Structural characterization of native pectins

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Structural characterization of native pectins

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Abstract

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Key words Pectin, structural elements, homogalacturonan, xylogalacturonan, rhamnogalacturonan, degradation, oligosaccharides, CE-MS, model

Pectin is of interest as cell wall component, food additive, and health promoting substance. Revealing the precise structure of pectin is necessary to optimize its industrial and medical application as well as to increase understanding of its physiological function in plants and technological function in food processing. Although the structure of the different elements of which pectin is composed is rather well known, the exact build up of the polymer remains under debate. Therefore, the aim of the research described in this thesis was to develop new approaches to identify linkages involved in the connection of pectin's different structural elements, which would lead to an improved understanding of the pectin structure.

Oligosaccharides, obtained by controlled acid hydrolysis of apple pectin, were characterized using optimized LC-MS and NMR approaches. These oligosaccharides indicated that in apple pectin both homogalacturonan (HG) and xylogalacturonan (XGA) were covalently linked to rhamnogalacturonan I (RGI). A newly developed method coupled capillary electrophoresis (CE) on-line to mass spectrometry (MS). This approach allowed annotation of the signals in CE electropherograms of complex apple XGA and RGI digests. Furthermore, oligosaccharides present in low amounts could be localized and identified by their corresponding mass fragmentation. Using selective degradation by β-elimination, it was demonstrated that arabinans containing a rudimentary RGI backbone fragment play an important role within the complement fixing activity of white cabbage (*Brassica oleracea)* pectin. By combining β-eliminative breakdown with CE-LIF and CE-UV-MS^n analysis, detailed information on the neutral sugar side chain structure and distribution was obtained of some enzyme resistant structural elements of soy pectin.

Based on the results obtained in this research and data reported in literature, a pectin model was proposed which positions HG both in–line and as a side chain of RGI. In addition, new insights in the position of XGA with respect to HG and RGI were obtained. A further increase of our knowledge on pectin and polysaccharide structures can be achieved by combining the "combined approaches" described in this thesis with other chemical and novel enzymatic degradation methods and by implementing these methods more quantitatively.

Contents

Chapter 1

General introduction

Part of this chapter will be published as,
Voragen, A.G.J., G.J. Coenen, R.P. Verhoef, and H.A. Schols. Pectin, a multifunctional food component. In P. A. Williams, & G. O. Philips. Gums and Stabilisers for the food industry. Cambridge: The Royal Society of Chemistry.

Pectin, a versatile polysaccharide

Pectin is one of the major plant cell wall components and probably the most complex macromolecule in nature, as it can be composed out of as many as 17 different monosaccharides containing more than 20 different linkages (Mohnen, 1999; O'Neill *et al.*, 2004; Ridley *et al.*, 2001).

Plant functionality of pectin

In a plant, pectin is present in the primary cell wall and middle lamella and is deposited in the early stages of growth during cell expansion (Crombie *et al.*, 2003). Its functionality to a plant is quite divers. Firstly, pectin plays an important role in the formation of higher plant cell walls (Fry, 1988), which lend strength and support to a plant and yet are very dynamic structures (Crombie *et al.*, 2003). In general, the polymeric composition of primary cell walls in dicotyledonous plants consists of approximately 35% pectin, 30% cellulose, 30% hemicellulose, and 5% protein (Fry, 1988). In cell walls of some fruits and vegetables, the pectin content can be substantially higher and the protein content lower (Fischer & Bennett, 1991). Secondly, pectin influences various cell wall properties such as porosity, surface charge, pH, and ion balance and therefore is of importance to the ion transport in the cell wall (McNeil *et al.*, 1984). Furthermore, pectin oligosaccharides are known to activate plant defense responses: they elicit the accumulation of phytoalexin which has a wide spectrum of anti-microbial activity (Hahn *et al.*, 1981; Jin & West, 1984; Nothnagel *et al.*, 1983). Finally, pectin oligosaccharides induce lignification (Robertsen, 1986) and accumulation of protease inhibitors (Bishop *et al.*, 1984) in plant tissues.

