only the essential enzymes, without undesirable side activities, can be applied in the process.

The variety of raw fruit and vegetable materials, and the various stages of ripening of fruits, make it very difficult for the producer to standardize the enzyme application. In case of the enzymic production of pineapple juice for instance, gellifying polysaccharides, mainly galactomannans, appeared to hamper ultrafiltration of the juice. Therefore, a new enzyme preparation was developed, rich in enzymes degrading these polysaccharides, and low in pectinases, since only a small amount of pectin was naturally present (Grassin and Fauquembergue, 1996). This illustrates the importance of knowledge of the composition of cell walls in fruit and vegetables, in order to be able to select the proper enzymes for a specific product/process. As may follow from the above, the enzymic degradation of pectin, one of the major polysaccharides of the primary cell walls of dicotyledons like apple, is very important in fruit and vegetable processing. This thesis deals with new pectolytic enzymes, and therefore a short update on pectin structure and enzymic degradation is given in the next paragraphs.

PECTIN IN THE PLANT CELL WALL

Pectins comprise about one third of the mass of the primary cell walls of most flowering plants (dicotyledons and nongraminaceous monocotyledons), and form an important domain in the cell wall. Although present also in monocotyledons and lower plants, pectins have mostly been studied in dicotyledons. They are some of the most complex polymers known, and are thought to perform many functions in the plant cell wall. These include determining cell wall porosity; providing charged surfaces that modulate wall pH and ion balance; and serving as recognition molecules that signal appropriate developmental responses to symbiotic organisms, pathogens, and insects (Carpita and Gibeaut, 1993).

Recent cell wall models (McCann and Roberts, 1991; Talbott and Ray, 1992; Carpita and Gibeaut, 1993) depict the three major domains in the wall, i.e. the cellulose-xyloglucan framework (about 50% of the mass), the pectic polysaccharides, and the structural proteins, as three structurally independent, although interacting, domains. This is in contrast with early models where the matrix

polymers were thought to be covalently crosslinked in order to maintain coherence (Keegstra et al., 1973). A model for the primary cell wall of most flowering plants as described by Carpita and Gibeaut (1993) is shown in Figure 1. The stretch-resistant load-bearing *cellulose-xyloglucan network* is embedded in the compression-resistant *pectin matrix*. Synthesis and assembly of each of these domains has found to be independent of the other, and the components of each of these domains can change independently depending on developmental state or in response to special kinds of stress. The current models usually still miss many vital components of the cell wall, namely the many enzymes involved in polysaccharide metabolism, and the proteins (e.g. the hydroxyproline-rich protein extensin) and lignin that are incorporated during cessation of elongation and further differentiation.

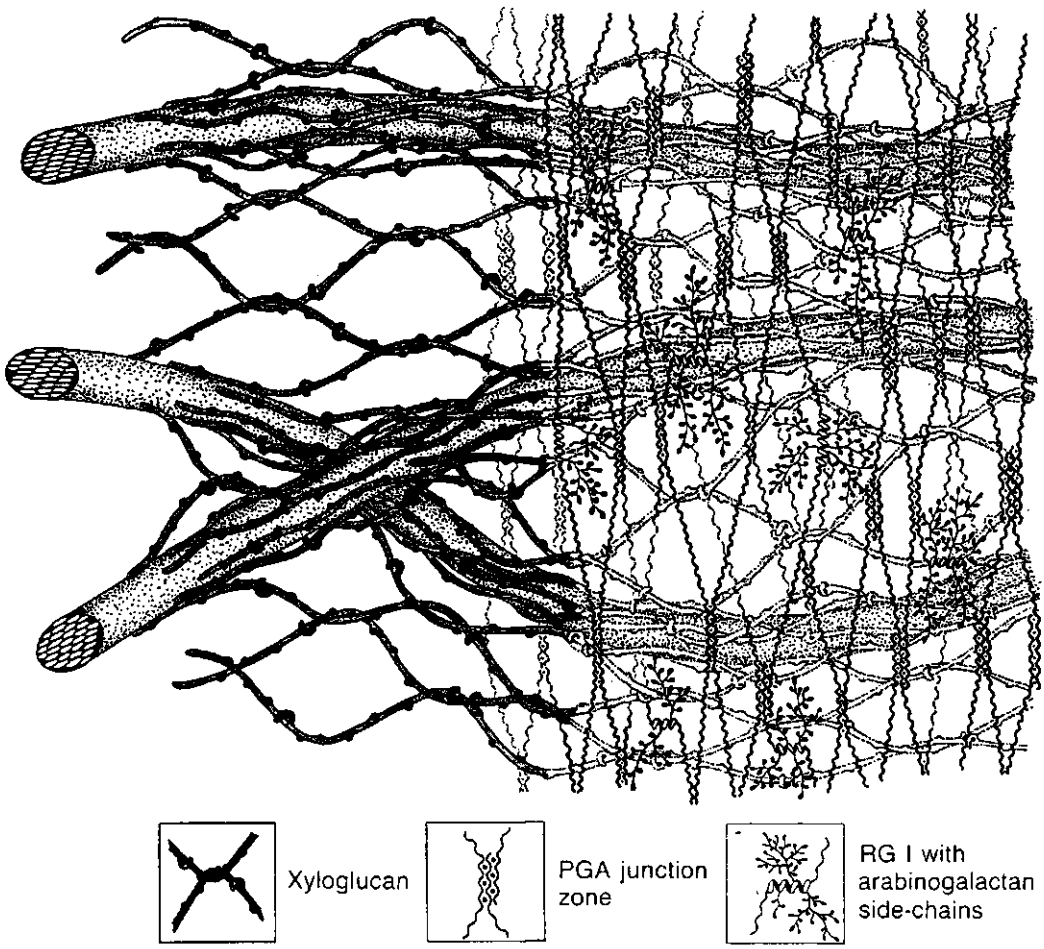

Figure 1. *The type I primary cell wall of most flowering plants. Representation of a single layer of the wall just after formation of a meristem in dividing cells. Several layers such as this coalesce to form a wall (from Carpita and Gibeaut, 1993). PGA, polygalacturonic acid.*

New methods have provided better insight in plant cell wall architecture. These methods include the use of monoclonal antibody probes specific for particular cell wall epitopes; the fast-freeze, deep-etch, rotary-shadowed replica technique, that enables direct visualization of the cell wall in the electron microscope in as close to the *in vivo* state as possible; and fourier transform infra-red microspectroscopy (FTIR) of thin cell samples mounted on a microscope (McCann et al., 1995), by which both the presence and the orientation of specific chemical bonds in a specific area can be detected. Changes in the cell wall architecture during cell expansion and cell elongation are being monitored and new models described. It is hypothesized for instance, that upon cell elongation, an entire pectin network is replaced when newly synthesized highly esterified pectin is deposited in the wall, while the 'older' unesterified pectin might move into the middle lamella. The esterification may change the rheological properties of the pectic network, but may also permit access of enzymes with wall-modifying activities to the cellulose/xyloglucan network (McCann and Roberts, 1994).

Several different types of polysaccharides in the plant cell wall can be considered as pectic material. The most well-known pectic polysaccharide is *homogalacturonan* (HG[1]), a homopolymer of (1,4)-linked α-GalA residues. HGs are able to form gels, a property widely utilized in the food industry, and in all likelihood a property that determines some of the functions of pectin in the primary cell walls (Voragen et al., 1995). Methylesterification of the carboxyl groups is the most common modification of HGs. In the plant, the middle lamella between neighboring cell walls consists predominantly of low esterified pectin. Here, the pectin chains can range up to 700 nm in length (McCann and Roberts, 1996). For comparison, the middle lamella is typically 10 to 20 nm wide, so these pectins must at least be constrained to lie parallel to the plasma membrane. Although generally HGs are not found to contain much acetyl groups, acetylation of HGs has been reported to occur on C-3 in potato and bamboo (Ishii, 1997), and especially sugar beet HG pectin is known for its high acetyl content (Voragen et al., 1995).

The HG type of pectin contains essentially no side chains, and is therefore also referred to as "smooth regions" of pectin. In contrast with this, the second major type of pectic polysaccharide, *rhamnogalacturonan* (RG), contains many side chains, and is often referred to as "hairy regions". One of the most intensively studied RGs, isolated from suspension-cultured cells from many plant sources, was named *RG-I* (O'Neill et al., 1990). It has a strictly alternating RG backbone, i.e. repeats of the disaccharide [(1,2)-α-L-rhamnosyl-(1,4)-α-D-galactosyluronic acid-(1,)]. Arabinosyl- and galactosyl-rich side chains are attached to C-4 of the Rha units, although the proportion of Rha residues with attached side chains varies from app. 20% to 80% depending on the source of the polysaccharide (Albersheim et al., 1996). Arabinans, galactans, and two forms of arabinogalactans (type I and type II), can be regarded as three other pectin associated polymers. Although reported to occur not linked to RG in the cell wall of pea (Talbott and Ray, 1992), these polysaccharides are mostly found to be covalently attached to RGs. RGs are

[1] See List of Abbreviations

typically highly acetylated (Schols and Voragen, 1994) at positions C-2 and C-3 of GalA, and not on Rha units (Komalavilas and Mort, 1989; Ishii, 1997).

A third type of pectic polysaccharide in the plant cell wall, with a relatively low molecular mass, is *RG-II*. Although the name suggests an alternating RG backbone, RG-II consists of a HG backbone of about nine units, to which four side chains are attached. Rha is present in these side chains, beside several rare "diagnostic" monosaccharides such as apiose, 2-O-methyl-L-Fuc, 2-O-methyl-D-Xyl, aceric acid (3-C-carboxy-5-deoxy-L-Xyl), KDO (3-deoxy-D-manno-octulosonic acid), and DHA (3-deoxy-D-lyxo-heptulosaric acid) (Whitcombe et al., 1995). RG-II appears to have the same structure in every plant from which it has been isolated.

RG and HG types of pectic polymers are thought to be interconnected, since treatment of cell wall material with polygalacturonase, only able to cleave HG regions, solubilizes RG types of polysaccharides (De Vries et al., 1982; O'Neill et al., 1990; Schols et al., 1995b), and on the other hand treatment of cell wall material with RGase, only able to cleave RG regions, solubilizes HG regions (Renard et al., 1993). Recently, it has been shown that the pectic polysaccharide RG-II is present in cell walls as a mixture of monomers and dimers. The dimers are covalently crosslinked by borate diesters. Therefore, it is possible that these dimeric RG-II-borates are the "load-bearing", acid-labile linkages of the cell-wall pectin matrix that are hydrolyzed by a decrease in wall pH during auxin-induced cell expansion (O'Neill et al., 1996). Almost certainly the principal form of crosslinking between pectic molecules in the cell wall is the formation of Ca^{2+} binding junction zones of low esterified HG pectins. This type of crosslinking in the middle lamella is assumed to control cell wall porosity (McCann and Roberts, 1991). Furthermore, covalent linkages between different pectin chains in the cell wall might be formed by dimerization of ferulic acid residues, which are found to be present in the hairy regions of e.g. sugar-beet (Rombouts and Thibault, 1986).

Hardly any Xyl is found in the pectic polysaccharides from suspension-cultured plant cells (O'Neill et al., 1990), in contrast with an enzyme-resistant pectic fraction isolated from apple juice produced using the liquefaction process (Schols et al., 1990b). This highly branched pectin structure was tentatively named "*modified hairy regions*" (*MHR*), since the enzymes of the liquefaction process might have modified it. A structural model for this MHR was postulated (Schols and Voragen, 1996). In Figure 2 a hypothetical structure of apple pectin and MHR is given. Two of the three different subunits identified had the characteristics of RG-I, one a strictly alternating RG chain with single unit Gal branches attached to Rha (subunit III in Fig. 2), the other containing higher amounts of GalA compared to Rha, and predominantly long arabinan side chains (subunit II in Fig. 2). The third subunit (subunit I in Fig. 2) was identified as a *xylogalacturonan*, with a degree of substitution of its GalA residues with Xyl of about 0.7 (Schols et al., 1995a). The three structural units of MHR were released from each other using one enzyme, *rhamnogalacturonase* (*RGase*, Schols et al., 1990a), which suggests that xylogalacturonan and RG chains are interconnected. Yu and Mort (1996) also found indications for the presence of a highly methylesterified xylogalacturonan,

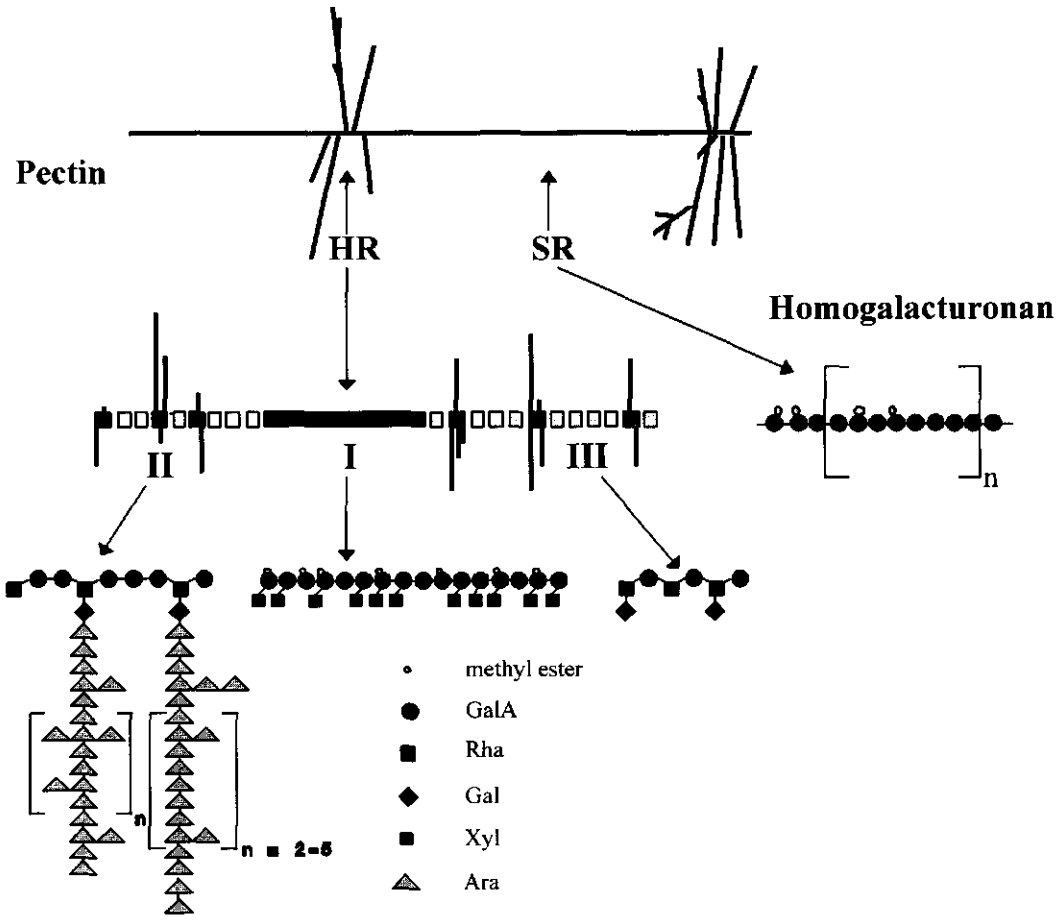

Figure 2. *Hypothetical structure of apple pectin and of the prevailing population of MHR isolated herefrom. SR, smooth regions; HR, hairy regions. Subunits I to III are described in the text. The distribution of acetyl groups is not presented, but there is evidence that the major part of the acetyl groups are located within subunit III. No information is available on the presence of methyl esters in subunit II (From Schols and Voragen, 1996).*

associated with RG regions, in cotton suspension-cultured cell walls and in the cell walls of watermelon.

ENZYMES ACTIVE TOWARD PECTIN

Until recently, major reviews on pectinases only described the enzymes known with activity toward smooth HG regions of pectin (Rombouts and Pilnik, 1980; Whitaker, 1990). This group of HG degrading enzymes can be divided in esterases and depolymerases. *Pectin methylesterase* has been found in higher plants, numerous fungi and some yeasts and bacteria. Pectin methylesterase releases methanol which was originally esterified to the carboxyl group of GalA. Only recently, also a *pectin acetylesterase* has been purified from *Aspergillus niger* (Searle-Van Leeuwen et al.,

1996), able to remove the acetylester that is be attached to the C-2 or C-3 of GalA, e.g. from sugar-beet pectin (Pilnik and Voragen, 1993).

The HG depolymerizing enzymes known are hydrolases or lyases. Of the hydrolases, only enzymes active toward low esterified pectin have been found, so-called *polygalacturonases*. Polygalacturonases have been isolated from higher plants, numerous plant-pathogenic and saprophytic fungi and bacteria, and from some yeasts. Polygalacturonases can act in an endo-manner, cleaving one or more bonds per random encounter, or in an exo-manner, cleaving a chain in a zipper fashion, usually acting from the nonreducing end of the saccharide chain until it is fully degraded. Earlier reports claim the existence of a hydrolase able to cleave highly esterified HG chains (see references in Whitaker, 1990, Table 3), but this has been ascribed to the co-purification of a pectin methylesterase and polygalacturonase, or to the activity of pectin lyase (see below). Recently, however, polymethylgalacturonase has been reported again (Ohtsuki et al., 1995) of Cry j 2, a major allergen of Japanese cedar pollen. The purified enzyme showed no lyase activity. The release of methyl groups from pectin was not determined, which would indicate a contamination with pectin methylesterase. Nevertheless, the authenticity of the enzyme is illustrated by the fact that it was only active toward methylesterified pectin, with a maximum activity when the degree of methoxylation was between 50 and 60, and not at all toward polygalacturonic acid, which is the optimal substrate for polygalacturonases.

The HG lyases, on the other hand, can be divided into those acting toward low methylesterified pectin, the *pectate lyases*, and those acting toward high methylesterified pectin, the *pectin lyases*. No lyases have been found in plants up to date (Sutherland, 1995), and common sources are bacteria and plant pathogenic fungi. They cleave the HG backbone by β-elimination, introducing a double bond between C-4 and C-5 of the newly formed nonreducing end. Pectate lyase requires the presence of a Ca^{2+} ion for its action, which binds directly to the enzyme, although its precise role remains to be clarified (Jurnak et al., 1996). The exo-pectate lyases and oligogalacturonate lyases are special in that they attack their substrate from the reducing end, contrary to exopolygalacturonases and oligogalacturonate hydrolases (Whitaker, 1990). An exo-pectin lyase has been reported in an *Aspergillus* sp. (Sutherland, 1995).

While pectin methylesterases have been described since the 1940s, and the distinction between pectin hydrolases and lyases was made since the 1960s, only recently enzyme activity toward the RG part of pectin has been found. Schols et al. (1990a) were the first to describe such an enzyme, named *RGase*, with activity toward the strictly alternating RG subunit of MHR (Fig. 2). During the course of this PhD-thesis work, a number of reports dealing with RGase activity have been published (An et al., 1994; Kofod et al., 1994; Sakamoto et al., 1994; Azadi et al., 1995; Gross et al., 1995; Ishii, 1995; Suykerbuyk et al., 1995). RG structures cause fouling of the ultrafiltration membranes in processing of straight press apple juices (Will and Dietrich, 1992), and liquefaction apple juices (Schols et al., 1991). The enzyme preparations used in industry at the time apparently were not capable of RG degradation. By screening many commercial enzyme preparations, only one was

found capable of degrading MHR, produced by *Aspergillus aculeatus* (Pectinex Ultra SP from Novo Nordisk), from which the above mentioned RGase was purified (Schols et al., 1990a). Treatment of pear and apple juices with RGase indeed affected the UF flux rate positively (Stutz, 1993). Nowadays many pectolytic enzyme preparations are enriched in RGase activity.

AIM AND OUTLINE OF THIS THESIS

From the above, the technological relevance of RG degrading enzymes is obvious. Furthermore, using RGase, Schols et al. were able to elucidate some structural features of MHR, and it is clear that new RG degrading enzymes might be helpful in further elucidation of RG structures from various sources. The commercial mixture Pectinex Ultra SP from *A. aculeatus* contained the first reported enzyme capable of degrading these structures. Furthermore this preparation was also able to degrade a highly branched RG structure isolated from soy (Adler-Nissen et al., 1984), although not by RGase. Therefore, Pectinex Ultra SP was chosen to screen for other yet unknown RG degrading activities. The complexity of apple MHR (Fig. 2) made this a very suitable substrate to screen for new enzyme activities. Moreover, RGase is only able to degrade one type of subunit (III) present in MHR, while Pectinex Ultra SP can degrade MHR almost completely after incubating for prolonged time with repetitive additions of enzyme, which indicates that there are more relevant enzymes present in the preparation.

During the research period of which the results are described in this thesis, three new RG degrading enzymes were isolated from Pectinex Ultra SP and characterized. Chapter 2 describes the first of these enzymes, the RG-rhamnohydrolase, an exo-enzyme able to remove Rha units from the nonreducing end of RG chains. Chapters 3 and 4 deal with the second new enzyme, the RG-lyase, able to cleave RG regions by β-elimination instead of hydrolysis like RGase does. RGase is now more specifically named RG-hydrolase. A staining method that appeared to be specific for RGases, applicable in plate assays, is described in Chapter 5. The third new enzyme is RG-galacturonohydrolase, an exo-enzyme able to remove the nonreducing GalA unit from RG chains (Chapter 6). In Chapter 7, in order to obtain more information about the mode of action of RG-hydrolase and RG-lyase, single RG oligomers were degraded with RG-hydrolase and RG-lyase. The reaction products were characterized using the two new exo-enzymes. In the general discussion in Chapter 8, beside an overview of the thesis work, the current state of the art on enzymes degrading the branched RG part of pectin is given.

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Chapter 2

Rhamnogalacturonan α-L-rhamnopyranosylhydrolase. A novel enzyme specific for the terminal nonreducing rhamnosyl unit in rhamnogalacturonan regions of pectin

This chapter has been published in *Plant Physiol* **106**: 241-250 (1994) by the authors Margien Mutter, Gerrit Beldman, Henk A. Schols, Alphons G.J. Voragen.

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-rhamnopyranosylhydrolase (pnp-rhamnohydrolase). From the data collected, the enzyme seemed specific for the α-(1,2)- or α-(1,6)-linkage to ß-Glc. Pnp-rhamnohydrolase had a molecular mass of 87 kD (SDS-PAGE[1]), a pH optimum of 5.5 to 6; a temperature optimum of 60 °C and a specific activity toward 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. 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. RG-rhamnohydrolase liberated Rha from the nonreducing end of the RG chain and appeared specific for the α-(1,4)-linkage to α-GalA. The enzyme was hindered when this terminal Rha residue was substituted at the 4-position by a ß-Gal. 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-rhamnopyranosylhydrolase, 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-Rha 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)-Rha-α-(1,4)-GalA (Lau et al., 1985; Colquhoun et al.,

[1] See List of Abbreviations

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., 1994b). 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, 1994b). Schols et al. (1990a, 1994b) 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-Rha-(1,4)-α-GalA-(1,2)-α-Rha-(1,4)-α-GalA (Colquhoun et al., 1990; Schols et al., 1994b). In these oligomers, a ß-Gal unit can be 4-linked to the terminal Rha residues or the (1,2)-linked Rha 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 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

Modified hairy regions (MHR) were isolated from apple liquefaction juice and were subsequently saponified (MHR-S) according to the method of Schols et al. (1990a), 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 *A. 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 (RG oligomers, 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 x 26 mm) in series using a flow rate of 2.5 mL min^{-1} and 0.1 M NaOAc 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 NaOAc pH 5 with ß-galactosidase from *Aspergillus niger* (van de Vis, 1994) 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.

Rhamnosides of Non-RG Origin

Other Rha containing substrates were p-nitrophenyl-α-L-rhamnopyranoside (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-ß-(1,4)-galactan (isolated from

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 from MHR-S, before and after degalactosylation.*
Identification of oligomers was done as described by Schols et al. (1994b).

RG hexamer:

α-Rha-(1,4)-α-GalA-(1,2)-α-Rha-(1,4)-GalA
↓ ↓
β-Gal-(1,4) β-Gal-(1,4)

RG octamer (mixture of two):

α-Rha-(1,4)-α-GalA-(1,2)-α-Rha-(1,4)-α-GalA-(1,2)-α-Rha-(1,4)-GalA
↓ ↓ ↓
β-Gal-(1,4) β-Gal-$(1,4)_n$ β-Gal-$(1,4)_m$

with either n=1 and m=0, or n=0 and m=1.

Degalactosylated RG hexamer:
α-Rha-(1,4)-α-GalA-(1,2)-α-Rha-(1,4)-GalA

Degalactosylated RG octamer:
α-Rha-(1,4)-α-GalA-(1,2)-α-Rha-(1,4)-α-GalA-(1,2)-α-Rha-(1,4)-GalA

potato fiber according to the method of Labavitch et al., 1976), larchwood arabino-ß-(1,3)/(1,6)-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 methoxylation, 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) 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-α-D-Man_p, pnp-β-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 NaOAc 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 10-15% polyacrylamide gel (Pharmacia). A low molecular mass kit (Pharmacia) from 14.4 to 94.0 kD was used for calibration. The pI's were deduced from a pH 3-9 isoelectric focusing gel using the standards from the broad pI 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 of degalactosylated RGoligo for RG-rhamnohydrolase and 0.02% w/v of pnp-Rha for pnp-rhamnohydrolase. Incubations took place for 30 min and preincubation (in stability experiments) occurred for 1 h at 40 °C in 50 mM NaOAc 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 RG-rhamnohydrolase and 0.70 µg mL^{-1} for 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 RG-rhamnohydrolase and 4.86 µg mL^{-1} for pnp-rhamnohydrolase. After preincubation an aliquot of the preincubated solution was added to substrate solution in NaOAc buffer (pH 5) resulting in a total

buffer salt concentration of approximately 0.15 M for RG-rhamnohydrolase and 0.2 M for pnp-rhamnohydrolase. Final protein concentrations in incubation mixtures were 0.29 μg mL^{-1} for RG-rhamnohydrolase and 1.87 μg mL^{-1} for 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 RG-rhamnohydrolase and 0.35 μg mL^{-1} for pnp-rhamnohydrolase.

The stability of the enzymes at different temperatures was measured by preincubation of the enzymes in 50 mM NaOAc buffer (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 RG-rhamnohydrolase and 0.70 μg mL^{-1} for pnp-rhamnohydrolase.

The influence of buffer salt concentration on the activity of the enzymes was determined by incubating enzymes in NaOAc buffer (pH 5) with molarities in the range 50 mM to 1.2 M. Final protein concentrations were 0.18 μg mL^{-1} for RG-rhamnohydrolase and 2.81 μg mL^{-1} for pnp-rhamnohydrolase. The higher protein content for pnp-rhamnohydrolase compared to RG-rhamnohydrolase was necessary since 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 trifluoroacetic acid as described by De Ruiter et al. (1992).

The molecular mas 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 x 250 mm) and a Dionex pulsed electrochemical detection (PED) detector in the pulsed amperometric detection (PAD) 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 NaOAc 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

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
GalA	33	34	29	34

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.

Degalactosylation of RG Substrates

The rhamnohydrolase with activity toward RG substrates appeared to be hindered by Gal side chains attached via a ß-linkage to the 4-position of Rha. Therefore, RG substrates were treated with a ß-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 ß-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 ß-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 ß-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

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 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

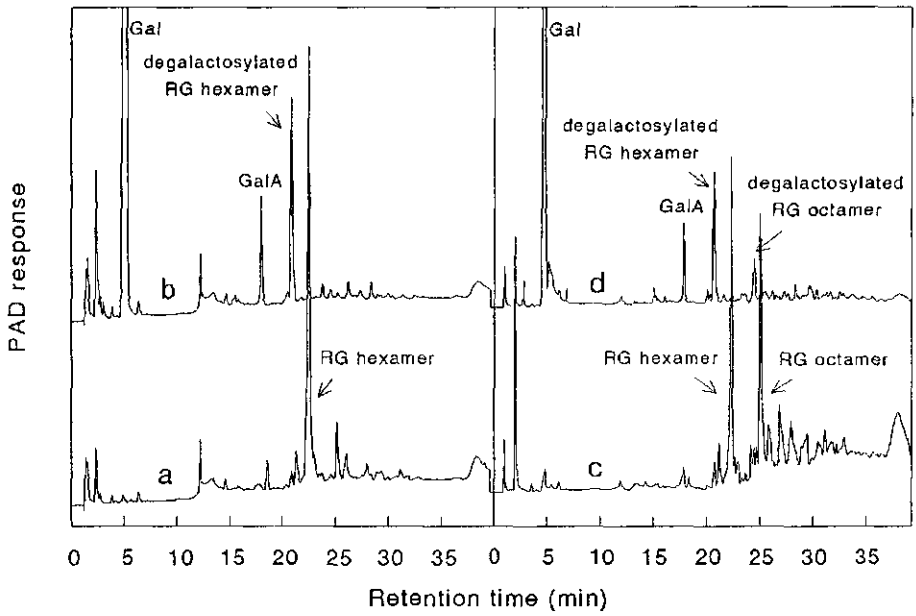

Figure 1. *HPAEC of the RG hexamer (a), the degalactosylated RG hexamer (b), RGoligo (c), and degalactosylated RGoligo (d). Solutions of the RG hexamer were 0.03% w/v and of RGoligo 0.1% w/v (on a total sugar basis) in 50 mM NaOAc buffer (pH 5.0). The structures of the RG oligomers after Schols et al.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

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. (1994b), is presented in Table I.

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

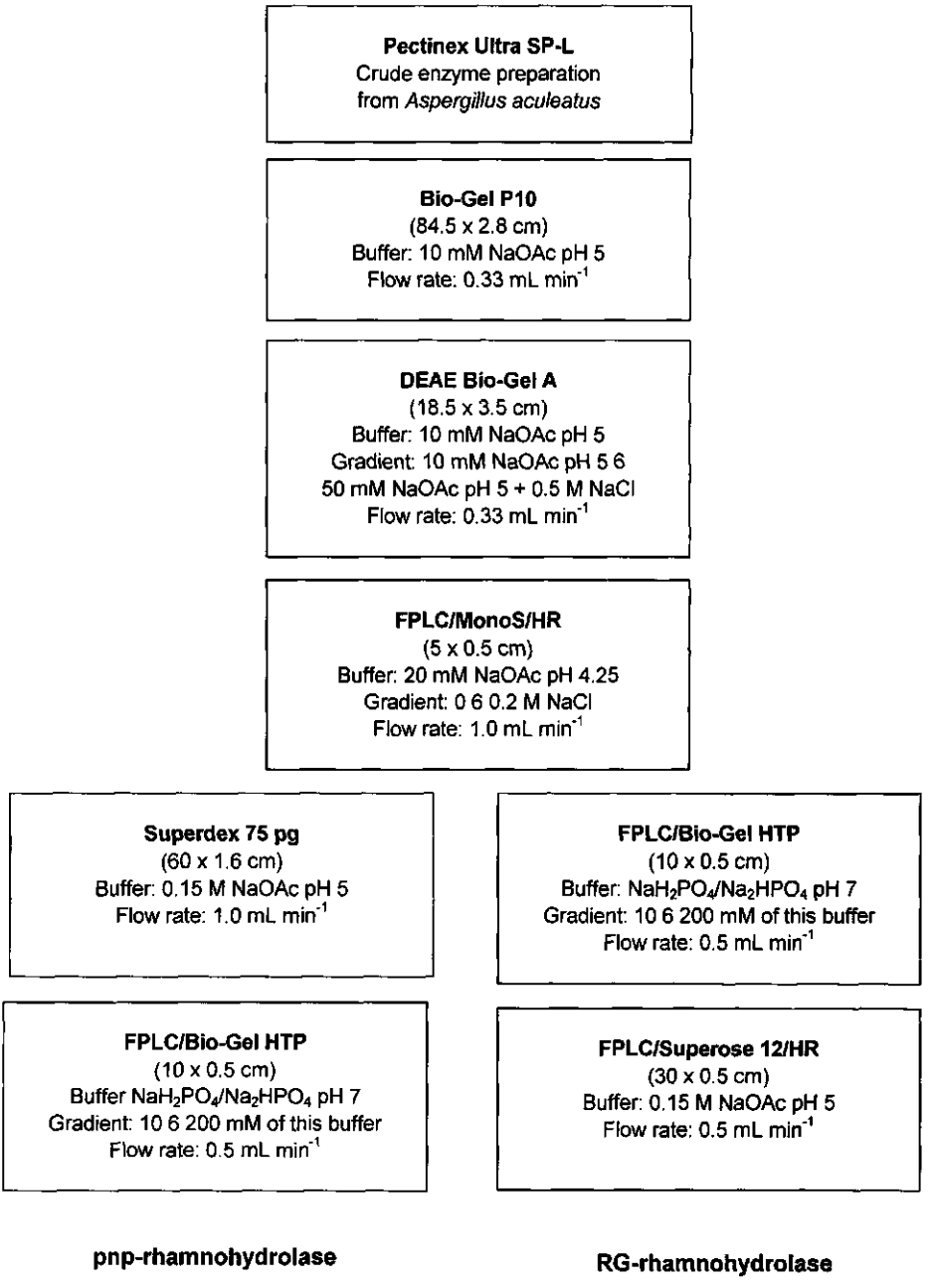

Figure 2. *Scheme of the purification of the pnp-rhamnohydrolase and the RG-rhamnohydrolase from a crude preparation of A. aculeatus. FPLC, fast protein liquid chromatography.*

(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 pnp-rhamnohydrolase involved Superdex 75 prep grade to remove some low molecular mass protein material. The larger part of the protein did not bind to the subsequent Bio-Gel HTP column, in contrast with pnp-rhamnohydrolase. Pnp-rhamnohydrolase eluted at 60 mM sodium phosphate buffer. The protein in the HTP-bound fraction accounted for less than 0.1 % in weight of the originally desalted protein (774 mg). Characterization of pnp-rhamnohydrolase was performed using this HTP-bound fraction.

Purification of RG-rhamnohydrolase

Further purification of RG-rhamnohydrolase involved Bio-Gel HTP. The major part of the protein did not bind onto this column, in contrast with of RG-rhamnohydrolase. RG-rhamnohydrolase eluted at 160 mM sodium phosphate buffer. Characterization of RG-rhamnohydrolase was performed using this HTP-bound fraction, which also accounted for less than 0.1 % of the originally desalted protein (774 mg).

