STRUCTURAL CHARACTERIZATION OF PECTIC HAIRY REGIONS
ISOLATED FROM APPLE CELL WALLS

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Henk Schols

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Structuurkenmerken van vertakte pectine fragmenten
afkomstig van de celwanden van appel

STELLINGEN

1) De conclusie van Will en Dietrich (1992) dat alle galacturonzuur eenheden van appel MHR veresterd zouden zijn omdat een methyleringsgraad van 40% en een acetyleringsgraad van 60% gevonden werd, is onjuist.

   F. Will, and H. Dietrich; Isolation, purification and characterization of neutral polysaccharides from extracted apple juices. *Carbohydr. Polym.*, 18 (1992) 109-117.

2) Het is in strijd met de regels dat een referent de gegevens van een te refereren manuscript citeert in een eigen publikatie vóórdat dit manuscript geaccepteerd *en* verschenen is.

   - J.M. Brillouet, L. Saulnier en M. Moutounet; Les polysaccharides pectiques et les enzymes de dégradation. *Revue Francaise d'oenologie*, 122 (1990) 43-54.
   - H.A. Schols, C.C.J.M. Geraeds, M.J.F. Searle-van Leeuwen, F.J.M. Kormelink, and A.G.J. Voragen; Rhamnogalacturonase: a novel enzyme that degrades the hairy regions of pectins. *Carbohydr. Res.*, 206 (1990) 105-115.

3) De termen 'Rhamnogalacturonan I" en 'Hairy Regions' beschrijven niet altijd dezelfde sterk vertakte structuren binnen een pectine molecuul.
   Dit proefschrift.

4) Met het huidige aanbod van databanken en literatuur-zoeksystemen is opzet niet uit te sluiten bij het ontbreken van essentiële literatuurreferenties in een publikatie.

5) Het is bij de weergave van een model voor de fijnstructuur van polysacchariden niet eenvoudig om weer te geven welk deel wel goed onderbouwd is en welk deel minder goed.
   - *Dit proefschrift.*
   - I.J. Colquhoun, M.-C. Ralet, J.-F. Thibault, C.B. Faulds en G. Williamson: Structure identification of feruloylated oligosaccharides from sugar-beet pulp by NMR spectroscopy. *Carbohydr. Res.*, 263 (1994) 243-256.

6) Mais et al. (1994) zijn in de beschrijving van de synthese van hun gelabeld retinoïde erg slordig.

   D.E. Mais, E.M. Berger, L. Zhang en M.F. Boehm; Biochemical characterization of a novel RXR-selective, high specific activity radioligand. *Med. Chem. Res.*, 4 (1994) 406-413.

7) Gezien de grote overeenkomsten in structuur tussen appel MHR en pectines fracties geïsoleerd uit geneeskrachtige kruiden zou een medicinale werking van appel MHR verwacht mogen worden.

H. Yamada; Chemical and Pharmacological studies on efficacy of Japanese and Chinese herbal medicines. *Kitasato Arch. of Exp. Med.*, 65 (1992) 159-180.

8) Door het afschaffen van de dienstplicht verdwijnt een zekere vorm van ongelijkheid doordat onderzoeksinstellingen niet langer *hoger opgeleide* dienstweigeraars kunnen werven als goedkoop onderzoeker.

9) De besluitvaardigheid van de politiek met betrekking tot de versteviging en verhoging van de rivierdijken bleek recht evenredig met de waterstand.

10) Er blijkt soms een (te) groot verschil te bestaan in de inzet en deskundigheid van verschillende referenten bij de beoordeling van wetenschappelijke artikelen: waar de ene referent zich zoveel moeite en inspanning getroost dat een vermelding als co-auteur niet zou misstaan kan het voorkomen dat een andere referent hooguit genoemd zou mogen worden onder vermelding van het woordje 'ondanks'.

11) De huidige bezuinigingen aan de Landbouwuniversiteit hadden wellicht lager uit kunnen vallen, indien de ingebruikname van het automatiseringsprogramma ten behoeve van de boekhouding en projectadministratie verlopen was volgens de gewekte verwachtingen.

Stellingen behorende bij het proefschrift:
'Structural characterization of pectic hairy regions
isolated from apple cell walls'
Henk Schols
Wageningen, 16 juni 1995

"Pectine bestaat gedeeltelijk uit gedeeltelijk uit 'repeating units' bestaande 'repeating units'".

J. De Vries, Proefschrift Landbouwhogeschool, Wageningen, 1983.

Aan Anita

VOORWOORD

Het schrijven van een voorwoord is altijd een hachelijke zaak omdat het meestal één van de meest gelezen (en begrepen) bladzijden van een proefschrift is en omdat mensen uit je directe omgeving vaak nieuwsgierig zijn 'hoe' je 'wie' bedankt.

'Het schrijven van een proefschrift' is iets wat pas vrij laat in mijn gedachten opkwam en mede door de ongebruikelijke route die ik gevolgd heb is het overduidelijk dat dit nooit had gekund zonder de hulp en steun van vele anderen.

Allereerst wil ik Peter Folstar en Prof. Pilnik bedanken voor het 'binnenhalen' van een beginnend analist, nog tijdens de stage periode en zelfs zonder een officieel sollicitatie gesprek.

Direct hierna gaat mijn dank vooral uit naar mijn promotor Fons Voragen. Ons 'tweemans-winkeltje', (sinds 1980), is danig uit de hand gelopen! Jij hebt ondertussen een goed lopend 'grootwinkelbedrijf', terwijl ik er trots op ben dat je mij mee hebt willen laten groeien tot '(mede)filiaalhouder'. Je hebt me ingeleid in de suikerchemie en me steeds aangemoedigd en ruimte gegeven om iets nieuws aan te pakken. Uiteindelijk ben jij het vooral geweest die me wist te overtuigen dat het promoveren iets was wat ook ik zou kunnen en bovenal de inspanning waard zou zijn. Het onderwerp voor het proefschrift lag redelijk voor de hand, want de structuur van pectines had al sinds lang onze gemeenschappelijke interesse.
Al kwam het de snelheid van promoveren niet altijd ten goede, de vrijheid om te mogen samenwerken met andere onderzoeksgroepen heb ik altijd erg gewaardeerd.

De collega's van de sectie Levensmiddelenchemie zal ik niet allemaal bij name noemen (want dan zou ik ze *allemaal* willen noemen), maar ik vind het een voorrecht om deel uit te mogen maken van zo'n inspirerende en vooral ook communicatieve omgeving die het me mogelijk maakte om de toch niet-alledaagse overstap van analist naar gepromoveerd onderzoeker te kunnen maken.

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Enzymen zijn tijdens mijn onderzoek veelvuldig gebruikt als analytisch gereedschap bij het ophelderen van de fijnstructuur van pectines en dit had zeker niet gekund zonder de goede wisselwerking met de 'enzymhoek': Marjo Searle, Ben van den Broek, Màrgien Mutter, Toos Kroef en Gerrit Beldman.

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Jean-Paul Vincken; als 'room-sharing' altijd zou leiden tot 'onze' mate van relativeren en vruchtbaar brainstormen over al het (on)mogelijke, dan zou het absoluut verplicht gesteld moeten worden. Verder was je een kritisch lezer van de voor-eerste versie van menig artikel en hoofdstuk en in deze een waardig opvolger van Gerhard de Ruiter. Gerhard opende met zijn stage in Engeland de samenwerking met Ian Colquhoun en samen zorgden zij voor de opheldering van de structuur van enkele oligosacchariden, wat een essentieel onderdeel van het beschreven onderzoek vormde.

I would like to thank dr. Ian Colquhoun for his valuable contribution in revealing the structure of (complex mixtures) of pectic oligomers by NMR spectroscopy.

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ABSTRACT

Cell wall pectic substances have a great influence on the production and quality aspects of apple juice. Apple juices were characterized by their polysaccharide content and composition. A pectic fraction, retained by ultrafiltration of a liquefaction juice, was isolated and termed MHR (modified hairy regions). MHR, resistant to further enzymic degradation by the liquefaction enzymes, was characterized using chemical and enzymic methods. Next to a high arabinose content, this fraction was characterized by a high rhamnose to galacturonic acid ratio and a high acetyl content. Rhamnogalacturonase (RGase), an enzyme able to hydrolyze galacturonic acid-(1→2)-rhamnosyl linkages within the rhamnogalacturonan backbone, was purified and characterized.

Next to high molecular weight degradation products, rhamnogalacturonase released characteristic oligosaccharides from MHR. Various 2D-NMR techniques were used to identify these RGase oligomers, which consisted of a tetrameric or hexameric backbone of alternating rhamnose and galacturonic acid residues with a galactose residue substituted at C-4 of part of the rhamnose moieties.

Analogous MHR fractions were isolated from potato fibre, pear, carrot, leek, and onion tissue, although variations occurred with respect to the arabinose and xylose content. All MHR fractions were degraded by RGase in a comparable fashion resulting in the same type of oligomers in an almost equal ratio.

Comparison of the MHR with non-modified pectic hairy regions of apple cell wall, isolated in a mild and defined way, revealed great resemblance indicating that the modifications of the MHR during enzymic liquefaction were only minor. The oligomers liberated by RGase from the various pectic hairy regions differed in absolute as well as in relative amount.

After degradation by RGase of the highest molecular weight population of apple MHR, three subunits were characterized representing a xylogalacturonan segment, residual stubs of the backbone rich in arabinose and regions of alternating rhamnose-galacturonic acid residues of unknown length. The substitution of xylose residues and methylestergroups over the galacturonan backbone was studied by NMR and was found to be random.

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Cell walls of plant raw materials determine to a large extent quality attributes of fresh fruits and vegetables (e.g. ripeness, texture) and their processing characteristics in the manufacture of foods (juices, nectars, purees, preserves). They also influence the extractability of important constituents of plant raw materials like sugar, oil, proteins, etc. Detailed knowledge of the major constituents making up the cell walls (e.g. pectic substances, hemicelluloses, cellulose, and structural proteins) and the ultra-structure of the cell wall is important to control and improve processing and utilization of plant products. It is obvious that cell wall modifying enzymes, endogenous to plant raw materials and active during ripening and storage, as well as enzymes added as processing aids, play a key role in these phenomena.
Interest in cell wall polysaccharides, cell wall synthesis and ultra-structure, and cell wall modifying enzymes has obtained extra momentum through the potential to genetically modify cell wall synthesis and structure, and levels of enzymes, endogenous to the plant as well as to food-grade micro-organisms suitable to produce the desired enzymes. This may result in a hyper-production of technological relevant enzymes *in vivo* in plant materials, and in micro-organisms to formulate tailored enzyme cocktails.

During our studies on quality aspects of apple juices obtained by the enzymic liquefaction process, we were confronted with a haze problem, which found its origin in the release of enzyme resistant fragments of pectin materials into the juice. Our investigations on the structure of these fragments led to the discovery of a new pectic enzyme. This new enzyme turned out to be an essential analytic tool of invaluable importance for structural studies of pectic substances in general.

The literature review below will give a short introduction to apple juice manufacture, followed by background information on plant cell walls, pectic substances and corresponding enzymes, and finally, the aims of this thesis will be given.

Apple juice manufacture

Apple juice is one of the most important fruit juices in Europe. The traditional way to produce apple juices on a industrial scale starts with crushing the fruits resulting in a mixture of disordered tissue, intact and fragmented cells, and cell liquid. From this pulp, the juice is usually separated by various types of presses and separators. Depending on the variety, age, and storage conditions of the apples used, pressing properties of the apple pulp might be poor resulting in low yields and turbid juices. Nowadays, technical enzyme preparations are frequently used in the fruit-processing industry[1-3] to increase processing capacity and yield, and to obtain clear juices which can be concentrated to ± 70 °Bx. Figure 1 schematically shows flow diagrams for the various processes which are being practised and arrows indicate where in the different processes specific enzyme mixtures are added. The oldest and most
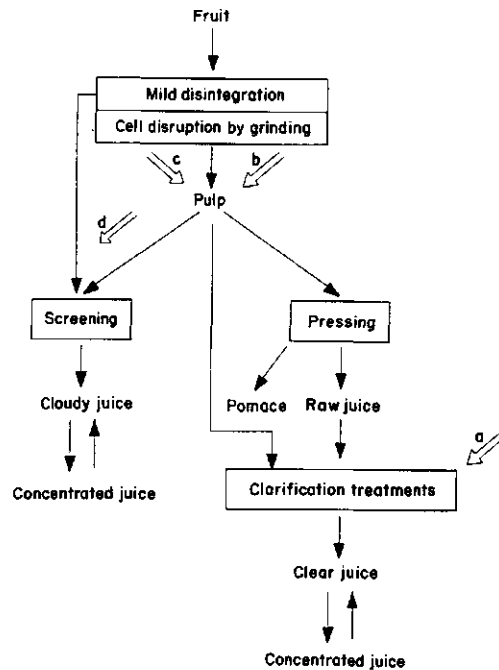
Fig. 1. Flow diagram of fruit juice manufacture. Arrows indicate eventual enzyme treatments by (a) pectinases for clarification; (b) pectinases for pulp enzyming; (c) pectinases and C1 cellulases for liquefaction; and, (d) polygalacturonase, pectin lyase, or pectate lyase for maceration (from ref.[2]).

widely applied use of enzymes in fruit juice manufacture is enzymic clarification of juices obtained by straight pressing. These juices are viscous due to dissolved pectin molecules and contain complexes of solubilized carbohydrates and proteins forming a persistent cloud. Enzymes are added to overcome these problems and to enable juice concentration.

The composition of the enzyme cocktails used to treat fruit pulps prior to pressing depends on the type and age of the fruit involved and on the type of processing and final product desired. Maceration is obtained by the action of single pectic enzymes and treatment results in a suspension of intact cells by limited degradation of pectins (generally low esterified) in the middle lamella of the cell wall. Whether only maceration will occur or a more intensive type of disintegration of the cell wall, indicated as pulp enzyming in figure 1, depends on the intensity of the enzyme treatment and on the enzyme mixture used[4]. Pulp enzyming is also used to treat the press-cake obtained after straight pressing of fruit pulp to increase juice yield and to obtain juices from soft fruits like black currants, strawberries, raspberries, bananas, where pressing can not be used easily.

An even more exhaustive way of cell wall degradation is achieved by the enzymic liquefaction process. Using a broad spectrum of polysaccharide degrading enzymes (pectic, hemicellulolytic and cellulolytic enzymes), almost the entire fruit tissue can be solubilized

resulting in a high juice yield without a pressing step.

The use of enzymes in juice manufacture has an effect on the quality of the (clear) juices obtained[3]. Haze problems, caused by retrogradated linear arabinans, have appeared in concentrated apple and pear liquefaction juices[5,6]. Juices obtained after enzyme treatment of the pulp were found to contain rather high amounts of uronides originating from (partially) degraded pectic substances[7]. Part of these pectin fragments were present in the unsaturated form as released by pectin lyase (PL)[7]. It was shown that these unsaturated oligogalacturonides are very reactive precursors for browning reactions[8]. Next to oligomeric reaction products, also the presence of polymeric material, retained by ultrafiltration, has been reported[7].

Above mentioned observations demonstrate important roles of cell wall polysaccharides and their enzymic degradation in juice manufacturing and of degradation products on juice quality. For this reason, the composition and architecture of the (primary) cell wall will be discussed in more detail with focus on pectic polysaccharides.

The plant cell wall.

The cell wall forms a single continuous extracellular matrix through the body of the plant and the walls of many cells together form the skeleton of plant tissues. The cell matrix consists of various types of polysaccharides, proteins and lignin in varying amounts, organized in such a way that the cell wall is chemically rather stable and physically robust. Primary walls can best be described as reinforced, multi-component gels[9]. Cell walls control cell growth by influencing cell size and shape, but also act as a pre-existing structural barrier to invasion of micro-organisms[10]. When infection or wounding of the wall occurs, the cell may respond by thickening, lignification or suberization of the cell, production of phenolic acids and extensins, pigments, etc.[9]

After the characterization of the various (polysaccharide) components of the cell walls, hypotheses were formulated to explain how these polymers were assembled in such a flexible and multi-tasking architecture. Early models of the primary wall have been described by Albersheim and colleagues[11,12], mainly based on identified polysaccharide and protein structures usually obtained after (mild) sequential extraction procedures. More recent models have been published by McCann and Roberts[13], Carpita and Gibeaut[14], and Talbott and Ray[15]. As illustrated in Fig. 2, the model of McCann and Roberts[13] is mostly based on cellulose microfibrils being cross-linked by hemicellulosic polymers. An important representative of this latter group is formed by the xyloglucans, probably cross-linking to two or more cellulose microfibrils by tight hydrogen-bonding. This cellulose-xyloglucan domain is embedded in an independent matrix of pectic polysaccharides which are often referred to as "lubricating" or "cementing" agent[16]. Pectins present in the middle lamella are Ca^{2+}-cross-linked to each other and are thought to limit cell wall porosity[13]. From immunocytochemical localization tests, Moore et al.[17] concluded that rhamnogalacturonan I (RG-I) is only localized in the middle lamella. Other important polysaccharides like arabinans, galactans and arabinogalactans are reported to be associated with the pectic substances in the wall[9,18].
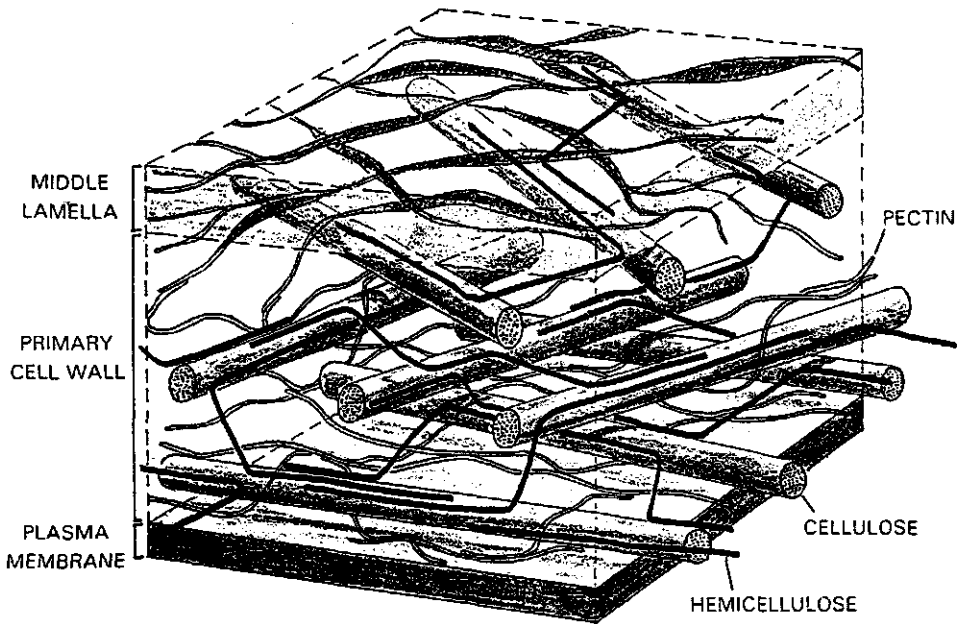
Fig. 2   A simplified and schematic representation of how different polysaccharides might be spatially
          arranged in the onion cell wall (from ref.[13])

Contradictory, Talbott and Ray[15] stated that for pea cell walls, at least 60% of these arabinan
and galactan polymers occur in the hemicellulose fraction, not covalently or ester-linked to
pectic substances. Differences between the most recent models mainly refer to the depiction
of the pectin[13-15], sometimes making slightly more distinction between the various classes of
pectins like homogalacturonan and RG-I[14]. Conflicting with the models described, Renard[19]
proposed a model in which part of the pectin is anchored to xyloglucan, cellulose or other
cell wall compounds, as was proposed in the earlier models[11,12].

Since the pectic substances present in the plant cell walls play an important role during the
processing of fruit and vegetables[7], these polymers will be discussed in more detail.

Pectic substances.

Pectin is defined as a heteropolysaccharide of dominantly galacturonic acid residues in
which varying proportions of the acid groups are present as methoxyl esters, while a certain
amount of neutral sugars might be present as side chains. Also the expression "protopectin"
is frequently used and comprises the native pectins that can not be extracted by non-
degradative methods. The location of pectin in the cell wall middle lamella complex has been

known since the earliest work on this material[20].

The pectic polysaccharides are probably the most complex class of wall polysaccharides[9] and comprise a family of acidic polymers like homogalacturonans, rhamnogalacturonan and several neutral polymers like arabinans, galactans and arabinogalactans attached to it[9,18,22].

The pectic backbone consists of a (rhamno)galacturonan backbone, in which "smooth" α-D-$(1\rightarrow)4$-galacturonan regions are interrupted by ramified ("hairy") regions. Hairy refers to the many side chains which are attached to α-L-$(1\rightarrow2)$-linked rhamnose residues present in the backbone interrupting the galacturonosyl residues[9,18,21].

Homogalacturonans.—Homogalacturonan segments are defined as polymers consisting predominantly of α-$(1\rightarrow4)$-linked galacturonosyl residues[10]. Pure homogalacturonans rarely occur[9], although Zitko and Bishop[22] described a homogalacturonan extracted from sunflower seed heads. One of the reasons for the limited number of reports on pure galacturonans might be the poor solubility of these polymers[10], although solubility depends on the degree of methylation (DM). On the other hand, more and more indications are found that homogalacturonans are covalently linked to RG-I and other cell wall polymers[9,10,21,23]. Homogalacturonans are usually extracted from plant material by mild acid treatment[23,24] or by the use of enzymes[18,24-26]. Depending on the extraction method used, some modification of the polymer may occur. Powell et al.[27] suggested that the pectin isolated from citrus, apple and sunflower using acid hydrolysis conditions, consisted of repetitive galacturonate sequences of constant length ($\pm$ 25 residues) separated by a single rhamnose unit. Uninterrupted homogalacturonan regions with a degree of polymerisation (DP) of approximately 70 have been isolated from suspension-cultured cell walls of carrots[28]. Suspension-cultured *Rosa* cell walls have been reported to contain a homogalacturonan with a DP > 100[29]. These DP values (70-100) have been confirmed recently for homogalacturonan segments from apple, beet and citrus[23,30]. More research will be needed to generalize these observations for pectins in common.

Another important feature of galacturonans is the esterification of galacturonic acid residues with methanol and/or acetic acid. The DM is defined as the number of moles of methanol per 100 moles of galacturonic acid. Pectins are called high methoxyl pectins when the value for DM is 50 or higher. In the other cases, the pectin is called low methoxyl pectin. For native apple pectins a random distribution of the methoxyl groups over the galacturonan chain was found[31-33]. For commercially extracted pectins, the distribution was found to be slightly different since the relative amounts of mono-, di-, and trigalacturonides in endo PG-digests suggested small blocks of unesterified galacturonosyl residues[30]. Kiyohara et al.[34] found galacturonic acid oligomers up to octamer after PG-digestion of homogalacturonans from *Angelica acutiloba* Kitagawa, suggesting a more blockwise distribution. Acetyl groups are usually only present in low amounts in pectins from e.g. apple and citrus, but are present in much higher amounts in pectins from sugar beet[35] and potato[36].

Rhamnogalacturonan I.—The group of Albersheim devoted a high number of publications to RG-I type of pectic substances which is conveniently reviewed by O'Neill et al.[18]. RG-I was the major polysaccharide solubilized from suspension-cultured sycamore cell walls after

TABLE I.   Some structurally characterized oligomers, as obtained after releasing the neutral glycosyl-residue
           side chains of rhamnogalacturonan I from sycamore cells by treatment of lithium in ethylene-
           diamine[38]

β-D-Galp-(1→6)-β-D-Galp-(1→4)-β-D-Galp-(1→4)-Rhamnitol

α-L-Fucp-(1→2)-β-D-Galp-(1→4)-β-D-Galp-(1→4)-Rhamnitol

L-Araf-(1→5)-α-L-Araf-(1→2)-α-L-Araf-(1→3)-β-D-Galp-(1→4)-Rhamnitol

Araf-[Araf]$_{0-3}$-Rhamnitol

Araf-[Araf]$_{0-3}$-Arabinitol

Galp-[Galp]$_{0-3}$-Rhamnitol

Galp-[Galp]$_{0-2}$-Galactitol

treatment with PG and represented 7-14% of the cell wall[18]. The RG-I polymer is composed
of alternating rhamnose and galacturonic acid residues. The length is unknown, but it could
contain as many as 300 rhamnose and 300 galacturonic acid residues[10]. Polymers containing
this backbone are present in most if not all higher plant cell walls[18]. Next to the rhamnose and
galacturonic acid residues in the backbone, RG-I is composed of arabinofuranosyl-,
galactopyranosyl- and minor quantities of fucopyranosyl residues[18]. The elucidation of the
chemical structure of RG-I is mainly based on chemical degradation reactions resulting in a
wide range of characteristic oligosaccharides[18]. These characteristic oligomers are liberated
by treating RG-I in ethylenediamine with metallic lithium, which resulted in a selective
cleavage of all glycosidic linkages in which a galacturonic acid is involved[37,38]. After
reduction of the neutral sugar on the reducing end, a family of structures was obtained (Table
I) representing side chains consisting of neutral sugar residues like arabinose and galactose
linked to a rhamnose residue which was originally flanked by two galacturonic acid residues.
Side chains may contain up to 15 glycosyl residues[9]. RG-I is considered to represent a family
of polysaccharides, all having a similar backbone of alternating rhamnose and galacturonic
acid residues, although the sugar composition, length and number of side chains probably
varies[18,39]. Acetyl groups are reported to be present in RG-I type of polymers[18], although the
exact position is not determined conscientiously. Komalavilas and Mort[40] reported the
isolation of a galacturonic acid-rhamnose dimer using HF-hydrolysis of which 30-40% of the
galacturonosyl residues carried an O-acetyl group at C-3. No indications are given in
literature for esterification of the carboxyl group of galacturonosyl residues in RG-I with
methanol.

   Rhamnogalacturonan II.—Next to RG-I, the group of Albersheim also described
rhamnogalacturonan II (RG-II) isolated from suspension-cultured sycamore cells[41].
   RG-II accounts for 4% or less of the primary cell walls of sycamore cells[18]. Subsequently,

A) Main Chains

→4-α-D-GalAp-[(1→4)-α-D-GalAp-](1→4)-α-D-GalAp-(1→2)-α-L-Rhap-[(1→4)-α-D-GalAp-(1→2)-α-L-Rhap-](1→4)-α-D-GalAp-(1→2)-α-L-Rhap-(1→

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        ↑                    ↑        n≈ to 70                                4      n′=300        4
        R4                   R3(?)                                            ↑                     ↑
                                                                             R2                    R1,R3(?)
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                                                        |  Rhamnogalacturonan I (RGI)*  |
                                                        |  R2                           |
                                                        |                               |
B) Major Side Chains                                    |  →5)-L-Araf-(1→                |
                                                        |  D-Galp-(1→                    |
                                    α-L-Araf            |  →3)-D-Galp-(1→                |
                                       ↑                |  →4)-D-Galp-(1→                |
      R1 arabinans                     ↓               |  →6)-D-Galp-(1→                |
                                       2                |  →6)                           |
→5-α-L-Araf-(1→5)-α-L-Araf-(1→5)-α-L-Araf-(1→5)-α-L-Araf-(1→  |     -D-Galp-(1→          |
              3           3                              |  →3)                           |
              ↑           ↑                              |                               |
              1           1                              |  →6)                           |
        (α-L-Araf)n    α-L-Araf                          |     -D-Galp-(1→                |
                                                        |  →3)                           |
                                                        |                               |
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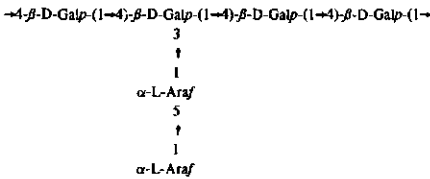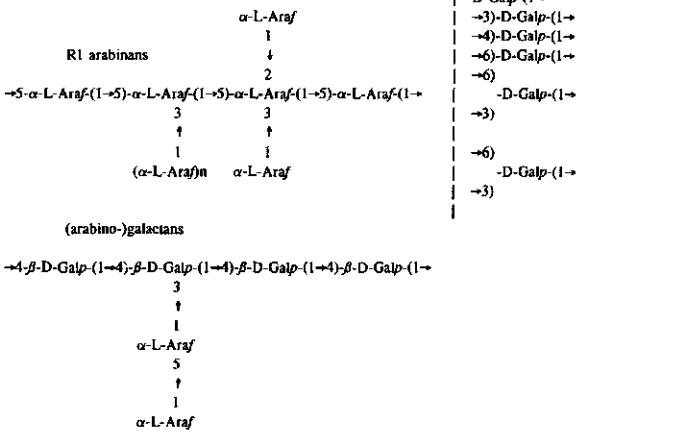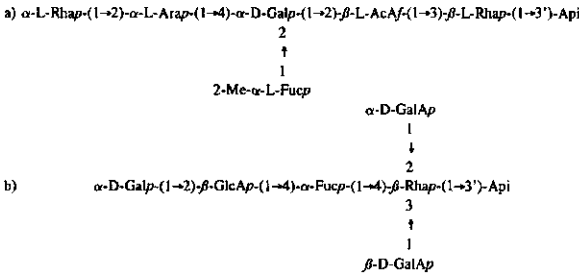
(arabino-)galactans

→4-β-D-Galp-(1→4)-β-D-Galp-(1→4)-β-D-Galp-(1→4)-β-D-Galp-(1→
```
              3
              ↑
              1
           α-L-Araf
              5
              ↑
              1
           α-L-Araf
```

R3 Rhamnogalacturonan II (RGII)**

a) α-L-Rhap-(1→2)-α-L-Arap-(1→4)-α-D-Galp-(1→2)-β-L-AcAf-(1→3)-β-L-Rhap-(1→3')-Api
```
                              2
                              ↑
                              1
                        2-Me-α-L-Fucp
                                           α-D-GalAp
                                              |
                                              ↓
                                              2
b)         α-D-Galp-(1→2)-β-GlcAp-(1→4)-α-Fucp-(1→4)-β-Rhap-(1→3')-Api
                                              3
                                              ↑
                                              1
                                         β-D-GalAp
```

R4 Apiogalacturonan (*Lemna minor*) (ref 57,58)

D-Apif-(1→3)-D-Apif-(1→3[or2])

C) Minor Side Chains

```
    β-D-Xylp-(1→3)-
      L-Araf-(1→3)-
    β-D-Galp-(1→2)-β-D-Xylp-(1→3)-
    α-L-Fucp-(1→2)-β-D-Xylp-(1→3)-
    β-D-GlcAp-(1→4)-L-Fucp-(1→
    β-D-GlcAp-(1→6)-D-Galp-(1→
```
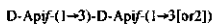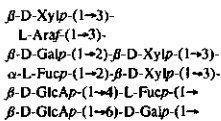
Fig. 3.   Diagrammatic representation of main chains (A) and side chains (B and C) of pectic polysaccharides (from ref[9]). There is also variable methyl esterification and *O*-acetylation of the (1→4)-α-D-linked galacturonosyl residues. *RG-I[10]; side chains (R2) may have at least 15 glycosyl residues. **RG-II[10]; two heptasaccharides which account for 60% of the glycosyl residues; position of attachment to main chain not determined.

RG-II was characterized in more detail and based on the presence of typical RG-II monosaccharides in the cell walls of bean, apple, tomato, cabbage, and potato provide strong evidence that RG-II is present in the primary walls of all of these plants[18]. RG-II is composed of approximately 30 glycosyl residues[10,38] and may consist of a backbone of 7-9 galacturonosyl residues, branched at C-2 and C-3[42,43]. These side chains might exist of the more common sugar residues rhamnose, fucose, arabinose, galactose, galacturonic acid and glucuronic acid, next to more rare sugars like 2-O-methyl-fucose, 2-O-methyl-xylose, apiose, aceric acid, 2-keto-3-deoxy-D-manno-octulosonic acid (KDO) and 3-deoxy-D-lyxo-2-heptulosaric acid (DHA)[10]. A problem in the recognition and analysis of some of these rare sugars is the acid-catalysed degradation of e.g. KDO and the different elution behaviour on GLC of e.g. methyl-sugars after conversion to their alditol acetates[18]. A schematic illustration of a complex pectin molecule according to the concept of the group of Albersheim and including homogalacturonan, RG-I and RG-II type of regions is given by Bacic et al.[9] (Fig. 3), which also suggested that RG-II might be present as side chains of the rhamnogalacturonan backbone. Although quite some relevant information is available on the structures present in both the backbone and the side chains, the figure illustrates clearly that only little is known on the length and structural features of the side chains and their distribution over the backbone.

Hairy regions.—Barrett and Northcote[44] were one of the first researchers who recognized that in addition to long sequences of esterified galacturonosyl residues, pectinic acid from apple fruit tissue also contained more neutral portions containing a high proportion of arabinofuranose residues attached to them. At the same time that the first models on RG-I and RG-II type of pectic fragments were published[41,45], the group of Pilnik also developed a model for pectic molecules[21,32,33,46,47]. Where RG-I is considered to consist always of alternating rhamnose and galacturonic acid (ratio rhamnose:galacturonic acid is 1)[18], De Vries et al.[22] also described pectic fragments isolated from apple having a ratio of 0.22. This observation has been confirmed later for e.g. onion[48] (ratio 0.28) and grape berries[49,50] (ratio 0.14-0.4). The ratio of rhamnose:galacturonic acid in these pectic fragments, obtained after (exhaustive) degradation of homogalacturonans by pure pectic enzymes, is much lower than a straight alternating RG-I would suggest. The hypothetical model as proposed by De Vries et al.[21]
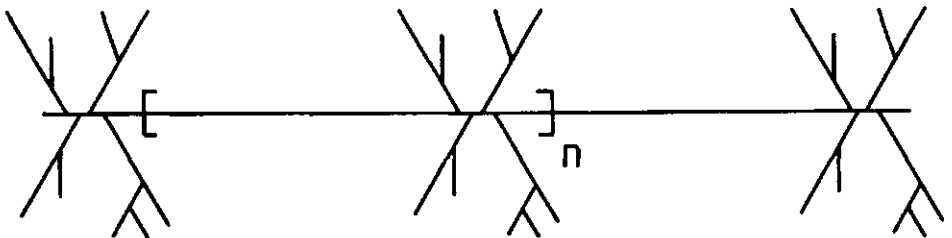

Fig. 4   Schematic structure of apple pectin[3]: rhamnogalacturonan backbone with regions rich in neutral sugar side chains according to De Vries et al.[21]

included both the homogalacturonan regions and the more branched regions and it is suggested that only up to 10% of the galacturonosyl residues were included in the "hairy" regions containing almost all of the neutral sugar residues. A schematic representation is given in Fig. 4. From a neutral sugar distribution curve, constructed for a large number of pectin fractions obtained by ion-exchange and size-exclusion chromatography from carefully extracted apple pectins, it was deduced that three main types of molecules exist having one, two, or three hairy regions respectively[21,51] (not shown in the figure). No studies sofar were directed to reveal the sequence of the rhamnose and galacturonic acid residues in these hairy regions.

Other galacturonic acid-containing plant cell wall polysaccharides.—Next to the presence of homogalacturonan, RG-I and RG-II, speculations are made[18,47,51] on the existence of other galacturonic acid containing polysaccharides. The presence of xylogalacturonans in which terminal xylose is linked directly to the galacturonosyl residues has been reported for mountain pine pollen[52], soy beans[53], and kidney beans[54,55]. The presence of terminal xylose residues linked directly to galacturonic acid moieties in pectic substances from apple has been also suggested[44,47]. Apiogalacturonan regions are proposed to be present in pectins extracted from eel grass[56] and duckweed[57,58]. Mild acid hydrolysis of the apiogalacturonan from duckweed gave three products, apiose, apiobiose and homogalacturonan[57,58].

Side chains containing glucuronic acid, galacturonic acid linked to galactose or rhamnose residues, and galactose side chains linked via a galacturonosyl residue to the C-4 of rhamnose in the backbone have been described to be present in pectic fragments from leaves and roots of several plants having an anti-complementary activity[59-62]. A rhamnogalacturonan isolated from the stipules of the umbrella tree is reported to include a side chain of 4-*O*-methyl-glucuronic acid-(1→4)-galacturonic acid dimer linked to C-4 of the rhamnose moiety of the backbone[63]. Structural analysis of the rhamnogalacturonan cores from the leaves of *Panax ginseng* suggested the presence of di-rhamnosyl units since the reduced dimer rhamnose-(1→2)-rhamnose-ol was detected[61].

Whether these structures are common in rhamnogalacturonans from various sources and whether or not they should be included into the accepted models and how these models should be altered is unknown at the moment.

Pectic enzymes

Pectic enzymes are classified according to their mode of attack on the galacturonan part of the pectin molecule[3]. Many detailed reviews have been dedicated to pectin degrading enzymes[3,64-66] and therefore these enzymes will be discussed in this chapter only briefly.

In general, the group of pectic enzymes can be divided in esterases and depolymerases. Pectin methylesterase (PE) de-esterifies pectins resulting in low-methoxyl pectins and releases methanol which was originally esterified to the carboxyl group of galacturonic acid. The action of PE may be blockwise (plant PE) or random (fungal PE)[3,65]. Polygalacturonases (PG's) catalyze the hydrolytic cleavage of the *O*-glycosyl bond of α-D-(1→4)-

polygalacturonans. The pattern of degradation proceeds in either a random (endo-PG) or terminal fashion (exo-PG)[65]. Lyases that degrade galacturonan polymers and oligomers (either methyl esterified or not) are enzymes that cleave the glycosidic bond by the mechanism of β-elimination[65,67] generating a 4,5-unsaturated galacturonosyl residue on the nonreducing end. Pectate lyase or pectic acid lyase (PAL) split glycosidic linkages next to free carboxyl groups, where pectin lyases (PL's) are specific for highly esterified regions within the pectin.

Sakai[25] described an additional class of pectic enzymes: the protopectinases. They are defined as enzymes able to degrade protopectin to form soluble pectins[25]. However, from the characterization of the various protopectinases[25,68], it can be concluded that the group of protopectinase includes PG's, arabinanases and galactanases. For this reason, it should be stated that a separate group for the individual enzymes active on protopectin is not justified.

Apple cell wall pectic polysaccharides.

In the sixties and seventies, scientists became more and more aware of the importance of pectic substances in cell walls of various fruits. Although analytical techniques used at that time were by far inferior to the advanced techniques used today, valuable information was obtained on the general structure of pectins and its role during ripening. Barrett and Northcote[44] distinguished already in 1965 between long pectinic acid segments and segments containing more neutral sugars. Using partial hydrolysis, followed by paper-chromatography, they also recognized the presence of aldobiouronic acids in apple pectic material, consisting of galacturonic acid and rhamnose, galactose, or xylose. The role of pectins during ripening of apple fruits was investigated by Knee by studying the metabolism of polymethylgalacturonate[69,70], and by comparison of polysaccharide compositions of apple fruits ripened on and off the tree[71]. Knee also studied the possible interaction of (pectic) polysaccharides and glycoproteins by sequential extraction procedures[72]. Conclusions were mainly based on relative amount and sugar composition of the various fractions.

In the eighties, the approach of sequential extraction was further developed[46,73], and size-exclusion and anion-exchange chromatography became standard techniques to distinguish between different populations[21,46,74,75]. Stevens and Selvendran[74] showed that hot-water-soluble pectins contained more arabinose as compared to oxalate-extractable pectin. They also separated the hot-water-soluble pectin into three major components by DEAE-chromatography, which mainly differed in their arabinose:galacturonic acid ratio. Aspinall and Fanous[76] investigated pectic polysaccharides from apple cell walls using the same approach. Next to arabinose-rich pectins, they also isolated a neutral arabinose-rich fraction(>85 mol% arabinose). Using glycosidic linkage analysis and ^{13}C NMR spectroscopy, they characterized both the neutral arabinan and the arabinan-parts of the pectic molecules to consist of α-(1→5)-linked arabinan chains being highly branched by single arabinose residues or short arabinose side chains.

A new stage of looking more closely to polysaccharide structures is characterized by the use of enzymes to degrade cell wall polysaccharide structures[77,78]. This was illustrated by the work of De Vries, who extensively studied apple pectic substances using sequential extraction

methods and chromatographic separation techniques[46] as mentioned above, but also used purified pectic enzymes[21] as an analytical tool to obtain specific fragments in a defined way. De Vries studied pectic substances from both ripe and unripe apples using purified and well defined enzymes like PL and PAL[21,32,33,47]. As discussed above, De Vries worked out the findings of Barrett and Northcote[44] and he proposed a more detailed model dealing with the intra- and intermolecular distribution of the neutral side chains and the methoxyl esters over the pectic molecules. Pectins, extracted from apples, were considered to consist of homogalacturonans ("smooth regions"), interrupted by ramified or "hairy regions". From the findings of De Vries, it was concluded that next to highly branched arabinan side chains, part of the pectic molecules carried dominantly $(1\rightarrow3,6)$-linked galactan side chains, while other pectic fractions contained higher proportions of $(1\rightarrow4)$-linked galactans.

Studies on apple "protopectins", not extracted by the methods used by De Vries, were carried out by Renard[75,79,80]. The residue remaining after extraction of apple cell wall polysaccharides by chelating agents, was treated with chemical agents like hypochlorite[75] and with (combinations of) purified enzymes. It was concluded[79,80] that a wide range of hemicellulolytic as well as cellulolytic enzymes were necessary to solubilize significant proportions of galacturonic acid containing polymers from the insoluble residue. A hypothetical model was proposed[19] in which the pectin molecules were attached to the xyloglucan-cellulose matrix. These connections might include arabinans and (arabino)galactans, but the existence of an unknown "anchor" was suggested because of the limited solubilization of pectins by arabinanases and galactanases.

Since the enzymes used were unable to cleave within the ramified regions, structural differentiation between the hairy regions of the various pectins was not possible, and the information obtained dealt mainly with characteristics for the entire pectin fraction.

Aim and outline of this thesis

The aim of this thesis is (i) to investigate the presence of enzyme-resistant polysaccharides in various enzyme-treated and non-enzyme-treated juices, (ii) the elucidation of the structural characteristics of the polymeric fragments retained by ultrafiltration of liquefaction juices and (iii) comparison of the structural features found for this pectic polysaccharide fraction with the features of unmodified pectic molecules in the apple cell wall. This knowledge is thought to be essential to understand the role of plant cell wall polysaccharides during processing and will facilitate the introduction of new tailor-made enzymes, and application-directed strategies in fruit and vegetable processing.

Quality characteristics of apple juices, manufactured by direct pressing and by using enzymes, are compared with emphasis on polysaccharides (Chapter 2). The characterization of an enzyme resistant fraction, isolated by ultrafiltration, and further termed MHR (modified hairy regions) is given (Chapter 3). Rhamnogalacturonase, an enzyme able to degrade the rhamnogalacturonan backbone of MHR and releasing oligomeric structures next to polymeric fragments is described in Chapter 4. The identification by NMR spectroscopy of the oligomers is described in detail in Chapter 5. In Chapter 6 it is shown that similar MHR

fractions could be isolated from various fruits and vegetables using the same liquefaction process, illustrating that these polysaccharide structures occur widely spread in plant material. The degradation of all these different MHR fractions by rhamnogalacturonase is compared and the separation and characterization by NMR of five new oligomers is described (Chapter 7). Unmodified hairy regions were obtained from carefully extracted pectins from apple tissue and a comparison is made of their structural features with MHR isolated from apple juice (Chapter 8). Next to the characteristic rhamnogalacturonase oligomers, also the presence of high molecular weight subunits is recognized (Chapter 9). Based on the results obtained, our hypothetical model was updated in chapter 10.

References

1   W. Pilnik, in P. Dupuy (Ed) *Use of Enzymes in Food Technology*, Lavoisier, Paris, 1982, pp. 425-450.
2   W. Pilnik, and A.G.J. Voragen, *ACS Symp. Ser.*, 405 (1989) 250-269.
3   W. Pilnik, and A.G.J. Voragen, in P.F. Fox (Ed), *Food Enzymology*, Vol. 1, Elsevier Applied Science, London, 1991, pp. 303-336.
4   W. Pilnik, and F.M. Rombouts, in J.M.V. Blanshard, and J.R. Mitchell (Eds), *Polysaccharides in Food*, Butterworths, London, 1979, pp. 109-126.
5   A.G.J. Voragen, F. Geerst, and W. Pilnik, in P. Dupuy (Ed), *Use of Enzymes in Food Technology*, Lavoisier, Paris, 1982, pp. 497-502.
6   S.C. Churms, E.H. Merrifield, A.M. Stephen, D.R. Wolwyn, A. Polson, K.J. van der Merwe, H.S.C. Spies, and N. Costa, *Carbohydr. Res.*, 113 (1983) 339-344.
7   A.G.J. Voragen, H.A. Schols, H.A.I. Siliha, and W. Pilnik, *ACS Symp. Ser.*, 310 (1986) 230-247.
8   A.G.J. Voragen, H.A. Schols, and W. Pilnik, *Z. Lebensm. Unters. Forsch.*, 187 (1988) 315-320.
9   A. Bacic, P.J. Harris, and B.A. Stone, *The Biochemistry of Plants*, Vol 14, *Carbohydrates*, Academic, London, 1988, pp. 297-369.
10   M. McNeil, A.G. Darvil, S.C. Fry, and P. Albersheim, *Ann. Rev. Biochem.*, 53 (1984) 625-663.
11   K. Keegstra, K.W. Talmadge, W.D. Bauer, and P. Albersheim, *Plant Physiol.*, 51 (1973) 188-197.
12   K.W. Talmadge, K. Keegstra, W.D. Bauer, and P. Albersheim, *Plant Physiol.*, 51 (1973) 158-173.
13   M.C. McCann, and K. Roberts, in C.W, Lloyd (Ed) *The Cytoskeletal Basis of Plant Growth and Form*, Academic, London, 1991, pp. 109-129.
14   N.C. Carpita, and D.M Gibeaut, *Plant J.*, 3 (1993) 1-30.
15   L.D. Talbott, and P.M. Ray, *Plant Physiol.*, 98 (1992) 357-368.
16   D.A. Rees, and N.J. Wight, *Biochem. J.*, 115 (1969) 431-439.
17   P.J. Moore, A.G. Darvill, P. Albersheim and L.A. Staehelin, *Plant Physiol.*, 82 (1986) 787-794.
18   M. O'Neill, P. Albersheim, and A.G. Darvill, in P.M. Dey (Ed.), *Methods in Plant Biochemistry*, Vol. 2, *Carbohydrates*, Academic, London, 1990, pp. 415-441.
19   C.M.G.C. Renard, *Etude des polysaccharides parietaux de la pomme, extraction et caracterisation par des methodes chimiques et enzymatiques*, Ph.D. Thesis, University of Nantes, France, 1989.
20   Z.I. Kertesz, *The Pectic Substances*, Interscience, NY, 1951.
21   J.A. De Vries, F.M. Rombouts, A.G.J. Voragen, and W. Pilnik, *Carbohydr. Polym.*, 2 (1982) 25-33.
22   V. Zitko, and C.T. Bishop, *Can. J. Chem.*, 44 (1966) 1275-1282.
23   J.-F. Thibault, C.M.G.C. Renard, M.A.V. Axelos, P. Roger, and M-J. Crépeau, *Carbohydr. Res.*, 238 (1993) 271-286.
24   C. Rolin, and J. De Vries, in P. Harris (Ed.), *Food Gels*, Elsevier Applied Science, London, 1990, pp. 401-434.

25   T. Sakai, in G. Winkelmann (Ed), *Microbial Degradation of Natural Products*, VCH, Weinheim, 1992, pp. 57-81.

26   T. Sakai, and S. Yoshitake, *Agric. Biol. Chem.*, 48 (1984) 1941-1950.

27   D.A. Powell, E.R. Morris, M.J. Gidley, and D.A. Rees, *J. Mol. Biol.*, 155 (1982) 517-531.

28   H. Konno, Y. Yamasaki, and K. Katoh, *Phytochem.*, 25 (1986) 623-627.

29   G. Chambat, and J.-P. Joseleau, *Carbohydr. Res.*, 85 (1980) C10-C12.

30   T.P. Kravtchenko, M. Penci, A.G.J. Voragen, and W. Pilnik, *Carbohydr. Polym.*, 20 (1993) 195-einde.

31   J. De Vries, M. Hansen, J. Soderberg, P.E. Glahn, and J.K. Pedersen, *Carbohydr. Polym.*, 6 (1986) 165-176.

32   J.A. De Vries, F.M. Rombouts, A.G.J. Voragen, and W. Pilnik, *Carbohydr. Polym.*, 3 (1983) 245-258.

33   J.A. De Vries, A.G.J. Voragen F.M. Rombouts, and W. Pilnik, *Carbohydr. Polym.*, 4 (1984) 3-14.

34   H. Kiyohara, J.-C Cyong, and H. Yamada, *Carbohydr. Res.*, 182 (1988) 259-275.

35   F.M. Rombouts, and J.-F. Thibault, *Carbohydr. Res.*, 154 (1986) 177-187.

36   A.G.J. Voragen, H.A. Schols, and W. Pilnik, *Food Hydrocolloids*, 1 (1986) 65-70.

37   T. Ishii, J. Thomas, A. Darvill, and P. Albersheim, *Plant Physiol.*, 89 (1989) 421-428.

38   J.M. Lau, M. McNeil, A.G. Darvill, and P. Albersheim, *Carbohydr. Res.*, 168 (1988) 245-274.

39   M. McNeil, A.G. Darvill, and P. Albersheim, *Plant Physiology*, 70 (1982) 1856-1591.

40   P. Komalavilas, and A.J. Mort, *Carbohydr. Res.*, 189 (1989) 261-272.

41   A.G. Darvill, M. McNeil, and P. ALbersheim, *Plant Physiol.*, 62 (1978) 418-422.

42   T.T. Stevenson, A.G. Darvill, and P. Albersheim, *Carbohydr. Res.*, 179 (1988a) 269-288.

43   T.T. Stevenson, A.G. Darvill, and P. Albersheim, *Carbohydr. Res.*, 182 (1988b) 207-226.

44   A.J. Barrett, and D.H. Northcote, *Biochem. J.*, 94 (1965) 617-627.

45   M. McNeil, A.G. Darvill, and P. Albersheim, *Plant Physiol.*, 66 (1980) 1128-1134.

46   J.A. De Vries, A.G.J. Voragen, F.M. Rombouts, and W. Pilnik, *Carbohydr. Polym.*, 1 (1981) 117-127.

47   J.A. De Vries, F.M. Rombouts, A.G.J. Voragen, and W. Pilnik, *Carbohydr. Polym.*, 3 (1983) 193-205.

48   S. Ishii, *Phytochemistry*, 21 (1982) 778-780.

49   L. Saulnier, and J.-F. Thibault, *Carbohydr. Polym.*, 7 (1987) 345-360.

50   L. Saulnier, J.-M. Brillouet, and J.-P. Joseleau, *Carbohydr. Res.*, 182 (1988) 63-78.

51   J. De Vries, in G.O. Philips, D.J. Wedlock, and P.A. Williams (Eds), *Gums and Stabilizers for the Food Industry 4*, IRL Press, Oxford, (1988) pp. 25-29.

52   O. Bouveng, *Acta Chemica Scand.*, 19 (1965) 953-963.

53   T. Kikuchi, and H. Sugimoto, *Agr. Biol. Chem.*, 40 (1976) 87-92.

54   Y. Matsuura, *Nippon Nôgeikagaku Kaishi*, 58 (1984) 253-259.

55   Y. Matsuura, *Nippon Nôgeikagaku Kaishi*, 58 (1984) 1111-1115.

56   Y.S. Ovodov, *Pure Appl. Chem.*, 42 (1975) 351-369.

57   D.A. Hart, and P.K. Kindel, *Biochem. J.*, 116 (1970a) 569-579.

58   D.A. Hart, and P.K. Kindel, *Biochemistry*, 9 (1970b) 2190-2196.

59   H. Kiyohara, H. Yamada, and Y. Otsuka, *Carbohydr. Res.*, 167 (1987) 221-237.

60   H. Kiyohara, and H. Yamada, *Carbohydr. Res.*, 187 (1989) 117-129.

61   Q.-P. Gao, H. Kiyohara, and H. Yamada, *Carbohydr. Res.*, 196 (1990) 111-125.

62   H. Yamada, H. Kiyohara, and Y. Otsuka, *Carbohydr. Res.*, 170 (1987) 181-191.

63   P.-E. Jansson, B. Lindberg, M. Ogunlesi, and T. Orebamno, *Carbohydr. Res.*, 214 (1991) 281-287.

64   W. Pilnik, and F.M. Rombouts, in G.G. Birch, N. Blakebrough, and K.J. Parker (Eds), *Enzymes and Food Processing*, Applied Science Publishers LTD, London, 1981, pp. 105-128.