Pectin as food ingredient

Pectin is used in foods mainly as gelling, stabilizing, or thickening agent in products such as jam, yoghurt drinks, fruity milk drinks, and ice cream (Laurent & Boulenguer, 2003). Most of the pectin used by food industry originates from citrus or apple peel (Pilgrim *et al.*, 1991). In products that naturally contain pectin, e.g. fruit and vegetables, important quality changes during storage and processing are related to changes in pectin structure. Native or added pectic enzymes can play an important role in these changes (Pilnik & Voragen, 1970).

Health aspects of pectins

Plant products, fresh, extracted or processed, constitute a large part of the human diet. As a fiber naturally present in these food products, pectic substances fulfill a nutritional function (Bock & Krause, 1978; Cummings, 1979). Next to its nutritional status, pectin

increasingly gains interest as a possible health promoting polysaccharide and several studies have been conducted to prove its health promoting function. One study showed the beneficial influence of vegetable pectin-chamomile extract on shortening the course of unspecific diarrhoea and relieving associated symptoms (Becker *et al.*, 2006). Another study revealed that carrot soup contains pectin derived oligosaccharides that block the adherence of various pathogenic micro-organisms to the intestinal mucosa *in vitro*, which is an important initial step in the pathogenesis of gastrointestinal infections (Guggenbichler *et al.*, 1997; Kastner *et al.*, 2002). Furthermore, pectins were shown to have immuno-regulatory effects in the intestine, to change the ileal microbial activity, to change the morphology of the small intestinal wall (Langhout *et al.*, 1999; Lim *et al.*, 1997), to lower the blood cholesterol level (Kay *et al.*, 1978; Mokady, 1973; Trumbo *et al.*, 2002), and to slow down the absorption of glucose in the serum of diabetic and obese patients (Jenkins *et al.*, 1976; Trumbo *et al.*, 2002; Williams *et al.*, 1980). To better understand the bio-functionality of pectic polysaccharides scientific elucidation of the structures responsible for the beneficial effect is very important (Yamada *et al.*, 2003).

Pectin structural elements

Pectin is defined as a hetero-polysaccharide predominantly containing galacturonic acid (GalA) 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 (Kertesz, 1951). De Vries (1982) recognized a pattern of “smooth” homogalacturonic regions and ramified “hairy” regions, in which most of the neutral sugars are located. Over the years many pectin structural elements have been described and all pectins are believed to essentially contain the same repeating elements, although the amount and chemical fine structure of these elements varies (De Vries *et al.*, 1981; McNeill *et al.*, 1979; Schols & Voragen, 1996). A schematic representation of the composition of these structural elements is given in figure 1, which will be further discussed below.

Homogalacturonan

Homogalacturonan (HG) is the major type of pectin in cell walls, accounting for approximately 60% of the total pectin amount (Mohnen *et al.*, 1996; O'Neill *et al.*, 1990). The HG polymer consists of a backbone of α-1,4-linked GalA residues (McNeil *et al.*, 1984). The minimum estimated length of this backbone is, for citrus, sugar beet, and apple pectin 72-100 GalA residues (Thibault *et al.*, 1993). GalA moieties within this backbone may be methyl esterified at C-6 (Gee *et al.*, 1959; Mort *et al.*, 1993) and/or *O*-acetylated at *O*-2 and/or *O*-3 (Ishii, 1995; Rombouts & Thibault, 1986a). The methyl-esterification in particular has gained a lot of attention in pectin research, because it strongly determines