An endo-polygalacturonase (endo-PG) was still present in the HTP-bound fraction of RG-rhamnohydrolase. This endo-PG could be separated from 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 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 pI's and

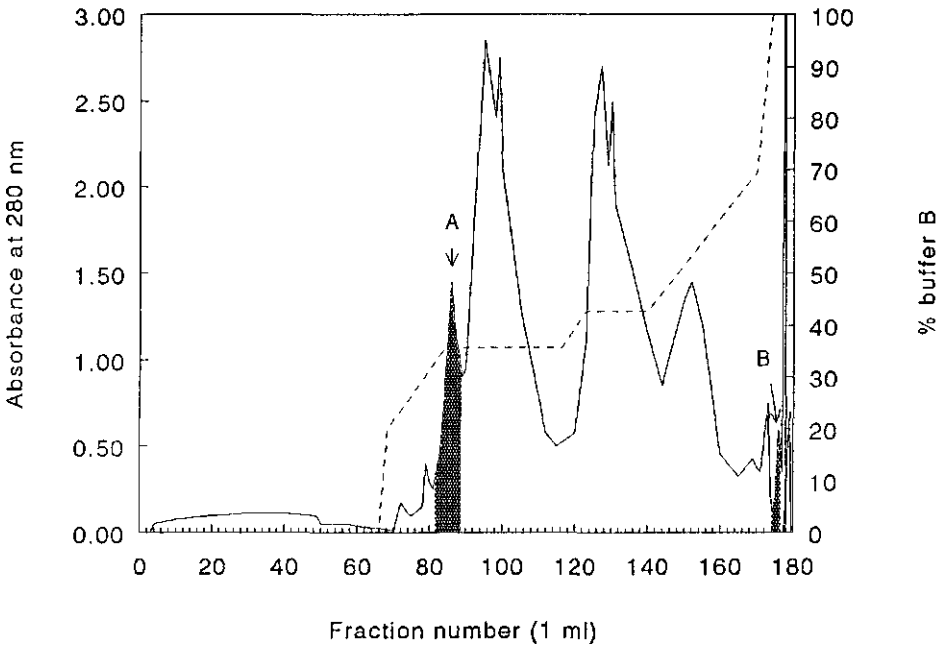

Figure 3. *Chromatography of the unbound fraction from the DEAE Bio-Gel A column on a MonoS HR cation-exchange column, using a sodium chloride gradient from 0 to 0.2 M in 20 mM NaOAc (pH 4.25; see Fig. 2). Symbols: _____, A_{280}; __ __ __ __, sodium chloride gradient (% buffer B); **A**, activity toward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

molecular masses, is currently under investigation. Although the GalA-releasing enzyme was still co-eluting with 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. 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 pnp-rhamnohydrolase. Two other bands were found at 67 and 49 kD. The pI 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 NaOAc buffer. NaOAc concentrations (at pH 5) above 0.3 M strongly inhibited the enzyme: at 0.44 M only 15% of the activity

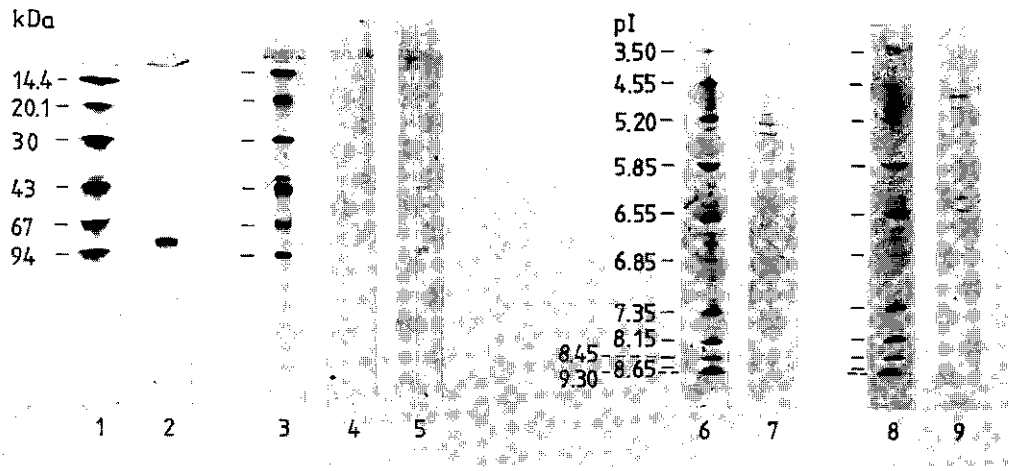

Figure 4. *SDS-PAGE, Coomassie staining. Lane 1, molecular mass standards; lane 2, MonoS-bound RG-rhamnohydrolase; lane 3, molecular mass standards; lane 4, HTP-bound RG-rhamnohydrolase; lane 5, HTP-bound pnp-rhamnohydrolase. IEF, Coomassie staining. Lane 6, broad pI standards; lane 7, HTP-bound RG-rhamnohydrolase;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

at 50 mM was left. Pnp-rhamnohydrolase was stable for 1 h in50 mM NaOAc 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 NaOAc 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. 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 NaOAc buffer (pH 5) an activity of 13 units mg^{-1} protein toward pnp-Rha was found for pnp-rhamnohydrolase.

Characterization of RG-rhamnohydrolase

By gel filtration using a Superose 12 column 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 RG-rhamnohydrolase, and a minor band of 71 kD. The endo-PG that was

Table IV. *Side activities of pnp-rhamnohydrolase and RG-rhamnohydrolase*
++, Activity visible after 30 min incubation; +, activity visible after 24 h incubation; -, activity not visible after 24 h 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 NaOAc 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-α-D-Man_p	-	-
pnp-β-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	-	-

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 pI range of 4.9 to 5.4 were found for the HTP-bound RG-rhamnohydrolase.

RG-rhamnohydrolase was optimally active at pH 4 in McIlvaine buffers and at 60 °C in NaOAc buffer. NaOAc 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 NaOAc buffer (pH 5). Similarly to 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 NaOAc 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 NaOAc buffer (pH 5)

an activity of 60 units mg^{-1} protein toward degalactosylated RGoligo was found for 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 pnp-rhamnohydrolase and RG-rhamnohydrolase were incubated with several Rha containing substrates (Table V). Preliminary experiments showed that at a concentration of 300 µM terminal, nonreducing, unbranched Rha in degalactosylated RGoligo, initial reaction rates could be measured (results not shown) under the conditions described in Table V.

From Table V it can be seen that 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, pnp-rhamnohydrolase seemed specific for the α-(1,2)- or α-(1,6)-linkage to ß-Glc. One of the two Rha units in α-chaconine is also α-(1,2)-linked to ß-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 pnp- rhamnohydrolase toward

Table V. *Action of pnp-rhamnohydrolase and RG-rhamnohydrolase toward 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-solanine, α-chaconine and degalactosylated substrates in 50 mM NaOAc 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 no 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 ß-D-Glc_p	2.2	68.9	0	0
Hesperidin	α-(1,6) to ß-D-Glc_p	2.2	62.9	0	0
α-Solanine	α-(1,2) to ß-D-Gal_p	0	0	0	0
α-Chaconine	α-(1,2) and α-(1,4) to ß-D-Glc_p	0	0	0	0
RGoligo	α-(1,4) to α-D-Gal_pA	0	0	3.2	1.3
degal.[a] 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] degal. for degalactosylated

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 NaOAc buffer (pH 5). No reasonable explanation could be given for this observation, since the stability experiments in NaOAc buffer did not indicate inactivation.

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. RG-rhamnohydrolase appeared to be hindered by the Gal side chains attached via a ß-(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 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 ß-galactosidase.

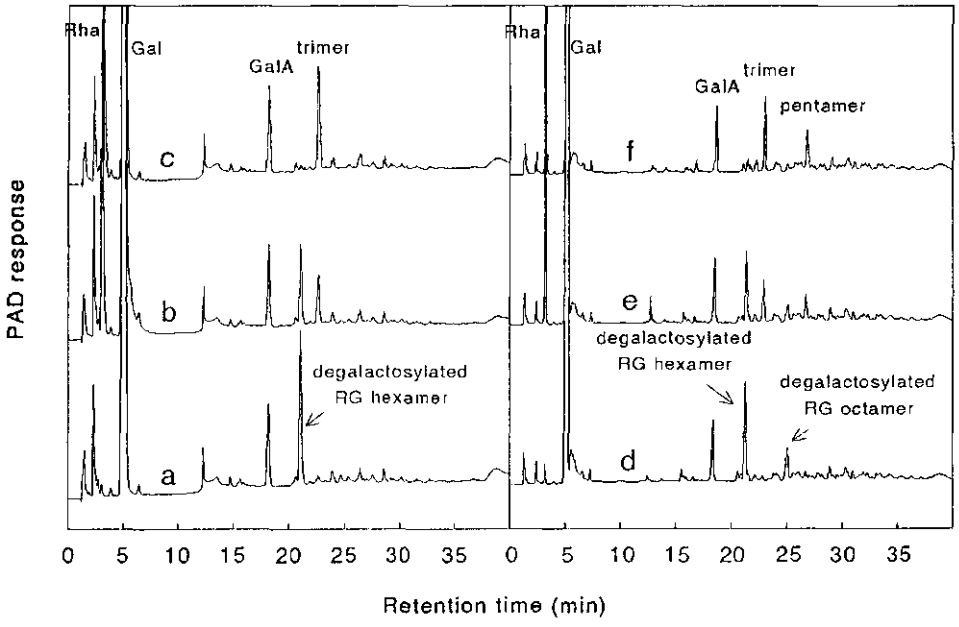

Figure 5. *HPAEC of the degalactosylated RG hexamer (a), the degalactosylated RG hexamer after 0.5 h of incubation with RG-rhamnohydrolase (b), the degalactosylated RG hexamer after 12 h of incubation with RG-rhamnohydrolase (c), degalactosylated RGoligo (d), degalactosylated RGoligo after 0.5 h of incubation 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 a total sugar basis) in 50 mM NaOAc 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-GalA-(1,2)-α-Rha-(1,4)-GalA and α-GalA-(1,2)-α-Rha-(1,4)-α-GalA-(1,2)-α-Rha-(1,4)-GalA respectively. PAD, pulsed amperometric detection.*

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 RG-rhamnohydrolase had no preference for high or low molecular mass substrates.

Since the nonreducing end in RGoligo consists of Rha, 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 RG-rhamnohydrolase. The released Rha eluted at 3 min. The peak at 18 min resulted from GalA which was released by the ß-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-GalA-(1,2)-α-Rha-(1,4)-GalA and α-GalA-(1,2)-α-Rha-(1,4)-α-GalA-(1,2)-α-Rha-(1,4)-GalA, 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-Rha-(1,4)-GalA, which eluted earlier from the column than monoGalA (Schols et al., 1994a). 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, pnp-rhamnohydrolase, is an α-L-rhamnopyranosylhydrolase 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 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 ß-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 glycoalkaloids 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, RG-rhamnohydrolase seems equally active toward low and high molecular mass RG fragments. The systematical name for the enzyme should be RG α-L-rhamnopyranosylhydrolase, abbreviated RG-rhamnohydrolase. The results show that the enzyme splits off the terminal, nonreducing Rha from RG fragments. The galactosyl residue ß-(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 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 RGs. 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, 1994b) 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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Chapter 3

Rhamnogalacturonase B from *Aspergillus aculeatus* is a rhamnogalacturonan α-L-rhamnopyranosyl-(1,4)-α-D-galactopyranosyluronide lyase

This chapter has been published in *Plant Physiol* **110**: 73-77 (1996) by the authors Margien Mutter, Ian J. Colquhoun[1], Henk A. Schols, Gerrit Beldman, and Alphons G. J. Voragen.

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[2], 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 a RG α-L-rhamnopyranosylhydrolase (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 modified hairy regions (MHR) of apple pectin differed in elution behaviour using HPAEC. Comparison of the primary structures, deduced from the

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[2] See List of Abbreviations

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 x 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. ^1H 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 AG (Buchs, Switzerland). 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 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 isoelectric focusing 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).

HPSEC was performed using three Bio-Gel TSK columns in series (40XL, 30XL and 20XL) as described (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 x 250 mm) column and a Dionex pulsed electrochemical 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 behaviour 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.

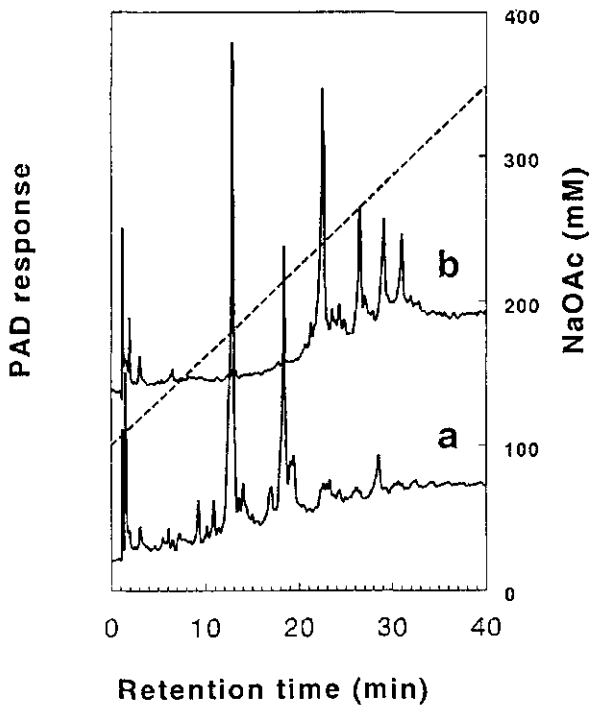


Figure 1. *Typical HPAEC chromatograms of MHR-S after degradation by RGase A (a); MHR-S after degradation by RGase B (b); _ _ _ _, NaOAc gradient.*

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 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 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-unsaturated-GalA (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

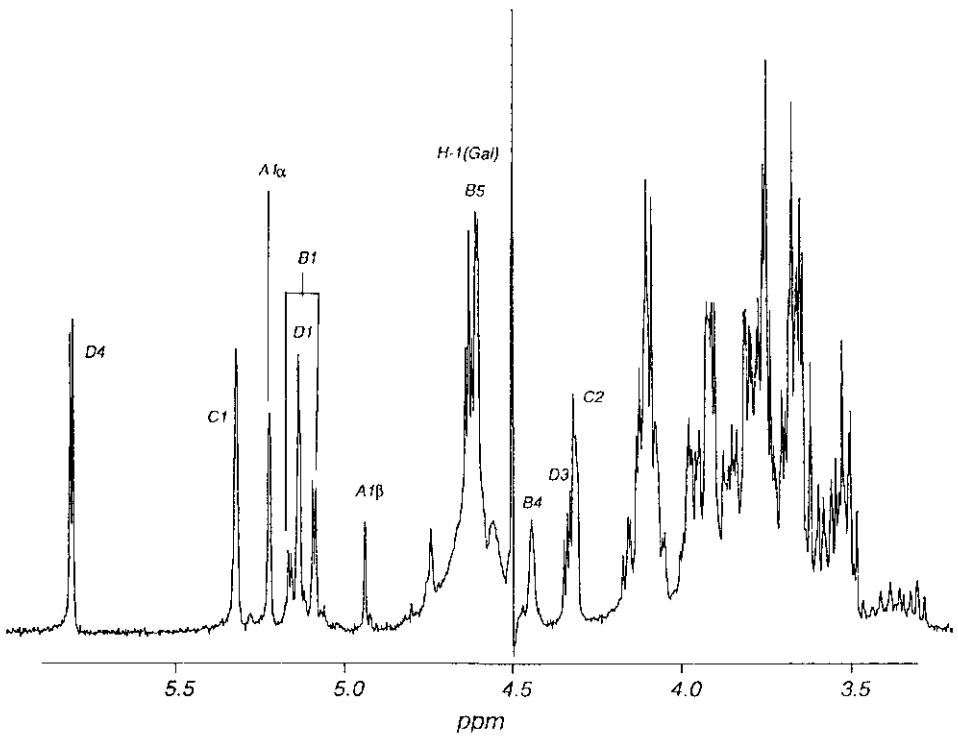
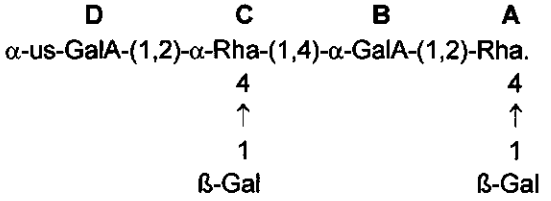


Figure 2. *The 400 MHz ^{1}H NMR spectrum (50 °C) of the smallest RGase B oligosaccharide. Residues are coded as in the text. The two B1 doublets are for GalA linked to α- and β-Rha-reducing end groups. ppm, Parts per million.*

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 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 ß 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 ß 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 absorbance 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

Table I. *^1H Chemical shifts for the smallest RGase B oligosaccharide*
n.d., Not determined. -, Not present.

Unit		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.

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

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.

	pH 6[a]		pH 8[b]	
Substrate	- 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 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 $CaCl_2$.

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 a 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 α-L-rhamnopyranosyl-(1,4)-α-D-galactopyranosyluronide lyase, abbreviated RG-lyase, is suggested for RGase B. RGase A should then be named RG α-D-galactopyranosyluronide-(1,2)-α-L-rhamnopyranosyl hydrolase, abbreviated 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 (Novo Nordisk Ferment Ltd., 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 (not shown). 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.

ACKNOWLEDGMENTS

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Chapter 4

Characterization of recombinant Rhamnogalacturonan α-L-rhamnopyranosyl-(1,4)-α-D-galactopyranosyluronide lyase from *Aspergillus aculeatus*. An enzyme that fragments rhamnogalacturonan I regions of pectin

This chapter has been submitted to *Plant Physiol* by the authors Margien Mutter, Ian J. Colquhoun[1], Gerrit Beldman, Henk A. Schols, Edwin J. Bakx, and Alphons G.J. Voragen.

The four major oligomeric reaction products from saponified modified hairy regions (MHR-S[2]) from apple, produced by recombinant rhamnogalacturonan (RG) α-L-rhamnopyranosyl-(1,4)-α-D-galactopyranosyluronide lyase (rRG-lyase) from *Aspergillus aculeatus*, were isolated and characterized by ^{1}H NMR spectroscopy. They contain an alternating RG backbone with a degree of polymerization of 4, 6, 8 and 10, and with an α-Δ-(4,5)-unsaturated GalA (us-GalA) at the nonreducing end and a Rha at the reducing end. Rha units are substituted at C-4 with β-Gal. The V_{max} of rRG-lyase toward MHR-S at pH 6 and 31 °C was 28 units mg^{-1}. rRG-lyase and RG-hydrolase cleave the same alternating RG I subunit in MHR. Both these enzymes fragment MHR by a multiple attack mechanism. The catalytic efficiency of rRG-lyase for MHR increases with decreasing degree of acetylation. Removal of Ara side chains improves the action of rRG-lyase toward MHR-S. In contrast, removal of Gal side chains decreased the catalytic efficiency of rRG-lyase. Native RG-lyase was purified from *A. aculeatus*, characterized and found to be similar to the rRG-lyase expressed in *Aspergillus oryzae*.

Highly branched RG structures are found associated with pectin in the cell walls of many different plants (O'Neill et al., 1990; Schols and Voragen, 1994; and Yamada, 1994). Polygalacturonases, pectin lyases and pectate lyases fragment the homogalacturonan (HG) regions of pectin but do no degrade RGs. However, these enzymes do release high molecular weight branched RGs from cell walls (De Vries et al., 1982; O'Neill et al., 1990; Schols et al., 1995b). The availability of RG degrading enzymes is of great value to the structural elucidation of RG structures in the plant cell wall. Furthermore, the usefulness of these enzymes has been

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[1] See List of Abbreviations

indicated in the processing of fruit, where it is important that the commercial pectolytic enzyme preparations solubilize and hydrolyze the branched RG structures, that otherwise remain as colloidally dissolved polymers in the juice and lead to problems during filtration and clarification (Voragen et al., 1992; Will and Dietrich, 1994).

The first enzyme with activity toward RG, rhamnogalacturonase (RGase), was found by Schols et al. (1990a). Subsequently, an RG-acetylesterase (Searle-Van Leeuwen et al., 1992), an RG-rhamnohydrolase (Mutter et al., 1994: Chapter 2), and an RG-galacturonohydrolase were identified (Mutter et al., 1996b). These enzymes were all purified from the commercial enzyme preparation, Pectinex Ultra-SP, produced by *A. aculeatus*. A second RGase (RGase B) was discovered in the authors' laboratory and later cloned and expressed in *A. oryzae* (Kofod et al., 1994). This recombinant RGase B is a specific RG α-L-rhamnopyranosyl-(1,4)-α-D-galactopyranosyluronide lyase, abbreviated rRG-lyase (Azadi et al., 1995; Mutter et al., 1996a: Chapter 3). The current report describes the characterization of all four major oligomers formed by rRG-lyase from MHR (Schols et al., 1990b) by ^{1}H NMR spectroscopy. The recombinant enzyme is further characterized with respect to the influence of various MHR substituents on kinetic parameters. Native RG-lyase from the commercial *A. aculeatus* preparation is purified and compared with the recombinant enzyme.

MATERIALS AND METHODS

Substrates

MHR were isolated from apple liquefaction juice, produced using Rapidase C600, according to Schols et al. (1990b). MHR were then saponified to yield MHR-S (Schols et al., 1990b), since RG-lyase and RG-hydrolase were hindered by acetyl groups. The sugar composition of MHR-S was reported previously (Chapter 2). MHR-S were also obtained from leek and carrot (Schols and Voragen, 1994).

Isolation and characterization of intermediate-sized (RGmed) and polymeric (RGpoly) degradation products of MHR-S produced by RG-hydrolase (previously RGase or RGase A) were described in Chapter 2.

Substrates used to determine side activities were polygalacturonic acid (Fluka Chemie AG, Buchs, Switzerland), highly methoxylated pectin (degree of methoxylation [DM] 92.3) prepared in our laboratory according to the procedure of Van Deventer-Schriemer and Pilnik (1976), larchwood arabino-β-(1,3)/(1,6)-galactan ("stractan", Meyhall Chemical AG, Kreuzlingen, Switzerland), xylan ex oat spelts (Koch and Light Ltd., Haverhill, England), carboxymethylcellulose (Akucell AF-0305, Akzo, Arnhem, The Netherlands), soluble starch (Merck AG, Darmstadt, Germany), a linear arabinan kindly provided by British Sugar (Peterborough, UK), potato arabino-β-(1,4)-galactan (isolated from potato fiber according to Labavitch et al., 1976), and a hexamer of galacturonic acid as described before (Voragen, 1972).

The p-nitrophenylglycosides used for screening of glycosidase activities were obtained from Koch and Light Ltd. (Haverhill, England) and from Sigma chemical

company (St. Louis, MO): pnp-α-L-Ara_f, pnp-α-L-Ara_p, pnp-α-L-Gal_p, pnp-β-D-Gal_p, pnp-α-D-Xyl_p, pnp-β-D-Xyl_p, pnp-α-L-Rha_p.

Isolation of rRG-lyase Oligomers from MHR-S

MHR-S was degraded (2.5 g, 4.8% w/v, 16.5 h, 40 °C, in 5 mM NaOAc buffer pH 6) by rRG-lyase (0.23 mg). The degradation products were separated on a column of Sephadex G-50, as described by Schols et al. (1990b), using demineralized water for elution. Fractions were assayed by automated colorimetric methods for uronic acids and total neutral sugars as described in Chapter 2. Fractions were analyzed by high-performance anion-exchange chromatography (HPAEC) and those containing the rRG-lyase MHR oligomers (fractionated as 5, 6 and 7, see Fig. 1a) were further purified by preparative HPAEC, essentially as described by Schols et al. (1994), using a Dionex (Sunnyvale, CA) PA-100 (22 x 250 mm) column 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.

The neutral sugar composition of the Sephadex G-50 fractions was determined by hydrolyzing lyophilized material in 1 *M* sulfuric acid (3 h at 100 °C) and subsequently converting the released neutral sugars to their alditol acetates (Englyst and Cummings, 1984). These were analyzed on a 15 m x 0.53 mm i.d. wide bore capillary DB 225 column in a Carlo Erba 4200 gas chromatograph (Milan, Italy). The temperature program was set at: 180 °C for 1 min, from 180 to 220 at 2.5 ° C/min and 220 °C for 3 min. The system was equipped with a flame ionization detector set at 275 °C. Inositol was used as the internal standard. Uronic acid was determined using an automated colorimetric method based on the method described by Ahmed and Labavitch (1977).

Preparative Deacetylation of MHR

Eight batches of MHR (approximately 60 mg each) were treated for 1 h at 40 °C as 0.27% w/v solutions in 50 mM NaOAc buffer (pH 5) with different amounts (between 1 µg and 40 µg, and one batch with an excess amount of 4 mg) of a recombinant RG-acetylesterase from *Aspergillus aculeatus* (Kauppinen et al., 1995). After inactivation for 20 min at 100 °C, the batch treated with 4 mg enzyme was centrifuged to remove denaturated protein, and all batches were dialyzed one night against running tap water and seven days against destilled water (at 4 °C), and lyophilized. The degree of acetylation (DA) and DM were determined by HPLC according to Voragen et al. (1986).

Preparative Removal of Ara and Gal from MHR-S by Enzymes

MHR-S (in batches of approximately 50 mg) were treated with various enzymes and enzyme combinations in excess amounts (2-5 mg). Recombinant α-arabinofuranosidase, recombinant arabinanase and recombinant β-(1,4)-galactanase

from *A. aculeatus* (Christgau et al., 1995) were experimental batches and kindly provided by Novo Nordisk A/S (Copenhagen, Denmark). A β-galactosidase from *Aspergillus niger* was purified following the procedure of Van de Vis (1994). To remove Ara, MHR-S was treated with the arabinofuranosidase (MHR-S deAra-1); with the endo-arabinanase (MHR-S deAra-2); and with the combination of these two enzymes (MHR-S deAra-3). In order to remove Gal, MHR-S was treated with the β-galactosidase (MHR-S deGal-1); with the β-(1,4)-galactanase (MHR-S deGal-2); and with the combination of these two enzymes (MHR-S deGal-3). The removal of all Ara and Gal containing side chains was attempted with a combination of all formerly mentioned enzymes (MHR-S deAra-deGal). Reaction mixtures were incubated at 40 °C for 24 h in 0.18 to 0.23% w/v solutions in 50 mM NaOAc (pH 5). After inactivation for 20 min at 100 °C the incubation mixtures were centrifuged to remove precipitated material. After dialysis the samples were lyophilized. Sugar composition was determined after methanolysis and subsequent hydrolysis with trifluoroacetic acid as described by de Ruiter et al. (1992).

Enzyme Purification

Native RG-lyase was partially purified from the commercial preparation Pectinex Ultra SP-L produced by *A. aculeatus*, kindly provided by Novo Nordisk Ferment Ltd. (Dittingen, Switzerland). Purification steps involved Bio-Gel P10; DEAE Bio-Gel A (Bio-Rad Laboratories, Richmond, CA) and MonoS HR 5/5 (Pharmacia LKB Biotechnology, Uppsala, Sweden) and were performed as described in Chapter 2. Concentration between DEAE and MonoS chromatography steps was carried out by ultrafiltration using a YM 10K membrane from Amicon Corporation (Danvers, MA). Fractions were screened for RG-lyase activity by incubation with MHR-S, followed by high-performance size-exclusion chromatography (HPSEC) and HPAEC (gradient A, see Analytical methods).

Recombinant RG-lyase from *A. aculeatus* was purified essentially as described by Kofod et al. (1994), starting from lyophilized crude culture supernatant of an *A. oryzae* transformant (A 1560) producing rRG-lyase, kindly provided by Novo Nordisk A/S (Copenhagen, Denmark). Native RG-lyase was characterized and found to be essentially the same enzyme as rRG-lyase. Since rRG-lyase was available in large amounts it was used in all further experiments.

RG-hydrolase was purified using the method of Schols et al. (1990a).

Enzyme Incubations and Enzyme Assays

Determination of Side Activities of Native RG-lyase from A. aculeatus

Native RG-lyase (0.5 μg mg^{-1} substrate) was screened for contaminating glycanase activities by incubation for 24 h at 40 °C with 0.24% w/v solutions of selected substrates in 50 mM NaOAc buffer (pH 5). The digests from the glycanase assays were analyzed by HPSEC and HPAEC (Gradient A). For contaminating glycosidase activities, screening was performed by incubating RG-lyase (12 μg mg^{-1} substrate) for

1 h at 30 °C with 0.02% w/v solutions of pnp-glycosides in 50 mM NaOAc buffer (pH 5). 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}.

Influence of pH and Temperature on Native RG-lyase from A. aculeatus

The optimum pH for RG-lyase was determined by incubating RG-lyase (1.25 µg mg^{-1} substrate) for 3 h at 40 °C in 0.24% w/v substrate solutions in McIlvaine buffers (mixtures of 0.1 M citric acid and 0.2 M sodium hydrogenphosphate) in the pH range 2 to 8. The same buffers were used for preincubation of RG-lyase (4.5 h at 40 °C) to measure the stability of RG-lyase at different pH values. After preincubation, 1.5 M NaOAc buffer (pH 5) was added to adjust the pH, and substrate solution was added to start incubation for 3 h at 40 °C. The optimum temperature was determined by incubating RG-lyase (1.25 µg mg^{-1} substrate) for 3 h in 0.24% w/v substrate solutions in 50 mM NaOAc buffer (pH 5) at different temperatures in the range 3 to 60 °C. The stability of RG-lyase at different temperatures was measured by preincubation for 4.5 h in 50 mM NaOAc buffer (pH 5). After adjusting the temperature, substrate solution was added to start incubation for 3 h at 40 °C. Incubation mixtures were inactivated by heating for 10 min at 100 °C. Temperature and pH optima and stability were determined from the total amount of oligomeric fragments in digests based on peak area (HPAEC, gradient A). The pH optimum was also determined from the increase in A_{235}.

Determination of Kinetic Parameters of rRG-lyase toward MHR Derived Samples

General reaction conditions were: 50 mM NaOAc buffer (pH 6), 31 °C, 0.42 µg rRG-lyase mL^{-1}. Substrate concentrations in case of MHR samples with different DA's (Fig. 3 and Table III) ranged between 0.03% w/v and 0.6% w/v. Substrate concentrations in case of various enzyme treated MHR-S samples (Table V) ranged between 0.01% w/v and 0.4% w/v. RG-lyase activity was calculated from the increase in A_{235} as measured in duplicate every 30 or 60 s using a Beckman DU-62 spectrophotometer equipped with a Soft-Pac Kinetics Module. 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). Data analysis for calculation of kinetic parameters, using non-linear regression, was performed by the program Enzfitter (Biosoft, Cambridge, UK)

Activity of rRG-lyase and RG-hydrolase toward Other MHR Subunits

Recombinant RG-lyase (0.20 µg mL^{-1} reaction mixture) and RG-hydrolase (0.086 µg mL^{-1} reaction mixture) were both incubated with the following substrates: combined Sephadex G-50 Fractions 1+2 (see Table I), combined Sephadex G-50 Fractions 3+4, RGpoly, and RGmed. The latter two substrates were obtained from earlier work (Chapter 2). The enzymes were incubated with 0.1% w/v substrate solutions in 50 mM

NaOAc (pH 5) for 20 h at 40 ° C. Mixtures were inactivated by heating for 5 min at 100 °C and analyzed using HPSEC and HPAEC (gradient B).

Determination of the Degree of Multiple Attack of rRG-lyase

MHR-S solutions of 0.23% w/v in 50 mM NaOAc buffer (pH 6) + 0.01% w/v NaN_3 were incubated at 40 °C for 1 h using varying amounts of rRG-lyase per mg MHR-S. After inactivation for 10 min at 100 °C, samples were analyzed using HPSEC. For RG-hydrolase the experiment was carried out similarly, with the exception of the use of 50 mM NaOAc buffer pH 4 instead of pH 6.

Gel Electrophoresis and Dot-Blotting

SDS-PAGE was carried out as described in Chapter 2. The pI's were determined using zymography as described in Chapter 5.

RG-hydrolase and native RG-lyase were applied onto nitrocellulose membranes (Bio-Rad), which were previously washed in TBS (20 mM Tris buffer pH 7.5 containing 500 mM NaCl). Unreacted binding sites were blocked by incubation for 45 min at room temperature in TBS containing 3% w/v gelatin. For immunoblotting, the membrane was incubated for 1.5 h at room temperature with a polyclonal rabbit antiserum raised against purified RG-hydrolase as described (Harlow and Lane, 1988), diluted 1 : 2500 in TBS containing 0.05% w/v Tween 20 and 1% w/v gelatin. Bound rabbit antibodies were visualized by incubation with an alkaline phosphatase labeled goat anti-rabbit antibody (Sigma).

Determination of Molecular Mass of Native RG-lyase Using SEC

A Superose 12 HR 10/30 column (Pharmacia) was calibrated with endopolygalacturonase (43 kD), RG-rhamnohydrolase (84 kD), RG-hydrolase (53 kD), and several partially purified proteins with molecular masses of 78, 76, 52, 45 and 32 kD, as characterized by SDS-PAGE. A buffer of 150 mM NaOAc (pH 6) was used for elution. Retention of RG-hydrolase (17.1 µg) and native RG-lyase (2.4 µg) on this column was monitored by collecting fractions and determining their activity toward MHR-S (analysis by HPSEC and HPAEC [Gradient A] as described below).

Analytical Methods

HPSEC

The molecular mass distribution of substrates before and after enzyme treatment was determined by HPSEC on three Bio-Gel TSK columns in series (40XL, 30XL and 20XL) as described by Schols et al. (1990a). Pectin standards of 100; 82; 77.6; 63.9; 51.4; 42.9; 34.6; 10 kD and GalA and diGalA were used for calibration of the system. Software ("GPC/PC") from Spectra Physics (San Jose, CA) was used for determination of the number-average molecular mass.

HPAEC

HPAEC was performed using a Dionex Bio-LC system equipped with a Dionex CarboPac PA-100 (4 x 250 mm) column and a Dionex PED detector in the pulsed amperometric detection (PAD) mode. Gradients of NaOAc in 100 mM NaOH (1 mL min^{-1}) were used as follows:
Gradient A: 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;
Gradient B: 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.

^{1}H NMR Spectroscopy

^{1}H NMR spectra of the oligosaccharides in deuterated H_2O were obtained at 400 MHz using a JEOL GX400 spectrometer. Sample temperature was 27 °C (or 50 °C to reveal signals at δ 4.75) and chemical shifts were determined using acetone (δ 2.217 with respect to tetramethylsilane) as internal reference. Phase-sensitive two-dimensional NMR experiments (correlation spectroscopy [COSY], rotating frame Overhauser effect spectroscopy [ROESY] and homonuclear Hartmann-Hahn spectroscopy [HOHAHA]) were carried out as described previously (Colquhoun et al., 1990). For the two-dimensional experiments the spectral width was 2500 Hz in both dimensions, matrix size 2048(t_2)x256(t_1), spin-locking times were 200 ms (ROESY) and 110 ms (HOHAHA), spin-lock power 6.25 kHz (both experiments). Data processing was carried out using FELIX software (Molecular Simulations).

RESULTS AND DISCUSSION

Isolation of rRG-lyase Oligomers Produced from MHR-S

Structural characterization of the oligomers that rRG-lyase releases from MHR-S will lead to a better understanding of the mode of action of the enzyme. Therefore, MHR-S was incubated on preparative (gram) scale with rRG-lyase. The digest was separated into seven fractions using a Sephadex G-50 column (Fig. 1a). The sugar composition of the seven fractions is shown in Table I. Fractions 5, 6 and 7 contained oligomeric reaction products (**I** to **IV** in Fig. 1b) that could be detected using HPAEC, and contained Gal, GalA and Rha as predominant sugars. Thus, the oligomers generated by rRG-lyase treatment of MHR-S and the oligomers produced by RG-hydrolase treatment of MHR-S are composed of the same sugar residues.