65   J.K. Burns, in R.G. Walter (Ed), *The Chemistry and Technology of pectin*, Academic, London, 1991, pp. 165-188.

66   T. Sajjaanantakul, and L.A. Pitifer, in R.G. Walter (Ed), *The Chemistry and Technology of pectin*, Academic, London, 1991, pp. 135-164.

67   L. Rexova-Benkova, and O. Markovic, *Adv. Carbohydr. Chem. Biochem.*, 33 (1976) 323-385.

68   T. Sakai, K. Ikemoto, and Y. Ozaki, *Agric. Biol. Chem.*, 53 (1989) 1213-1223.

69   M. Knee, *Phytochemistry*, 17 (1978) 1261-1264.

70   M. Knee, *Phytochemistry*, 17 (1978) 1257-1260.

71   M. Knee, *Phytochemistry*, 12 (1973) 1543-1549.

72   M. Knee, *Phytochemistry*, 12 (1973) 637-653.

73   R.R. Selvendran, and P. Ryden, *Methods in Plant Biochemistry*, Vol. 2, *Carbohydrates*, Academic Press, London, 1990, pp 549-579.

74   B.J. Stevens, and R.R. Selvendran, *Carbohydr. Res.*, 135 (1984) 155-166.

75   C.M.G.C. Renard, A.G.J. Voragen, J.-F. Thibault, and W. Pilnik, *Carbohydr. Polym.*, 12 (1990) 9-25.

76   G.O. Aspinall, and H.K. Fanous, *Carbohydr. Polym.*, 4 (1984) 193-214.

77   A.G.J. Voragen, R. Heutink, and W. Pilnik, *J. Applied Biochem.*, 2 (1980) 452-468.

78   X. Rouau, and J.-F. Thibault, *Carbohydr. Polym.*, 4 (1984) 111-125.

79   C.M.G.C. Renard, M.J.F. Searle-van Leeuwen, A.G.J. Voragen, J.-F. Thibault, and W. Pilnik, *Carbohydr. Polym.*, 14 (1991) 295-314.

80   C.M.G.C. Renard, H.A. Schols, A.G.J. Voragen, J.-F. Thibault, and W. Pilnik, *Carbohydr. Polym.*, 15 (1991) 13-32.

CHAPTER 2

THE EFFECT OF THE MANUFACTURING METHOD ON THE CHARACTERISTICS OF APPLE JUICE

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Abstract

Apple juices, obtained by straight pressing, pulp enzyming or liquefaction and clarification by the conventional process using bentonite, gelatin, and silicasol or by ultrafiltration, were analyzed for sugar and acid content, colour, polyphenol content, ultrafiltration fluxes and polysaccharide content and composition. The amount and composition of the high-M_w polysaccharide fractions found indicate the importance of the juice processing method and enzyme preparation chosen. Polysaccharides solubilized from the cell walls were found to contain galacturonic acid and arabinose as the major constituent sugars. Ultrafiltration enabled the removal of high-M_w polymers but neutral arabinans were still found to be present in ultrafiltered juice.

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Introduction

Technical enzyme preparations are widely used in the fruit-processing industry to facilitate juice release, to increase juice yields and to clarify juices[1,2]. In apple juice processing, they are applied in (a) depectinization of the pressed juice; (b) pulp treatment prior to pressing (mash fermentation or pulp enzyming); (c) treatment of the pressed-cake remaining after the first press cycle to enhance the juice yield in the second cycle; (d) treatment of the apple mass remaining after water extraction prior to pressing and (e) liquefaction of the apple pulp.

Quality characteristics of juices obtained by conventional pressing, pulp enzyming, water extraction and liquefaction have been studied by many investigators. In particular, differences in phenolics, acidity and aroma have been described[3-7]. During the last decade, much effort has been directed at studying the possible use of ultrafiltration in the clarification to turbid juices[8-10]. Also the effect of ultrafiltration on juice browning[11] and retention of volatiles during ultrafiltration[12,13] have been studied. Voragen et al.[14] observed that juices obtained by liquefaction were more liable to non-enzymatic browning which they ascribed to the reactivity of unsaturated galacturonides present in higher concentrations in these juices. In this last decade there has also been an increased incidence of haze in apple and pear juice concentrates[15,16]. This haze has been identified as microcrystalline arabinans[17,18]. The attention was therefore directed to the amount and nature of enzymatically solubilized cell wall polysaccharides present in juices, since they might be precursors for this type of haze[15,19-22].

In this article, results are presented from a study on the effect of juice manufacture method on juice characteristics like acidity, °Brix, uronide content, polyphenol content, and solubilized polysaccharides (content, neutral sugar composition and M_w distribution). Juices were prepared by pressing, pulp enzyming or liquefaction using technical enzyme preparations and were clarified by the conventional process using bentonite, gelatin, and Klar-sol or by ultrafiltration.

Experimental

Enzyme preparations.—Technical and experimental enzyme preparations used to obtain pulp enzyming and liquefaction juices were e.a. Pex, Rapidase C600 and Hemicellulase reg.II (Gist brocades, Delft, The Netherlands) and Pectinex Ultra SP (Novo Ferment AG, Basel, Switzerland). Enzymes used for clarification of the straight pressed juices were Pectinol S (destarching) and Pectinex 3 x L (depectination; Röhm, GmbH, Darmstadt, FRG).

Preparation of apple juices.—Batches of about 170 kg golden Delicious apples (harvested in 1985, fresh and stored under controlled atmosphere) were ground in an Amos rasp mill to a particle size of 7 mm.

Straight pressed juices were prepared by pressing the mash with an Ensink continuous belt press; the press cakes were wetted with water (ratio 3:2) and pressed again. The juices from the first and second press cycle were combined and heated to 85°C to precipitate thermo-labile compounds (e.g. proteins). The juices were treated with the clarifying enzymes (100 mg/kg, 45°C) until they showed a negative alcohol test (2-4 h).

Pulp enzyming juices were produced by treating the mash with enzymes (200 mg/kg, 1 h 20°C) prior to pressing. The press cakes obtained were also wetted and pressed again. The juices of both cycles were combined, and held at ambient temperature until the juice showed a negative alcohol test. The juice was then heat-treated (85°C, 15 min) to inactivate the enzymes.

Liquefaction juices were produced by treating the apple mash with enzyme (300 mg/kg) for 4 h at 45°C. After passing a decanter (Pennwalt Sharpless P-600) water was added to the sediment (ratio 3:2) and decanted again. The combined juices were held at ambient temperature until a negative alcohol test was obtained and then heat treated (85°C, 15 min) to inactivate the enzymes.

Clarification.—Two methods were used to clarify the turbid juices. (a) Conventional clarification using NaCalit 2000 (50 g/100 L; Erbslöh & Co), Gelatin Laine (15 g/100 L; Chevallier Appert) and Klar-Sol 30 (25 mL/100 L; Erbslöh & Co). (b) Ultrafiltration using tubular UF module B-1 equipped with BX 1 non-cellulosic membranes with a molecular weight cut-off of 60 000 (Paterson Candy International LTD, Whitchurch, UK). The material retained was diluted sixfold using 10 L water each time. All juices were prepared in duplicate. They were concentrated to about 72 °Brix using a horizontal film evaporator (Sako type KH10). All clarified samples were dialyzed successively against running tap water and then distilled water until non-retained material showed a negative phenol/sulphuric acid test[23]. Finally these products were freeze-dried.

Analytical methods.—The colour of juices and juice concentrates ($\pm$ 72 °Brix) were measured with the Hellige Neo-Komparator (IFU[24]).

Polyphenols were determined by HPLC using a LiChrosorb 10 RP18 column (250 x 4.6 mm, Merck Darmstadt (FRG) and elution with a methanol/water gradient (1.5 mL/min; both solvents contained 4% acetic acid) starting with 90% water for 9 min followed by a gradient of 2.67% methanol/min until 50% methanol was reached and this composition was held for an additional 8 min. A Kratos 773 spectrophotometer set at 280 nm was used to monitor the eluent.

The uronide content was measured by the *m*-hydroxybiphenyl assay[25]. To determine the uronide content in undialyzed juices, the values were corrected for the presence of high concentrations of sucrose, glucose and fructose (correction factor 1.6)[26]. The sugar composition of the polysaccharides was analyzed gas chromatographically as alditol acetates after hydrolysis with 2 M trifluoroacetic acid as described by Jones and Albersheim[27].

The degree of methylation and acetylation of pectic fractions obtained after dialysis were determined by HPLC as described by Voragen et al.[28]

High-performance size-exclusion chromatography (HPSEC) was performed on a SP8800 HPLC (Spectra Physics) equipped with three Bio-Gel TSK columns (each 300 x 7.5 mm) in series (40XL, 30XL, and 20XL; Bio-Rad Labs) in combination with a TSK XL guard column (40 x 6 mm) and elution at 30°C with 0.4 M acetic acid/sodium acetate (pH 3.0) at 0.8 mL/min. The eluate was monitored using a Shodex SE-61 refractive index detector. The system was calibrated with pectins having M_w values in the range 1000-100 000 (as determined by viscosimetry) and oligomers obtained after degradation of polygalacturonic acid by yeast endo-polygalacturonase. Acidic and neutral polysaccharides were separated by the

same HPSEC system with water as eluent.

Results

A number of characteristics of the juices obtained by straight pressing, pulp enzyming and liquefaction are presented in Table I.

Juice yield, °Brix.—Juice yield depended on the age of apples used and, for straight pressed juice, ranged from 94% for fresh apples to 87% for stored apples. The high yield was realized by adding water, followed by a second press cycle. When enzymes were used for juice release, less effect of storage time on the yield was observed. Variations in the yield of juice obtained by pulp enzyming and liquefaction were mainly caused by the different enzyme preparations used. When the refraction (°Brix) of the juices was measured, however, it was found that the liquefaction process gave higher values, indicating that more sugars were released in the juice. It should be mentioned that the °Brix values of liquefaction juices clarified by ultrafiltration were about 5% lower than the conventionally clarified juices as a result of the removal of a relatively large amount of material. Table II shows the yield of dry material in the released juices obtained from 100 kg dry pulp material. Straight pressing was found to give the lowest yield in crude juice (before clarification), pulp enzyming and liquefaction increased the yield to 7-13% and 17-21% respectively. When the juices were clarified however, the increase in dry material in the clarified juices obtained by the pulp enzyming method and the liquefaction process as compared to straight pressing were similar (5-11% versus 13%).

pH, acid content.—The pH of the juices and the total amount of acids (Table I) were found to be influenced by storage time of the apples and by the manufacturing method. Longer storage of the apples resulted in higher pH values and lower total acid content in the juices

TABLE I.   Effect of juice manufacturing method on juice characteristics

Characteristic	Value for juice obtained by		
	Straight pressing	Pulp enzyming	Liquefaction
Yield(w/w% of pulp)	87-94	94-95	91-94
°Brix	10.8-11.4	11.5-12.0	12.1-13.0
pH	3.4-3.7	3.4-3.7	3.4-3.5
Total acid (%)	0.29-0.46	0.29-0.47	0.35- 0.44
Colour (IFU units)	3.5	4.5	5.5
Ultrafiltration (L/m^2h)			
start	180	180	90
end	120	120	60

TABLE II. Yield of juice dry matter from dry pulp material

Method	Yield (kg/100 kg)					
	Crude juice	Conventionally clarified		Ultrafiltration clarified		
		Juice	Sediment	Juice	Sediment	Retained
Straight pressing	66.3	64.0	2.3	64.0	1.6	0.7
Pulp enzyming						
Pex	71.6	68.5	3.1	68.3	2.3	1.0
Hemi II	70.8	67.0	3.8	66.9	2.6	1.2
Ultra SP	74.8	72.0	2.8	71.3	2.1	1.3
Liquefaction						
Ultra SP	77.7	74.8	3.0	72.3	2.6	2.7
Rapidase C600	80.0	76.1	4.0	72.5	3.4	4.2

obtained by the three manufacturing processes. When apples were stored for the same period of time, enzyme treatment lowered the pH and increased the total acid content of the juices. In general, it can be stated that enzyme treatment of stored apples results in juices with pH and total acid content comparable to straight pressed juices of fresh apples.

   Colour.—The colour of the juices was found to depend on the method of manufacture and ranged from 3.5 IFU units for straight pressed juice to 5.5 IFU units for liquefaction juices. The colour of juice concentrates was measured directly after the concentration step and after 2-6 month storage at 4°C. No significant changes were found for the various concentrates, unlike Rosch[11] who found that ultrafiltered clarified juice concentrates were more stable towards browning reactions during storage.

TABLE III. The effect of juice manufacturing method on the polyphenol soluble polymeric cell wall composition

Polyphenol	Content (mg/L)		
	Straight pressing	Pulp enzyming	Liquefaction
Catechin	0	0	6-35
Chlorogenic acid	41-63	6-13	48-85
Caffeic acid	5-10	0-2	8-11
Epi-catechin	5-10	0	32-65
p-Coumaric acid	2-4	0-2	3-6
Ferulic acid	0-1	0-2	2-4
Phloridzine	10-16	2-11	40-67

Ultrafiltration fluxes.—During ultrafiltration of the various juices, the flow characteristics of the different juices were studied. For straight pressed juices and pulp enzyming juices, similar flow rates through the ultrafiltration membrane were measured; the flow decreased gradually by about 33% after 40-60 min of operation. The same effect was found for liquefaction juice, however, due to the presence of high amounts of small particles, the initial flow rate was only half that of the former juices (Table I).

Polyphenols.—Table III shows the polyphenol content of the various juices. The content of various polyphenols was strongly influenced by the juice release method but hardly by the method of clarification. As can be seen in Fig. 1, juices obtained by pulp enzyming had a low content of all polyphenols as compared to straight pressed juices. During enzyme treatment (1 h, 20°C) the polyphenols are probably oxidized[29] and condensed to polymers which are bound to the press cake. After longer incubation times (4 h) at increased temperature (45°C) as used for liquefaction, the total amount of polyphenols was increased as compared to the straight pressed juices, probably due to the increased release of polyphenols from the cell walls by the enzymic degradation.

Solubilized cell wall material.—Another point of interest involves the amount and composition of the cell wall polysaccharides released in the juices by the enzymes and the effect of ultrafiltration in their removal. To establish this effect all juices and the material retained by ultrafiltration were dialyzed and freeze-dried. The retained products were weighed
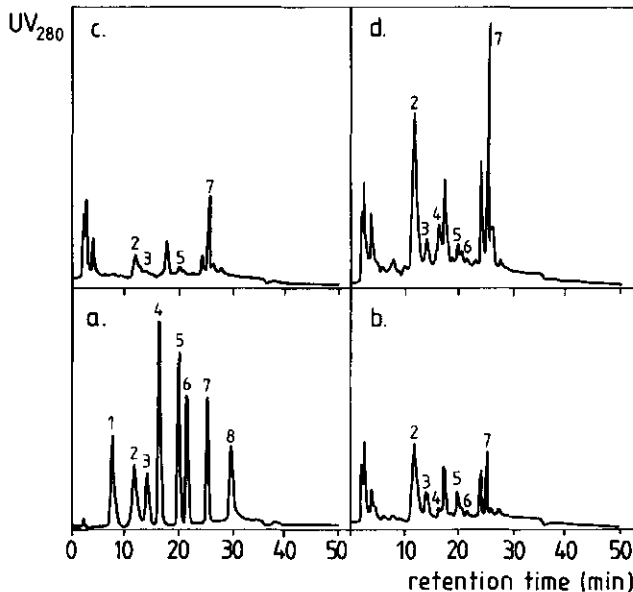

Fig.1.   Polyphenol analysis by HPLC: elution patterns of a reference mixture (a) and of juices obtained after straight pressing (b), pulp enzyming with Ultra SP (c), and liquefaction with Rapidase C600 (d). 1, Catechine; 2, chlorogenic acid; 3, caffeic acid; 4, epi-catechine; 5, *p*-coumaric acid; 6, ferulic acid; 7, phloridzine; 8, quercetine.

and analyzed for their sugar composition. The results were compared with those obtained for the material retained after ultrafiltration. Table IV summarizes the polysaccharide content and arabinose content of the material retained after dialysis of all juices, clarified both conventionally and by ultrafiltration and of the material retained after ultrafiltration. A significant effect of the method of juice manufacture on the amount of polysaccharides released can be observed. After conventional clarification, the polysaccharide content of the straight pressed juice was found to be 150 mg/L, for pulp enzyming juice and liquefaction juice the data ranged between 1100-1850 mg/L and 2800-3800 mg/L, respectively, depending on the enzyme preparation used. These data illustrate that the enzymes solubilize polysaccharides from the cell walls in amounts that depend on the activities present in the technical enzyme preparation and on the conditions of enzyme treatment (time, temperature). Ultrafiltration was found to reduce the polysaccharide content of the juices by 50-85% depending on the enzyme preparation used. In liquefaction juices, particularly the polymer content was strongly reduced and might explain the reduced Brix values found for this type of juice. The amounts of polymeric arabinose detected in the juices indicate that the enzyme preparations were not able to degrade the arabinans solubilized from the cell wall to dialysable fragments. The data clearly show that the total sugar and arabinose content depend both on the juice releasing method and the method of clarification. The amount of arabinose present in the juices also depends on the enzyme preparation used. By ultrafiltration the arabinose-containing polysaccharides can be largely removed, resulting in a reduction in arabinose content between 50-98%, indicating that most arabinose is present in high-M_w fragments.

TABLE IV.   Arabinose content and total neutral sugar content of high-M_w material (retained after dialysis) in conventionally and ultrafiltration clarified juices and in the material retained by ultrafiltration

Juice	Amount (mg) in juice obtained from 1 kg					
	Conventional		UF-permeate		UF-retentate	
	Ara	Total	Ara	Total	Ara	Total
Straight pressing	30	150	6	75	15	65
Pulp enzyming						
Pex	360	1100	90	550	200	800
Hemi II	85	1850	40	750	60	1700
Ultra SP	500	1100	100	400	140	400
Liquefaction						
Ultra SP	1100	2800	120	550	800	1600
Rapidase C600	2100	3800	50	430	1450	2900

UF-permeate and UF-retentate indicate material not retained and retained, respectively, after ultrafiltration. Total sugars were calculated as the sum of neutral sugars, analyzed by GLC as alditol acetates, and uronides as measured by the m-hydroxybiphenyl assay.

TABLE V. Neutral sugar composition of high-M_w material retained by dialysis in conventionally and ultrafiltration-clarified juices and in the material retained after ultrafiltration

Juice		Content (mol%) of								
		Rha	Ara	Xyl	Man	Gal	Glc	UA	OAc	OMe
Straight pressing										
	Conv	3	21	2	1	19	2	53	15	52
	UF	2	8	2	1	3	5	79	nd[a]	nd
	UF-ret	6	23	2	2	43	2	22	27	34
Pulp Enzyming										
Pex	Conv	5	38	5	0	9	1	42	18	72
	UF	2	21	2	0	2	1	72	7	55
	UF-ret	4	30	5	0	11	1	49	14	31
Hemi II	Conv	3	6	2	1	4	1	84	6	76
	UF	2	7	2	1	2	1	86	5	78
	UF-ret	2	4	2	1	5	0	86	5	51
Ultra SP	Conv	5	52	5	0	7	1	30	13	31
	UF	4	32	2	0	3	1	58	8	23
	UF-ret	4	40	8	0	13	1	34	14	32
Liquefaction										
Ultra SP	Conv	4	43	6	0	6	1	40	28	59
	UF	3	16	2	0	2	1	76	10	53
	UF-ret	4	54	8	0	8	1	25	51	62
RapC600	Conv	4	54	8	0	8	0	26	39	39
	UF	2	18	2	0	2	2	74	10	32
	UF-ret	4	54	8	0	9	1	24	54	43

UA indicates uronide content; OAc and OMe are both calculated on the uronide content; Conv indicates conventionally-clarified juice, UF juice clarified by ultrafiltration and UF-ret material retained after ultrafiltration; [a] not determined.

Sugar composition.—Table V presents the sugar composition of the products retained by dialysis after being clarified conventionally and by ultrafiltration and material retained after clarification by ultrafiltration. These data confirm our previous observation that the juice recovery method and the technical enzyme preparation used determine to a large extent the amount and nature of the polysaccharides in the juice. The most important sugars making up the apple cell walls are arabinose, glucose and galacturonic acid[30]. The polysaccharides in the juice retained by dialysis were found to be enriched in arabinose. Together with galacturonic acid, this sugar makes up 70 to 90% of the polysaccharides. The differences in degradation mechanism of the different enzyme preparations are clearly demonstrated when the uronide:arabinose ratios of the conventionally clarified juices are compared. These values are 2.5 for straight pressed juice, 0.6-14 for pulp enzyming juices and 0.5-0.9 for liquefaction juices. The dialyzed juices retained from the ultrafiltered material were enriched in uronides,

while the material retained after ultrafiltration enriched in arabinose with the uronides occurring in oligomeric fragments. The differences in uronide content, degree of methylation and degree of acetylation of these juices reflect the different pectolytic activities in the enzyme preparations. The uronide contents were found to vary between 26-86% in the dialyzed juices, the degree of methylation of the pectic material retained by dialysis of conventionally clarified juice was 52% for straight pressed juice and ranged from 30-70% for pulp enzyming juices and from 39-59% for liquefaction juices. The degree of acetylation ranged from 6% for pulp enzyming juices to 39% for one of the liquefaction juices, depending on the enzyme preparation used. In general more pectic substances rich in acetyl groups are released by the liquefaction process. The results obtained for the material returned by ultrafiltration after liquefaction with Rapidase C600 are in full agreement with data reported earlier by Voragen et al.[20] and Schols et al.[22] for the same polysaccharide fraction from Golden Delicious apples harvested in 1981.

Uronic acids in the juice.—Table VI presents the uronide content of the juices. To obtain an indication of the percentage of uronides having a low molecular weight, juices were dialyzed and the difference in uronide content before and after the dialysis step was considered as the percentage of low-M_w uronides. As can be seen, the percentage of dialysable uronide-containing material differed with the method of manufacture and enzyme preparation used. In straight pressed juice only 200 mg galacturonide/L juice was released and 60% of these pectic substances were low-molecular-mass fragments. During pulp enzyming the total uronide content increased and especially after ultrafiltration there are significant differences in the content of low-M_w uronide fragments (Hemi II versus Ultra SP: 26% of 800 mg/L versus 88% of 1600 mg/L). In liquefaction juice the uronide content is higher than in pulp enzyming juice and most uronides were dialysable. The more intensive the enzyme treatment, the higher the uronide content in the juice. In this study, we did not

TABLE VI.   Uronide content of conventionally and ultrafiltration clarified juices

Juice	Uronide (mg/L) in juice clarified:	
	conventionally	by ultrafiltration
Straight pressing	200 (60%)	200 (60%)
Pulp enzyming		
Pex	2600 (73%)	1700 (80%)
Hemi II	2700 (50%)	800 (26%)
Ultra SP	1900 (63%)	1600 (88%)
Liquefaction		
Ultra SP	3400 (65%)	2400 (86%)
Rapidase C600	3600 (59%)	2400 (90%)

Figures in parenthesis indicate the percentage of uronides which can be removed by dialysis.
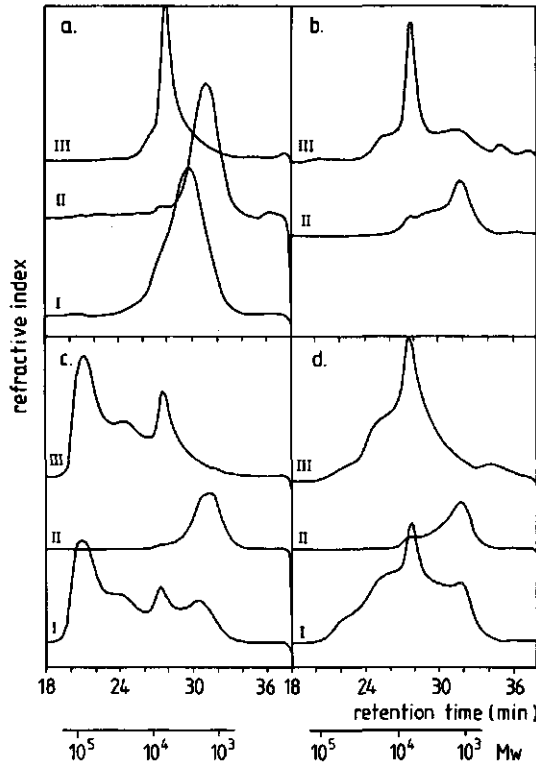
Fig. 2. High-performance size-exclusion chromatography of juice dialysates of conventionally clarified juice (I), ultrafiltration clarified juice (II), and the corresponding material retained after ultrafiltration (III). a, pulp enzyming (Hemi II); b, pulp enzyming (Ultra SP); c, liquefaction (Rapidase C600); d, liquefaction (Ultra SP). The HPSEC system is calibrated with pectin standards.

differentiate between saturated and unsaturated oligomers, while Voragen et al.[14] described different behaviour in browning reactions in models of apple juice concentrates between both types of oligogalacturonides.

Molecular weight distribution.—To examine the molecular weight distribution of the polysaccharides, the dialyzed juices and material retained by ultrafiltration were also analyzed by HPSEC. Typical elution profiles are shown in Fig. 2, which indicate that the conventionally clarified juices contained different M_w populations (Fig. 2; lines I): after ultrafiltration the low-M_w fraction was not retained while the high-M_w was. The HPSEC elution patterns of the clarified juices obtained by the pulp enzyming process using Hemi II (Fig. 2a) and Ultra SP (Fig. 2b) are shown. The M_w distribution of the fractions obtained by pulp enzyming with these two enzyme preparations are comparable but, as can be seen in Table V, the sugar composition is rather different, especially for the arabinose. The fraction obtained by the enzyme preparation Hemi II is relatively poor in arabinose, probably because

this enzyme preparation is able to degrade the solubilized arabinans to smaller, dialysable oligomers or that Hemi II lacks enzymes able to solubilize arabinose-rich fragments in the juice.

During the liquefaction process, higher-M_w fragments are solubilized and thus removed by the ultrafiltration process. Remarkable differences can be seen between the two enzymes Rapidase C600 (Fig. 2c) and Ultra SP (Fig. 2d). The material retained after ultrafiltration when using Rapidase C600 has been studied in detail[22]. The authors also screened for enzymes able to degrade the material further and isolated a novel enzyme, rhamno-galacturonase, from the enzyme preparation Ultra SP[31].

Neutral arabinans.—Voragen et al.[20] used conventional gel permeation chromatography on a Sephacryl S500 column with water as eluent to separate a neutral arabinan fraction from the acidic polysaccharide. Using the HPSEC system described above and water as eluent, we were able to reproduce the results obtained on Sephacryl S500 material in only 45 min. Under these conditions uronide-containing polymers, making up more than 90% of the polysaccharides present in the sample, are excluded from the column matrix by charge effects and therefore elute in the column void. As shown in Fig. 3, a small fraction may elute at a retention time of 28-30 min ($M_w \pm 10\ 000$). Detection of acidic polysaccharides at 210 nm revealed that this fraction contained no uronides (result not shown).

From Fig. 3, it can also be seen that no neutral fraction is present in the ultrafiltered juice prepared by pulp enzyming using Hemi II (lines I); the same is true for material retained after ultrafiltration and in all straight pressed juices (not shown). Juices obtained with Rapidase C600 by the liquefaction process and then ultrafiltered were found to contain a neutral fraction in the fraction not retained (lines II) as well as in the retained fractions (lines IV). The same is true for juices prepared with Ultra SP by the liquefaction process (lines III). These neutral fractions eluting at 28-30 min were degraded by a pure endo-α-1,5-arabinanase
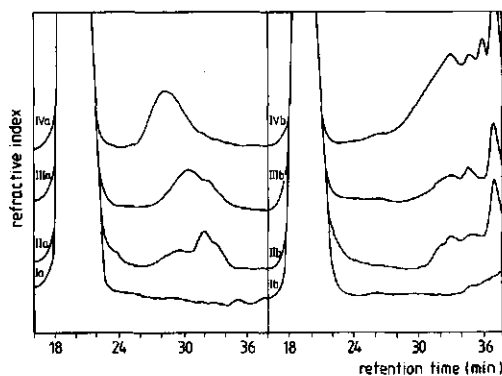

Fig. 3. Elution patterns of dialysates of juices and material retained after ultrafiltration using the HPSEC system with water as eluent, before (a) and after (b) endo-arabinanase treatment for 4 h at 40°C. Material not retained by ultrafiltration after pulp enzyming with Hemi II (I), liquefaction with Rapidase C600 (II) and liquefaction with Ultra SP (III), and material retained by ultrafiltration after liquefaction with Rapidase C600 (IV).

after incubation for 4 h at 40°C and therefore can be identified as (partly) linear α-1,5-linked arabinans. This is illustrated by Fig. 3 line II b, III b, and IV b respectively; the neutral sugar fractions eluting at 28-30 min have disappeared and elute at longer retention times (smaller M_w values). It is obvious that ultra filtration is not able to remove all of the neutral arabinan present in the juice. Also some differences in the elution patterns of the different liquefaction juices (lines II and III) can still be seen.

Conclusions

Juice recovery from apples can be increased by enzyme treatment of the apple mash, especially by the liquefaction process in which the cell wall is totally solubilized. Juices obtained by the various manufacturing processes differed in pH and total acid content depending on the enzyme treatment and storage time of the apples. The polyphenol content of straight pressed juices was found to be higher than that of juices obtained by pulp enzyming in which polyphenols were allowed to oxidize and condense on the pulp particles. In the case of liquefaction juices, the intensive enzyme treatment released polyphenols in to the juices so that the content was even higher then in straight pressed juices.

The content of polysaccharides solubilized in the juices increased when enzymes were used and significant differences between the different enzyme preparations were observed. Ultrafiltration was found to remove 50%, 50-65%, and 80-90% of the solubilized polysaccharides from the straight pressed juices, pulp enzyming and liquefaction juices respectively, depending on the enzyme preparation used.

The sugar composition of the solubilized cell wall material revealed that arabinose and galacturonic acid were the predominant sugars in the juices, even those retained by ultrafiltration. The uronide arabinose ratio, however, differed between the various juices: a ratio of 10 was found for ultrafiltered straight pressed juice and a ratio of 0.5 was found for conventionally clarified liquefaction juice (Rapidase C600).

Enzyme treatment increased the total amount of pectic material in the juice. Under liquefaction conditions, 60-90% of the uronides are of low molecular weight (removable by dialysis). From the amount of solubilized material, the sugar composition, and the percentage of dialysable uronide fragments, it can be concluded that the enzyme preparations used differed in their action pattern.

Most of the arabinose occurs in an associated form with pectic substances and can be removed by ultrafiltration. By HPSEC it was shown that most juices contained a neutral fraction ($M_w \pm 10\ 000$) composed predominantly of arabinose which is readily degraded using a endo-arabinanase. Only 40-70% of the neutral arabinan fraction can be removed by ultrafiltration. The acidic high-M_w material, rich in arabinose, and present particularly in liquefaction juice must be considered as a possible precursor of haze formation. This fraction can be largely removed from liquefaction juices by ultrafiltration but a rapid reduction in the flux, due to fouling of the membranes, was observed.

References

1   A.G.J. Voragen and W. Pilnik, *ACS Symp. Ser.*, 389 (1989) 23-247.
2   E. Steinbuch and W. van Deelen, *Confructa*, 30 (1986) 15-23.
3   W. Pilnik and L. de Vos, *Flüssiges Obst*, 37 (1970) 430-432.
4   P. Schreier, F. Drawert, G. Steiger, and W. Mick. *J. Food Sci.*, 43 (1978) 1797-1800.
5   M. Haug, F. Kielmeyer, and K. Gierschner, *Flüssiges Obst*, 49 (1982) 542-546.
6   P. Dürr, U. Schöbinger, and M. Zellweger, *Lebensm. Wiss. Technol.*, (1981) 81-85.
7   J. Weiss and H. Sämann, *Mitteilung Klosterneuburg*, 36 (1985) 48-53.
8   G. Baumann, B. Strobel, and K. Gierschner, *Flüssiges Obst*, 53 (1986) 246-261.
9   K. Vogt, *Flüssiges Obst*, 54 (1987) 425-429.
10 U. Schöbinger, *Flüssiges Obst*, 55 (1988) 614-620.
11 E. Rosch, *Confructa*, 29 (1985) 27-31.
12 Z.R. Yu, B.H. Chiang, and L.S. Gwang, *J. Food Sci.*, 51 (1986) 841-849.
13 M.A. Rao, T.E. Acree, H.J. Cooley, and R.W. Ennis, *J. Food Sci.*, 51 (1987) 375-377
14 A.G.J. Voragen, H.A. Schols, and W. Pilnik, *Z. Lebensm. Unters. Forsch.*, 187 (1988) 315-320.
15 P. Ducroo, *Flüssiges Obst*, 84 (1987) 265-269.
16 U. Schöbinger, P. Dürr, and R. Waldvogel, *Schweiz. Z. Obst und Weinbau*, 123 (1987) 678-683.
17 S.C. Churms, E.H. Merrifield, A.M. Stephen, D.R. Walwyn, A. Polson, K.J. van der Merwe, H.S.C. Spies, and N. Costa, *Carbohydr. Res.* 113 (1983) 339-344.
18 A.G.J. Voragen, F. Geerst, and W. Pilnik, in P. Dupuy (ed.) *Use of Enzymes in Food Technology*, Lavoisier, Paris, 1982, pp. 497-502.
19 A.G.J. Voragen, H. Wolters, T. Verdonschot-Kroef, F.M. Rombouts, and W. Pilnik, *Proc. Fruit Juice Symp. The Hague*, (1986) pp. 453-462.
20 A.G.J. Voragen, H.A. Schols, H.A.I. Siliha, and W. Pilnik, *ACS Symp. Ser.*, 310 (1986) 230-247.
21 A.G.J. Voragen, F.M. Rombouts, M.J.F. Searle-van Leeuwen, H.A. Schols, and W. Pilnik, *Food Hydrocolloids*, 1 (1987) 423-437.
22 H.A. Schols, M.A. Posthumus, and A.G.J. Voragen, *Carbohydr. Res.*, 206 (1990) 117-129 (this thesis, chapter 3).
23 M. Dubois, K.A. Gilles, J.K. Hamilton, D.A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350-356.
24 Internationale Fruchtsaft-Union, *IFU-Analysen* 27 (1965)
25 A.E. Ahmed and J.M. Labavitch, *J. Food Biochem.*, 1 (1977) 361-365.
26 P.K. Kintner and J.P. van Buren, *J. Food Sci.*, 47 (1982) 756-759.
27 T.M. Jones and P. Albersheim, *Plant Physiol.*, 49 (1972) 926-936.
28 A.G.J. Voragen, H.A. Schols, and W. Pilnik, *Food Hydrocolloids*, 1 (1986) 65-70.
29 J. Van Buren, L. De Vos, and W. Pilnik, *J. Agri. Food Chem.*, 24 (1976) 448-451.
30 A.G.J. Voragen, R. Heutink, and W. Pilnik .*J Appl. Biochem.*, 2 (1980) 452-468.
31 H.A. Schols, C.C.J.M. Geraeds, M.J.F. Searle-van Leeuwen, F.J.M. Kormelink, and A.G.J. Voragen, *Carbohydr. Res.*, 206 (1990) 105-115 (this thesis, chapter 4).

CHAPTER 3

STRUCTURAL FEATURES OF HAIRY REGIONS OF PECTINS ISOLATED FROM APPLE JUICE PRODUCED BY THE LIQUEFACTION PROCESS

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Abstract

A high molecular weight pectic fraction, released from the cell walls of apple tissue by pectolytic, hemicellulolytic, and cellulolytic enzymes, was isolated from apple juice prepared by the liquefaction process. The fraction, termed modified hairy regions (MHR), was characterized as a highly branched rhamnogalacturonan with arabinose-rich side chains and represented 0.26% of the fresh apple. Arabinose was the most abundant sugar (55 mol%) in MHR, which had a high rhamnose:galacturonic acid ratio (0.29) and degrees of methylation and acetylation (42% and 60%, respectively). The sugar and linkage compositions, distribution of molecular weights, susceptibility to β-elimination mediated by alkali and 4-methylmorpholine N-oxide, and the effect of various technical and pure enzymes on MHR and chemically and enzymically modified MHR have been studied. From the results and ^{13}C NMR data a tentative structure for MHR is proposed.

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Introduction

The polysaccharide composition and the structure of apple cell walls have been the subject of many investigations. Pectin, hemicellulose, and cellulose fractions have been obtained and characterized[1-4]. The changes in the cell wall composition at different stages of ripening of the apple have been studied[5-7]. Fractionation procedures including mild extraction with water, buffer, chelating agents, alkali, chlorite, and various enzymes have been used[3,8-10]. Much effort has been expended on linkage analysis of the cell wall-polysaccharides [3,11,12]. De Vries et al.[8,9] investigated the water-, acid-, and oxalate-soluble pectin fractions from apple cell walls and their degradation with pectate and pectin lyases. A new model was proposed in which the pectins were considered to contain homogalacturonan regions comprising 90% of the galacturonic acid residues and so-called "hairy" or ramified regions which contained most of the neutral sugars. These hairy regions are also present in pectins isolated from carrots[13] and the pulp of grape berries[14,15].

We now report on the characterization of a cell wall polysaccharide fraction isolated from the apple juice of Golden Delicious apples obtained by the so-called liquefaction process in which the juice is released from the apple pulp by the combined action of pectolytic and cellulolytic enzymes. This polysaccharide fraction, previously designated ultrafiltration retentate[16], resembled the hairy regions of apple pectin[9]. Since the enzyme preparation used in the liquefaction process contains various pectolytic, hemicellulolytic and cellulolytic activities, it is probable that the polysaccharide fraction was altered during extraction and therefore it has been renamed modified hairy regions (MHR).

Experimental

Isolation of MHR.—Golden Delicious apples (100 kg, harvest 1981 and 1985) were crushed in an Amos rasp mill (particle size 3.5 mm) and treated with an experimental enzyme preparation (Rapidase C600, 0.02%) from Gist Brocades (Delft, The Netherlands) for 4 h at 45°C. After centrifugation (Pennwalt Sharpless P600 Decanter) and aroma-stripping, the liquid was processed in an ultrafiltration tubular system equipped with a BX3 polysulfone membrane with a molecular weight cut-off of 60 000 (Paterson Candy Ltd.). The retentate was diluted with 60 L of water, ultrafiltered, the residue was dialyzed, centrifuged (15 000g, 20 min), and lyophilized.

The MHR-fraction represents 0.26% of the fresh apple and 1.68% of the apple solids.

Chemical treatment of MHR.—The methyl esters and O-acetyl groups in MHR, were saponified with 0.05 M NaOH (1.7% MHR, 0°C, 24 h), followed by dialysis and lyophilization, to yield MHR-S (91% of MHR).

The arabinan structures were removed by treatment with acid. The pH of a solution of MHR (1 g) in water (350 mL) was adjusted to 0.4 with aqueous 37% hydrochloric acid. The solution was heated for 2 h at 80°C, then dialyzed and freeze-dried to give MHR-HCl ($\pm$ 45% of MHR).

MHR was treated[17] with 4-methylmorpholine N-oxide for 0.5 and 4 h. Each mixture

was dialyzed against water and lyophilized. The yields of products were 78% and 66%, respectively.

Analytical methods.—Uronic acid was determined by the colorimetric *m*-hydroxybiphenyl assay[18]. Neutral sugars were determined by GLC after pretreatment (1 h, 30°C) with aqueous 72% H_2SO_4 followed by hydrolysis with M H_2SO_4 (3 h, 100°C) and conversion of the products into alditol acetates[19]. The alditol acetates were analyzed on a glass column (3 m x 2 mm i.d.), packed with Chrom WAW 80-100 mesh coated with 3% OV275 in a Carlo Erba Fractovap 2300 GC.

Methanolysis (24 h, 85°C) with methanolic M HCl followed by trimethylsilylation of the resulting methyl glycosides[20] was performed in order to identify uronic acid residues and to confirm the absence of fucose. Methanolysis was also used to check the complete liberation of all sugar residues by hydrolysis with M H_2SO_4 (3 h, 100°C) or with 2 M trifluoroacetic acid (1 h, 121°C). No differences between these methods were observed.

Linkage analysis was established by methylation (Hakomori[21]), followed by hydrolysis with 2 M trifluoroacetic acid, and conversion[22] of the products into partially methylated alditol acetates. Carboxyl reduction was effected by the method of Taylor and Conrad[23]. After three treatments, >90% of the uronic acids had been reduced (*m*-hydroxybiphenyl assay). The products of methylation analysis of the polysaccharides were identified and quantified by capillary GLC and GLC-MS. For capillary GLC, a wall-coated OV-225 column (10 m x 0.32 mm; 0.23 μm layer) and a Carlo-Erba Fractovap 4160 gas chromatograph were used with the temperature programme 150°C for 1 min, 150°→200°C at 2°C/min, 200°C for 2 min. GLC-MS was performed with a VG MM 7070F mass spectrometer coupled to a Pye 204 GC equipped with a packed column (1.5 m) of 3% OV-225 on Chromosorb WHP. In order to differentiate 2- and 4-substituted xylose residues, reduction to alditols was also carried out with sodium borodeuteride.

The degrees of methylation and acetylation were determined[24] by HPLC.

Size-exclusion chromatography.—A solution of MHR (50-100 mg), dissolved in water (1-3 mL) was applied to a column (100 x 2.2 cm) of Sephacryl S500 (Pharmacia; separation range for dextrans: 4 x 10^4-2 x 10^7 Da) or a column (100 x 2.6 cm) of Sephacryl S200 (separation range for dextrans 10^3-8 x 10^4 Da) and eluted with a 0.05 M sodium phosphate buffer (pH 7.0). Fractions were assayed by automated methods[25,26] for uronic acid and total neutral sugars. Neutral sugar values (as "anhydroarabinose") were corrected for the contribution of the uronic acid in the orcinol assay.

High-performance size-exclusion chromatography (HPSEC) was performed on a SP8800 HPLC (Spectra Physics) equipped with three Bio-Gel TSK columns (each 300 x 7.5 mm) in series (40XL, 30XL, and 20XL; Bio-Rad Labs) in combination with a TSK XL guard column (40 x 6 mm) and elution at 30°C with 0.4 M acetic acid/sodium acetate (pH 3.0) at 0.8 mL/min. The eluate was monitored using a Shodex SE-61 Refractive Index detector. The system was calibrated with pectins with molecular weights in the range 10000-100 000 (as determined by viscosimetry) and oligomers obtained after degradation of polygalacturonic acid by yeast endo-polygalacturonase[27]. The software was obtained from Spectra Physics.

NMR spectroscopy.—The ^{13}C NMR spectra were obtained with Jeol GX-400 (100.4

MHz for MHR-HCl) and Bruker CXP 300 spectrometers (75 MHz for MHR) at 90°C in 3% solutions in D_2O (internal acetone; 31.55 ppm relative to Me_4Si). Data were acquired with broad-band decoupling and acquisition times of 0.65 s and 69 283 transients (MHR-HCl) and 0.65 s and 5724 transients (MHR).

Enzymic hydrolysis.—0.2% Solutions of MHR and MHR-S in 0.05 M sodium acetate buffer (pH 5.0) were incubated with 3.33 nkats of enzyme/ml for 4 h at 30°C. Treatment with pectate lyase was carried out in 0.1 M glycine buffer (pH 9.4) containing 0.5 mM of $CaCl_2$. The activities of the hydrolases were measured by the increase in the reducing end groups[28]. Lyase action was determined[29] by the increase in absorbance at 235 nm. The following enzymes were used: two endo-$(1\rightarrow4)$-β-glucanases (EC 3.2.1.4; a specific glucanase active only on cellulose substrates and a non-specific glucanase that was also active on xylans[30]), endo-$(1\rightarrow4)$-β-galactanase (EC 3.2.1.89), endo-polygalacturonase (EC 3.2.1.15) of fungal and yeast origin, endo-pectate lyase (EC 3.2.2.2), endo-pectin lyase (EC 4.2.2.10), and fungal pectin methylesterase (EC 3.1.1.11).

Enzymic degradation of MHR by arabinanases was performed as follows. A solution of MHR (1 g) in 0.1 M sodium acetate (pH 5.0; 70 mL) was incubated with 1620 nkats of α-L-arabinofuranosidase type B (EC 3.2.1.55, activity measured against p-nitrophenyl-α-L-arabinofuranoside) and 12 nkats endo-$(1\rightarrow5)$-α-L-arabinanase (EC 3.2.1.99, activity measured against linear α-$(1\rightarrow5)$-L-arabinan[31]) for 48 h at 35°C. After dialysis and freeze-drying, 45% of MHR was recovered as MHR-ARA.

The effect on MHR of 45 commercial enzyme preparations involved incubation of 0.5% solutions in 0.05 M sodium acetate buffer (pH 5.0) with 0.01% of the enzyme preparation. Digests were analyzed by HPSEC.

Results and discussion

Characterization of MHR.—A fraction (MHR, modified hairy regions), that represented 0.26% of the fresh apple, was isolated and appeared to be a high-molecular-weight polysaccharide that was resistant to further degradation by the liquefying enzyme preparation. The sugar compositions of MHR and modified MHR are shown in Table I. MHR contained mainly arabinose but was also rich in rhamnose, xylose, galactose, and galacturonic acid. The absence of glucuronic acid residues was proved by methanolysis and analysis of the resulting methyl glycosides after trimethylsilylation. This analysis also confirmed that rhamnose was the only 6-deoxyhexose present. The rhamnose:galacturonic acid ratio of 0.29 was high in comparison with those of other apple pectin fractions (0.029 and 0.038, respectively, for water- and oxalate-soluble pectin fractions[3] and 0.004-0.026 for cold-buffer-soluble pectin fragments[8]). High rhamnose:galacturonic acid ratios have been reported for enzyme-treated pectin fractions of apples[9] (0.22), carrots[13] (1.0), and grape berries[15] (0.8).

The sugar composition revealed that MHR polysaccharide resembled the so-called "hairy regions" as described by de Vries et al.[9] (rhamnose:galacturonic acid ratio 0.22, arabinose 50%, xylose 5%, galactose 13%, rhamnose 5% and galacturonic acid 23%).

TABLE I. Sugar composition (mol%) of modified MHR

Sugar	MHR	MHR-S	MHR-HCl	MHR-ARA
Rha	6 (5.0) *	6 (4.3)	13 (4.0)	16 (3.7)
Ara	55 (42.6)	55(35.3)	5 (1.3)	7 (1.5)
Xyl	8 (5.7)	7 (4.6)	18 (4.8)	8 (1.7)
Man	0 (0)	0 (0)	0 (0)	2 (0)
Gai	9 (9.7)	10 (8.5)	19 (6.4)	15 (4.0)
Glc	1 (0.6)	1 (0.7)	1 (0.3)	0 (0)
GalA	21 (19.6)	21(18.6)	44 (16.2)	52(15.7)
OMe	42 (1.5)	0 (0)	33 (1.0)	14 (0.4)
OAc	60 (4.0)	0 (0)	10 (0.6)	65 (3.5)
Total sugars	83%	79%	73%	60%
Rha:GalA	0.29	0.29	0.30	0.31

* Gram quantities per residue in the MHR substrates originating from 100 grams of MHR.

However, MHR contained less methyl esters, and the presence of acetyl groups was not reported by De Vries et al.[9] MHR was isolated after treatment of the entire cell wall with various polysaccharide-degrading enzymes present in the experimental enzyme preparation used. De Vries et al.[9] obtained their hairy regions by extraction of a pectin fraction under mild conditions followed by degradation with a pure pectate lyase.

The relatively large proportion of MHR isolated, indicated that it may originate from the soluble pectins from the apple cell wall and the insoluble proto-pectin fraction. Voragen et al.[32] reported that the concentration of uronic acids in liquefaction juice increased 7 times as compared to juice obtained by conventional pressing, whereas the arabinan content was 20 times higher[16]. These data illustrate the degradation and solubilization of insoluble cell wall polysaccharides by the action of enzymes in the liquefaction process. MHR could be isolated in 100 g quantities whereas De Vries et al.[9] obtained only mg quantities of the hairy regions. Arabinan-rich pectic hairy regions, isolated after treatment of cell walls with pectolytic enzymes, have been described for carrot cell walls[13] (40 mol% arabinose) and grape berries[14,15] (30-50 mol% arabinose).

McNeil et al.[33,34] and Lau et al.[35] have described rhamnogalacturonan I, isolated from suspension-cultured sycamore cells, that had a rhamnose:galacturonic acid ratio of 0.5 and a backbone that consisted partly of alternating rhamnose and galacturonic acid residues.

No protein or polyphenols were detected in MHR and, based on the uronic acid content, a degree of methylation of 35% was calculated. The degree of acetylation was 60% and such a value has not been reported hitherto for apple pectin fractions; commercial apple pectins have values of 3-4%[24].

The methyl esters and O-acetyl groups could be saponified to give MHR-S without

changing the sugar composition (Table I) and with only a slight loss of sugars (max 10%).

Exhaustive treatment of MHR with a combination of endo-arabinanase and arabinofuro-nosidase B removed most of the arabinose without loss of acetyl groups (Table I, MHR-ARA). Also, 70% of the xylose residues and 60% of the galactose residues could be removed enzymically. These results indicated that the acetyl groups were linked to the galacturonosyl residues. MHR-ARA also had a decreased methoxyl content. The low sugar content can be explained by the addition of large amounts of enzyme protein (40 mg) which may account for 10-15% of this fraction. Arabinose could also be removed from MHR by treatment with hydrochloric acid. The product (MHR-HCl) had a low content of arabinose, but 75% of all the other sugars were retarded. The degree of methylation was reduced by 33% and only 15% of the acetyl groups survived.

The molecular weight and the homogeneity of MHR were determined using HPSEC (Fig. 1). MHR consisted of three distinct populations (A-C) with different molecular weights, which were isolated by fractionation (Sephacryl S500 and S200). Data on these fractions are shown in Table II. The rhamnose:galacturonic acid ratios for A-C were 0.21, 0.35, and 0.30, respectively, and there were differences in the xylose and methoxyl contents. Although variations were observed in the sugar composition (minor) and hydrodynamic volumes (major), MHR was considered to be a mixture of similar polysaccharides.

However, by elution from Sephacryl S500 with water, Voragen et al.[32] isolated a neutral fraction that comprised 6-7% of the MHR and which was identified as an arabinan (degree of branching 18%). Since our investigations were concerned with the structure of the backbone of MHR and since this arabinan did not interfere in the chemical and enzymic analyses, it was not removed. This arabinan is not present in MHR-ARA and
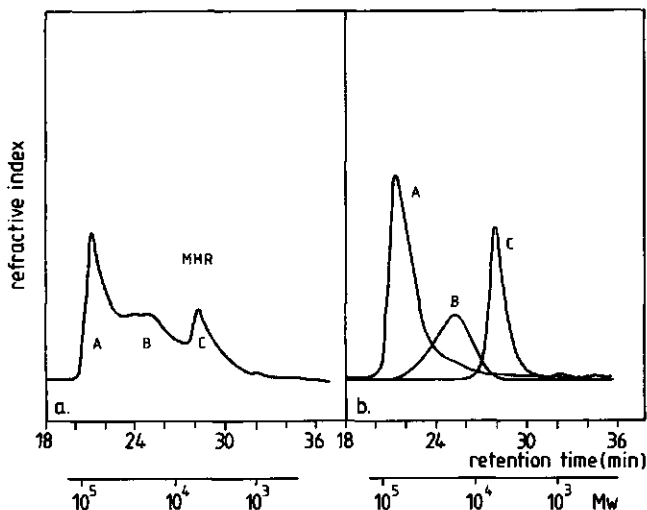

Fig. 1.   High-performance size-exclusion chromatography of (a) MHR, (b) the fractions of MHR isolated by chromatography over Sephacryl S200 and S500

TABLE II. Sugar composition (mol%) of the fractions of MHR isolated by chromatography over Sephacryl S200 and S500

Sugar	MHR	A	B	C
Rha	6	5	6	10
Ara	55	50	59	47
Xyl	8	11	5	3
Man	0	0	0	0
Gal	9	10	13	7
Glu	1	0	0	0
GalA	21	24	17	33
OMe	42	28	84	100
OAc	60	55	57	21
Rha:GalA	0.29	0.21	0.35	0.30

MHR-HCl.