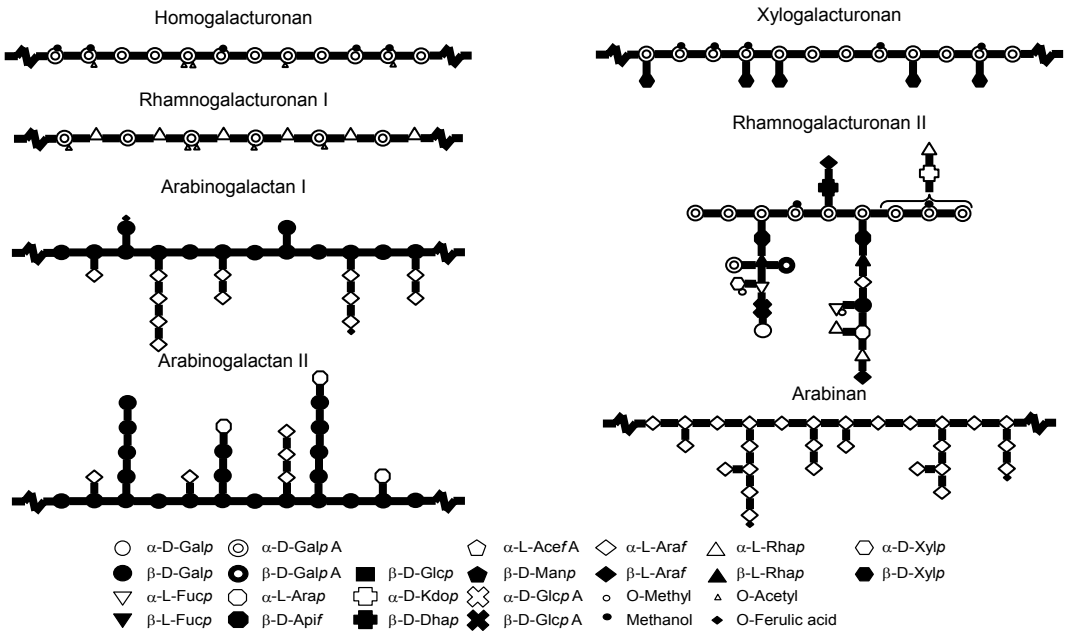

Figure 1: Schematic representation of pectin structural elements (Hilz, 2007).

the physical properties of pectin. For instance, blocks of more than 10 non-esterified GalA residues yield pectin molecules that are sensitive to Ca^{2+} crosslinking (Daas *et al.*, 2001).However, not only the amount of methyl-esterification is important, but also the distribution of these esters is. The suggestion made by Rees and Wight (1971) that HG elements could be interspersed with single L-rhamnose residues, resulting in a kink of the molecule, was convincingly argued against by Zhan *et al*. (1998). These authors could not isolate this internal rhamnose (Rha) from an *endo*-polygalacturase digest of citrus pectin, indicating a scarcity or complete lack of interspersing single rhamnose residues. Furthermore, based on molecular modeling, the presence of a kink in the molecule caused by interspersing Rha is further undermined (Pérez *et al.*, 2000).

Xylogalacturonan

Homogalacturonan substituted with β-D-Xyl*p*-(1→3) single unit side chains is called xylogalacturonan (XGA) (Albersheim *et al.*, 1996; Le Goff *et al.*, 2001; Schols *et al.*, 1995a). The degree of xylosidation can vary between 25% (watermelon) and 75% (apple) (Le Goff *et al.*, 2001; Schols *et al.*, 1995a; Yu & Mort, 1996). Part of the GalA residues in XGA are methyl-esterified and the methyl esters are found to be equally distributed among the substituted and unsubstituted GalA residues (Schols *et al.*, 1995a; Yu & Mort, 1996). Although XGA has been mainly identified in reproductive tissues such as fruits and seeds (Albersheim *et al.*, 1996; Schols *et al.*, 1995a), Zandleven *et al.* (2007) recently demonstrated the presence of this element in various tissues of *Arabidopsis thaliana*.