The trend in sugar composition of the Sephadex G-50 fractions over the elution profile of the rRG-lyase digest of MHR-S resembles that of the Sephacryl S-200 fractions of the RG-hydrolase digest of MHR-S population A (the highest molecular mass population) (Schols et al., 1995a). From the work of these authors on the characterization of the degradation products of apple MHR population A, a detailed model for the chemical structure of this population came forward. Three subunits were distinguished: subunit I, xylogalacturonan type of polymers

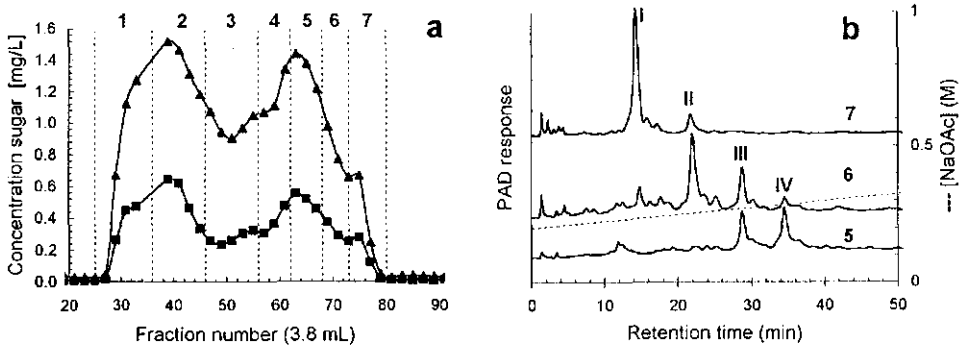


Figure 1. *a, SEC of the rRG-lyase digest of MHR-S on a Sephadex G-50 column using demineralized water for elution. Fractions were pooled as indicated in the figure (1 to 7) (▲: neutral sugars; ■: uronic acid); b, HPAEC elution patterns of Fractions 5, 6 and 7.*

(recognizable in G-50 Fractions 1 and 2); subunit II, RG backbone stubs with a Rha:GalA ratio < 1 and arabinan side chains (recognizable in G-50 Fractions 3 and 4); and subunit III, strictly alternating RG fragments with Gal side chains (recognizable in G-50 Fractions 5, 6 and 7). Subunit III and RG I as described by Albersheim et al. (1996) are essentially the same type of polysaccharide. The comparable S-200 fractions described by Schols et al. (1995a) were richer in Ara, but their starting material already contained more Ara. The results suggest that rRG-lyase attacks the same type of subunit in MHR as RG-hydrolase does, namely the strictly alternating RG I regions with single unit Gal side chains attached to the C-4 of Rha. This was confirmed by the fact that rRG-lyase was not active toward the higher molecular mass MHR-S degradation products (RGpoly, RGmed) liberated by RG-hydrolase after producing RG oligomers (not shown). Similarly, RG-hydrolase was not active toward the higher molecular mass MHR-S degradation products (combined Sephadex G-50 Fractions 1+2 and 3+4) liberated by rRG-lyase after producing oligomers (not shown).

Fractions 5, 6 and 7 were further fractionated using preparative HPAEC (Fig. 1b). Seven HPAEC fractions (7.1-7.7) were obtained from Fraction 7; eight HPAEC fractions were obtained from Fraction 6 (6.1-6.8), and 18 HPAEC fractions (5.1-5.18)

Table I. *Sugar composition in mol% of Sephadex G-50 fractions of the rRG-lyase degradation products from MHR-S*

Sugar	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 6	Fraction 7	MHR-S
Rha	4	6	9	12	18	22	27	16
Fuc	1	4	1	0	1	0	1	0
Ara	18	26	43	33	18	11	4	20
Xyl	27	21	11	4	2	2	4	11
Man	2	1	0	1	1	1	1	0
Gal	14	13	14	21	30	33	35	18
Glc	2	2	1	0	0	0	0	2
GalA	32	27	21	29	30	31	28	33

from Fraction 5. Fraction 7.2 (containing oligomer **I**); Fraction 6.5 (containing oligomer **II**); and Fraction 5.12 (containing the two largest oligomers **III** and **IV**, that could not be separated by HPAEC) were available in amounts sufficient for ^{1}H NMR spectroscopic analysis.

Characterization of rRG-lyase Oligomers from MHR-S Using NMR

Fraction 7.2. Figures 2a and 2b show the ^{1}H NMR spectra of Fractions 7.2, 6.5 and 5.12. Figure 2a is the region to low field of the residual water signal and Figure 2b shows all the peaks to high field of this position, except for a group of doublets at ~ 1.3 ppm. The spectrum of Fraction 7.2 was discussed previously (Chapter 3). Assignments made with the help of two-dimensional NMR spectra allowed the primary structure of the major oligosaccharide in Fraction 7.2 to be determined as **I**. The feature that most clearly distinguished the degradation products of rRG-lyase from those of RG-hydrolase was the doublet *a* (δ 5.81) in Figure 2a, which was assigned to H4 of a α-Δ-(4,5)-unsaturated-GalA (us-GalA) residue at the nonreducing terminus of the oligosaccharide. Other assignments of

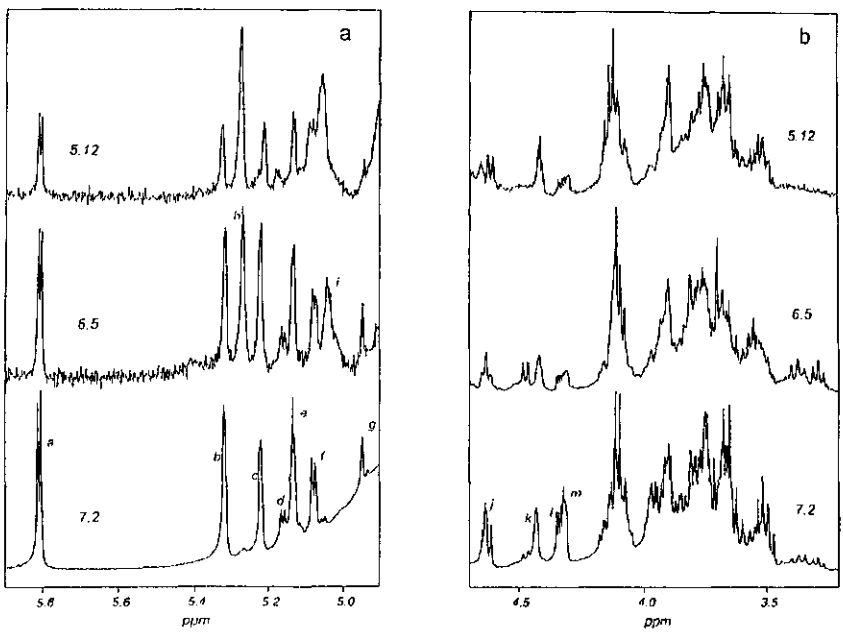


Figure 2. *The 400 MHz ^{1}H NMR spectrum of Fractions 5.12, 6.5 and 7.2 (27 °C). RE = reducing end, NR = nonreducing end.*

2a: Low field region. Key: a, H4 us-α-GalA (NR); b, H1 α-Rha (linked to NR); c, H1 α-Rha (RE); d, H1 α-GalA (linked to β-RE); e, H1 us-α-GalA (NR); f, H1 α-GalA (linked to α-RE); g, H1 β-Rha (RE); h, H1 α-Rha (unit C, Table II); i, H1 α-GalA (unit D, Table II).

2b: High field region. Key: j, H1 β-Gal and H5 α-GalA; k, H4 α-GalA; l, H3 us-α-GalA (NR); m, H2 α-Rha (linked to NR). An impurity (lactate?) is present in all the spectra with signals at δ 4.11 (quartet) and 1.33 (doublet).

well resolved signals are indicated in the figure captions. They established that a Rha residue (H1 signals *c* and *g*) was at the reducing end whilst the relative intensities of the remaining anomeric signals (*d*, *f* for α-GalA; *b* for α-Rha) showed that in addition to the terminal residues the oligosaccharide in Fraction 7.2 had one internal Rha-GalA disaccharide unit. The linkage positions were confirmed by a ROESY experiment. H1 signals of β-Gal residues were also identified (doublets *j* at δ 4.63 in Figure 2b). These residues were linked to C-4 of the Rha units, as found for the oligosaccharides resulting from RG-hydrolase treatment of MHR-S (Colquhoun et al., 1990). Fraction 7.2 consisted almost entirely of the hexasaccharide I. The integration of the β-Gal H1 signal was difficult (because of proximity to the irradiated water signal and overlap with GalA H5). The presence of unsubstituted Rha units would be indicated by Rha H4 signals below δ~3.5 or H6 signals below δ~1.28 (Colquhoun et al., 1990). Since neither of these signals was observed we conclude that all the Rha residues are substituted at C-4. The two weak triplets at δ 3.3-3.4 have a different origin as discussed for Fraction 6.5.

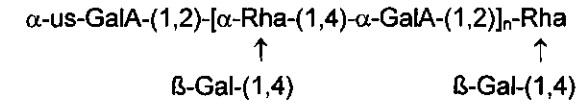
Fractions 5.12 and 6.5. The anomeric signals discussed above were also clearly identifiable in spectra of Fractions 5.12 and 6.5. A detailed two-dimensional NMR study (COSY, ROESY and HOHAHA) was made of Fraction 6.5 to determine the ^{1}H chemical shifts of the major oligosaccharide and these are presented in Table II. The basic features were very similar to those reported for Fraction 7.2 (see above) but with the addition of a further internal Rha-GalA unit. It is seen from Table II that the Rha H1 and H2 chemical shifts are sensitive to whether the neighboring residue is GalA or us-GalA. Otherwise the chemical shifts of protons in comparable residues were nearly the same. In particular, the Rha H4 chemical shifts (δ 3.6 - 3.7) showed again that all the Rha residues carried a β-Gal substituent. The two new H1 resonances are labeled *h* (Rha) and *i* (GalA) for Fraction 6.5 in Figure 2a.

Table II. *^{1}H Chemical shifts of oligosaccharide II (Fraction 6.5).*
n.d., Not determined. -, Not present.

		Chemical Shift (δ)					
Unit	**Code[a]**	H-1	H-2	H-3	H-4	H-5	H-6
Rha	Aα	5.22	3.97	4.09	3.71	3.95	1.34
	Aβ	4.95	4.06	3.86[b]	3.60[b]	3.52[b]	1.35[b]
α-GalA	B	5.08,5.16[c]	3.92,3.97[c]	4.13,4.15[c]	4.42	4.64	-
α-Rha	C	5.27	4.13	4.09	3.67	3.85	1.30
α-GalA	D	5.04	3.93	4.12	4.42	4.64	-
α-Rha	E	5.32	4.31	4.09	3.62	3.85	1.29
us-α-GalA	F	5.14	3.80	4.34	5.81	-	-
β-Gal[d]		4.63	3.51	3.66	3.90	n.d.	n.d.
?β-Xyl[e]		4.48	3.29	3.55	3.78	3.37,4.10	-

[a] Backbone sugar units are coded A-F from reducing end to nonreducing end
[b] Tentative assignment (weak signals)
[c] Two values are for unit B linked to Aα and Aβ respectively
[d] Three residues, linked to A, C, and E. Not distinguished, δ values differ by < 0.01 ppm
[e] Detected in Fraction 6 but not part of oligosaccharide **II** (see text)

Two signals appeared in the same positions, but with greater relative intensity, in the spectrum of Fraction 5.12. Signal *h* had intensity 1.1 (for Fraction 6.5) and 2.5 (for Fraction 5.12) with respect to signal *b* (known to represent a single proton). This suggests that Fraction 6.5 contains a mixture of **II** (major) and **III** (minor) with Fraction 5.12 containing a mixture of **III** and **IV** in roughly equal proportions. Thus it appears that the four major peaks found in the HPAEC analysis (Fig. 1b) can be identified with the oligosaccharides **I**-**IV**:



n = 1: **I**
2: **II**
3: **III**
4: **IV**

Four major oligomers are identified, in contrast with Kofod et al. (1994), who reported only two major oligosaccharides released by rRG-lyase from MHR-S. However, the mass spectrometry data of Azadi et al. (1995) showed that rRG-lyase released partially galactosylated oligomers with an RG backbone of degree of polymerization (DP) 4, 6 and 8 from sycamore RG I.

The overall sugar analysis (Table I) indicates some deviation from the expected 1:1:1 ratios for Rha:Gal:GalA, as well as the presence of additional sugars, especially Ara. The presence of a very large number of minor components can be seen from the HPAEC elution patterns (Fig. 1b) and is most marked for Fraction 6 (between 2 and 20 min). Although the great majority of the NMR signals observed could be attributed to **I** to **IV**, there were additional signals in the spectrum of Fraction 6.5 which could not be assigned to these RG oligosaccharides. None of these additional signals could definitely be assigned to Ara residues, but one complete network of signals was identified as originated from a β-Xyl residue (Table II). These signals were most prominent in Fraction 6.5, weak in Fraction 7.2 and absent from 5.12. Well-resolved signals belonging to the network are the H1 doublet at δ 4.48 and two triplets at δ 3.3 to 3.4 (Fig. 2b, Fraction 6.5). The chemical shifts and the coupling pattern indicated that the signals might arise from a β-Xyl residue. A further unassigned signal at δ 4.75 (singlet or unresolved doublet) was present, again strong in Fraction 6.5 and weak in the other two samples. This chemical shift is characteristic of H1 of GalA, branched at C-3 with β-Xyl, as in xylogalacturonan subunits of the pectic hairy regions (Schols et al., 1995a). It suggests that some oligosaccharides of the xylogalacturonan type may be present in Fraction 6.5, but there is no evidence that they are linked to the well characterized RG oligosaccharides.

Influence of MHR Acetyl Groups on the Activity of rRG-lyase

The effects of the acetyl substituents, reported to occur on the RG part of pectin (Schols et al., 1990b; Ishii, 1995), on the V_{max} and the K_m of rRG-lyase for MHR was investigated. The DA (calculated as the number of moles acetyl groups per 100 moles GalA residues) of MHR is 57, while treatment of MHR with NaOH resulted in an almost complete removal of acetyl groups (DA of MHR-S is 1). The RG-acetylesterase was able to remove 72% of the acetyl groups originally present in MHR. This is in agreement with the findings of Searle-Van Leeuwen (1992). According to Kauppinen et al. (1995) only 60% of the available acetyl groups could be removed, which they subscribe to the use of a different MHR batch. The DM (calculated as the number of moles of methoxyl groups per 100 moles of GalA residues) of MHR treated with excess RG-acetylesterase and of parental MHR were similar (DM 23), confirming that no methoxyl groups were removed by the RG-acetylesterase. K_m and V_{max} values of rRG-lyase were determined using MHR, MHR-S, and MHR batches with different DA values. The results are shown in Figure 3.

It is clear that the affinity of rRG-lyase decreases drastically when the DA increases: the K_m is over ten times higher for MHR than for MHR-S. It must be noted that MHR-S (data point at DA 1 in Fig. 3) is the only sample that has all methoxyl groups removed as a result of the saponification. However, the minor difference in V_{max} of rRG-lyase toward MHR-S and toward MHR DA 16 suggests that the methoxyl groups do not have a large influence on rRG-lyase action, similar to RG-hydrolase (Schols et al., 1990a). It can be concluded from Figure 3 that the affinity for highly acetylated MHR will be extremely low. It is remarkable that the affinity of rRG-lyase for MHR (K_m 0.35% w/v) is not much different from that of RG-acetylesterase toward MHR (K_m 0.3-1.0% w/v) (Kauppinen et al., 1995). The effect of the DA on the V_{max} of rRG-lyase is only moderate, but the 60% lower V_{max} of rRG-lyase toward MHR compared to MHR DA 16 shows that it becomes catalytically more difficult to cleave the RG backbone when more acetyl groups are present. The

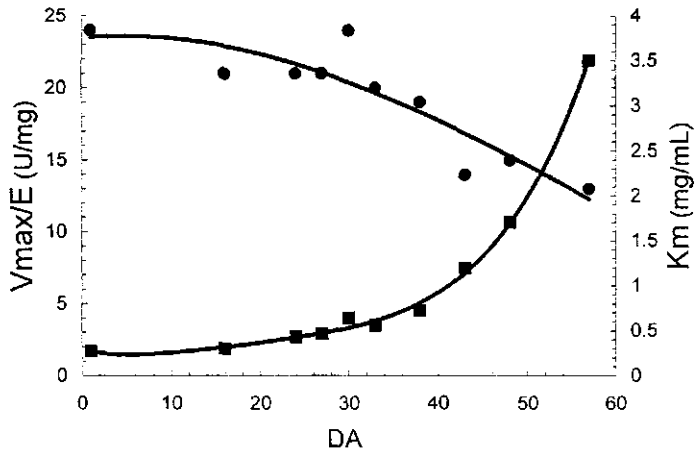


Figure 3. *Influence of the degree of acetylation (DA) of MHR on the V_{max} (●) and K_m (■) of rRG-lyase.*

Table III. *Specificity constant of rRG-lyase toward MHR samples in various stadia of deacetylation (K_m and V_{max} see Fig. 3)*

Substrate	$(V_{max}/[E])/K_m$ (units mL mg^{-2})
MHR DA 57	3.7
MHR DA 48	8.8
MHR DA 43	12
MHR DA 38	26
MHR DA 33	36
MHR DA 30	38
MHR DA 27	46
MHR DA 24	49
MHR DA 16	70
MHR-S DA 1[a]	89

[a] This is saponified MHR, therefore both acetyl and methoxyl esters have been removed

V_{max} of rRG-lyase toward MHR is even higher than that of RG-acetylesterase toward MHR: 13 versus 0.8-1.3 units mg^{-1} (Kauppinen et al., 1995). The overall effect of the kinetic parameters can be expressed as the "specificity constant": k_{cat}/K_m, which equals $(V_{max}/[E])/K_m$ (Table III) (Fersht, 1985). The calculated constants indicate that rRG-lyase is 25 times more specific for MHR-S than for MHR, and that rRG-lyase is 20 times more specific for MHR DA 16 than for MHR DA 57. For comparison, the majority of alginate lyases are also known to be hindered by the presence of O-acetyl groups on the C-2 and C-3 positions of D-mannuronosyl residues. Xanthan lyases, on the other hand, are not hindered by the presence of acetyl groups (Sutherland, 1995).

Influence of MHR Side Chains on the Activity of rRG-lyase

The influence on rRG-lyase activity of the Ara and Gal containing side chains (together comprising 38 mol%), attached to the RG part of MHR, was investigated. MHR-S was treated with several glycolytic enzymes and enzyme combinations. No enzymes were available able to remove the β-(1,3)-linked Xyl from the xylogalacturonan part of MHR (Schols et al., 1995a). However, from the data collected so far, rRG-lyase was not likely to act toward the xylogalacturonan part of the hairy regions. After the enzyme treatment, the molecular mass distribution of the MHR-S samples (as determined using HPSEC, not shown) had not changed, which indicated that the backbone of the samples was not degraded.

In Table IV the sugar composition of the enzyme treated substrates is presented. Calculations were performed to check if other sugars than Ara (e.g. in case of deAra-1) were removed. Thereto, for each batch the increase in mol% of other sugars was calculated assuming that only the Ara-, or Gal-, or Ara+Gal (in case of deAra-deGal)-content decreased. From the difference between these theoretical data and the sugar composition data actually found, it could be concluded that from most enzyme treated MHR-S batches also some Glc was removed, in case of the deGal-samples also some Ara was removed, and some Rha was removed when the β-galactosidase was used (results not shown), presumably by contaminating enzyme activities. From Table IV it can be seen that not all Ara

Table IV. *Sugar composition of the enzyme treated MHR-S fractions in mol%*

Sugar	MHR-S	MHR-S deAra-1	MHR-S deAra-2	MHR-S deAra-3	MHR-S deGal-1	MHR-S deGal-2	MHR-S deGal-3	MHR-S deAra-deGal
Rha	14	18	16	19	12	15	15	14
Ara	19	9	9	5	16	17	17	7
Gal	18	20	19	19	13	16	14	13
Glc	5	2	4	4	4	5	3	3
Xyl	14	19	17	21	13	18	19	27
GalA	30	32	35	32	42	29	32	36

could be removed, not even by the combination of the arabinofuranosidase and the arabinanase (maximum 74% of Ara removed). Attempts to remove Gal were even less successful (maximum 28% of Gal removed). Apparently other types of these enzymes with other specificities are required. The fact that MHR-S can be completely degraded by the *A. aculeatus* preparation Pectinex Ultra SP, be it after prolonged incubation, suggests that all required enzyme activities for side chain and backbone degradation are present in the preparation, but probably as very minor constituents, like the RG-rhamnohydrolase (Chapter 2) or the rRG-lyase.

The V_{max} and the K_m of rRG-lyase toward these substrates were determined. Since the RG region of MHR is the true substrate for rRG-lyase, and owing to the enzyme treatments the various MHR-S batches contained varying amounts of RG, the K_m values initially obtained were adjusted to represent the same RG content (based on the Rha content, Table IV) as MHR-S. These corrected values are presented in Table V. The K_m values for the substrates treated with endo-arabinanase (MHR-S deAra-2 and -3) and for the substrate treated with the β-(1,4)-galactanase (MHR-S deGal-2) were lower than the value for MHR-S. Treatment with arabinofuranosidase only (MHR-S deAra-1), with the β-galactosidase only (MHR-S deGal-1), with the combination of β-galactosidase and β-(1,4)-galactanase (MHR-S deGal-2), and with the combination of all four enzymes (MHR-S deAra-deGal) did not significantly influence the K_m. It is inconsistent that the K_m for the latter two samples had not decreased, since for the modification of these samples the same enzymes were used as for MHR-S deAra2, -deAra3 and -deGal2.

The improved action of rRG-lyase after removal of Ara side chains from MHR-S by endo-arabinanase, suggests that the Ara side chains sterically hinder the rRG-

Table V. *V_{max} and K_m of rRG-lyase toward enzyme treated MHR-S samples*
The initially obtained K_m values for the enzyme treated MHR-S batches were thus adjusted to represent the same RG content (based on the Rha content) as the original MHR-S.

Substrate	V_{max}/[E] (units mg^{-1})	K_m (mg mL^{-1}))	(V_{max}/[E])/K_m (units mL mg^{-2})
MHR-S	28 (± 1.3)	0.33 (± 0.09)	85
MHR-S deAra-1	26 (± 0.8)	0.32 (± 0.03)	81
MHR-S deAra-2	25 (± 0.8)	0.20 (± 0.03)	125
MHR-S deAra-3	24 (± 0.1)	0.20 (± 0.004)	120
MHR-S deGal-1	20 (± 0.5)	0.32 (± 0.02)	63
MHR-S deGal-2	16 (± 0.4)	0.25 (± 0.02)	64
MHR-S deGal-3	18 (± 0.9)	0.35 (± 0.05)	51
MHR-S deAra-deGal	17 (± 1.6)	0.39 (± 0.11)	44

lyase. Although the arabinofuranosidase is able to remove the same amount of Ara from MHR-S, the K_m is not affected. This could be explained by the fact that the arabinofuranosidase, able to cleave off terminal α-(1,2)-, α-(1,3)- and α-(1,5)-linked Ara units (Beldman et al., 1993), cannot pass a Gal unit if present e.g. in the subbranches of the longer Ara side chains, thus leaving large stretches of these longer side chains intact that might sterically hinder the rRG-lyase. The endo-arabinanase on the other hand is able to attack the longer side chains at locations closer to the RG backbone, so that only small or no side chains will be left. Improved action of rRG-lyase toward RG I regions when Ara side chains were removed was also reported by Azadi et al. (1995). They found that neither rRG-hydrolase or rRG-lyase were able to fragment sycamore RG I unless most Ara units had been removed by trifluoroacetic acid hydrolysis.

The V_{max} of rRG-lyase toward the treated MHR-S substrates was slightly lower when Ara was removed, but the Gal removal, although marginal, decreased the V_{max} markedly. Apparently, Gal had been removed near regions where cleavage could occur. The results suggest that the single unit Gal side chains attached to C-4 of Rha might play an important role in the cleavability of the Rha-GalA linkage by rRG-lyase. The effectivity of cleavage by rRG-lyase of the various enzyme treated MHR-S batches was expressed in the specificity constant $(V_{max}/[E])/K_m$ (Fersht, 1985). Clearly, the substrates treated with endo-arabinanase were cleaved with the highest catalytic efficiency.

Degree of Multiple Attack of rRG-lyase

When the time course of degradation of MHR-S by rRG-lyase was followed, the rapid shift in molecular mass of MHR-S upon HPSEC was accompanied by a rapid increase in oligomers formed as detected using HPAEC (not shown). The same set of four oligomers was formed from the start, in a ratio that did not significantly change during progress of the degradation. Therefore, it was hypothesized that rRG-lyase fragmented MHR-S with a certain degree of multiple attack. In a multiple attack mechanism, once the enzyme forms an enzyme-polymer complex, in which there is suitable geometry for catalysis, the enzyme may catalyze the hydrolysis of several bonds before it dissociates and forms a new active complex with another polymer chain. The multiple attack mechanism can be seen as a general concept including *single chain* ("zipper" *fashion*) *and multichain* (*random attack*) *mechanisms* as extreme special cases (Robyt and French, 1967).

The degree of multiple attack may be defined as the average number of catalytic events, following the first, during the lifetime of an individual enzyme-substrate complex (Robyt and French, 1967). For calculation of the degree of multiple attack the ratio (r) has to be calculated. This is the ratio between the total amount of linkages that are split, i.e. the sum of both newly produced polymer and oligomer fragments, and the number of effective encounters, i.e. newly produced polymer fragments. This ratio gives the number of bonds that is broken per effective encounter. Since the first bond broken releases a polymer fragment, the average

number of *subsequent* bond broken (r-1) is numerically equal to the degree of multiple attack (Robyt and French, 1967).

As a measure for the total amount of linkages split, Robyt and French (1967) determined the increase in reducing value of the total amylose digest during degradation. To obtain the number of effective encounters, they determined the increase in reducing value of the 67% ethanol precipitate of the digest, which contains polymers. In this study, instead of ethanol precipitation to separate polymers from oligomers, HPSEC was performed of MHR-S digests, and a separation between oligomers and polymers was made at the retention time of 29 min, corresponding with a molecular mass of ~ 3000 D (~ DP 20). Under the conditions used by Robyt and French (1967) the smallest polymer that could be precipitated by 67% ethanol had an average DP of 20 as well. Subsequently, the number-average molecular mass was calculated, using GPC/PC software, of the total digest and the thus defined polymer fraction. From the number-average molecular mass and the carbohydrate content of the total digest, the total number of molecules present in a sample was calculated. The area percentage that the polymers formed from the total HPSEC chromatogram (the refractive index detection correlates with the carbohydrate concentration) was used to determine the carbohydrate content of polymers in a sample. From this carbohydrate content, and the number-average molecular mass, the number of polymers in a sample was calculated. Finally, the parameter r was calculated from the increase in the total number of molecules in the digest, divided by the increase in the number of polymers in the samples during degradation.

While Robyt and French (1967) used a fixed concentration of enzyme mg^{-1} substrate and took samples at increasing incubation times, in this study samples of progressing degradation were obtained by using increasing amounts of enzyme mg^{-1} substrate with a fixed incubation time. In Figure 4a the number of total molecules and the number of polymers present in the samples are shown. Up to a concentration of 60 ng rRG-lyase mg^{-1} substrate, the number of polymers still increased. Above this concentration, the number of polymers decreased, which means that degradation has advanced thus far, that all newly formed fragments fall into the category of oligomers. Obviously this results in a rapidly increasing r-1 value, as shown in Figure 4b. Therefore, true r-1 values can only be obtained from samples where the number of polymers is still increasing. The average degree of multiple attack, r-1, calculated from the data points up to 60 ng rRG-lyase mg^{-1} substrate was 2.5. For RG-hydrolase a similar experiment was carried out and the degree of multiple attack was found to be almost twice as high: 4.0 (not shown). For different α-amylases values of multiple attack between 1.9 and 6.0 were measured (Robyt, 1984). Since the method of Robyt and French is hardly used in literature reports, no comparison with other than starch degrading enzymes could be made.

A major difference with the experiments of Robyt and French is that instead of long (average DP of 1000) starch homopolymers, the MHR as used here is a heterogeneous substrate, with a very broad distribution in molecular mass (major populations ranging between 80000 and 7000 D, i.e. roughly between DP 500 and 40. The alternating RG sequences in MHR might not be very long, in which case the

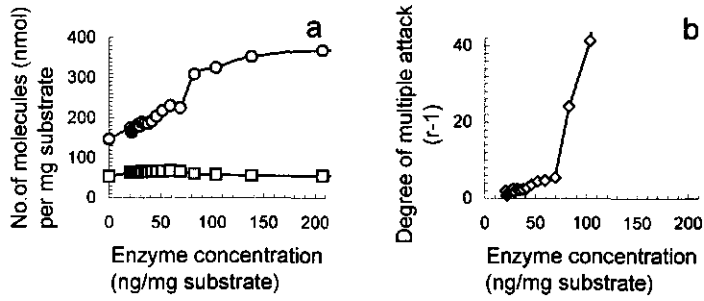


Figure 4. *Various parameters for determination of the degree of multiple attack (r-1) of rRG-lyase toward MHR-S; 4a: the number of total molecules (○) and polymers (□) present; 4b: the (r-1) values calculated from r, see text for explanation.*

degree of multiple attack as determined is underestimated. However, the values obtained for rRG-lyase and RG-hydrolase do give us valuable information about the length of these RG sequences in MHR. They show that the average length of alternating RG sequences in MHR would have to allow for the release of four (RG-hydrolase) and 2.5 (rRG-lyase) consecutive oligomers, respectively. It is known which oligomers are released from MHR-S by RG-hydrolase (major products with RG backbones of DP 4 and 6, Chapter 2), and therefore the RG sequences in MHR would have to be on average twenty (4+4+6+6) sugar residues long to release two oligomers of DP4 and two of DP 6. For rRG-lyase, the average sequence length would have to be twenty (4+6+8) sugar residues, to release three oligomers of DP 4, 6 and 8. The length of the RG I regions in MHR must therefore be rather longer than the average of thirteen units, as suggested by Schols and Voragen (1996) in their model.

Comparison of Native and rRG-lyase from *A. aculeatus*

The crude enzyme mixture Pectinex Ultra SP, produced by *A. aculeatus*, was first desalted on a Bio-Gel P10 column. Desalted protein was applied to a DEAE Bio-Gel A anion-exchanger at pH 5. RG-lyase was present in the unbound protein. On subsequent chromatography of the unbound protein using a MonoS HR 5/5 cation-exchanger at pH 4.25, RG-lyase eluted in a distinct peak at 40 mM NaCl. Pooled RG-lyase fractions represented 0.1% in weight of the originally desalted protein.

The characteristics of native RG-lyase are summarized in Table VI. SDS-PAGE of RG-lyase revealed a major protein band at 76 kD and a minor one at 57 kD. Upon chromatography of RG-lyase using a calibrated Superose 12 column, RG-lyase eluted in the tail (57 kD) of the major protein peak. The nature of the major protein is yet unknown, since no activity toward various glycans and pnp-glycosides could be detected. The pI of RG-lyase, determined using zymography (Chapter 5), was 5.1 to 5.3. RG-lyase was found to be most active between 50 and 60 °C and was stable for 4.5 h up to 40 °C. A pH optimum of 6 was found, and the enzyme was

Table VI. *Characteristics of native RG-lyase from A. aculeatus, compared with rRG-lyase from A. aculeatus (Kofod et al., 1994)*

Characteristic	native RG-lyase	rRG-lyase[a]
Molecular mass (kD)	57	55
pI	5.1-5.3	5.2
pH optimum	6	6
Stable 4.5 h at pH	> 6	> 6
Temperature optimum	50-60 °C	50 °C
Temperature stability	stable 4.5 h up to 40 °C (100% activity)	activity after 2 h 60 ° C is 70%
Recognition by antiserum raised against RG-hydrolase	negligible (dot-blot)	no (Western blot)

[a] Data from Kofod et al. (1994)

stable at pH 6 and higher (measured up to pH 8). The pH optimum determined from the increase in A_{235} was the same as derived from HPAEC peak area. Dot-blotting was performed to investigate whether native RG-lyase showed immunological cross-reactivity to a polyclonal rabbit antiserum raised against purified native RG-hydrolase. The antiserum reacted only in negligible amounts with RG-lyase but strongly with RG-hydrolase, suggesting that the two polypeptides are structurally different, representing two different enzymes.

Recombinant RG-lyase from *A. aculeatus* was purified from the culture supernatant of an *A. oryzae* transformant. The reaction products that are formed from MHR-S upon incubation with native RG-lyase from *A. aculeatus* or rRG-lyase showed similar HPSEC and HPAEC elution patterns (not shown). The data on temperature and pH optimum and stability of rRG-lyase, as determined by Kofod et al. (1994), are shown in Table VI. Small differences in characteristics can be explained by inter-laboratory assay variation and the different assays and assay conditions employed. It can be concluded that rRG-lyase and native RG-lyase from *A. aculeatus* are essentially the same enzyme.

The importance of RG degrading enzymes like RG-lyase will most certainly be established in the future for example in the field of the oligosaccharins. RG I generated from cultured *Acer* cell walls by pectinase digestion has been demonstrated to have wound-signal activity (Ryan et al., 1981). From mucilage of germinated cress seeds us-GalA-(1,2)-Rha disaccharides were isolated (as the sodium salt and named lepidimoide), which appeared to promote *Amaranthus* hypocotyl elongation (Hasegawa et al., 1992). Interestingly, in Pectinex Ultra SP, from which RG-lyase was purified, enzyme activities have been discovered by the authors that are able to degrade all four RG-lyase MHR-S oligomers completely into Gal and one unknown product, which, regarding the elution behavior, is tentatively identified as the us-GalA-(1,2)-Rha dimer. This implies that a β-galactosidase and a new lyase have been active, and that more new enzyme activities can still be purified from the *A. aculeatus* preparation. From these observations and the literature reports on this subject it is anticipated that RG-lyase will be very useful in the investigation of the biological activity of Δ-(4,5)-unsaturated-RG oligosaccharides.

CONCLUSIONS

rRG-lyase releases α-Δ-(4,5)-unsaturated RG oligosaccharides I to IV from MHR-S, which are completely galactosylated and have backbone DP's of 4, 6, 8 and 10, confirming the results of Azadi et al. (1995), who found partially galactosylated oligomers with RG backbones of DP 4, 6 and 8. These oligomers are larger than the major products released from MHR-S by RG-hydrolase (backbone DP's of 4 and 6, Colquhoun et al., 1990; Chapter 2). Apparently rRG-lyase has more subsites than RG-hydrolase. This is confirmed by the observation that RG-hydrolase is able to cleave smaller linear RG oligomers than rRG-lyase (Mutter et al., 1996b). The degree of multiple attack toward MHR-S of RG-lyase (2.5) and RG-hydrolase (4) reveals that in MHR the alternating RG I sequences have to be at least twenty units long, in contrast with the model of Schols and Voragen (1996), where the RG I regions are suggested to be on average thirteen units long. The catalytic efficiency of rRG-lyase for MHR increases with decreasing degree of acetylation. Removal of Ara side chains improves the action of rRG-lyase toward MHR-S. In contrast, removal of Gal side chains decreases the catalytic efficiency of rRG-lyase. Native RG-lyase was purified from *A. aculeatus*, characterized and found to be similar to the rRG-lyase expressed in *A. oryzae* (Kofod et al., 1994; Mutter et al., 1996a).