Table III shows the results of the methylation analyses of MHR and MHR-HCl after reduction of the galacturonic acid residues. The MHR-associated arabinans were less branched (27%) than arabinans (40-45%) isolated from apple cell walls that had not been treated with enzyme[16].

Methylation analyses of MHR showed the rhamnose residues to be branched at C-4. However, only 73% of the rhamnose residues were recovered as methylated alditol acetates. Of the xylose residues, 65% were terminal and the remainder were (1→4)-linked. Almost 50% of the galactose units were (1→4), (1→3), (1→6), or (1→3,6)-linked and 45% were terminal. The high proportion (45%) of terminal galactose residues compared to that (4-5%) in the hairy regions reported by De Vries et al.[12] indicates that, due to endo-galactanase activity in the liquefaction process, longer galactan sequences are removed. The residual short side chains were not degraded by the galactanases.

All of the galacturonosyl residues were (1→4)-α-linked with 20% branched at C-3. The high proportion (6.5%) of terminal xylose units cannot be explained by the presence of a highly branched xylan. No xyloglucans were present (no glucose derivatives were detected in the methylation analysis). Part of the terminal xylose residues might be accounted for by the presence of a rhamnogalacturonan that carries side chains of single xylose units as described by De Vries et al.[12,43]. Methylation analysis of MHR-HCl revealed only 50% of the rhamnose residues to be branched at C-4 and all xylose residues to be terminal. However, the recovery of the sugar residues in the methylation analysis (especially of xylose and galacturonic acid) was not in full agreement with the sugar composition as analyzed as alditol acetates. Methylation analysis of MHR-ARA did not give trustworthy results.

The ^{13}C NMR spectrum of MHR (Fig. 2a) resembled those of branched arabinans. The

TABLE III. Glycosidic linkage composition of MHR and MHR-HCl

Sugar residue		Glycosidic linkage composition[a]	
		MHR	MHR-HCl
Rhamnose	1,2-Rha$p$[b]	-	14.3
	1,2,4-Rha$p$	4.4	15.0
		(4.4%)	29.3%
Arabinose	T-Ara$f$	10.7	3.7
	1,2-Ara$f$	0.4	-
	1,3-Ara$f$	6.3	-
	1,5-Ara$f$	30.8	1.9
	1,3,5-Ara$f$	8.8	-
	1,2,5-Ara$f$	1.9	-
	1,2,3,5-Ara$f$	3.0	
		(61.9%)	(5.6%)
Xylose	T-Xyl$p$	6.5	6.1
	1,4-Xyl$p$	2.9	-
	1,2-Xyl$p$	0.3	-
		(9.7%)	(6.1%)
Galactose	T-Gal$p$	3.6	16.2
	1,3-Gal$p$	1.5	1.4
	1,4-Gal$p$	1.1	3.8
	1,6-Gal$p$	0.3	4.8
	1,2,4-Gal$p$	0.3	-
	1,3,6-Gal$p$	1.1	2.3
	1,2,3,4-Gal$p$	-	1.1
		(7.9%)	(29.6%)
Galacturonic acid[c]	1,4-Gal$p$A	12.8	24.2
	1,3,4-Gal$p$A	3.2	4.9
		(16%)	(29.1%)
ratio terminal / branching		0.98	1.07

[a] Linkage types in mol%; the figures after methylation analysis are given in brackets.
[b] 1,2-linked Rha etc. T connotes a terminal group. [c] After carboxyl reduction.

arabinose side chains (55% of the total sugar residues) were highly flexible and gave strong signals, the chemical shifts of which were in agreement with data reported[36]. The backbone structure, consisting of galacturonic acid and rhamnose, was rigid, which resulted in the broadening and weakening of signals. This effect has been reported for pectic substances[37,38]. The signals of the backbone are more pronounced in the ^{13}C NMR spectrum of MHR-HCl (Fig. 2b) since there were no signals for arabinose. The peak at 53.73 ppm is characteristic for the COOMe group of galacturonate residues[38] and there were no peaks for acetyl groups. The signals for C-6 in the region 16-18 ppm revealed
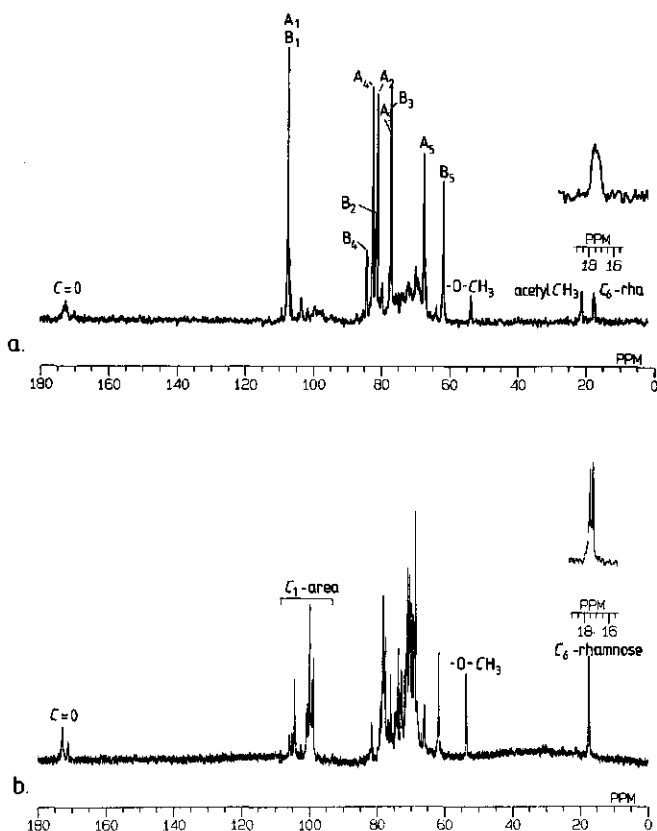
Fig. 2. ^{13}C NMR spectra of (a) MHR and (b) MHR-HCl. A (1→5)-α-Araf: 108.82 (C-1), 82.14 (C-2), 78.05 (C-3), 83.63 (C-4), 68.20 (C5) ; B Araf: 108.78 (C-1), 82.57 (C-2), 77.88 (C-3), 85.33 (C-4), 62.50 ppm (C-5).

two differently linked rhamnose units. In general, ^{13}C NMR spectra of polysaccharides are difficult to assign completely.

MHR was fairly resistant to base-catalysed β-elimination under the conditions described by Thibault[39], but was degraded on treatment[17] with 4-methylmorpholine N-oxide. Table IV shows the recovery of the sugars, methyl esters, and acetyl groups. After treatment for 0.5 h, ±80% of the sugar residues were recovered but, after 4 h, xylose, galacturonic acid, and methyl esters in particular were lost, probably during dialysis. These observations confirm the presence of xylogalacturonan regions and that demethylation proceeds faster than deacetylation.

Enzymic degradation of MHR.—MHR was resistant (Table V) to most pectic enzymes and specific and non-specific endo-(1→4)-β-D-glucanases. Fungal endo-(1→4)-poly-α-D-galacturonase cleaved ±6% of the galacturonosyl linkages. Pectin esterase was not able to

TABLE IV. Recovery of the sugar residues, methyl esters, and acetyl groups
of MHR after treatment with 4-methylmorpholine N-oxide

Sugar residue	Recovery (%) after treatment.	
	0.5 h	4 h
Rha	83	77
Ara	80	77
Xyl	67	18
Gal	77	77
GalA	73	38
OMe	55	0
OAc	80	57

make MHR a more suitable substrate for either fungal or yeast poly-galacturonases. Endo-$(1\rightarrow4)$-β-D-galactanase hydrolyzed 10% of the galactosyl linkages. Mild saponification of MHR gave MHR-S which was degraded slightly be endo-polygalacturonase. Of 45 different commercial enzyme preparations, only five could degrade MHR to some extent, e.g. Hemicellulase Reg. II, Gist Brocades (Fig. 3, enzyme A). Arabinanases were responsible for this degradation, since the presence of arabinose and its oligomers was established by HPLC according to Voragen et al.[40] These five enzyme preparations were active towards linear and branched arabinans but not towards MHR-HCl and MHR-ARA. Arabinofuranosidase activity was observed by the release of small amounts of arabinose monomer when MHR was incubated with Rapidase C600. The lack of arabinose oligomers after incubation with MHR or pure linear arabinan indicated that Rapidase C600 had poor endo-arabinanase activity. Incubation with polysaccharides isolated from plant cell walls revealed that this enzyme mixture contained a wide spectrum of pectolytic and cellulolytic enzymes in addition to endo-xylanases and endo-galactanases.

HPSEC of MHR after treatment with enzyme A (Fig. 3), showed an almost identical behaviour of the MHR backbone that remained after removal of the arabinan residues which make up >50% of MHR. This phenomenon can be explained by assuming a minor change in hydrodynamic volume of MHR after removal of 50% of its constituent sugars. Only one of the enzyme preparations (Ultra SP, Novo Ferment AG) degraded the rhamnogalacturonan backbone of MHR (Fig. 3, enzyme B). Most of the molecules of high molecular weight were degraded to oligomeric fragments but some were resistant to enzyme B. A novel enzyme was isolated from the crude enzyme preparation B which catalysed the degradation of the backbone of MHR (see following paper[41]).

Although a heterogeneous molecular-weight was established for MHR, a possible structure is proposed (Fig. 4). The presence of a region with alternating rhamnose and galacturonic acid residues was demonstrated by ^{13}C NMR spectroscopy of tetra-, penta-
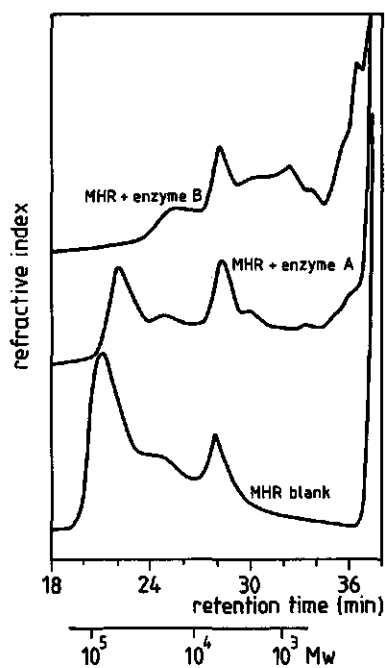
Fig. 3. High-performance size-exclusion chromatography of MHR, before and after degradation with commercial enzyme preparations: A, Hemicellulase Reg II; B, Ultra Sp.

TABLE V. Degradation of MHR by various purified enzymes during 4 h at 30°C

Enzyme	Degradation (%)	
	MHR	MHR-S
Pectin esterase (PE)	0	0
Yeast Polygalacturonase(PG)	0	3.4 [a]
Fungal Polygalacturonase	6.0	14.5
Yeast PG + PE	0	3.1
Fungal PG + PE	6.6	13.8
Pectate lyase	2.3	4.6
Pectin lyase	0	0
Endo-galactanase	10.3 [b]	17.4
Cellulase/xylanase	0	0

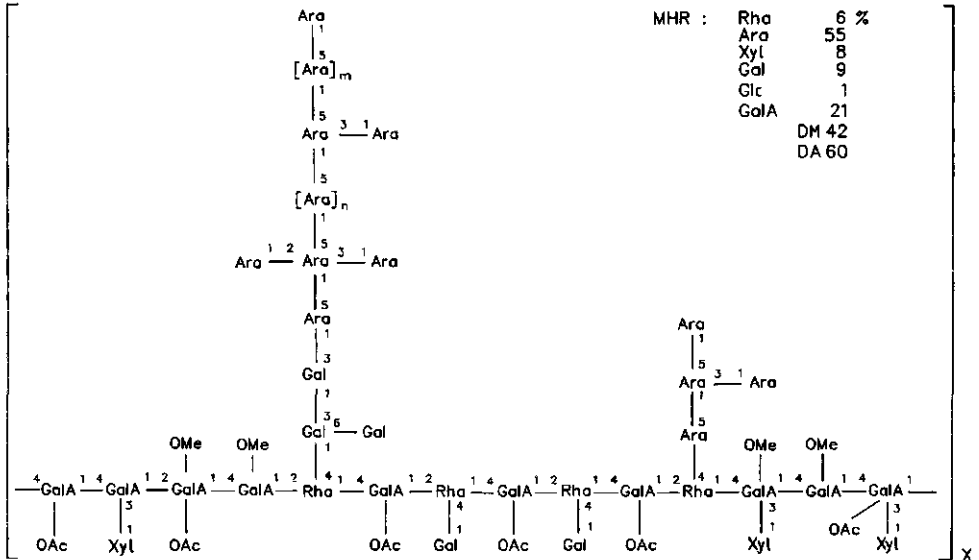[a] GalA-linkages split. [b] Gal-linkages split.

Fig. 4   Possible structure of MHR.

and hexasaccharide fragments produced with the above novel enzyme[42].

The position of the *O*-acetyl groups at C-2 and/or C-3 of the galacturonic acid was confirmed by the enzymic degradation studies with rhamnogalacturonase as described in the following paper[41]. Most of the xylose residues were linked to the galacturonic acid backbone and almost all of the rhamnose residues were branched with either galactose or arabinose side chains. These arabinan side chains were (1→5)-α-linked with a degree of branching which was ±50% lower than that of native apple arabinans[16]. It is recognized that structures other than that in Fig. 4 are possible. It is concluded that the complex hairy regions are composed of different repeating units as earlier stated by De Vries[43].

Acknowledgments

References

1   A.G.J. Voragen, J.P.J. Timmers, J.P.H. Linssen, H.A.Schols, and W. Pilnik, *Z. Lebensm. Untersuch. Forsch.*, 174 (1983) 251-256.
2   G.O. Aspinall and K. Fanous, *Carbohydr. Polym.*, 4 (1984) 193-214.
3   B.H.J. Stevens and R.R. Selvendran, *Carbohydr. Res.*, 135 (1984) 155-166.
4   M. Knee, *Phytochemistry*, 12 (1973) 637-653.
5   M. Knee, *Phytochemistry*, 12 (1973) 1543-1549.
6   M. Knee, A.H. Fielding, S.A. Archar and F. Laborda, *Phytochemistry*, 14 (1975) 2213-2222.
7   J.A. De Vries, A.G.J. Voragen, F.M. Rombouts, and W. Pilnik, *Carbohydr. Polym.*, 4 (1984) 3-13.
8   J.A. De Vries, A.G.J. Voragen, F.M. Rombouts, and W. Pilnik, *Carbohydr. Polym.*, 1 (1981) 117-127.
9   J.A. De Vries, F.M. Rombouts, A.G.J. Voragen, and W. Pilnik, *Carbohydr. Polym.*, 2 (1982) 25-33.
10 C.M.G.C. Renard, A.G.J. Voragen, J.-F. Thibault, and W.Pilnik, *Carbohydr. Polym.*, 12 (1989) 9-25.
11 A.G.J. Voragen, H.A. Schols, and W. Pilnik, *Z. Lebensm. Unters. Forsch.* 83 (1986) 105-110.
12 J.A. De Vries, C.H. den Uijl, A.G.J. Voragen, F.M. Rombouts, and W.Pilnik, *Carbohydr. Polym.*, 3 (1983) 193-205.
13 H. Konno, Y. Yamasaki, and K. Katoh, *Phytochemistry*, 25 (1986) 623-627.
14 L. Saulnier and J.-F. Thibault, *Carbohydr. Polym.*, 7 (1987) 345-360.
15 L. Saulnier, J.-M. Brillouet, and J.-P. Joseleau, *Carbohydr. Res.*, 182 (1988) 63-78.
16 A.G.J. Voragen, F.M. Rombouts, M.J.F. Searle-van Leeuwen, H.A. Schols, and W. Pilnik, *Food Hydrocolloids*, 1 (1987) 423-437.
17 J.-P. Joseleau, F. Chambat, and B. Cumpitaze-Hermoza, *Carbohydr. Res.*, 90 (1981) 339-344.
18 A.E. Ahmed and J.M. Labavitch, *J. Food Biochem.*, 1 (1977) 361-365.
19 H.N. Englyst and J.H. Cummings, *Analyst*, 109 (1984) 939-942.
20 G.J. Gerwig, J.P. Kamerling, and J.F.G. Vliegenthart, *Carbohydr. Res.*, 129 (1984) 149-157.
21 S. Hakomori, *J. Biochem.*, 55 (1964) 205-208.
22 K.W. Talmadge, K. Keegstra, W.D. Bauer, and P.Albersheim, *Plant Physiol.*, 51 (1973) 158-173.
23 D. Taylor and H.E. Conrad, *Biochemistry*, 11 (1972) 1383-1388.
24 A.G.J. Voragen, H.A. Schols, and W. Pilnik, *Food Hydrocolloids*, 1 (1986) 65-70.
25 J.-F. Thibault, *Lebensm. Wiss. Technol.*, 12 (1979) 247-251.
26 M. Tollier and J. Robin, *Ann. Technol. Agric.*, 28 (1979) 1-15.
27 A.G.J. Voragen, H.A. Schols, J.A. De Vries, and W. Pilnik, *J. Chromatogr.*, 244 (1982) 327-336.
28 R.G. Spiro, *Methods Enzymol.*, 8 (1966) 3-26.
29 F.M. Rombouts and J.-F. Thibault, *Carbohydr. Res.*, 154 (1986) 189-203.
30 G. Beldman, A.G.J. Voragen, F.M. Rombouts, M.J.F. Searle-van Leeuwen, and W. Pilnik, *Biotechnol. Bioeng.*, 31 (1988) 160-167.
31 F.M. Rombouts, A.G.J. Voragen, M.J.F. Searle-van Leeuwen, C.C.J.M. Geraeds, H.A. Schols, and W. Pilnik, *Carbohydr. Polym.*, 9 (1988) 25-47.
32 A.G.J. Voragen, H.A. Schols, H.A.I. Siliha, and W. Pilnik, *ACS Symp. Ser.*, 310 (1986) 230-247.
33 M. McNeil, A.G. Darvill, and P. Albersheim, *Plant Physiol.*, 66 (1980) 1128-1134.
34 M. McNeil, A.G. Darvill, and P. Albersheim, *Plant Physiol.*, 70 (1982) 1586-1591.
35 J.M. Lau, M. McNeil, A.G. Darvill, and P. Albersheim, *Carbohydr. Res.*, 137 (1985) 111-125.
36 P. Ryden, I.J. Colquhoun, and R.R. Selvendran, *Carbohydr. Res.*, 185 (1989) 233-237.
37 R. Pressey and D.S. Himmelsbach, *Carbohydr. Res.*, 127 (1984) 356-359.
38 M.H.J. Keenan, P.S. Belton, J.A. Matthew, and S.J. Howson, *Carbohydr. Res.*, 138 (1985) 168-170.
39 J.-F. Thibault, *Phytochemistry*, 22 (1983) 1567-1571.
40 A.G.J. Voragen, H.A. Schols, M.J.F. Searle-van Leeuwen, G. Beldman, and F.M. Rombouts, *J.*

Chromatogr., 370 (1986) 113-120.

41   H.A. Schols, C.C.J.M. Geraeds, M.J.F. Searle-van Leeuwen, F.J.M. Kormelink, and A.G.J. Voragen, *Carbohydr. Res.*, 206 (1990) 105-115 (this thesis, chapter 4).

42   I.J. Colquhoun, G.A. De Ruiter, H.A. Schols, and A.G.J. Voragen. *Carbohydr. Res.*, 206 (1990) 131-144 (this thesis, chapter 5).

43   J.A. De Vries, in G.O. Philips, D.J. Wedlock, and P.A. Williams (Eds), *Gums and Stabilizers for the Food Industry 4*, IRL Press, Oxford, 1988, pp. 25-29.

CHAPTER 4

RHAMNOGALACTURONASE: A NOVEL ENZYME THAT DEGRADES THE HAIRY REGIONS OF PECTINS

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F.J.M. Kormelink, and A.G.J. Voragen

Abstract

A rhamnogalacturonase (RGase), that could degrade the modified hairy regions (MHR) prepared from apple cell walls, was isolated and purified from a technical preparation of *Aspergillus aculeatus*. The RGase cleaved galactopyranosyluronic-rhamnopyranosyl linkages. No activity was observed towards other cell wall polysaccharides or p-nitrophenyl glycosides. The optimal conditions for RGase were pH 3-4 and 40-50°C. MHR was degraded by RGase and methylation analysis and ^{13}C NMR spectroscopy indicated the products to have a tetrasaccharide backbone of alternating rhamnose and galacturonic acid residues. Some oligomers had a galactose residue (1→4)-linked to rhamnose. The potential value of this new enzyme is considered.

Introduction

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

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

Experimental

Substrates.—Beet arabinan and xylan from oat spelts were purchased from Koch-Light, $(1\rightarrow5)$-α-L-arabinan (haze arabinan) was isolated from turbid apple juice concentrate[12,13], CM-cellulose (Akucell AF 0305) was obtained from Akzo (Arnhem), polygalacturonic acid was purchased from ICN (Cleveland), high-methoxyl pectin (degree of methylation 90%) was prepared in our laboratory[14], and galactan was isolated from potato fibre (obtained from Avebe) according to the modified method of Labavitch et al.[15] as described by Rombouts et al.[16]

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

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

Enzyme assays.—The Nelson-Somogyi assay[17] to measure the increase in reducing end groups did not have the sensitivity needed and high-performance size-exclusion chromatography (HPSEC)[11] was the only method suitable for measuring the action of the enzyme by monitoring the change in the molecular weight distribution.

Enzyme fractions were screened for contaminating activities by measuring the increase in the reducing end groups[17] after incubation of the enzyme fractions for 1 h at 30°C with a

0.1% solution of the substrate in 0.05 M sodium acetate buffer (pH 5.0). The digests were analyzed by HPSEC.

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

Purification of the enzyme.—Fractionation of the crude enzyme preparation included chromatography on Bio-Gel P10 (100-200 mesh), DEAE Bio-Gel A, and Bio-Gel HTP (Bio-Rad). Gradient elution was performed with a LKB 11300 Ultrograd Gradient Mixer with the sensor set at an A_{280} value of 0.01.

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

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

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

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

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

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

NMR spectroscopy.—The ^{13}C and ^{1}H NMR spectra were recorded and interpreted as described.[22].

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

Results and discussion

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

Enzyme purification.—The scheme for the isolation of RGase from Ultra SP is shown in Fig. 1 and the corresponding elution patterns are shown in Fig. 2. Ultra Sp was first desalted on Bio-Gel P10 and the fractions which were active against MHR were combined and applied to a column of DEAE Bio-Gel A. Elution was performed with the gradient mixer controlled by the level sensor (A_{280}) set at 0.01 which made it possible to elute protein peaks with a minimum of contamination. In this step, part of the polygalacturonase activity could be separated from the RGase fraction. The fractions that contained the RGase activity were
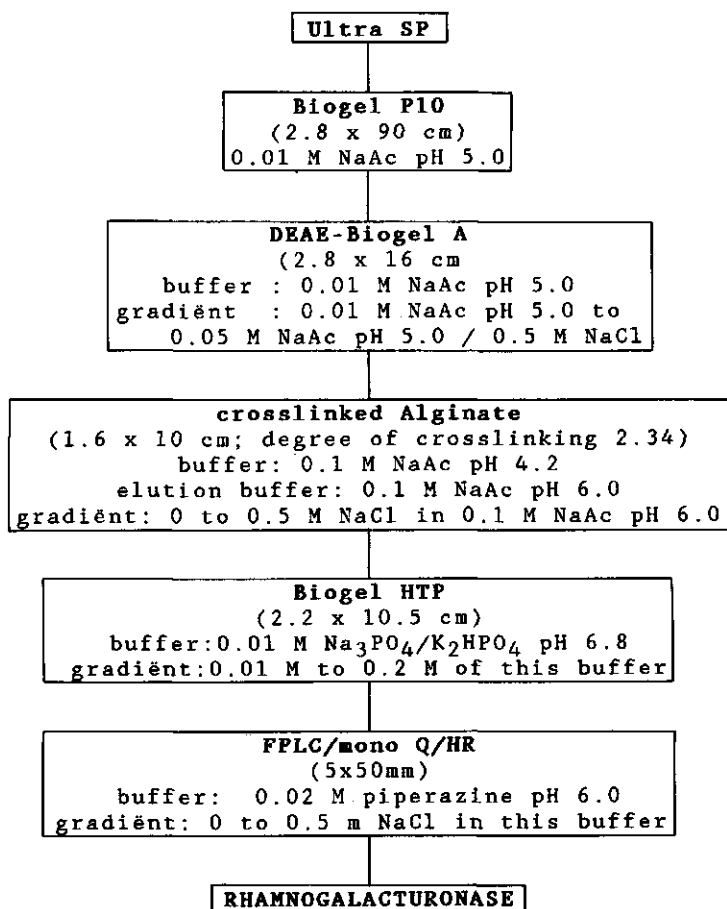
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                    ┌──────────────┐
                    │  Ultra SP    │
                    └──────────────┘
                            │
            ┌───────────────────────────────┐
            │         Biogel  P10            │
            │       (2.8  x  90  cm)         │
            │   0.01 M NaAc pH 5.0           │
            └───────────────────────────────┘
                            │
        ┌─────────────────────────────────────────────┐
        │             DEAE-Biogel A                    │
        │             (2.8 x 16 cm                     │
        │   buffer  :  0.01 M NaAc pH 5.0              │
        │   gradiënt :  0.01 M NaAc pH 5.0 to          │
        │      0.05 M NaAc pH 5.0 / 0.5 M NaCl         │
        └─────────────────────────────────────────────┘
                            │
    ┌─────────────────────────────────────────────────────┐
    │              crosslinked Alginate                    │
    │ (1.6 x 10 cm; degree of crosslinking 2.34)           │
    │          buffer: 0.1 M NaAc pH 4.2                   │
    │      elution buffer: 0.1 M NaAc pH 6.0              │
    │ gradiënt: 0 to 0.5 M NaCl in 0.1 M NaAc pH 6.0      │
    └─────────────────────────────────────────────────────┘
                            │
        ┌─────────────────────────────────────────────┐
        │             Biogel HTP                       │
        │           (2.2 x 10.5 cm)                    │
        │ buffer:0.01 M Na3PO4/K2HPO4 pH 6.8           │
        │ gradiënt:0.01 M to 0.2 M of this buffer      │
        └─────────────────────────────────────────────┘
                            │
        ┌─────────────────────────────────────────────┐
        │             FPLC/mono Q/HR                   │
        │             (5x50mm)                         │
        │ buffer:   0.02 M piperazine pH 6.0           │
        │ gradiënt: 0 to 0.5 m NaCl in this buffer     │
        └─────────────────────────────────────────────┘
                            │
            ┌───────────────────────────────┐
            │     RHAMNOGALACTURONASE       │
            └───────────────────────────────┘
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Fig. 1.   Scheme for the isolation of RGase from a crude *Aspergillus aculeatus* preparation (Ultra SP); (0.01 % NaN_3 was added as buffer preservative in all steps).
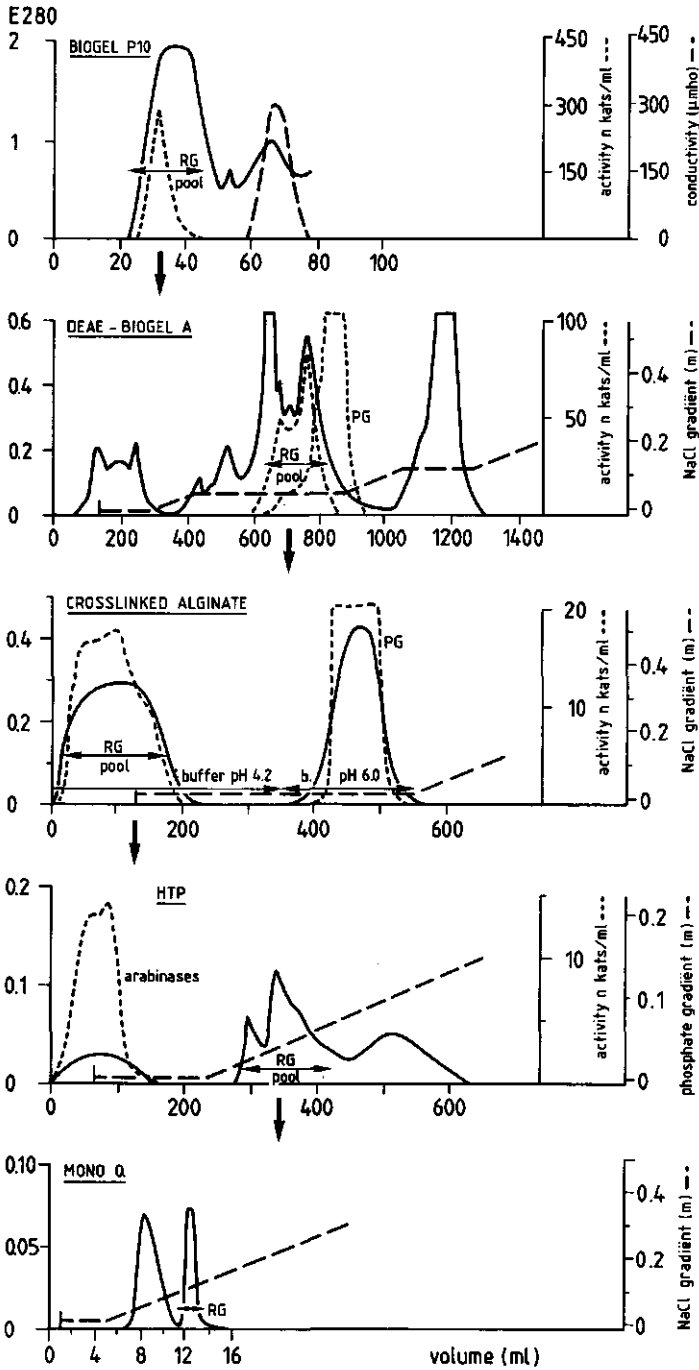
Fig. 2. Elution patterns on various colums used in Fig. 1 for the purification of RGase (RG).

combined and dialyzed against 0.1 M sodium acetate (pH 4.2). The remaining poly-galacturonase activity was removed by using a column of cross-linked alginate. The combined fractions that contained the MHR-degrading activity were dialyzed against sodium phosphate (pH 6.8) and applied to a column of Bio-Gel HTP. The first peak was active against MHR when measuring the reducing end groups, but this was due to the presence of arabinanases. The RGase containing fractions were practically free of other activities. As the RGase was labile in phosphate buffers, the HTP-step was performed without delay in order to avoid inactivation. This sensitivity toward phosphate buffers was described by Karr and Albersheim[23] for their wall-modifying enzyme (WME). For this reason, the fractions from the HTP column were assayed directly for RGase activity and the active fractions were dialyzed immediately against 0.1 M sodium acetate (pH 5.0).

Final purification involved fractionation using an FPLC system equipped with a mono Q column.

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

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

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

The specific activity of RGase at pH 5.0 and 30°C was calculated from the change in the molecular weight distribution as determined by HPSEC. Using commercial software, M_n was
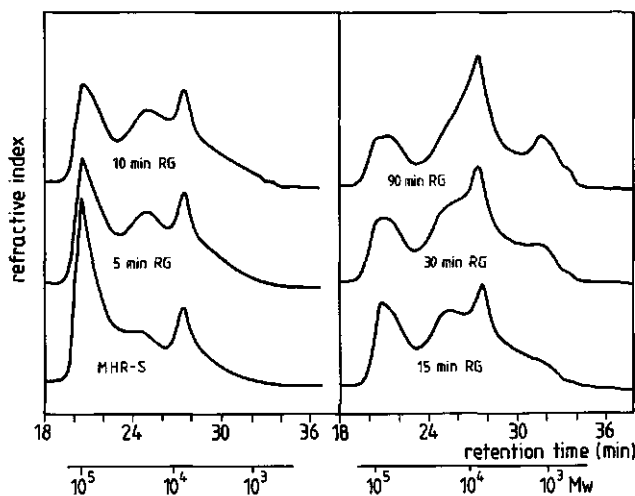

Fig. 3.   High-performance size-exclusion chromatography of saponified MHR (MHR-S) after treatment with RGase at 30°C and pH 5 for various times.
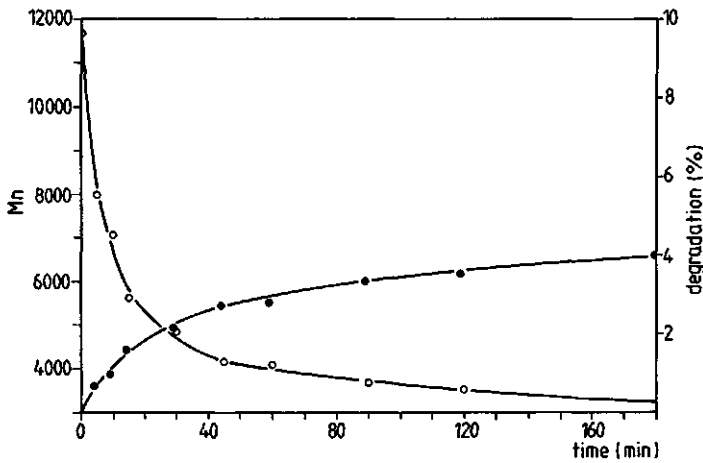
Fig. 4.   Decrease of the average M_n (-o-) and the percentage of degradation (-•-) of MHR-S on incubation
with RGase at 30°C and pH 5.

calculated from which, together with the substrate concentration, the number of glycosidic
linkages cleaved was estimated and expressed in activity units. The value obtained ($\pm$ 5
μkats/mg) gives only the order of magnitude, since the content of protein was low, and it
applies only to the MHR-S substrate. Moreover, it should be borne in mind that the column
system was calibrated with pectin standards.

    Figure 3 shows the HPSEC patterns of MHR-S after incubation with RGase (75 ng of
protein). The fraction with the highest M_w in MHR was degraded from the beginning of the
reaction and was converted into fractions of lower M_w. The second peak (10 000-30 000 Da)
was shifted towards the third peak (7000 Da) which represented a fraction that was resistant
to RGase. Low molecular weight degradation products ($\pm$ 1500 Da) were formed (see
below).
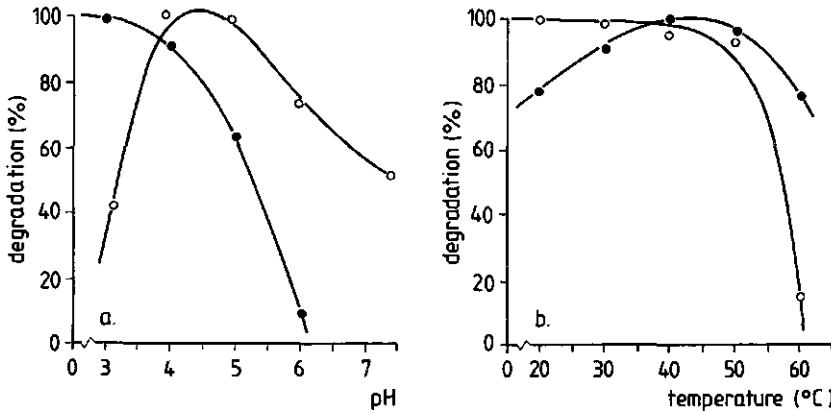
Fig. 5.   The pH and temperature curve (-•-) and pH and temperature stability curve (-o-) for RGase.

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

RGase was optimally active in the pH range 3-4 (Fig. 5). At pH 5, the activity was reduced to $\pm50\%$ and, at pH 6 to 20%. In the range 20-60°C; the activity of the enzyme was always $>75\%$ of the maximal activity which was measured between 40 and 50°C (fig. 5).

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

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

Characterization of products of RGase action.—RGase was active only on MHR substrates. Figure 6 shows the degradation before and after saponification. MHR and MHR-ARA underwent little or no degradation. After removal of the methyl esters and *O*-acetyl groups, however, the resulting MHR-S and MHR-ARA-S were degraded readily to resistant poly-meric fractions and oligomers. The difference in the patterns of elution of MHR-ARA and
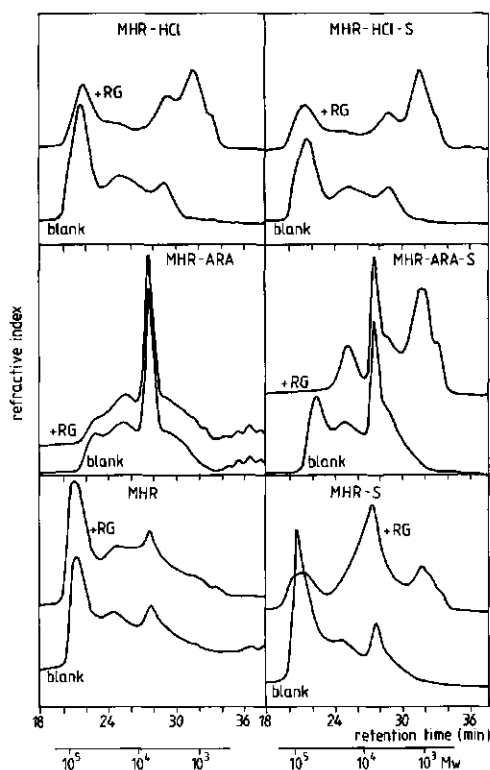

Fig. 6.   High-performance size-exclusion chromatography of MHR substrates, before and after incubation with
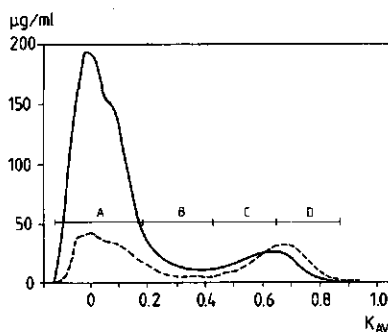   RGase at 30°C and pH 5 for 3 h.

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

MHR-ARA-S must be ascribed to their preparation from different batches. MHR-HCl with
degrees of methylation and acetylation of 33% and 10%, respectively, was degraded in about
the same way as saponified MHR-HCl, indicating that the methyl esters did not hinder the
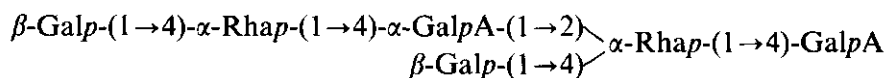RGase or that they are located in the regions which were not degraded by the enzyme.
    In order to characterize the oligomers, enzyme digests of MHR-S and MHR-HCl were
eluted from Sephadex G50 and the fractions were assayed for neutral sugars and uronic acids.
The elution pattern of the MHR-S digest is shown in Fig. 7. A similar pattern was obtained
for the MHR-HCl digest and the sugar compositions of the fractions are presented in Table
I. Almost all of the xylose was present in the void fraction A, the xylose:galacturonic acid
ratio of which was high. The arabinose residues were also recovered from the MHR-S digest
in fraction A, and galactose was present in all fractions. Rhamnose, galactose, and galacturo-

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

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

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

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

$$\beta\text{-Gal}p\text{-}(1 \to 4)\text{-}\alpha\text{-Rha}p\text{-}(1 \to 4)\text{-}\alpha\text{-Gal}p\text{A-}(1 \to 2)$$
$$\beta\text{-Gal}p\text{-}(1 \to 4)$$
$$\searrow \alpha\text{-Rha}p\text{-}(1 \to 4)\text{-Gal}p\text{A}$$
$$\nearrow$$

1

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

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

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

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

References

1 G.O. Aspinall, in J. Preis (ed.), *The Biochemistry of Plants, A Comprehensive Treatise*, Vol 3. Academic Press, New York, 1980, pp. 473-499.

2 G.O. Aspinall, in G.O. Aspinall (ed.) *The Polysaccharides*, Vol 2. Academic Press. New York, 1982, pp. 98-169.

3 R.R. Selvendran and M.A. O'Neill, *Methods Biochem. Anal.*, 32 (1987) 25-153.

4 A.G.J. Voragen, J.P.J. Timmers, J.P.H. Linssen, H.A. Schols, and W. Pilnik, *Z. Lebensm. Untersuch. Forsch.*, 174 (1983) 251-256.

5 A.G.J. Voragen, H.A. Schols, H.A.I. Siliha, and W. Pilnik, *ASC Symp. Ser.*, 310 (1986) 230-247.

6 S.C. Fry, in *The Growing Plant Cell Wall: Chemical and Metabolic Analysis*, Wiley, New York, 1988, pp. 102-141.

7 M. Knee, *Phytochemistry*, 12 (1973) 1543-1549.

8 J.A. De Vries, F.M. Rombouts, A.G.J. Voragen, and W. Pilnik, *Carbohydr. Polym.*, 2 (1982) 25-33.

9 H. Konno, Y. Yamasaki, and K. Katoh, *Phytochemistry*, 25 (1986) 623-627.

10 L. Saulnier and J.-F. Thibault, *Carbohydr. Polym.*, 7 (1987) 345-360.

11 H.A. Schols, M.A. Posthumus, and A.G.J. Voragen, *Carbohydr. Res.*, 206 (1990) 117-129 (this thesis, chapter 3).

12 A.G.J. Voragen, F. Geerst, and W. Pilnik, in P. Dupuy (ed.) *Use of Enzymes in Food Technology*, Lavoisier, Paris, 1982, pp. 497-502.

13 S.C. Churms, E.H. Merrifield, A.M. Stephen, D.R. Waldwijn, A. Polson, K. Van der Merwe, H.S.C. Spies, and N. Costa, *Carbohydr. Res.*, 113 (1983) 339-344.

14 W.H. van Deventer-Schriemer and W. Pilnik, *Lebensm. Wiss. Technol.*, 9 (1976) 42-44.

15 J.M. Labavitch, L.E. Freeman, and P. Albersheim, *J. Biol. Chem.*, 251 (1976) 5904-5910.

16 F.M. Rombouts, A.G.J. Voragen, M.J.F. Searle-van Leeuwen, C.C.J.M. Geraeds, H.A. Schols, and W. Pilnik, *Carbohydr. Polym.*, 9 (1988) 25-47.

17 R.G. Spiro, *Methods Enzymol.*, 8 (1966) 3-26.

18 F.J.M. Kormelink, J.W. van de Vis, M.J.F. Searle-van Leeuwen, and A.G.J. Voragen, *Food Hydrocolloids*, 4 (1991) 481-487.

19 J. Sedmak and S.E. Grossberg, *Anal. Biochem.*, 79 (1977) 544-552.

20 J.-F. Thibault, *Lebensm. Wiss. Technol.*, 12 (1979) 247-251.

21 M. Tollier and J. Robin, *Ann. Techn. Agric.*, 28 (1979) 1-15.

22 I.J. Colquhoun, G.A. De Ruiter, H.A. Schols, and A.G.J. Voragen, *Carbohydr. Res.*, 206 (1990) 131-144 (this thesis, chapter 5).

23 A.L. Karr Jr. and P. Albersheim, *Plant Physiol.*, 46 (1970) 69-80.

24 M. McNeil, A.G. Darvill, and P. Albersheim, *Plant Physiol.*, 66 (1980) 1128-1134.

25 M. McNeil, A.G. Darvill, and P. Albersheim, *Plant Physiol.*, 70 (1982) 1586-1591.

26 J.M. Lau, M. McNeil, A.G. Darvill, and P. Albersheim, *Carbohydr Res.*, 137 (1985) 111-125.

27 J.L. Adler-Nissen, H. Guertler, G.W. Jensen, H.A.S. Olsen, S. Riisgaard, and M. Schnelein, U.S. Pat. 4,478,939 (1984); *Chem. Abstr.*, 102, (1985) 111481.

28 J.A. De Vries, in G.O. Philips, D.J. Wedlock, and P.A. Williams (Eds), *Gums and Stabilizers for the Food Industry 4*, IRL Press, Oxford, 1988, pp. 25-29.

CHAPTER 5

IDENTIFICATION BY NMR SPECTROSCOPY OF OLIGOSACCHARIDES OBTAINED BY TREATMENT OF THE HAIRY REGIONS OF APPLE PECTIN WITH RHAMNOGALACTURONASE

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Abstract

2D NMR methods have been used to determine the composition of a mixture of oligosaccharides obtained by enzymic degradation of the modified hairy (ramified) regions of apple pectin with a new rhamnogalacturonase. The structures of the oligosaccharides were based on the unit α-Rha$p$-(1→4)-α-GalA-(1→2)-α-Rha$p$-(1→4)-GalA. A β-Gal$p$ unit was 4-linked to approximately half of the terminal Rha$p$ residues and to half of the (1→2)-linked Rha$p$ residues. The sample contained a mixture of a tetrasaccharide, two pentasaccharides, and one hexasaccharide.

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Introduction

Powerful new 2D NMR techniques which greatly assist analysis of the structure of oligosaccharides are now available including homonuclear Hartmann-Hahn[1] (HOHAHA) and rotating-frame n.0.e. spectroscopy[2] (ROESY), and $^{13}C/^1H$ shift-correlation using heteronuclear multiple quantum coherence[3] (HMQC). Early applications of these methods[4-6] involved compounds of known structure. We now report the use of 1D and 2D NMR methods to determine the structures of several closely related oligosaccharides present in a mixture.

The mixture of oligosaccharides was obtained[8] by enzymic degradation of the hairy (ramified) regions isolated from pectic polysaccharides of apple. The overall sugar composition was known, but there was no reliable information on the size of the oligomers.

The accompanying paper[7] describes the isolation from apple juice of the pectic polysaccharides referred to as the modified hairy region (MHR). The fraction examined here was produced[8] by enzymic degradation of MHR using a new rhamnogalacturonase (RGase). Identification of the degradation product(s) was required in relation to the determination of the structure of MHR and the specificity of the enzyme.

MHR was rich in arabinose (55 mol%) and galacturonic acid (21 mol%) and rhamnose, xylose, and galactose were also present in reasonable proportions[7]. The rhamnose:galacturonic acid ratio was particularly high for this type of polysaccharide. MHR was resistant to most technical enzyme preparations and to pectolytic, hemicellulolytic, and cellulolytic enzymes. The availability of the new RGase made it possible to obtain oligosaccharide fractions suitable for study by 2D NMR spectroscopy.

Experimental

The mixture of oligosaccharides studied was that designated fraction D in the preceding papers.

NMR experiments.—The ^{13}C- (100.4 MHz) and ^1H NMR spectra (399.65 MHz) were obtained with a Jeol GX-400 spectrometer on a solution of fraction D in D_2O (15 mg/0.5 mL; pD 4.7) in a 5 mm tube at 24°C. The chemical shifts of the methyl group of internal acetone were taken to be 2.217 (^1H) and 31.07 ppm (^{13}C) with respect to the signals for Me_4Si.

COSY, ROESY and HOHAHA experiments.—All 2D homonuclear correlation experiments were performed[9] in the phase-sensitive mode. A 2048(t_2) x 512(t_1) x 2 data matrix was used with spectral widths of 2.5 x 2.5 kHz, which gave digital resolution of 2.44 and 4.88 Hz in f_2 and f_1 respectively. A double quantum filter[10] was used in the COSY experiment so that all signals could be phased to the pure absorption mode. In the ROESY experiment, the spin-locking (mixing) period was preceded and followed by 90° pulses[11]. A continuous spin-locking field of 2.5 kHz was applied during the mixing time of 200 ms. The HOHAHA experiment[2] involved a repeated MLEV-17 sequence during the mixing time (110 ms) and an r.f. field strength of 6.25 kHz. The carrier frequency was placed at 3.3 ppm.

$^{13}C/^1H$ Shift-correlation experiments.—These experiments were performed using both the conventional sequence (with ^{13}C detection) and the HMQC method (which has ^1H detection,

and therefore high sensitivity). The HMQC experiment included a BIRD sequence and was as described[3] except that ^{13}C decoupling was not available in the acquisition period and the data were processed in the absolute-value mode. A 2048 (t_2, ^{1}H) x 128 (t_1, ^{13}C) data matrix was used, with spectral widths of 2000 (^{1}H) x 5556 Hz (^{13}C) which, after zero filling in t_1, gave digital resolutions of 1.95 (^{1}H) and 21.7 Hz (^{13}C). Delay times were 0.7 s between scans (measured from the start of the acquisition period) and 0.4 s from the end of the BIRD sequence to the first pulse of the HMQC sequence. A conventional ^{13}C-{^{1}H} dual probe was used and the 90° pulse lengths were 8 (^{13}C) and 16 μs (^{1}H).

The resolution in the ^{13}C dimension in the HMQC experiment was not sufficient for an assignment of all the ^{13}C signals in the region 60-85 ppm so that a conventional phase-sensitive ^{13}C/^{1}H shift correlation experiment[12] was performed. The data matrix was 4096 (t_2, ^{13}C) x 64 (t_1, ^{1}H) x 2 points giving digital resolutions of 2.7 Hz for ^{13}C and 3.9 Hz for ^{1}H after zero-filling. The HMQC experiment was carried out with only 96 scans per t_1 value whereas the ^{13}C/^{1}H shift correlation with ^{13}C detection required 2000 scans per t_1 value in order to obtain an adequate signal-to-noise ratio.

Results and discussion

1D NMR (^{1}H and ^{13}C) spectra.—The sugar composition[8] of fraction D was close to 2 rhamnose:2 galacturonic acid:1 galactose, and the NMR data showed that the residues were pyranoid. The absolute configurations were assumed to be the same as those invariably found[13] in polysaccharides from plant cell walls, i.e. L-Rha, D-GalA, and D-Gal. The ^{1}H NMR spectrum of fraction D is shown in Fig. 1. The region for anomeric protons can be divided into two parts, namely, 5.0-5.3 ppm which contains 7 d with J values in the range 1.5-4 Hz, and 4.5-4.7 ppm which contains 3 d, each with a coupling constant of 8 Hz, together with a fourth signal (4.67 ppm) for which the structure was not clear. Resonances for H-1α are expected to be in the first region and those of H-1β in the second region. Other signals which can be assigned readily occurred in the range 1.22-1.32 ppm and arose from H-6,6,6 of the Rha units. Although there appeared to be 2 t in this region, H,6,6,6 gave a dm which, actually, must be 4 d. This finding indicates the presence of four different Rha units in fraction D.

The ^{13}C NMR spectrum of fraction D (Fig. 2) contains four peaks in the region 17-18 ppm (C-6 of the Rha units) and confirms that four different Rha units were present, but the unequal intensities of the lines suggest that there was a mixture of compounds in the fraction. Two lines at 61.73 and 61.76 ppm (assigned to C-6 of Gal not 6-linked) indicate that there were two different Gal units. The regions (92 -105 ppm) for C-1 resonances contains nine lines, although broadening of two of these lines (at 98.2 and 98.6 ppm) could arise from overlap of resonances with similar chemical shifts. A comparison of the chemical shifts for the C-1 resonances of fraction D with literature data for galactose, rhamnose[14,15] and galacturonic acid[16] suggested the preliminary assignments in Table I. If fraction D contained a single compound, the number of C-1 resonances would indicate it to have been an octamer but the integrated ^{13}C NMR spectrum showed that this was not the situation.
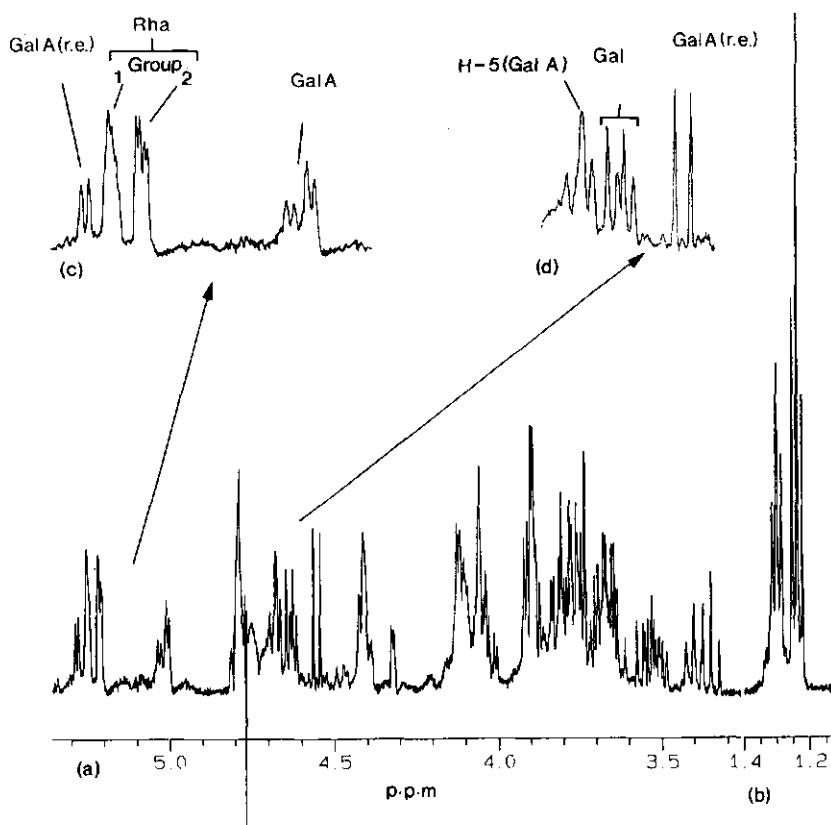
Fig. 1.   (a) ¹H NMR spectrum (400 MHz) of fraction D, (b) H-6 (Rha) region, (c) H-1α region, and (d) H-1β
region.