Rhamnogalacturonan I

The rhamnogalacturonan I (RGI) backbone is composed of [→2)-α-L-Rha*p*-(1→4)-α-D-Gal*p*A-(1→] repeats (Albersheim *et al.*, 1996; McNeil *et al.*, 1980). Sycamore cells that are cultured in suspension can have as many as 300 repeats of this disaccharide (Albersheim *et al.*, 1996; McNeil *et al.*, 1980). In contrast, in sugar beet pectin oligosaccharides with a maximum length of only 20 residues of alternating Rha and GalA units were isolated. However, it is unclear whether the acid hydrolysis extraction might have caused backbone breakdown, thus underestimating the RGI backbone length (Renard *et al.*, 1995).

The rhamnosyl residues of RGI can be substituted at *O*-4 with neutral sugars side chains (Colquhoun *et al.*, 1990; Lau *et al.*, 1987; McNeil *et al.*, 1980). These side chains are mainly composed out of galactosyl and/or arabinosyl residues. Both single unit [β-D-Gal*p*-(1→4)] as well as polymeric substitutions, such as arabinogalactan I (AGI) and arabinan (50 glycosyl residues or more) have been identified (Lau *et al.*, 1987; Lerouge *et al.*, 1993) in the side chains. The proportion of branched Rha residues varies from ~20% to ~80% depending on the source of the polysaccharide (Albersheim *et al.*, 1996).

The GalA residues of RGI are presumably not methyl esterified, because RGI is not degraded under β-eliminative circumstances (Kravtchenko *et al.*, 1992). On the other hand, a flax RGI fraction has been reported to contain 40% methyl esters (Rihouey *et al.*, 1995). The GalA residues in the RGI backbone may be highly *O*-acetylated on position *O*-2 and/or *O*-3 of the GalA residues (Carpita & Gibeaut, 1993; Komalavilas & Mort, 1989; Schols *et al.*, 1990b; Schols & Voragen, 1994).

Rhamnogalacturonan hydrolase digestion of apple modified hairy regions (MHR) yielded specific populations, consisting out of [→2)-α-L-Rha*p*-(1→4)-α-D-Gal*p*A-(1→] repeats, with alternatively 0, 1 or 2 galactose substitutions to the rhamnose moieties. The ratio between these alternative substituted oligosaccharides suggests that hairy regions are composed, in part, of different repeating units (Colquhoun *et al.*, 1990). Structural characterization of oligosaccharides released from sugar beet by dilute acid treatment showed single-unit β-D-Glc*p*A-(1→3) side chains attached to one of the GalA residues (Renard *et al.*, 1999).

Rhamnogalacturonan II

Rhamnogalacturonan II (RGII) is a highly conserved structure in the plant kingdom. The structure is characterized as a distinct region within HG, containing clusters of four different side chains with very peculiar sugar residues, such as apiose, aceric acid, 3-deoxy-lyxo-2-heptulosaric acid (DHA), and 3-deoxy-manno-2-octulosonic acid (KDO). These side chains are attached to a HG fragment of approximately nine GalA residues, of

which some are methyl esterified (O'Neill *et al.*, 2001; Ridley *et al.*, 2001; Vidal *et al.*, 2000). The structure of RGII seems to be highly conserved in the plant kingdom. RGII can complex together with Boron, forming a borate–diol ester, which can crosslink two HG molecules (Ishii & Matsunaga, 2001; Ishii *et al.*, 1999). Only the apiofuranosyl residues of the 2-*O*-methyl-D-xylose-containing side chains in each of the subunits of the dimer participate in the cross-linking (Ishii *et al.*, 1999).

Arabinan

Arabinan consist of a 1,5-linked α-L-Ara*f* backbone, which usually is substituted with α-L-Ara*f*-(1→2)-, α-L-Ara*f*-(1→3)-, and/or α-L-Ara*f*-(1→3)-α-L-Ara*f*-(1→3)- side chains (Beldman *et al.*, 1997; Carpita & Gibeaut, 1993; Mohnen, 1999; Ridley *et al.*, 2001; Schols *et al.*, 1990b).