ACKNOWLEDGMENTS

Thanks are due to Jan van Iersel for his contribution to the isolation of RG-lyase MHR oligomers; to Marjo Searle-Van Leeuwen for purification of the β-galactosidase from *A. niger*; to Ben van den Broek for his work on the immunoblotting; to Marcel Mischler, Yvette Schnell, and Dr. Kurt Dörreich from Novo Nordisk Ferment Ltd (Dittingen, Switzerland) for purification of RG-hydrolase; and to Dr. Lene Venke Kofod and Dr. Hans-Peter Heldt-Hansen from Novo Nordisk (Denmark) for supplying crude rRG-lyase and the antiserum raised against RG-hydrolase.

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Chapter 5

Application of Cu^{2+} staining for detection of rhamnogalacturonase activity toward modified hairy regions of pectin

This chapter has been submitted to *Analytical Biochemistry* by the authors Margien Mutter, Gerrit Beldman, Valérie L.C. Klostermann, Yvette Schnell[1], Kurt Dörreich[1], Henny Berends, Henk A. Schols, Alphons G.J. Voragen.

An easy method for the detection of rhamnogalacturonan (RG) endo-degrading enzymes (RGases[2]), more specifically RG-hydrolase and RG-lyase, is described. In this method, applied in plate assays and zymography, copper acetate is used as staining reagent. Detection of enzyme activity is based on a white precipitate of Cu^{2+} ions with xylogalacturonan molecules. The latter are released from the pectin substrate used, the modified hairy regions (MHR), when RGases degrade the connected RG regions. Therefore, this method requires ramified pectin substrates, that contain beside RG regions also xylogalacturonans, as present in apple, onion, pear, and watermelon. Beside in the enzyme preparation from *Aspergillus aculeatus*, the presence of RGase activity could also be established in experimental preparations from *Aspergillus niger* and *Aspergillus japonicus*. Using IEP zymography, pI's of RG-hydrolase (4.4) and RG-lyase (5.0 and 5.2) could be determined, even in partially purified enzyme fractions. Image analysis was shown to be very useful to record and analyze the IEP zymography results.

Since the discovery of the RG degrading enzyme, RGase, by Schols et al. (1990a), an increasing number of reports dealing with this new type of pectin degrading activity have been published (Matsuhashi et al., 1992; Düsterhöft et al., 1993; An et al., 1994; Kofod et al., 1994; Sakamoto et al., 1994; Azadi et al. 1995). Recently a new type of RGase, an RG α-L-rhamnopyranosyl-(1,4)-α-D-galactopyranosyluronide lyase (RG-lyase), has been identified (Mutter et al., 1996: Chapter 3). The hydrolyzing RGase (Schols et al., 1990a) is now named RG-hydrolase. In this chapter the word RGase is used as a general description of endo-acting RG degrading enzymes, i.e. both RG-hydrolase and RG-lyase. The most accurate analytical tool for the detection of RGase activity so far has been high-performance size-exclusion chromatography (HPSEC) combined with high-performance anion-exchange chromatography (HPAEC). HPSEC is used to detect MHR backbone degradation, and the typical oligomers produced by RG-hydrolase or RG-lyase can be distinguished using HPAEC (Chapter 3).

[1] Novo Nordisk Ferment Ltd., Neumatt, CH-4243 Dittingen, Switzerland
[2] See List of Abbreviations

This report describes a rapid and sensitive staining method for plate assays and zymography, using copper acetate, which appeared to be very specific for the degradation products of modified hairy regions (MHR) of apple pectin by the action of RGases. Schols and Voragen (1996) postulated a model for apple MHR, in which three structural units are interlinked. Subunit I consists of xylogalacturonan molecules (Schols et al., 1995). RG regions with a Rha : GalA ratio < 1 and arabinan side chains attached to Rha are named subunit II. Strictly alternating RG regions with single unit β-Gal side chains attached to C-4 of Rha are named subunit III, and can be cleaved by RG-hydrolase and RG-lyase (Schols et al., 1990a; Chapter 4). Saponified MHR (MHR-S) was used in this method since both RG-hydrolase and RG-lyase are hindered by the presence of acetyl groups (Searle-Van Leeuwen et al., 1992; Kauppinen et al., 1995; Chapter 4). The copper acetate staining was used for detection of RGase activity in crude enzyme mixtures, and in isoelectric focusing (IEF) zymograms of (partially) purified enzyme fractions. The use of image analysis to visualize the combined results of IEF zymography is demonstrated.

MATERIALS AND METHODS

Enzymes

RG-hydrolase was purified from Pectinex Ultra SP according to Schols et al. (1990a), and RG-lyase was purified from the same source as described in Chapter 4. A partially purified exogalacturonase from *Aspergillus aculeatus*, able to degrade xylogalacturonan, was similar to the one described by Beldman et al. (1996).

Simultaneous fractionation of both RGases was commenced from 1000 mL of the commercial mixture Pectinex Ultra SP produced by *A. aculeatus*. Purification involved anion-exchange chromatography on a DEAE Sepharose FF column, cation-exchange chromatography on a SP Sepharose FF column, and anion-exchange chromatography on a Q-Sepharose hp column.

Enzyme preparations coded SP258, Pectinex AP18 ('AP18'), Novoferm 6 from *Aspergillus niger* and SP281 from *Aspergillus japonicus* were experimental preparations from Novo Ferment Ltd (Dittingen, Switzerland). Hemicellulase reg. II produced by *A. niger* was a gift from Gist-Brocades (Delft, The Netherlands).

Part of the samples was concentrated by ultrafiltration prior to zymography using Microcon 10 (kD) microconcentrators (Amicon Corporation, Danvers, MA).

Enzyme Assays

Incubations were carried out with 0.25% w/v solutions of apple MHR-S (described in Mutter et al., 1994: Chapter 2) in 50 mM NaOAc buffer pH 5 containing 0.01% w/v NaN_3 at 40 °C for 24 h. Enzymes were added in amounts sufficient for complete degradation of suited substrate in at most 6 h. Screening of column fractions during enzyme purification was performed essentially according to this procedure. Enzyme digests were analyzed using HPSEC and HPAEC. Distinction between RG-hydrolase

or RG-lyase activity was established using HPAEC by detection of the typical RG degradation products (Chapter 3).

Gel Electrophoresis

Electrophoresis was carried out with a PhastSystem (Pharmacia LKB Biotechnology, Uppsala, Sweden), according to the instructions of the supplier. The pI's were deduced from pH 3 to 9 or pH 4 to 6.5 IEF gels, using standards from the broad or narrow pI calibration kit (Pharmacia). The gels were stained with Coomassie Brilliant Blue R-250.

Plate Assays

For plate assays, 4 mm thick 1% w/v agarose gels (electrophoresis grade, Bethesda Research Laboratories, Gaithersburg, MD) containing 0.5 % w/v MHR-S in 50 mM NaOAc buffer pH 6 were cast in petri dishes. Enzyme solutions (4 µl) were applied on top of the gels and incubation took place for 60 min at 30 °C, after which a saturated copper acetate solution was poured onto the gels. Gels were photographed as soon as possible but preferably within one hour.

Zymography

IEF gels with a pH range from 3 to 9 or 4 to 6.5 (PhastGel IEF Pharmacia) were used for zymography. After focusing the gel was cut lengthwise. The half with sample and a standard protein mixture was stained with Coomassie Brilliant Blue R-250. The other half containing only sample was carefully overlaid on top of a 4 mm thick 1% w/v agarose gel containing 0.5% w/v MHR-S in 50 mM NaOAc buffer pH 6, which had been cast in a transparent plastic box (59 x 47 mm, height 17 mm). The gel was subsequently smoothed to remove air bubbles, and the exact contact area of the IEF gel with the substrate gel was marked on the outside of the plastic box. After incubating for 30 min at 30 °C, the IEF gel was removed and a saturated copper acetate solution was poured onto the substrate gel. Using a dark background and oblique illumination from above, the appearance of white bands in the transparent blue substrate gel was photographed or scanned (see below) directly (preferably within one hour). The Coomassie stained half of the IEF gel (containing standard proteins) was compared directly with the copper acetate stained gel.

To record the results of this comparison, as well as to visualize them, image analysis was used. Coomassie stained gels and substrate gels with activity patterns were digitized with a MagiScan (Applied Imaging, Newcastle, UK) using a bright field transmitted and a dark field reflected method respectively. The two digital data sets were combined using image editing software (Adobe PhotoShop or Aldus PhotoStyler for Windows 3.1), and subsequently printed (Canon-CLC300 color laser printer). By this way activity stained bands and Coomassie stained bands could be matched. This allowed accurate determination of pI's of enzymes active in the zymogram.

Cu^{2+} Precipitation of MHR Samples

A 2.5% w/v solution (in 40 mM NaOAc buffer pH 5) of RGpoly, which is the polymeric fraction after size-exclusion chromatography (SEC) separation of the MHR-S degradation material produced by RG-hydrolase (Chapter 2), was mixed with saturated copper acetate solution to induce precipitation. The resulting mixture was centrifuged and pellet and supernatant were separated. After addition of a fresh amount of copper acetate solution to the supernatant no further precipitation occurred. To the pellet 0.5 M disodium-EDTA was added to dissolve the precipitated material. After dialyzing to remove the Cu^{2+} ions and EDTA, supernatant and pellet were lyophilized. Uronic acid content was determined with an automated colorimetric method using *m*-hydroxydiphenyl (Ahmed and Labavitch, 1977). Neutral sugars were determined by GLC after hydrolysis with 2 M TFA and subsequent derivatization of monomeric sugars to alditol acetates essentially as described in Chapter 4.

HPLC Methods

HPSEC and HPAEC were performed as described in Chapter 2.

RESULTS

Detection of RG-hydrolase and RG-lyase activity in several experimental technical preparations and purified enzymes in the plate assay is shown in Figure 1. No.'s 1 and 5 correspond with enzyme fractions containing RG-hydrolase, and no.'s 1, 3 and 7 with enzyme fractions containing RG-lyase. Whenever RGase activity was present in a sample, a white opalescent zone was observed. Enzymes were not inactivated by the presence of copper acetate, and after a few hours the boundaries of the white zones became diffuse and the white color spread throughout the gel (not shown). Additionally, in case of Pectinex Ultra SP (no. 1 in Fig. 1), after several hours the original white zone turned blue again.

The white opalescent area in the substrate gel was thought to result from a precipitate formed by Cu^{2+} ions with substrate degradation products produced by the RGases. The substrate used in the plate assays and zymograms was the saponified apple MHR as described in Chapter 2. It has been shown that both RG-hydrolase (Schols et al., 1990a) and RG-lyase (Chapter 4) act toward the strictly alternating RG regions with single unit Gal side chains, subunit III, in MHR. Action of these RGases sets free the other two types of subunit molecules. Kravtchenko et al. (1992) found that precipitation with Cu^{2+} ions, usually applied in the purification of the homogalacturonan (HG) type of pectin (Hwang et al., 1992), can be used to separate HG type pectins from pectins highly ramified with neutral sugar side chains. Therefore, it is most likely that the xylogalacturonan molecules, structurally similar to HGs, will precipitate with the Cu^{2+} ions. To prove this, the polymeric RG-hydrolase degradation products of apple MHR-S after SEC separation (RGpoly, Chapter 2), which contained 90% of all Xyl recovered in the SEC pools, were treated with copper acetate on preparative scale. A precipitate was formed and recovered by centrifugation. EDTA

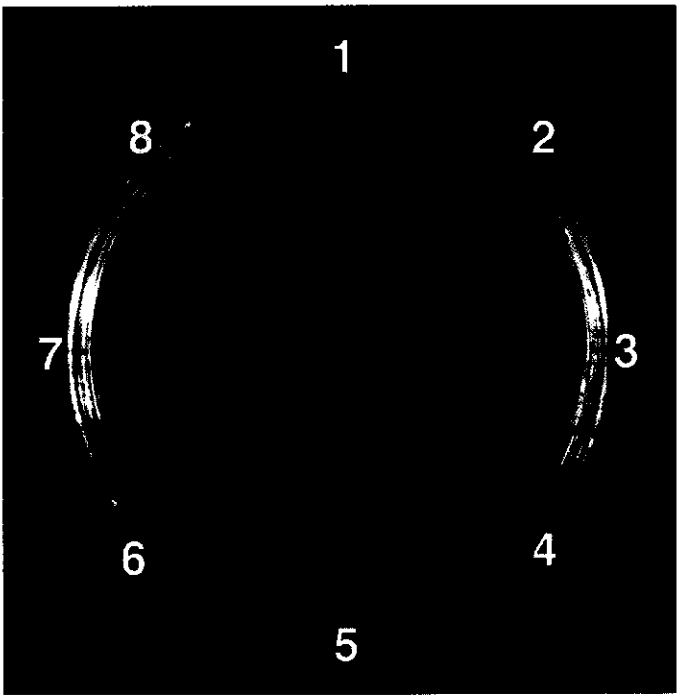

Figure 1. *Plate assay, 1 = Pectinex Ultra SP; 2 = Hemicellulase reg. II; 3 = purified RG-lyase; 4 = Novoferm 6; 5 = purified RG-hydrolase; 6 = partially purified exogalacturonase; 7 = partially purified RG-lyase; 8 = SP258.*

Table I. *Sugar composition (in mol%) of the polymeric degradation products of MHR-S produced by RG-hydrolase after SEC separation before (Original material) and after precipitation with copper acetate and subsequent centrifugation (Pellet and Supernatant). Amount of pellet/supernatant expressed as weight percentage of total recovered sugars.*

Sugar (mol%)	MHR-S + RG-hydrolase (polymers after SEC, Mutter et al., 1994)		
	Original material	Pellet	Supernatant
Rha	10	12	5
Ara	25	7	51
Xyl	20	30	5
Gal	13	6	22
Glc	2	2	2
GalA	30	43	15
% in weight of recovered sugars		70	30

could successfully be used to remove the Cu^{2+} ions from the precipitate, resulting in a clear solution. In Table I the sugar compositions of the untreated material, the Cu^{2+} precipitate and the supernatant are presented. From this table it can be seen that more than 90% of all Xyl and almost 90% of all GalA is recovered in the pellet. This shows that especially the xylogalacturonan molecules were precipitated with the Cu^{2+} ions and probably are responsible for the white precipitate in the plate assays. The supernatant on the other hand is enriched in Ara and Gal and has a higher Rha : GalA ratio, and presumably contains the arabinan rich RG stubs of the MHR backbone (subunit II).

The specificity of the Cu^{2+} staining was investigated using purified enzymes and several experimental enzyme preparations (Table II). Enzymes were not only tested in plate assays, but were also incubated with MHR-S in solution. The resulting digests were analyzed using HPSEC to determine a shift in molecular mass, indicating endo-type of degradation of the RG backbone in MHR-S. HPAEC was used to determine which mono- and oligomeric sugars were released: Rha and GalA and/or RG oligomers from the RG backbone; and Ara, Gal and Xyl and/or arabinan and galactan oligomers from the side chains (Schols et al., 1990b; Chapter 2). From Table II it can be seen that exclusively the enzymes able to degrade the RG backbone in MHR-S were positive in the plate assay. Enzymes only able to degrade MHR-S side chains were not positive in the plate assay. The exogalacturonase from *A. aculeatus*, which gradually degrades the xylogalacturonan in MHR-S in an exo-fashion, producing dimers of β-Xyl-(1,3)-GalA, was also not positive in the plate assay. The HPSEC elution patterns in Figure 2 show that the highest molecular mass populations of MHR-S have disappeared after degradation of MHR-S by Pectinex Ultra SP. HPAEC of the digests revealed that Pectinex Ultra SP released RG oligomers, arabinan oligomers, the dimer β-Xyl-(1,3)-GalA, and furthermore GalA, Rha, Ara and Gal from MHR-S. On

Table II. *Activity of various commercial enzyme preparations and purified enzymes from various sources toward MHR-S.*

Enzymes	Source	Cu^{2+} staining[a]	RG backbone degradation[b]	Side chain degradation[b]
RG-hydrolase	*A. aculeatus*	+	+	-
RG-lyase	*A. aculeatus*	+	+	-
Exogalacturonase	*A. aculeatus*	-	-	-[c]
Endo-arabinanase	*A. niger*	-	-	+
Arabinofuranosidase B	*A. niger*	-	-	+
Endo-ara.+ Ara.fur. B	*A. niger*	-	-	+
β-galactosidase	*A. niger*	-	-	+
Pectinex Ultra SP	*A. aculeatus*	+	+	+
Hemicellulase reg. II	*A. niger*	-	-	+
SP281	*A. japonicus*	+	+	+
SP258	*A. niger*	-	-	+
AP18	*A. niger*	±	+	+
Novoferm 6	*A. niger*	-	-	±

[a] Determined by Cu^{2+} staining after incubation of enzymes on agarose-MHR-S gels in plate assays

[b] Concluded from combined results from HPSEC and HPAEC analyses of MHR-S digests

[c] Dimers of β-Xyl-(1,3)-GalA are formed, resulting in backbone degradation upon prolonged incubation, see text

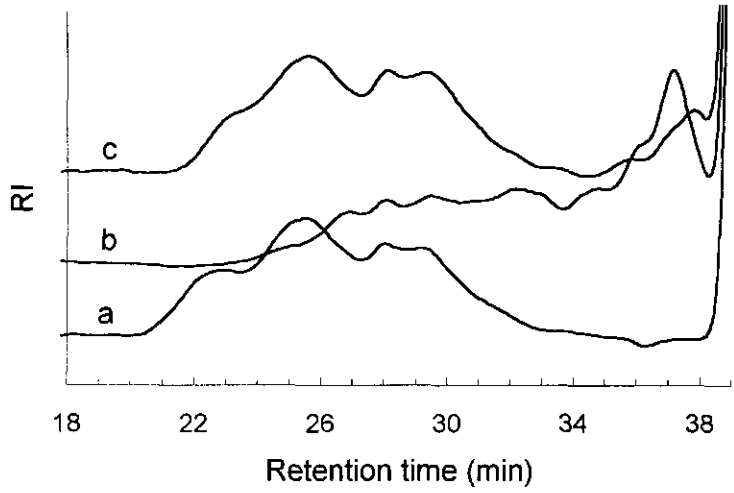

Figure 2. *HPSEC elution patterns of a, MHR-S; b, MHR-S after extensive treatment with Pectinex Ultra SP; and c, MHR-S after extensive treatment with Hemicellulase reg. II.*

the other hand, the molecular mass distribution of MHR-S had not changed much after degradation with Hemicellulase reg. II, while some material is detected in the oligomer/monomer region (36-38 min). As concluded from HPAEC analysis, Hemicellulase reg. II did not produce any RG oligomers or arabinan oligomers, but only released small amounts of the dimer β-Xyl-(1,3)-GalA, Ara, GalA, Gal, Xyl and some Rha. Although small amounts of RG backbone sugars were released, this did not cause a major shift of the molecular mass (HPSEC). These sugars are presumably produced by exo-enzymes such as the exogalacturonase (Beldman et al., 1996), RG-rhamnohydrolase (Chapter 2) and RG-galacturonohydrolase (Chapter 6).

Already in early stages of the purification of RGases from *A. aculeatus*, Cu^{2+} staining in IEP zymography showed that multiple RGases were present in Pectinex Ultra SP, based on differences in pI. During the first purification steps, RG-hydrolase and RG-lyase were always coeluting (not shown). Of pooled enzyme fractions, containing both RGases, zymography was performed using IEF gels. The result is shown in Figure 3, where image analysis was used to superimpose the image of Coomassie stained protein bands in an IEF gel on the image of the activity pattern of the corresponding zymogram. The chosen software settings resulted in a green color against a blue background for the Coomassie stained protein bands in Figure 3. Activity in the zymogram is indicated by the white area at those places. Where the color of the Coomassie stained protein bands is very intense, and coincides with activity in the zymogram, the color is red. In Figure 3 the results from Coomassie staining of the IEF gel and the results from the zymogram could be compared directly, enabling a very accurate determination of the pI of the active enzymes in the protein mixture. Figure 3 shows that in this enzyme fraction different RGases are present with

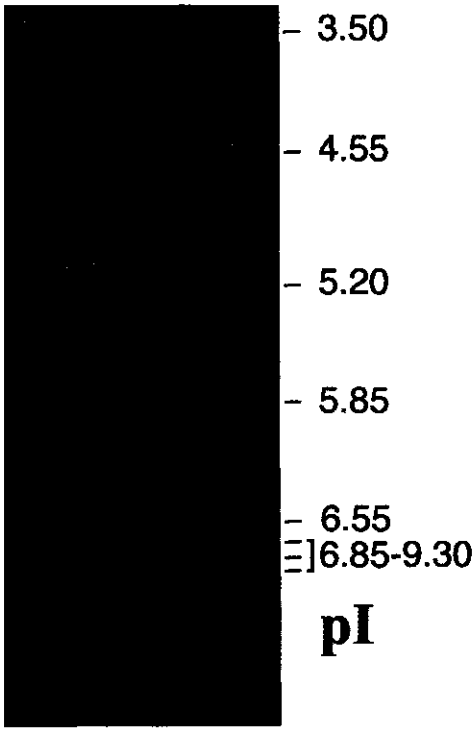

Figure 3. *Overlay produced by image analysis of a Coomassie stained IEF gel (pI 4-6.5) and the corresponding zymogram, of pooled protein fractions containing both RG-hydrolase and RG-lyase (eluting from the SP-Sepharose FF column at 124 mM NaCl, 20 mM NaOAc pH 4.25).*

pI's of 4.3 - 4.4 and 5.1. On subsequent chromatography of the pooled RGase containing fractions on a Q-Sepharose column, three RGase peaks were obtained (not shown). Two of them contained RG-lyase as determined by HPAEC, and were shown to have pI's of 5.2 and 5.0 respectively using IEF zymography. Earlier (Chapter 4), a pI range of 5.1 to 5.3 was found for RG-lyase from *A. aculeatus*, and Kofod et al. (1994) found a pI of 5.2 for their recombinant RG-lyase. The third RGase with a pI of 4.4 was identified as a partially purified RG-hydrolase. Kofod et al. (1994) reported a pI of 4.5 for their recombinant RG-hydrolase. An RG-hydrolase from *Trametes sanguinea* (Sakamoto et al., 1994) had characteristics similar to the RG-hydrolase from *A. aculeatus*, except for the high pI of 8.1.

DISCUSSION

The described plate assay and the IEF zymogram technique using copper acetate staining have been shown to be specific for RGases capable of endo-degradation of the RG backbone of modified hairy regions (MHR) of pectin. The xylogalacturonan molecules of MHR-S, released when RGases degrade the RG subunits, are involved in the precipitate formed after copper acetate addition. This precipitate is responsible for a white opalescent area in the blue gel. Since the staining involves xylogalacturonans, this particular method requires the use of ramified pectins containing these structures. Schols and Voragen (1994) found that beside apple MHR, also onion and pear MHR presumably contained xylogalacturonan, while carrot, leek and potato hardly contained any Xyl. Yu and Mort (1996) reported the presence of small amounts of xylogalacturonan in suspension cultured cotton cell walls, and large amounts of xylogalacturonan in the cell walls of watermelon.

Presumably the complex of Cu^{2+} with xylogalacturonan can not be degraded as such by enzymes. However, when beside RGases a mixture of other enzymes was present in the sample, as in case of Pectinex Ultra SP, after several hours the white color disappears. This suggests that the complexation of Cu^{2+} with xylogalacturonan is an equilibrium reaction, and that the uncomplexed xylogalacturonan is degraded by the relevant enzymes, causing a shift in the equilibrium reaction. An enzyme such as the exogalacturonase (Beldman et al., 1996) might be responsible, since it is capable of degrading xylogalacturonan in an exo-manner. Therefore, when used in combination with an RGase, this method could also be used for detection of enzymes able to degrade xylogalacturonan.

Nanograms of RGase could be detected in the plate assay, while submicrogram quantities were detected using the zymogram method. For the zymogram method higher enzyme concentrations were needed in order to be able to detect enzymes with Coomassie staining. Application of silver staining would enable the use of approximately ten times less enzyme. However, the limiting factor will probably be the amount of enzyme that migrates from the IEF gel into the substrate gel during incubation.

In the screening for RGases this simple method is time-saving, since for the detection of RGase activity in enzyme fractions the time-consuming HPLC analyses can be skipped. In the separation of RG-hydrolase from RG-lyase activity, the Cu^{2+} staining used in IEP zymography is a valuable tool to evaluate purification steps, since these enzymes from *A. aculeatus* differ in pI. The use of image analysis in IEP zymography was shown to be very useful to record and visualize the results, and helpful for an accurate determination of pI. Using this staining method, it could be shown (Table II) that beside *A. aculeatus* also *A. niger* and *A. japonicus* contain RGases. An RG-hydrolase from *A. niger* has been cloned by Suykerbuyk et al. (1997).

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Chapter 6

Rhamnogalacturonan α-D-galactopyranosyluronohydrolase. An enzyme that specifically removes the terminal nonreducing galacturonosyl residue in rhamnogalacturonan regions of pectin

This chapter has been submitted to *Plant Physiol* by the authors Margien Mutter, Gerrit Beldman, Stuart M. Pitson[1], Henk A. Schols, and Alphons G.J. Voragen.

A new enzyme, rhamnogalacturonan (RG[2]) α-D-galactopyranosyluronohydrolase (RG-galacturonohydrolase), able to release a galacturonic acid residue from the nonreducing end of RG chains and not from homogalacturonan (HG), was purified from an *Aspergillus aculeatus* enzyme preparation. RG-galacturonohydrolase acted with inversion of anomeric configuration, initially releasing β-GalA. The enzyme cleaved smaller RG substrates with the highest catalytic efficiency. A K_m of 85 μM and a V_{max} of 160 units mg^{-1} was found toward a linear RG fragment with a DP of 6. RG-galacturonohydrolase had a molecular mass of 66 kD, a pI of 5.12, a pH optimum of 4, and a temperature optimum of 50 °C. The enzyme was most stable between pH 3 and 6 (for 24 h at 40 °C) and up to 60 °C (for 3h).

D-Galacturonic acid is the major constituent sugar of pectins in plant cell walls. Most of the GalA residues are present in the HG regions of pectin. It is hypothesized that in cell wall pectin, HG regions occur interspersed with RG regions, which are rich in neutral sugar side chains (De Vries et al., 1981; Thibault et al., 1993; Schols et al., 1995). Ongoing research on HG degrading enzymes such as polygalacturonases, pectin lyases and pectate lyases, has been accompanied in the last decade by an increase in reports on enzymic degradation of the hairy RG regions of pectin. The application of pectin degrading enzymes in general lies in the fruit and vegetable processing industry, where processing and quality can be improved using these enzymes (Pilnik and Voragen, 1993). Furthermore, interest lies in the field of the enzymic degradation *in vivo* of HG and RG as a potential source of plant signaling molecules (Van Cutsem and Messiaen, 1994). Purified enzymes have also gained significance as analytical tools in structural studies because of their high specificity (Voragen et al., 1993).

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[2] See List of Abbreviations

A series of enzymes, all highly specific for hairy RG regions of pectin, have been purified and characterized. These include RG-hydrolase (Schols et al., 1990a), RG-acetylesterase (Searle-Van Leeuwen et al., 1992), RG-rhamnohydrolase (Mutter et al., 1994: Chapter 2), RG-lyase (Azadi et al., 1995; Mutter et al., 1996: Chapter 3) and xylogalacturonan exogalacturonase (Beldman et al., 1996). The current paper describes the purification and characterization of the latest enzyme in this series, named RG-galacturonohydrolase. The mode of action, substrate specificity, and several possible applications of RG-galacturonohydrolase are discussed.

MATERIALS AND METHODS

Substrates

Isolation and characterization of saponified MHR (MHR-S) is described in Chapter 2. The mixture of oligosaccharides **1** and **2** (structures in Table I), and the purified hexasaccharide **1**, generated by treatment of MHR-S with RG-hydrolase and subsequent SEC purification, is described in Chapter 2. Preparation of a mixture of oligosaccharides **7**, **8**, **9**, and **10** (Table I), and the purified hexasaccharide **7**, generated by treatment of MHR-S with RG-lyase and subsequent SEC fractionation, is described in Chapters 3 and 4.

Linear RG fragments (**11** to **16** in Table I) were kindly provided by Dr. C.M.G.C. Renard (Institut National de la Recherche Agronomique, Nantes, France), and were prepared by controlled acid hydrolysis of sugar-beet cell walls and isolated by ion-exchange chromatography and size-exclusion chromatography (Renard et al., 1995; Renard et al., accepted).

Substrates used to determine if other saccharidases than RG-galacturonohydrolase were present in the finally purified enzyme fraction included polygalacturonic acid (PGA) (Fluka Chemie AG, Buchs, Switzerland), methoxylated pectin (degree of methoxylation [DM] 92.3) prepared in our laboratory according to Van Deventer-Schriemer and Pilnik (1976), larchwood arabino-β-(1,3)/(1,6)-galactan ("stractan", Meyhall Chemical AG, Kreuzlingen, Switzerland), potato arabino-β-(1,4)-galactan (isolated from potato fiber according to Labavitch et al., 1976), a linear arabinan from sugar-beet kindly provided by British Sugar (Peterborough, UK), xylan from oat spelts (Koch and Light Ltd., Haverhill, England), carboxymethylcellulose (Akucell AF type 2805, Akzo, Arnhem, the Netherlands), Avicel cellulose (type SF, FMC, Serva, Heidelberg), and soluble starch (Merck AG, Darmstadt, Germany). The p-nitrophenyl (pnp)-glycosides, used for screening of glycosidase side activities of RG-galacturonohydrolase, were obtained from Koch and Light Ltd. and from Sigma chemical company (St. Louis, MO): pnp-α-L-Ara_f, pnp-α-D-Gal_p, pnp-β-D-Gal_p, pnp-α-D-Xyl_p, pnp-β-D-Xyl_p, pnp-α-D-Man_p, pnp-β-D-Man_p, pnp-α-L-Fuc_p, pnp-β-D-Fuc_p, pnp-α-D-Glc_p, pnp-β-D-Glc_p, pnp-α-L-Rha_p, pnp-β-D-Glc_pA, and pnp-β-D-Gal_pA.

Further substrates used for determination of the substrate specificity of RG-galacturonohydrolase included pectin (DM 35; Obipectin, Bischofszell, Switzerland), an α-(1,4)-linked GalA dimer, tetramer, heptamer, a Δ-(4,5)-unsaturated GalA (us-GalA) α-(1,4)-linked tetramer (Voragen, 1972; Tjan et al., 1974), and a pectate lyase

(from *Pseudomonas fluorescens GK5*, Rombouts et al., 1978) digest of PGA + 1 mM $CaCl_2$.

Enzymic Modification of RG Oligomers

The mixture of oligosaccharides **1** and **2** and the purified hexasaccharide **1** were degalactosylated using a β-galactosidase purified from Pectinase 29 (a gift from Gist-Brocades, Delft, The Netherlands), produced by *Aspergillus niger*, essentially according to Van de Vis (1994). Substrates were incubated in 50 mM NaOAc buffer (pH 5) at 40 °C. Inactivation took place by heating at 100 °C for 10 min. Enzyme doses and incubation times were adjusted to ensure that the maximal degradation possible was obtained. In a similar manner the degalactosylated substrates were derhamnosylated using a partially purified RG-rhamnohydrolase from *A. aculeatus*, separated from RG-galacturonohydrolase by immobilized metal ion affinity chromatography (IMAC), see "Results and Discussion". Released Rha, Gal and GalA were determined using high-performance anion-exchange chromatography (HPAEC) (gradient B, see Analytical Methods).

Enzyme Purification

RG-galacturonohydrolase was purified from the commercial mixture Pectinex Ultra SP produced by *A. aculeatus* starting from 1000 mL preparation. Purification involved desalting by dialysis, anion-exchange chromatography on a DEAE Sepharose Fast Flow column, cation-exchange chromatography on a SP Sepharose Fast Flow column, anion-exchange chromatography on a Q-Sepharose High Performance column, and IMAC using Chelating Sepharose Fast Flow and High Performance quality (Pharmacia LKB Biotechnology, Uppsala Sweden). Purification procedures were carried out essentially as described in Chapter 2. Further details are given in "Results and Discussion". Enzyme fractions were screened for RG-galacturonohydrolase activity on the mixture of oligosaccharides **5** and **6** (Table I), and for RG-rhamnohydrolase activity on the mixture of **3** and **4** (Table I) using HPAEC (gradient B).

Enzyme Assays

Determination of Side Activities of RG-galacturonohydrolase

RG-galacturonohydrolase (2.3 μg mg^{-1} substrate) was screened for contaminating glycanase activities by incubation for 1 and 24 h at 40 °C with 0.23% w/v substrate solutions in 50 mM NaOAc buffer (pH 5). Inactivation took place by heating 10 min at 100 °C. The digests from the glycanase assay were analyzed by high-performance size-exclusion chromatography (HPSEC) and HPAEC (gradient C). Glycosidase activities were determined by incubating RG-galacturonohydrolase (29 μg mg^{-1} substrate) for 1 h at 30 °C with 0.02% w/v solutions of pnp-glycosides in 50 mM NaOAc buffer (pH 5). After addition of 0.5 M glycine-OH buffer (pH 9), 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 $M^{-1}cm^{-1}$.

Influence of pH and Temperature on RG-galacturonohydrolase

The influence of pH on RG-galacturonohydrolase activity was determined by incubating RG-galacturonohydrolase (0.011 µg mg^{-1} substrate) for 30 min at 40 °C in 0.047% w/v substrate (mixture of oligosaccharides **5** and **6**) solutions in 0.1 M McIlvaine buffers with pH's varying between 2.1 and 8.1. The stability of RG-galacturonohydrolase with pH was determined by preincubating the enzyme for 1 h and 24 h at 40 °C in McIlvaine buffers. Afterwards 0.15 M NaOAc buffer (pH 5) was added to adjust the pH, and substrate solution was added to start incubation of 30 min at 40 °C. The optimum temperature for RG-galacturonohydrolase (0.19 µg mg^{-1} substrate) was determined by incubating 0.047% w/v substrate (mixture of oligosaccharides **5** and **6**) solutions in 50 mM NaOAc buffer (pH 5) for 30 min at temperatures in the range 2 to 80 °C. The temperature stability was determined after preincubation of enzyme solutions for 30 min, 1 h, 3 h and 24 h at 8, 40 and 60 °C in 50 mM NaOAc buffer (pH 5). After cooling, substrate was added and incubation took place for 30 min at 40 °C. Incubation mixtures were inactivated by heating for 10 min at 100 °C. Incubation mixtures and blanks were analyzed by HPAEC (gradient A).