If the reducing GalA residue is taken as a single unit, then, there are also one α-GalA, two
α-Rhap units with different linkages, and one β-Galp unit. Thus, fraction D contains a
mixture of oligosaccharides each of which is smaller than an octasaccharide. In order to
confirm the assignments in Table I and reveal further details of the structure of the
oligosaccharides in fraction D, 2D NMR spectroscopy was used.

  COSY experiments.—The COSY spectrum of fraction D is shown in Fig. 3. The coupling
networks beginning with the H-1 resonances at 5.28 and 4.55 ppm were discerned readily.
The chemical shifts of the resonances in these networks accord with those found for the α and
β reducing end units in (1→4)-linked oligomers of α-galacturonic acid[17,18]. The J values,
obtained from the fine structure of the cross-peaks, were also those expected[17] for
galacturonic acid residues. The ¹H chemical shifts for the resonances of these reducing end
units are presented in Table II, together with other data. Taken with the assignments of the
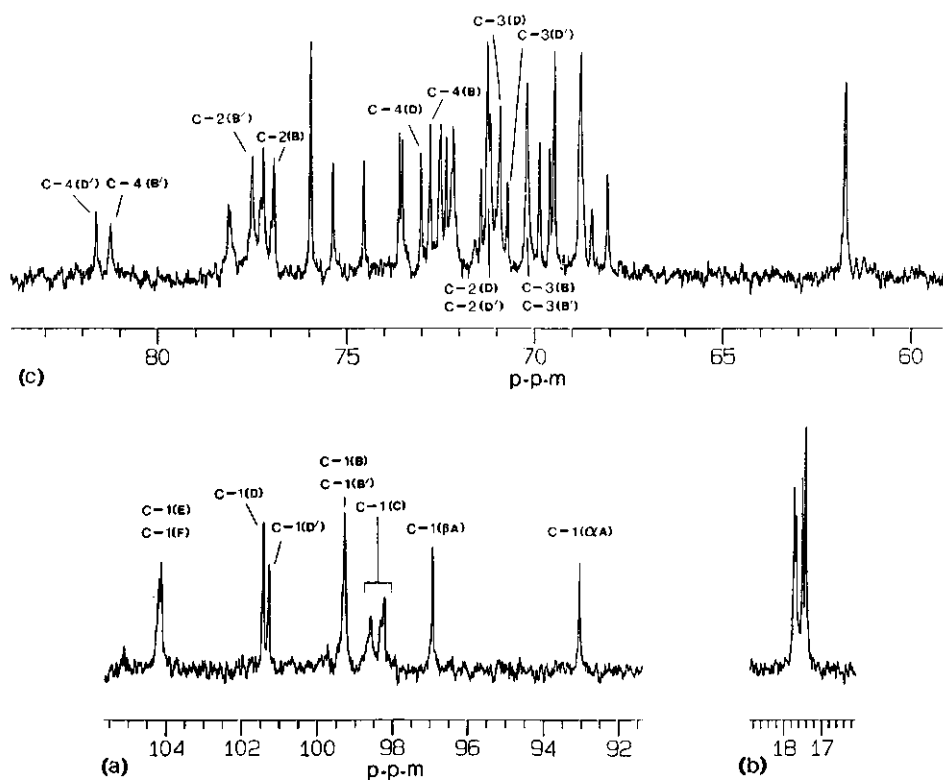¹³C NMR spectrum, the ¹H data confirm that the reducing moiety in each oligosaccharide is

Fig. 2.   ¹³C NMR spectrum (100 MHz) of fraction D: (a) C-1 region (b) C-6 (Rha) region, and (c) C-2/6
region, excluding GalA C-6. The labelling scheme is shown in Fig.6.

GalA.

A second type of GalA unit was identified from the COSY spectrum. A coupling network
began with the 2 d for H-1 at 5.04 and 5.01 ppm, but separate signals could not be observed
from the fine structure of the cross-peaks for H-2 or for any of the succeeding protons in the
ring. The chemical shifts and J values of the resonances of the protons in this network were
similar to those obtained [17,18] for the central unit of a (1→4)-linked α-galacturonic acid trimer,
indicating that fraction D contained (1→4)-linked α-GalA in addition to the reducing end unit.
Thus, the signal at 4.67 ppm did not arise from H-1 but from H-5 of the (1→4)-linked α-
GalA residue. The observation of 2 d for H-1, but identical chemical shifts for the H-2
resonance etc., suggested the presence of two types of (1→4)-linked α-GalA unit that differed
only in the nature of the sugar linked to C-1. The 2 d at 4.63 and 4.62 ppm (each with J 7.8
Hz) can be assigned to H-1 of β-Gal units. It was not possible to obtain the chemical shifts
of the resonances of H-5 and H-6 in the Gal residues from the COSY spectrum as the coup-

TABLE I. ^{13}C NMR data for the anomeric region of fraction D

	Chemical Shift (ppm)[a]	Integral
β-Galp	104.21, 104.14	1. 9
α-Rhap	101.45, 101.29	2.1
α-Rhap	99.32	2.3
α-GalA	98.57, 98.23	2.3
β-GalA (r.e.)[b]	96.94	1.1
α-GalA (r.e.)[b]	93.04	1.0

[a] ±0.01 ppm. [b] r.e. = reducing end

ling network could only be traced as far as H-4. The δ-values for the resonances of H-5 and H-6 in Table II were obtained from the ^{13}C/^{1}H correlation spectrum (see below). In common with the (1→4)-linked GalA residues, two signals were observed from H-1 of the Gal units, but only one signal for each of H-2, H-3, and H-4 which indicates again that there were two Gal units that differed only in the sugar unit attached at position 1.

Assignment of the ^{1}H resonances for the Rha units was more complex but provided the key to the determination of the oligosaccharide structure. The H-1 resonances of the Rha units fall into two groups, with equal integrated intensities, centred at 5.25 ppm (group 1) and 5.21 ppm group 2). Each group contains 2 d with $J \pm 1.5$ Hz. The H-6 resonances also gave two pairs of d centred at 1.23 and 1.30 ppm. Observation of H-1/H-2 cross-peaks in the COSY spectrum allowed allocation of the chemical shifts of the H-2 resonances to groups 1 (4.11 ppm) and 2 (4.06 ppm) but further tracing of the connectivity network revealed that there were two quite different chemical shifts for the H-3 and H-4 resonances within both groups 1 and 2 (Table II). The chemical shifts of the H-3 (f_1 axis) and H-4 (f_2 axis) resonances may be obtained from the H-3/H-4 cross-peaks labelled a-d in Fig. 3. Cross-peaks a,c originated from H-1 (group 1) and b,d from H-1 (group 2). The difference in the chemical shifts of the H-4 resonances within group 1 (or group 2) can be attributed to the presence or absence of a sugar unit 4-linked to Rha. The provisional assignments in Table I can be extended by suggesting that the four types of Rha unit present are terminal, 2- and 4-linked, and 2,4-linked. However, since the difference in chemical shifts of the H-3 resonances is almost as great as that for the H-4 resonances, the linkage may be at 0-3 rather than at 0-4. The effects of the formation of glycosidic linkages on the chemical shifts of the resonances of the protons at, or close to, the linkage site have been documented in studies of disaccharides[19,20]. Of particular relevance is the finding[20] that the chemical shifts of the H-3 and H-4 resonances in the L-Rha residue of β-D-Glcp-(1→4)-α-L-Rhap-OMe are shifted downfield by ± 0.2 ppm in comparison with the values for the corresponding protons in α-L-Rha-OMe. The observation of effects of similar magnitude for fraction D supports the proposal that some of the Rha units are 4-linked.

HOHAHA and ROESY experiments. —It was not possible to complete the assignment of the
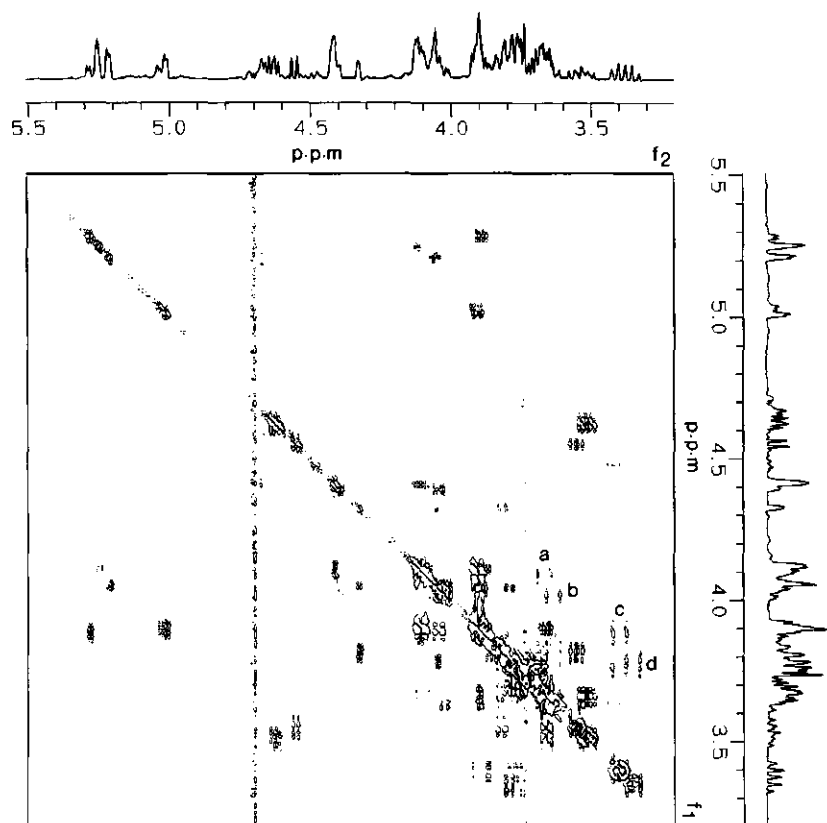
Fig. 3.   COSY spectrum of fraction D. Cross-peaks relating H-3 and H-4 for the various Rha units are labelled
          a,c (group 1) and b,d (group 2).

Rha resonances from the COSY spectrum because the cross-peaks that involved H-5
overlapped. This is a common problem which requires the use of additional 2D techniques
such as RELAY, multiple RELAY, or HOHAHA spectroscopy[21]. The HOHAHA experiment
is particularly useful since magnetisation may be transferred between all the protons of a
coupled network. Where none of the J values are close to zero, this network will include all
the protons of a given sugar unit. Therefore, in the 2D experiment, a cross-section (row or
column) taken through the chemical shift of a particular proton resonance in the sugar unit
provides a correlation with the chemical shifts of all other resonances in the unit. Although
the full 2D spectrum may be extremely complex, cross-sections can usually be taken through
the positions of isolated resonances, e.g. in the regions for anomeric or methyl protons.

   Figure 4 shows a part of the 2D HOHAHA spectrum of fraction D, in which the methyl
signals are correlated with the other resonances of the Rha units. The correlation of the
methyl signals to the H-1 region is seen clearly and one down-field Me d belonged to a group

TABLE II. Chemical shifts[a] (ppm) for the resonances of fraction D

Unit		H-1	H-2	H-3	H-4	H-5	H-6
GalA(r.e.)[b]	αA	5.28	3.89	4.05	4.39	4.43	
	βA	4.55	3.55	3.82	4.32	4.05	
α-Rha$p$ [c]	Bd	5.26	4.11	3.87	3.40	3.78	1.24
	B'	5.25	4.12	4.09	3.67	3.85	1.31
α-GalA	C	5.04,5.0	13.91	4.11	4.41	4.67	
α- Rha$p$ [d]	D[f]	5.22	4.05	3.79	3.35	3.76	1.23
	D'	5.21	4.07	4.02	3.64	3.84	1.29
β-Gal$p$	E,F	4.63,4.62	3.51	3.66	3.90	3.68	3.74[g]

[a] ± 0.01 ppm. [b] r.e. = reducing end. [c] Group 1 (see text). [d] B, 2-linked, B', 2,4-linked.
[e] Group 2. [f] D, terminal; D', 4-linked. [g] H-6,6'.

1 Rha unit whereas the other was associated with group 2. The same inference was true for the upfield 2 d. The complete ^{1}H assignments for the Rha units, obtained from the COSY and HOHAHA experiments, are given in Table II.

The problem of determining the sequence of sugar residues in an oligosaccharide by NMR methods has been approached in several ways. Although the delayed-COSY experiment has been used[22] to detect the small couplings between anomeric and aglyconic protons, the method is not generally applicable. Indirect evidence for the location of glycosylation sites may be
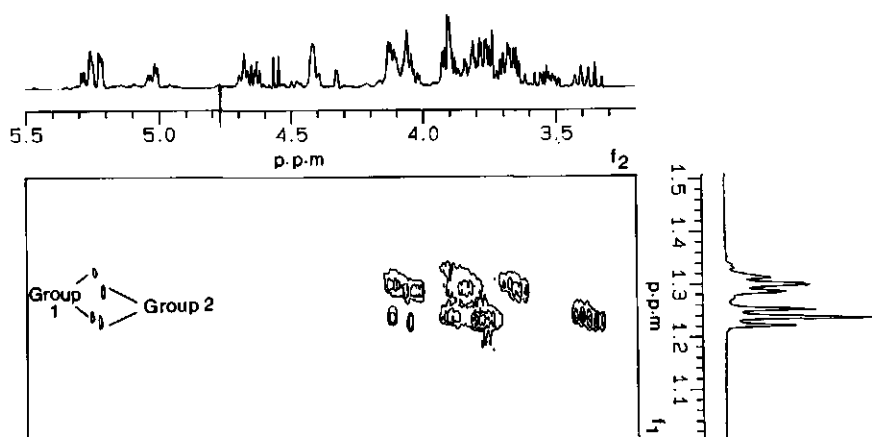

Fig. 4.   Partial HOHAHA spectrum of fraction D, showing correlations between H-6 (Rha) and H-1/5 (Rha).

obtainable from a comparison of ^{1}H chemical shifts for the resonances of the oligosaccharide with those for the component monosaccharides[19,20], but the sequence can be determined more readily from n.0.e. data[23], ^{13}C glycosylation shifts[24], or by detection of long range ^{1}H-^{13}C couplings across the glycosidic linkage[6].

Theoretical and experimental studies[25,26] of the conformations of oligosaccharides have shown that, for most types of glycosidic linkage, the internuclear distance for anomeric and aglyconic protons is in the range 2.5-3.0 Å at the minimum-energy conformation. An inter-residue n.0.e. should be observable for two protons separated by such a short distance, and observation of the n.0.e. can be used to establish which residues are linked together and the linkage positions. In addition, short range contacts ($\pm$ 2.5Å) between protons within the same ring give rise to intra-residue n.0.e.s. For example, in a β-Gal$p$ residue, n.0.e.s. are observable from H-1 to H-2,3,5. In NOESY and ROESY spectra, cross-peaks that arise from inter-residue n.0.e.s. can be distinguished from intra-residue cross-peaks if all of the resonances have been assigned previously and there is no overlap of peaks. A correct assignment of all the inter-residue cross-peaks that involve anomeric protons suffices for a complete determination of the oligosaccharide sequence. However, a cross-peak may arise from the close approach of an anomeric proton to a proton in another residue which is not the aglyconic proton. Correctly interpreted, the existence of such a cross-peak may provide information on the overall conformation of the oligosaccharide but, if misinterpreted, it may indicate a linkage where none exists. Therefore, it is advisable to supplement the evidence from NOESY (or ROESY) experiments with ^{13}C NMR data.

The rate of tumbling of oligosaccharides in aqueous solution is such that the condition $\omega_L \tau_C \sim 1$ is frequently met, where ω_L is the spectrometer operating frequency (rad/s) and τ_C the correlation time for molecular re-orientation. Under these circumstances the n.0.e. values are small, irrespective of the internuclear distances, and it may not be possible to use the NOESY technique in order to determine the sequence. The ROESY experiment[1] was developed to overcome the problem of making n.0.e. measurements on molecules that have unfavourable rates of tumbling. The principles of the experiment[1] and its application to oligosaccharides[5] have been discussed elsewhere.

Both NOESY and ROESY experiments were carried out on fraction D. Some of the cross-peaks in the ROESY spectrum (Fig. 5) were also observed in the NOESY spectrum but the signal-to-noise ratio of the latter was considerably worse for the same total acquisition time. The assignment of resonances to individual sugar units (Table II) permitted identification of intra- and inter-ring cross-peaks in the ROESY spectrum. The only cross-peaks which involved H-1 of the reducing end GalA unit arose from intra-ring n.0.e.s, but inter-ring cross-peaks involving H-1 of all of the other sugar units were detected, and these are indicated in Fig. 5 and summarised in Table III. Cross-peaks b-e arose from interactions across glycosidic linkages and show that the backbone of the oligosaccharide has an alternating structure of rhamnose and galacturonic acid units, namely, α-Rha$p$-(1$\rightarrow$4)-α-GalA-(1$\rightarrow$2)-α-Rha$p$-(1$\rightarrow$4)-GalA. The group 1 units are the 2-linked Rha residues. The central α-GalA-(1$\rightarrow$2)-α-Rha$p$ linkage brings H-1 of the GalA and Rha units into close proximity, giving rise to cross-peak a. The group 2 resonances arise from terminal Rha units. A comparison of the intensities of the signals in the anomeric region for the reducing end GalA
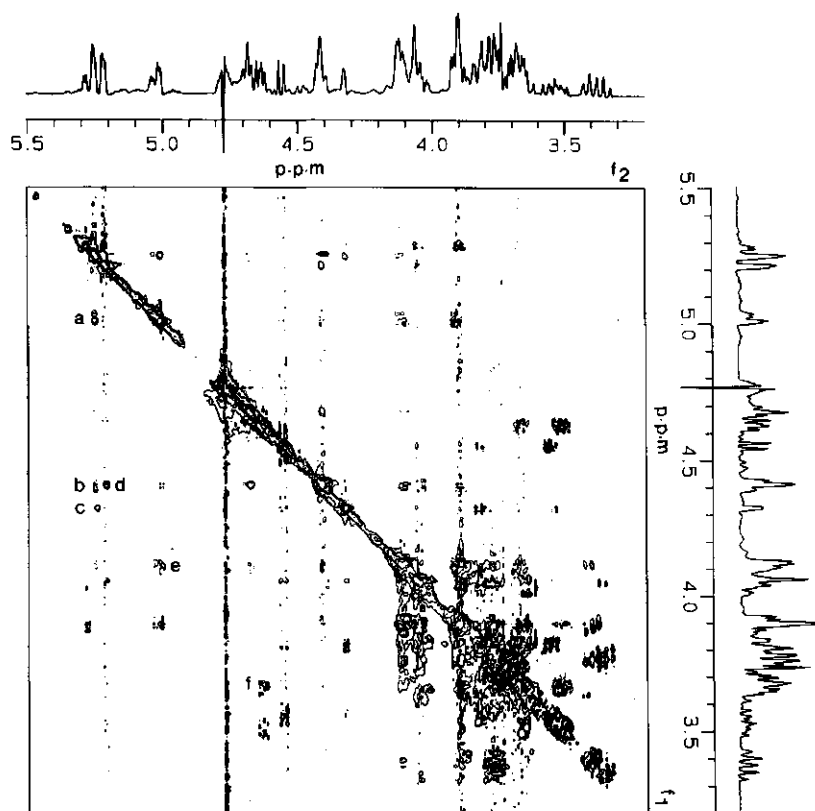
Fig. 5.   ROESY spectrum of fraction D. Positive and negative cross-peaks are not distinguished. Peaks a-f
(all negative) are identified in Table III. Peaks in the lower right corner (3.3-4.2 ppm) are all
positive.

unit and the remaining Rha and GalA units makes it clear that the basic structure must be a
tetrasaccharide.

The remaining problem is to establish the positions of the linkages of the galactose
residues. A cross-peak (f) was detected in the ROESY spectrum which could possibly connect
H-1 of β-Gal$p$ with H-4 of certain Rha$p$ units (3.64-3.67 ppm). However, intra-ring n.0.e.s.
are also expected between H-1 and H-3,5 of β-Gal$p$ and H-3,5 of β-Gal$p$ have chemical shifts
of ± 3.67 ppm. Therefore the ROESY method does not provide definitive evidence for the
position of the linkage of the β-Gal$p$ unit.

In addition to the ROESY cross-peaks listed in Table III, which were all negative (i.e.
opposite in sign to the diagonal), a large number of positive cross-peaks were detected in the
region 3.3-4.1 ppm. Such cross-peaks arise from coherent magnetisation transfer through the
coupled network as in the HOHAHA experiment. The intensity of these peaks precluded

Table III.   Inter-residue cross-peaks identified in the ROESY spectrum of fraction D

Cross-peak label	Location of the anomeric proton	Connected proton
a	α-Rhap (group 1)	H-1 α-GalA
b	α-Rhap (group 1)	H-4 α-GalA (r.e.)[a]
c	α-Rhap (group 1)	H-4 β-GalA (r.e.)[a]
d	α-Rhap (group 2)	H-4 α-GalA
e	α-GalA	H-2 α-Rhap (group 1)
f	β-Galp	H-4 α-Rhap (group 1,2)[b]?

[a] r.e. = reducing end.   [b] δ 3.65.

observation of any n.0.e. effects in this region. In the region for anomeric protons, however, cross-peaks that arise *via* the Hartmann-Hahn mechanism are not observed providing that the carrier frequency is set to one side of the main spectral region. Therefore, the ROESY experiment should be a generally useful method for the determination of the structures of oligosaccharides.

13C/1H Shift-correlation experiments.—A full assignment of the ^{13}C NMR spectrum of fraction D was undertaken, using C/H shift correlation methods, in order to confirm the structure proposed above and to establish conclusively the positions of the linkages of the Gal residues. In view of the small amount of sample available, an initial attempt to assign the spectrum was made using the high-sensitivity HMQC sequence[3]. The spectrometer did not have the capability for applying ^{13}C broad-band decoupling during the ^{1}H acquisition period, so that the resonances in the ^{1}H dimension appeared at the position of the ^{13}C satellite signals. This method of operation has the advantage that $^{1}J_{C,H}$ values are obtained readily. For example, $J_{C-1,H-1}$ of both types of Rha residue was 170 Hz, confirming that they were α.

The HMQC experiment confirmed the assignments of the C-1 resonances in Table I, but the lack of ^{13}C decoupling was a handicap in assigning the heavily-crowded region (60-85 ppm). Although many correlations were detected, it was not possible to assign the spectrum in full because of the added complexity caused by $^{1}J_{C,H}$ couplings and because of the poor digital resolution in the ^{13}C dimension. The assignments were completed by obtaining a conventional (^{13}C detected) C/H correlation spectrum in which (neglecting zero filling) the ^{13}C digital resolution was improved by a factor of 16. Consideration of the full assignments of the chemical shifts of the ^{13}C resonances, given in Table IV, proves that the oligosaccharides in fraction D had the structures shown in Fig. 6. The intensities of the signals show that not all of the Rha units were linked to Gal so that fraction D consisted of a mixture of the basic Rha:GalA tetrasaccharide plus two pentasaccharides and one hexasaccharide that contained one and two Gal units, respectively.

The chemical shifts for the ^{13}C resonances of ring A accorded with those determined[18] for the reducing end unit in several oligomers of (1→4)-linked α-galacturonic acid, the largest difference being found for C-3α,βA which was 1.4 ppm higher than in the oligomers. The

TABLE IV. Chemical shifts[a] (ppm) of the ^{13}C resonances for fraction D

Unit	C-1	C-2	C-3	C-4	C-5	C-6
GalA(r.e.)[b]						
α A	93.04	68.76	70.94	78.10, 78.16	71.40	±175.5
β A	96.94	72.29	74.52	77.55, 77.50	75.35	174.8
α-Rha$p$						
B[c]	99.32	76.86	70.17	72.73	69.88	17.39[f]
B'	99.32	77.44	70.17	81.18	68.47	17.66[g]
α-GalA						
C	98.23, 98.57	68.76	71.14	77.19	72.14	-175.5
α-Rha$p$						
D[d]	101.45	71.25	70.87	72.99	69.60	17.48
D'	101.29	71.22	70.70	81.59	68.10	17.71
β-Gal$p$						
E, F[e]	104.14	72.45	73.49	69.45	75.94	61.73
	104.21	72.50	73.57	"	"	61.76

[a] ±0.01 ppm. [b] r.e. = reducing end. [c] B 2-linked; B', 2,4-linked. [d] D, terminal; D', 4-linked.

[e] Rings E and F are not specifically assigned. [f] Assignments for B,D may need to be reversed.

[g] Assignments for B',D' may need to be reversed.

C-4α,βA resonances were split into two lines of equal intensity because only half of the neighbouring Rha units were substituted with a Gal unit, and there was a long-range effect on the chemical shifts. The chemical shifts of the ^{13}C resonances in oligosaccharides and polysaccharides can be predicted from data for the appropriate monosaccharides and the substituent shifts produced by the immediate neighbours[24]. The present work illustrates the small, but measurable, influences which more remote residues can have on the chemical shift. The ^{13}C NMR data for ring C may also be compared with those for the (1→4)-linked α-GalA units in oligomers of galacturonic acid. The resonance of C-3C was 1.4 ppm higher, but the shifts of C-1C and C-4C resonances were 1.5 ppm lower than for the equivalent carbons in the oligomer. Presumably, these changes reflect configurational[20] and conformational[27] effects caused by replacement of two neighbouring α-D-GalA residues by two α-L-Rha$p$ residues. The resonances of ring C were rather broad, probably because their chemical shifts are slightly different in each of the four molecules (see below).

The data in Table IV allows the four different types of Rha unit to be identified as 2-linked (ring B), 2,4-linked (B'), terminal (D), and 4-linked (D'). The resonances at 101.45 and 101.29 ppm were assigned to C-1D and C-1D', respectively, whereas the C-1B and C-1B' resonances overlapped at 99.3 ppm. The relative intensity of the ^{13}C signals of the first pair was 1.4:1, and this gives the ratio of terminal Rha (ring D) to Gal-substituted Rha (D') units.
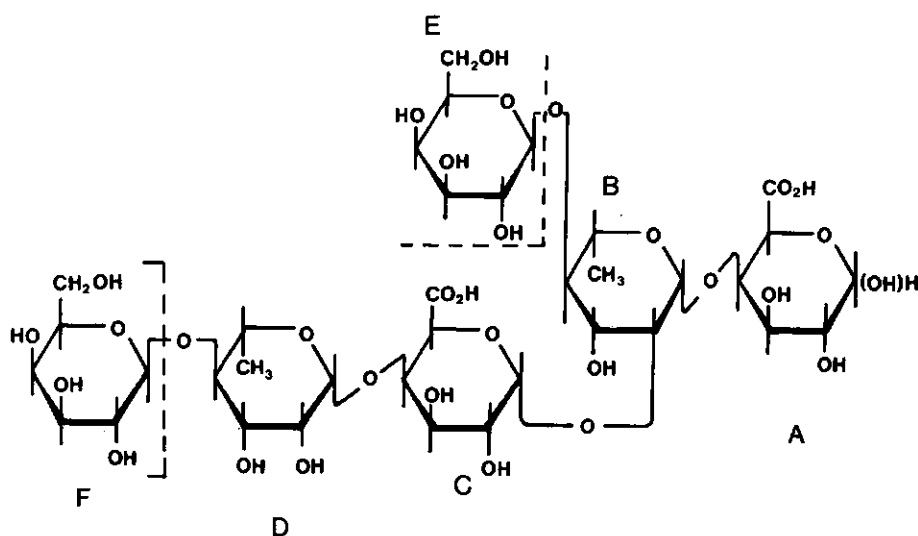
Fig. 6.   Structures of the oligomers identified in fraction D. Molecules with zero, one, and two Gal units were present.

A resolution-enhanced ^{13}C NMR spectrum revealed that the resonance at 99.3 ppm was not a single peak, so that measurements of signal intensities were not possible.

The ^{13}C glycosylation shifts showed conclusively that the Gal residues were 4-linked to the Rha units, and not 3-linked, the possible alternative suggested by ^{1}H NMR data. There was a down-field shift of 8.6 ppm for both the C-4D' and C-4B' resonances as compared with those of C-4D and C-4B. By contrast, the shifts of the C-3 resonances hardly changed. Linkage of the group 1 Rha residues (B,B') at C-2 was also demonstrated by the down-field shifts of ± 6 ppm observed for the resonances of C-2B,B' in comparison with those for C-2D,D'. Two sets of ^{13}C resonances were found for C-1,2,3,6 of Gal that corresponded to the two possible locations (E,F) for these residues. However, the resonances could not be assigned specifically. The chemical shifts were fully consistent with those expected for terminal β-Gal$p$ residues.

Evidence that all four oligosaccharides might be present came from detailed examination of the C-1C resonance. Resolution enhancement split each of the resonances at 98.23 and 98.57 ppm into two components, suggesting four possible environments for C-1C. It seems reasonable to infer that the larger splitting (0.34 ppm) reflects the presence or absence of ring E, whereas the smaller splitting (0.03 ppm) reflects the more remote influence of ring F. Precise quantification of the oligosaccharides was difficult because of overlap in the ^{1}H NMR spectrum and poor signal-to-noise ratio in the ^{13}C NMR spectrum. However, it was estimated that <50% of the Rha units were substituted with Gal residues.

Methylation analysis (using the methods described in Part I[7]) confirmed the conclusions of the NMR study concerning linkage positions, but gave somewhat higher values for the degree

of substitution of the Gal. Thus, the distribution of rhamnosyl units was terminal (18%), 4-linked (31%), 2-linked (21%), and 2,4-linked (30%). Of the galactose residues, 75% were terminal with the remainder 3- (4%), 4- (16%) and 6-linked (5%). The longer chains were not detectable in the NMR spectra.

Structures similar to those determined for the oligosaccharides of fraction D have been described for rhamnogalacturonan I, isolated from sycamore cells[28,29]. The backbone of this polysaccharide had the same alternating sequence of Rha and GalA residues and possessed a variety of side chains 4-linked to ± 50% of the Rha residues.

Elucidation of the structures of the oligosaccharides in Fraction D is an important contribution to the model[8] for the modified hairy regions. The isolation, in reasonable quantity, of these oligosaccharides with the same basic tetrasaccharide unit suggests that the complex hairy regions from apple pectin may be more regular than has been assumed hitherto. It is proposed that the hairy regions are composed, in part, of different repeating units.

References

1   A. Bax and D.G. Davis, *J. Magn. Reson.*, 65 (1985) 355-360.
2   A. Bax and D.G. Davis, *J. Magn. Reson.*, 63 (1985) 207-213.
3   A. Bax and S. Subramanian, *J. Magn. Reson.*, 67 (1986) 565-569.
4   S.W. Homans, R.A. Dwek, J. Boyd, N. Soffe, and T.W. Rademacher, *Proc. Natl. Acad. Sci. U.S.A.*, 84 (1987) 1202-1205.
5   J. Breg, D. Romijn, J.F.G. Vliegenthart, G. Strecker, and J. Montreuil, *Carbohydr. Res.*, 183 (1988) 19-34.
6   L. Lerner and A. Bax, *Carbohydr. Res.*, 166 (1987) 35-46.
7   H.A. Schols, M.A. Posthumus, and A.G.J. Voragen, *Carbohydr. Res.*, 206 (1990) 117-129 (this thesis, chapter 3).
8   H.A. Schols, C.C.J.M. Geraeds, M.J.F. Searle-van Leeuwen, F.J.M. Kormelink, and A.G.J. Voragen, *Carbohydr. Res.*, 206 (1990) 105-115 (this thesis, chapter 4).
9   O.J. States, R.A. Haberkorn, and D.J. Ruben, *J. Magn. Reson.*, 48 (1982) 286-292.
10   M. Rance, 0.W. Sørensen, G. Bodenhausen, G. Wagner, R.R. Ernst, and K. Wüthrich, *Biochem. Biophys. Res. Commun.*, 117 (1983) 479-485.
11   C. Griesinger and R.R. Ernst, *J. Magn. Reson.*, 75 (1987) 261-275.
12   M. Ohuchi, M. Hosono, K. Furihata, and H. Seto, *J. Magn. Reson.*, 72 (1987) 279-297.
13   M. McNeil, A.G. Darvill, S.C. Fry, and P. Albersheim, *Annu. Rev. Biochem.*, 53 (1984) 625-663.
14   K. Bock, C. Pedersen, and H. Pedersen, *Adv. Carbohydr. Chem. Biochem.*, 42 (1984) 193-225.
15   J. H. Bradbury and G.A. Jenkins, *Carbohydr. Res.*, 126 (1984) 125- 156.
16   M. Rinaudo, G. Ravanat, and M. Vincendon, *Makromol. Chem.*, 181 (1980) 1059-1070.
17   S.B. Tjan, A.G.J. Voragen, and W. Pilnik, *Carbohydr. Res.*, 34 (1974) 15-23.
18   C. Chouly, I.J. Colquhoun, and G.A. De Ruiter, unpublished results.
19   A. De Bruyn, M. Anteunis, and G. Verhegge, *Bull. Soc. Chim. Belg.*, 84 (1975) 721-734.
20   I. Backman, B. Erbing, P.-E. Jansson, and L. Kenne, *J. Chem. Soc., Perkin Trans. 1*, (1988) 889-898.
21   F. Inagaki, D. Kohda, C. Kodama, and A. Suzuki, *FEBS Lett.*, 212 (1987) 91-97.
22   G. Massiot, C. Lavaud, L. Le Men-Oliver, G. Van Binst, S.F. Miller, and H.M. Fales, *J. Chem. Soc., Perkin Trans. 1*, (1988) 3071-3079.
23   T.A.W. Koerner, J.H. Prestegard, P.C. Demou, and R.K. Yu, *Biochemistry*, 22 (1983) 2687-2690.

24   P.-E. Jansson, L. Kenne, and G. Widmalm, *Carbohydr. Res.* 168 (1987) 67-77.

25   H. Thøgersen, R.U. Lemieux, K. Bock, and B. Meyer, *Can. J. Chem.*, 60 (1982) 44-57.

26   K. Bock, *Pure Appl. Chem.*, 55 (1983) 605-622.

27   K. Bock, A. Brignole, and B.W. Sigurskjold, *J. Chem. Soc., Perkin Trans.* 2, (1986) 1711-1713.

28   M. Mc Neil, A.G. Darvill, and P. Albersheim, *Plant Physiol.*, 70 (1982) 1586-1591.

29   J.M. Lau, M. McNeil, A.G. Darvill, and P. Albersheim, *Carbohydr. Res.*, 137 (1985) 111-125.

CHAPTER 6

OCCURRENCE OF PECTIC HAIRY REGIONS IN VARIOUS PLANT CELL WALL MATERIALS AND THEIR DEGRADABILITY BY RHAMNOGALACTURONASE

H.A. Schols and A.G. J. Voragen

Abstract

Pectic polysaccharide fractions of high molecular weight, resistant to further degradation by pectolytic, hemicellulolytic, and cellulolytic enzymes, were isolated from potato fibre and from pear, carrot, leek, and onion tissue by the liquefaction process. The fractions, referred to as modified hairy regions (MHR), were characterized by the determination of their sugar composition, linkage type composition, degree of esterification (methyl ester and *O*-acetyl groups), and molecular weight distribution. Galacturonic acid, galactose and rhamnose were found to be the major sugar residues in most of the MHR preparations, while arabinose was the main sugar in pear MHR. The rhamnose:galacturonic acid ratio ranged between 0.44 for pear MHR to 0.63 for MHR from leek. High degrees of acetylation (DA) were calculated assuming that acetyl groups were only attached to galacturonic acid residues. All MHR fractions had a similar molecular weight distribution which was rather heterogeneous. It was observed that all MHR preparations were degraded by RGase in a similar fashion. In all digests, a characteristic population of reaction products having a molecular weight of ± 1000-2000, representing rhamnogalacturonase oligomers, was present.
It was concluded that pectic hairy regions with comparable structural features are common to a variety of fruit and vegetable tissues.

Introduction

For many years pectins and pectin-related substances have been investigated minutely for their structural features[1] and their functions within the plant cell wall[2]. O'Neill et al.[1] stated that so far only three pectic polysaccharides have been isolated from the primary cell walls of plants. These are homogalacturonan, rhamnogalacturonan I (RG-I), and a substituted galacturonan referred to as rhamnogalacturonan II (RG-II). RG-I was pictured as a long repeating sequence of alternating L-rhamnose and D-galacturonic acid residues with a variety of L-arabinosyl-, D-galactosyl-, and L-fucosyl-containing side chains[1]. RG-II has an extremely complex glycosyl-residue and glycosyl-linkage composition, including such rare sugars as aceric acid, 2-keto-3-deoxyoctulosonic acid, 2-O-methyl-L-fucose, 2-O-methyl-D-xylose, and D-apiose. For apple pectin, a general model was proposed by De Vries[3], containing a main "smooth region" [a linear α-(1→4)-galacturonan] and "hairy regions" containing most of the neutral sugars. These hairy regions or polysaccharide fractions having similar structures have been reported to be present in pectins isolated from grapes[4], carrot[5-8], pear[9,10], kiwi[11,12], onion[13-15], potato[16-18] and many other plant tissues.

Recently, we determined the structure of the hairy regions of apple pectin isolated from apple juice manufactured by the liquefaction process[19]. This pectic fraction, referred to as modified hairy regions (MHR), was characterized as a branched, highly acetylated rhamnogalacturonan, carrying side chains of arabinose, galactose or xylose. The length of these side chains varied. Also, we described the isolation and characterization of an enzyme rhamnogalacturonase (RGase)[20], which was able to hydrolyse up to 4% of all glycosidic linkages present in apple MHR. After degradation of MHR with RGase, various fractions could be isolated, including residual, high molecular weight galacturonan polymers rich in xylose and arabinose and oligomeric fragments consisting of rhamnose, galactose and galacturonic acid[20]. NMR analysis of a low molecular weight fraction[21] revealed the presence of a basic alternating tetramer α-Rha$p$-(1→4)-α-GalA-(1→2)-α-Rha$p$-(1→4)-GalA. A β-Gal$p$ unit was linked to C-4 of approximately half of the terminal Rha$p$ and to α-(1→2)-linked Rha$p$ residues.

Based on these results, a hypothetical model was proposed[19] for MHR, which was updated recently[22] by the introduction of subunits of xylogalacturonan and of stubs of the rhamnogalacturonan backbone rich in arabinose.

It is suggested that rhamnogalacturonan-rich hairy regions play a role in the manufacture of fruit juices, produced with the use of enzymes. Schols et al.[23] claimed the hairy regions to be the origin of haze formation in juices manufactured with the assistance of enzymes. Will and Dietrich[24] reported that rhamnogalacturonans are responsible for membrane fouling in the clarification of fruit juices by ultrafiltration.

In this paper, we report the isolation and characterization of MHR fractions after enzymic liquefaction of the dicotyledonic tissue of potato (Solanum tuberosum), carrot (Daucus carota), and pear (*Pyrus communis*), and monocotyledonic tissue of leek (*Allium porrum*) and onion (*Allium cepa*). All MHR preparations were characterized chemically and enzymically using RGase.

Experimental

Isolation of MHR.—Leek, carrots, onions, and pears (Conference) were bought at the local market. Potato fibres (remaining after starch removal) were a gift of Avebe, The Netherlands. The carrots were steam-peeled (30-40 s, 10 bar; PKC, Pöttesfeld/Neuwied). All fresh products were sliced (<3 cm), steam-blanched (100°C, 3 min), and then milled to a particle size of 4 mm (Fryma perforated disc mill). Pears were directly milled without blanching. Approximately 8 kg of the pulp obtained was mixed with 2 kg of water in a thermostated vessel (Terlet, Zutphen, The Netherlands). Potato fibres (0.2 kg) were suspended in 10 kg of water. The pH of the products was adjusted to 5.0 ($\pm$0.3), the enzyme (0.1% Rapidase C600; Gist Brocades, Delft, The Netherlands) was added, and the mixtures were held at 45°C for 4 h under continuous stirring. After inactivation of the enzyme (30 min, 90°C) and centrifugation (15 000g, 15 min), the supernatant solutions were subjected to ultrafiltration using a tubular system (Kidney dialysis system; Nephross[TM] Andante HF; molecular weight cut-off 5000; Organon Teknika, The Netherlands). The ultrafiltration retentates were diluted three times with distilled water, ultrafiltered again, and lyophilized.

Saponification of the MHR.—The methyl ester and O-acetyl groups in MHR were removed by treatment with 0.05 M NaOH (1.7% MHR, 0°C, 24 h), followed by dialysis and lyophilization, to yield MHR-S.

Analytical methods.—Samples were hydrolysed by 2 M trifluoroacetic acid at 121°C for 1 h and neutral sugars were converted into their alditol acetates[19] to determine the sugar composition. The uronic acid content was determined colorimetrically using *m*-hydroxybiphenyl[19]. Methylation analysis and the reduction of uronides were performed as described previously[19]. Protein content was determined as described[25].

Chromatography.—High-performance size-exclusion chromatography (HPSEC) on three Bio-Gel TSK columns in series (40XL, 30XL, and 20XL; calibration was performed using pectins) and size-exclusion chromatography (SEC) over Sephacryl S200 and S500 were carried out as described[19].

Enzymic hydrolysis.—Solutions of the various MHR and MHR-S samples (0.25%) in 0.05 M NaOAc (pH 5.0) were incubated with RGase for 20 h at 30°C as described[20]. After incubation the enzymes were inactivated (5 min, 100°C) and the digests were analyzed by HPSEC.

Results

Characterization of MHR from various sources.—Tissues of leek, onion, carrot and pear and potato fibres were treated with a technical enzyme preparation under conditions used in the liquefaction process[19]. From the juices obtained, pectic polysaccharides were isolated by ultrafiltration. Since these pectic substances were isolated similarly to the

MHR from apple pulp[19], we used the same name for the isolated pectic polymers. The isolated MHR from potato fibre accounted for 4.1% of the starting material and ± 0.04% of the initial fresh tissue. For the other plant materials under investigation, the MHR fractions represented 0.13-0.25% of the fresh weight.

The sugar composition of MHR from the various sources are shown in Table I, together with that of apple MHR for comparison. Although there are differences between the composition of the materials, they all have rhamnose, arabinose, galactose and galacturonic acid as main sugar residues. Arabinose is the major sugar in the MHR from pear tissue (40 mol%), while onion MHR contained only 7 mol% of arabinose. The lower arabinose levels in some MHR explain the relative higher amounts of the other sugar residues. Xylose was almost absent in carrot, leek, and potato MHR. The rhamnose content varied between 12 mol% for pear MHR to 22 mol% for MHR from carrot and leek. The galacturonic acid content fluctuated between 27 (pear MHR) and 39 mol% (carrot MHR). All MHR preparations had a remarkably high rhamnose-galacturonic acid ratio, which varied from 0.44 to 0.63. The sugar composition of the MHR from apples varied slightly from the data published before[19] where sulfuric acid hydrolysis was used. TFA hydrolysis used in this study gave a slightly higher rhamnose content. Obviously, also the rhamnose-galacturonic acid ratio increased: 0.44 instead of the value 0.29 as found before. It can also be observed from Table I that the percentage of xylose showed great variation depending on the origin of the tissue. As shown in Table I, all newly isolated MHR preparations had a high degree of acetylation (mol of acetic acid per 100 mol of galacturonic acid) similar to data previously published for apple MHR[19]. The

Table I. Sugar composition (mol%) of modified hairy regions isolated from various sources

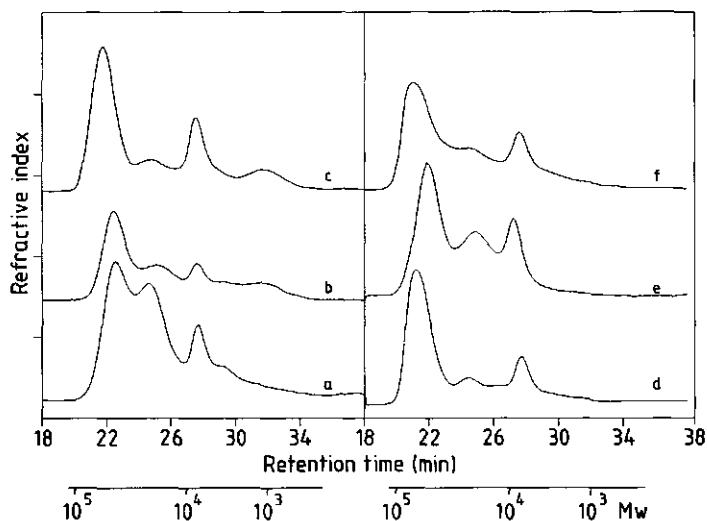| Sugar	Source					
	Pear	Carrot	Leek	Onion	Potato	Apple
Rha	12	22	22	21	16	9
Ara	40	12	18	7	16	51
Xyl	7	1	1	3	2	8
Man	0	1	0	1	1	0
Gal	14	24	24	30	24	10
Glc	0	1	0	0	6	0
GalA	27	39	35	38	35	22
OMe	21	6	11	6	13	42
OAc	33	44	77	54	90	60
Rha:GalA	0.44	0.56	0.63	0.55	0.46	0.41
Total carbohydr.(%w/w)	75	72	69	74	67	86
Protein (%w/w)	1	3	7	6	4	1

Fig. 1. High-performance size-exclusion chromatography elution patterns of MHR isolated from various sources. a: carrot; b: onion; c: leek; d: potato fibre; e: pear; f: apple.

degree of acetylation (DA) varied from 33% for pear MHR to 90% for the MHR fraction isolated from potato fibre. These DA values are high, although for the corresponding rhamnogalacturonan I isolated from suspension cultured cotton cells it was reported[26] that 30% of the galacturonosyl residues carried an acetyl group. In most studies in which similar pectin fragments have been studied, acetyl groups were lost during alkaline extraction or the presence of acetyl groups was neglected completely. The degree of methylation (DM) is significantly lower (6-42%) than the degree of acetylation. The DM not only depends on the type of starting material, but is also influenced by the action of pectin methyl esterase during the liquefaction process. From Table I, it can be seen that all MHR fractions tested contained low amounts of protein.

The molecular weight and the molecular weight distribution of MHR were determined by HPSEC as shown in Fig. 1. The various MHR preparations all had a fairly similar elution pattern in which three populations (A, B, and C) can be recognized. Only the relative amounts of the three populations varied. Next to these three populations, in most elution patterns, a rather broad fourth peak was present in minor quantities. The different populations were isolated using chromatography over Sephacryl S500 and S200 an. characterized by determining their sugar composition and degrees of esterification (Table II). Since population D of most MHR could not be isolated in sufficient quantities, their characteristics were not included in Table II. Sometimes, regularities could be recognized in the sugar composition of the corresponding populations. For both apple and pear MHR, the xylose content decreased with decreasing molecular weight; for onion MHR, this trend was not observed. The number of rhamnose residues in MHR from pear, carrot and apple

Table II. Sugar composition (mol%) of the fractions of MHR from various sources, isolated over Sephacryl S200 and S500

MHR		Rha	Ara	Xyl	Man	Gal	Glc	GalA	OMe	OAc	Rha:GalA
Pear											
Population	A	11	41	10	0	9	0	29	22	39	0.38
	B	14	38	3	0	22	0	23	24	32	0.61
	C	17	25	3	0	7	1	47	29	20	0.36
Carrot											
Population	A	23	12	2	0	30	0	33	2	60	0.70
	B	23	11	2	0	28	1	35	2	34	0.66
	C	27	14	2	0	12	0	45	7	12	0.60
	D	26	19	1	1	9	2	42	nd[a]	nd	0.62
Leek											
Population	A	20	21	1	0	23	1	34	7	99	0.59
	B	20	18	1	2	27	2	30	11	69	0.67
	C	21	17	1	1	12	0	48	15	27	0.44
	D	18	15	2	1	11	3	50	nd	nd	0.36
Onion											
Population	A	23	3	4	0	29	1	40	4	69	0.58
	B	20	3	5	1	27	2	42	10	52	0.48
	C	21	8	4	1	19	1	46	15	36	0.46
Potato											
Population	A	21	22	1	0	28	1	27	3	155	0.78
	B	23	20	1	1	26	1	28	8	136	0.82
	C	21	14	1	1	9	2	52	32	29	0.40
Apple											
Population	A	8	49	10	0	9	0	24	28[b]	55[b]	0.33
	B	8	55	5	0	11	0	21	84[b]	57[b]	0.38
	C	10	58	2	0	6	1	23	100[b]	21[b]	0.43

[a] Not determined. [b] Degree of methylation and acetylation as determined before[19]

was higher in the high molecular weight populations than in the lower molecular weight populations. The relative proportion of arabinose varied significantly. MHR from pear, carrot, leek, and potato contained relatively more arabinose in the higher molecular weight populations, while MHR from onion and apple showed a higher proportion of arabinose in the lower molecular weight fractions. In general, it could be concluded that the high molecular weight populations were relatively rich in galactose and acetyl groups, and relatively low in galacturonic acid and methanol. Notable is the high degree of acetylation of the populations of potato MHR. Despite the observed differences in molecular weight and sugar composition of the populations within MHR from one source, MHR can be considered to be a mixture of similar polysaccharides.