Arabinogalactan I

Arabinogalactan I (AGI) is composed out of a 1,4 linked β-D-Gal*p* backbone with α-L-Ara*f* residues attached to *O*-3 of the galactosyl residues (Carpita & Gibeaut, 1993; Mohnen, 1999; Ridley *et al.*, 2001). *O*-6 substitution of the galactan backbone with β-galactose is also found (Vis, 1994). The AGI backbone can be terminated with a α-L-Ara*p*-(1→4) at the non-reducing end (Huisman *et al.*, 2001). Internal -(1→5)-α-L-Ara*f* linked arabinofuranose (Huisman *et al.*, 2001) and (1→3)-β-D-Gal*p* linked galactopyranose (Hinz *et al.*, 2005) residues have as well been identified.

Arabinogalactan II

Arabinogalactan II (AGII) is composed of a 1,3 linked β-D-Gal*p* backbone, containing short side chains of α-L-Ara*f*-(1→6)-[β-D-Gal*p*-(1→6)]$_n$ (n=1, 2 or 3) (Carpita & Gibeaut, 1993; Mohnen, 1999; Ridley *et al.*, 2001). The galactosyl residues of the side chains can be substituted with α-L-Ara*f*-(1→3) residues.

AGII is mainly associated with proteins (3-8%), so called arabinogalactan proteins (AGPs). The protein part is rich in proline/hydroxyproline, alaline, serine and threonine (Gaspar *et al.*, 2001). The major part of AGPs (>90%) consists of polysaccharides. Pectin and AGII often co-extract and are subsequently difficult to separate from each other (Vincken *et al.*, 2003). It has even been demonstrated that a small fraction of carrot tap root cell wall AGPs is linked to pectin (Immerzeel *et al.*, 2006).

Enzymes used in structure elucidation of pectins

Polysaccharide degrading enzymes are suitable tools to study the structure of pectin (Schols & Voragen, 1996). The main reason is the specificity of these enzymes in comparison to chemical methods, which are less-specific. Pectic enzymes are classified according to the mode of attack on their specific structural element of the pectin molecule (Benen *et al.*, 2002). Many detailed reviews have been dedicated to pectin degrading enzymes (Beldman *et al.*, 1996; Beldman *et al.*, 1997; Benen *et al.*, 2002; Fischer & Bennett, 1991; Prade *et al.*, 1999) and therefore only the enzymes involved in the examination of polymeric pectin fragments described in this thesis (represented in figure 2) are briefly discussed in this chapter.

Endo-polygalacturonase (EndoPG; EC 3.2.1.15)

Endo-polygalacturonases (EndoPG's) cleave the α-1,4-D galacturonan linkages in HG segments. EndoPG's generally prefer non-esterified substrate and show decreasing activity with increasing degree of methyl-esterification (Parenicovà *et al.*, 2000). The enzyme randomly attacks its substrate and produces a number of GalA oligosaccharides (Osteryoung *et al.*, 1990).