Other Substrate Degradation Studies

Details regarding further experiments are presented in "Results and Discussion" in the table of interest. Enzyme activities were expressed as units: one unit corresponds to the release of 1 µmol GalA min^{-1} under the conditions described.

Determination of Molecular Mass and pI

SDS-PAGE and isoelectric focusing were carried out as described in Chapter 2. Isoelectric focusing gels (Pharmacia) with a pH range from 3 to 9 and from 4 to 6.5 were used with the appropriate standards. The proteins were silver stained.

Determination of the molecular mass of RG-galacturonohydrolase activity using size-exclusion chromatography (SEC) was carried out using a Superose 12 HR 10/30 column (Pharmacia). The column was calibrated with endopolygalacturonase (43 kD), RG-rhamnohydrolase (84 kD), RG-hydrolase (53 kD), and several partially purified proteins with molecular masses of 78, 76, 52, 45 and 32 kD, as characterized by SDS-PAGE. A buffer of 150 mM NaOAc (pH 6) was used for elution. Retention of RG-galacturonohydrolase and of RG-rhamnohydrolase on this column was monitored by collecting fractions and determining their activity toward the mixture of oligosaccharides **5** and **6** and the mixture of **3** and **4**, respectively, as detected using HPAEC (Gradient B).

Preparative isoelectric focusing was performed using a Rotofor preparative IEF cell (Biorad Laboratories, Richmond, CA). Bio-Lyte pH 4-6 was used as ampholyte, and 0.1 M NaOH and 0.1 M H_3PO_4 were used as electrolytes in the cathode and

anode chambers respectively. Partially purified protein fractions (70 mg), containing RG-galacturonohydrolase and RG-rhamnohydrolase, were pooled from the SP Sepharose Fast Flow separation and dialyzed against distilled water. Ampholyte was added (to 1.7% w/v) and the sample was applied to the system. Focusing required 3.5 h at 4 °C at 12 Watt constant power supply, after which the fractions from the twenty compartments were collected immediately. After pH measurement, fractions were screened for RG-galacturonohydrolase and RG-rhamnohydrolase activity on enzymically modified RG oligomers using HPAEC (gradient B).

Stereochemical course of hydrolysis

RG-galacturonohydrolase (ca. 1 unit in water) was desalted (into water) using a NAP-5 column (Pharmacia) prior to lyophilization, since the enzyme was inactivated when it was lyophilized in the presence of the buffer salts. After desalting, the enzyme was lyophilized once from deuterated H_2O (99.96 atom % D, Cambridge Isotope Laboratories, Andover, MA), to exchange labile ^1H atoms for D. The substrate, being 12 mg of a mixture of linear RG oligomers (**13** and **14** in Table I) produced by acid hydrolysis according to Renard et al. (1995), was lyophilized three times from D_2O. The RG oligomers were dissolved in 0.7 mL D_2O just prior to ^1H NMR analysis and the solution was equilibrated at 30 °C in a 5 mm NMR tube before recording the initial spectrum. RG-galacturonohydrolase (50 µL in D2O) was then added and the stereochemical course of hydrolysis followed by recording ^1H NMR spectra at 30 °C in a Bruker DPX-400 spectrometer at intervals during the incubation as described earlier (Pitson et al., 1996).

Analytical Methods

HPSEC was used to determine the molecular mass distribution of substrates before and after enzyme treatment. Three Bio-Gel TSK columns in series (40XL, 30XL and 20XL) were used as described by Schols et al. (1990a). Pectin standards of 100; 82; 77.6; 63.9; 51.4; 42.9; 34.6; 10 kD and GalA and the dimer of GalA were used for calibration of the system. Software ("GPC/PC") from Spectra Physics (San Jose, CA) was used for determination of the number-average molecular mass.

HPAEC was performed using a Dionex (Sunnyvale, CA) Bio-LC system equipped with a Dionex CarboPac PA-100 (4 x 250 mm) column and a Dionex PED detector in the pulsed amperometric detection (PAD) mode. Gradients of NaOAc in 100 mM NaOH (1 mL min^{-1}) were used as follows:

gradient A: 0 to 7 min, 100 to 200 mM; 7 to 10 min, 200 to 1000 mM; 10 to 15 min, 1000 mM; 15 to 30 min, 100 mM;

gradient B: 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;

gradient C: 0 to 50 min, 0 to 450 mM; 50 to 55 min, 450 to 1000 mM; 55 to 70 min, 0 mM.

RESULTS AND DISCUSSION

Preparation of RG Substrates

A mixture of the hexasaccharide **1** and octasaccharide **2** (structures in Table I, according to Schols et al., 1994) was generated by treatment of MHR-S with RG-hydrolase, and subsequent purification of the degradation products by SEC (Chapter 2). This mixture was then treated with a β-galactosidase from *A. niger*, which generated a mixture of tetrasaccharide **3** and hexasaccharide **4** (Table I, Chapter 2). Final enzymic modification of this mixture was done with RG-rhamnohydrolase from *A. aculeatus* to produce trisaccharide **5** and pentasaccharide **6** (Table I, Chapter 2). This mixture was used during purification of RG-galacturonohydrolase to screen column fractions on.

Table I. *Explanation of codes of the RG oligosaccharides used in the characterization of RG-galacturonohydrolase*

GA, 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; uGA, α-us-GalA (1,2)-linked to Rha; G, α-Gal (1,4)-linked to Rha.

Code	Structure
1	R-GA-R-GA ↑ ↑ G G
2	R-GA-R-GA-R-GA ↑ ↑ ↑ G G_n G_m with either n=1 and m=0, or n=0 and m=1
3	R-GA-R-GA
4	R-GA-R-GA-R-GA
5	GA-R-GA
6	GA-R-GA-R-GA
7	uGA-R-GA-R ↑ ↑ G G
8	uGA-R-GA-R-GA-R ↑ ↑ ↑ G G G
9	uGA-R-GA-R-GA-R-GA-R ↑ ↑ ↑ ↑ G G G G
10	uGA-R-GA-R-GA-R-GA-R-GA-R ↑ ↑ ↑ ↑ ↑ G G G G G
11	GA-R-GA-R-GA-R (DP 6)
12	GA-R-GA-R-GA-R-GA-R (DP 8)
13	GA-R-GA-R-GA-R-GA-R-GA-R (DP 10)
14	GA-R-GA-R-GA-R-GA-R-GA-R-GA-R (DP 12)
15	GA-R-GA-R-GA-R-GA-R-GA-R-GA-R-GA-R (DP 14)
16	mixture of $(GA-R)_n$ with n > 8, with an average DP of approximately 20

The same sequential enzymic degradation procedure was performed on the hexasaccharide **1** (peak a1 in Fig. 1a) that was purified from a RG-lyase MHR-S digest (Chapter 2). The major product generated by β-galactosidase treatment of **1** is the degalactosylated tetrasaccharide **3** (peak b1 in Fig. 1b). We assume that the minor components (peaks b2 and b3 in Fig. 1b) are partially degalactosylated oligosaccharides. Moreover, small amounts of Rha (12 mol%) and GalA (11 mol%) were released which suggests that the β-galactosidase fraction also contained rhamnohydrolase and galacturonohydrolase activities. The main product generated by treating the degalactosylated tetrasaccharide **3** with RG-rhamnohydrolase was the trisaccharide **5** (peak c1 in Fig. 1c). At the same time, some additional Gal was released (not shown), which could explain that peak b2 (a galactosylated fragment) was not detected anymore. We assume that peak b3, another presumptive galactosylated fragment, was also converted to the trisaccharide. The structure of peak c2 in Figure 1c is unknown. Nevertheless, we considered the oligosaccharide fraction to be suitable for use in investigating the mode of action of RG-galacturonohydrolase.

The substrate specificity of RG-galacturonohydrolase was further determined using apple MHR-S, a mixture of oligomers **7** to **10** (Table I) and the purified hexasaccharide **7** generated by RG-lyase treatment of MHR-S and subsequent SEC purification, purified linear RG oligomers **11** to **15** (Table I), and a mixture of RG oligomers with an average DP of 20 (**16** in Table I) (Renard et al., accepted).

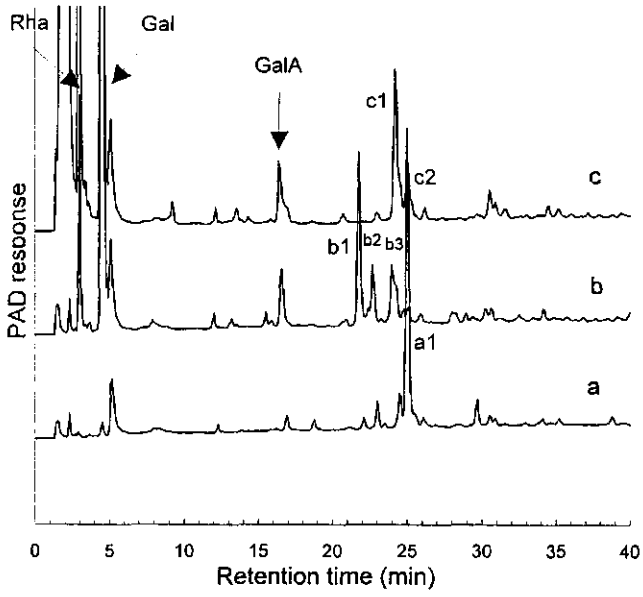

Figure 1. *HPAEC of a, the purified hexasaccharide **1** fraction (peak a1) (structures in Table I); b, this fraction after degalactosylation which generates as major product tetrasaccharide **3** (peak b1); c, and this fraction after degalactosylation and subsequent derhamnosylation which generates as major product trisaccharide **5** (peak c1).*

Purification of RG-galacturonohydrolase from *A. Aculeatus*

Purification was commenced from 1 L of Pectinex Ultra SP. The detailed fractionation scheme is shown in Figure 2.

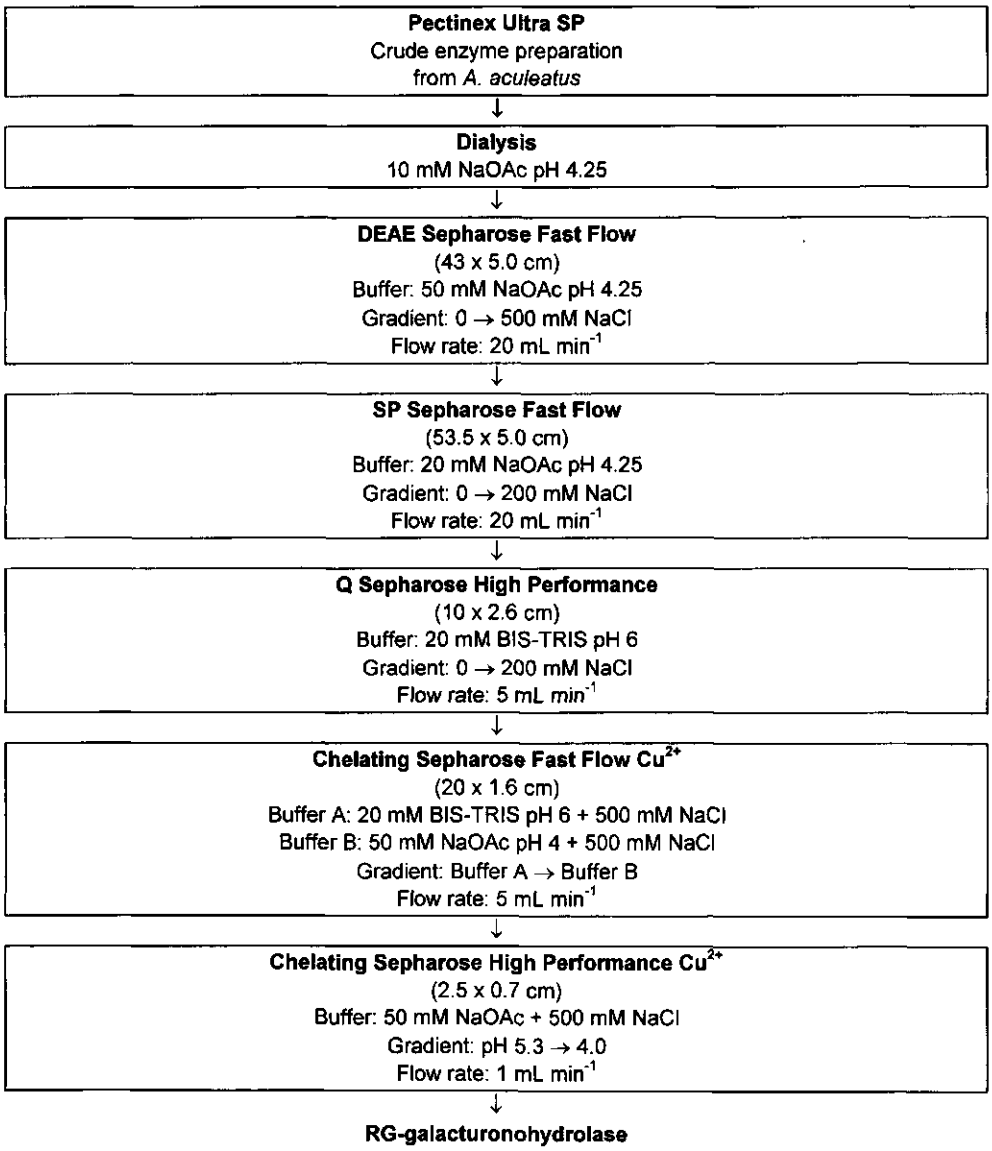

Figure 2. *Detailed purification scheme of RG-galacturonohydrolase from Pectinex Ultra SP produced by A. aculeatus*

After dialysis of the crude enzyme preparation, the desalted protein was applied to a DEAE Sepharose anion-exchanger at pH 4.25. Both unbound and bound protein contained RG-galacturonohydrolase activity, which was indicated by the release of GalA from the mixture of oligosaccharides **5** and **6** using HPAEC.

Since other enzymes of interest were also present in the unbound fraction, purification was continued with this fraction, which was applied to an SP Sepharose cation-exchanger at pH 4.25. RG-galacturonohydrolase activity eluted from the column at 124 mM NaCl. Pooled fractions were applied to a Q Sepharose anion-exchanger at pH 6. RG-galacturonohydrolase activity was not found in a distinct peak, but was present in all three major protein peaks eluting from the column at 24, 35 and approximately 60 mM NaCl, in amounts that could be related to the protein content of the fractions. RG-rhamnohydrolase was also present in all major fractions, but was most abundant in the 35 and 60 mM NaCl fractions. Many attempts were made to achieve separation of RG-galacturonohydrolase from RG-rhamnohydrolase. These included various different forms of SEC; ion-exchange chromatography; chromatofocusing, hydrophobic interaction chromatography; and affinity chromatography. None of these methods were effective. Finally, the best separation of the two enzymes was obtained using IMAC. The three fractions from the Q-Sepharose column were applied to a column of Chelating Sepharose Fast Flow, that was loaded with $CuCl_2$. For the 35 mM Q Sepharose fraction the elution pattern is shown in Figure 3. It can be seen that the majority of RG-rhamnohydrolase

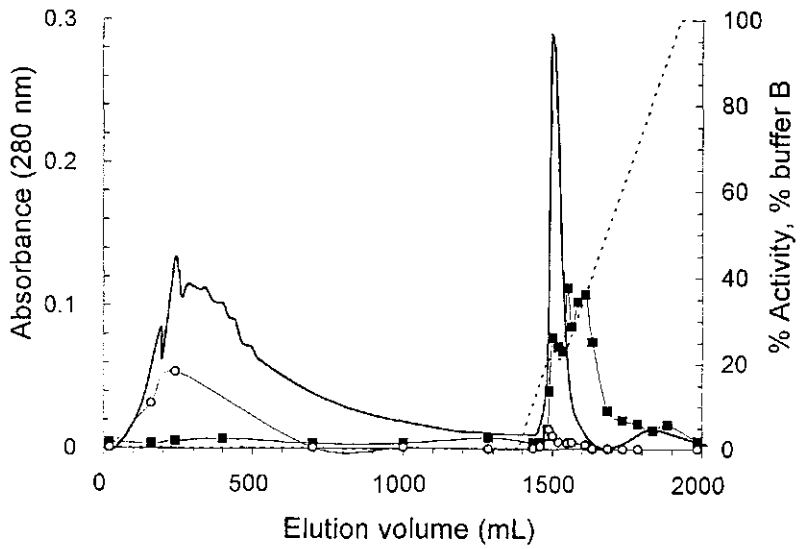

Figure 3. *Chromatography of the protein fraction, that was eluted from the Q Sepharose column at 35 mM NaCl, on a Chelating Sepharose Fast Flow column loaded with Cu^{2+} ions. For elution a pH gradient of pH 6 to 4 (buffer B) in 20 mM BIS-TRIS containing 500 mM NaCl was used (see Fig. 2). Symbols: _____, A_{280}; _ _ _ _, % buffer B; ■, RG-galacturonohydrolase activity; ○, RG-rhamnohydrolase activity (expressed as percentages of sugar released from the total amount present in the substrate).*

was not bound to the column, although a small amount eluted at the front of the major protein peak (at pH 5.56). RG-galacturonohydrolase eluted in the tail of the major protein peak. The unbound protein containing RG-rhamnohydrolase activity was pooled and used for derhamnosylation of substrates, as described above.

Column fractions with the highest RG-galacturonohydrolase activity were pooled, and all resulting fractions formed were again applied to a Chelating Sepharose column, this time using High Performance material, to remove traces of RG-rhamnohydrolase. Finally, nine pools containing RG-galacturonohydrolase activity were obtained, in total representing approximately 0.01 % w/w of the originally desalted protein of Pectinex Ultra SP. The proteins in all these pools showed the same molecular mass upon SDS-PAGE. The purest fraction, based on side activity determination (see below) was chosen for characterization.

Characteristics of RG-galacturonohydrolase

RG-galacturonohydrolase showed only one protein band on SDS-PAGE representing a molecular mass of 66 kD. Using a calibrated SEC column, a molecular mass of 62 kD (± 5 kD) was found for RG-galacturonohydrolase activity. The RG-rhamnohydrolase from which the RG-galacturonohydrolase was separated also had a molecular mass of 66 kD (not shown), in contrast with the previously described RG-rhamnohydrolase and co-eluting RG-galacturonohydrolase that had molecular masses of 84 kD (Chapter 2). Other experiments also indicated the presence of multiple RG-rhamnohydrolases and RG-galacturonohydrolases in Pectinex Ultra SP with different pI's and different behavior on a hydroxylapatite column (not shown). On isoelectric focusing a major band at pI 5.12 and minor bands at 5.00, 5.07 and 5.20 were found for RG-galacturonohydrolase. Preparative isoelectric focusing showed maximal activity of RG-galacturonohydrolase in the collected fractions with a pH of 4.9 and 5.0.

RG-galacturonohydrolase was tested toward various substrates, to screen for other glycanase and/or glycosidase activities. HPSEC was used to detect a shift in molecular mass of the polymeric substrate, and HPAEC was used to detect if sugar monomers or oligomers were released. No activity was found toward CM-cellulose, crystalline cellulose, xylan from oat spelts, soluble starch, potato arabino-β-(1,4)-galactan, larchwood arabino-β-(1,3)/(1,6)-galactan (stractan), linear arabinan, PGA with or without 1 mM $CaCl_2$ added and pectin with a DM of 92.3. The same was true for pnp-α-L-Ara_f, pnp-α-D-Gal_p, pnp-β-D-Gal_p, pnp-α-D-Xyl_p, pnp-β-D-Xyl_p, pnp-α-D-Man_p, pnp-β-D-Man_p, pnp-α-L-Fuc_p, pnp-β-D-Fuc_p, pnp-α-D-Glc_p, pnp-β-D-Glc_p, pnp-α-L-Rha_p, pnp-β-D-Glc_pA, and pnp-β-D-Gal_pA.

The optimum pH of RG-galacturonohydrolase was found at pH 4 in McIlvaine buffers (Fig. 4a) and the enzyme was most stable between pH 2.5 and 7 for 1 h at 40 °C and between pH 3 and 6 for 24 h at 40 °C (Fig. 4b). The optimum temperature was at 50 °C in NaOAc buffer (pH 5) (Fig. 4c) and RG-galacturonohydrolase was stable for at least 3 h at 60 °C (Fig. 4d).

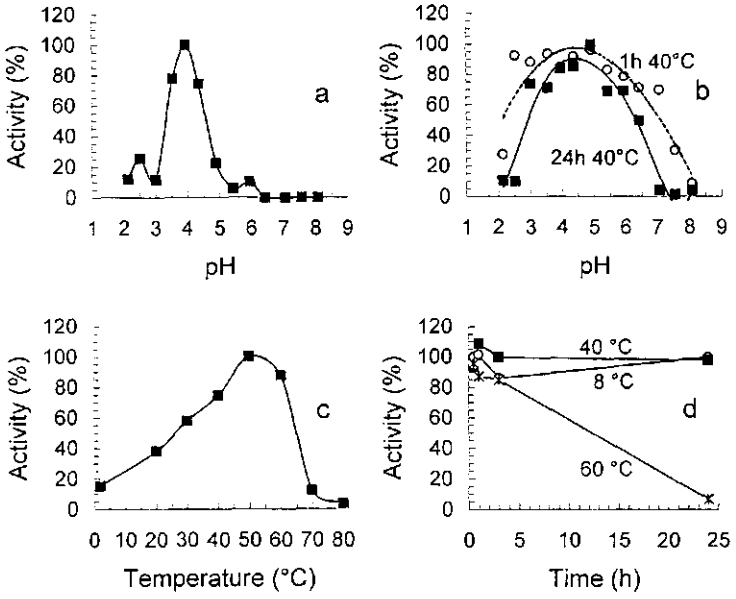

Figure 4. *a, Optimum pH of RG-galacturonohydrolase, 100% = enzyme activity at optimum pH; b, pH stability of RG-galacturonohydrolase, 100% = activity of untreated enzyme; c, optimum temperature of RG-galacturonohydrolase, 100% = enzyme activity at optimum temperature; and c, temperature stability of RG-galacturonohydrolase, 100% = activity of untreated enzyme.*

Mode of action of RG-galacturonohydrolase

Oligosaccharides **5** and **6** (Table I), present in the mixture used to test enzyme fractions during purification, contain a GalA at both the nonreducing and the reducing end, and it was not clear which GalA was removed by the RG-galacturonohydrolase. Therefore, incubations were performed with the purified hexasaccharide **1** and its derivative after degalactosylation: tetrasaccharide **3**, and subsequent derhamnosylation: trisaccharide **5**. These three oligomers all contain a GalA residue as reducing end sugar, but have either a Rha or GalA at the nonreducing end (see Table I). The activity of RG-galacturonohydrolase toward these substrates is presented in Table II as well as the percentages of GalA released from the total amount of GalA present in the oligomer after 45 h. In Figure 5a to 5c the corresponding HPAEC patterns are shown.

From Table II it is clear that the highest activity was obtained for trisaccharide **5**, which contains a GalA at the nonreducing end in contrast with the two other oligomers **1** and **3**. Figure 5c shows that peak c1, corresponding to trimer **5**, is degraded under formation of GalA and a new peak, d1, which must be the dimer α-Rha-(1,4)-GalA. The results therefore show that RG-galacturonohydrolase removes the GalA from the nonreducing end of RG fragments. The disaccharide α-Rha-(1,4)-

Table II. *Activity of RG-galacturonohydrolase toward structurally different RG fragments*
RG-galacturonohydrolase (0.42 µg mg^{-1} substrate) was incubated with 0.025% w/v substrate solutions in 50 mM NaOAc buffer (pH 5), for 1 h and 45 h at 40 °C. Incubation mixtures and blanks were analyzed on HPAEC (gradient C). Explanation of symbols in Table I.

Oligosaccharide	Activity (units mg^{-1})	% GalA released from total in 45 h
1: R-GA-R-GA ↑ ↑ G G	0.81	1.2
3: R-GA-R-GA	8.1	13
5: GA-R-GA	28	47

GalA, eluting at 13.5 min, is less retarded on the column than GalA, which can be attributed to the effect of Rha at the nonreducing end, as was already shown in Chapter 2. Peak d1 could completely be degraded into GalA and Rha upon subsequent degradation with RG-rhamnohydrolase (not shown). Only a trace of RG-galacturonohydrolase activity was found toward hexasaccharide **1**, and Figure 5a shows that peak a1, corresponding to **1** was not degraded. Although 13% of the total GalA was released from tetrasaccharide **3**, HPAEC (Fig. 5b) reveals that peak b1, corresponding to **3**, was not degraded, and therefore the GalA released must be released from contaminating oligomers present in the fraction, instead of from the reducing end of **3**.

The partial ^{1}H NMR spectra recorded just prior to, and at intervals after the addition of RG-galacturonohydrolase to a mixture of RG oligosaccharides **13** and **14**

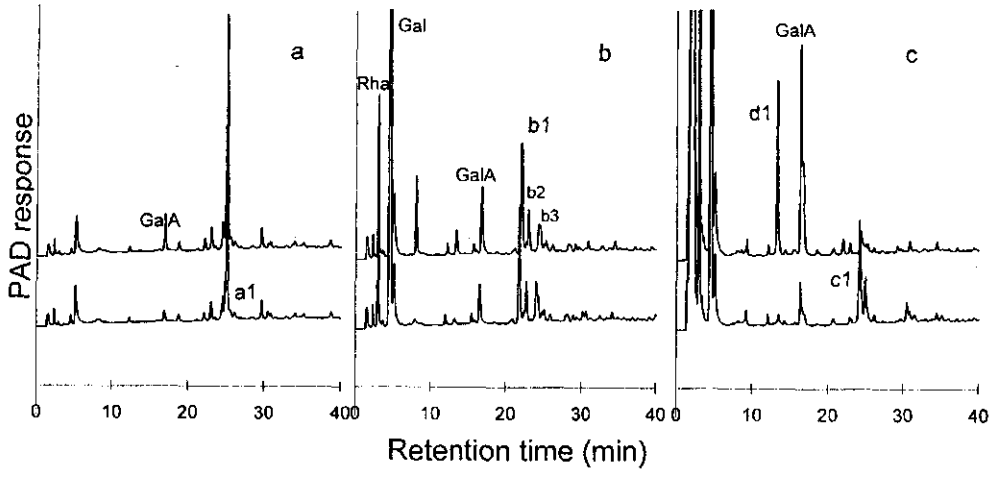

Figure 5. *HPAEC of a, hexasaccharide **1** fraction (peak a1) (structures in Table I) before (bottom) and after (top) 45 h incubation with RG-galacturonohydrolase; b, tetrasaccharide **3** (peak b1) before (bottom) and after (top) 45 h incubation with RG-galacturonohydrolase; c, trisaccharide **5** (peak c1) before (bottom) and after (top) 45 h incubation with RG-galacturonohydrolase.*

(Table I), illustrating the stereochemical course of the reaction, are shown in Figure 6. During the first few minutes of the incubation a doublet at ca. 4.61 ppm (J 7.9 Hz), assigned to H-1β of GalA (Rees & Wight, 1971; Tjan et al., 1974) appeared and rapidly increased in intensity. Later in the incubation a small doublet at 5.30 ppm (J 3.8 Hz), due to H-1α of GalA (Rees & Wight, 1971; Tjan et al., 1974) became noticeable and almost certainly arose from the mutarotation of the initially formed β-anomers. Other notable changes in the ^{1}H NMR spectra during the incubation includes an increase in the resonance at ca. 5.21 ppm, assigned to H-1 of terminal nonreducing end α-Rha residues, and a decrease in the resonance at ca. 5.25 ppm due to internal α-Rha residues (Colquhoun et al., 1990; Schols et al., 1994). This confirms that the GalA is removed from the nonreducing end. Therefore, all the data clearly indicate that RG-galacturonohydrolase catalyzes the hydrolysis of α-GalA-(1,2)-α-Rha linkages at the nonreducing end of the RG oligomers with inversion of anomeric configuration (*e* → *a*), and most likely operates via a single displacement reaction mechanism (Sinnott et al., 1990). This is similar to most other galacturonosyl hydrolases so far investigated (Biely et al., 1996; Pitson et al., submitted), although a digalacturonohydrolase (EC 3.2.1.82) from *Selenomonas ruminantium* was reported to catalyze glycosyl transfer (Heinrichová et al., 1992) and

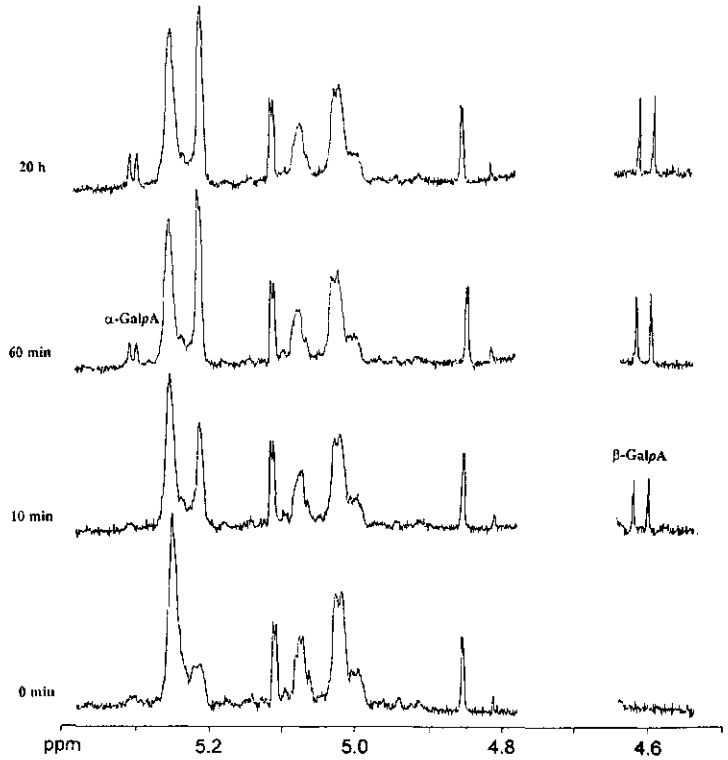

Figure 6. *Partial ^{1}H-NMR spectra showing the stereochemical course of hydrolysis of linear RG oligomers by the RG-galacturonohydrolase. H-1 resonances of the GalA released are indicated (α-GalA and β-GalA).*

therefore probably acts with net retention of anomeric configuration.

Substrate specificity of RG-galacturonohydrolase

The substrate specificity of RG-galacturonohydrolase was investigated using several different HG and RG substrates containing GalA at the nonreducing end (Table III). From Table III it is clear that hardly any GalA releasing activity was found toward the HG type of substrates, regardless of the degree of methoxylation, size, or presence of saturated or us-GalA residues at the nonreducing end. Thus, we conclude that RG-galacturonohydrolase is not active toward HG structures.

RG-galacturonohydrolase was active toward all RG types of substrate, except for those with an us-GalA at the nonreducing end. The fact that RG-galacturonohydrolase is active toward MHR-S shows that some nonreducing GalA must be present in MHR-S, although only 0.4% of the total GalA present was released. The slight activity toward the mixture of oligosaccharides **7** to **10** (Table I) might be explained by the presence of contaminating larger original MHR-S

Table III. *Activity of RG-galacturonohydrolase toward different GalA containing substrates* RG-galacturonohydrolase (0.76 µg µmol^{-1} substrate) was incubated with substrate solutions, adjusted to approximately 150 µM nonreducing GalA residues in 50 mM NaOAc buffer (pH 5), for 1 h and 45 h at 40 °C. Incubation mixtures and blanks were analyzed on HPAEC using gradient A and C, polymeric substrates also analyzed on HPSEC. Nonred., nonreducing; n.d., not determined. Explanation of oligosaccharide codes in Table I.

Substrate	Type of nonreducing end structure (only backbone shown)	Activity (units mg^{-1})	% GalA released from total in 45 h	% GalA released from nonred. ends in 45 h[a]
PGA	α-GalA-(1,4)-α-GalA-	0.4	0.1	1.8
pectin DM 35	α-GalA-(1,4)-α-GalA-	1.0	0.1	13
pectin DM 92.3	α-GalA-(1,4)-α-GalA-	0.0	0.0	0.0
Pectate lyase digest of PGA+ Ca^{2+}	α-us-GalA-(1,4)-α-GalA-	0.2	0.1	1.4
$GalA_7$	α-GalA-(1,4)-α-GalA-	0.0	0.0	0.0
$GalA_4$	α-GalA-(1,4)-α-GalA-	0.0	0.0	0.0
$GalA_2$	α-GalA-(1,4)-α-GalA-	0.4	n.d.	n.d.
us-$GalA_4$	α-us-GalA-(1,4)-α-GalA-	0.0	0.0	0.0
MHR-S	unknown	16	0.4	-[b]
Mixture of **5** and **6**	α-GalA-(1,2)-α-Rha-	10	19	61
Mixture of **7**, **8**, **9** and **10**	α-us-GalA-(1,2)-α-Rha-	1.2	1.5	3.0
Hexasaccharide **7**	α-us-GalA-(1,2)-α-Rha-	0.3	1.2	2.4
Mixture **16**	α-GalA-(1,2)-α-Rha-	19	8.8	97
15	α-GalA-(1,2)-α-Rha-	18	12	95
13	α-GalA-(1,2)-α-Rha-	17	16	95
11	α-GalA-(1,2)-α-Rha-	21	24	94

[a] For calculation of the amount of available nonreducing GalA units present, it was assumed that all nonreducing ends of chains contained GalA, and the number-average molecular mass of substrates was calculated from HPSEC patterns using GPC/PC software

[b] Could not be determined since the nature of the nonreducing end sugars of MHR-S chains are not known.

fragments in the oligomer mixture, containing GalA at the nonreducing end. From oligomers **11**, **13**, **15**, and mixture **16**, essentially all available GalA was released. From the mixture of oligosaccharides **5** and **6** only 61% of all available GalA residues could be released instead of the expected 100%. This could be due to the fact that the degalactosylation was not complete in the batch used, and therefore not all material originally present was modified into products with a nonreducing GalA available, as was assumed in the calculation.

Although the RG-galacturonohydrolase activity toward pectin with a DM of 35 was only 1 unit mg^{-1}, 13% of the available GalA residues could be released after 45 h. Van Rijssel et al. (1993) degraded citrus pectin (DM 62) with a PGA hydrolase from *Clostridium thermosaccharolyticum* and found that 5.7% w/w of this substrate could not be degraded by the enzyme. This so-called 'limit pectin' was rich in GalA, Rha, Ara and Gal (Rha : GalA = 0.54). To investigate the possibility that the released GalA from pectin with a DM of 35 and PGA in Table III originated from RG regions, the substrates were incubated with both RG-galacturonohydrolase and RG-rhamnohydrolase. Besides GalA, Rha was also released (not shown), indicating that accessible RG regions were indeed present.