Table III shows the results of the methylation analysis of the MHR from various sour-

Table III. Sugar glycosidic linkage composition of MHR isolated from various sources

Sugar linkage	Glycosidic linkage composition[a]				
	Pear	Carrot	Leek	Onion	Potato
Rhamnose					
T-Rha$p$[b]	1.7	1.7	1.0	0.9	1.0
1,2-Rha$p$[b]	7.7	13.1	11.4	9.8	5.0
1.3-Rha$p$	0.3	0.2	0.2	-	-
1,2,4-Rha$p$	4.6	14.7	12.6	19.5	18.1
	(14.3)	(25.2)	(25.2)	(30.2)	(24.1)
Arabinose					
T-Ara$f$	9.9	4.0	3.3	2.1	3.7
1,5-Ara$f$	33.4	11.0	19.2	1.8	22.4
1,3,5-Ara$f$	2.7	-	-	0.3	-
1,2,3,5-Ara$f$	1.0	0.4	0.5	-	-
	(47.0)	(15.4)	(23.0)	(4.2)	(26.1)
Xylose					
T-Xyl$p$	5.5	1.7	2.2	3.9	1.8
1,4-Xyl$p$	1.5	tr[d]	1.6	tr	-
1,2-Xyl$p$	0.3	-	-	-	-
	(7.3)	(1.7)	(3.8)	(3.9)	(1.8)
Galactose					
T-Gal$p$	5.0	12.7	11.3	15.4	16.5
1,2-Gal$p$	0.6	0.9	2.0	1.4	-
1,4-Gal$p$	3.0	5.6	2.7	5.0	5.0
1,6-Gal$p$	2.1	3.2	1.4	1.6	0.6
1,2,4-Gal$p$	-	-	1.4	1.6	1.4
1,3,4-Gal$p$	-	0.2	0.5	0.5	0.3
1,3,6-Gal$p$	3.2	1.9	1.7	0.8	-
	(13.9)	(24.5)	(21.0)	(26.3)	(23.8)
Glucose					
T-Glc$p$	0.7	3.0	-	-	0.7
1,4-Glc$p$	1.2	1.3	0.7	-	0.8
	(1.9)	(4.3)	(0.7)	(0)	(1.5)
Galacturonic acid[c]					
T-Gal$p$A	2.7	4.5	6.1	9.2	3.9
1,4-Gal$p$A	7.4	14.7	16.8	18.4	15.0
1,3,4-Gal$p$A	3.7	3.3	2.2	4.1	2.3
	(13.8)	(22.5)	(25.1)	(31.7)	(21.2)
Ratio					
terminal/branching	1.77	1.17	1.23	1.18	1.22

[a] Linkage types in mol% ; the totals after methylation analysis are given in brackets. [b] T - terminal ; 1,2-linked Rha etc. [c] Determined as galactose residues after carboxyl reduction. [d] Traces.
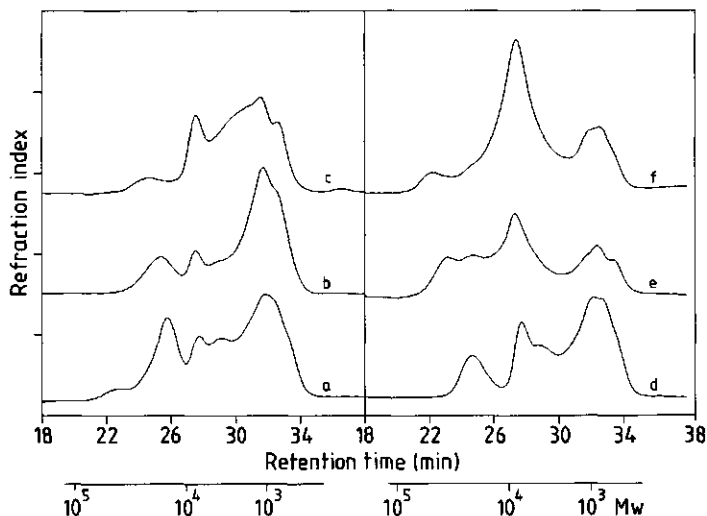
Fig. 2. High-performance size-exclusion chromatography elution patterns of MHR isolated from various
         sources after saponification and degradation with RGase at 30°C and pH 5 for 20 h. a: carrot; b:
         onion; c: leek; d: potato fibre; e: pear; f: apple.

ces. Despite differences in origin and sugar composition, no significant variations were
observed for the sugar linkage composition. Remarkably high values were found for
terminally linked rhamnose and galacturonic acid residues. This might be caused by some
degradation of the backbone during the methylation analysis. However, not all galac-
turonic acid residues were recovered after reduction and methylation. This also influenced
the molar ratio of the other sugar residues, which are somewhat higher as expected.
Rhamnose residues were mainly $(1\rightarrow2)$-linked, some of them branched at C-4. Xylose was
mainly present as terminally linked residues. Arabinose was found to be present as $(1\rightarrow5)$-
linked chains, occasionally branched at C-3. A rather high proportion of the galactose
residues were terminally or $(1\rightarrow4)$-linked, next to other types of linkages and branching
points. Most of the galacturonosyl residues recovered were $(1\rightarrow4)$-linked while 10-25% of
these residues were branched at C-3.

   Enzymic degradation of the various MHR.—Since MHR was isolated during a
liquefaction process using a crude enzyme preparation containing pectolytic,
hemicellulolytic and cellulolytic enzymes, it was expected to be resistant to further
enzymic degradation. In incubation experiments, it was indeed confirmed that both the
technical enzyme preparation Rapidase C600 and a variety of purified pectolytic,
hemicellulolytic and cellulolytic enzymes were not able to degrade the isolated MHR
polysaccharides, even after prolonged incubation times. Since apple MHR could be
degraded by a specific rhamnogalacturonase[19], the various MHR were also incubated with
RGase after removal of the ester groups by chemical saponification. Characteristic HPSEC
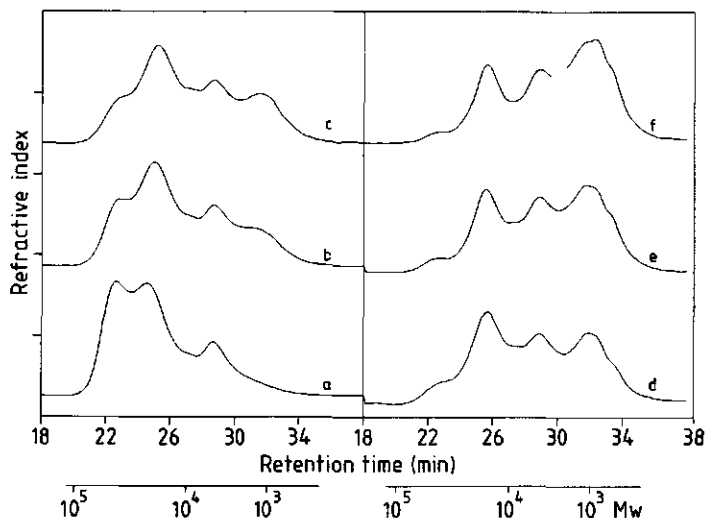
Fig. 3. High-performance size-exclusion chromatography elution patterns of saponified carrot MHR after treatment with RGase at 30°C and pH 5 for various times. a: blank; b: 5 min; c: 15 min; d: 60 min; e: 300 min; f: 20 h.

elution patterns are shown in Fig. 2, which indicated that RGase was active towards all MHR tested. However, small differences could be observed in the extent of degradation. The elution patterns showed small proportions of residual high molecular weight material, followed by a distinct population of fragments at a retention time of 27 min and a characteristic population of fragments at retention times of 32-33 min. The latter population represented oligomers, composed of rhamnose and galacturonic acid with structures similar to oligomers identified in RGase digests of apple MHR[20,21]. The total amount of RGase oligomers varied for the different MHR fractions and seemed to be dependent on the amount of rhamnogalacturonan in the MHR.

It was calculated with GPC software that RGase was able to hydrolyse all MHR preparations to similar levels as previously found for apple MHR[19]. The degrees of degradation (percentage of glycosidic bonds hydrolysed) for MHR from pear, leek, onion, carrot and potato were 7, 9, 9, 10 and 12% respectively.

RGase degradation of MHR monitored with time.—RGase was added to all saponified MHR samples, and the reaction products formed were monitored after fixed time intervals as shown for MHR of carrot in Fig. 3. The fraction with the highest molecular weight in MHR was degraded directly after addition of RGase with formation of fragments having lower molecular weight. The other two populations seemed to be less accessible for RGase and were not totally degraded, even after prolonged incubation times. The peaks representing the characteristic RGase oligomers became more prominent with time. The degradation by RGase of all MHR samples tested was similar.

Discussion

From the results presented, it can be concluded that by liquefaction of tissues of a wide variety of plants using the technical enzyme preparation Rapidase C600, a high M_w pectin fraction could be isolated, representing 0.04-0.25% of the starting material. The pectic fractions obtained from the various plant tissues show a remarkable analogy with the modified hairy regions described from apple tissue[19]. We therefore designated all fractions as modified hairy regions. They all have a high content of rhamnose and galacturonic acid. Similar to apple MHR and rhamnogalacturonan I[1] also the ratio between these sugars is high and varies between 0.44 and 0.63. Pectic polysaccharides with similar structures have been isolated before from the materials studied here[6,7,10,14,17]. However, the methods of isolation in those studies were rather diverse, which prohibits comparison of the results.

Using size-exclusion chromatography, it was shown that all MHR preparations, in spite of differences in sugar composition and degrees of esterification, had the same elution behaviour and consisted of at least three populations. The various populations were isolated and further characterized. The populations within the MHR of one source showed almost the same sugar composition as the starting MHR although differences occurred. For some MHR preparations, it can be noticed that with decreasing molecular weight, the proportion of ester groups or certain sugars decreases or increases (e.g. rhamnose in pear MHR increasing from 11 mol% in population A to 17 mol% in populations C. In most cases, the sum of the concentrations of a specific sugar in the subpopulations was consistent with the concentration in the total MHR.

Major variations were found in the DM and DA values for the different populations. The excessively high DA values for potato population A and B (155 and 137% respectively) gave rise to doubts on the reliability of these values. For this reason, the average DA and DM were calculated from the weight percentage of each individual population (as established by HPSEC) and the DM and DA values of the individual populations. This average value was compared with the measured DM and DA value for the total MHR fraction. In most cases there was good agreement. For the DA of leek MHR, a value of 78% was calculated, while the measured value was 77%. The extraordinary high values of 155% and 136% for population A and B of potato MHR resulted in a calculated value of 113% while the measured value was 90%. Since the values for the populations were reproducible and both calculated and measured DA values for potato MHR had the same order of magnitude, the high DA values were thought to be representative for these specific populations. Rhamnogalacturan I[1] was reported to be acetylated as well, although the position of the ester groups was not exactly established[1]. However, Komalavilas and Mort[26] reported that up to 30% of the galacturonic acid residues in an RG-I like polymer isolated from suspension cultured cotton cells had acetyl groups linked through O-3. In the near future, more information might become available on the position and distribution of the acetyl groups, since the presence of a rhamnogalacturonan acetylesterase in some industrial enzyme preparations has been reported[27]. This enzyme was found to be specific for rhamnogalacturonan regions and not for homogalacturonan (smooth) regions.

Methylation analysis resulted in qualitative rather than quantitative information. The efficiency of carboxyl-reduction was low and variable as reflected in the low yield of galactose residues after reduction. However, colorimetric determination of the carboxyl-reduced material showed that only small amounts of uronides were left when carboxyl-reduction was carried out three times. This could reflect a tendency of pectic polymers to undergo β-elimination during the methylation procedure. In general, the same types of sugar linkages were found in all MHR fractions.

Comparison of our results for pear MHR with data in the literature dealing with pectin fractions rich in neutral sugars[10,28] showed good agreement, although MHR contained more rhamnose and galactose residues, while the degree of methylation was rather low. This might be caused by enzymic demethylation during the liquefaction process, while Yoshioka et al.[28] suggested that "native" pectin molecules might be de-esterified during ripening from $\pm$ 90% to less than 40%.

Many recent publications deal with pectic substances extracted from carrot tissue both before[5,8,29-32] and after enzymic treatment[6,7]. A wide range of different pectic molecules have been described including fractions having a rhamnose-galacturonic acid ratio of 1[8]. This latter fraction was isolated without the use of enzymes, which is quite remarkable. Also, treatment of carrot cell wall polysaccharides with endo-pectin lyase[7] and endo-pectate lyase[6] resulted in the same type of hairy regions. It should be mentioned that some studies of carrot pectic material were carried out using suspension cultured cells[6,33], which might give different characteristics for the corresponding polysaccharides[34]. Although the presence of acetyl groups in hairy regions is not often mentioned in the literature, Massiot et al.[30] reported a DA value of 29%.

Pectins isolated from onion tissue have been reported[13-15] to be relatively rich in galacturonic acid. Treatment of onion cell wall polysaccharides with pectin lyase[14] resulted in fractions with similar characteristics to our MHR; a fraction was described[14] having a rhamnose-galacturonic acid ratio of 0.4.

The characteristics of potato MHR were compared with data in the literature regarding chemically[16,18] as well as enzymically[17,35] extracted pectins. The former pectins were rich in galacturonic acid and were believed[16,18] to represent homogalacturonan chains with some neutral sugar side-chains attached. Treatment of potato tissue resulted in rhamnogalacturonan rich polysaccharides[17,35] which resembled our MHR. Differences were found for the relative amount of galactose residues in the various fractions.

Enzymic degradation by RGase.—Since our isolation procedure of MHR included the use of a broad range of enzymes, our MHR preparations were resistant to a variety of pure carbohydrases present in our laboratory except towards rhamnogalacturonase. This enzyme was isolated specifically for its ability to degrade the backbone of apple MHR[19]. As discussed previously[20], no degradation by RGase was observed when the MHR fractions were not saponified before incubation. This is explained by the hindrance of RGase by *O*-acetyl groups.

Monitoring the degradation at various time intervals by HPSEC revealed that the elution patterns were about the same at all stages and showed great similarities in the various MHR. However, MHR from carrot tissue was found to be somewhat more resistant to

degradation then the other MHR fractions. Population B especially was degraded slowly and not so completely as the others. Although the degree of degradation (9-12%) seemed to be related to the amount of rhamnose and/or galacturonic acid in this type of polymer, no suitable correlation was found. Linear regression of the degree of degradation against the relative amount of rhamnose, galacturonic acid, or the sum of both resulted in correlation factors of $\pm$ 0.7. Since we do think that the degradation of MHR is coupled to the amount of rhamnose and/or galacturonic acid, the poor correlation might be explained by the assumption that only part of the rhamnose and galacturonic acid residues were present in an alternating form, while segments of the backbone have another sequence of both sugars. Previous results[22] based on studies of the degradation products of apple MHR by RGase action pointed to the presence of three different subunits: a xylogalacturonan, an arabinan-rich stub of the rhamnogalacturonan and sections in which the RGase oligomers are dominantly present. In the accompanying paper[36], the presence of these rhamnogalacturonase oligomers will be discussed in more detail. Although MHR from carrot, leek and potato were found to contain hardly any xylose, analogous subunits having various ratios of rhamnose to galacturonic acid might be present in all MHR. This idea is strengthened by the limited action of both polygalacturonase and rhamno-galacturonase towards the backbone of the various MHR.

In conclusion it can be stated that the enzymic liquefaction process resulted in populations of specific pectin fragments. Although slight alterations may occur during this isolation procedure, the pectic hairy regions obtained showed great similarity to fractions isolated in a different way, as described in the literature. Due to the identical isolation procedure, the (modified) hairy regions obtained in this study can be more readily compared. Rhamnogalacturonase proved to be a powerful tool for the elucidation of pectin structures, since it is able to hydrolyse selectively in the backbone of the MHR.

Such characteristics as the sugar composition, DA, DM, molecular weight distribution, and degradability by RGase showed great resemblances between MHR from pear, carrot, leek, onion and potato tissue. Since the occurrence of the MHR seemed to be widespread, as it is found to be present in tissues of both monocotyledons and dicotyledons, a specific role for such molecules in plant growth or defence should not be excluded.

There is still a difference of opinion about whether the hairy regions and homogalacturonans are connected to each other in the cell wall. The model of De Vries et al.[3] began from the principle that both molecules are interlinked; also, rhamnogalac-turonan I is usually isolated by the action of polygalacturonase[1]. On the other hand, Dick and Labavitch[10] and Dongowski and Anger[8] isolated their hairy regions without the use of enzymes.

More research is needed to reveal the exact structure of the (modified) hairy regions and their position within the cell wall framework.

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References

1 M O'Neill, P. Albersheim, and A. Darvill, in P.M. Dey (Ed), *Methods in Plant Biochemistry. Volume 2, Carbohydrates.* Academic, London, 1990, pp. 415-441.

2 N.C. Carpita and D.M. Gibeaut. *Plant J.,* 3 (1993) 1-30.

3 J. A. De Vries, F. M. Rombouts, A. G. J. Voragen, and W. Pilnik, *Carbohydr. Polym.,* 2 (1982) 25-33.

4 L. Saulnier, J-M. Brillouet, and J-P. Joseleau, *Carbohydr. Res.,* 182 (1988) 63-78.

5 B.J.H. Stevens, and R. R. Selvendran, *Carbohydr. Res.,* 128 (1984) 321-333.

6 H. Konno, Y. Yamasaki, and K. Katoh. *Phytochemistry,* 25 (1986) 623-627.

7 P. Massiot and J-F. Thibault, *Carbohydr. Res.,* 190 (1989) 121-136.

8 G. Donkowski and H. Anger. In F. Meuser, D.J.Manners, and W. Seibel (Eds), *Plant Polymeric Carbohydrates,* Royal Society of Chemistry, Cambridge, UK, 1993.

9 A.E. Ahmed and J.M. Labavitch, *Plant Physiol.,* 65 (1980) 1014-1016.

10 A.J. Dick and J.M. Labavitch, *Plant Physiol.,* 89 (1989) 1394-1400.

11 D.M. Dawson and L.D. Melton, *Carbohydr. Polym.,* 15 (1991) 1-11.

12 R.J. Redgwell, L.D. Melton, D.J. Brasch, and J.M. Coddington, *Carbohydr. Res.,* 226 (1992) 287-302.

13 A.T. Mankarios, M.A. Hall, M.C. Jarvis, D.R. Threlfall, and J. Friend, *Phytochemistry,* 19 (1980) 1731-1733.

14 S. Ishii, *Phytochemistry,* 21 (1982) 778-780.

15 R.J. Redgwell and R.R. Selvendran, *Carbohydr. Res.,* 157 (1986) 183-199

16 M.C. Jarvis, M.A. Hall, D.R. Threlfall, and J. Friend, *Planta,* 152 (1981) 93-100.

17 S. Ishii, *Phytochemistry,* 20 (1981) 2329-233.

18 P. Ryden and R.R. Selvendran, *Carbohydr. Res.,* 195 (1990) 257-272.

19 H.A. Schols, M.A. Posthumus, and A.G.J. Voragen, *Carbohydr. Res.,* 206 (1990) 117-129 (this thesis , chapter 3).

20 H.A. Schols, C.C.J.M. Geraeds, M.J.F. Searle-van Leeuwen, F.J.M. Kormelink, and A.G.J. Voragen, *Carbohydr. Res.,* 206 (1990) 105-115 (this thesis, chapter 4).

21 I.J. Colquhoun, G.A. de Ruiter, H.A. Schols, and A.G.J. Voragen, *Carbohydr. Res.,* 206 (1990) 131-144 (this thesis, chapter 5).

22 A.G.J. Voragen, H.A.Schols, and H. Gruppen, in F. Meuser, D.J. Manners, and W. Seibel (Eds.), *Plant Polymeric Carbohydrates,* Royal Society of Chemistry, Cambridge, UK, 1993, pp 3-15.

23 H.A. Schols, P.H. In 't Veld, W. van Deelen, and A.G.J. Voragen, Z. *Lebensm. Unters. Forsch.,* 192 (1991) 142-148 (this thesis, chapter 2).

24 F. Will and H. Dietrich, *Carbohydr. Polym.,* 18 (1992) 109-117.

25 J.P. Roozen and R. Ouwehand, *Voedingsmiddelentechnologie,* 11 (1978) 23-25.

26 P. Komalavilas and A.J. Mort, *Carbohydr. Res.,* 189 (1989) 261-272.

27 M.J.F. Searle-van Leeuwen, L.A.M. van den Broek, H.A. Schols, G. Beldman, and A.G.J. Voragen, *Appl. Microbiol. Biotechnol.,* 38 (1992) 347-349.

28 H. Yoshioka, K. Aoba, and Y. Kahimura, *J. Am. Soc. Hort. Sci.,* 117 (1992) 600-606.

29 D. Plat, N. Ben-Shalom, A. Levi, D. Reid, and E.E. Goldschmidt, *J. Agric. Food Chem.,* 36 (1988) 362-365.

30 P. Massiot, X. Rouau, and J-F. Thibault, *Carbohydr. Res.,* 172 (1988) 217-227.

31 P. Massiot, X. Rouau, and J-F. Thibault, *Carbohydr. Res.,* 172 (1988) 229-242.

32 N. Ben-Shalom, D. Plat, A. Levi, and R. Pinto. *Food Chem,* 44 (1992) 251-254.

33 T. Asamiza, N. Nakayama, and A. Nishi, *Planta*, 160 (1984) 469-473.

34 R.R. Selvendran, *J. Cell. Sci. Suppl*, 2 (1985) 51-88.

35 S. Ishii, *Phytopathol.*, 66 (1976) 281-289.

36 H.A. Schols, A.G.J. Voragen, and I.J. Colquhoun, *Carbohydr. Res.*, 256 (1994) 97-111 (this thesis, chapter 7).

CHAPTER 7

ISOLATION AND CHARACTERIZATION OF RHAMNOGALACTURONAN OLIGOMERS, LIBERATED DURING DEGRADATION OF PECTIC HAIRY REGIONS BY RHAMNOGALACTURONASE

H.A. Schols, A.G.J. Voragen, and I.J. Colquhoun[1]

Abstract

Digests of modified hairy regions of apple pectin (MHR) obtained after degradation by rhamnogalacturonase (RGase) were analyzed for oligomer composition using high-performance anion-exchange chromatography and pulsed amperometric detection. A series of oligomers which appear to be characteristic of RGase degradation could be recognized. These oligomers were isolated on a preparative scale by size-exclusion chromatography and preparative anion-exchange chromatography and analyzed for sugar composition. ^{1}H NMR spectroscopy showed that the oligomers consisted of between 4 and 9 sugar units with a backbone of alternating rhamnose and galacturonic acid residues, partly substituted with galactose residues linked to C-4 of the rhamnose moiety. The HPLC elution pattern showed that higher oligomers were also formed during incubation with RGase. These have the same basic structure but may contain other sugar units in addition to those given above. The oligomer composition of RGase digests of MHR isolated from apple, pear, leek, onion, carrot and potato was very similar. Using anion-exchange chromatography to monitor the degradation of MHR at increasing incubation times it was found that all the oligomers were present from the initial stages of the enzyme reaction and that the ratio between the different oligomers remained constant with time. Implications of these results for the structure of MHR and the mechanism of RGase action are discussed.

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Introduction

Although the structure of pectic substances has been the subject of many investigations, important questions about their structure remained unanswered. Pectin is a hetero-polysaccharide consisting of a linear chain of α-(1→4)-linked D-galacturonic acid residues, interrupted at intervals by α-(1→2)-linked L-rhamnopyranosyl residues, to which neutral sugar side chains are connected. Barrett and Northcote[1] first showed that neutral sugars are located in certain areas of the pectin molecule. In enzymic degradation studies of extracted pectins, De Vries et al.[2] found that 95% of the neutral sugars in a pectin were carried by a backbone containing only 5% of the galacturonosyl residues. These regions were named hairy regions. Similar regions were reported for pectins from grapes[3,4], carrots[5] and *Angelica acutiloba* Kitagawa[6]. Characteristic of these hairy regions is the relatively low galacturonic acid content (7-23%) and a high rhamnose content (5-15%) resulting in high ratio of rhamnose to galacturonic acid (0.2-1). The most important accompanying neutral sugars are usually arabinose and galactose; their relative amounts depend on the plant material under investigation.

In previous studies[7], we isolated a polysaccharide fraction from apple juice manufactured by liquefying ground apples with a mixture of pectolytic, hemicellulolytic, and cellulolytic enzymes. In this process, the polysaccharide fraction had been solubilized from the cell walls and was resistant to further degradation by the enzyme mixture. From its sugar and glycosidic linkage composition and from chemical degradation studies, it appeared that the polysaccharide had a structure very similar to the pectic hairy regions as described by De Vries et al.[2]. For obvious reasons we named this fraction "modified hairy regions" (MHR). Using MHR as a substrate, we discovered and isolated a new enzyme which was able to degrade 4% of the glycosidic linkages in the MHR-backbone and which we named therefore "rhamnogalacturonase" (RGase)[8]. Since it was not possible at that time to separate the various oligosaccharides present in the MHR digest, the low molecular weight products were characterized by NMR spectroscopy of the mixture[9]. The oligomers were found to have a basic structure of α-Rha$p$-(1→4)-α-GalA-(1→2)-α-Rha$p$-(1→4)-GalA. A β-Gal$p$ unit was 4-linked to approximately half of the terminal Rha$p$ residues and to half of the (1→2)-linked Rha$p$ residues. With this information, a detailed but still partly hypothetical structure was proposed[7] for MHR.

The accompanying paper[10] describes the characterization of MHR fractions, including their degradability by RGase as followed by changes in the molecular weight distribution of MHR by high-performance size-exclusion chromatography (HPSEC). It was shown that RGase was able to degrade the MHR fractions isolated from various sources. However, to study the low molecular weight degradation products of MHR in more detail, a chromatographic method capable of separating the oligomeric fragments was required.

In this paper, we report an HPLC method using anion-exchange chromatography to separate the oligomers liberated by RGase. Oligomers having a degree of polymerization (DP) between 4 and 9 were isolated on a preparative scale and identified using ^{1}H NMR spectroscopy. Using the HPLC method, the degradation of apple MHR by RGase was followed with time. Degradation products formed by RGase during enzymic degradation

of MHR from potato fibre, carrot, onion, leek and pear were compared with those from apple MHR.

Experimental

Isolation of the modified hairy regions and their degradation by RGase.—The MHR were isolated from juices obtained by liquefying the ground raw materials as described[7,10]. The degradation of MHR by RGase was performed after hydrolysis of methyl esters and *O*-acetyl groups by NaOH (resulting in MHR-S) as described by Schols et al.[8].

Chromatography.—High-performance anion-exchange chromatography (HPAEC) was performed on a Dionex Bio-LC system (Sunnyvale, CA) which included a quaternary gradient pump, eluent degas (He) module, a (4 x 250 mm) CarboPac PA1 column with matching guard column, and a pulsed electrochemical detector (PED) in the pulsed amperometric detection (PAD) mode. Samples were introduced into the system using a Spectra Physics SP8800 autosampler (San Jose, CA) and chromatograms were recorded with a Spectra Physics Winner system. The effluent was monitored using the PED detector (reference electrode Ag/AgCl) containing a gold electrode. Potentials of E1 0.1, E2 0.6 and E3 -0.6 V were applied for duration times T1 0.5, T2 0.1 and T3 0.1 s. The flow rate was 1.0 mL/min. The gradient was obtained by mixing solutions of 0.1 M NaOH and M NaOAc in 0.1 M NaOH. After an equilibration step of at least 15 min with 0.15 M NaOAc in 0.1 M NaOH, 20 μL of the sample was injected and a linear gradient to 0.5 M NaAc in 0.1 M NaOH within 40 min was started. The column was washed for 5 min with M NaOAc in 0.1 M NaOH and equilibrated again for 15 min with 0.15 M NaOAc in 0.1 M NaOH.

Isolation of oligomers from MHR digests.—Size-exclusion chromatography over Sephadex G50[8] was used to separate the oligomers from the high molecular weight fraction in the RGase digest of apple MHR-S.

The oligomeric fraction was further fractionated by preparative HPAEC using the Dionex system as described above. A CarboPac PA1 preparative column (9 x 250 mm) without guard column was used with a flow rate set at 5.0 mL/min. The gradient was slightly modified and 200 μL aliquots were injected. Following detection, the effluent was neutralized by the addition of M HAc (Peristaltic 2232 pump, Pharmacia/LKB, Uppsala, Sweden) and fractions were collected using a Helirac 2212 fraction collector (Pharmacia/LKB). Pooled fractions were dialyzed against distilled water and lyophilized.

Analytical methods.—Samples were hydrolyzed by 2 M trifluoroacetic acid at 121°C for 1 h and neutral sugars were converted into their alditol acetates as described[7], in order to determine the sugar composition. The uronic acid content was determined colorimetrically using *m*-hydroxybiphenyl as described[7].

NMR spectroscopy.—^{1}H NMR spectra were recorded for each fraction (1 mg or less in 0.5 mL of D_2O) at 400 MHz with a Jeol GX400 spectrometer. The residual water signal was suppressed by saturation during the 2 s delay between acquisitions. The number of transients was between 200 and 2000, depending upon the amount of sample available.

Acetone was added as internal reference and its chemical shift was set to 2.217 ppm with respect to Me$_4$Si.

Results

Analysis of oligomeric reaction products of RGase.—Most of the silica gel-based columns used in carbohydrate chromatography, including weak and strong anion-exchangers, were unable to retain the type of oligomer liberated by RGase. Also, chromatography on commercially available polystyrene diphenylbenzene resins was not very successful. On a 2% cross linked resin (Aminex HPX 22H), successfully used by Hicks and Hotchkiss[11] for the separation of maltodextrins and oligogalacturonides, we could only obtain limited resolution (results not shown). We also applied a newly developed technique which is based on the use of strong anion-exchange resins in combination with pulsed electrochemical detectors and which allows the use of gradient elution for improved separation of oligosaccharides[12-14].

Figure 1 shows the elution pattern of the RGase digest of apple MHR on a CarboPac PA1 column. Due to the high selectivity of the column under the conditions of the applied gradient, an elution pattern was obtained in which three major peaks and several minor peaks could be distinguished. Although not all components were separated completely, the separation could not be improved by changes in elution conditions. A possible explanation for this observation is the great similarity in structure of the various oligomers (see below). Direct quantification of the different oligomers by comparison of peak areas is not possible due to different response factors for different oligosaccharides[12,14-16].
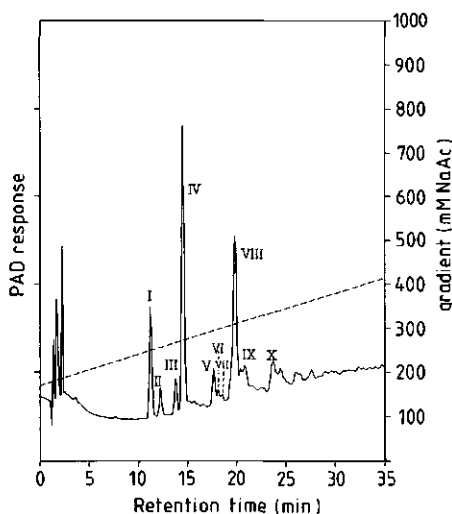

Fig. 1.   Elution profile on HPAEC of apple MHR-S after treatment with RGase at 30°C and pH 5.0 for 24h.

The estimated amounts of unknown oligomers can easily be in error. This is also the case when higher oligomers are quantified using the response factor of some monomeric sugar residues[12,15].

Sugar composition of the purified oligomers.—Preparative separation of the low molecular weight fraction was performed using a preparative CarboPac PA1 column which showed an equal or sometimes even better resolution between the different oligomers as compared to the analytical CarboPac PA1 column. The combined fractions were analyzed for their sugar composition. Since alditol acetates were prepared from very small quantities (30-100 μg), traces of xylose, mannose, and glucose were found, although these sugars were not detectable in the oligomer mixture before purification. For this reason, only the relative amounts of rhamnose, galactose and galacturonic acid are given in Table I. Using analytical HPAEC, it was shown that the preparative fractions I, II, V, VI, VII, and VIII (Table I) were essentially homogeneous. Others were found to be mixtures of closely eluting compounds. The preparative HPAEC isolation and subsequent pooling resulted in only a mixture of fractions III and IV, while fraction IX contained >70% of fraction VIII. All oligomer fractions except fractions I and V contained galactose, which is in agreement with earlier results[8,9]. However there was still insufficient information to deduce the complete structure and size of the oligomers, especially in the case of mixtures of fractions III/IV and VIII/IX. Confirmation of structure was also required since the order of elution of the different oligomers is not predictable and was found to depend on sugar residues and linkage types present as well as on the DP[14]. For this reason, further characterization of the oligomers was performed by NMR spectroscopy.

Oligomer structure from ¹H NMR spectra.—In previous work[9], an extensive series of 2D NMR experiments was used to deduce the structures of four rhamnogalacturonan oligosaccharides present as a mixture. To determine the linkage positions, it was necessary

TABLE I. Sugar composition (mol%) of oligomers, obtained after Sephadex G50 size exclusion chromatography and preparative HPAEC of the digest of apple MHR-S with RGase

Fraction	Major oligomer[a]	Rha	Gal	GalA
I	1	44	1	55
II	2	32	18	50
III/IV[b]	4	28	36	36
V	5	47	3	50
VI	5, 6	32	19	49
VII	6, ?	26	36	38
VIII	8a, 8b	29	35	36
IX	9	25	38	37
X	9, ?	34	46	21

[a] The numbers of the oligomers correspond with those given in Table II. [b] Components 3 and 4 pooled into one fraction.

to know the chemical shifts of protons in the extremely crowded spectral region from 3.5-4.2 ppm and, for this, 2D experiments were essential. With the structural information from the earlier study and the availability of fractions which contain only one major component, it has proved possible to use simple 1D ^{1}H NMR spectra to deduce the structures of further oligomers of this type. Characteristic signals in the less crowded regions of the spectrum provide sufficient information for structure determination, and allow the determination to be made on quantities of material which are insufficient for routine ^{13}C NMR studies. The signals for all the anomeric protons, together with those for H-4 and H-5 of the GalA units are found in the region from 4.3 to 5.4 ppm. Other well resolved signals are those between 3.3 and 3.5 ppm from H-4 of Rha units (unsubstituted by Gal) and those from H-6 of Rha (substituted and unsubstituted) between 1.22 and 1.31 ppm.

Figure 2 shows ^{1}H NMR spectra of two of these regions for fractions I, II, and III/IV

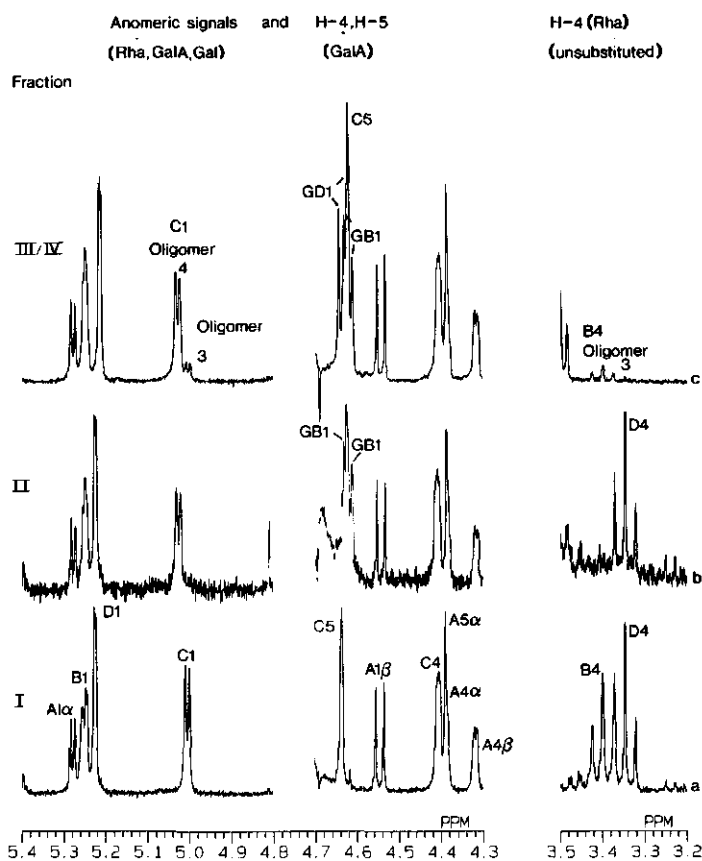Fig. 2. ^{1}H NMR spectra of fractions containing oligomers based on the (Rha-GalA)$_2$ backbone unit. Major oligomer components of the fractions are a) 1, b) 2 and c) 4. Key to individual assignments is given in Table III. The partly suppressed water signal (4.74 ppm) is not shown.

(mixture of two fractions). Structures of oligomers which have been identified are given in Table II and the lettering scheme for individual sugar units is given in Table III, together with selected chemical shifts. Fraction I contained only the tetramer **1** which is unsubstituted by β-Gal. Assignments (Fig. 2a) were obtained from earlier work[9] and the chemical shifts were in good agreement with those reported previously. The DP of **1** was established by integration of the anomeric signals of the non-reducing sugar rings with respect to the total integral ($\alpha + \beta$ forms) of the A1 resonances of the reducing end GalA unit. It may be noted that the chemical shift of the B1 resonance is affected by the anomeric form of the neighbouring A ring, and that there is apparent broadening of the B4 resonance (in comparison with D4) for the same reason.

Fraction II consisted entirely of the pentamer **2**, in which ring B is substituted with a β-Gal unit. The anomeric proton of this unit gave a doublet (J 7.6 Hz) at 4.625 ppm which overlapped with the C5 resonance of the GalA ring (Fig. 2b). It was found previously[9] that linkage of a β-Gal unit at O-4 of the Rha ring produced a downfield shift of 0.27 ppm for the H-4(Rha) resonance. The location of Gal on ring B, rather than ring D, was shown by the disappearance of the B4 triplet in Fig. 2b (shifted downfield from 3.399 ppm in Fig. 1a), whereas the D4 triplet was unaffected. Other chemical shift changes which accompany the linkage of the β-Gal unit to ring B are those of C1 and B6 (moved 0.02 and 0.06 ppm downfield respectively) and C5 (moved 0.01 ppm upfield), but the shift of B1 is hardly changed. Similar changes occur with the higher DP oligomers discussed below. It is also known[9] that the chemical shifts of B3 and B5 are appreciably affected by Gal substitution, but these shifts are not readily measurable from the 1D spectra used here.

TABLE II. Structures of identified oligomers, obtained after degradation of apple MHR-S by RGase

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

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

3	β-Gal$p$-(1 → 4)-α-Rha$p$-(1 → 4)-α-Gal$p$A-(1 → 2)-α-Rha$p$-(1 → 4)-Gal$p$A

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

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

6	α-Rha$p$-(1 → 4)-α-Gal$p$A-(1 → 2)-α-Rha$p$-(1 → 4)-α-Gal$p$A-(1 → 2)
	                                                                    α-Rha$p$-(1 → 4)-Gal$p$A
	                      β-Gal$p$-(1 → 4)

8a	β-Gal$p$-(1 → 4)-α-Rha$p$-(1 → 4)-α-Gal$p$A-(1 → 2)
	                                                  α-Rha$p$-(1 → 4)-α-Gal$p$A-(1 → 2)-α-Rha$p$-(1 → 4)-Gal$p$A
	           β-Gal$p$-(1 → 4)

8b	β-Gal$p$-(1 → 4)-α-Rha$p$-(1 → 4)-α-Gal$p$A-(1 → 2)-α-Rha$p$-(1 → 4)-α-Gal$p$A-(1 → 2)
	                                                                                    α-Rha$p$-(1 → 4)-Gal$p$A
	                      β-Gal$p$-(1 → 4)

9	β-Gal$p$-(1 → 4)-α-Rha$p$-(1 → 4)-α-Gal$p$A-(1 → 2)
	                                                  α-Rha$p$-(1 → 4)-α-Gal$p$A-(1 → 2)
	           β-Gal$p$-(1 → 4) α-Rha$p$-(1 → 4)-Gal$p$A
	                                 β-Gal$p$-(1 → 4)

TABLE III.   Selected ^{1}H chemical shifts[a] (ppm) for rhamnogalacturonan oligomers

Oligomer	Unit	Gal	Rha	GalA	Gal	Rha	GalA	Gal	Rha	GalA (r.e.)[b]
		GF	F	E	GD	D	C	GB	B	A
1	H-1					5.227	5.006		5.257α[c] 5.248β	5.280α 5.548β
	H-4					3.346	4.406		3.399	4.391α 4.320β
	H-5						4.637			4.391α
	H-6					1.227			1.242	
2	H-1					5.228	5.028	4.625	5.251	d
	H-4					3.346	4.412		e	
	H-5						4.627			
	H-6					1.228			1.301	
4	H-1				4.636	5.216	5.030	4.625	5.251	d
	H-4					e	4.409		e	
	H-5						4.626			
	H-6					1.291			1.301	
5	H-1		5.228	5.003		5.261	5.014		5.257	d
	H-4		3.345	4.407		3.394	4.407		3.403	
	H-5			4.642			4.656			
	H-6		1.227			1.243			1.243	
6	H-1		5.227	5.000		5.261	5.032	4.629	5.261	d
	H-4		3.343			3.392			e	
	H-6		1.227			1.243			1.303	
8a	H-1	4.637	5.216	5.044	4.624	5.262	5.014		5.262	d
	H-4		e			e			3.403	
	H-6		1.289			1.301			1.243	
8b	H-1	4.637	5.216	5.004		5.262	5.030	4.624	5.262	d
	H-4		e			3.394			e	
	H-6		1.289			1.243			1.302	
9	H-1	4.637	5.216	5.038	4.624	5.216	5.030	4.624	5.261	d
	H-4		e			e			e	
	H-6		1.289			1.303			1.303	

[a] ± 0.003 ppm. [b] r.e. = reducing end. [c] Effect of neighbouring r.e. [d] Ring A parameters same as **1**.
[e] Ring substituted by Gal, H-4 shifted to ± 3.67 ppm.

Fraction III/IV consisted mainly of the hexamer **4** plus a small amount (± 10%) of pentamer **3**. The presence of a second β-Gal unit was indicated by the addition of a second doublet at 4.636 ppm (Fig. 2c). In this region the two H-1 doublets of β-Gal overlap with the C5 resonance (4.626 ppm). Fig. 2c shows the disappearance of the D4 resonance, corresponding to complete Gal substitution at the O-4 position of ring D. However, small

residual signals from B4 (3.399 ppm) and C1 (5.006 ppm) are assigned to the pentamer **3**. Gal substitution of the terminal Rha residue shifts D1 upfield by 0.01 ppm, and D6 downfield by 0.06 ppm, but leaves other readily measurable resonances unchanged. Again, there are similar observations with higher DP oligomers. The oligosaccharides **1-4** were previously identified[9] in a mixture which contained all four species, but it was not possible to be very specific about the relative amounts of each component. Separation of individual components has allowed finer structural details (such as the exact position of Gal substitution) to be determined and has shown that the major constituents are the tetramer and hexamer.

Spectra of the fractions with a longer retention time on the HPAEC column are shown in Fig. 3. Fraction V (Fig. 3a) contained only the linear hexamer **5**, unsubstituted by Gal. The DP of the oligomer was obtained by integration of the anomeric resonances (as for **1**) but a comparison of Fig. 3a with Fig. 2a also shows clearly the new resonances assigned
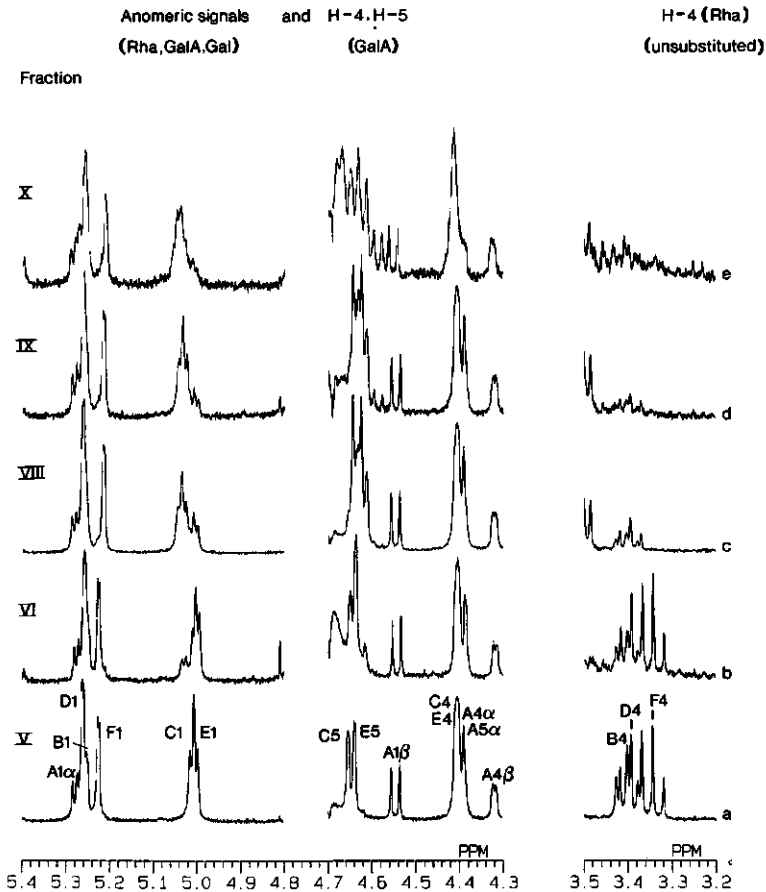

Fig. 3.   ¹H NMR spectra of fractions containing oligomers based on the (Rha-GalA)₃ backbone unit. Major oligomer components of the fractions are a) **5**, b) **5**, **6**, c) **8a**, **8b**, d) **9** and e) **9** + ?

to the additional GalA and Rha units. Resonances associated with the terminal reducing and nonreducing units have the same chemical shifts as in **1**. Thus F1, F4 and F6 have chemical shifts similar to those of the corresponding resonances from the terminal Rha residues of **1**. The B and D rings of **5** may be distinguished using the signals in the H-4(Rha) region since the B4 chemical shift is slightly affected by the anomeric form of the neighbouring A ring (*cf* **1**). This gives a slight broadening of the B4 resonance, which can therefore be assigned as the highest frequency triplet of the three H-4(Rha) multiplets.

The partial loss of B4 signal intensity in the spectrum of fraction VI (Fig. 3b) showed that VI did not consist of a single oligomer but was an approximately equimolar mixture of **5** and **6**. Substitution of ring B with a β-Gal unit changed the C1 chemical shift to 5.032 ppm and the loss of intensity of the high frequency H-1(GalA) doublet (5.014 ppm) suggested that the assignment of C1 and E1 in **5** was as indicated in Fig. 3a. Fraction VII (not shown) was rather similar to VI but contained a higher proportion of **6**, mixed with the other mono-Gal isomers.

Fraction VIII (Fig. 3c) consisted mainly of a mixture of the two isomers **8a** and **8b**. Disappearance of the F4 signal showed that the terminal Rha was completely substituted by β-Gal and this was confirmed by the change of the F1 shift to 5.216 ppm (as in **4**). Anomeric signals of the β-Gal units are assigned as the doublet centred on 4.637 ppm (Gal linked to terminal Rha; this doublet was not found in the spectrum of **6**) and the two doublets centred on 4.624 ppm (Gal linked to units B and D). Resonances C1 and E1 were shifted downfield by Gal substitution of rings B and D, respectively. If fraction VIII had consisted only of **8a** and **8b** the two downfield C1/E1 doublets would be expected to have the same intensity as the upfield pair. Integration shows a ratio of 2:1 rather than 1:1, so VIII must also contain some of the nonamer **9**. In fraction IX (Fig. 3d) this ratio has increased to 4:1 and from this it is estimated that the proportion of **9** is about 30% in fraction VIII and 60% in fraction IX.

It was thought that fraction X might contain oligomers based on a (Rha-GalA)$_4$ backbone unit. However the spectrum (Fig. 3e) was similar to that of **9**, and although H-1(Rha) and H-1(GalA) regions appeared more complex, integrations showed that the molecule was still based on the (Rha-GalA)$_3$ unit. Absence of signals from the 3.4 ppm H-4(Rha) region indicated a high level of Gal substitution (confirming the results of Table I). A doublet (J 7.2 Hz) on 4.59 ppm, which was just evident in Fig. 3d, had become more prominent in Fig. 3e. This signal could arise from the anomeric proton of β-Gal units which form part of longer galactan side chains. Shortage of material prevented further investigations of this fraction.

NMR studies of fractions V-IX have therefore shown the existence of a series of oligomers based on a hexameric rhamnogalacturonan unit, with progressively increasing amounts of galactose. Clean separation of the individual oligomers becomes more difficult as the molecular size increases. NMR provides a convenient and rapid alternative to conventional analytical methods when only small quantities of sample are available, even if the samples contain a mixture of closely related compounds.

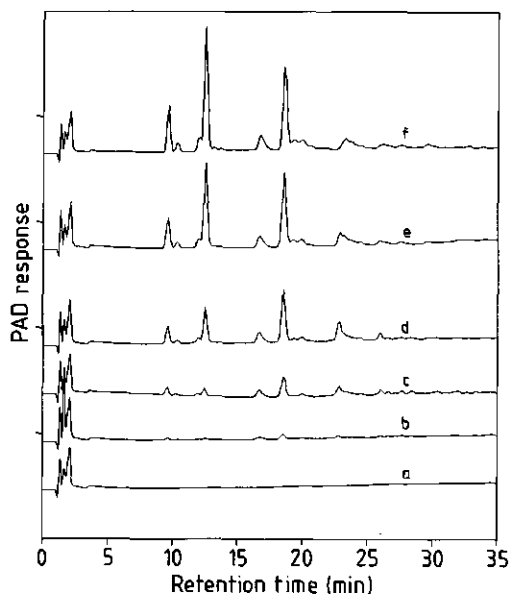*Time curves of the degradation of apple MHR by RGase.*—Now that the oligomers have

Fig. 4.   Elution profile on HPAEC of apple MHR-S after treatment with RGase at 30°C and pH 5.0 for
various incubation times. a: blank; b: 5 min; c: 15 min; d: 60 min; e: 300 min; f: 20 h.

been separated by HPAEC and their structures have been established by NMR
spectroscopy, the HPAEC method can be used to investigate the formation of these
reaction products during enzymic degradation of apple MHR. The degradation was
monitored by both HPSEC and HPAEC. The HPSEC elution patterns are similar to the
chromatograms previously published by Schols et al. (ref. 8, Fig. 3). The elution patterns
obtained on the anion-exchange column are shown in Fig. 4. The separation shown in Fig.
1 is significantly better than that shown in Fig. 4. The first separation was obtained on a
new CarboPac PA1 column and could not be reproduced for large series of digests.
However, the separation shown in Fig. 4 could be reproduced numerous times (more than
500 injections), even after using the column intensively for 1 year. From Fig. 4, it can be
seen that almost all of the oligomers were present from the initial stage of the RGase
action and that they increased together during the incubation time. The preponderant
oligomers in the initial stage were also the most important oligomers after prolonged
incubation times (Fig. 4; 5 min vs. 20 h of incubation). It can be seen from Fig. 4 that
the proportion of oligomer **4** is increasing slightly during incubation when compared to the
proportion of the other oligomers.

   Degradation of MHR from other sources by RGase.—Using the liquefaction process,
hairy regions could be isolated from a variety of fruit and vegetable tissues[10]. Striking
similarities were observed with respect to sugar composition, rhamnose-galacturonic acid
ratio, degree of acetylation, and degradability by RGase as monitored by HPSEC[10]. The
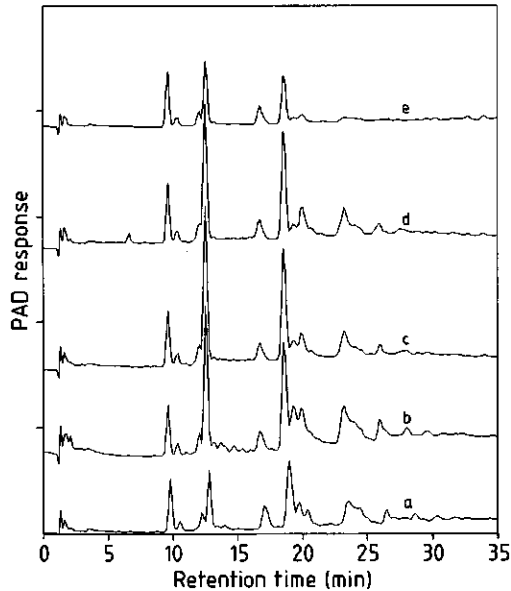
Fig. 5.   Elution profile on HPAEC of MHR-S, isolated from various sources after treatment with RGase
           at 30°C and pH 5.0 for 24 h. a: carrot; b: onion; c: leek; d: potato fiber; e: pear.

RGase digests of MHR originating from potato fibre, carrot, onion, leek, and pear tissue
were examined by HPAEC and the elution patterns were compared with those obtained for
apple MHR digests. Fig. 5 shows the elution patterns after 20 h of incubation. After this
incubation time, the degradation can be considered as complete[8,10]. The elution pattern of
pear MHR resembled that of apple MHR, and indicated a similar structure for both MHR
preparations. This is also indicated by the similarity in sugar composition[10]. In the digest
of onion MHR some oligomeric compounds were found which elute between hexamer **4**
and octamer **8**, as well as significant quantities of higher oligomers. These higher
oligomers seemed to be resistant to further degradation.

Discussion

It can be concluded that the newly developed HPAEC method is of great value in the
study of the fine structure of modified hairy regions, and the pattern of action of RGase.
Complete resolution of the oligomers eluting at higher concentrations of sodium acetate
could not be achieved, although Hotchkiss and Hicks[13] and Koizuma et al.[12] reported
separations with high resolution for oligogalacturonides and oligoglucosides up to a DP of
40-70. The poor resolution found for some of the RGase oligomers is probably caused by
the fact that these oligomers have a repeating unit which differs in substitution. In

contrast, the galacturonic acid oligomers and maltodextrins have simple linear structures. Structural diversity of the rhamnogalacturonans will result in (higher) oligomers having the same DP but with galactose residues linked at different positions in the molecule. They may not be readily separable.

Using the HPAEC method, we were able to isolate pure oligomers which could be partly characterized by their sugar composition. The different ratios of constituent rhamnose, galacturonic acid, and galactose residues indicated the presence of oligomers with different DP and different degrees of galactose substitution as found previously[9]. This was confirmed using NMR spectroscopy to identify components (1-4) based on the (Rha-GalA)$_2$ unit whilst additional oligomers with a basic (Rha-GalA)$_3$ unit were also recognized (5, 6, 8 and 9). The latter series has not been reported before in the literature. It should be mentioned that characterization of various oligomers from rhamnogalacturonan I, obtained after chemical degradation, has been reported[17,18] previously. The use of the specific rhamnogalacturonase however resulted only in the oligomers discussed, while the less specific chemical methods (e.g. β-elimination) resulted also in oligomers with side chains containing one or more arabinose and two galactose residues. In these studies[17,18] separation of the differently prepared oligomers was usually performed after reduction of the carboxyl group followed by permethylation of the free hydroxyl groups. This explains why the structure of the oligomers isolated differs enormously. However, the structure of the original rhamnogalacturonan as deduced from the information reported is quite similar. It can be concluded that isolation of the native oligomers and characterization by NMR as described here is more direct and the samples remain available for further experiments.