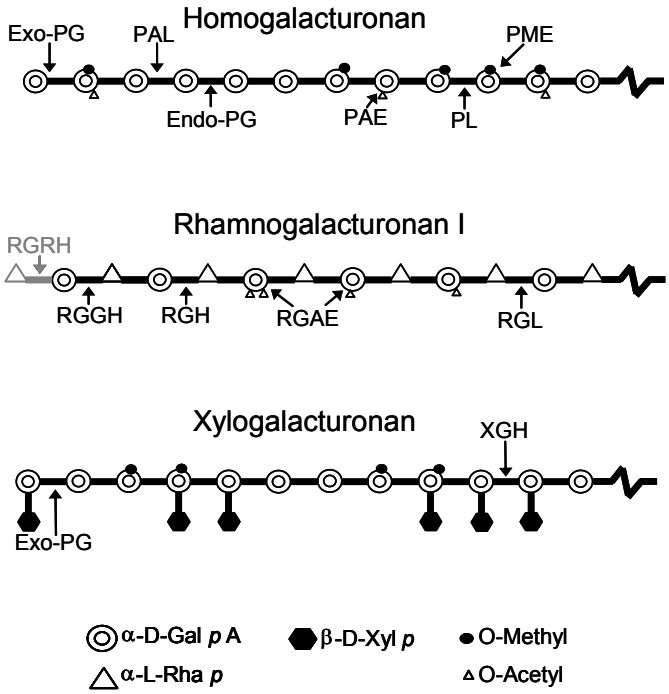

Figure 2: Mode of action of pectinases involved in the degradation of homogalacturonan, rhamnogalacturonan I and xylogalacturonan (see text for abbreviations). Terminal end of rhamnogalacturonan I is represented in grey to stress that indicated exo-activity only exists with a single sugar moiety. Figure has been adapted from Hilz *et al*. (2007).

Exopolygalacturonase (ExoPG; EC 3.2.1.67 and EC 3.2.1.82)

ExoPG attacks the substrate from the non-reducing end and is able to remove terminally (1→)–linked GalA residues from HG chains. The enzyme requires a non-esterified Gal*p*A unit at subsites -2, -1 and +1 (Kester *et al.*, 1999) and is tolerant for xylose substitution (able to remove a GalA-Xyl dimer), hence XGA is also an ExoPG substrate (Beldman *et al.*, 1996; Benen *et al.*, 2002).

Rhamnogalacturonan hydrolase (RGH; EC 3.2.1.-)

RGH hydrolyses the α-D-1,4-Gal*p*A-α-L-1,2-Rha*p* linkage in the RGI backbone, leaving Rha*p* at the non-reducing side (Schols *et al.*, 1990a). Within the products formed, the Rha residues can be substituted with single galactose units (Colquhoun *et al.*, 1990). The enzyme is intolerant for acetyl-esterification of the RGI backbone (Beldman *et al.*, 1996; Kauppinen *et al.*, 1995)

Rhamnogalacturonan lyase (RGL; EC 4.2.2.-)

Degradation by RGL occurs through eliminative cleavage of the RGI α-L-1,2-Rha*p*-α-D-1,4-Gal*p*A backbone leaving a 4-deoxy-β-L-threo-hex-4-enepyranosyluronic acid (unsaturated GalA) group at the non-reducing end (Azadi *et al.*, 1995; Mutter *et al.*, 1998b). Removal of arabinan side chains from saponified hairy regions of pectin resulted in an increased catalytic efficiency of *Aspergillus aculeatus* RGL, whereas the removal of galactan side chains decreases the enzyme efficiency (Mutter *et al.*, 1998b). The RGL activity increased after removal of acetyl groups (Mutter *et al.*, 1998b).

Rhamnogalacturonan rhamnohydrolase (RGRH)

RGRH is an exo-acting pectinase, which possesses a specificity to release terminal rhamnosyl residues (1→4)-linked to α-galacturonosyl residues (Mutter *et al.*, 1994). The enzyme is intolerant for (galactose) substitutions and has not yet been assigned to a glycosyl hydrolase family since no sequence information is available.

Rhamnogalacturonan galacturono hydrolase (RGGH)

RGGH is able to release a GalA moiety connected to a rhamnose residue from the non-reducing side of RGI chains but is unable to liberate GalA from HG (Mutter *et al.*, 1998a). Similar to RGRH no sequence information for RGGH is available.

Endo Xylogalacturonan hydrolase (XGH; EC 3.2.1.-)

XGH hydrolyses the α-1,4-D linkages of xylose substituted galacturonan moieties in xylogalacturonan (van der Vlugt-Bergmans *et al.*, 2000). XGH has a requirement for

xylosyl side chains and is therefore believed to cleave between two xylosidated Gal*p*A residues (van der Vlugt-Bergmans *et al.*, 2000). Removal of ester linkages of galacturonan by saponification increases the enzyme activity (Beldman *et al.*, 2003).