Kinetic Properties of RG-galacturonohydrolase

The kinetic properties of RG-galacturonohydrolase were studied toward MHR-S and oligosaccharides **11**, **12**, **14** and **16** (Table IV). From Table IV it can be seen that the V_{max} tended to increase with increasing DP. The affinity of RG-galacturonohydrolase for the substrate decreased (increasing K_m) with increasing DP. The overall effect of the kinetic parameters is expressed in the "specificity constant", k_{cat}/K_m (Fersht, 1985), which equals $(V_{max}/[E])/K_m$ (Table IV). The specificity constant increases with decreasing DP, indicating that the overall catalytic efficiency toward smaller substrates is higher: the constant of a linear RG fragment of DP 6 (**11**) is almost twenty times that of MHR-S. Therefore, RG-galacturonohydrolase is an exo-acting oligomerase. The K_m of RG- galacturonohydrolase for the smallest linear RG oligomers was of the same order of magnitude (75 µM for octasaccharide **12**) as the

Table IV *Kinetic parameters for RG-galacturonohydrolase*
RG-galacturonohydrolase (5.2 ng mL^{-1} incubation mixture) was incubated for 30 min at 40 °C with six different substrate concentrations between 0.030 and 1.3 mM for oligosaccharides **11**, **12**, **14**, and mixture **16** (structures in Table I) and between 0.83 and 5 mM for MHR-S, dissolved in 50 mM NaOAc buffer (pH 5). Incubation mixtures and blanks were analyzed using HPAEC (gradient A).

Substrate	K_m (%w/v)	K_m (mM)	V_{max} (units mg^{-1})	10^{-2} k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
MHR-S	1.4 (± 0.0025)	2.3[a] (± 0.0042)	2.5 10^2 (± 0.2)	2.8	1.2
mixture **16**	0.046 (± 0.0092)	0.14 (± 0.028)	2.1 10^2 (± 16)	2.3	16
14	0.040 (± 0.011)	0.21 (± 0.056)	2.2 10^2 (± 21)	2.4	11
12	0.0098 (± 0.0028)	0.075 (± 0.021)	1.4 10^2 (± 7.9)	1.5	20
11	0.0084 (± 0.0031)	0.085 (± 0.032)	1.6 10^2 (± 11)	1.8	21

[a] The number-average molecular mass of MHR-S was determined to be 6000 D, although the major populations of MHR-S range between 7 and 80 kD

K_m of another RG specific enzyme, RG-lyase, for MHR-S (approximately 55 μM, Chapter 4). The V_{max} of RG-galacturonohydrolase for the linear RG oligomers, however, is five to ten times higher (140 to 220 units mg^{-1}) than the V_{max} of RG-lyase for MHR-S (25 to 30 units mg^{-1}) (Chapter 4).

RG-galacturonohydrolase, in combination with RG-rhamnohydrolase, might be applied to remove RG fragments from acid extracted HGs as used in industry, to improve the gelling properties. The combination of these RG exo-enzymes can also be used in the complete saccharification of biomass as e.g. sugar-beet pulp, from which process the resulting Rha monomers can be used as a precursor of aroma compounds, such as furaneol (Micard et al., 1996). Finally, capable of modifying RG structures, RG-galacturonohydrolase and RG-rhamnohydrolase might become important in the study of biologically active RGs, such as sycamore RG-I that has been demonstrated to have wound-signal activity (Ryan et al., 1981). These exo-enzymes have not yet been found in plants. However, recently activity of another RG specific enzyme, the RG-hydrolase, has been indicated in apples, grapes and tomatoes (Gross et al., 1995).

CONCLUSIONS

From the commercial enzyme mixture Pectinex Ultra SP, produced by *A. aculeatus*, an RG-galacturonohydrolase has been purified. This enzyme hydrolyzes the GalA residue from the nonreducing end of RG structures with inversion of anomeric configuration. To date no such enzyme has been described in literature. Being highly specific for RGs, and not active toward HGs, RG-galacturonohydrolase can be considered the latest in a series of RG specific enzymes, after RG-hydrolase (RGase, Schols et al, 1990a), RG-acetylesterase (Searle-Van Leeuwen et al., 1992), RG-rhamnohydrolase (Chapter 2) and RG-lyase (Azadi et al., 1995; Chapter 3). Taking into account the substrate specificity and mode of action of the enzyme, the proposed systematic name is RG α-D-galactopyranosyluronohydrolase.

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Chapter 7

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

This chapter has been submitted to *Carbohydrate Research* by the authors Margien Mutter, Catherine M.G.C. Renard[1], Gerrit Beldman, Henk A. Schols, Alphons G.J. Voragen.

The mode of action of RG-hydrolase[2] 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, by modifying RG oligomers. ^{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 pattern it can be speculated that in hairy regions, the RG stretches have to be at least thirteen residues long for RG-hydrolase and sixteen residues long for RG-lyase in order to produce one tetramer.

Since Schols et al. (1990) described the enzyme RGase, able to degrade the RG backbone in hairy regions of pectin, several papers have been published dealing with RGase activity (Matsuhashi et al., 1992; Düsterhöft et al., 1993; An et al., 1994; Kofod et al., 1994; Sakamoto et al., 1994; Azadi et al., 1995; Gross et al., 1995; Suykerbuyk et al., 1995). 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 (Searle-van Leeuwen et al., 1992); RG-rhamnohydrolase (Mutter et al., 1994: Chapter 2); RG-lyase (Mutter et al., 1996a: Chapter 3), and RG-

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[2] See List of Abbreviations

galacturonohydrolase (Chapter 6). RGase as described by Schols et al. (1990) 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 (Schols and Voragen, 1996). Furthermore, they might become important for production and modification of (potentially) biologically active RG structures. Wound-signal activity has been demonstrated for RG I (Ryan et al., 1981), and hypocotyl elongation for us-GalA-(1,2)-Rha disaccharides (Hasegawa et al., 1992).

In this paper we describe the cleavage patterns of RG-hydrolase and RG-lyase toward a series of RG oligomers with different 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 series of different types of RG oligomers available as standards for 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.

MATERIALS AND METHODS

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 to 16, abbreviated **A4** to **A16** (see Table I) and a mixture of oligomers with a DP > 10, were isolated by ion-exchange chromatography and SEC by Renard et al. (accepted).

Saponified apple MHR was treated with RG-hydrolase, and the RG oligomers produced were isolated using SEC as described in Chapter 2. Removal of Gal from these oligomers, and subsequent derhamnosylation was carried out as described in Chapter 2. A similar isolation procedure was carried out with RG-lyase, and the resulting branched unsaturated RG oligomers were isolated as described in Chapters 3 and 4. From these unsaturated RG oligomers the Gal was removed

using 9.3 μg β-galactosidase mg^{-1} substrate, under the conditions described in Chapter 2.

Enzymes

RG-hydrolase and RG-lyase from *Aspergillus aculeatus* were purified using the method of Schols et al. (1990) and of Kofod et al. (1994) respectively.

β-galactosidase from *Aspergillus niger* was purified by Van de Vis (1994) and used for degalactosylation of RG oligomers.

Procedures for the purification of RG-rhamnohydrolase and RG-galacturonohydrolase from *A. aculeatus* are described in Chapter 2 and in Chapter 6 respectively.

Incubations with Enzymes

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 to 5 mL) were collected and analyzed. The GalA and neutral sugars (using a Rha standard) concentrations were measured by automated meta-hydroxy-diphenyl (Thibault, 1979) and orcinol assays (Tollier & Robin, 1979) 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 x 250 mm) column and a Dionex pulsed electrochemical detector in the pulsed amperometric detection (PAD) mode. A gradient of NaOAc in 100 mM NaOH (1 mL/min) was used as follows: 0 to 50 min, 0 to 450 mM; 50 to 55 min, 450 to 1000 mM; 55.1 to 70 min, 0 mM.

^{1}H 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.

RESULTS

Preparation of a Series of Standards of RG Oligomers

Five types of linear, strictly alternating RG oligomers were made available, coded **A** to **E**, differing in their reducing and/or nonreducing end sugars. The oligomers are listed in Table I. 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 I, will be used throughout the paper. The isolation and enzymic modification of RG oligomers types **A** to **E** are described below.

Type **A** oligomers are linear RG oligomers of the type G-(R-G)$_n$-R, with n = 1 to 7, i.e. **A4** to **A16** in Table I, and have been purified from sugar beet pulp and characterized (Renard et al., accepted). Type **A** oligomers were treated with RG-galacturonohydrolase (Chapter 6), to remove the nonreducing GalA to obtain oligomers of type **B**: (R-G)$_n$-R, with n = 1 to 7, i.e. oligomers **B3** to **B15** in Table I. **A4** was sequentially treated with RG-galacturonohydrolase and RG-rhamnohydrolase (Chapter 2) 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 (Schols et al., 1990; Schols et al., 1994). These oligomers were linearized by treatment with a β-galactosidase from *A. niger* (Van de Vis, 1994), to obtain type **C** oligomers: (R-G)$_n$-R-G, with n = 1 to 2, with as major products **C4** and **C6** (Chapter 2). Sequential degradation of purified **C4** (Chapter 2) with RG-rhamnohydrolase and RG-galacturonohydrolase finally gave **C2**.

The mixture of linearized type **C** oligomers was derhamnosylated with RG-rhamnohydrolase (Chapter 2) to form oligomers of type **D**: (G-R)$_n$-G, with n = 1 to 2, i.e. oligomers **D3** and **D5** in Table I. However, after this treatment, a third peak was distinctly present, eluting after the former two oligomers upon HPAEC. Plotting the

Table I. *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-us-GalA (1,2)-linked to Rha. Retention times upon HPAEC under the conditions described in "Materials and methods".

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

log (k')[3] (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 degalactosylation and derhamnosylation of the original oligomers. Previous results (Chapter 2) showed that the response factors decrease when the original oligomers are degalactosylated and derhamnosylated, the largest difference being introduced by degalactosylation. Therefore it was not expected that this third oligomer in a homologous series was only observed after these enzymic degradation steps. In the original mixture,

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

differently galactosylated oligomers with the same backbone size (DP 8) could have been present, but then it can be expected that after degalactosylation a major peak of DP 8 would appear, which was not the case. A good explanation could not yet be found.

Unsaturated RG oligomers with all Rha units C-4 substituted with Gal, were purified from saponified apple MHR after treatment with RG-lyase (Chapter 3 and 4). These oligomers were linearized using a β-galactosidase from *A. niger*, to obtain oligomers of type **E**: uG-(R-G)$_n$-R, with n = 1 to 4. The resulting oligomers **E4** to **E10** were used as standards.

Retention of RG Oligomers upon HPAEC

With isocratic HPAEC elution, a linear relationship between log (k') and DP is expected for a homologous series (Lee, 1990). In our case, however, a gradient of NaOAc was applied. In Figure 1 log (k') is plotted versus the DP of the various types of RG oligomers, and for comparison also for HG oligomers. Gradient elution probably explains why no linear relationship is found for the series in Figure 1. Instead, the increase in log (k') tended to become smaller when the DP increased. Only HG oligomers and type **D** oligomers, which latter contain the highest amount of GalA residues of all types of RG oligomers in Figure 1, have a reasonable correlation coefficient for a linear relationship between log (k') and DP (see caption of Fig. 1).

The HG oligomers had much higher capacity factors than RG oligomers, which can be explained by the fact that they consist exclusively of acidic GalA units, resulting in higher charge densities and therefore stronger retention on the CarboPac column than the RG oligomers. Differences in the end sugars greatly influenced the capacity factors of the RG oligomers. Types **A** and **C**, which have their nonreducing and reducing end sugars interchanged, behaved practically the same on the CarboPac PA-100 column. Type **B** oligomers, with a Rha unit at both ends, were less retained on the column than type **A** and **C**. Apparently the effect of the loss in acidity on any of the termini of the oligomer, resulting in diminished binding to the resin, is larger than the effect of the increase in DP, which within a homologous series generally results in better binding (Lee, 1990). This phenomenon was observed before (Chapter 2). Moreover, the capacity factors of oligomers of type **A** and **C** were rather close to those of type **B** oligomers that are one Rha unit larger, but which contain the same number of GalA residues. This shows that the number of acidic GalA units in an RG oligomer is the most important factor for binding to the CarboPac column. Consequently, RG oligomers with a GalA at both ends are even more retained. Oligomers **D3**, **D5**, and **D7** even eluted after oligomers of type **B** with DP's of 5, 7 and 9 respectively, so two units larger. An us-GalA at the nonreducing end as in **E4** causes much stronger retention on HPAEC compared to oligomer **A4**. This might be ascribed to the increased acidity of the nonreducing us-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 (Hotchkiss and Hicks, 1993).

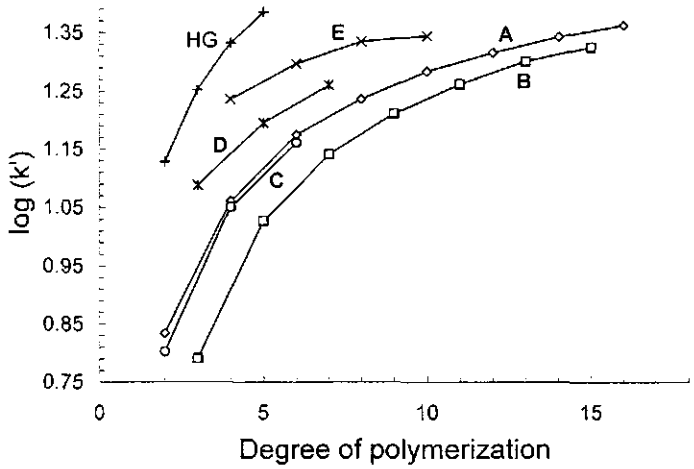

Figure 1. *Log (k') versus DP for HG oligomers (HG), and RG oligomers of type **A**, **B**, **C**, **D**, and **E** (see Table I). The r^2 calculated for a linear relationship between log (k') and DP was: **A**: 0.83; **B**: 0.86; **C**: 0.95; **D**: 0.98; **E**: 0.91; and for the HG oligomers: 0.97.*

In Figure 1, the curves of log (k') versus DP were similar for oligomers of type **A**, **B**, **C** and **D**. This means that the effect of an additional R-G unit seemed independent of the effects of the end sugars. Only major differences, like the us-GalA at the nonreducing end in case of type **E** oligomers, and of course a different composition as in case of the HG oligomers, resulted in different slopes.

Preparative Isolation and ^{1}H 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 to A-6, were identified by comparison with standards (see Table I) using HPAEC, and are shown in Table II. As the response factors of the various oligomers were not known, the data in Table II do not represent the absolute amount of the various oligomers present. In each fraction one oligomer is predominantly present (60 to 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

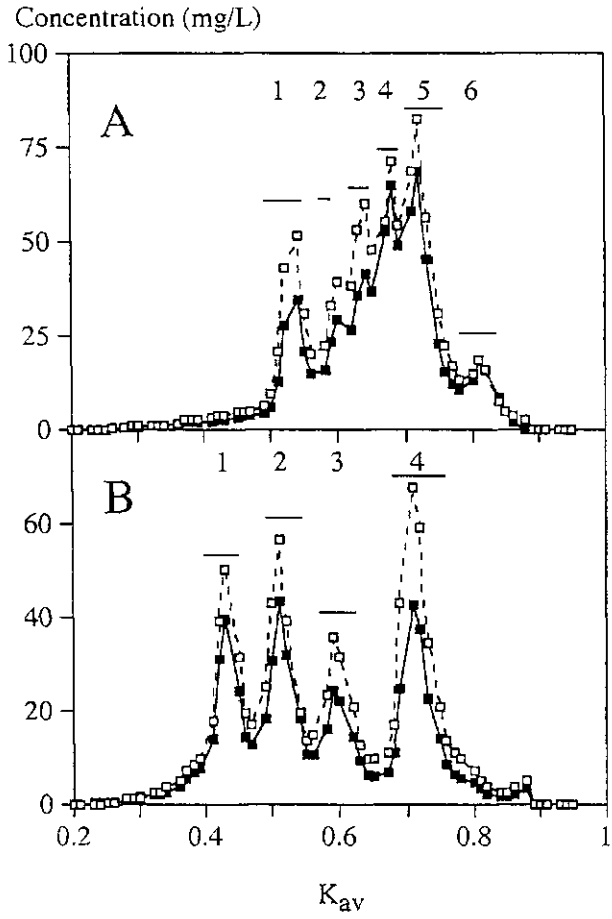

Figure 2. *Bio-Gel P-4 + P-6 fractionation of the digests of the mixture of type **A** oligomers with a DP > 10 made by RG-hydrolase (A) and RG-lyase (B).*___■____, *GalA;*___ ❑ ___, *Rha.*

Table II. *Composition of Bio-Gel fractions A1 to A6 (from the RG-hydrolase digest)*
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 I.

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

relatively more enzyme than was used for incubation of only 20 h in case of the purified RG oligomers (see below). Another RG-hydrolase digest was prepared under similar conditions as the mixture digest but by only 20 h incubation. In this case, no **D3** was formed. 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 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 to A-6 (Fig. 2a) and B-1 to 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 III) (Schols et al., 1994; Renard et al., accepted; Chapter 3). Identification was complicated by variations of the NMR spectra that occurred with varying pH values, especially of the H-5 signal of GalA (Tjan et al., 1974).

Fraction A-6, eluting at the highest K_{av} on Bio-gel P-4 + P-6, and therefore containing the smallest oligomers, showed predominantly the signals for both nonreducing end and reducing end GalA: in addition to the diagnostic signals given in Table III, the signals for the H-2 of the β anomer (a quadruplet at ~ 3.55 ppm) and the H-3 of the α anomer (at 4.02 ppm) were clearly visible. The other major signal in the anomeric region (5.27 ppm) originated from an internal Rha. Integration of the H-1 signals gave areas of 5 : 5 : 6 respectively for reducing end GalA, internal Rha and nonreducing end GalA. A signal with an area of 1 was present at 5.23 ppm (nonreducing end Rha). NMR spectroscopy of fraction A-6 thus confirmed the presence of predominantly the trimer **D3**, contaminated by some oligomer with Rha as nonreducing end sugar, probably **C4** (see below).

Fraction A-5 was the most abundant and purest oligomer, and gave a well-defined NMR spectrum which showed seven signals between 4.5 and 5.5 ppm: anomeric signals for reducing end (5.33 and 4.62 ppm) and internal (5.05 ppm) GalA, and for nonreducing end and internal Rha (5.23 and 5.17 ppm); two additional signals at 4.94 and 4.70 ppm were from H-5 of internal GalA and the α anomer of reducing end GalA, indicative of a low pH. Integration of the signals gave similar

Table III. *Diagnostic signals for identification of reducing and nonreducing end Rha and GalA residues of RG oligomers, and their presence in enzyme degradation products*
++: Major signal; +: present; +/-: weak signal; -: absent. Int, internal; r.e., reducing end; n.r.e., nonreducing end; U-n.r.e., us-GalA nonreducing end. Explanation of symbols in oligomer structure in Table I.

	GalA					Rha			Main oligomer
	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.	based on ^{1}H NMR
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**? $(R\text{-}G)_3\text{-}R$
B-4	-	-	-	++	++	++	++	-	**E4**: uG-R-G-R
B-3	+	?	-	+	+	++	+	-	

[a] Renard et al. (accepted) [b] Colquhoun et al. (1990), Schols et al. (1994) [c] Mutter et al. (1996a)

areas for the four types of residues and fraction A-5 was therefore interpreted as the tetramer **C4**, confirming the structure assignment made using HPAEC.

Fraction A-4 was a mixture of three oligomers upon HPAEC, with surface ratio's of the peaks of 3.5 : 1 : 1, which explained the low quality of its NMR spectrum. Predominant signals were those of internal GalA and Rha. The signals of reducing end Rha, if present, were too low to be distinguished from the noise. A low intensity quadruplet at ~3.6 ppm indicated presence of reducing end GalA, while the H-4 signal of a nonreducing end GalA was also present. Anomeric signals for reducing end GalA were masked either by the H-1 signal of internal Rha (α anomer) or by the wide water peak (β anomer). However, the NMR spectroscopy results did not contradict the presence of **D5** as major product in fraction A-4.

The spectrum of fraction A-3 indicated the pentamer **B5** i.e. with Rha at both reducing and nonreducing end. Though the H-1 signals for the α anomer of the reducing end Rha and the nonreducing end Rha were superimposed, distinctive signals were the H-1 of the β anomer and the split signals for the GalA directly linked to a reducing end Rha, indicative of a reducing end Rha. In addition to the intense signal at 5.23 ppm, presence of two triplets at 3.40 and 3.35 ppm (H-4 of internal and nonreducing end Rha, respectively) and absence of a signal at 4.30 ppm (H-4 of nonreducing end GalA) confirmed this identification. Integration of the anomeric

signals showed roughly a 1 : 2 ratio for internal to nonreducing end + reducing end Rha, and a 1 : 1 ratio for the two types of GalA, i.e. a pentamer.

Fraction A-2 was again clearly a mixture, showing signals for both reducing end GalA and Rha, though the earlier had higher intensities (relative intensities: 2 : 0.8). An intense signal for nonreducing end Rha (area 2) was also present at 5.2 ppm. Intensities of the signals indicated presence of three Rha (nonreducing 2, internal 3.6) and therefore presence as main oligomer of the hexamer **C6**. This confirms the assignment made using HPAEC.

The spectrum of fraction A-1 showed signals for reducing end and nonreducing end Rha as in fraction A-3, while integration of the anomeric signals indicated three GalA's i.e. the heptamer **B7**.

Of the degradation products of the RG-lyase, only fraction B-4 was pure enough for NMR spectroscopy identification. Its ^{1}H NMR spectrum confirmed that it was an unsaturated oligomer, with the strong signal for the vinylic H-4 at 6.1 ppm, accompanied by the H-1 signal at 5.17 ppm. The reducing end was Rha, with signals at 5.22 and 4.90 ppm, plus those of the neighboring GalA at 5.10 ppm. Another major signal in the anomeric region was that of internal Rha, while no signal was detected for H-1 of internal GalA. Intensity of the signals confirmed that B-4 was the tetramer **E4**.

NMR spectroscopy characterization confirmed the structure assignments that were made by comparing the HPAEC elution behavior of unknown degradation products with that of RG standards. 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 RG-hydrolase and RG-lyase.

Degradation of Linear RG Oligomers by RG-hydrolase

Oligomers **A4** to **A16** (for explanation of codes see Table I), and oligomers **B7** to **B15** were incubated with RG-hydrolase, under conditions that were sufficient to reach an end-point situation, i.e. 20 h. 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 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** etc., see "Discussion". Therefore, only the identification of degradation products from type **A** oligomers is described in detail below.

From **A10**[4] (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

[4] Previously, RG-hydrolase was reported to be most active toward DP 12 and higher (Renard et al., 1995), and RG-lyase toward DP 14 and higher (Mutter et al., 1996b). However, the DP assignment of oligomers was later

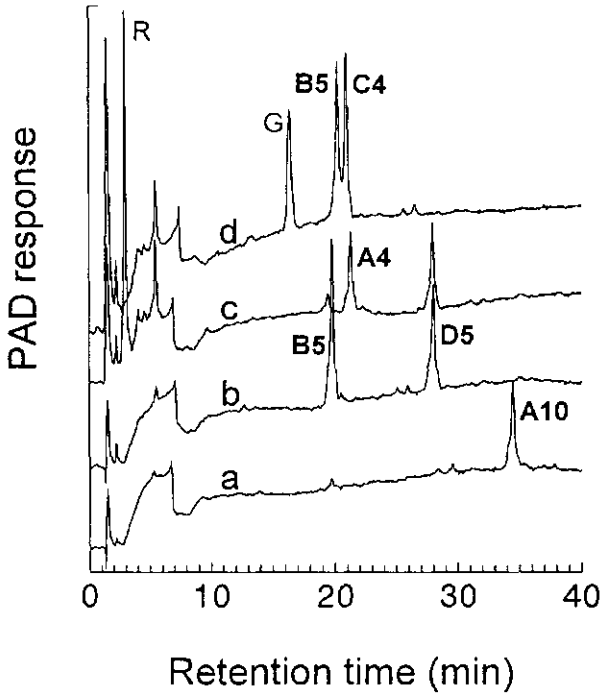

Figure 3. *HPAEC patterns of **A10** (a); after incubation with RG-hydrolase (b); after incubation with RG-hydrolase and subsequently RG-rhamnohydrolase (c); and after incubation with RG-hydrolase and subsequently RG-galacturonohydrolase (d). Explanation of codes in Table I.*

originate. The designation of the products was confirmed by subsequent incubation of the reaction mixture with either RG-rhamnohydrolase or RG-galacturonohydrolase. Figure 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 nonreducing Rha from **B5** by RG-rhamnohydrolase. After RG-galacturonohydrolase action, shown in Figure 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:

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

corrected after characterization by NMR spectroscopy (Renard et al., accepted) into two units smaller, i.e DP 10 instead of DP 12 etc.

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). The surface of the peak of **B7**, formed when RG-hydrolase cleaved five units from the nonreducing end, was 2.1 times that of **B5**, resulting from cleavage seven units from the nonreducing end. Though not enough material was present to isolate all degradation products on large scale, and therefore the HPAEC response factors could not be calculated, Hotchkiss and Hicks (1990) reported that smaller DP oligogalacturonides exhibited enhanced molar response, whereas larger DP oligomers had reduced molar response relative to GalA. Going from **A10** to **A16**, we observed a decrease of app. 40% of the molar response factor. Therefore it can safely be assumed here that the molar response factor of heptamer **B7** is at best as high as and probably lower than that of the corresponding pentamer **B5**. This means that cleavage at five units from the reducing end, producing **B7**, occurred at least 2.1 times as frequently as cleavage at seven units from the reducing end. This suggested that the RG-hydrolase preferred cleavage five units from the nonreducing end GalA. For the corresponding nonreducing end products **D5** and **D7**, a ratio of 1 to 0.7 was found. As the molar response factor of the heptamer can be expected to be lower than that of the pentamer, this ratio naturally did not give the same information as the ratio between **B7** and **B5**.

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 (1 : 0.7) 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 nonreducing 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.

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**. **B11** was not degraded, and in contrast with RG-hydrolase, the general trend was that type **B** oligomers were not cleaved at the same locations as the type **A** oligomers they originated from.

From **A12** (Fig. 4, line a) two peaks were formed (Fig. 4, 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. 4, 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 us-GalA from the nonreducing end of RG fragments. As expected, RG-rhamnohydrolase was not able to remove Rha from the oligomers in the digest.

The nonreducing us-GalA unit must result from the cleavage by RG-lyase, since the original **A12** contains a saturated GalA at the nonreducing end. The structures therefore can be positioned as follows to form the original **A12**:

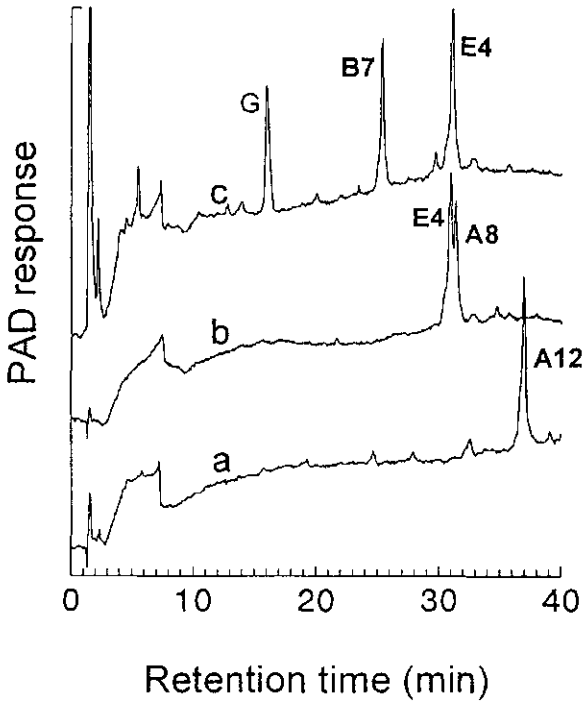

Figure 4. *HPAEC patterns of **A12** (a); after incubation with RG-lyase (b); and after incubation with RG-hydrolase and subsequently RG-galacturonohydrolase (c). Explanation of codes in Table I.*

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 nonreducing end).

From **B13** two peaks were formed that corresponded with **B9** and **E4** (not shown). Subsequent incubation of the reaction mixture with RG-rhamnohydrolase confirmed the designation of **B9**. To form the original **B13** the products can be positioned as follows:

R-G-R-G-R-G-R-G-R (**B9**) + uG-R-G-R (**E4**),

where cleavage has occurred at four units from the reducing Rha (or nine units from the nonreducing Rha).

From **A14** two major and two minor products were formed as concluded from the HPAEC pattern (not shown). The major peaks corresponded to **A10** and **E4**, the minor ones, together responsible for 15% of the total peak area, had similar retention times as **A8** and **E6**. Subsequent incubation with RG-galacturonohydrolase confirmed the assignments of **A10** and **A8**.

To form the original **A14** the products can be put together as follows:

G-R-G-R-G-R-G-R-G-R (**A10**) + uG-R-G-R (**E4**), and
G-R-G-R-G-R-G-R (**A8**) + uG-R-G-R-G-R (**E6**),

where RG-lyase cleaved preferentially at four units from the reducing end (or ten units from the nonreducing end) and to a smaller extent six units from the reducing end (or eight units from the nonreducing end).

B15 was cleaved into four products: **B9**, **E4**, **B11**, and **E6** (not shown) . Subsequent incubation of the reaction mixture with RG-rhamnohydrolase confirmed designations of **B9** and **B11**. To form **B15**, the products could be positioned as follows:

R-G-R-G-R-G-R-G-R (**B9**) + uG-R-G-R-G-R (**E6**), and
R-G-R-G-R-G-R-G-R-G-R (**B11**) + uG-R-G-R (**E4**).

The response factor for **B11** is most likely lower than for **B9**. However, since the amount of **B11** produced was higher than that of **B9**, it can be concluded that RG-lyase cleaved preferentially at four units from the reducing Rha residue (or 11 units from the nonreducing Rha residue).

Cleavage of **A16** by RG-lyase produced five peaks upon HPAEC (not shown). The largest peak corresponded to **E4**, three other peaks were identified as **A8**, **A10** and **E6**, and traces of a peak corresponding to **E8** were found. Incubation of the reaction mixture with RG-galacturonohydrolase confirmed the assignments of **A8** and **A10**. **A16** could be reconstituted as:

G-R-G-R-G-R-G-R-G-R (**A10**) + uG-R-G-R-G-R (**E6**), and
G-R-G-R-G-R-G-R (**A8**) + uG-R-G-R-G-R-G-R (**E8**).

This leaves the production of **E4** to be explained, since the corresponding second product **A12** was not found in the mixture after 20 h incubation. A time study showed that initially **A12** was indeed formed, but after 20 h it had practically disappeared and the amount of **E4** and **A8** had increased (not shown). RG-lyase apparently first produced **E4**, cleaving at four units from the reducing end. The resulting **A12** could then be further cleaved, as shown above, into **E4** and **A8**. This would explain that the amount of **E4** was more than twice as high (concluded from HPAEC peak areas) than that of **A8**, **A10** and **E6**. Since the amount of **A8** is an indirect measure for cleavage four units from the reducing end, and the amount of **A10** is a measure for cleavage six units from the reducing end, the HPAEC areas of these oligomers were compared. Although the response factor for **A10** is most likely lower than for **A8**, the peak surface of **A10** was more than 30% higher. Therefore, it was concluded that RG-lyase had a preference for cleavage six units from the reducing end.

DISCUSSION

RG-galacturonohydrolase and RG-rhamnohydrolase were very useful in the production of different types of RG oligomers, by modifying the nonreducing end of

available RG fragments. These RG oligomers were successfully used as standards for HPAEC analysis. The components of RG-hydrolase and RG-lyase digests of a mixture of type **A** oligomers were characterized by comparison of the retention times of HPAEC peaks with those of the available standards. SEC isolation of these degradation products and subsequent characterization by ^{1}H NMR spectroscopy, confirmed the HPAEC identification of those products that could be obtained in pure enough form. The RG-galacturonohydrolase was also used to remove the nonreducing GalA unit from purified type **A** oligomers, forming original type **B** oligomers. Subsequently, the components of the RG-hydrolase and RG-lyase digests of purified type **A** and **B** oligomers were characterized. Here, RG-galacturonohydrolase and RG-rhamnohydrolase could modify originally formed products into new RG oligomers for which standards were available. In this way, a double check on the structure assignment was established.

Initially, there were problems with the PAD detection of RG oligomers. In general, when the sensitivity of detection decreased due to a filthy, or unpolished gold surface of the PAD detector, or when the gold surface had sunk below the level of the surrounding electrode block due to intensive use, the response factor for RG oligomers decreased more than that for HG oligomers. This suggests that the RG oligomers were more sensitive to a detection system in sub-optimal condition than the HG oligomers. The ratio of molar response factors e.g. for **A8** (see Table I) : GalA could vary between a ratio of 8 : 1 in optimal condition of the PAD system, to a ratio of 0.8 : 1 for a PAD system in sub-optimal condition. Other factors of influence were the pulse train used, and the condition of the reference electrode. Furthermore, the working electrode filter as present in some versions of the Dionex PED detector, was found to filter away too much signal for acidic oligomers in general and was therefore removed from the Dionex systems in question. When the sensitivity of the gold electrode decreased, differences in selectivity between the different types of RG oligomers were also observed. Oligomers of type **A**: G-(R-G)$_n$-R, **E**: uG-(R-G)$_n$-R, and **B**: (R-G)$_n$-R showed in general lower response factors than oligomers of the type **C**: R-(G-R)$_n$-G and **D**: (G-R)$_n$-G. Apparently oligomers with a Rha at the reducing end are more sensitive for a gold electrode in bad condition than oligomers with a GalA at the reducing end. For the reasons described here, HPAEC-PAD was always performed using a freshly polished gold electrode, with no difference in level between the gold surface and the surrounding electrode block.