The structure of the identified oligomers confirmed earlier findings[8,9] that RGase is able to hydrolyze galacturonic acid-rhamnose linkages in the backbone in an endo-fashion. However, the variety, sequence and relative amounts in which the oligomers are formed in the course of the reaction are not typical of an endo-attack. All the oligomers identified are formed from the beginning and none of them appears to be degraded after prolonged incubation times. Even after an incubation time of 24 h, the hexamer 5 is still present (Fig. 1) and is apparently not degraded further. The relative amounts of 1 and 4 increased slightly compared with 5 and 8. Since there is no increase of small RGase oligomers at the expense of larger RGase oligomers, the increase in relative amounts of 1 and 4 must originate from polymeric material. The observed pattern of formation of the oligomers with time may be related to the structure of the rhamnogalacturonan regions in MHR. It is suggested that RGase is only able to hydrolyze linkages in the backbone after recognition of sequences of alternating rhamnose and galacturonic acid residues of a certain length. Depending on the structure of the backbone of MHR, this will result in the liberation of an oligomer with a tetrameric backbone, a hexameric backbone, or possibly even higher oligomeric fragments.

Although the oligomers identified consisted only of galacturonic acid, rhamnose and galactose, it is likely that higher oligomers might exist containing other sugar residues. Size-exclusion chromatography over Sephadex G50 showed[8] that a fraction containing higher oligomers (DP 10-20) with an arabinose content of 12 mol% was present in the

RGase digest of apple MHR. So far it is not known whether the arabinose is present as a single unit or as longer side chains (linear or branched).

The identified oligomers were also found in RGase digests of MHR from potato fibre, carrot, onion, leek, and pear. In spite of slight differences, there is a striking similarity in the elution patterns. Again, the ratio between the various oligomers formed in the course of the reaction remained rather constant, as described above for the RGase degradation products of apple MHR. Since all incubations were performed using the same substrate concentration, small differences in the total amount of liberated oligomers (Fig. 5) can be explained by the different sugar composition of the substrates. Depending on the source, some MHR fractions (apple and pear) contained considerably more arabinose, present as arabinan side chains of the rhamnogalacturonan. As a result of this, the actual substrate for RGase is present in lower concentrations. When HPSEC chromatograms of the various MHR preparations after RGase degradation were compared[10], the differences in amounts of oligomers formed were even more distinct. In contrast to HPSEC with refractive index detection, it should be realized that examination of the HPAEC elution patterns is not an appropriate method for absolute quantification of oligomers formed, because the response factor for the oligomers might be different. For this reason, HPSEC is a more valid method for judging the amount of oligomers formed after degradation with RGase.

These findings confirm that the structures of MHR isolated from a variety of sources are rather similar. As well as the similarity in sugar composition, degree of esterification, and degradability by RGase, the newly obtained elution patterns of the RGase digests can be seen as further evidence for the statement[7,10,19] that MHR is more regular in structure than generally assumed. In addition to the smaller oligomers such as 1, 4, and 8, a similar profile of higher oligomers is liberated from the various MHR substrates suggesting a high degree of structural homology between substrates. We believe that the breakdown pattern indicates that the rhamnogalacturonan degradation products do not originate from a long "homogeneous" backbone of rhamnose, and galacturonic acid residues as suggested by O'Neill et al.[20] for rhamnogalacturonan I. Since a variety of RG oligomers are liberated initially, and not degraded further, a more complex distribution of short rhamnogalacturonan units over the whole "hairy region" of the molecule is suggested. An adapted model of the modified hairy regions of apple pectin has been proposed[21] in which MHR consists of three subunits: a xylogalacturonan subunit interrupted by a subunit consisting of an arabinan-rich stub of the rhamnogalacturonan backbone and a subunit in which the identified rhamnogalacturonan oligomers are dominantly present. From this point of view, the various MHR under investigation are different from the RG-I as described by the Albersheim group[20], although interruption of the rhamnogalacturonan chain with two or more galacturonic acid (or rhamnose) residues is certainly possible and may explain the observed action of rhamnogalacturonase. In addition, RGase was found to be inactive on RG-I supplied by Albersheim's group (unpublished results). Since RGase incubation of the MHR fractions from diverse plant tissues resulted in comparable HPAEC elution patterns, the technique can be used to screen plant cell wall material for the presence of this type of pectic molecule. Now that the smaller oligomers liberated from MHR are well characterized, future research will be directed towards the high

molecular weight RGase degradation products of MHR, the segments of the rhamnogalacturonan backbone having various ratios of rhamnose to galacturonic acid, and the liberated "higher" oligomers with DP > 10.

References

1   A.J. Barrett and D.H. Northcote, *Biochem. J.*, 94 (1965) 617-627.

2   J.A. De Vries, F.M. Rombouts, A.G.J. Voragen, and W. Pilnik, *Carbohydr. Polym.*, 2 (1982) 25-33.

3   L. Saulnier and J.-F. Thibault, *Carbohydr. Polym.*, 7 (1987) 345-360.

4   L. Saulnier, J.-M. Brillouet, and J-P. Joseleau, *Carbohydr. Res.*, 182 (1988) 63-78.

5   H. Konno, Y. Yamasaki, and K. Katoh, *Phytochemistry*, 25 (1986) 623-627.

6   H. Kiyohara, J-C. Cyong, and H. Yamada, *Carbohydr. Res.*, 182 (1988) 259-275.

7   H.A. Schols, M.A. Posthumus, and A.G.J. Voragen, *Carbohydr. Res.*, 206 (1990) 117-129 (this thesis, chapter 3).

8   H.A. Schols, C.C.J.M. Geraeds, M.F. Searle-van Leeuwen, F.J.M. Kormelink, and A.G.J. Voragen, *Carbohydr. Res.*, 206 (1990) 105-115 (this thesis, chapter 4).

9   I.J. Colquhoun, G.A. de Ruiter, H.A. Schols, and A.G.J. Voragen, *Carbohydr. Res.*, 206 (1990) 131-144 (this thesis, chapter 5).

10 H.A. Schols and A.G.J. Voragen, *Carbohydr. Res.*, 256 (1994) 83-95 (this thesis, chapter 6).

11 K.B. Hicks and A.T. Hotchkiss Jr., *J. Chromatogr.*, 441 (1988) 382-386.

12. K. Koizuma, Y. Kubota, T. Tanimoto, and Y. Okada, *J. Chromatogr.*, 464 (1989) 365-373.

13 A.T. Hotchkiss Jr. and K.B. Hicks, *Anal. Biochem.*, 184 (1990) 200-206.

14 Y.C. Lee, *Anal. Biochem.*, 189 (1990) 151-162.

15 R.N. Ammeraal, G.A. Delgado, F.L. Tenbarge, and R.B. Friedman, *Carbohydr. Res.*, 215 (1991) 179-192.

16 J. van Riel and C. Olieman, *Carbohydr. Res.*, 215 (1991) 39-46.

17 J.M. Lau, M. McNeil, A.G. Darvill, and P. Albersheim, *Carbohydr. Res.*, 168 (1987) 245-274.

18 H. Kiyohara and H. Yamada, *Carbohydr. Res.*, 187 (1989) 117-129.

19 J.A. De Vries, in G.O. Phillips, D.J. Wedlock, and P.A. Williams (Eds), *Gums and Stabilisers for the Food Industry 4*, IRL Press, Oxford, 1988, pp 25-29.

20 M. O'Neill, P. Albersheim, and A. Darvill, in P.M. Dey and J.B. Harborne (Eds), *Methods in Plant Biochemistry*, Vol. 2 (1990) pp 415-441.

21 A.G.J. Voragen, H.A.Schols, and H. Gruppen, in F. Meuser, D.J. Manners, and W. Seibel (Eds.), *Plant Polymeric Carbohydrates*, Royal Society of Chemistry, Cambridge, UK, 1993, pp 3-15.

CHAPTER 8

DIFFERENT POPULATIONS OF PECTIC HAIRY REGIONS OCCUR IN APPLE CELL WALLS

H.A. Schols, E. Vierhuis, E.J. Bakx, and Alfons G. J. Voragen

Abstract

Apple Alcohol Insoluble Solids were extracted in sequence by buffer at 20°C and at 70°C, EDTA/oxalate, and mild alkali yielding four populations of pectins. These pectins and the insoluble residue were characterized by their sugar composition, degree of esterification (methyl ester and *O*-acetyl groups), molecular weight distribution, and their degradability by the combination of endopolygalacturonase (PG) and pectin esterase (PE) and by rhamnogalacturonase (RGase) after chemical saponification. After PG/PE treatment, the remaining high molecular weight material representing the pectic hairy regions was isolated and characterized. Clear differences were found in the sugar composition of the fractions obtained, while only small variations were observed in the sugar linkage composition. The pectic hairy regions were further degraded by RGase and the digests separated into high molecular weight and oligomeric degradation products. Both the absolute amount of RGase oligosaccharides released as well as the degree of galactose substitution of the oligomers increased when more severe extraction conditions were used. Relatively more RGase oligomers were released from the low molecular weight hairy regions as compared to the high molecular weight fraction. Typical high molecular weight fragments isolated from the RGase digests of various hairy regions included residual segments of the rhamnogalacturonan backbone rich in arabinose and a polymer presumably enriched in xylogalacturonan segments.

Introduction

Pectins are important constituents of plant cell walls and are considered to consist partly of homogalacturonan regions ("smooth regions") which are interrupted by ramified regions[1-3], referred to as hairy regions in this study. The homogalacturonan segments are reported to have a length of 72-100 galacturonic acid residues[3] and part of the galacturonic acid residues are methyl esterified. The ramified regions, usually obtained after extensive degradation of the homogalacturonan regions by polygalacturonase (PG), may consist completely of alternating rhamnose and galacturonic acid residues as has been demonstrated in rhamnogalacturonan I (RG-I) isolated from sycamore cells[2]. Hairy or ramified regions have also been isolated from enzyme treated tissue or pectin fractions from e.g. carrot[4,5], grape berries[6], apple[1,7,8], onion[9], and the roots of *Bupleurum falcatum* L[10]. In most cases the galacturonic acid to rhamnose ratio in these fractions was found to be much higher than a straight alternating RG-I would suggest.

The structure of apple pectins and the hairy regions thereof and their enzymic degradability have been studied extensively[1,7,8,11-15]. The most striking feature of apple pectin is that large amounts of arabinose residues are present in highly branched side chains[12]. De Vries et al.[16] reported the presence of two types of (arabino) galactans in apple pectic hairy regions: $(1\rightarrow3,6)$-linked galactan side chains preferentially present in the cold buffer extracted pectins and $(1\rightarrow4)$-linked galactans present in pectins extracted under more harsh conditions.

The structure of the hairy regions, isolated from apple and various other fruits and vegetables using the enzymic liquefaction process, has been studied in detail[17-19]. Since a wide spectrum enzyme preparation was used, the pectin fraction was designated MHR (modified hairy regions). Using rhamnogalacturonase[20] (RGase), structural subunits present in MHR could be recognized. Next to the characteristic RGase oligomers[19,21], also high molecular weight degradation products present in the digest were isolated and characterized. Evidence was obtained[22] that apple MHR consisted of a xylogalacturonan subunit, a subunit representing residual stubs of the backbone rich in arabinan side chains and a rhamnogalacturonan part of unknown length.

Although studies on the fine-structure of apple MHR, available in high amounts, increased our knowledge on pectin structures present in the cell wall, it should always be realized that structures present in MHR might be the results of enzymic alterations during the cell wall liquefaction process. The method of isolation did also result in an accumulation of (modified) hairy regions originating from completely different pectin molecules (varying in extractability or even insoluble pectins) into one single fraction.

In this paper, the distribution of hairy regions over different populations of pectic molecules present in the cell wall is described. After a brief characterization of differently extracted pectins, attention is paid to the different hairy regions, their degradability by RGase and the presence and distribution of subunits within these hairy regions.

Experimental

Isolation of various pectin fractions.—Golden Delicious apples were extracted with ethanol as described by De Vries et al.[11] to yield Alcohol Insoluble Solids (AIS). The AIS were subsequently extracted using 0.05 M NaOAc buffer pH 5.2 at room temperature and at 70°C, then 0.05 M EDTA/0.05 M NH$_4$-oxalate in 0.05 M NaOAc pH 5.2 (70°C) as described[11], followed by an extraction with 0.05 M NaOH (0°C). Fractions obtained were dialyzed and lyophilized to yield Cold Buffer Soluble Solids (CBSS), Hot Buffer Soluble Solids (HBSS), Chelating Agents Soluble Solids (ChSS), Alkali Soluble Solids (ASS), and the Residue (RES) respectively.

Isolation of the hairy regions (HR).—Solutions of the various pectin fractions (0.2%) in 0.05 M NaOAc pH 5.0 were incubated (20 h, 40°C) with the following purified enzymes, free from relevant side-activities: RGase from *Aspergillus aculeatus*[17,20], PG from *Kluveromyces fragiles*[17] and PE from *Aspergillus niger*[17]. The pectins, present in CBSS, HBSS, and ChSS still contained methyl esters which limited the action of PG (not shown). For this reason, purified fungal PE was added in sufficient amounts to de-esterify all pectins within 5 h. After incubation, enzymes were inactivated (5 min, 100°C), and the digests were analyzed by HPSEC (high-performance size-exclusion chromatography) and HPAEC (high-performance anion-exchange chromatography). Fractions were also incubated on semi-preparative scale using ± 1 g substrate in 200 mL 0.05 M NaOAc pH 5.0. Since the ASS fraction was almost insoluble in the buffer, 2 mM EDTA was added to the buffer and the mixture was heated (5 min, 100°C) to enhance solubilization. The digests were concentrated and applied onto a Sephacryl S300 column as described below resulting in the fractions HR-I and HR-II.

To check whether the homogalacturonan regions in CBSS, HBSS, ChSS were completely removed by the mixture of PG and PE, the HR fractions were chemically saponified as described below and subjected to an additional incubation with PG. All digests were analyzed by HPSEC and HPAEC.

Saponification of the hairy regions.—The methyl esters and *O*-acetyl groups in the HR were removed by treatment with 0.05 M NaOH as described[17,18], followed by neutralization. After adjustment of the solutions to the desired buffer pH and molarity, they were directly used in enzymic degradation studies.

Analytical methods.—Samples were hydrolyzed by M H$_2$SO$_4$ (100°C, 3 h) as described[17] and neutral sugars were converted to their alditol acetates[17] to determine the sugar composition. The insoluble AIS and RES fractions were first pre-hydrolyzed using aqueous 72% H$_2$SO$_4$ (30°C, 1 h) to include cellulosic material[17]. The uronic acid content was determined colorimetrically using *m*-hydroxybiphenyl[17]. Methylation analysis was performed as described previously[17].

Chromatography.—HPSEC was performed on three Bio-Gel TSK columns in series (40XL, 30XL, and 20XL) as described[17]. For HPAEC analysis, a Dionex Bio-LC system which included a quaternary gradient pump, eluent degas (He) module, and pulsed electrochemical detector (PED) in the pulsed amperometric mode (PAD), completed with a Spectra Physics SP8800 autosampler and a Spectra Physics Winner data handling system

was used as described[19]. A CarboPac PA100 column (4 x 250 mm) with matching guard column (Dionex) was used at a flow rate of 1.0 mL/min. The gradient was obtained by mixing solutions of 0.1 M NaOH and M NaOAc in 0.1 M NaOH. After an equilibration step of 15 minutes with 0.1 M NaOAc in 0.1 M NaOH, 20 μl of the sample was injected and a linear gradient to 0.35 M NaOAc in 0.1 M NaOH within 40 minutes was started. The column was washed for 5 min with M NaOAc in 0.1 M NaOH and equilibrated again for the next injection.

Size-exclusion chromatography on a preparative scale of CBSS-HR-II, HBSS-HR-I and ChSS-HR-II was performed on a column (100 x 26 mm) of Sephacryl S300 (separation range for dextrans 2 x 10³-4 x 10⁵ Da) using a Hiload System (Pharmacia) and 0.05 M NaOAc (pH 5.0) as eluent (2.5 mL/min). Fractions (2.5 mL) were collected and analyzed for neutral sugars and uronides as described; arabinose and galacturonic acid were used as standards, respectively. Pooled fractions were dialyzed and lyophilized before analysis.

Results

Characterization of the various pectin fractions.—Starting from the Alcohol Insoluble Solids isolated from Golden Delicious apples, four different pectin fractions were obtained: cold buffer soluble solids (CBSS), hot buffer soluble solids (HBSS), chelating agent soluble solids (ChSS) and alkali soluble solids (ASS), next to the remaining insoluble residue (RES). All fractions were analyzed for their sugar composition and degrees of esterification (Table I). It can be seen that the proportion of galacturonic acid residues varied between 45 and 80 mol% in the pectin fractions, while the RES fraction is relatively poor in this sugar (10 mol%). The degree of methylation (DM) is rather high for CBSS, HBSS and ChSS, while no ester groups were present in ASS and RES as a result of saponification. The ratio neutral sugars to galacturonic acid is low for the easily extractable pectins (CBSS and HBSS) and higher for the pectins extracted with chelating agents (ChSS) and alkali (ASS). This higher ratio for ChSS and ASS is mainly due to the increased amounts of arabinose and galactose in these extracts.

Since most arabinose and galactose side chains are reported[1,22] to be arranged in blocks (the so-called hairy regions), the localization of these blocks in the pectic backbone is studied by the use of RGase. Fig. 1A shows the HPSEC elution patterns of the saponified pectins without any enzyme treatment. It can be seen that CBSS, HBSS, and ChSS consisted of high molecular weight pectins, although a broad molecular weight range is covered. No ASS and RES material was eluted from the column. This was expected for the RES fraction (insoluble in buffer and diluted alkali), but according to the isolation scheme and the characteristics as shown in Table I, ASS should contain soluble pectic material as well. Although ASS pectins were almost insoluble in the incubation buffer, the addition of a small amount of chelating agent (2 mM EDTA) solubilized most material, but these pectins were still not eluted from the HPSEC column material. Peaks appearing at 34 minutes did not represent carbohydrates and were caused by some residual amounts of EDTA (ChSS fraction) or other salts present in the sample. Small differences were ob-

TABLE I. Sugar composition (mol%) of pectins, extracted from apple AIS.

Sugar	Pectin fraction					
	AIS	CBSS	HBSS	ChSS	ASS	RES
Rha	2 (1.19) [a]	1 (0.10)	2 (0.09)	3 (0.23)	3 (0.13)	1 (0.52)
Fuc	1 (0.87)	tr (0.01)	tr (0.01)	tr (0.01)	tr (0.01)	2 (0.79)
Ara	12 (8.05)	10 (0.70)	15 (0.69)	28 (1.92)	33 (1.23)	9 (3.96)
Xyl	8 (5.13)	1 (0.07)	2 (0.09)	4 (0.26)	5 (0.17)	11 (4.95)
Man	2 (2.00)	1 (0.08)	2 (0.09)	1 (0.06)	1 (0.04)	3 (1.60)
Gal	7 (5.71)	5 (0.41)	6 (0.35)	8 (0.65)	11 (0.53)	6 (3.45)
Glc	38 (31.2)	2 (0.16)	2 (0.13)	1 (0.12)	2 (0.08)	58 (31.5)
GalA	30 (26.8)	80 (7.71)	71 (4.37)	55 (4.99)	45 (2.31)	10 (5.82)
OMe	83(4.00)	73(1.02)	72(0.58)	82 (0.74)	0	0
OAc	19(1.70)	3(0.07)	5(0.08)	13 (0.23)	0	0
total sugars	81%	93%	83%	63%	45%	88%

[a] Gram quantities per residue in the fractions originating from 100 g AIS.

served when these patterns were compared with the elution patterns of the non-saponified material (results not shown). The saponified pectins were eluted at somewhat shorter retention times (i.e. higher molecular weights), probably due to ionic effects of the free carboxylgroups.

Enzymic degradation of various pectin fractions.—The HPSEC elution patterns of the various pectins after treatment by RGase are shown in Fig. 1B. Small amounts of oligomers were liberated and appeared at 32-33 min. Using HPAEC, it was shown that these oligomers represented the characteristic RGase oligomers as reported before[19]. The release of RGase oligomers will be discussed in detail below. Next to the formation of oligomers, changes in the elution patterns can be observed in the polymeric range of the various pectin fractions. Some material is apparently resistant to further degradation by RGase, while another part shifts to lower molecular weight ranges. In general, RGase action resulted in the release of small amounts of the typical RGase oligomers; CBSS, however, showed almost the same HPSEC elution behaviour before and after enzyme treatment. In the HBSS and ChSS fraction, RGase action resulted in a conversion of part of the high molecular weight material into material of lower molecular weights. In the ASS and the RES fraction, RGase was able to solubilize material which appeared near 26 min.

The HPSEC elution patterns of the different pectins after PE/PG treatment are shown in Fig. 1C. As expected for CBSS, HBSS, ChSS, and ASS pectin, this enzymic degradation resulted in a drastic shift in retention time for a major part of the material. Degradation products were mainly monogalacturonic acid and some galacturonic acid oligomers in the
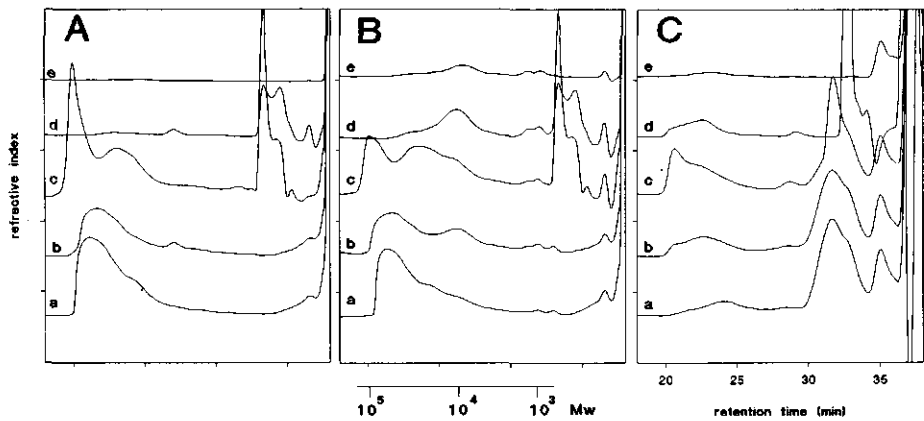
Fig. 1. High-performance size-exclusion chromatography elution patterns of various chemically saponified
extracts from apple AIS before degradation (A), after treatment by RGase (B) and after treatment
with PG and PE (C). a: CBSS; b: HBSS; c: ChSS; d: ASS; e: RES.

range of DP 2-7 (HPAEC, results not shown) which were eluted under HPSEC conditions
at retention times of 30 min or more. Smaller amounts of galacturonosyl oligomers were
found in the digest of ASS and RES. Residual high molecular weight material, resistant to
the combination of PG and PE, was recognized to be present in all digests, although the

TABLE II.   Sugar composition (mol%) of pectic hairy regions population I and II, obtained after
treatment of different pectin fractions of apple AIS with pectin esterase and
polygalacturonase and size-exclusion chromatography.

Sugar	CBSS-HR		HBSS-HR		ChSS-HR		ASS-HR		RES-HR	
	I	II	I	II	I	II	I	II	I	II
Rha	4	4	5	5	4	4	3	4	3	5
Fuc	2	1	tr	tr	tr	tr	tr	tr	tr	tr
Ara	49	44	52	49	47	66	52	55	52	54
Xyl	5	2	5	3	10	4	5	6	4	6
Man	4	1	1	1	tr	0	1	2	0	0
Gal	18	29	19	21	14	9	20	17	29	16
Glc	4	1	1	1	tr	0	2	3	0	0
GalA	14	19	18	20	25	16	18	14	12	19
Rha:GalA	0.29	0.36	0.28	0.25	0.16	0.25	0.17	0.29	0.25	0.26
Ara:Gal	2.72	1.62	2.74	2.33	3.36	7.33	2.60	3.24	1.79	3.38
rel. amount of HR[a]	1.0	4.8	5.2	4.9	10.6	2.5	3.2	2.2	0.7	0.6

[a] The amount of HR-I was arbitrarily set to 1.0

relative amounts varied. From the HPSEC elution patterns, it was calculated that 14%, 27% and 53% of the polymers present in the original material were eluted at retention times below 30 min (>5000 Da) for the PE/PG digests of CBSS, HBSS, and ChSS pectins respectively. The PE/PG resistant polymeric material was found to be present in reasonable amounts in the digest of ASS and, in minor amounts, in the digest of RES.

Isolation and characterization of pectic hairy regions.—The polymeric material remaining after PE/PG treatment is believed to represent the so-called pectic hairy regions. To study these pectin segments, they were produced and isolated in larger amounts. Digests having identical elution behaviour during HPSEC analysis as described above were obtained and were fractionated preparatively using Sephacryl S300 (results not shown). Also on Sephacryl S300 the digest showed two different high molecular weight populations, next to large amounts of uronide oligomers eluting in the included volume of the column. These two different high molecular weight populations were pooled separately, dialyzed and lyophilized to yield hairy regions I and II (HR-I and HR-II). As can be seen from Table II, the relative amounts of the various HR fractions which have been isolated differed significantly and are in agreement with the relative amounts per fraction as shown in Fig. 1C. From the sugar composition of the fractions (Table II), it can be seen that the relative amounts of galacturonic acid in all HR-I and HR-II fractions is significantly lower than in the original pectin fraction. The hairy regions are especially rich in arabinose (44-66 mol% compared with 12 mol% in AIS) and galactose residues (9-29 mol% compared with 7 mol% in AIS). Rhamnose and xylose residues were present in minor amounts (3-5 and 2-10 mol% respectively), while glucose, mannose, and fucose residues were present only in some of the fractions. Although small differences in the sugar composition between the HR-I and HR-II for each pectin extract were found, no specific trends in the sugar composition of either HR-I or HR-II could be observed. Unfortunately, not enough material was obtained to allow examination of the degree of methyl esterification and *O*-acetylation in the HR-I and HR-II fractions.

Figure 2a and b show the HPSEC elution patterns of the HR-I and HR-II fractions. Clear differences in retention times can be seen between the corresponding fractions I and II. Sometimes a broader molecular weight distribution could be observed within one group of hairy regions (Fig. 2A lines d and e). It should be mentioned that the first distinct peak for ASS-HR-I and RES-HR-I is eluted in the "void volume" of the column set used and might not represent a distinct population. No differences in elution pattern were observed when HR fractions were saponified prior to analysis by HPSEC.

It was confirmed that PE/PG had degraded all the homogalacturonan regions by chemical saponification of the HR fractions prior to re-incubation by PG. HPSEC analysis showed little change (results not shown), while only very limited amounts o₁ monogalacturonic acid could be detected by HPAEC.

Methylation analysis was carried out without reducing the galacturonosyl residues. The results obtained were in good agreement with those published before[12,16,17,23]. They showed that in the HR fractions isolated from CBSS, HBSS, and ChSS, half of the rhamnose residues were (1→2)-linked while the other half were also branched through C-4. In ASS-HR, a higher proportion of rhamnose was branched. The glycosidic linkage composition
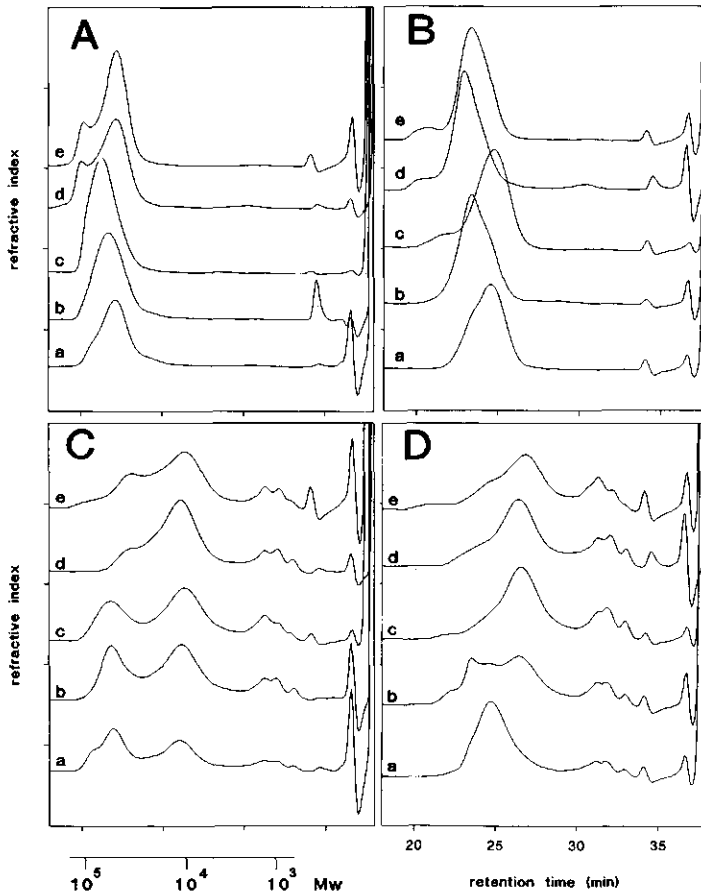
Fig. 2.   High-performance size-exclusion chromatography elution patterns of HR-I and HR-II fractions
           isolated from various extracts from apple AIS before degradation (respectively A and B) and after
           treatment with RGase (respectively C and D). a: CBSS; b: HBSS; c: ChSS; d: ASS; e: RES.

found for arabinose is typical of a highly branched arabinan chain, while no principal
differences were observed for the various HR fractions. In general, half of the $(1\rightarrow3)$- and
$(1\rightarrow5)$-linked arabinose residues carried one or two terminally linked arabinose residues,
with the exception of ChSS-HR-II which is more linear. Galactose residues were found to
be involved in many different types of linkages. In CBSS-HR, they were mainly $(1\rightarrow3)$-
,$(1\rightarrow6)$- and $(1\rightarrow3,6)$-linked, while in the other HR fractions $(1\rightarrow4)$-linked galactose
residues were also present in about equal amounts. A relatively high proportion of all
galactose residues were terminally linked. Xylose was mainly present as terminally linked
residues, although the proportion of terminally linked xylose decreased for the HR
fractions from ASS and RES.

TABLE III. Structures of identified oligomers, obtained after degradation of apple MHR-S by RGase[19].

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

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

3	β-Gal$p$-(1 → 4)-α-Rha$p$-(1 → 4)-α-Gal$p$A-(1 → 2)-α-Rha$p$-(1 → 4)-Gal$p$A

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

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

6	α-Rha$p$-(1 → 4)-α-Gal$p$A-(1 → 2)-α-Rha$p$-(1 → 4)-α-Gal$p$A-(1 → 2)
	                                                      α-Rha$p$-(1 → 4)-Gal$p$A
	                                β-Gal$p$-(1 → 4)

8a	β-Gal$p$-(1 → 4)-α-Rha$p$-(1 → 4)-α-Gal$p$A-(1 → 2)
	                                    α-Rha$p$-(1 → 4)-α-Gal$p$A-(1 → 2)-α-Rha$p$-(1 → 4)-Gal$p$A
	                  β-Gal$p$-(1 → 4)

8b	β-Gal$p$-(1 → 4)-α-Rha$p$-(1 → 4)-α-Gal$p$A-(1 → 2)-α-Rha$p$-(1 → 4)-α-Gal$p$A-(1 → 2)
	                                                                  α-Rha$p$-(1 → 4)-Gal$p$A
	                                              β-Gal$p$-(1 → 4)

9	β-Gal$p$-(1 → 4)-α-Rha$p$-(1 → 4)-α-Gal$p$A-(1 → 2)
	                                    α-Rha$p$-(1 → 4)-α-Gal$p$A-(1 → 2)
	                  β-Gal$p$-(1 → 4) α-Rha$p$-(1 → 4)-Gal$p$A
	                                              β-Gal$p$-(1 → 4)

RGase degradation of HR.—Using HPSEC, the HR-I and HR-II fractions were also examined for their degradability by RGase (Fig. 2C and 2D). RGase treatment resulted in partial degradation of the high molecular weight material, next to the formation of oligomers appearing at 32-33 min. The HR fractions, originating from pectins extracted under more severe conditions, seemed to be degraded more readily. HR-I fractions of CBSS, HBSS, and ChSS were incompletely degraded and next to the oligomers also a new population appeared at 26 min. About half of the material was eluted at the same time as the non-treated material. The HR-I fractions of ASS and RES were converted almost completely into lower molecular weight fragments. Also most of the HR-II fractions shifted readily to longer elution times. RGase acted differently on CBSS HR-II as compared to the other HR-II fractions. Although RGase was also able to liberate oligomers from CBSS-HR-II, the bulk of the material was eluted at about the same retention time as the non-treated material.

Characterization of oligomeric degradation products.—RGase digests of HR-I and HR-II were also analyzed by HPAEC and as can be seen in Fig. 3A and 3B, characteristic RGase oligomer profiles were obtained. In addition to peaks already identified[19] (Table III), minor quantities of unknown oligomers eluted at higher buffer concentrations could be observed. It can be seen that relatively low amounts of oligomers could be released from CBSS-HR-I and HR-II, while these amounts increased for HR-digests obtained from the more difficult-extractable pectins. Another clear observation is that RGase liberated relative high amounts of oligomer **1** and **5** (without galactose substitution) from the HR-I and HR-II fractions of CBSS and HBSS and from the HR-II fraction of ChSS pectin. RGase treatment of the other HR fractions resulted in the release of higher amounts of oligomers containing two rhamnose residues carrying a galactose branch (oligomer **4** and **8a/b**). Except for the HR-I and HR-II fraction of the ChSS pectin, only minor differences
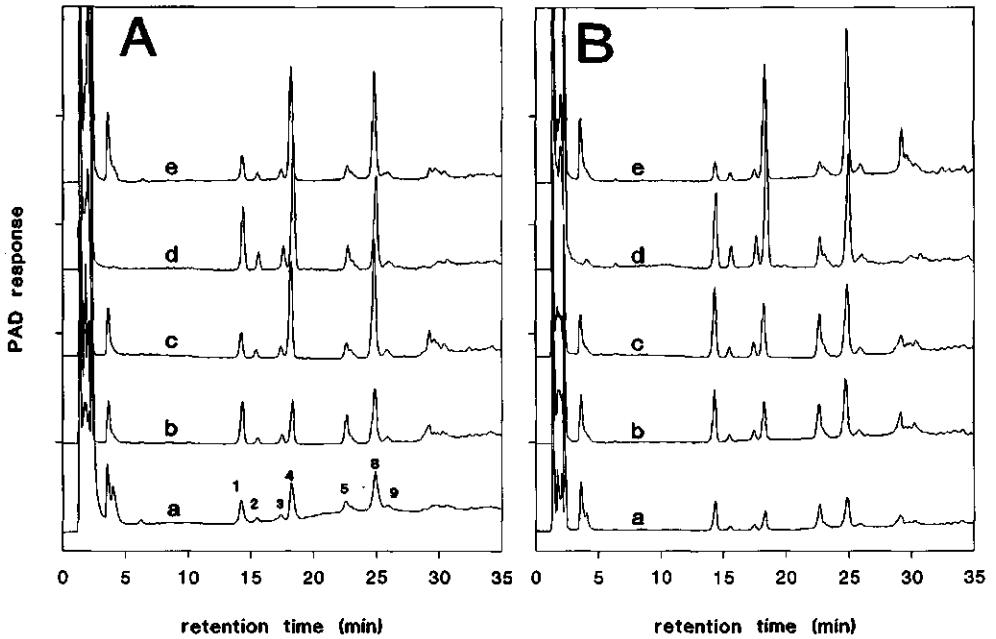
Fig. 3. High-performance anion-exchange chromatography elution patterns of HR-I (A) and HR-II (B)
         isolated from various extracts from apple AIS after treatment with RGase. a: CBSS; b: HBSS; c:
         ChSS; d: ASS; e: RES.

TABLE IV. Relative amounts of the various oligomers liberated during the incubation of different apple
           hairy regions by rhamnogalacturonase (expressed in μg GalA per mg sugars in the hairy
           regions).

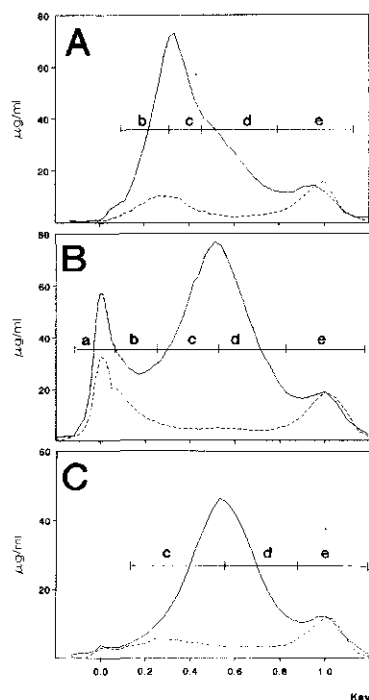| Pool		Oligomer							
		1	2	3	4	5	8a/b	9	total
CBSS	I	4	1	1	7	2	9	1	25
	II	6	1	1	4	7	9	1	29
HBSS	I	8	1	2	7	7	12	1	38
	II	12	2	3	9	12	16	2	56
ChSS	I	7	2	3	20	7	28	3	70
	II	18	2	4	13	16	24	3	80
ASS	I	15	4	6	31	9	25	3	93
	II	19	5	8	37	12	33	6	120
RES	I	5	1	2	23	5	24	3	63
	II	4	1	2	26	6	37	3	79

Fig. 4. Size-exclusion chromatography on Sephacryl S300 of the RGase digest of CBSS-HR-II (A), HBSS-HR-I (B), and ChSS-HR-II (C). ———, neutral sugars; ---- , uronic acids.

could be observed in the HPAEC elution profile of the RGase digests of HR-I and HR-II of the same pectin fraction. To be able to draw conclusions from data instead of judging only the chromatograms, the RGase oligomers were quantified using galacturonic acid as standard (Table IV). It can be seen that on a sugar weight basis, the relative proportion of each oligomer increased from the CBSS fraction to the ASS fraction, while for the HR fractions originating from the RES fraction this proportion was somewhat lower. It also can be concluded that more oligomers were formed from the HR-II fractions as compared to the higher molecular weight material of the HR-I fractions.

   Characterization of polymeric degradation products.—Although not enough material has been isolated yet to be able to study all polymeric fragments which remained after digestion of the HR fractions by RGase, some digests were fractionated on Sephacryl S300. We have chosen for the examination of CBSS-HR-II which hardly shifted towards lower molecular weights after RGase treatment; HBSS-HR-I which was divided into two high molecular weight populations by RGase and for ChSS-HR-II shifting as one peak to lower molecular weights after degradation by RGase (Fig. 2C and 2D). Elution patterns as determined from neutral sugar and uronide content in eluent fractions after chromatography on Sephacryl S300 are shown in Fig. 4. As expected, great resemblance

TABLE V. Sugar composition (mol%) of degradation products, obtained after treatment of different hairy regions fractions of apple AIS with RGase.

Sugar	CBSS-HR-II			HBSS-HR-I				ChSS-HR-II	
	b	c	d	a	b	c	d	c	d
Rha	5	2	3	5	4	2	2	2	3
Fuc	1	tr	tr	1	tr	tr	tr	tr	tr
Ara	35	49	70	23	32	70	71	62	73
Xyl	4	2	2	14	9	2	2	8	1
Man	2	1	2	2	2	1	1	2	1
Gal	37	34	7	24	23	13	13	7	7
Glc	2	1	3	3	3	2	2	1	2
GalA	15	9	14	28	26	10	10	19	12

was noticed between these elution patterns and the HPSEC patterns as shown in Fig. 2C and 2D. From the elution patterns on Sephacryl S300, it can be seen that the polysaccharide fraction eluting in the columns void volume contained quite some galacturonic acid residues. The same is true for the RGase oligomers eluting at the included volume (Kav = 1.0). Fractions were pooled as indicated and all except pool *e* containing the oligomers were examined for their sugar composition (Table V). From both Fig. 4A and Table V, it appeared that pool *c* of the degraded CBSS-HR-II is (at least partly) an intermediate of pool *b* and *d*. The same trends can be observed even more clear for the sugar composition of the pools obtained after degradation of HBSS-HR-I by RGase. The highest molecular weight pools (*a* and *b*) consisted of arabinose, xylose, galactose and galacturonic acid, next to rhamnose and some other minor sugar residues. The ratio arabinose:galactose is about 1:1 as was found for is in CBSS-HR-II-b. The pools *c* and *d*, representing the population eluting at 26 min under HPSEC conditions (Fig. 2D), consisted mainly of arabinose residues (70 mol%), next to some galacturonic acid, galactose, and rhamnose residues. Both pool *c* and *d* of the digest of ChSS-HR-II were rich in arabinose and the composition of pool *d* resembled that of the other pools *d*. The first half of the peak on Sephacryl S300 (pool *c*) contained somewhat more xylose residues as compared to pool *d*.

Discussion

Extracts.—Using rather mild extraction conditions around 75% of all galacturonic acids present in the AIS of Golden Delicious apples could be recovered in four extracts. These fractions, CBSS, HBSS, ChSS, and ASS, represent 10, 7, 13, and 10% of AIS on weight base respectively. Although the ASS fraction was obtained by alkali extraction at low

temperature, it is believed that by this treatment only ester linkages are saponified and hydrogen bonds detached without changing the polysaccharide structures. Fractions CBSS, HBSS, and ChSS comprised 64% of the galacturonic acid present in the AIS. Renard and Thibault[24] extracted 66% of the galacturonic acids in one step using buffer with or without CDTA or EDTA at higher temperature. Aspinall and Fanous[12] recovered 62% of the galacturonic acid present in apple CWM (cell wall material) in their hot water extract and 25% after extraction by oxalate. The total yield of polysaccharides extracted using buffer and chelating agents (CBSS + HBSS + ChSS) was 30% and this value is comparable with data reported before: 32%[24] and 27%[12]. Although CDTA is recommended more and more in literature to replace EDTA in complexing calcium ions to extract pectins[25], we still used EDTA since we preferred to compare our results with those of De Vries et al.[11]. It has been demonstrated[24] that EDTA did not differ much in complexation behaviour from CDTA (at pH 4.5) and only slightly higher concentrations of EDTA are needed to complex the same amounts of calcium ions[24]. For this reason, in our experiments the extractions were carried out three times after which no more material could be extracted.

Trends in the sugar composition of the extracts showed some correlation with the severity of extraction. Galacturonic acid was the main sugar residue in all extracts, while increasing amounts of rhamnose, arabinose, xylose, and galactose were found in the sequence CBSS, HBSS, ChSS, and ASS. However, the relative proportion of the neutral sugars differed in each extract. The ratio of relative amounts of individual sugars present in CBSS compared to ASS was 1:3 for rhamnose; 1:3.3 for arabinose; 1:5 for xylose, and 1:2.2 for galactose. In general, it can be concluded that pectins contained more rhamnose and more neutral sugars when more severe extraction conditions were used for the extraction. The sugar composition showed good agreement with published data[11-13], although sometimes different extraction procedures were followed.

From Table I, it was calculated that the pectins in CBSS, HBSS en ChSS containing 64% of the galacturonic acid residues contained 58% of the methyl esters but only 22% of the acetyl groups present in AIS. From the data it can be concluded that galacturonic acid residues in the pectic material in ASS and RES, had an average DM value of 96 and a DA value of 40, assuming that all the acetyl groups were present on galacturonic acid residues. It can be concluded that the pectins which were more difficult to extract (ChSS, ASS) were more branched with neutral sugars and were more methylated and acetylated than the more easily extractable material. Some galacturonic acid residues (22%) could not be extracted at all and remained in the residue, together with most of the fucose, xylose, and glucose residues.

Distribution of hairy regions over pectin molecules.—HPSEC analysis revealed that the extracted pectins were all of high molecular weight, since they were eluted near the void of the columns used. ASS pectin showed solubility problems and no proper HPSEC elution pattern could be obtained. The ASS fraction was only soluble in EDTA/buffer, and no peaks appeared after analysis by HPSEC, indicating that the pectins precipitated completely on the column material. The hairy regions are considered to be present in blocks along the pectin molecules. To verify this for our fractions, RGase was used to hydrolyze linkages between rhamnose and galacturonic acid residues in the hairy regions.

After treatment of the chemically saponified pectin extracts by RGase, it was demonstrated that RGase was able to release the characteristic RGase oligomers from all intact pectins. However, amounts of oligomers produced and the shifts in molecular weight as measured by HPSEC were different for the various pectins. Albeit that the formation of some RGase oligomers, the HPSEC elution pattern of the CBSS RGase digest resembled that of the untreated fraction. Larger differences in the elution patterns were observed for untreated and RGase treated HBSS and ChSS pectin, while RGase solubilized material from the "insoluble" ASS and RES fraction (HPSEC: 26 min). Since RGase is active only on hairy regions, the different effects of RGase on the elution patterns of the various pectins indicate differences in the proportion and distribution of hairy regions in these pectins. If RGase is active on the hairy regions present in the middle of a pectin molecule, a drastic decrease in the molecular weight of the molecules should be noticed. Since this is not the case for CBSS pectin, it may be suggested that the pectins in CBSS consist of long homogalacturonan chains having hairy regions at the end of these chains. For HBSS and ChSS pectin, a more complex structure may be present.

Hairy regions.—All extracts and the residue were treated by PG, or, for those pectins which still contained methyl esters, by the combination PE and PG, to degrade the homogalacturonan segments. The major part of the pectins was degraded to low molecular weight material while some high molecular weight material remained. In our laboratory, it has been shown that PE is able to saponify only part of the methyl esters present, however, this proved to be no limitation for PG to degrade all homogalacturonan segments present since re-incubation of the chemically saponified HR fractions with PG did not result in further degradation as monitored both by HPSEC and HPAEC. The observations described were in full agreement with the study of De Vries et al.[1] who used PL instead of PG or PE/PG. As expected, for CBSS, HBSS, and ChSS, a correlation was found between the amount of the PE/PG resistant material (hairy regions) and the amount of neutral sugars in the intact pectins. As a result of the higher rhamnose content in the pectins extracted under more severe conditions, it can be assumed that more rhamnogalacturonan-type regions are present in these pectins.

By HPSEC, the hairy regions could be roughly separated into two fractions. The relative amounts of these fractions varied for the different pectin extracts. The largest quantity of the highest molecular weight fraction was found in the digest of ChSS, while this fraction was scarcely present in the digest of CBSS.

The hairy regions of all extracts and the residue were also obtained on a preparative scale by chromatography of the PE/PG digests on Sephacryl S300. By analogy with the HPSEC patterns, two different fractions of hairy regions (HR-I and HR-II) were obtained. Since isolation of the hairy regions involved many steps, no exact data can be presented on their amount per gram of pectin extract. However, the amounts obtained differed significantly for the two HR fractions of the various pectins. PG did not liberate high quantities of hairy regions from the insoluble residue, probably indicating that some neutral sugar chains present in the hairy regions are covalently linked to the hemicellulosic or cellulosic matrix. It is less clear why only limited amounts of HR were released from the ASS fraction.

The sugar compositions of the fractions were representative of apple hairy regions as described. No logical regularity in the relative amounts of the sugars present in HR-I and HR-II was recognized. Sometimes a higher arabinose, galactose or galacturonic acid content was found for HR-I, but in other cases the opposite was true. The ratio of arabinose to galactose residues varied markedly for the various HR fractions and fluctuated from 1.6:1 for CBSS-HR-II to 7.3:1 for ChSS-HR-II. Xylose was usually present in higher concentrations in the HR-I fractions. The rhamnose to galacturonic acid ratio varied from 0.16 for ChSS-HR-I to 0.36 for CBSS-HR-II. These values are in the same range as reported before for apple hairy[1] (0.22) and apple modified hairy regions[17] (0.29). Great resemblance is also observed when mol% values of the sugars are compared with those given by De Vries et al.[1] (hairy regions of type E pectin: arabinose 50%, xylose 5%, galactose 13%, rhamnose 5%, and galacturonic acid 23%), although we found slightly lower amounts of galacturonic acid residues and slightly higher amounts of galactose. Comparison of our HR fractions with MHR[17] show that MHR has a lower galactose content and is relatively richer in xylose residues. This might be due to enzyme treatment during the isolation of MHR.

Methylation analysis revealed that the sugar linkage composition of all HR fractions was very similar to that reported earlier for neutral sugars, present in apple pectic substances[12,16,17,23] and are not repeated here. Rhamnose residues were branched via C-4 as described[16,17]. In contradiction with De Vries et al.[16], no (1→3)-linked rhamnose residues were found in our HR fractions. Arabinose was present in highly branched chains, as has been reported before[12,16,23]. Probably due to enzymic modification during the isolation of their modified hairy regions, Schols et al.[17] found more linear arabinan side chains. Xylose residues were mainly terminally linked, again in agreement with the literature[16,17]. The glycosidic linkages of galactose residues in the CBSS-HR fractions indicated the presence of (1→3,6)-galactans (type II arabinogalactan) as described by others[16,26]. Type I (arabino) galactans (1→4)-linked) predominated in the other fractions. Relative high proportions of terminally linked galactose were observed to be present in all HR fractions and this is in full agreement with earlier findings[1,17,19,21].

Enzymic degradation of HR by RGase.—The action of RGases isolated from *Aspergillus aculeatus* and *Trametes sanguinea* IFO 6490 towards native pectins from apple and beet has been reported before[23,27]. Since these experiments were performed on insoluble cell wall material[23,27] or on material solubilized after extensive heat treatment under alkaline conditions[27], it was hardly possible to use the data obtained to reveal the structure of individual pectins. As illustrated above in our experiments, RGase was active towards extracted pectins liberating typical RGase oligomers as deduced from their HPSEC and HPAEC elution behaviour.

From the HPSEC elution patterns of the RGase digested HR fractions, which were isolated in a defined way, it was concluded that differences exist between the various HR fractions and thus between the various pectins. Sometimes, the HR-population shifted completely to smaller molecular weight material, while in the case of CBSS-HR-II incubation by RGase resulted in the release of RGase oligomers while the remaining polysaccharide was eluted in an very similar fashion as the starting material. This might

suggest that, as argued before for CBSS-pectin, CBSS-HR-II contained some alternating rhamnose and galacturonic acid residues in its backbone, not in the middle of the Hairy Region segment, but more likely at the ends of it.

Differences are also observed when the elution patterns of the oligomers obtained by HPAEC are compared. First of all, it should be stated that the same type of oligomers were formed from the non-modified HR fractions as from MHR[19,20]. This indicated that the single-unit side chains of galactose are not a result of the action of enzymes during the liquefaction process, but that these are present in the original pectins and hairy regions. However, the relative amount of the oligomers carrying galactose residues varied for the type of pectin of which the hairy regions originated from. The relative amount of each oligomer was estimated using galacturonic acid as standard. Although PAD response depends on size, sugar residues, and linkage types present[19], quantification, even without having the proper standard compounds, enables us to draw conclusions more readily than only judging the elution pattern. It should be remarked that especially the concentrations of the higher oligomers will be underestimated. Parallel to the increasing amount of HR obtained when more severe extraction conditions were used, the amount of oligomers released per same quantity of HR also increased significantly. Relatively more linear oligomers having a tetrameric or hexameric backbone of alternating rhamnose and galacturonic acid residues were found for the easily extractable pectins (CBSS and HBSS). The ratio of oligomers having a tetrameric rhamnogalacturonan unit to oligomers having a hexameric unit is similar for all HR-digests, while dissimilarities were observed for the ratio single branched tetrameric rhamnogalacturonan unit to doubly branched tetramer unit: the presence of the latter was more pronounced in the digests of HR-I fractions. Estimation of similar ratios for the hexameric backbone unit was not carried out since separation and quantification of the oligomers containing one galactose residue is difficult under the conditions used. From structural studies on apple MHR, Voragen et al.[22] proposed that subunits consisting of alternating rhamnose and galacturonic acid residues (with part of the rhamnose residues being branched having a galactose residues at C4) are present in apple MHR (subunit III)[22]. These subunits were degraded (partly) by RGase into the characteristic RGase oligomers[20,22]. It can be concluded that these rhamnogalacturonan segments are present in all pectic molecules investigated in this study, but variations in the relative amounts of the various RGase oligomers within these segments are present.