Cleavage of RG oligomers by RG-hydrolase, as summarized in Figure 5, occurred between a GalA unit on the nonreducing side and a Rha unit on the reducing side of glycosidic linkage being split. The fact that initially **B9** was formed in case of **A14**, and **B11** in case of **A16**, showed that first cleavage was close to the nonreducing end. RG-hydrolase cleaved type **A** oligomers preferentially at five units from the nonreducing GalA, and type **B** oligomers at four units from the nonreducing Rha. The smallest oligomer that could be cleaved by RG-hydrolase was **B9**, with a Rha unit at both the nonreducing and reducing end of the chain. When the DP increased, the cleavage possibilities also increased (Fig. 5): beside cleaving four units from the first nonreducing Rha, RG-hydrolase also cleaved six units from the first nonreducing Rha. However, except for **B11**, cleavage at four units from the

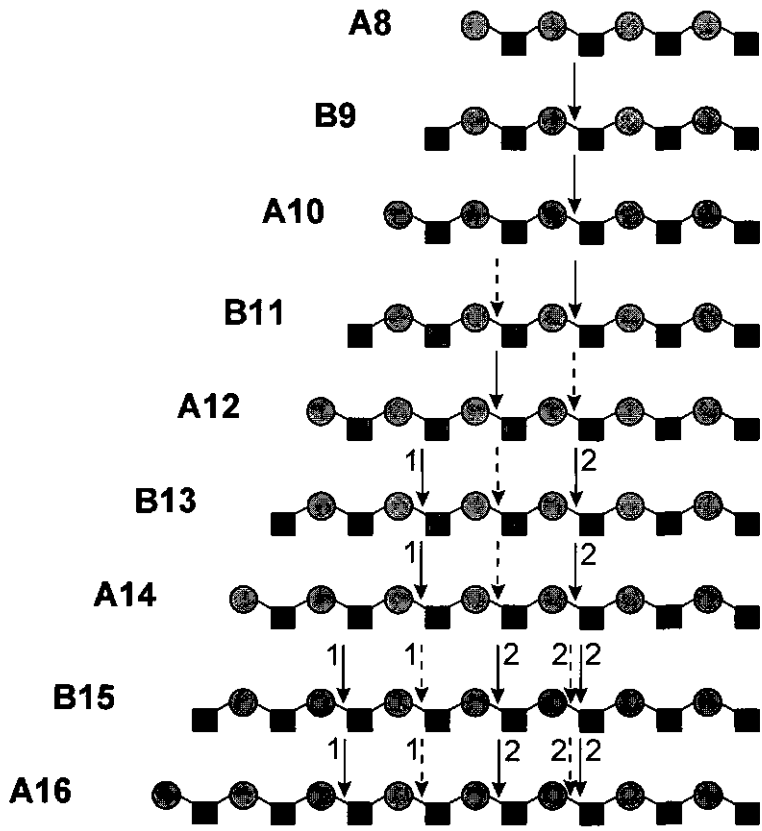

Figure 5. *Cleavage patterns of RG oligomers by RG-hydrolase. Symbols: ●: α-GalA (1,2)-linked to Rha; ■: α-Rha (1,4)-linked to GalA, or Rha at the reducing end. The solid arrows indicate preferential cleavage, the dotted arrows indicate least preferred cleavage. The numbers refer to 1, first cleavage; and 2, second cleavage.*

nonreducing Rha was preferred.

RG-hydrolase cleaved the type **B** oligomers at the same locations as the type **A** oligomers they resulted from. Going from **B9** to larger oligomers in Figure 5, 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. Unfortunately, no such oligomers, of sizes large enough for the RG-hydrolase to be able to cleave them, were available.

From apple MHR-S, RG-hydrolase releases primarily galactosylated equivalents of **C4** and **C6**, and small amounts of **C4** and **C6**. MHR-S is thought to consist of three different subunits (Schols and Voragen, 1996). Subunit I consists of

xylogalacturonan molecules, subunit II consists of RG regions with a Rha : GalA ratio < 1 and long arabinan side chains, and subunit III consists of the galactosylated strictly alternating RG regions, toward which RG-hydrolase and RG-lyase are active. The results obtained in the present study on the action pattern of RG-hydrolase toward purified RG oligomers might enable some predictions about the length of the subunit III RG regions in MHR-S, from which oligomers such as **C4** and **C6** can be released. If the possible influence of Gal side chains is neglected, it can be speculated that from an RG region of minimum thirteen residues, with Rha at both ends, such as **B13** (Fig. 5), at both ends connected to other MHR subunits, RG-hydrolase is able to release the tetramer **C4** after two cleavages. From RG regions of DP 15 and higher, RG-hydrolase is able to release the hexamer **C6** after two cleavages (Fig. 5). Previously it was shown that RG-hydrolase had a degree of multiple attack of 4.0 toward apple MHR-S (Chapter 4). This means that during the lifetime of an individual enzyme-substrate complex, on average four linkages are cleaved following the first. If RG-hydrolase acts toward galactosylated RG regions in MHR-S with the same mode of action as toward the linear RG oligomers, then the enzyme can have a degree of multiple attack of 4 toward an RG region with a length of 25 residues, if only the tetramers are released. This suggests that the RG regions in MHR-S must be rather longer than suggested by Schols and Voragen (1996), i.e. around thirteen units.

RG-lyase cleaved RG fragments between a Rha on the nonreducing side and a GalA unit on the reducing side of glycosidic linkage being split, as summarized in Figure 6. Initial formation of **A12** from **A16**, showed that first cleavage was close to the reducing end. **A12** was the smallest oligomer cleaved by RG-lyase, at four units from the reducing Rha unit. **A14** and **B15** were preferentially cleaved at four units from the reducing end, but could also be cleaved six units from the reducing end. **A16** was the only oligomer that was preferentially cleaved at six units from the reducing end of the RG chain. The presence of oligomer **E8** in the RG-lyase digest of the mixture of type **A** oligomers with a DP > 10 suggests that larger RG oligomers, for instance of DP 18 and 20, can probably even be cleaved eight units from the reducing end. Similarly to what was observed for RG-hydrolase, the cleavage options increased with increasing DP of RG oligomers. However, contrary to RG-hydrolase, RG-lyase did not cleave the type **B** oligomers at the same sites as the type **A** oligomers they originated from. Going from **A12** to larger oligomers in Figure 6, the cleavage options increased when besides an additional nonreducing Rha unit also an additional GalA was present, i.e. in **A14** and **A16**. 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 twelve 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 eleven residues. Unfortunately, no oligomers with a GalA at the reducing end, of sizes large enough for the RG-lyase to be able to cleave them, were available.

From apple MHR-S RG-lyase releases as major products the completely galactosylated equivalents of **E4**, **E6**, **E8**, and **E10**. The results obtained in the

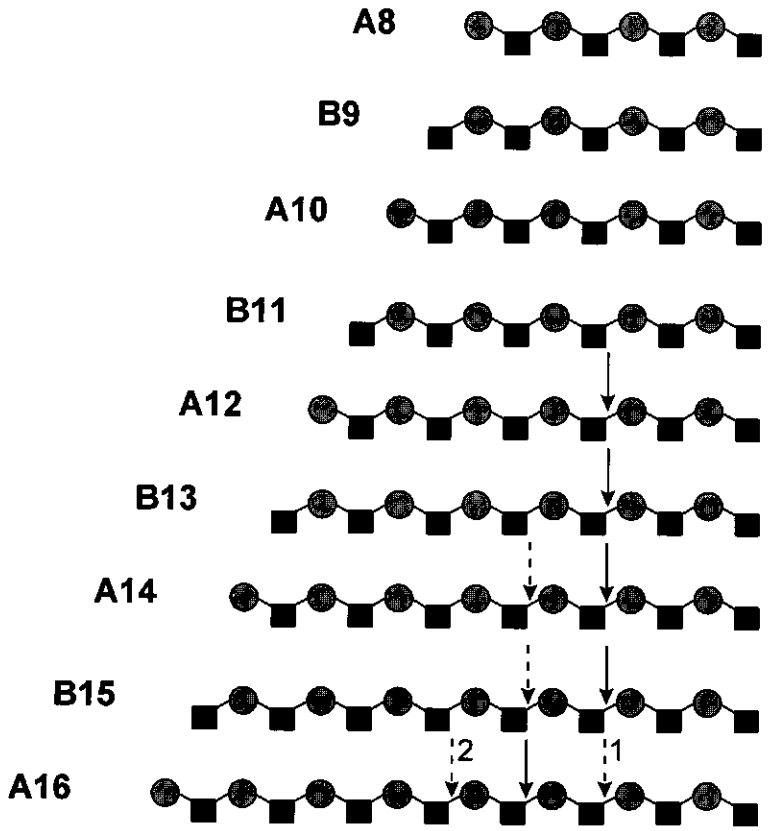

Figure 6. *Cleavage patterns of RG oligomers by RG-lyase. Symbols: ●: α-GalA (1,2)-linked to Rha; ■: α-Rha (1,4)-linked to GalA, or Rha at the reducing end. The solid arrows indicate preferential cleavage, the dotted arrows indicate least preferred cleavage. The numbers refer to 1, first cleavage; and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

present study on the action pattern of RG-lyase toward purified RG oligomers might enable some predictions about the length of the subunit III RG regions in MHR-S, from which the typical RG-lyase oligomers can be released. However, earlier it was found (Chapter 4) that removal of Gal from MHR-S with β-galactosidase resulted in a decrease in V_{max} of RG-lyase. Therefore, the length of (partly) galactosylated RG regions, needed to produce the typical oligomers, could be shorter than for bare RG regions. Anyhow, if the possible influence of Gal side chains is neglected, it can be speculated that from an RG region of minimum sixteen residues, such as **A16**, at both ends connected to other MHR subunits, RG-lyase is able to release the tetramer **E4** after two cleavages (Fig. 6). Previously it was shown that RG-lyase acted toward apple MHR-S with a degree of multiple attack of 2.5 (Chapter 4). This means that during the lifetime of an individual enzyme-substrate complex, on average 2.5 linkages are cleaved following the first. If RG-lyase acts toward galactosylated RG regions in MHR-S with the same mode of action as toward the

linear RG oligomers in this study, then a degree of multiple attack of 2.5 can be found toward an RG region with a minimum length of 22 residues, if only tetramers are released. Again, this suggests that the average length of RG regions in MHR-S must be rather longer than approximately thirteen units as suggested by Schols and Voragen (1996).

The pattern of action of RG-hydrolase and RG-lyase toward RG oligomers implies that small stubs of RG must still be attached to the other MHR subunits after enzyme action. If an RG chain is pictured forming a link between for instance two xylogalacturonan chains in MHR-S, then in case of RG-hydrolase on both the nonreducing and reducing side RG stubs of five units would be left, and in case of RG-lyase on the nonreducing side RG stubs of eight units, and on the reducing side stubs of four, six or perhaps eight units. This could explain why after RG-hydrolase and RG-lyase action, beside GalA, still a considerable amount of Rha is present in the larger MHR-S degradation products (Chapter 2, Chapter 4).

Both RG-hydrolase and RG-lyase are able to degrade RG chains devoid of Gal side chains. However, from apple MHR-S predominantly galactosylated oligomers are released by RG-hydrolase (Chapter 2) and RG-lyase (Chapter 4). Therefore, in apple MHR-S, the RG stretches must be predominantly galactosylated, and if linear or partly galactosylated regions are present, they are too short for the enzymes to release oligomers from. Nevertheless, Schols et al. (1995) showed that different populations of pectic hairy regions occur in apple cell walls. From alkali soluble pectin extracts for instance, a comparatively large amount of the tetramer **C4** was released by RG-hydrolase. This suggests that in native pectin, RG regions with less Gal substitution than the enzymically obtained MHR preparation are present.

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Chapter 8

General Discussion

NEW RG DEGRADING ENZYMES

The drive behind the initiation of this thesis, was first of all our interest in possible new pectolytic enzyme components in the commercial enzyme preparation *Pectinex Ultra SP*. From this preparation, produced by the fungus *Aspergillus aculeatus*, the first rhamnogalacturonan (RG) degrading enzyme was isolated: *RGase* (Schols et al., 1990a), later named *RG-hydrolase*[1] (Mutter et al., 1996; Chapter 3). Furthermore, preliminary experiments at that time indicated the presence of other yet unknown enzyme activities. Pectinex Ultra SP as such is currently used for mash treatment (pulp enzyming) of apples and pears in juice production, but can be the basis of enzyme formulations for other pectinase applications e.g. in citrus, grape and olive processing. Industrially used polysaccharide degrading enzyme preparations are mostly mixtures of many enzymes produced under specified conditions by the microorganism in question. In addition to the few major activities, the presence of unknown enzymic side activities can result in desired or undesired effects in fruit and vegetable processing. Therefore, knowledge of these unknown enzymes can help to explain and solve problems, or improve processing. RG-hydrolase for instance was effective in reducing ultrafiltration problems, that resulted from fouling of ultrafiltration membranes by high molecular mass RGs, released from raw materials by processing enzymes (Stutz, 1993).

The newest trend in industrial enzyme application will be the use of *tailor-made* enzyme preparations (Heldt-Hansen et al., 1996). These preparations contain only the essential enzymes, in the proper ratio, needed for a specific combination of raw material and process to make a specific product. Beside knowledge of the composition of raw materials in order to select the appropriate enzymes, enzyme producers need to have a reliable and adequate system for production of single pure enzymes in high quantities. Rapid progress in DNA recombinant technology enabled the cloning and expression of selected recombinant single enzyme components. The usual way of obtaining monocomponent enzymes is to identify the enzyme component in the enzyme mixture, purify it, determine the amino acid sequence, use this information to construct a labeled DNA-probe, isolate by hybridization the gene from a cDNA or genomic library constructed from the fungus in question, and finally transform the gene into an expression host for production of high amounts of the enzyme. The most time-consuming process in the cloning of new enzymes is usually

[1] See List of Abbreviations

the purification and the amino acid sequence determination required for the synthesis of labeled oligonucleotide probes. One of the latest developments is a method called *expression cloning* (Dalbøge and Heldt-Hansen, 1994). In this method, a cDNA library in *E. coli* is transformed into *Saccharomyces cerevisiae*, and the transformants are screened for enzymes using sensitive and reliable plate assays. Positive colonies are then selected, the genes cloned, and transformed into *Aspergillus oryzae* for high level expression. The great advantage of this method compared to traditional cloning is primarily that it requires no prior knowledge of the structure of the protein of interest. The steps of enzyme purification, amino acid sequencing, construction of probes and hybridization can be excluded. Using expression cloning, many new enzyme genes have been cloned from *A. aculeatus* (Kofod et al., 1994b; Kauppinen et al., 1995; Christgau et al., 1994; Christgau et al., 1995; Christgau et al., 1996). However, using this method, only those genes can be found, coding for enzymes for which appropriate plate screening assays are available. These plate assays usually rely on a change in visual appearance of a gel with substrate incorporated, as a result of - mostly endo-acting - enzymes. For enzymes for which no (satisfactory) plate assay has been developed yet, still the conventional, time-consuming, route has to be followed.

In this thesis two new exo-enzymes are described, *RG-rhamnohydrolase* (Mutter et al., 1994; Chapter 2) and *RG-galacturonohydrolase* (Chapter 6), for which no plate assays have been developed yet. It is difficult to develop plate assays for exo-enzymes, since the enzymically catalyzed changes of the substrate are only minor, and e.g. the attachment of a chromophore to the substrate could even hamper enzyme action. Therefore, these enzymes could not have been found using expression cloning. A third new enzyme, *RG-lyase*, was isolated using conventional purification (Chapter 4), and at about the same time the enzyme was cloned using expression cloning (Kofod et al., 1994b). The enzyme activity could be picked up because a plate assay for RGases had been developed. These three new enzymes from Pectinex Ultra SP were fractionated by various forms of chromatography. Enzyme activity of the obtained fractions was tested on appropriate substrates. In this project *apple modified hairy regions (MHR)*, and *RG oligomers* derived therefrom, were used as model substrates for RG types of pectic polysaccharides (see Fig. 2, Chapter 1). Since RG-hydrolase was hindered by acetyl groups, saponified MHR (MHR-S) was always used to exclude the possibility that new enzyme activities could not be detected because they were hindered by ester groups. Enzyme-substrate incubation mixtures were then analyzed using HPLC in order to detect changes in molecular mass of MHR-S (HPSEC), and release of oligomers or monomers from MHR-S or from RG oligomers (HPAEC). Enzymes of interest were purified, and the most relevant characteristics will be discussed in the following paragraphs.

RG-rhamnohydrolase (Chapter 2) removes a terminal Rha unit from the nonreducing end of RG chains. To date, no enzyme with the same substrate specificity has been reported elsewhere in literature. RG-rhamnohydrolase is specific for the α-(1,4) linkage of Rha to α-GalA, as present in RG structures. It is not active toward the model substrate commonly used for rhamnohydrolases, p-

nitrophenyl rhamnopyranoside, in which the C-1 of Rha is linked to p-nitrophenylate. The enzyme is not able to cleave Rha α-(1,2), Rha α-(1,4) and Rha α-(1,6) linkages to β-Glc; or Rha α-(1,2) linkages to β-Gal, as present in some flavanone glycosides and glycoalkaloids. In contrast with RG-rhamnohydrolase, another rhamnohydrolase that was purified from the same source, *pnp-rhamnohydrolase* (Chapter 2), is active toward p-nitrophenyl rhamnopyranoside, but not toward any of the RG substrates tested. This pnp-rhamnohydrolase is less specific than RG-rhamnohydrolase, as it cleaves besides the pnp-Rha, both Rha α-(1,2) and Rha α-(1,6) linkages to β-Glc. An important conclusion from this work is that a pnp-glycoside, frequently used in the screening for new glycosidases, is not a good model substrate for all glycosylhydrolases.

The second RG-degrading exo-enzyme, *RG-galacturonohydrolase* (Chapter 6), is not active toward homogalacturonan (HG) structures, independent of their size or presence of methoxyl groups. The enzyme is only able to remove the terminal nonreducing GalA unit from RG structures. It can not remove a Δ-(4,5)-unsaturated GalA (us-GalA) from the nonreducing end of RG or HG chains. Again, this was the first literature report of such an enzyme. The influence of an acetyl group, attached to C-2 or C-3 of GalA, on enzyme activity was not investigated, since from all substrates used the esters were removed during preparation. Small substrates, e.g. $(G\text{-}R)_3$ (for explanation of symbols: see List of Abbreviations), are cleaved with higher specificity than larger oligomers or MHR-S. RG-galacturonohydrolase catalyzes hydrolysis with inversion of anomeric configuration and thus most likely operates via a single displacement reaction mechanism of hydrolysis. Also RG-hydrolase, RG-rhamnohydrolase, pnp-rhamnohydrolase, and an exogalacturonase able to degrade xylogalacturonan (Beldman et al., 1996), all from *A. aculeatus*, were found to catalyze hydrolysis with inversion of anomeric configuration (Pitson et al., submitted). Members of a given glycosyl hydrolase family, i.e. classified by amino acid sequence similarity (Henrissat and Bairoch, 1996), do appear to exhibit the same stereoselectivity, since they share a common fold and active-site topology (Davies and Henrissat, 1995). Of these inverting hydrolases from *A. aculeatus*, only for RG-hydrolase the amino acid sequence is known (Kofod et al., 1994b; Suykerbuyk et al., 1995), by which it can be classified into glycosyl hydrolase family 28 (SWISS-PROT Protein sequence data bank; *http://expasy.ch/cgi-bin/lists?glycosid.txt*), also known as the "polygalacturonase family".

Recently also the crystal structure of *RG-hydrolase* has been solved (Petersen et al., 1997). It is the first reported three-dimensional structure of an enzyme belonging to the polygalacturonase family. The enzyme folds into a large right-handed parallel β-helix structure, and the core of the molecule is composed of 13 complete turns of β strands, which line up to form four parallel β-sheets. The enzyme represents a fairly new fold, which was first observed in 1993 for the pectate lyase C (PelC) structure from *Erwinia chrysanthemi* in 1993 (Jurnak et al., 1996). All of the known enzymes with this fold are involved both in recognition of large polysaccharides and usually also in cleavage of them, and have a large groove that appears to be suited for this. The distance between the predicted catalytic residues in RG-hydrolase is 10.1 Å (Petersen et al., 1997), which is in agreement with an

inverting mechanism of hydrolysis (Davies and Henrissat, 1995), and indeed established for RG-hydrolase by Pitson et al. (submitted). Two other members of family 28, *Aspergillus niger* endopolygalacturonase I and II, act with the same stereochemical outcome (Biely et al., 1996).

Already in the early stages of the research project, an enzyme activity was discovered that readily degraded the backbone of MHR-S, as does RG-hydrolase. However, upon degradation, completely different oligomers were released as concluded from their retention behavior upon HPAEC. This new endo-enzyme was purified from *A. aculeatus* (Chapter 4), and also found by expression cloning (Kofod et al., 1994b). The amino acid sequence of this enzyme, initially named *RGase B*, was quite different from that of RG-hydrolase (Kofod et al., 1994b), and in fact the enzyme can not yet be classified into any of the 62 glycosyl hydrolase families known to date (SWISS-PROT Protein sequence data bank; *http://expasy.ch/cgi-bin/lists?glycosid.txt*). A print-out of the information on RGase B in the SWISS-PROT protein sequence database is given in Figure 1. The oligomers produced by RGase B from MHR-S were isolated and characterized (Chapter 3; Chapter 4). Surprisingly, these RG oligomers contained an us-GalA residue on the nonreducing end, and appeared to be the result of lyase action. This probably explains the low homology with RG-hydrolase, since these enzymes have completely different cleavage mechanisms. RGase B was therefore named *RG-lyase*, and as a consequence the RGase described by Schols et al. (1990a) was renamed *RG-hydrolase*, but is still frequently referred to as RGase A in literature. Like the other RG degrading enzymes, RG-lyase is only active toward RG structures, and not toward HGs. Similarly to RG-hydrolase, RG-lyase is hindered by acetyl ester groups attached to GalA residues. This appeared to be mainly due to the low affinity of RG-lyase for high acetylated substrates: the affinity for MHR was less than 10% of that for MHR-S (Chapter 4), while the V_{max} toward MHR was only 55% of that toward MHR-S. RG-acetylesterase, purified from *A. aculeatus* (Searle-Van Leeuwen et al., 1992), and later also cloned (Kauppinen et al., 1995), was able to remove 72% of the MHR acetyl groups, after which the affinity of RG-lyase for the substrate became the same as for MHR-S, although the V_{max} was still slightly lower. Furthermore, removal of Ara, present in side chains of MHR-S, improves the action of RG-lyase, especially because this increases the affinity of the enzyme for the substrate, suggesting sterical hindrance by the side chains. Removal of Gal, on the other hand, has a negative effect on RG-lyase action, predominantly by decreasing the V_{max}. This suggests that the single Gal unit attached to C-4 of Rha positively effects the cleavability of the Rha-GalA linkage by RG-lyase.

RG-hydrolase and RG-lyase are both active toward the strictly alternating RG subunit of MHR (subunit III in Fig. 2). This explained why action of both enzymes gives the same result in a *plate assay* with MHR-S as substrate (Chapter 5). In this assay, enzymes are incubated with an agarose gel incorporated with substrate (MHR-S), after which addition of *copper acetate* results in a white opalescent area where RGases have been active. This white color was shown to result from a precipitate of Cu^{2+} ions with xylogalacturonans (subunit I in Fig. 2). These latter molecules are released when RGases degrade the RG subunits of MHR-S.

SWISS-PROT: Q00019

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ID   RHGB_ASPAC     STANDARD;      PRT;   527 AA.
AC   Q00019;
DT   01-FEB-1997 (REL. 35, CREATED)
DT   01-FEB-1997 (REL. 35, LAST SEQUENCE UPDATE)
DT   01-FEB-1997 (REL. 35, LAST ANNOTATION UPDATE)
DE   RHAMNOGALACTURONASE B PRECURSOR (EC 3.2.1.-) (RGASE B) (RHG B).
GN   RHGB.
OS   ASPERGILLUS ACULEATUS.
OC   EUKARYOTA; FUNGI; ASCOMYCOTINA; PLECTOMYCETES; EUROTIALES.
RN   [1]
RP   SEQUENCE FROM N.A.
RC   STRAIN=KSM 510;
RX   MEDLINE; 95050740. [NCBI, Geneva]
RA   KOFOD L.V., KAUPPINEN S., CHRISTGAU S., ANDERSEN L.N.,
RA   HELDT-HANSEN H.P., DOERREICH K., DALBOEGE H.;
RL   J. BIOL. CHEM. 269:29182-29189(1994).
CC   -!- FUNCTION: PECTINOLYTIC ENZYME THAT HAS A POSITIVE EFFECT IN THE
CC       APPLE HOT-MASH LIQUEFACTION PROCESS. HYDROLYSES LINKAGES IN THE
CC       BACKBONE OF THE HAIRY REGIONS OF PECTINS. RHG B IS MORE ENDO-
CC       ACTING THAN RHG A. OPTIMAL PH IS 6.0, ENZYMATIC ACTIVITY BECOMES
CC       UNSTABLE BELOW THIS VALUE; OPTIMAL TEMPERATURE IS 50 DEGREES
CC       CELSIUS.
DR   EMBL; L35500; G558315; -. [EMBL / GenBank / DDBJ] [CoDingSequence]
DR   PRODOM [Domain structure / List of seq. sharing at least 1 domain]
DR   SWISS-2DPAGE; GET REGION ON 2D PAGE.
KW   HYDROLASE; GLYCOSIDASE; GLYCOPROTEIN; SIGNAL.
FT   SIGNAL        1     19       POTENTIAL.
FT   CHAIN        20    527       RHAMNOGALACTURONASE B.
FT   CARBOHYD    350    350       POTENTIAL.
SQ   SEQUENCE   527 AA;  56190 MW;  9806ADF7 CRC32;
     MLKASLLSFV AFTAQVAHAA FGITTSSSAY VIDTNAPNQL KFTVSRSSCD ITSIIHYGTE
     LQYSSQGSHI GSGLGSATVT ATQSGDYIKV TCVTDTLTQY MVVHNGDPII HMATYITAEP
     SIGELRFIAR LNSDLLPNEE PFGDVSTTAD GTAIEGSDVF LVGSETRSKF YSSERFIDDQ
     RHCIAGDAHR VCMILNQYES SSGGPFHRDI NSNNGGSYNA LYWYMNSGHV QTESYRMGLH
     GPYSMYFSRS GTPSTSIDTS FFADLDIKGY VAASGRGKVA GTASGADSSM DWVVHWYNDA
     AQYWTYTSSS GSFTSPAMKP GTYTMVYYQG EYAVATSSVT VSAGSTTTKN ISGSVKTGTT
     IFKIGEWDGQ PTGFRNAANQ LRMHPSDSRM SSWGPLTYTV GSSALTDFPM AVFKSVNNPV
     TIKFTATSAQ TGAATLRIGT TLSFAGGRPQ ATINSYTGSA PAAPTNLDSR GVTRGAYRGL
     GEVYDVSIPS GTIVAGTNTI TINVISGSSG DTYLSPNFIF DCVELFQ
```

Figure 1. *The information on the enzyme RGase B, later named RG-lyase (Mutter et al., 1996), as listed in the SWISS-PROT Protein sequence data bank, on http://expasy.ch/cgi-bin/lists?glycosid.txt.*

Nanograms of RGases can be detected in the plate assay. The Cu^{2+} staining was also used for zymography using isoelectric focusing, by which the pI's of RG-hydrolase and RG-lyase could be determined already in partially purified enzyme fractions. For the screening of the *A. aculeatus* cDNA library in yeast, a different assay was developed in which MHR was treated with dilute acid to remove side chains sugars, then dyed and crosslinked into insoluble grains (Kofod et al., 1994b). Nanograms of RGase activity could then be visualized by solubilization of the grains. The Cu^{2+} staining is coupled to the use of substrates that contain xylogalacturonan, like apple, onion and pear MHR (Schols and Voragen, 1994), and is time-saving in the screening of column fractions during purification of RGases, since time-consuming HPLC analysis can be skipped.

MODE OF ACTION OF RG-HYDROLASE AND RG-LYASE

To learn more about the *mode of action* of *RG-hydrolase* and *RG-lyase*, the enzymic degradation products of a series of purified RG oligomers of different DP's were characterized (Chapter 7). These RG oligomers were produced from sugar beet by acid hydrolysis (Renard et al., accepted), and since the pseudoaldobiuronic linkage R-G is more easily cleaved by acid hydrolysis than the aldobiuronic linkage G-R, these oligomers were of the type $(G\text{-}R)_n$ (type **A** in Chapter 7). In this study, the two exo-enzymes *RG-rhamnohydrolase* and *RG-galacturonohydrolase* appeared to be essential in the modification of various available RG structures to produce a large number of different types of RG oligomers. These oligomers differed in reducing and nonreducing end sugars, and were used as standards to compare the retention times of unknown HPAEC peaks with. Secondly, these exo-enzymes were used to provide a double-check on structure assignment, since they modified the originally formed oligomers in the digest into products for which HPAEC standards were available. In this way, it was established that the smallest oligomer that can be degraded by RG-hydrolase is $(R\text{-}G)_4\text{-}R$ (DP 9), and cleavage occurs preferentially at four units from the first nonreducing Rha unit between a GalA and Rha unit (R-G-R-G↓R-G-R-G-R). The smallest oligomer cleaved by RG-lyase on the other hand is larger: $(G\text{-}R)_6$ (DP 12), cleaved preferentially at four units from the reducing end between a Rha and GalA unit (G-R-G-R-G-R-G-R↓G-R-G-R). Larger oligomers seem to be cleaved preferentially six units from the reducing end. It must be noted that the influence of the reducing end sugar (Rha) could not be investigated, since no enzymes were available nor reported in literature, capable of removing Rha from the reducing end.

$(R\text{-}G)_6\text{-}R$ (DP 13) is cleaved twice by RG-hydrolase, first cleaving four units from the nonreducing end, and secondly cleaving the resulting nonamer again four units from the nonreducing end (R-G-R-G↓^{1}R-G-R-G↓^{2}R-G-R-G-R) (Chapter 7). From a larger oligomer, $(R\text{-}G)_7\text{-}R$ (DP 15), beside the tetramer also the hexamer is released. This suggests that when the RG oligomers are pictured in between two other MHR subunits, the linear RG stretches would have to be thirteen units long for RG-hydrolase in order to be able to produce a tetramer, and fifteen units in order to be able to produce a hexamer. From MHR-S, RG-hydrolase releases predominantly oligomers with a backbone of four and six sugar units, with most Rha units substituted on C-4 with β-Gal (Schols et al., 1990b; Chapter 2). The study of the mode of action of RG-hydrolase toward purified RG oligomers suggests that when long RG stretches are degraded, initially both tetra- and hexamer can be released, but as the RG stretches become smaller, only tetramer can be released. However, when the degradation of MHR-S by RG-hydrolase is followed in time, although initially somewhat more hexamer is produced, and after 20 h the amount of tetramer released is slightly higher than the amount of hexamer, the ratio between the amounts of galactosylated tetramer and hexamer remains rather constant (Schols et al., 1994). Since no differences in start and end products are observed, most RG regions in MHR are most likely not very long.

The largest pure RG oligomer available in the study (Chapter 7), $(G\text{-}R)_8$ (DP 16), is the only oligomer that can be cleaved twice by RG-lyase. This is done by first cleaving four units from the reducing end, and secondly cleaving the resulting duodecamer again four units from the reducing end (G-R-G-R-G-R-G-R$\downarrow^2$G-R-G-R$\downarrow^1$G-R-G-R). This suggests that when the RG oligomers are pictured in between two other MHR subunits, the linear RG stretches would have to be sixteen units long for RG-lyase in order to be able to produce a tetramer. However, RG-lyase releases from MHR-S completely galactosylated oligomers with a backbone of four, six, eight and ten sugar residues (Chapter 3 and 4). This suggests that linear RG stretches in MHR-S are larger than sixteen units. The presence of linear RG stretches of DP 6 to 20 in apple, beet and citrus pectin has been shown by Renard et al. (1995). These DP values are probably underestimated due to degradation during the preparation (acid hydrolysis). As essentially no linear oligomers are released from MHR-S by RG-hydrolase (Chapter 2) or RG-lyase (Chapter 3; Chapter 4), either no linear RG stretches are present, or they are too short for the release of oligomers. The V_{max} of RG-lyase decreases when Gal is removed by treatment with a β-galactosidase (Chapter 4). This suggests that it is easier for RG-lyase to cleave galactosylated regions than linear RG regions, and therefore the minimal length required for RG-lyase action might be shorter for galactosylated RG regions than for linear RG stretches.

Further speculations about the length of RG regions in MHR can be made using the following observations. It was shown that RG-hydrolase acts toward MHR-S with a degree of multiple attack of 4 (Chapter 4). This means that during the lifetime of an enzyme-substrate complex, on average four linkages are cleaved following the first cleavage. This implies that in MHR-S, the average length of RG regions would have to allow for five subsequent cleavages by which four oligomers are produced. Since the galactosylated tetramer and hexamer are produced in almost equal amounts based on HPAEC response, let us assume that from such an average RG region two galactosylated tetramers and two galactosylated hexamers are released. If the observed cleavage pattern of linear RG oligomers is also valid for galactosylated RG regions in MHR-S, then nine sugar backbone residues (R-G-R-G-R-G-R-G-R) would have to be present to allow the first cleavage (Chapter 7), and an additional twenty more for the release of two tetramers and two hexamers, which is an RG sequence of 29 sugar backbone residues, as illustrated in Figure 2. In this figure a degree of multiple attack of 4 can only be obtained if the enzyme starts to cleave close to the nonreducing end of the RG chain. If it starts in the middle, then the degree of multiple attack is lower than 4. Therefore, it is likely that also longer RG stretches are present in MHR. Furthermore, even if RG-hydrolase would cleave exactly as described in Figure 2, if we assume that the value of 4 is an average, there must be RG regions present in MHR that are longer than 29 backbone residues, otherwise the degree of multiple attack as observed would be limited by the length of the RG regions. Schols and Voragen (1996) suggest an average length of thirteen RG backbone units, ranging between six and 24 units in their MHR model (Chapter 1, Fig. 2). The average length of RG regions in MHR, as

predicted from the results in this thesis, is much longer than suggested by Schols and Voragen.

RG-lyase on the other hand cleaves MHR-S with a degree of multiple attack of 2.5 (Chapter 4). RG-lyase needs longer RG stretches to produce oligomers of the same size as RG-hydrolase (Chapter 7). Although galactosylated RG regions might be cleaved more easily than linear RG regions (Chapter 4), it is again assumed that the cleavage pattern on linear RG oligomers (Chapter 7) is also valid for galactosylated RG regions in MHR-S. To obtain an average degree of multiple attack of 2.5, two possible cleavage sequences of the RG backbone sequence of 29 sugar residues in Figure 2 are illustrated. For the first cleavage of RG-lyase, the RG region would have to be twelve backbone sugar units long (G-R-G-R-G-R-G-R-G-R-G-R) (Chapter 7). In the example in Figure 2, in the first cleavage pattern one oligomer of backbone DP 10 and one of DP 6 is released (12+10+6=28), and in the second pattern one oligomer of DP 8 and two of DP 4 (12+8+4+4=28). An additional nonreducing Rha (which makes the sequence 29 units) does not effect the cleavage pattern (Chapter 7). The fact that for RG-lyase a degree of multiple attack of 2.5 can be obtained on the same RG length where RG-hydrolase can have a degree of multiple attack of 4 on, suggests that the degree of multiple attack of RG-lyase (and maybe also of RG-hydrolase) is in fact limited by the length of RG regions in MHR. The availability of linear RG polymers (e.g. 100 sugar backbone residues and more) on which the action pattern of RG-hydrolase and RG-lyase can be studied would be very important to confirm this.

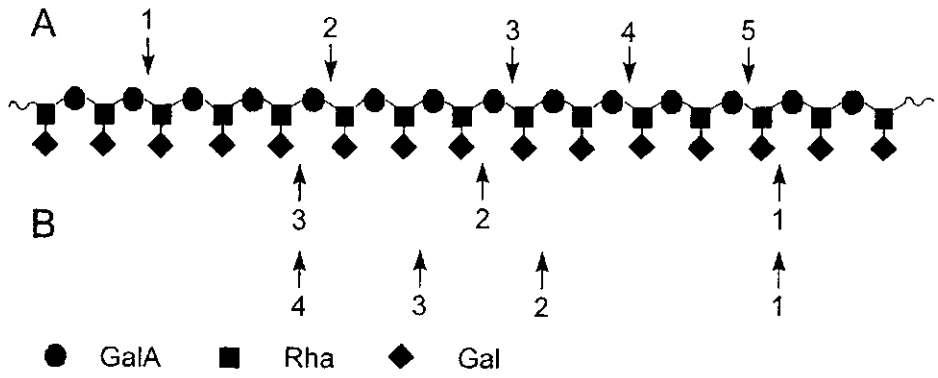

Figure 2. *Suggested cleavage pattern by RG-hydrolase (A) and RG-lyase (B) of a hypothetical alternating RG sequence with Gal side chains of 29 backbone sugar residues, neighboring two other subunits in MHR. For RG-lyase two cleavage patterns are shown, with an average degree of multiple attack of 2.5. The numbers indicate the order in which the linkages are cleaved. The degree of multiple attack of RG-hydrolase and RG-lyase was determined toward MHR-S, from which galactosylated RG oligomers are produced, while the cleavage pattern was obtained from studies toward linear RG oligomers.*

ENZYMES ACTIVE TOWARD PECTIC HAIRY REGIONS: STATE OF THE ART

In this thesis *MHR* has been used as a model substrate for pectic hairy regions. The RG-II type of pectin, described by the group of Albersheim (O'Neill et al., 1990), is not represented in MHR, but no enzymes specifically active toward RG II have been reported in literature yet. Three MHR subunits are distinguished: subunit I, which are xylogalacturonan molecules; subunit II, consisting of RG backbone stubs with less Rha compared to GalA and side chains containing predominantly Ara; and subunit III, which are the strictly alternating RG regions with single Gal side chains (Schols and Voragen, 1996). In the paragraphs below, the enzymes known to date, acting toward the various types of molecules as represented in the three types of subunits of MHR, are described. The site of attack of the various enzymes toward MHR is given in Figure 3.

The sugar composition of the MHR batch used throughout this thesis (Chapter 2) differs from that of Schols et al. (1990b). Therefore, the model of Schols and Voragen (1996) has been adjusted accordingly. In the detailed representation of the subunits in Figure 3, each sugar represents 1 mol%, and the three subunit-representations together add up to 100 mol% of sugars in the correct ratio as in MHR-S: 16 mol% Rha, 20 mol% Ara, 11 mol% Xyl, 18 mol% Gal, 33 mol% GalA (Chapter 2; 2 mol% Glc not represented). The degree of acetylation of MHR is 57 (Chapter 4), and all acetyl esters in Figure 3 are located on the GalA residues in RG regions. The degree of methoxylation is 23, and these methoxyl esters are all located on the xylogalacturonan in Figure 3. Schols et al. (1995) found an average molecular mass of xylogalacturonan of 20,000 to 30,000 Da. Six units of the detailed representation of xylogalacturonan in Figure 3 have a molecular mass of app. 25.000 D. Therefore, all subunit-representations were used six times in the overview of the distribution of the three subunits in MHR in Figure 3 (top). The average length of the RG regions in Figure 3 is 40 backbone residues (ranging between 20 and 60 residues), which would allow for an average degree of multiple attack of 4 for RG-hydrolase and 2.5 for RG-lyase (see above). The distribution of subunits in Figure 3 (top) has been chosen randomly, except for the presence of subunits III neighboring subunit I, since this explains the release of xylogalacturonan molecules from MHR-S when RG-hydrolase degrades the RG regions, in a model where all subunits are part of one backbone.

In Figure 3 the action of RG-hydrolase is indicated, which hydrolyzes the G-R linkage in an endo-fashion in strictly alternating RG structures, as in subunit III of MHR-S. Since the discovery of *RG-hydrolase* in an *A. aculeatus* preparation by Schols et al. (1990a), this enzyme has been found in other fungi including *A. niger* (An et al., 1994; Suykerbuyk et al., 1997), the *Irpex lacteus* preparation Driselase (Düsterhöft et al., 1993; Ishii, 1995), *Trametes sanguinea* (Sakamoto et al., 1994; Sakamoto and Sakai, 1994), and recently the enzyme has been cloned from *Botrytis cinerea* (K.C. Gross, personal communication). Furthermore, the presence of RG-hydrolase is indicated in tomatoes, apples and grapes (Gross et al., 1995).

The second type of RG cleaving enzyme described, *RG-lyase* (Chapters 3 and 4), is endo-acting toward the same MHR subunit, although at a different location

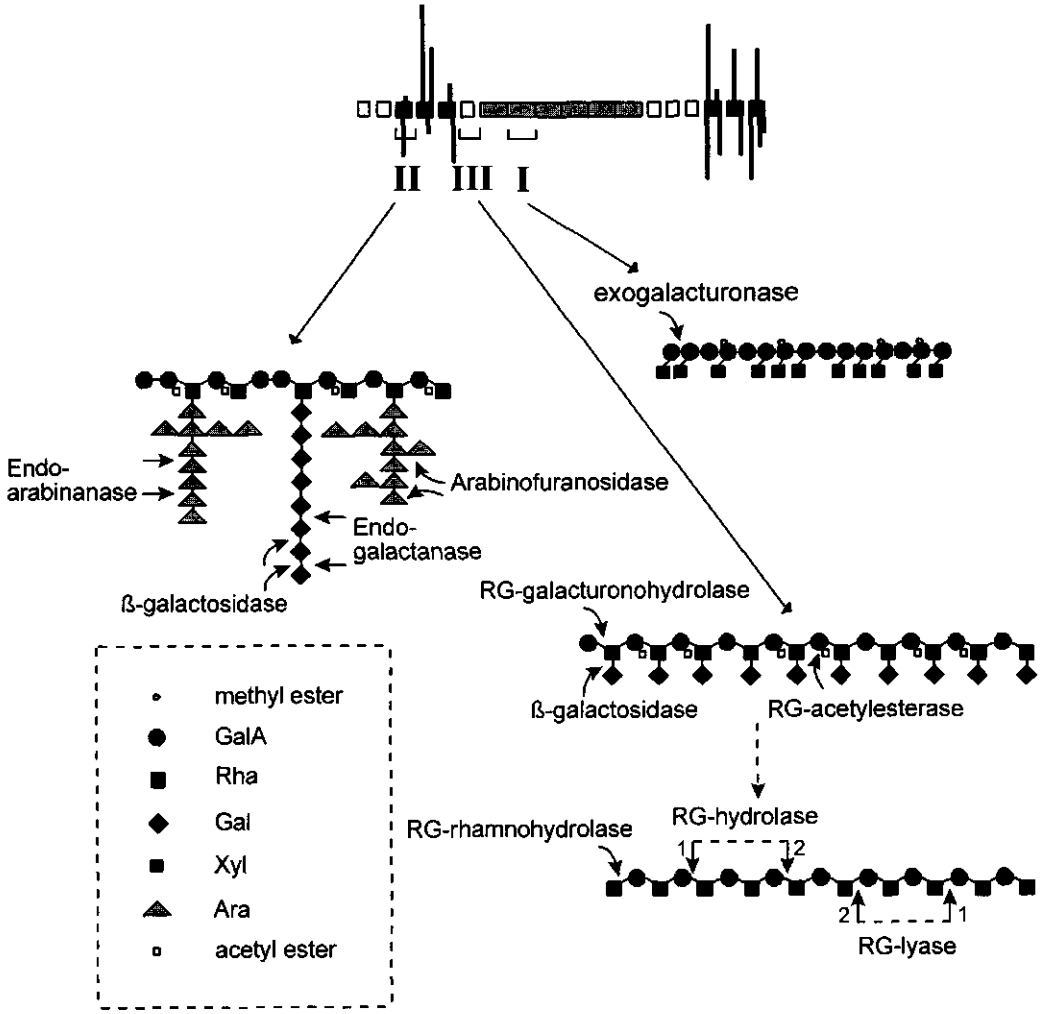

Figure 3. *Location of action of the RG degrading enzymes known to date toward the three subunits of apple MHR. Structural model for MHR from Schols and Voragen (1996), with adjustments according to the data obtained for the MHR batch used in this thesis, see text.*

than RG-hydrolase (Fig. 3): it cleaves the R-G linkage by β-elimination instead of hydrolysis. RG-lyase was cloned by Kofod et al. (1994b). Azadi et al. (1995) also showed that this cloned enzyme was a lyase, although no detailed characterization of the degradation products was given.

The two new exo-enzymes described, *RG-rhamnohydrolase* and *RG-galacturonohydrolase*, were also both active toward strictly alternating RG

structures, removing a Rha and GalA unit respectively from the nonreducing ends. Both enzymes are active toward MHR-S, although they release less than 0.5% of the total amount of Rha or GalA present (Chapter 6). No enzymes have been reported yet acting toward the reducing end of RG chains, although indications have been found during this research for the presence of a galacturonohydrolase, able to cleave off reducing end GalA residues.

Recently, an *exogalacturonase* has been purified from *A. aculeatus* (Beldman et al., 1996), that is not hindered by single unit Xyl side chains attached to C-3 of GalA. The enzyme releases GalA and the dimer of β-Xyl-(1,3)-GalA from gum tragacanth and MHR. Therefore, if the model of MHR is right, there must be xylogalacturonan molecules located at the nonreducing end of MHR chains, in contrast with the chosen representation in Figure 3. Saponification of these substrates increases the action of this enzyme. This exogalacturonase is the only enzyme known to date able to degrade the xylogalacturonan subunit I of MHR. Contrary to the RG degrading enzymes, this enzyme is not highly specific for its substrate, since it was also able to degrade polygalacturonic acid and pectin.

As can be seen in Figure 3, no enzymes able to degrade the backbone of subunit II are known yet. To the backbone of MHR, various substituents are attached (Fig. 3). The single unit Gal residues attached to C-4 of Rha in subunit III can be removed by a *β-galactosidase* from *A. niger* (Van de Vis, 1994; Chapter 2). No enzyme has been found capable of removing the single unit β-Xyl residues attached to C-3 of GalA in subunit I. *Arabinanases*, *arabinofuranosidases*, *galactanases* and *β-galactosidases* have been described with activity toward the side chains attached to C-4 of Rha units in RG regions, as presented in Figure 3 (Rombouts et al., 1988; Guillon et al., 1989; Lerouge et al., 1993). However, so far it has not been possible to remove all Ara and Gal units from MHR in order to obtain a bare backbone (Guillon et al., 1989; Lerouge et al., 1993; Chapter 4). Apparently additional enzymes with other specificities are required in the total degradation of MHR side chains. As with RG-rhamnohydrolase and RG-galacturonohydrolase, that are both very specific for the Rha-GalA and the GalA-Rha linkage respectively, it can be speculated that these additional enzymes are RG-arabinofuranosidases and RG-galactosidases, that are highly specific for the Ara-Rha and the Gal-Rha linkage, respectively. Even the β-galactosidase from *A. niger* was not able to completely remove all Gal from RG oligomers (Chapter 2). Since Pectinex Ultra SP is capable of complete MHR degradation, be it after prolonged incubation, the necessary enzymes seem to be present in this preparation, although probably as minor constituents.

Evidence has been found that the RG regions are predominantly substituted with acetyl groups on C-2 and/or C-3 of GalA (Lerouge et al., 1993; Ishii, 1995), while the xylogalacturonans contain predominantly carboxymethylesters on their GalA residues (Schols et al., 1995), similarly to HGs. Common pectin esterases were present in the enzyme mixture used in the preparation of MHR, and apparently these enzymes can not remove the carboxymethylesters from xylogalacturonans. No xylogalacturonan specific methylesterase has been reported yet. On the other hand, *RG-acetylesterases* specific for RG regions have been isolated from *A. aculeatus*

(Searle-Van Leeuwen et al., 1992) and from *A. niger* (Searle-Van Leeuwen et al., 1996).

PERSPECTIVE FOR APPLICATIONS

New pectic hairy regions degrading enzymes, especially endo-enzymes, are indispensable tools in the *structure elucidation* of this type of polysaccharide. This was already illustrated by the importance of the enzyme RG-hydrolase for the structural characterization of MHR (Schols and Voragen, 1996). Sakamoto and Sakai (1995) proposed a tentative structure for the hairy regions of sugar beet pectin based on information obtained by sequential degradation by enzymes, among which RG-hydrolase, in combination with sugar composition and ^{1}H NMR analysis. Furthermore, RGases can be used to determine the distribution of hairy regions in commercially extracted pectin, and perhaps improve functional properties of these pectins.

On lab-scale, RG-hydrolase has also been used for *pectin extraction* from cell wall material (Renard et al., 1993). The use of RGases, preferable in combination with RG-rhamnohydrolase and RG-galacturonohydrolase, in extraction might even be preferred to the traditional acid extraction, that usually leaves significant amounts of hairy regions (2-4 mol% Rha) attached to the molecules (Kravtchenko et al., 1992). The most well-known application of HG types of pectin, extracted e.g. from sugar-beet pulp, apple pomace and citrus peels, is as *gelling agents*. In the future, hairy types of pectins might also be used for that purpose, e.g. in this research a 2% w/v solution of MHR-S was observed to become highly viscous upon dialyzation against tap water. Possibly this is the result of interaction between calcium ions in tap water and xylogalacturonan molecules, since the latter were found to be involved in a Cu^{2+} precipitate (Chapter 5). Furthermore, extracted pectin can be used as *dietary fiber*, which is thought to have beneficial effects on constipation and diseases such as colon cancer, coronary heart disease, and cholesterolemia. Its incorporation into the diet has been recommended (Voragen et al., 1995).

From an enzyme as RG-lyase the same benefits can be expected in solving ultrafiltration problems in *juice manufacture* as from RG-hydrolase. Application trials with cloned monocomponent enzymes have shown that RG-hydrolase in combination with RG-acetylesterase and galactanase was found to improve the cloudiness and cloud stability of cloudy apple juice (Heldt-Hansen et al., 1996). High molecular mass substances released by the enzymes were thought to increase the viscosity of the juice, resulting in a stabilization of the cloud particles. It can be expected that in the processing of raw materials with higher pH values than most fruits, RG-lyase is preferred above RG-hydrolase, since the former is optimally active at such higher pH values (pH 6 and higher). This may apply e.g. to rape seeds, carrots, potatoes, olives, soy and sugar beet (Kofod et al., 1994a). RG-lyase was found to be superior to RG-hydrolase in releasing polysaccharides from soy cell wall material when used in combination with RG-acetylesterase, together capable of releasing almost 50% of the original material (Kofod et al., 1995). The authors

speculated that RG-hydrolase was more sensitive to sterical hindrance of arabinan and galactan side chains than RG-lyase.

The enzymes RG-rhamnohydrolase and RG-galacturonohydrolase might gain industrial importance in the total *saccharification* of biomass, e.g. sugar-beet pulp which is the main by-product of the sugar refining industry (Micard et al., 1996). GalA, that can be transformed into tensioactive agents by esterification with fatty acids, can already be obtained in high amounts from HG regions by the action of endo- and exopolygalacturonases. Rha, which can be used as a precursor of aroma (such as furaneol, applied in caramel, roasted, and fruit flavors) can be released from RGs by a combined action of RG-rhamnohydrolase and RG-galacturonohydrolase. The importance of these exo-enzymes as *analytical tools* in structure determination has already been demonstrated in the study of the mode of action of RG-hydrolase and RG-lyase (Chapter 7). Another analytical application of these enzymes lies in the determination of the sugar composition of polysaccharides. The disadvantage of traditional methods, involving acid hydrolysis, is the simultaneous conversion of the released sugar monomers, e.g. GalA into lactones in irreproducible amounts (Quigley and Englyst, 1994). Specific degradation of polysaccharides into monomers using enzymes would therefore be preferable. However, the complete scala of e.g. pectin degrading enzymes would be necessary, and this is not available yet. However, it has been reported that enzymic prehydrolysis prior to the traditional chemical hydrolysis enables a much higher release and better determination of GalA (Quemener et al., 1993).

A developing research area where RG degrading enzymes become important, is that of biologically active poly- and oligosaccharides. RGs have been reported to have different pharmacological effects in humans, predominantly involved in improved immune defense (Yamada, 1994; Tomoda et al., 1994; Steinmassl and Anderer, 1996). In plants, RG-I generated from suspension-cultured sycamore cell walls by pectinase digestion has been demonstrated to have wound-signal activity (Ryan et al., 1981). From mucilage of germinated cress seeds, α-us-GalA-(1,2)-Rha disaccharides were isolated as the sodium salt and named lepidimoide, and appeared to promote *Amaranthus* hypocotyl elongation (Hasegawa et al., 1992). RG-lyase is not able to produce dimers, but can release unsaturated tetra- up to decamers of this type. In addition, during this thesis research, indications have been found for an enzyme that cleaves the unsaturated RG tetra-, hexa-, nona- and decamer into one product. Based on its HPAEC elution behavior this product is tentatively identified as the α-us-GalA-(1,2)-Rha dimer.

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Summary

In this thesis three new RG[1] degrading enzymes from the commercial preparation Pectinex Ultra SP, produced by the fungus *Aspergillus aculeatus*, were purified and characterized. Pectinex Ultra SP is industrially used for mash treatment of apples and pears in juice production, increasing juice yields. The enzyme RG-hydrolase was purified from this preparation already in 1990, and found to be a crucial enzyme in improving ultrafiltration problems in juice production, and in elucidating the structure of modified hairy regions (MHR) of apple pectin. MHR and oligomeric derivatives thereof were used as model substrates throughout the project to screen for new enzymes that could degrade highly branched pectin structures. Indications for more new enzymes such as RG-hydrolase in Pectinex Ultra SP led to the initiation of the research project of which the results are described in this thesis.

The enzyme RG-rhamnohydrolase is described in Chapter 2. This enzyme removes the terminal nonreducing Rha residue from RGs, which are part of MHR. Beside RGs, other MHR subunits are xylogalacturonans and RGs with a Rha:GalA ratio < 1 and Ara side chains. RG-rhamnohydrolase is specific for the α-(1,4) linkage of Rha to α-GalA, and is not active toward Rha α-(1,2), α-(1,4) and α-(1,6) linkages to β-Glc, or α-(1,2) linkages to β-Gal. In contrast, a pnp-rhamnohydrolase purified from the same source is not active toward RGs, but cleaves the Rha α-(1,2) and α-(1,6) linkages to β-Glc. The enzyme RG-galacturonohydrolase is described in Chapter 6. This enzyme removes the terminal nonreducing GalA residue that is α-(1,2)-linked to Rha in RGs. It is not active toward HGs. RG-galacturonohydrolase hydrolyzes the GalA residue with inversion of anomeric configuration and therefore most likely operates via a single displacement reaction mechanism, as do RG-rhamnohydrolase, pnp-rhamnohydrolase, RG-hydrolase and an exogalacturonase, all purified from *Aspergillus aculeatus*.

The third RG degrading enzyme described in this thesis is RG-lyase (Chapters 3 and 4). In contrast with RG-hydrolase, which cleaves the GalA-Rha linkage of RGs by hydrolysis, RG-lyase cleaves the Rha-GalA linkage by a β-elimination mechanism, introducing an α-Δ-(4,5)-unsaturated GalA residue at the nonreducing end of one of the two newly formed fragments. RG-lyase cleaves the same strictly alternating RG I regions in MHR-S, which contain β-Gal residues attached to C-4 of Rha, as RG-hydrolase does. Whereas RG-hydrolase releases galactosylated oligosaccharides with RG backbones of DP 4 and 6 from MHR-S, RG-lyase generates unsaturated galactosylated RG oligosaccharides with RG backbones of DP 4, 6, 8 and 10 (Chapter 4). From these observations it can be concluded that RG-lyase has more subsites in its active site than RG-hydrolase. The affinity of RG-lyase for MHR increases when the degree of acetylation decreases,

[1] See List of Abbreviations

and also when the Ara side chains are removed by an endo-arabinanase. Removal of Gal from MHR-S by a β-galactosidase on the other hand has a negative effect on RG-lyase action, by decreasing the V_{max}. From an enzyme such as RG-lyase the same benefits can be expected in solving ultrafiltration problems in juice manufacture as from RG-hydrolase. It can be expected that in the processing of raw materials that have higher pH values than most fruits, such as rape seeds, carrots, potatoes, olive, soy and sugar beet, RG-lyase is preferred above RG-hydrolase, since RG-lyase is optimally active at pH values of 6 and higher.

An easy method to detect RG-hydrolase and RG-lyase activity in a plate assay using MHR-S as substrate is described in Chapter 5. This method is based on the formation of a white precipitate of Cu^{2+} ions with xylogalacturonan molecules, which latter molecules are released from MHR-S when RG I regions are degraded by RG-hydrolase or RG-lyase. The Cu^{2+} staining was also applied in IEF zymography, by which the pI of RG-hydrolase and RG-lyase could be determined even in partially purified enzyme fractions. This method is time-saving in the screening of column fractions during purification of RGases, since time-consuming HPLC analysis can be skipped.

The exo-acting RG-rhamnohydrolase and RG-galacturonohydrolase were essential analytical tools in the study of the mode of action (Chapter 7) of the endo-acting RG-hydrolase and RG-lyase toward linear RG oligosaccharides. Firstly, these exo-enzymes helped to prepare a series of different RG oligomers, that could be used as standards to compare unknown HPAEC peaks with. Secondly, these exo-enzymes were used to confirm structure assignments, by modifying initially formed oligomers, produced by RG-hydrolase or RG-lyase out of linear RG oligosaccharides, into new products for which HPAEC standards were available. The smallest RG oligosaccharide that can be fragmented by RG-hydrolase is R-G-R-G↓R-G-R-G-R (DP 9), at four units from the nonreducing end, while the smallest oligosaccharide fragmented by RG-lyase is of G-R-G-R-G-R-G-R↓G-R-G-R (DP 12), at four units from the reducing end (Chapter 7).

RG-hydrolase and RG-lyase both act with a multiple attack mechanism toward MHR-S, which means that once the enzyme forms an active enzyme-substrate complex, it catalyzes the hydrolysis of several bonds before it dissociates and forms a new active complex with another substrate chain (Chapter 4). From the degree of multiple attack of RG-hydrolase (4) and RG-lyase (2.5), and the mode of action toward purified RG oligomers (Chapter 7), the average length of RG I regions in MHR-S was estimated to be at least 29 sugar residues, which is rather longer than suggested in an earlier model.

Samenvatting

In dit proefschrift zijn drie nieuwe rhamnogalacturonaan afbrekende enzymen beschreven, die gezuiverd werden uit het commerciële enzympreparaat Pectinex Ultra SP, geproduceerd door de schimmel *Aspergillus aculeatus*. Pectinex Ultra SP wordt als zodanig industrieel toegepast voor een enzymatische voorbehandeling van pulp in appel- en perensapbereiding, hetgeen leidt tot een hogere sapopbrengst. Het enzym RG-hydrolase[1] werd al in 1990 uit dit preparaat gezuiverd en bleek een cruciaal enzym, enerzijds voor het verbeteren van ultrafiltratieproblemen in de sapindustrie, en anderzijds voor de structuuropheldering van het sterk vertakte appelpectine "modified hairy regions" (MHR). Dit MHR en oligomeren hieruit gemaakt zijn tijdens het gehele onderzoek steeds als modelsubstraten gebruikt om enzymen te vinden die op vertakte pectines werken. Aanwijzingen dat meer van dergelijke nieuwe enzymen zoals RG-hydrolase aanwezig waren in Pectinex Ultra SP leidde tot het opzetten van het onderzoeksproject waarvan de resultaten zijn beschreven in dit proefschrift.

Het enzym RG-rhamnohydrolase wordt beschreven in Hoofdstuk 2. Dit enzym verwijdert de eindstandige Rha suiker aan het niet-reducerende uiteinde van rhamnogalacturonaanstructuren, zoals bijvoorbeeld aanwezig in MHR. Andere bouwstenen van MHR zijn xylogalacturonaanmoleculen, en rhamnogalacturonanen met een Rha:GalA verhouding < 1 en Ara zijketens. RG-rhamnohydrolase is specifiek voor de α-(1,4) binding van Rha met α-GalA, en splitst geen Rha α-(1,2), α-(1,4) and α-(1,6) bindingen met β-Glc, of α-(1,2) bindingen met β-Gal. In tegenstelling tot het RG-rhamnohydrolase splitst het pnp-rhamnohydrolase, dat eveneens uit Pectinex Ultra SP werd opgezuiverd, geen Rha af van rhamnogalacturonanen, maar kan wel de Rha α-(1,2) en α-(1,6) bindingen met Glc verbreken, zoals aanwezig in respectievelijk naringine en hesperidine.

In Hoofdstuk 6 wordt het RG-galacturonohydrolase beschreven, een enzym dat de eindstandige GalA suiker verwijdert, die α-(1,2) verbonden is met α-Rha aan het niet-reducerende uiteinde van rhamnogalacturonanen. Het enzym is in het geheel niet actief op homogalacturonanen. RG-galacturonohydrolase hydrolyseert het GalA met inversie van de anomere configuratie, hetgeen inhoudt dat het enzym werkt via een S_N1-hydrolyse-mechanisme. Eenzelfde inversie van de anomere configuratie wordt gevonden voor de andere *Aspergillus aculeatus* enzymen RG-hydrolase, RG-rhamnohydrolase, pnp-rhamnohydrolase, en een exogalacturonase.

Het derde rhamnogalacturonaan-afbrekende enzym beschreven in dit proefschrift is RG-lyase (Hoofdstukken 3 en 4). In tegenstelling tot RG-hydrolase, dat de GalA-Rha binding splitst door hydrolyse, splitst RG-lyase de Rha-GalA binding door β-eliminatie, waarbij aan het niet-reducerende uiteinde van één van de

[1] Zie "List of Abbreviations"

nieuw gevormde fragmenten een α-Δ-(4,5)-onverzadigde GalA wordt gevormd. RG-lyase werkt in op dezelfde strikt alternerende rhamnogalacturonanen in MHR als RG-hydrolase. Terwijl RG-hydrolase uit MHR-S gegalactosyleerde oligosacchariden met een hoofdketen van vier en zes suikereenheden vrijmaakt, produceert RG-lyase gegalactosyleerde onverzadigde RG oligomeren met hoofdketens van vier, zes, acht en tien suikereenheden (Hoofdstuk 4). Hieruit kan worden afgeleid dat RG-lyase meer substraatbindingsplaatsen in het katalytisch centrum heeft dan RG-hydrolase. De affiniteit van RG-lyase voor MHR neemt toe wanneer de acetyleringsgraad afneemt, en ook wanneer Ara-zijketens met behulp van endo-arabinanase verwijderd worden. Aan de andere kant heeft verwijdering van Gal met behulp van een β-galactosidase een negatief effect op de RG-lyase werking, doordat de V_{max} afneemt. Van een enzym als RG-lyase kan dezelfde positieve werking verwacht worden op ultrafiltratieproblemen in de sapbereiding als RG-hydrolase. Bovendien kan RG-lyase zelfs de voorkeur hebben in de verwerking van grondstoffen die een hogere pH-waarde hebben dan de meeste vruchten, zoals raapzaad, wortels, aardappels, olijven, soja en suikerbiet, omdat RG-lyase het beste werkt bij een pH van 6 en hoger.

Een eenvoudige methode om RG-hydrolase en RG-lyase activiteit aan te tonen in agarplaten met MHR-S als substraat is beschreven in hoofdstuk 5. Deze methode is gebaseerd op de vorming van een wit neerslag van Cu^{2+} ionen met xylogalacturonanen, welke laatstgenoemde vrijgemaakt worden uit MHR-S wanneer de rhamnogalacturonanen afgebroken worden door RG-hydrolase of RG-lyase. Deze koperkleuring werd ook gebruikt voor IEF zymografie, waarmee de pI van RG-hydrolase en RG-lyase al kon worden bepaald in gedeeltelijk gezuiverde enzymfracties. De methode bespaart tijd bij het screenen van kolomfracties tijdens de zuivering van RGases, omdat geen tijdrovende HPLC analyse hoeft te worden uitgevoerd.

De exo-enzymen RG-rhamnohydrolase en RG-galacturonohydrolase waren essentiële analyse-hulpmiddelen in een studie naar het werkingsmechanisme van RG-hydrolase en RG-lyase op lineaire rhamnogalacturonaanoligomeren (Hoofdstuk 7). Op de eerste plaats werden de exo-enzymen gebruikt om een serie van verschillende soorten rhamnogalacturonaanoligomeren te maken, die als standaarden konden dienen om onbekende HPAEC pieken mee te vergelijken. Op de tweede plaats werden de exo-enzymen gebruikt voor bevestiging van structuurtoewijzingen, door de reactieprodukten die door RG-hydrolase en RG-lyase gemaakt werden vanuit de lineaire rhamnogalacturonaanoligomeren, verder af te breken waardoor weer nieuwe producten ontstonden die met standaarden konden worden vergeleken. Het kleinste oligomeer dat gesplitst kan worden door RG-hydrolase is R-G-R-G↓R-G-R-G-R (DP 9), vier suikereenheden vanaf het niet-reducerende uiteinde, terwijl het kleinste fragment dat gesplitst kan worden door RG-lyase G-R-G-R-G-R-G-R↓G-R-G-R (DP 12) is, vier suikereenheden vanaf het reducerende uiteinde (Hoofdstuk 7).

RG-hydrolase en RG-lyase blijken beiden te werken via een zogenaamd "multiple attack" mechanisme waarbij het enzym, na vorming van een actief enzym-substraat complex, na de eerste binding nog een aantal andere opeenvolgende

bindingen splitst, alvorens het dissocieert en een nieuw complex vormt met een andere substraatketen (Hoofdstuk 4). Dit in tegenstelling tot een mechanisme waarbij na vorming van een actief enzym-substraat complex alle bindingen in het substraat achtereenvolgens worden verbroken, als in een ritssluiting, of een mechanisme waarbij het enzym na vorming van een actief complex éénmaal knipt, vervolgens het substraat loslaat en met een nieuwe substraatketen een actief complex aangaat en knipt etc., op een willekeurige manier. Uit het gemiddelde aantal opeenvolgende bindingen dat in MHR-S gesplitst wordt na de eerste binding door RG-hydrolase (4) en RG-lyase (2.5) (Hoofdstuk 4), in combinatie met gegevens omtrent het waargenomen werkingsmechanisme van de twee enzymen op lineaire rhamnogalacturonaanoligomeren (Hoofdstuk 7), kan voorspeld worden dat de gemiddelde lengte van de rhamnogalacturononanen zoals ze in MHR voorkomen minstens 29 suikereenheden moet zijn. Dit is aanzienlijk langer dan voorgesteld in een eerder MHR model.

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Curriculum vitae

Margien Mutter werd op 5 januari 1967 geboren te Nijmegen. In juni 1985 behaalde zij het diploma ongedeeld VWO aan het Elshof College te Nijmegen. In september van hetzelfde jaar begon ze aan de studie Levensmiddelentechnologie aan de toenmalige Landbouwhogeschool te Wageningen. De doctoraalfase omvatte de hoofdvakken Levensmiddelenchemie (dr. H.A. Schols, prof. dr. ir. A.G.J. Voragen) en Organische Chemie (dr. J.F.J. Engbersen). Aansluitend werd een half jaar stage gelopen bij TNO Voeding te Zeist (dr. K.D. Bos). In januari 1991 sloot zij haar studie met lof af.

Van maart 1991 tot oktober 1995 werkte zij als assistent in opleiding (AIO) bij de sectie Levensmiddelenchemie en -microbiologie van de Landbouwuniversiteit Wageningen. Het project werd gefinancierd door Novo Nordisk A/S (Bagsvaerd, Denemarken). Het onderzoek uitgevoerd in deze periode staat beschreven in dit proefschrift, en werd begeleid door prof. dr. ir. A.G.J. Voragen, dr. G. Beldman en dr. H.A. Schols. Tijdens de AIO-periode werden haar twee dochters geboren, en in de aansluitende periode waarin het proefschrift geschreven werd, kreeg zij een zoon.

List of Abbreviations

Ara,	L-arabinofuranose;
$CaCl_2$,	calcium chloride;
COSY,	correlation spectroscopy;
D,	Dalton;
DA,	degree of acetylation: number of moles acetyl groups per 100 moles GalA residues;
DM,	degree of methoxylation: number of moles methoxyl groups per 100 moles GalA residues;
DP,	degree of polymerization;
[E],	enzyme concentration;
f,	furanose;
Fuc,	L-fucopyranose;
G,	α-GalA (1,2)-linked to Rha, or GalA at the reducing end;
Gal,	D-galactopyranose;
GalA,	D-galactopyranosyluronic acid;
Glc,	D-glucopyranose;
GlcA,	D-glucopyranosyluronic acid;
H_3PO_4,	phosphorous acid;
HG,	homogalacturonan;
HOHAHA,	homonuclear Hartmann-Hahn spectroscopy;
HPAEC,	high-performance anion-exchange chromatography;
HPLC,	high-performance liquid chromatography;
HPSEC,	high-performance size-exclusion chromatography;
HTP,	hydroxylapatite;
IEF,	isoelectric focusing;
IMAC,	immobilized metal ion affinity chromatography;
k_{cat},	catalytic constant, k_{cat}/K_m is the specificity constant;
K_m,	Michaelis-Menten constant;
Man,	D-mannopyranose;
MHR,	modified hairy regions of pectin;
MHR-S,	saponified MHR;
NaCl,	sodium chloride;
NaN_3,	sodium azide;
NaOAc,	sodium acetate;
NaOH,	sodium hydroxide;
NMR,	nuclear magnetic resonance;
p,	pyranose;
PAD,	pulsed amperometric detection;
pI,	isoelectric point;
pnp,	p-nitrophenyl;
pnp-Rha,	pnp-α-L-rhamnopyranoside;
pnp-rhamnohydrolase,	pnp-Rha rhamnohydrolase;
R,	α-Rha (1,4)-linked to GalA, or Rha at the reducing end;
RG,	rhamnogalacturonan;
RGase,	rhamnogalacturonase;
RG-galacturono-hydrolase,	RG α-D-galactopyranosyluronohydrolase;

RG-hydrolase,	RG α-D-galactopyranosyluronide-(1,2)-α-L-rhamnopyranosyl hydrolase (Chapter 3), previously RGase (Schols et al., 1990a) or RGase A (Kofod et al., 1994);
RG-lyase,	RG α-L-rhamnopyranosyl-(1,4)-α-D-galactopyranosyluronide lyase (Chapter 3), previously RGase B (Kofod et al., 1994);
RGmed,	intermediate-sized fragments produced from MHR-S by RGase;
RGoligo,	low molecular mass fragments produced from MHR-S by RGase, consisting of RG oligomers;
RGpoly,	high molecular mass fragments produced from MHR-S by RGase;
RG-rhamnohydrolase,	RG α-L-rhamnopyranosylhydrolase;
Rha,	L-rhamnopyranose;
ROESY,	rotating frame Overhauser effect spectroscopy;
SDS-PAGE,	sodium dodecyl sulfate-polyacrylamide gel electrophoresis;
SEC,	size-exclusion chromatography;
uG,	α-us-GalA (1,2)-linked to Rha;
us-GalA,	α-Δ-(4,5)-unsaturated GalA;
V_{max},	maximum reaction rate;
Xyl,	D-xylopyranose.