Using chromatography on Sephacryl S300, some regularities were observed for the HR fractions. In all three digests, an arabinose-rich polymer was recognized resembling the subunit II as described[22,29] for apple MHR population A. This type of arabinan-rich remnant of the backbone was also reported by Renard et al.[23] after incubation of CDTA-insoluble solids of apple with RGase (1% rhamnose, 73% arabinose, 2% xylose, 12% galactose, and 7% galacturonic acid). The a and b pools obtained after chromatography over S300 were relatively enriched in xylose residues and had a relatively low rhamnose to galacturonic acid ratio. Although the relative amount of xylose was at maximum 14 mol%, it suggests the presence of a xylogalacturonan. Barrett and Northcote[28] had reported already in the early sixties the occurrence of an aldobiouronic acid of xylose and

galacturonic acid, isolated after partial hydrolysis of apple pectinic acid. The existence of a xylogalacturonan polymer in apple MHR population A has been demonstrated[29].

It can be concluded that pure enzymes can be used as tool to study small differences in the fine structure of pectins. This was illustrated by differences in the PE/PG and RGase degradability of the pectin extracted by cold buffer when compared to that extracted by hot buffer. Many researchers have combined these pectins into one single fraction. Another example of the complexity of the pectins is that RGase incubation of HR fractions having a rather similar sugar composition resulted in clear differences in relative amounts of the RGase oligomers obtained (both relatively to each other and in absolute amounts).

More research is being carried out to characterize the proposed subunits present in (other) HR fractions in more detail, to establish their distribution over the rhamnogalacturonan backbone, and to study the distribution of the hairy regions over the pectic molecules.

Acknowledgments

References

1   J.A. De Vries, F.M. Rombouts, A.G.J. Voragen, and W. Pilnik, *Carbohydr. Polymers*, 2 (1982) 25-33.
2   M. O'Neill, P. Albersheim, and A.G. Darvill, in P.M. Dey (Ed.), *Methods in Plant Biochemistry*, Vol. 2, *Carbohydrates*, Academic, London, 1990, pp 415-441.
3   J.-F. Thibault, C.M.G.C. Renard, M.A.V. Axelos, P. Roger, and M.-J. Crépeau, *Carbohydr. Res.*, 238 (1993) 271-286.
4   H. Konno, Y. Yamasaki, and K. Katoh, *Phytochemistry*, 25 (1986) 623-627.
5   P. Massiot, and J.-F. Thibault, *Carbohydr. Res.*, 190 (1989) 121-136.
6   L. Saulnier, and J.-F. Thibault, *Carbohydr. Polym.*, 7 (1987) 345-360.
7   A.G.J. Voragen, R. Heutink, and W. Pilnik, *J. Applied Biochem.*, 2 (1980) 452-468.
8   C.M.G.C. Renard, A.G.J. Voragen, J.-F. Thibault, and W. Pilnik, *Animal Feed Sc. Techn.*, 32 (1991) 69-75.
9   S. Ishii, *Phytochemistry*, 21 (1982) 778-780.
10 M. Hirano, H. Kiyohara, T. Matsumoto, and H. Yamada, *Carbohydr. Res.*, 251 (1994) 145-162.
11 J.A. De Vries, A.G.J. Voragen, F.M. Rombouts, and W. Pilnik, *Carbohydr. Polym.*, 1 (1981) 117-127.
12 G.O. Aspinall, and H.K. Fanous, *Carbohydr. Polym.*, 4 (1984) 193-214.
13 B.J. Stevens, and R.R. Selvendran, *Carbohydr. Res.*, 135 (1984) 155-166.
14 C.M.G.C. Renard, A.G.J. Voragen, J.-F. Thibault, and W. Pilnik, *Carbohydr. Polym.*, 12 (1990) 9-25.
15 X. Rouau, and J.-F. Thibault, *Carbohydr. Polym.*, 4 (1984) 111-125.
16 J.A. De Vries, F.M. Rombouts, A.G.J. Voragen, and W. Pilnik, *Carbohydr. Polym.*, 3 (1983) 193-205.
17 H.A. Schols, M.A. Posthumus, and A.G.J. Voragen, *Carbohydr. Res.*, 206 (1990) 117-129 (this thesis,

chapter 3).

18 H.A. Schols, and A.G.J. Voragen, *Carbohydr. Res.*, 256 (1994) 83-95 (this thesis, chapter 6).

19 H.A. Schols, A.G.J. Voragen, and I.J. Colquhoun, *Carbohydr. Res.*,256 (1994) 97-111 (this thesis, chapter 7).

20 H.A. Schols, C.C.J.M. Geraeds, M.J.F. Searle-van Leeuwen, F.J.M. Kormeling, and A.G.J. Voragen, *Carbohydr. Res.*, 206 (1990) 105-115 (this thesis, chapter 4).

21 I.J. Colquhoun, G.A. de Ruiter, H.A. Schols, and A.G.J. Voragen, *Carbohydr. Res.*, 206 (1990) 131-144 (this thesis, chapter 5).

22 A.G.J. Voragen, H.A. Schols, and H. Gruppen, in F. Meuser, D. J. Manners, and W. Seibel (Eds.), *Plant Polymeric Carbohydrates*, Royal Society of Chemistry, Cambridge, UK, 1993, pp 3-15.

23 C.M.G.C. Renard, J.-F. Thibault, A.G.J. Voragen, L.A.M. van den Broek, and W. Pilnik, *Carbohydr. Polym.*, 22 (1993) 203-210.

24 C.M.G.C. Renard, and J.-F. Thibault, *Carbohydr. Res.*, 244 (1993) 99-114.

25 R.R. Selvendran, and P. Ryden, *Methods in Plant Biochemistry*, Vol. 2, *Carbohydrates*, Academic Press, London, 1990, pp 549-579.

26 F. Will, and H. Dietrich, *Carbohydr. Polym.*, 18 (1992) 109-117.

27 T. Sakamoto, and T. Sakai, *Carbohydr. Res.*, 259 (1994) 77-91.

28 A.J. Barrett and D.H. Northcote, *Biochem. J.*, 94 (1965) 617-627.

29 H.A. Schols, E.J. Bakx, D. Schipper, and A.G.J. Voragen, *Carbohydr. Res.*, accepted for publication (this thesis, chapter 9).

CHAPTER 9

A XYLOGALACTURONAN SUBUNIT PRESENT IN THE MODIFIED HAIRY REGIONS OF APPLE PECTIN

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Abstract

The digest of chemically saponified population A of the modified hairy regions (MHR) of apple pectin obtained after treatment by rhamnogalacturonase (RGase) was fractionated using size-exclusion chromatography. Two new subunits could be recognized: a xylogalacturonan unit and residual stubs of the rhamnogalacturonan backbone containing arabinan side chains, next to the previously reported rhamnogalacturonan regions with alternating rhamnose and galacturonic acid residues (ratio of rhamnose to galacturonic acid is 1).

Starting from chemically saponified MHR, the isolated xylogalacturonan subunit eluted as only one single peak over an anion-exchange resin, although slight differences in relation to the ratio xylose to galacturonic acid within the peak were observed. Xylogalacturonan was also obtained from non-saponified MHR by removing the *O*-acetyl groups (hindering degradation by RGase) enzymatically using rhamnogalacturonan acetylesterase (RGAEase) prior to RGase action. Fractionation (SEC) of the digest resulted in a xylogalacturonan fraction having similar characteristics as compared to the chemically saponified xylogalacturonan, whereas anion-exchange chromatography showed the presence of three distinct populations with only minor variations in their sugar composition. The data obtained suggest variations in the degree of methylation of the xylogalacturonan molecules. A xylogalacturonan subunit being still methyl esterified was further characterized by NMR spectroscopy. It was shown that xylose residues were β-(1→3)-linked to part of the galacturonic acid residues within a rather high molecular weight xylogalacturonan. From NMR data obtained, a degree of methylation of 39 was calculated and the methyl esters were found to be equally divided over the substituted galacturonic acid and unsubstituted galacturonosyl residues. The xylogalacturonan was accompanied by a typical rhamnogalacturonan having galactose residues linked to (part of) the rhamnose residues.

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[1] Gist brocades, Delft

Introduction

Pectic substances, which represent one of the most important plant cell wall polysaccharides, have been subject of many studies, but still little is known about their fine structure. De Vries et al.[1] proposed a hypothetical model in which pectin consists of alternating smooth homogalacturonan regions and highly branched hairy or ramified regions. Starting from apple juice obtained by an enzymic liquefaction process, we described[2] the isolation of a pectic fraction having similar characteristics to the hairy regions described before by De Vries et al.[1] Since the apple juice "hairy regions" might have been altered by the action of enzymes present in the technical enzyme preparation, the pectic fraction was designated as modified hairy regions (MHR)[2]. The structure of the MHR could be described in more detail by making use of the enzyme rhamnogalacturonase (RGase)[3] which is able to hydrolyze specifically galactopyranosyluronic-rhamnopyranosyl linkages. The modified hairy regions were judged to differ from rhamnogalacturonan I as described to be present in e.g. suspension cultured sycamore cells[4]. Our (modified) hairy regions contained a higher proportion of galacturonic acid as compared to rhamnose, whereas RG-I was suggested to consist of strictly alternating rhamnose and galacturonic acid residues[4]. Pectic molecules like the (modified) hairy regions[1,2,5-8] and RG-I[4] have been isolated from many sources and seem to be ubiquitous in plant cell wall material. Both the (modified) hairy regions and RG-I are built from the same sugar residues having the same types of sugar linkages. A variety of oligomers derived from these types of pectic polymers, consisting of alternating rhamnose and galacturonic acid residues, have been reported[8-14]; side chains of arabinose and/or galactose residues, mainly attached to the rhamnose residues, might be present.

There is a lack of information on the sequences of the various sugar residues within the pectic molecule. Chemical hydrolysis and fragmentation reactions frequently used in structure elucidation are often hampered by poor selectivity, making successful recombination of the identified fragments toward the native polymeric molecule rather difficult. Reliable opportunities are offered by enzymes which can be used as analytical tools in the elucidation of the fine structure of polysaccharides[14-16]. The availability of pure, well characterized enzymes, active toward pectic hairy regions or RG-I type of polysaccharides is increasing and includes rhamnogalacturonases[3,17], rhamnogalacturonan acetylesterase[18], rhamnogalacturonan rhamnopyranohydrolase[19], next to enzymes acting on the side chains of the pectic molecules like arabinanases[20] and galactanases[19,21]. To be able to recognize distinct areas within the polymer, enzymes acting in an endo manner should be preferred.

Based on RGase degradation studies, Voragen et al.[15] suggested the existence of three different subunits within ramified pectic molecules extracted by a technical enzyme preparation: a xylogalacturonan unit, residual stubs of the pectic backbone containing arabinan side chains and rhamnogalacturonan chains of unknown length.

This paper deals with the isolation of the three different subunits and further characterization of the xylogalacturonan subunit. The substitution pattern of xylose residues and methyl esters over the galacturonan backbone could be established by 2D

NMR spectroscopy.

Experimental

Substrate.—Modified hairy regions (MHR) were isolated from apple and fractionated by gel permeation chromatography in populations A, B, C, as described previously[2]. MHR population A was used for further studies. Prior to enzymic degradation with RGase, it was first saponified. For this purpose, 25 mg of MHR was treated with 0.1 M NaOH (1 mL; 0°C) for 16 h, followed by neutralization (1 mL 0.1 M HAc), and dilution with buffer (9 mL 0.05 M NaOAc pH 5.0).

Enzymic degradation of MHR population A.—Saponified MHR population A in NaOAc buffer (1 mL) obtained as described was incubated with RGase (1 μL; $\pm$ 100 ng of protein), purified from *Aspergillus aculeatus*[3], for 16 h at 30°C. The enzyme was inactivated by heating (5 min, 100°C) and the digests were analyzed by high-performance size-exclusion chromatography (HPSEC) and high-performance anion-exchange chromatography (HPAEC). For semi-preparative fractionation of the digest, 350 mg of apple MHR population A (saponified) in 0.05 M NaOAc pH 5.0 (0.4%) was incubated for 20 h using 2 mL RGase.

Alternatively, 150 mg apple MHR population A (0.04% in 0.05 M NaOAc pH 5.0) was directly incubated for 18 h at 40°C with 1 mL RGase in combination with 10 μg rhamnogalacturonan acetylesterase[18] (RGAEase; kindly provided by Novo-Nordisk, Denmark).

Analytical methods.—The neutral sugar composition and uronic acid content of all fractions were determined after hydrolysis with 2 M trifluoroacetic (1h, 121°C)[5] or with M H_2SO_4 (3 h, 100°C) without pre-hydrolysis as described[2].

Chromatography.—HPSEC was performed on three Bio-Gel TSK columns in series (40XL, 30XL, and 20XL) as described[2]. For HPAEC analysis, a Dionex Bio-LC system which included a quaternary gradient pump, eluent degas (He) module, and pulsed electrochemical detector (PED) in the PAD mode, completed with a Spectra Physics SP8800 autosampler and a Spectra Physics Winner data handling system was used as described[13]. A CarboPac PA100 column (4 x 250 mm) with guard column (Dionex) was used at a flow rate of 1.0 mL/min. The gradient was obtained by mixing solutions of 0.1 M NaOH and M NaOAc in 0.1 M NaOH. After 15 min equilibration with 0.1 M NaOH, 20 μL of the sample was injected and a linear gradient to 0.43 M NaOAc in 0.1 M NaOH within 35 minutes was started. Finally, the column was washed for 5 min with M NaOAc in 0.1 M NaOH.

Size-exclusion chromatography was performed on a column (105 x 26 mm) of Sephacryl S200 (Pharmacia; separation range for dextrans 10^3-8 x 10^4Da) or Sephacryl S300 (separation range for dextrans 2 x 10^3-4 x 10^5 Da) using a Hiload System (Pharmacia) and 0.1 M NaOAc (pH 5.0) as eluent. Fractions (2.5 mL) were collected and analyzed for neutral sugars and uronides as described[2]; arabinose and galacturonic acid were used as standards, respectively. Pooled fractions were dialyzed and lyophilized

before analysis.

Anion-exchange chromatography was performed on a column (250 x 20 mm) of DEAE-Sepharose Fast Flow (Pharmacia). Sample (18-25 mg) was applied on the column at a flow of 1 mL/min (0.005 M NaOAc, pH 5.0); after 60 minutes the flow was increased to 5 mL/min and a gradient (in 80 min) to M NaOAc (pH 5.0) was started. Elution with M NaOAc was continued for another 8 min, followed by a gradient up to 2 M NaOAc in 8 min. Fractions (5 mL) were collected and analyzed as described. Pooled fractions were dialyzed and lyophilized before analysis.

NMR spectroscopy.—All of the NMR experiments were recorded on a Bruker AMX-600 spectrometer at 350-370K. Both the homonuclear and heteronuclear experiments were performed using a 5 mm triple resonance probe equipped with gradients. The polysaccharide fraction RGase/RGAEase S300 I-*c* (see Fig. 4; $\pm$ 6 mg) was dissolved in 0.5 mL D_2O. In each experiment except for the HSQC experiment, the residual HDO signal was saturated during the 0.5 s recycle delay. Phase cycling was applied for phase-sensitive detection[22]. The ^{1}H spectral width in all the homonuclear spectra was 4000 Hz. The TOCSY experiments involved the clean-TOCSY sequence using the MLEV17 sequence[23] for isotropic mixing; the delays during the mixing time were chosen equal to the 90° pulse width for the powerlevel of the spin-lock, which was 20 μs. The total spin-lock mixing time in the TOCSY experiment ranged from 17 to 120 ms. The mixing time in the NOESY and ROESY experiment was 100 ms. For all the homonuclear 2D experiments, a total of 256 1K FIDs, 400 scans each, were collected, zero filled once in the F2 dimension and twice in the F1 dimension. A squared sine-bell function shifted by $\pi/3$ was applied in both dimensions. In the HSQC experiment, the ^{1}H spectral width was 18500 Hz, ^{13}C spectral width 9300 Hz and ^{13}C decoupling during acquisition was achieved by the GARP-1 scheme[24]. Residual water signal and artefacts were suppressed by using 3 gradients as described[25,26]. 256 4K FIDs of 480 scans were collected with a recycle delay of 0.95s and the zero filling and digital filtering was identical to the homonuclear experiments.

Results

Degradation of MHR population A.—In previous studies[2] it was shown that MHR consisted of three populations of polysaccharides differing in molecular weight. Degradation studies of MHR by RGase were hampered by the finding that degradation products of the high molecular weight population A co-eluted with non-degraded molecules of the populations B and C. For this reason, we only used the most important population A in this study. Population A has been isolated by chromatography over Sephacryl S500 and S200[2]. Previous studies also revealed that the enzyme RGase is hindered by the presence of ester groups; therefore MHR population A was saponified prior to enzymic degradation. Figure 1 shows the HPSEC elution profiles of the saponified population A before and after degradation by RGase. From the elution pattern it can be seen that the molecular weight of the polysaccharide fraction shifted completely to
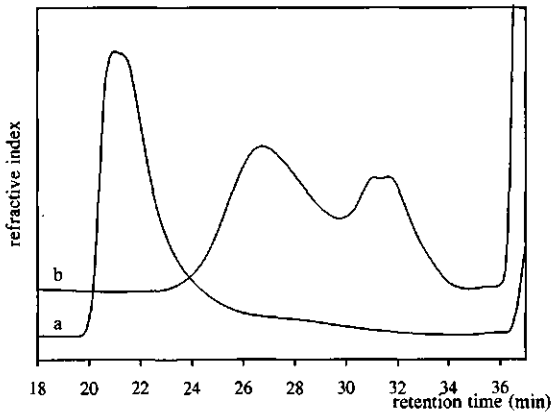
Fig. 1.   High-performance size-exclusion elution pattern of chemically saponified MHR population A, before (a) and after (b) treatment with RGase at 30°C for 16 h.

lower molecular weight values within 16 h.

Fractionation of RGase digest on Sephacryl S200.—To be able to study the higher molecular weight material present in the RGase digest of MHR population A, the incubation mixture was separated by SEC on a Sephacryl S200 column. Using this mode of chromatography, three different peaks could be distinguished (Fig. 2), contrary to HPSEC analysis (Fig. 1) which displayed only two populations. The most rational explanation is probably the differences in separation range of both column materials.
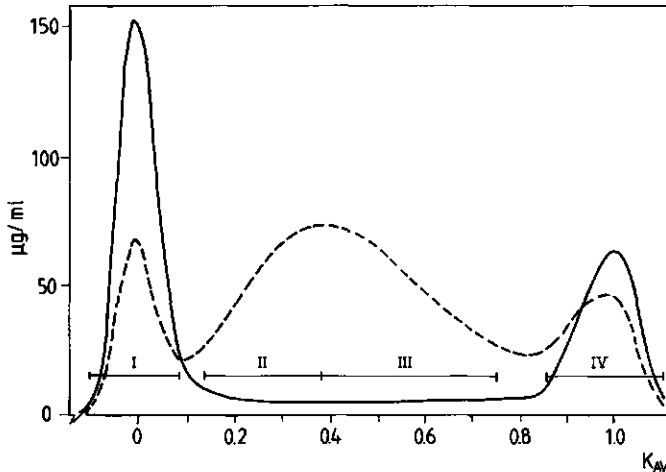

Fig. 2.   Size-exclusion chromatography on Sephacryl S200 of MHR population A after degradation with RGase: ————, uronic acids; ---- , neutral sugars.

TABLE I. Sugar composition (mol%) of MHR population A and fractions I-IV, obtained after Sephacryl
          S200 size-exclusion chromatography of the digest of MHR population A with RGase

Sugar	MHR pop.A	I	II	III	IV
Rha	5	4	1	3	23
Ara	50	8	84	81	11
Xyl	11	38	3	1	0
Man	0	0	0	0	0
Gal	10	4	3	5	29
Glu	0	5	3	3	0
GalA	24	41	6	7	37
DM	28				
DA	55				
Xyl:GalA	0.5	0.9	0.5	0.16	0.0

Sephacryl S200 covered a relative narrow range, resulting in a better resolution. The fractions were pooled as indicated in the figure and analyzed for their sugar composition (Table I) which was compared to the sugar composition of the parental material. It can be seen that xylose and galacturonic acid were the main sugars in the void fraction; together they accounted for about 80% of the sugar residues in this fraction. This is in sharp contrast with the starting material in which arabinose prevails, while xylose and galacturonic acid only make up 35% of the constituent sugars. Minor constituent sugars in fraction I were rhamnose, arabinose, and galactose. The glucose determined in the fractions I-III could not be detected in the starting material. Since 70% of the xylose residues present in the original MHR fraction were terminally linked, while 20% of all galacturonic acid residues were branched through C-3[2], it was assumed that this fraction I represents xylogalacturonan-rich molecules.

Fractions II and III covered a broad molecular weight range and both consisted of arabinose-rich polysaccharides (>80% arabinose). Minor amounts of rhamnose, xylose, galactose and galacturonic acid were determined in this fraction. Apparently, these fractions represent arabinans, connected to some residual stubs of the pectic backbone. Fractions II and III contained rhamnose and galacturonic acid in a ratio which resembles that of the starting material.

Fraction IV (Fig. 2) represented oligomeric fragments and corresponded with the material eluting at 31-33 min in Fig. 1. The oligomeric reaction products were analyzed by HPAEC analysis (results not shown) and exactly the same oligomeric reaction products consisting of alternating rhamnose and galacturonic acid with or without galactose residues linked to the rhamnose moiety were found to be present in the digest as identified earlier[13]. A slightly different ratio for the various oligomers was observed as compared to the ratios reported before for the digest of the whole apple MHR[13].
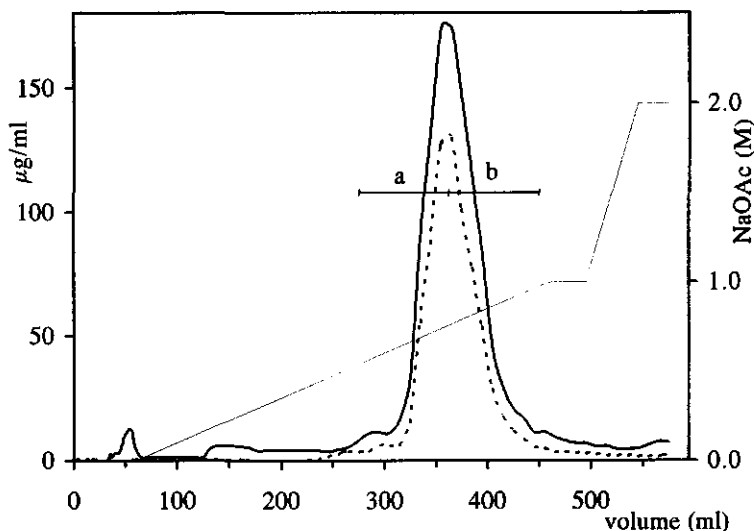
Fig. 3.   Anion-exchange chromatography on DEAE Sepharose of fraction I of the Sephacryl S300
fractionation of saponified MHR population A after degradation with RGase: ————, uronic
acid; ---- , neutral sugars; thin line, NaOAc gradient.

Anion-exchange chromatography of the xylogalacturonan fraction.—To facilitate the
isolation of appropriate amounts of the xylogalacturonan fraction, a new and larger batch
of population A was incubated with RGase and separated on a Sephacryl S300 column.
Sephacryl S300 material was used to improve the separation between the xylogalac-
turonan-rich pool I and the arabinose rich molecules. As anticipated, a better separation
between fraction I and II was obtained, however, the resolution between fraction III
(arabinose-rich molecules) and IV (the RGase oligomers) decreased (not shown).

To check whether the xylogalacturonan fraction of the Sephacryl S300 column was
homogeneous according to charge, this pool I was further fractionated on a DEAE
Sephacryl Fast Flow column. As can be seen in Fig. 3, all carbohydrates eluted
practically in one single peak. For this reason, it can be stated that no major differences in
charge between the various molecules were present. Therefore pool RGase S300-I is
considered to be rather homogeneous, both in molecular weight and charge density,
suggesting the presence of only one type of polymer. This was also examined by
comparing the sugar composition of the starting material RGase S300-I with the sugar
composition of the DEAE-fractions thereof (Table II). The composition of both pool I-*a*
and I-*b* indicates the presence of a xylogalacturonan polymer, although small proportions
of rhamnose, arabinose, galactose and glucose were found to be present. The relatively
higher content of galacturonic acid in pool I-*b* as compared to pool I-*a* might explain the
longer retention on the DEAE material.

Isolation of the non-saponified xylogalacturonan fraction.—It has been shown[2] that MHR

TABLE II.   Sugar composition (mol%) of fraction RGase-I obtained after chromatography over
Sephacryl S300 of the RGase digest of apple MHR population A and fractions I-*a* and I-*b*
obtained after DEAE Sepharose anion-exchance chromatography hereof

Sugar	RGase S300-I	I-*a*	I-*b*
Rha	3	3	2
Ara	6	5	5
Xyl	28	34	25
Man	1	2	3
Gal	4	3	3
Glu	3	1	4
GalA	55	52	58
Xyl:GalA	0.5	0.7	0.4

population A is rich in *O*-acetyl groups (55 moles of acetyl per 100 moles of galacturonic acid), while a considerable number of methoxyl groups are present (28 moles per 100 moles of galacturonic acid), the latter obviously being esterified to the carboxyl group of the galacturonic acid residues. Since RGase is hindered by the presence of *O*-acetyl groups[3], experiments so far were carried out using the saponified MHR population A. To investigate whether methoxyl groups are present in the xylogalacturonan regions, the *O*-acetyl groups were hydrolysed specifically using RGAEase. Prior to use, the RGAEase preparation was examined carefully for side activities on MHR, but no activity other than acetyl release was found. The degradation of MHR population A by RGase and RGAEase was monitored by HPSEC. It was observed that in spite of the slow degradation (presumably caused by the limiting amount of RGAEase) the expected HPSEC pattern as in Fig. 1 was obtained. It should be stated however, that the elution behaviour of the degraded MHR population A was not always consistent. Sometimes, HPSEC analysis of a RGase digest of MHR population A revealed that *all* polymeric material was degraded to smaller fragents, while *after* inactivation of the enzyme (5 min, 100°C), a high molecular weight fragment (RT=20) could be observed by HPSEC analysis. We observed that solutions containing degradation products rich in xylose and galacturonic acid residues (i.e. xylogalacturonan) were prone to aggregation (as monitored by HPSEC) and even precipitation sometimes occurred. Although quite some effort was directed to solve these problems, the only way to prevent these undesirable effects was to carry out the experiments, chromatographic separations, etc. in a tight time schedule under well standardized conditions.

The RGase/RGAEase digest of MHR population A was separated on Sephacryl S300 resulting in similar results as described for the RGase digest after chemical saponification. However, when the xylogalacturonan fraction was applied on a DEAE column, a different elution pattern was obtained (Fig. 4). At least three distinct populations of molecules could be recognized, of which the last eluting population, representing 68% of the poly-
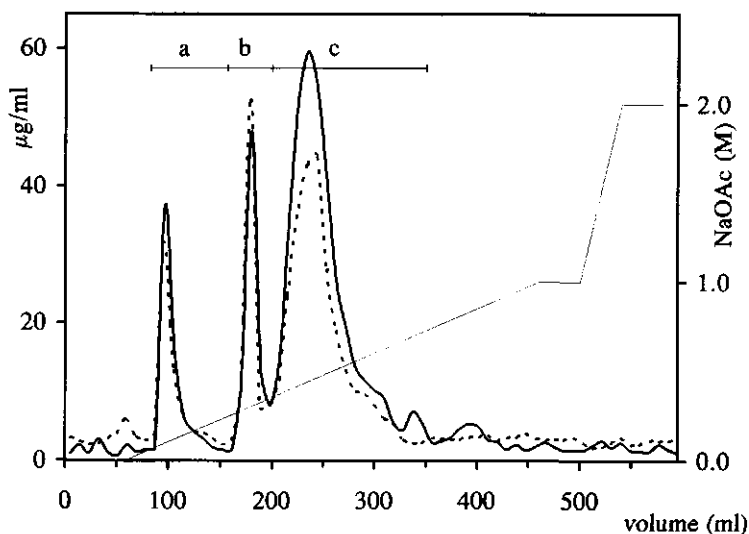
Fig. 4. Anion-exchange chromatography on DEAE Sepharose of fraction I of the Sephacryl S300 fractionation of MHR population A after degradation with RGase and RGAEase: ————— , uronic acid; ---- , neutral sugars; thin line, NaOAc gradient.

saccharides, was much less retained on the anion-exchange resin as compared to the xylo-galacturonan obtained from the chemically saponified MHR population A (eluting at 0.5 M and 0.75 M NaOAc respectively; Fig. 3 versus Fig. 4). The sugar composition of the pools, as shown in Table III, confirmed that the same type of material was isolated as be-

TABLE III. Sugar composition (mol%) of fraction RGase/RGAEase-I obtained after chromatography over Sephacryl S300 of the RGase/RGAEase digest of apple MHR population A and fractions I-*a*, I-*b*, and I-*c* obtained hereof after DEAE Sepharose anion-exchance chromatography

Sugar	RGase/RGAEase I	I-*a*	I-*b*	I-*c*
Rha	4	3	3	5
Ara	11	11	14	6
Xyl	34	26	22	34
Man	1	4	2	1
Gal	5	5	4	4
Glu	2	9	5	3
GalA	44	42	50	47
Xyl:GalA	0.8	0.6	0.4	0.7

fore. The starting material (RGase/RGAEase pool I) was rather similar to the corresponding fraction obtained from the saponified material (RGase pool I; Table II). Most important differences were the relatively higher arabinose and xylose content and the lower galacturonic acid content in pool RGase/RGAEase I, which also resulted in a somewhat higher xylose:galacturonic acid ratio. The three populations obtained after DEAE-column chromatography of pool RGase/RGAEase I showed all to be rich in xylose and galacturonic acid residues (together responsible for about 70% of all sugars), next to varying amounts of other sugars. The glucose, found in all fractions, was not detected in the starting MHR population A and might originate from the column material. The DEAE-pools RGase/RGAEase I-*a* and I-*b* were relatively enriched in arabinose as compared to pool I-*c*, while the xylose content varied slightly. Unfortunately, not enough material was obtained to determine the amount of methoxyl esters and to differentiate fur-
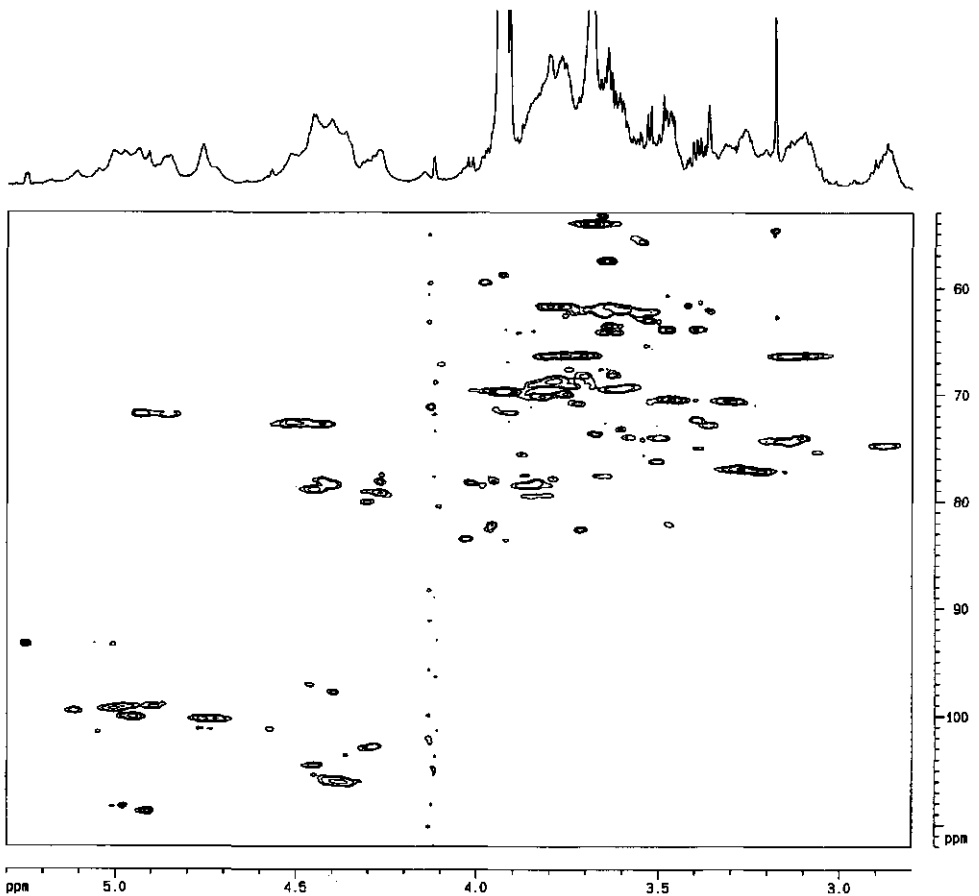

Fig. 5.   {¹H, ¹³C} HSQC spectrum of a partially methyl esterified xylogalacturonan fraction
(RGase/RGAEase S300-I-*c*) with high resolution ¹H spectrum on top.

ther between the three pools. Since pool RGase/RGAEase I-*c* appeared to be the most important pool and is considered to be a homogeneous and rather pure xylogalacturonan still being esterified, this fraction was chosen for further characterization by NMR spectroscopy.

Xylogalacturonan structure from NMR spectra.—In Fig. 5 the ^{1}H (top) and 2D ^{1}H/^{13}C HSQC spectra are shown. The broad lines in the region of 5.3-2.8 ppm in these complex spectra suggest that there is a high molecular weight carbohydrate polymer present, apart from some low molecular weight impurities (mainly lactic acid, probably due to contamination during dialysis; results not shown). Despite the high molecular weight of this complex material, the relative good quality of the TOCSY and ROESY spectra (spectra not shown) and the good dispersion in the HSQC spectrum allowed us to assign the ^{1}H and ^{13}C chemical shifts of the main components in this xylogalacturonan fraction. The assignment was much complicated by the presence of methyl esters at part of the galacturonosyl residues, since the ^{1}H/^{13}C signals of C-1 and C-5 are split into at least four

TABLE IV. [13]C and ^{1}H chemical shifts (ppm) of monomeric residues of galacturonic acid and xylose as found for the partially methyl esterified xylogalacturonan fraction (RGase/RGAEase S300-I-*c*)

	GalA[a]		GalA[b]		GalA[monomer]	
C-		H-	C-	H-	C-	H-
1	99.1;100.0;99.1[1]	5.10;4.95;4.89	100.2	4.75	92.9	5.21
2	69.4	3.61	70.2	3.82	68.7	3.72
3	68.7	3.79	78.2	3.85	70.1	3.82
4	79	4.4	78.4	4.4	71.5	4.18
5	71.8;71.8;72.7;72.7	4.93;4.85;4.52;4.48	2	2	72.2	4.31

	Xyl[a]		Xyl[b]		Xyl[c]		Xyl[monomer]	
	C-	H-	C-	H-	C-	H-	C-	H-
1	105.9	4.40	106.0	4.36	102.8	4.21	97.3	4.54
2	74.3	3.15	74.7	2.88	74.0	3.11	74.9	3.20
3	77.1	3.22	76.9	3.28	76.9	3.28	76.6	3.40
4	70.4	3.47	70.5	3.30	70.4	3.47	70.0	3.60
5	66.2	3.70;3.10	66.2	3.70;3.10	66.2	3.78;3.14	65.9	3.90 3.30

GalA[a], (1→4)-linked galacturonosyl residue. GalA[b], (1→4)-linked galacturonosyl residue, branched at C-3. Xyl[a], xylose residue, linked to a non-esterified galacturonosyl residue. Xyl[b], xylose residue, linked to an esterified galacturonosyl residue. Xyl[c], xylose residue having deviating C/H-1 chemical shifts for reasons unknown. [1], due to the partial methyl esterification, it was not possible to assign all resonances to specific galacturonic acid residues. [2], the chemical shifts of C/H-5 of the substituted galacturonic acid can not be discriminated from the chemical shifts of the unsubstituted galacturonic acid residues.
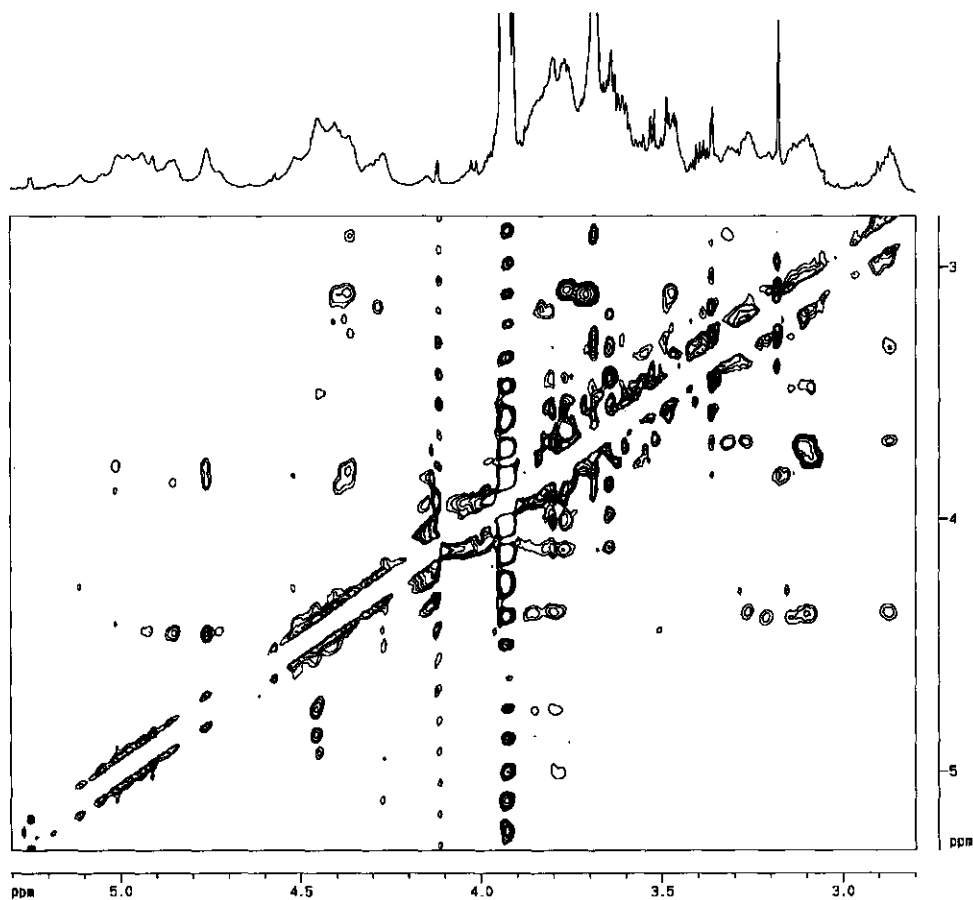
Fig. 6.   {¹H, ¹H} ROESY spectrum of a partially methyl esterified xylogalacturonan fraction
(RGase/RGAEase S300-I-c) with high resolution ¹H spectrum on top.

resonances[27]; the same holds for C-4 (Schipper, unpublished results). These multiple
resonances are caused by the fact that the difference in shielding by a free carboxyl group
and an esterified carboxyl group is also felt in preceding and following monomeric units[27].
The degree of methylation (mol methanol per 100 mol galacturonic acid residues) was
determined by integration of the C-5/H-5 resonances in the HSQC spectrum and a DM
value of 39 was calculated. From the ¹H and the HSQC spectra, it could be concluded that
no O-acetyl groups were present in the fraction investigated.

After assignment of the monomeric units, the type and substitution pattern of the
different residues can be extracted from the ¹³C chemical shifts by comparison with model
compounds[28]. In table IV, it can be seen that the xylose[a] chemical shifts only differ from

those of monomeric xylose at C-1 indicating that all xylose is terminally linked. The chemical shifts of the galacturonic acid residues differ from the chemical shifts of the free galacturonic acid (apart from C-1) at C-4. Galacturonic acid residue[b] also shows a downfield shift of $\pm$ 8 ppm at C-3 indicating the presence of a substituent at this position. From 2D NOESY (spectrum not shown) and ROESY experiments (Fig. 6), it was established that a β-xylose residue was linked to part of the galacturonosyl residues at C-3. The resonance of the C-2/H-2 of xylose are also doubled (Table IV). This is again caused by the presence of methyl esters: xylose H-2 protons are more shielded by the methyl esterified carboxyl group of galacturonic acid. This was confirmed by comparison of the spectra with those from a sample of saponified xylogalacturonan. Thirty six percent of all xylose residues (as determined using two-dimensional integration) was linked to a methyl esterified galacturonosyl residue. The presence of a second (minor) substituent could be detected. The C-1/H-1 chemical shifts differ slightly from the main xylose substituent (Table IV), but the remaining chemical shifts definitely point to a xylose residue (xyl[c]). The exact nature of the chemical shift differences is not known, but might have to do with sequence specific substitution patterns like the presence of a xylose substituted galacturonic acid moiety, flanked on both sides by methyl esterified galacturonic acids. Also a triad of substituted galacturonosyl residues might cause these type of difference in shielding.

From two dimensional integration of the C-1 resonances of the galacturonic acid and xylose moieties, the xylose to galacturonic acid ratio was calculated to be 0.7 which was in good agreement with the overall sugar composition (Table III). Other residues present and assignable in the HSQC spectrum are α-rhamnose, β-galactose and α-arabinose. Their chemical shifts agree with those published earlier by Colquhoun et al.[29] on RGase oligomers released from apple MHR. Although not proved, they might originate from residual rhamnogalacturonan fragments flanking the xylogalacturonan.

Discussion

Recently, hairy regions as well as RG-I have been identified on the basis of their sugar composition, sugar linkage composition and on the structure of chemically and enzymically obtained oligomers[7,9-14]. The use of pure and defined enzymes like RGase has the advantage that higher molecular weight fragments could also be obtained without the formation of potential artefacts. The oligomers isolated from the RGase digest of apple MHR population A were analyzed by HPAEC and were found to be similar to the characteristic oligomers as described before[13,29]. All oligomers consisted of alternating rhamnose and galacturonic acid residues in the backbone (4-6 residues) having galactose residues connected to some or all of the rhamnose residues. Small differences were observed in the relative amounts of the various oligomers as compared to the oligomers present in the digest of the whole MHR[13]. In the RGase digest of MHR population A, the hexamer $Rha_2GalA_2Gal_2$ and the octamer $Rha_3GalA_3Gal_2$ were more predominantly present, while the RGase oligomers without or with only one galactose substitution were

present in relatively low concentrations.

Chromatography of the digest over Sephacryl S200 revealed the presence of intermediate molecular weight fragments ($\pm$ 6000-15 000 Da) consisting for more than 80% of arabinose residues (pool II and III). The accompanying sugar residues rhamnose and galacturonic acid (ratio rhamnose to galacturonic acid varying from 1:6 to 1:2.3) are characteristic for a rhamnogalacturonan backbone. Almost all of the arabinose residues present in the MHR population A were recovered in these pools. The fragments present in pool II and III are therefor considered to represent residual stubs of the pectic backbone containing arabinan side chains. Since RGase was unable to degrade these polymeric fractions further, it can be concluded that the rhamnose and galacturonosyl residues are either not present in the desired alternating order or these residues are too highly branched for RGase to split in the backbone.

Next to the rhamnogalacturonan part which was degraded to rhamnogalacturonase oligomers and the arabinose-rich residual stubs of the backbone, the presence of a third type of subunit was indicated. From its sugar composition, we hypothesize that this is a xylogalacturonan (pool RGase S200 I).

Since separation based on size-exclusion chromatography does not prove the homogeneity of the xylogalacturonan population, it was still uncertain whether the other sugar residues present in the Sephacryl S200 pool I were part of the xylogalacturonan polymer or belonged to another polysaccharide having the same hydrodynamic volume. Anion-exchange chromatography showed only one peak, although slight variations in sugar composition within this peak occur. For this reason, it can be concluded that the xylogalacturonan subunits, next to galacturonic acid and xylose, still contain some other sugar residues. Irregularities between various xylogalacturonan molecules might exist since the xylose to galacturonic acid ratio varied slightly (0.7 and 0.4 respectively).

HPSEC analysis of the digest of the non-saponified MHR population A treated with the combination of RGase and RGAEase showed that the degradation was comparable with the degradation of the chemically saponified substrate with RGase only. This indicates that the sequences of alternating rhamnose and galacturonic acid residues of MHR population A where RGase acts are either not methyl esterified or that RGase is not hindered by these methyl ester groups. Anion-exchange chromatography over DEAE-Sepharose revealed the presence of at least three distinct xylogalacturonan fractions in the digest obtained with RGase and RGAEase, having only small differences in sugar composition. All three populations were much less strongly bound to the column material as compared to the saponified xylogalacturonan molecules, although differences between the three fractions could not be explained by the differences in galacturonic acid content. Unfortunately, not enough material was available to determine the degree of methylation of these fractions, but the elution behaviour on the anion-exchange column of the pools RGase/RGAEase I-*a*, I-*b* and I-*c* strongly suggests the presence of various amounts of methyl esters linked to the galacturonosyl residue. Since no direct information was obtained to which position of the galacturonic acid residue (C-2 or C-3) the xylose residue is linked, the xylogalacturonan fraction RGase/RGAEase S300 I-*c* was studied by NMR spectroscopy. Since the NMR data proved to be unequivocal, no sugar linkage type composition was

determined for this fraction.

The galacturonic acid residues, present in the fractions RGase/RGAEase I-*c*, were found to be present as (1→4)-linked residues; two-thirds of them carried a terminal β-linked xylose residues attached to C-3 of the galacturonic acid residue. The ratio of galacturonic acid residue to xylose residues as estimated by NMR are in excellent agreement with the sugar composition and indicate that a certain variation in xylose substitution within various xylogalacturonan molecules may occur. The structural features of the xylogalacturonan are in good agreement with the structure proposed before[15] and also with data on the sugar linkage composition of the parental MHR fraction[2] which showed that 70% of the xylose residues in apple MHR are terminally linked. Since NMR spectroscopy revealed that xylose was found to be present as terminal residues only, this may suggest that apple MHR populations B and C[2] contained most of the (1→3)- and (1→2)-linked xylose residues found for the complete MHR fractions although the relative amount of xylose in population B and C is much lower as compared to population A[2].

A xylogalacturonan polymer has not been described in full detail to be present in apple pectic substances before, although Barrett and Northcote[30] already in the sixties described the isolation of an aldobiouronic acid consisting of a xylose and a galacturonic acid residue from apple tissue. Other researchers reported also the presence of a terminally linked xylose in pectic substances from apple[2,31], probably linked directly to the galacturonosyl residues as was concluded from base-catalysed β-elimination reactions. Xylogalacturonans polymers having a xylose present as single unit side chains have been reported earlier[32-35]. Bouveng[32] described a rather pure xylogalacturonan, originating from the pollen of the Mountain Pine, with a ratio for xylose:galacturonic acid of 0.5 in which the xylose was also linked to C-3 of the galacturonosyl residues in the backbone. Matsuura[34,35] described the isolation and characterization of a xylogalacturonan from kidney bean cotyledons having a xylose to galacturonic acid ratio of 0.5. Kikuchi and Sugimoto[33] described a xylogalacturonan fragment (DP = 21), isolated after mild acid hydrolysis of an acidic polysaccharide in soy sauce. The xylose to galacturonic acid ratio was reported to be 0.2. The same authors[33] established that the xylose residue was in the β-form in their fraction by using a pure β-xylosidase from *Aspergillus sojae*.

As already suggested by the elution behaviour on the DEAE-ion-exchange column, the xylogalacturonan fraction, obtained after incubation with RGase in combination with RGAEase, was shown to be partly esterified at the carboxyl group of the galacturonic acid residues. The NMR-data suggests that the methoxyl groups are (within the experimental error) equally distributed among substituted and unsubstituted galacturonic acid residues. The other pools of RGase/RGAEase-I obtained after chromatography on DEAE-Sepharose bound less strongly to the column material than the xylogalacturonan investigated by NMR (Fig. 4) although the sugar compositions were rather similar. This might indicate that these xylogalacturonans were more esterified than pool RGase/RGAEase S300 I-*c*. The presence of these populations indicates that there is not an equal distribution of methoxyl groups over the various xylogalacturonan molecules and that distinct groups of esterified xylogalacturonan molecules exist. Whether there is a correlation between the DM value and the degree of xylose-substitution is indefinite. Matsuura[34,35] mentioned that the

xylogalacturonan isolated from kidney bean cotyledons was highly esterified, although no values were given and no suggestions were made about the distribution of the ester groups over the backbone.

It can be concluded that the modified hairy regions of apple pectin are more regular in structure than was reported before. The presence of a xylogalacturonan subunit replaces the xylose substitution of solitary galacturonosyl residues as presumed before[2]. The elution behaviour of the xylogalacturonan fractions on HPSEC suggested a rather high molecular weight (20 000-30 000 Da), while such a high molecular weight is confirmed by the broad lines in the NMR spectra. Next to the xylogalacturonan originating from apple MHR which was isolated from apple tissue using the enzymic liquefaction process, we recently isolated also polymeric xylogalacturonan-rich fractions from the hairy regions of apples which were only treated by polygalacturonase and pectinesterase[36].

Many similarities have been reported for MHR fractions originating from various plant materials[5] and this might also be the case for the presence for the different subunits described here. However, the relative amounts of the subunits might vary considerably as illustrated by the low xylose content in e.g. carrot and leek MHR and the relatively low arabinose content in MHR from onions[5].

References

1   J.A. De Vries, F.M. Rombouts, A.G.J. Voragen, and W. Pilnik, *Carbohydr. Polym.*, 2 (1982) 25-33.
2   H.A. Schols, M.A. Posthumus, and A.G.J. Voragen, *Carbohydr. Res.*, 206 (1990) 117-129 (this thesis, chapter 3).
3   H.A. Schols, C.C.J.M. Geraeds, M.J.F. Searle-van Leeuwen, F.J.M. Kormeling, and A.G.J. Voragen, *Carbohydr. Res.*, 206 (1990) 105-115 (this thesis, chapter 4).
4   M. O'Neill, P. Albersheim, and A. G. Darvill, in P.M. Dey (Ed.), *Methods in Plant Biochemistry*, Vol. 2, *Carbohydrates*, Academic, London, 1990, pp 415-441.
5   H.A. Schols and A.G.J. Voragen, *Carbohydr. Res.*, 256 (1994) 83-95 (this thesis, chapter 6).
6   P. Odonmazig, D. Badga, A. Ebringerová, and J. Alföldi, *Carbohydr. Res.*, 226 (1992) 353-358.
7   H. Kiyohara, J.-C. Cyong, and H. Yamada, *Carbohydr. Res.*, 182 (1988) 259-275.
8   H. Kiyohara, J.-C. Cyong, and H. Yamada, *Carbohydr. Res.*, 193 (1989) 201-214.
9   M. McNeil, A.G. Darvill, and P. Albersheim, *Plant Physiol.*, 70 (1982) 1586-1591.
10 H. Kiyohara, H. Yamada, and Y. Otsuka, *Carbohydr. Res.*, 167 (1987) 221-237.
11 H. Yamada, H. Kiyohara, and Y. Otsuka, *Carbohydr. Res.*, 170 (1987) 181-191.
12 J.R. Thomas, A.G. Darvill, and P. Albersheim, *Carbohydr. Res.*, 185 (1989) 279-305.
13 H.A. Schols, A.G.J. Voragen, and I.J. Colquhoun, *Carbohydr. Res.*,256 (1994) 97-111 (this thesis, chapter 7).
14 J. An, L. Zhang, M.A. O'Neill, P. Albersheim, and A.G. Darvill, *Carbohydr. Res.*, 264 (1994) 83-96.
15 A.G.J. Voragen, H.A. Schols, and H. Gruppen, in F. Meuser, D. J. Manners, and W. Seibel (Eds.), *Plant Polymeric Carbohydrates*, Royal Society of Chemistry, Cambridge, UK, 1993, pp 3-15.
16 N.K. Matheson and B.V. McCleary, *Adv. Carbohydr. Chem. Biochem.*, 1986, 147-176.
17 L.V. Kofod, S. Kauppinen, S. Christgau, L.N. Anderson, H.P. Heldt-Hansen, K. Dörreich, and H. Dalboge, *J. Biol. Chem.*, 269 (1994) 29182-29189.
18 M.J.F. Searle-van Leeuwen, L.A.M. van den Broek, H.A. Schols, G. Beldman, and A.G.J. Voragen, *Appl. Microbiol. Biotechnol.*, 38 (1992) 347-349.

19   M. Mutter, G. Beldman, H.A. Schols, and A.G.J. Voragen, *Plant Physiol.*, 106 (1994) 241-250.

20   P. Lerouge, M.A. O'Neill, A.G. Darvill, and P. Albersheim, *Carbohydr. Res.*, 243 (1993) 359-371.

21   C.M.G.C. Renard, H.A. Schols, A.G.J. Voragen, J.-F. Thibault, and W. Pilnik, *Carbohydr. Polym.*, 15 (1991) 13-32.

22   D.J. States, R.A. Haberkorn, and D.J. Ruben, *J. Magn. Reson.*, 48 (1982) 286-292.

23   C. Griesinger, G. Otting, K. Wuthrich, and R.R. Ernst, *J. Am. Chem. Soc.*, 110 (1988) 7870-7872.

24   A.J. Shaka, P.B. Baker, and R. Freeman, *J. Magn. Reson.*, 64 (1985) 547-552.

25   A. Bax, and S.S. Pochapsky, *J. Magn. Reson.*, 99 (1992) 638-643.

26   S. Medvedeva, J.-P. Simorre, B. Brutscher, F. Guerlesquin, and D. Marion, *FEBS Letters*, 333 (1993) 251-256.

27   H. Grasdalen, O.E. Bakøy, and B. Larsen, *Carbohydr. Res.*, 184 (1988) 183-191.

28   J.H. Bradbury and G.A. Jenkins, *Carbohydr. Res.*, 126 (1984) 125-156.

29   I.J. Colquhoun, G.A. de Ruiter, H.A. Schols, and A.G.J. Voragen, *Carbohydr. Res.*, 206 (1990) 131-144 (this thesis, chapter 5).

30   A.J. Barrett, and D.H. Northcote, *Biochem. J.*, 94 (1965) 617-627.

31   J.A. De Vries, F.M. Rombouts, A.G.J. Voragen, and W. Pilnik, *Carbohydr. Polym.*, 3 (1983) 193-205.

32   O. Bouveng, *Acta Chemica Scand.*, 19 (1965) 953-963.

33   T. Kikuchi, and H. Sugimoto, *Agr. Biol. Chem.*, 40 (1976) 87-92.

34   Y. Matsuura, *Nippon Nôgeikagaku Kaishi*, 58 (1984) 253-259.

35   Y. Matsuura, *Nippon Nôgeikagaku Kaishi*, 58 (1984) 1111-1115.

36   H.A. Schols, E. Vierhuis, E.J. Bakx, and A.G.J. Voragen, *Carbohydr. Res.*, accepted for publication (this thesis, chapter 8).

CHAPTER 10

CONCLUSIVE REMARKS

Motivation for this research

Problems during apple juice manufacture like reduced ultrafiltration efficiency or the quality defect of haze formation during storage of concentrates, may originate from polymeric cell wall polysaccharide fragments released in the juice by multi-component enzyme preparations used during manufacture[1]. Since only a limited number of studies were carried out on polysaccharides present in apple juices prepared by different technologies, we started such an investigation with the hope to be able to explain the problems and to find solutions to avoid or to solve them. Our special interest went to polymeric pectin fragments, resistant to the attack of a wide range of technical enzyme preparations.

Preliminary results indicated that high amounts of the building units arabinose and galacturonic acid residues were present in apple juices when technical enzymes, being complex mixtures of many enzyme activities, were used to treat the apple mash[2]. In our studies towards the effect of ultrafiltration on quality characteristics of juices prepared in different ways, we also started to investigate in more detail the high molecular weight polysaccharides, which were retained by the ultrafiltration membrane.

The aim of this thesis was (i) to investigate the presence of enzyme-resistant polysaccharides in various enzyme-treated and non-enzyme-treated juices, (ii) the elucidation of the structural characteristics of these polysaccharides retained by ultrafiltration and (iii) comparison of the structural features found for this pectic polysaccharide fraction with those of unmodified pectic molecules of the apple cell wall.

Enzyme treated apple juices

Apple juices were prepared at 150-200 kg scale either by direct pressing of the pulp or by the use of enzymes (pulp enzyming and liquefaction). The clarified juices (conventionally using gelatine/bentonite versus ultrafiltration) were judged on characteristics like colour, acidity, and polyphenol content. Special attention was paid to the amount of solubilized polysaccharides in general and more specifically to the amount of the building units arabinose and galacturonic acids present (Chapter 2). As expected, the enzymes solubilized high amounts of cell wall polysaccharides in the juice. Depending on the enzyme mixture used, major variations were found for the sugar composition of the solubilized material. As at that time, the amount of individual sugar residues present in the apples used for juice production was not determined, the recovery of individual sugars units like arabinose and galacturonic acid present in the solubilized polymers was calculated using data obtained for AIS of another batch of Golden Delicious (Chapter 8). The given values are only indicative. It was calculated that in the straight pressed juice, 2% of all arabinose residues was recovered as polymeric

material (retained after dialysis); for pulp enzyme juice, these values varied from 5-28% and for liquefaction juice from 62-120%. Of galacturonic acid, 2% (straight pressing), 23-31% (pulp enzyming) and 40-43% (liquefaction) of the residues present in the pulp were recovered in the juice. From this figures, it is clear that enzyme treatment resulted in an enhanced solubilization of arabinose-rich polymers. Depending on the enzyme cocktail used, also high proportions of galacturonic acid were solubilized from the cell wall; either as low molecular weight material or as polymer. The arabinose was found to be present as free arabinans as well as linked to pectic polysaccharides as already demonstrated by Voragen et al.[2]. When apple juice was obtained by pressing followed by extensive water extraction of the press cake, Will and Dietrich[3] determined pectin concentrations up to 10 times the amount of pectin present in normal press juice. This indicates that under the conditions used by industry to prepare press juices, only a limited amount of the potentially soluble polysaccharides are extracted. Polymeric pectic material was found to be present in all enzymically prepared apple juices, although the amount and sugar composition differed significantly for the various enzyme preparations used. These polysaccharides may reach a concentration up to 5 gram per litre juice and may lead to problems during processing and storage of the juice (concentrate).

Modified hairy regions

Knowledge of the sugar composition of the polymers present in the juice is not sufficient to translate these data to their structure. To know the structure would be of great importance to understand their role in the architecture of the cell wall, why they were solubilized and why they appear to be enzyme resistant. The precise structure might enable us to predict where problems may occur in juice processing and facilitate screening for enzymes able to degrade the polysaccharides during juice manufacture or juice clarification, to solve or even to avoid these problems.

The ultrafiltration retentate of liquefaction juice obtained after treating the pulp with the technical enzyme preparation Rapidase C600 was studied in more detail (Chapter 3). The polysaccharide fraction in this retentate was found to be heterogeneous with respect to molecular weight (HPSEC) and was characterized by a high arabinose content, a high rhamnose to galacturonic acid ratio and a high degree of acetylation of the galacturonic acid residues. The arabinose residues in apple MHR proved to be present in rather long side chains attached to the pectin backbone (Chapter 3) and were more linear than was expected from native apple arabinans (Aspinall and Fanous[4]; this thesis, chapter 9). The characteristics of this pectic polysaccharide resemble those reported by De Vries[5] for apple pectin hairy regions. For this reason and considering the possibility of minor enzymic modifications during the juice manufacture, the fraction was named MHR (modified hairy regions). Based on these data, a first hypothetical model for MHR has been proposed (Chapter 3). The same type of sugar residues and the same type of linkages were found for MHR as described to be characteristic for RG-I, although the ratio of rhamnose to galacturonic acid was rather different. Similar polysaccharide fractions, isolated from pectins present in extracted apple juice by the use of purified pectic enzymes (PG and PE), have been described by Will and

Dietrich[3].

MHR degrading enzymes

Examination of more than 40 different enzyme preparations showed the presence of arabinan-degrading activity in 5 of them (Kroef, 1985, unpublished results), next to minor galactose-releasing activities in some others. We also observed that one of the enzyme preparations tested was able to hydrolyze linkages within the backbone of MHR. The enzyme responsible for this activity was isolated from the technical preparation Pectinex Ultra SP (obtained from *Aspergillus aculeatus*) and was further characterized (Chapter 4). The structure of the oligomeric reaction products was elucidated by NMR spectroscopy (Chapter 5 and 7). They all consisted of a backbone of alternating rhamnose and galacturonic acid residues, substituted with a galactose residue linked to C-4 of (part of) the rhamnose moieties. Since the enzyme was able to cleave galactopyranosyluronic-rhamnopyranosyl linkages within the rhamnogalacturonan backbone, it was designated rhamnogalacturonase (RGase). It was demonstrated that RGase was unable to hydrolyze the original MHR, but needs the removal of acetyl groups, probably linked to C-2 and/or C-3 of the galacturonosyl residues (Chapter 4). Following our findings, RGases of the same type were found to be present in enzyme preparations of *Trametes sanguinea*[6,7], *Irpex lacteus* (Sigma; enzyme preparation Driselase)[8], and *Aspergillus niger*[9]. From the technical enzyme preparation Ultra SP, Searle-van Leeuwen et al.[10] isolated a rhamnogalacturonan acetylesterase (RGAEase), specific for the removal of acetyl groups esterified to the galacturonic acid residues of the rhamnogalacturonan backbone. Mutter et al.[11] isolated and characterized a rhamnogalacturonan α-L-rhamnopyranohydrolase, which is only able to remove rhamnose from chains of alternating rhamnose and galacturonic acid residues. Also the existence of an enzyme able to remove galacturonic acid residues from this type of oligomers is suggested[11]. A rhamnogalacturonase having different pH and temperature optima and able to liberate a different set of rhamnogalacturonan oligomers has been purified recently from *Aspergillus aculeatus* (Mutter et al., unpublished results). This enzyme has been isolated by Kofod et al.[12] by expression cloning in yeast. It can be stated that, in analogy to the enzymic degradation of the homogalacturonan regions of pectin, a whole array of enzymes specific for the degradation of pectic hairy regions, is present in nature.

Next to these enzymes acting on the backbone of MHR, several enzymes acting on the side chains of the pectic molecules like arabinanases[13-15], galactanases[15,16], and galactosidases[11,15] have been described and will not be discussed in detail here.

The availability of these novel enzymes, next to the known pectic enzymes, offer new opportunities to use them as analytical tools in revealing the structure of pectic substances based on their ability to degrade oligo- and polysaccharides in a defined way. This in contrast with chemical degradation methods frequently used, which usually have a poor selectivity. To be able to recognize different structural units within the polymer, endo-acting type of enzymes are preferred.

Pure pectic enzymes, specific for the homogalacturonan regions of pectin, can be used to

completely degrade the smooth regions and so enable the isolation of the hairy regions
(Chapter 8). Depending on the distribution of the methoxyl groups over the homogalacturonan
backbone, degradation products will differ in proportion, size, and degree of methyl
esterification, thus giving valuable information on the intramolecular distribution pattern of
methoxyl groups along the homogalacturonan backbone[17,18].

Information on the distribution of hairy regions over the pectin molecule can be derived
from the degradation products obtained after RGase/RGAEase treatment of the extracted
pectins. When hairy regions are located in the middle of the pectin molecule, different
degradation products are expected as compared to the situation where the hairy regions are
located at the extremity of the intact pectin molecule. After chromatographic separation of
the different degradation products, information may become available on the length of the
smooth regions. Oligomeric and polymeric subunits within the hairy regions can be
distinguished by degradation studies of isolated hairy regions with RGase or other backbone
degrading enzymes.

Structural elucidation of apple MHR using RGase

The above outlined approach of the use of enzymes in structural analysis was followed to
elucidate the structure of apple MHR (Chapter 4). A first hypothetical model was based on
identified structures of the typical RGase oligomers having an alternating sequence of
rhamnose and galacturonic acid residues (Chapter 5 and 7), on data about the sugar and
linkage composition, and chemical and enzymic degradation studies. A drawback of this first
model was the limited number of sugar residues which could be depicted, as a result of which
long and individual subunits could not be shown. To be able to differentiate between various
subunits of MHR, the degradation of MHR by RGase was studied more closely and the three
MHR populations A, B, and C having a different M_w were incubated individually with
RGase. It was found that only apple MHR populations having the highest molecular weights
(A and B) were degraded by RGase to lower molecular weight fragments, while population
C was not degraded at all (results not shown). No explanation for this phenomenon is
available yet, although it is suggested that the backbone of population C might be too highly
branched for RGase to cleave linkages. The structure of the rhamnogalacturonan backbone
may also deviate from a strictly alternating sequence, resulting in short chains of e.g.
galacturonic acid (or rhamnose). The presence of a dimer of rhamnose-(1→2)-rhamnose in a
pectic fragment has been reported by Gao et al.[19]

To facilitate interpretation of the results, apple MHR population A was isolated and treated
with RGase rather than treating the mixture of three populations. The degradation products
formed were further fractionated and characterized (Chapter 9). The recognition of a
xylogalacturonan, next to residual stubs of the pectic backbone having arabinan side chains,
and the typical RGase oligomers encouraged us to propose an adapted model for apple
MHR[20].

A conscientious examination of the relative amounts of the three subunits is now under
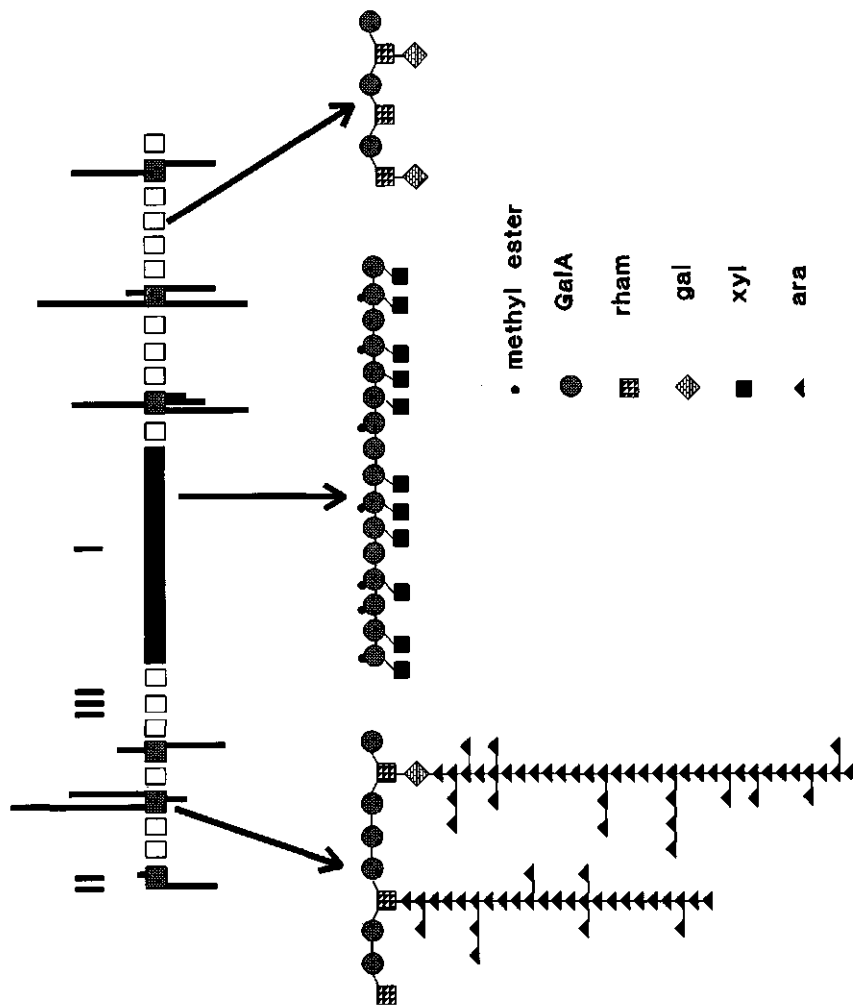investigation. First indications are given here and are based on the relative amounts of the

Fig. 1. Hypothetical structure of the prevailing population of apple MHR having the highest molecular weight. Subunit I, xylogalacturonan; subunit II, stubs of the backbone rich in arabinan side chains; subunit III, rhamnogalacturonase oligomers. The distribution of acetyl groups is not presented, but there is evidence[10,21] 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.

subunits as determined after Sephacryl S200 chromatography of the RGase digest of MHR population A, and on estimation of the molecular weight of population A and its degradation products by HPSEC using pectin standards. It is assumed that population A, the xylogalacturonan subunit I, the rhamnogalacturonan stubs rich in arabinan side chains (subunit II), and the RGase oligomers as released from the rhamnogalacturonan regions (subunit III) have molecular weights of 80, 20-30, 8-12, and 1-1.5 kDa respectively. On weight basis, the ratio of subunits I, II, and III was calculated to be 2:3:1. Using these figures, it can be calculated that in one molecule of MHR population A, there is only one xylogalacturonan segment present, next to 5-6 segments of subunit II, while on average 12 RGase oligomers are present.

It is speculated that the length of the xylogalacturonan backbone might be 75-100 galacturonosyl units. Variations were found for the degree of xylose substitution to C-3 of the galacturonosyl residues in the different xylogalacturonan fractions (40-90%). Also different populations with respect to the methyl esterification of the xylogalacturonan were observed. The xylogalacturonan fraction which was investigated by NMR showed to have a DM of ± 40%. By anion-exchange chromatography using a DEAE-column calibrated with pectins with known DM, also a DM value around 40 to 50% was calculated for this fraction. For the other two xylogalacturonan fractions, DM values of approximately 70 and 85-90 were calculated.

The length of the arabinan side chains present in subunit II may vary between 30 and 50 arabinose residues and from this estimation, it can be deduced that the backbone of this subunit is only 8-10 sugar residues long (ratio of rhamnose to galacturonic acid is 0.2 to 0.4). It is not known yet how the arabinan side chains are connected to the rhamnogalacturonan backbone. They might be connected directly to a rhamnose residue in the backbone or via one or more galactose residues which are linked to a rhamnose residue[22].

Based on the presented findings, an updated model of apple MHR population A is presented in Fig. 1. The sequence in which the various subunits are arranged is purely speculative. On the other hand, whereas RGase is characterized to be a typical endo-acting enzyme (based on HPSEC analysis), the release of RGase oligomers is not representative for an endo-attack (Chapter 7). This might indicate that the regions of alternating rhamnose and galacturonic acid residues within the MHR backbone are not extremely long, and probably are interrupted with other subunits resistant to hydrolysis by RGase.

Apple hairy regions

Based on sugar residue and linkage composition, it has been noticed that apple MHR was similar to the hairy regions, isolated from apple cell walls by mild extraction methods and by using pure enzymes, as described by De Vries et al.[5] However, it was realized that the MHR fraction might originate from different pectic materials located in different sites of the cell wall structures. Another question which arises and remains to be solved is whether hairy regions originating from different pectin populations are similar. The presence of high proportions of terminally linked galactose residues in apple MHR, mainly present as single

unit side chains of rhamnose residues, gave rise to the question whether this is caused by the enzymic liquefaction process or whether they are naturally present in the native hairy regions (HR). Using mild extraction conditions, various pectin populations have been obtained and characterized. Degradation by purified PG and PE resulted in different populations of HR (Chapter 8). The sugar composition resembled that of MHR, although slightly higher proportions of galactose were determined. Again, 20-40% of the galactose residues were found to be terminally linked. RGase was able to release the same set of oligomers from the HR as was described for the MHR, although the ratio between the oligomers differed for the various HR-fractions. More branched RGase oligomers were released from pectins which were extracted under more harsh conditions. From these observations, it was concluded that also native pectins contained hairy regions having a relative high proportion of rhamnose residues substituted with a single galactose moiety. The ratio of the building blocks of subunit III, the RGase oligomers as illustrated in Fig. 1, may vary for the individual hairy regions. Next to the oligomeric reaction products, also the larger RGase degradation products of apple HR were studied in more detail. The subunit consisting of a rhamnogalacturonan segment being rich in arabinan side chains was present in several digests. Both the high arabinose content (50-70%) and the ratio of rhamnose:galacturonic acid (1:5) are in good agreement with those found for subunit II of MHR. Some other fractions were clearly enriched in xylose, indicating the presence of xylogalacturonan segments.

Although the hairy regions isolated from the different pectin extracts were shown to consist of principally the same building blocks, the arrangement of these blocks might be different. This is suggested by the different behaviour of the hairy regions on HPSEC before and after RGase treatment. For some HR-fractions, the molecular weight shifted completely to lower values and typical RGase oligomers were formed when degraded by RGase. In other HR-fractions, the molecules eluted at the same hydrodynamic volume as the starting material although oligomers were formed as well. This suggests that the RGase oligomers in some hairy regions are located on the extremities of the molecules, whereas in other hairy regions they are thought to be distributed more randomly over the molecule. This confirms De Vries's statement "Pectins can be characterized as partially consisting of repeating units, partially consisting of repeating units"[23].

In addition, it can be stated that the distribution of the latter repeating units over the pectin molecules is rather diverse.

MHR from various plant materials

To investigate whether MHR-fragments are unique for apple tissue or can be found in cell walls of plant material of other origin, the same liquefaction process was used to degrade the cell wall polysaccharides of a variety of other plant materials like leek, onion, carrot, pear and potato (Chapter 6). From all juices obtained, a similar MHR fraction could be isolated, although variations were found with respect to arabinose and xylose content.

Typical characteristics of all MHR fractions isolated were the high ratio between rhamnose and galacturonic acid residues and a high acetyl content, similar to those found for apple

MHR. Galactose residues were present in all MHR (10-30 mol%) of which 35-70% appeared to be terminally linked. All isolated MHR fractions had a broad molecular weight distribution; minor differences were found for the sugar composition of the various M_w populations. In all cases, the DA value decreased and the DM increased with decreasing molecular weight.

The model presented in Fig. 1 might also be valid for the MHR fractions isolated from other plant materials. Degradation by RGase of the MHR fractions of the different sources resulted in the same series of RGase oligomers (Chapter 7), next to rather high molecular weight fragments (Chapter 6). The ratio between the subunits I, II, and III varied. Especially the presence of the xylogalacturonan subunit seems to depend on the origin of the MHR.

As observed before for population C of the apple MHR, also the populations C (and D if present) of the MHR from other plant material could not be degraded by RGase, whereas the populations A and B were degraded readily in most cases.

Pectic fractions having similar characteristics as compared to our MHR have been reported in literature before (see also Chapter 6). When the published data are evaluated, indications are present that also the above mentioned subunits were present (although not recognized as such by the authors). Regions of alternating rhamnose and galacturonic acid residues (ratio $\pm$ 1:1) were found in pectin fractions isolated from carrot tissue which was not treated by pectolytic enzymes[24,25], suggesting that "free" RG-I or hairy regions subunit III-like segments might be present in the cell wall. On the other hand, also pectin fractions isolated from carrot tissue having a much higher proportion of galacturonic acid content as compared to rhamnose have been described[25,26], most likely representing hairy regions to which homogalacturonan segments are still attached. Also the presence of acetyl groups in pectins from carrot has been reported[26,27]. Treatment of carrot cell wall polysaccharides with PL[27] and PAL[25] resulted in fragments, having similar characteristics as described for the hairy regions in our study: a high content in neutral sugars and a high ratio of rhamnose:galacturonic acid. Also terminally linked galactose residues were reported to be present in these rhamnogalacturonan fractions[27] and may suggest the presence of structures like we described for our subunit III. Next to a pectin fraction isolated from non-enzyme treated carrot tissue containing arabinose (64%), rhamnose (4%) and galacturonic acid (5%)[26], more arabinose-rich fractions were isolated from carrot cell walls after enzyme treatment[25,27]. These fractions are rather similar to our subunit II as described for apple MHR. Other fractions described by Massiot et al.[27], were relatively enriched in galactose resulting in a ratio of arabinose to galactose residues of 1:5 or 1:2. The relatively high galactose content was also found for carrot MHR with a ratio of 1:2 for arabinose to galactose.

From the above mentioned findings, it can be concluded that our subunit model as proposed for apple MHR is not generally valid. The xylogalacturonan subunit might be absent in some MHR fractions, since we only determined 1% xylose in carrot MHR. Contrarily, Massiot et al.[27] isolated pectic fractions with a xylose content up to 10%, next to a xylose to galacturonic acid ratio of 0.3. More than 50% of all xylose residues present were terminally linked, while in some other fractions *all* xylose moieties were terminally linked. This may indicate that xylogalacturonan also exist in pectins extracted from carrots, although the quantity might depend on the procedure of isolation. Subunit II, as proposed to be present in apple MHR,

might as well be present in hairy regions of other origin, although major differences may exist in the neutral sugar composition of the side chains.

Is a general model for plant cell wall pectic substances feasible?

One of the challenges for scientists is to express their findings in a model which has a more general validity. De Vries[23] stated that all polysaccharides containing galacturonic acid may be constructed of the building blocks: homogalacturonan, apiogalacturonan, xylogalacturonan, rhamnogalacturonan, and galactogalacturonan. As illustrated in this thesis, enzymes capable to split within these regions facilitate a more closer view into the quite complex ramified

TABLE I. Suggested subunits present in most pectic substances, next to possible variations within an individual subunit

subunit	diversity based on:
homogalacturonan (ref[28,29])	* length of the homogalacturonan sequences between individual rhamnose residues * degree and distribution of methyl esterification * degree and distribution of acetyl esterification
RG-I[30]	* nature of neutral sugars (and acidic sugars?) present in side chains * length, sugar, and linkage composition and degree of branching of side chains * distribution of side chains over the alternating rhamnose-(1→4)-galacturonic acid backbone
RG-II[30]	* proportion of common and rare sugars like Ome-xylose, KDO, DHA. * distribution of neutral sugars in the side chains * number, type and distribution of uronic acids in the (side) chains * attachment and distribution of RG-II chains over the pectic molecule
rhamnogalacturonan (this thesis)	* rhamnose:galacturonic acid ratio * relative proportion of neutral sugars (mainly arabinose and galactose) * length, degree of branching and distribution of side chains * degree and distribution of acetyl esterification (and methyl esterification?)
xylogalacturonan (this thesis)	* degree of xylose substitution * degree of methyl esterification (and acetylation?) * distribution of substituents over the backbone
apiogalacturonan (ref[31,32])	* degree of apiose substitution * length of apiose chains * methyl esterification? * distribution of substituents over the backbone

regions of pectins, and even might enable us to propose a more general model for pectic substances originating from plant cell walls.

Our results obtained for apple pectic substances and the modified hairy regions of plant material from several other sources suggest that the model for apple MHR, as proposed in Fig. 1, may be used as a starting point to develop such a model. Principal differences in relative amounts between the various subunits may exist, which is substantiated by the fact that no indications were found for the presence of RG-II or apiogalacturonan type of polymers in apple. Suggestions for major variations, which might be present within one given subunit, are listed in Table I.

In literature, pectic molecules are sometimes termed RG-I, but also the more general term rhamnogalacturonan is used. Since it became clear[30] that RG-I is used for pectic segments which have a strictly alternating sequence of rhamnose and galacturonic acid, the more general name "rhamnogalacturonan" might be given to pectic fragments having a low rhamnose:galacturonic acid ratio. To be able to differentiate between rhamnogalacturonans and homogalacturonans having individual rhamnose moieties in between long sequences of galacturonic acid, rhamnogalacturonans are considered to have a ratio varying between 0.05 and 1. Segments of the backbone of at least 20 galacturonic acid residues with only one solitary rhamnose residue can then be termed homogalacturonans. Although the definition of rhamnogalacturonan would include RG-II type of polysaccharides, it is recognized that the term RG-II is used for a rather characteristic segment of pectic molecules. Due to the rare sugars present in RG-II, no confusion is expected to occur.

RG-I and hairy regions; the same type of polysaccharide?

The fact that two polysaccharides consisting of the same sugar residues, and having the same sugar linkage composition, were given different names in literature might cause confusion. Where the group of Albersheim[30] started their exploration of pectic ramified regions from suspension cultured sycamore cells, the group of Pilnik and Voragen (De Vries[33], Renard[34], this thesis) studied apple pectic molecules. Pectic material originating from the sycamore cells was shown to consist only of homogalacturonan regions and of RG-I and RG-II segments. This concept is also recognized to be valid for pectins from other sources. However, there have been several reports in literature[23,30], where the combination of these three segments alone failed to fully described the pectin under investigation. This was also found in this study. We believe that our definition of "hairy regions", together with the homogalacturonan or "smooth" regions, cover the structure of pectin as found in various sources. In our view, RG-I can be considered to be part of these hairy regions, although it is clearly shown that they may be present predominantly in pectins from e.g. sycamore cells.

Consequences for fruit juice manufacture.

The principal aim of this thesis is to contribute to the basic knowledge of the structure of

pectic cell wall polysaccharides from apple and at the same time, to form a bridge between fundamental knowledge and real problems in the fruit juice industry.

MHR-type polysaccharides have obtained increased interest since Will and Dietrich[3] clearly showed that this type of rhamnogalacturonan material caused membrane fouling during filtration experiments which resulted in a marked reduction in flux rate. Since we started our study 8 years ago, the liquefaction process is increasingly used by the juice industry and membrane fouling and reduced flux rates are a serious problem. Our study might be helpful in overcoming the ultrafiltration problem. RGase is able to partially degrade the MHR material, however, RGase needs the activity of the accessory enzyme rhamnogalacturonan acetylesterase. This latter enzyme is only present in low concentrations in technical enzyme preparations and the time used for the liquefaction process might be too short to complete the degradation of MHR-type polymers. An increased dose of RGase and RGAEase might be helpful, but on the other hand, one can hypothesize over the polymeric xylogalacturonan and rhamnogalacturonan chains which will remain after RGase treatment and might cause membrane fouling as well. Since the technical enzyme preparations nowadays are enriched in arabinan-degrading activities, problems due to haze formation caused by linearized arabinan molecules, have been overcome.

The described research gives a good illustration of how to address such new problems and research is already in progress in our laboratory to identify new enzymes able to degrade the various subunits of MHR-like polysaccharides.

References

1   W. Pilnik, and A.G.J. Voragen, in P.F. Fox (Ed), *Food Enzymology*, Vol. 1, Elsevier Applied Science, London, 1991, pp. 303-336.
2   A.G.J. Voragen, H.A. Schols, H.A.I. Siliha, and W. Pilnik, *ACS Symp. Ser.*, 310 (1986) 230-247.
3   F. Will, and H. Dietrich, *Carbohydr. Polym.*, 18 (1992) 109-117.
4   G.O. Aspinall, and H.K. Fanous, *Carbohydr. Polym.*, 4 (1984) 193-214.
5   J. A. De Vries, F. M. Rombouts, A. G. J. Voragen, and W. Pilnik, *Carbohydr. Polym.*, 2 (1982) 25-33.
6   M. Sakamoto, Y. Shirane, I. Naribayashi, K. Kimura, N. Morishita, T. Sakamoto, and T. Sakai, *Eur. J. Biochem.*, 226 (1994) 285-291.
7   T. Sakamoto, and T. Sakai, *Carbohydr. Res.*, 259 (1994) 77-91.
8   E.-M. Düsterhöft, A.W. Bonte, J.C. Venekamp, and A.G.J. Voragen, *World J. Microbiol. Biotechnol.*, 9 (1993) 544-554.
9   J. An, L. Zhang, M.A. O'Neill, P. Albersheim, and A.G. Darvill, *Carbohydr. Res.*, 264 (1994) 83-96.
10 M. J. F. Searle-van Leeuwen, L. A. M. van den Broek, H. A. Schols, G. Beldman, and A. G. J. Voragen, *Appl. Microbiol. Biotechnol.*, 38 (1992) 347-349.
11 M. Mutter, G. Beldman, H. A. Schols, and A. G. J. Voragen, *Plant Physiol.*, 106 (1994) 241-250.
12 L.V. Kofod, S. Kauppinen, S. Christgau, L.N. Anderson, H.P. Heldt-Hansen, K. Dörreich, and H. Dalbøge, *J. Biol. Chem.*, 269 (1994) 29182-29189.
13 A.G.J. Voragen, F.M. Rombouts, M.F. Searle-van Leeuwen, H.A. Schols, and W. Pilnik, *Food Hydrocolloids*, 1 (1987) 423-437.
14 F.M. Rombouts, A.G.J. Voragen, M.F. Searle-van Leeuwen, C.C.J.M. Geraeds, H.A. Schols, and W. Pilnik, *Carbohydr. Polym.*, 9 (1988) 25-47.
15 P. Lerouge, M. A. O'Neill, A. G. Darvill, and P. Albersheim, *Carbohydr. Res.*, 243 (1993) 359-371.
16 C.M.G. C. Renard, H.A. Schols, A.G.J. Voragen, J.-F. Thibault, and W. Pilnik, *Carbohydr. Polym.*, 15

(1991) 13-32.

17 T.P. Kravtchenko, M. Penci, A.G.J. Voragen, and W. Pilnik, *Carbohydr. Polym.*, 20 (1993) 195-205.

18 H. Kiyohara and H. Yamada, *Carbohydr. Polym.*, 25 (1994) 117-122.

19 Q.-P. Gao, H. Kiyohara, and H. Yamada, *Carbohydr. Res.*, 196 (1990) 111-125.

20 A.G.J. Voragen, H.A. Schols, and H. Gruppen, in F. Meuser, D. J. Manners, and W. Seibel (Eds.), *Plant Polymeric Carbohydrates*, Royal Society of Chemistry, Cambridge, UK, 1993, pp 3-15.

21 H.A. Schols, C.C.J.M. Geraeds, M.J.F. Searle-van Leeuwen, F.J.M. Kormeling, and A.G.J. Voragen, *Carbohydr. Res.*, 206 (1990) 105-115 (this thesis, chapter 4).

22 J.M. Lau, M. McNeil, A.G. Darvill, and P. Albersheim, *Carbohydr. Res.*, 168 (1988) 245-274.

23 J.A. De Vries, in G.O. Philips, D.J. Wedlock, and P.A. Williams (Eds.), *Gums and Stabilizers for the Food Industry 4*, IRL Press, Oxford, 1988, pp. 25-29.

24 G. Dongowski and H. Anger. in F. Meuser, D. J. Manners, and W. Seibel (Eds.), *Plant Polymeric Carbohydrates*, Royal Society of Chemistry, Cambridge, UK, 1995.

25 H. Konno, Y. Yamasaki and K. Katoh. *Phytochemistry*, 25 (1986) 623-627.

26 P. Massiot, X. Rouau and J.-F. Thibault, *Carbohydr. Res.*, 172 (1988) 229-242.

27 P. Massiot and J.-F. Thibault, *Carbohydr. Res.*, 190 (1989) 121-136.

28 J.-F. Thibault, C.M.G.C. Renard, M.A.V. Axelos, P. Roger, and M-J. Crépeau, *Carbohydr. Res.*, 238 (1993) 271-286.

29 T. P. Kravtchenko, *Studies on the structure of industrial high methoxyl pectins*, Ph.D. Thesis, Wageningen Agricultural University, The Netherlands, 1992.

30 M. O'Neill, P. Albersheim, and A. G. Darvill, in P.M. Dey (Ed.), *Methods in Plant Biochemistry*, Vol. 2, *Carbohydrates*, Academic, London, 1990, pp 415-441.

31 Yu.S. Ovodov, *Pure Appl. Chem.*, 42 (1975) 351-369.

32 D.A. Hart, and P.K. Kindel, *Biochem. J.*, 116 (1970) 569-579.

33 J.A. De Vries, *Structural features of apple pectic substances*, Ph.D. Thesis, Wageningen Agricultural University, The Netherlands, 1983.

34 C.M.G.C. Renard, *Etude des polysaccharides parietaux de la pomme, extraction et caracterisation par des methodes chimiques et enzymatiques*, Ph.D. Thesis, University of Nantes, France, 1989.

SUMMARY

The aim of the research described in this thesis was (i) to investigate the polysaccharides present in various enzyme-treated and non-enzyme-treated juices, (ii) the elucidation of the structural characteristics of a pectic polysaccharide from liquefaction juice remaining after ultrafiltration and (iii) comparison of the structural features found for this polymeric pectin fraction with those of unmodified pectic molecules in the apple cell wall.

Chapter 1 describes briefly recent insights on the interaction of the various polysaccharides within the primary plant cell wall. Pectic polysaccharides, of major importance during the production of apple juices, are described in detail. Attention is paid to pectin degrading enzymes and a short overview is given on the structural features of apple pectic substances.

In chapter 2, the characterization of apple juices obtained by straight pressing, pulp-enzyming, and liquefaction is described. The sugar, acid, soluble polysaccharide and polyphenol content, colour and ultrafiltration fluxes are given. The amount of solubilized polysaccharides in the juice depended on the technology and enzyme mixture used and contents up to 5 gram per litre juice were found. The composition and the molecular weight distribution of the solubilized carbohydrates varied significantly, although galacturonic acid and arabinose were the major constituent sugars in all solubilized polysaccharides. Arabinans were present both as free polymers as well as linked to pectic molecules. Only part of the free arabinans could be removed by ultrafiltration. Endo-arabinanase treatment revealed that these arabinans were linearized during solubilization and for this reason might cause haze-problems during storage of the concentrated juices.

A high molecular weight pectin fraction, released from the cell walls of apples by the enzymic liquefaction process and remaining as ultrafiltration retentate, is described in detail in chapter 3. Arabinose was the most important sugar residue (55 mol%), next to galacturonic acid (21 mol%), rhamnose (6 mol%), xylose (8 mol%) and galactose (9 mol%). The sugar composition and the ratio of rhamnose to galacturonic acid moieties resembled those described earlier for hairy regions and the pectin fraction was termed modified hairy regions (MHR). About half of the galacturonic acid residues were methyl esterified (42%), while 62 moles of acetyl ester groups were present on every 100 moles of galacturonic acid. It is shown that MHR consists of three distinct populations differing in molecular weight, although only minor differences were observed for the sugar composition. Chemical and enzymic characterization resulted in a hypothetical model for MHR.

An enzyme able to degrade the backbone of MHR was purified and characterized (chapter 4). Since the enzyme cleaved galactopyranosyluronic-rhamnopyranosyl linkages within the rhamnogalacturonan backbone, it was termed rhamnogalacturonase (RGase). Optimal conditions for the purified RGase were pH 3-4 and 40-50°C. RGase was only able to hydrolyse approximately 4% of all sugar linkages when the acetyl groups were removed prior to incubation. Separation by size-exclusion chromatography of the RGase digest of MHR resulted in an oligomeric fraction which was mainly composed of rhamnose, galactose, and galacturonic acid.

The identification of a mixture of four RGase oligomers by various 2D NMR techniques

is described in chapter 5. All structures were based on the unit α-Rha$p$-(1→4)-α-Gal$p$A-(1→2)-α-Rha$p$-(1→4)Gal$p$A. A β-Gal$p$ unit was 4-linked to approximately half of the terminal Rha$p$ residues and to half of the (1→2)-linked Rha$p$ residues.

In chapter 6 various MHR fractions obtained from potato fibre, pear, carrot, leek and onion tissue, using the same enzymic procedure as was used to obtain apple MHR, were compared. Next to principal differences in arabinose and xylose content, all MHR fractions were characterized by a high rhamnose to galacturonic acid ratio and a high acetyl content. A similar heterogenous molecular weight distribution was found for all MHR, while RGase was able to degrade all preparations in a similar fashion.

The separation by high-performance anion-exchange chromatography (HPAEC) of the RGase oligomers is described in chapter 7. Next to the four identified oligomers, the presence of five other oligomeric structures was recognized. After size-exclusion chromatography and preparative HPAEC, the structure of the purified oligomers was revealed by ^{1}H NMR. RGase was able to liberate oligomers from apple MHR in the range of tetramer to nonamer, all having a tetrameric or hexameric backbone of alternating rhamnose and galacturonic acid residues, partly substituted with galactose residues linked to C-4 of the rhamnose moiety. Monitoring the degradation of apple MHR at increasing incubation times revealed that all oligomers were released straight from the initial stage of the reaction and the ratio between the various oligomers remained constant with time. Corresponding HPAEC elution patterns were obtained for RGase digests of MHR isolated from potato, pear, carrot, leek, and onion MHR, indicating that the identified structures were universally present in pectic hairy regions isolated from various plant material.

In chapter 8 the sugar composition of carefully extracted pectins from apple cell walls is compared. Non-modified hairy regions were isolated from these fractions by enzymic degradation of the homogalacturonan regions by purified polygalacturonase and pectinesterase. Differences in yield, sugar composition and degradability by RGase are described for the various hairy regions. More linear RGase oligomers were found in digests of the hairy regions of easy-extractable pectins, while more galactose-substitution occurred in oligomers liberated from the hairy regions originating from pectins extracted under more severe conditions. RGase degradation products also included polymeric fragments representing residual stubs of the backbone rich in arabinose. Also accumulation of xylose and galacturonic acid appeared in one of the high molecular weight fractions.

In chapter 9 subunits present in the highest molecular weight fraction of apple MHR are described in detail. MHR was degraded by RGase after chemical saponification to remove the estergroups, but also rhamnogalacturonan acetylesterase was used to enable RGase to act. Next to the typical RGase oligomers, residual stubs of the backbone carrying arabinose side chains and furthermore xylogalacturonan segments were recognized. Various xylogalacturonan fractions were isolated and differences in xylose substitution and elution behaviour on a anion-exchange column were determined. A xylogalacturonan studied by NMR was shown to have a xylose to galacturonic acid ratio of 0.7 while both the xylose residues and the methyl esters were distributed randomly over the backbone.

Finally, in chapter 10, an adapted model is presented for the prevailing population of apple MHR having the highest molecular weight, taking into account the presence, size and relative

amounts of the subunits. The universal validity of this model for pectic hairy regions from other plant sources was verified on the basis of our data and data published by other authors. It was concluded that such a model in essence may be possible, although a particular subunit might be absent in pectins from a given source, while other subunits prevail. Possible differences within the various subunits are given. Finally, the similarity and difference between the pectic hairy regions and rhamnogalacturonan I as described in literature is summarized.

SAMENVATTING

Doel van het in dit proefschrift beschreven onderzoek was (1) het onderzoeken van enzym-resistente polysacchariden aanwezig in appelsappen, verkregen middels persen en met behulp van enzymen, (2) het ophelderen van de structuurkenmerken van de polysacchariden die achterbleven na ultrafiltratie van een sap verkregen door enzymatische vervloeiïng en (3) vergelijking van de gevonden fijnstructuur van deze hoog-moleculaire pectine fractie met structuren zoals die gevonden zijn voor niet gemodificeerde pectine moleculen uit de appel-celwand.

Hoofdstuk 1 geeft een korte beschrijving van een recent celwandmodel welke de samenhang van de verschillende polysacchariden weergeeft voor de primaire celwand. Pectine speelt een belangrijke rol bij de produktie van appelsap en daarom worden de verschillende pectine-structuren gedetailleerd besproken. De verschillende enzymen die actief zijn op pectine worden genoemd en er wordt een kort overzicht gegeven van de huidige kennis van de structuur van appelpectine.

Karakteristieken van appelsappen, verkregen middels persen, pulp-enzymering en vervloeiing, zoals suiker-, zuur-, polysaccharide- en polyfenolgehalte en kleur en ultrafiltratie-snelheid worden beschreven in hoofdstuk 2. De hoeveelheid opgeloste polysacchariden bleek afhankelijk te zijn van het gebruikte proces en het gebruikte enzympreparaat en kon oplopen tot 5 gram per liter sap. De suikersamenstelling en de molecuulgewichtsverdeling van de opgeloste polysacchariden varieerde beduidend, maar arabinose en galacturonzuur waren steeds de belangrijkste bouwstenen. Arabanen bleken zowel voor te komen als vrij polymeer als gebonden aan pectine moleculen, terwijl maar een deel van de vrije arabanen uit het sap verwijderd kon worden middels ultrafiltratie. Enzymbehandeling met endo-arabanase toonde aan dat deze arabanen gelineariseerd waren tijdens de sapbereiding en daarom mogelijk een troebeling zouden kunnen veroorzaken in appelsapconcentraten.

Hoofdstuk 3 beschrijft een hoog-moleculaire pectinefractie die vrijgemaakt werd uit de appelcelwand tijdens het vervloeiïngsproces en achterbleef tijdens ultrafiltratie. Arabinose was de belangrijkste suikerbouwsteen (55 mol%); verder werd galacturonzuur (21 mol%), rhamnose (6 mol%), xylose (8 mol%) en galactose (9 mol%) gevonden. De suikersamenstelling en de verhouding tussen rhamnose en galacturonzuur kwamen sterk overeen met waarden zoals beschreven voor de sterk vertakte pectine fractie, de zogenaamde "hairy regions" en de geïsoleerde fractie wordt vervolgens steeds aangegeven met MHR ("modified hairy regions"). Van de galacturonzuur eenheden was 42% veresterd met een methoxylgroep, terwijl er 62 acetylgroepen aanwezig waren per 100 moleculen galacturonzuur. Aangetoond werd dat MHR bestaat uit drie populaties met verschillend molecuulgewicht, waarbij slechts kleine verschillen werden gevonden in de suikersamenstelling. Op basis van de chemische en enzymatische karakterisering van MHR werd een model voor de fijnstructuur opgesteld.

Een enzym dat in staat bleek om in de hoofdketen van MHR te splitsen werd opgezuiverd en verder gekarakteriseerd (hoofdstuk 4). Het enzym werd rhamnogalacturonase (RGase) genoemd omdat het zeer selectief de hydrolyse katalyseert van de glycosidische binding tussen

galacturonzuur en rhamnose in de rhamnogalacturonaan-hoofdketen. De hoogste activiteit van het enzym werd gemeten bij pH 3-4 en bij 40-50°C. RGase bleek slechts te kunnen splitsen (max. 4% van alle bindingen) als alle acetylesters verwijderd werden. Scheiding van de reaktieprodukten van MHR na afbraak met RGase met behulp van gelpermeatie-chromatografie resulteerde in een oligomeren-bevattende fractie. De oligomeren bleken vooral opgebouwd te zijn uit rhamnose, galactose en galacturonzuur.

De identificatie van een viertal RGase oligomeren met behulp van 2D NMR is beschreven in hoofdstuk 5. Alle oligomeren hadden eenzelfde hoofdketen van α-Rha$p$-(1→4)-α-Gal$p$A-(1→2)-α-Rha$p$-(1→4)Gal$p$A. Zowel de helft van de eindstandig gebonden rhamnose eenheden als de helft van de (1→4)-gebonden rhamnose eenheden was gesubstitueerd via C-4 met een β-Gal$p$ eenheid.

In hoofdstuk 6 wordt de isolatie beschreven van andere MHR fracties uit aardappelvezel en peer-, wortel-, prei- en ui-weefsel, verkregen met hetzelfde enzymproces als gebruikt werd om appel MHR te isoleren. Naast verschillen in het arabinose- en xylosegehalte werden de MHR fracties vooral gekenmerkt door de hoge verhouding tussen rhamnose en galacturonzuur en een hoog acetylgehalte. Een zelfde brede molecuulgewichtsverdeling als voor appel MHR werd ook gevonden voor de andere MHR fracties, terwijl ook de afbraak met RGase een vergelijkbaar beeld te zien gaf.

De scheiding van RGase oligomeren met behulp van HPAEC ("high-performance anion-exchange chromatography") wordt beschreven in hoofdstuk 7. Naast pieken van de vier geïdentificeerde oligomeren werden in het chromatogram nog een vijftal andere pieken zichtbaar. Met behulp van gelpermeatie chromatografie en preparatieve HPAEC werden deze componenten opgezuiverd en de structuur bepaald met behulp van ^{1}H NMR. RGase bleek een serie van oligosacchariden vrij te maken van tetrameer tot nonameer, allemaal met een hoofdketen van vier of zes alternerende eenheden van rhamnose en galacturonzuur. Galactose-substitutie kwam voor op C-4 van een deel van de rhamnose bouwstenen. Bij het volgen van de afbraak van appel MHR met RGase in de tijd werd duidelijk dat alle oligomere afbraakprodukten direct vanaf het begin gevormd werden en tijdens de gehele reactie steeds in ongeveer dezelfde verhouding aanwezig waren. Met behulp van HPAEC werden overeenkomstige elutiepatronen gevonden voor de gevormde oligomeren na afbraak van aardappel-, peer-, wortel-, prei- en ui-MHR, hetgeen erop wijst dat de geïdentificeerde structuren universeel voorkomen in pectine "hairy regions".

In hoofdstuk 8 wordt de suikersamenstelling vergeleken van een aantal pectine fracties die uit de appelcelwand geïsoleerd zijn zonder de structuur wezenlijk te veranderen. Hairy regions werden bereid uit deze pectinefracties door middel van afbraak van het homogalacturonaan door polygalacturonase en pectine-esterase. Verschillen tussen de "hairy regions" werden aangetoond in de opbrengst, suikersamenstelling en enzymatische afbreekbaarheid met RGase. De gemakkelijk extraheerbare pectines bleken vooral de meer lineaire RGase oligomeren in de "hairy regions" te bevatten, terwijl de "hairy regions" van de moeilijk-extraheerbare pectines vooral de galactose-gesubstitueerde RGase-oligomeren bevatten. Na afbraak met RGase bleken de hoogmoleculaire MHR-afbraakprodukten hoofdketenfragmenten met arabinose-rijke zijketens te bevatten, terwijl xylose en galacturonzuur sterk geconcentreerd voorkwamen in één van de andere populaties.

In hoofdstuk 9 worden de subeenheden beschreven die voorkomen in de hoogst-moleculaire fractie van appel MHR. MHR werd afgebroken met RGase na chemische verwijdering van alle estergroepen, maar ook werd gebruik gemaakt van de combinatie van RGase en rhamnogalacturonaan-acetylesterase. Naast de karakteristieke RGase oligomeren werden arabinose-rijke fragmenten van de hoofdketen en een xylogalacturonaan herkend. Van deze xylogalacturonanen werden meerdere fracties geïsoleerd die verschilden in xylose-substitutie en elutiegedrag over een anionenwisselingskolom. Eén van deze fracties werd nader bestudeerd met behulp van NMR en had een verhouding tussen xylose en galacturonzuur van 0.7 en een veresteringsgraad van 39%. De verdeling van de xylose-zijketens en de methylesters over de hoofdketen bleek willekeurig.

In hoofdstuk 10 wordt op basis van de verkregen resultaten een nieuw model gepresenteerd voor de meest belangrijke populatie uit appel-MHR die tevens het hoogste molecuulgewicht had. Hierbij werd rekening gehouden met de grootte en het aantal van de verschillende subeenheden. De discussie wordt aangegaan of er een universeel model mogelijk is voor pectine "hairy regions" uit allerlei plantemateriaal zoals beschreven in de literatuur. Er wordt geconcludeerd dat zo'n model inderdaad mogelijk is, hoewel niet alle subeenheden (in gelijke hoeveelheden) hoeven voor te komen in alle pectines. Mogelijke variaties binnen de verschillende subeenheden worden besproken. Als laatste wordt ingegaan op de verschillen en overeenkomsten tussen de "hairy regions" en de in de literatuur beschreven "rhamnogalacturonan I".

CURRICULUM VITAE

Hendrik Arie (Henk) Schols werd op 28 december 1956 geboren te Heenvliet (ZH). In 1973 behaalde hij het MAVO-diploma aan de Bahûrim-Mavo te Brielle. In datzelfde jaar werd via een voorbereidend jaar begonnen met de studie Chemisch Hoger Beroeps Onderwijs aan het "Van 't Hoff Instituut" te Rotterdam. Na de HBO-opleiding werd doorgegaan met het Chemisch Hoger Technisch Onderwijs aan hetzelfde instituut. In oktober 1978 werd het HTS-diploma chemie behaald met als hoofdvakken organische chemie, biochemie en analytische chemie. Al tijdens zijn stage-periode bij de sectie Levensmiddelenchemie en -microbiologie van de vakgroep Levensmiddelentechnologie van de Landbouwhogeschool kreeg hij in december 1977 een vaste aanstelling als analist bij dezelfde sectie. Het in dit proefschrift beschreven onderzoek werd, naast de andere werkzaamheden, in de periode 1986-1994 uitgevoerd.

Sedert 1 januari 1994 is hij als toegevoegd onderzoeker aan bovengenoemde sectie verbonden.