Cross links

Although individual structural elements have been studied and their structures have been characterized, the knowledge on the interconnections between different structural elements with each other and with other polysaccharides is limited. Figure 3 represents a number of covalent and non-covalent linkages, which have been observed in pectin polymers, and are possibly involved in intra- or intermolecular linkages.

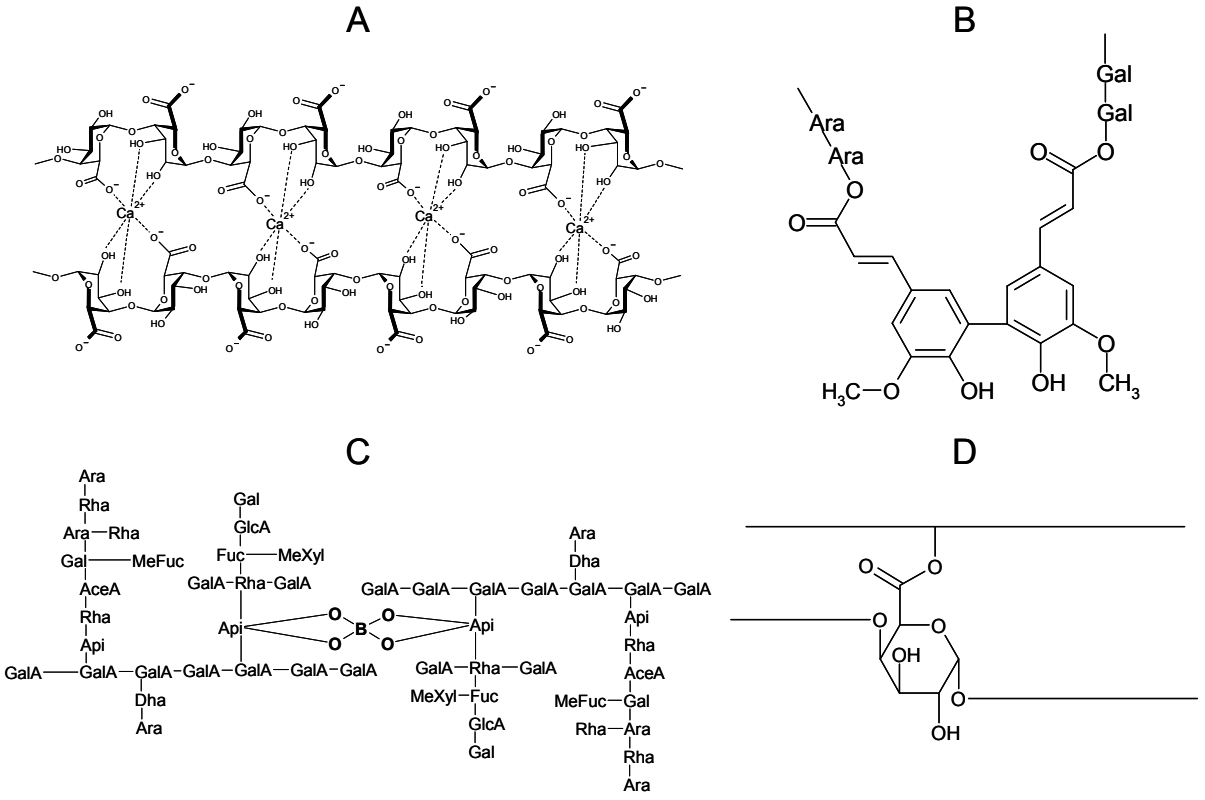

Figure 3: Pectin cross links as described in literature. A) Calcium-pectin-crosslink as egg box model (Morris *et al.*, 1982), B) 5-5-diferulic esterified with neutral sugar side chains of pectin (Ralet *et al.*, 2005), C) Rhamnogalacturonan II diester (Ishii *et al.*, 1999), D) uronyl ester of pectin with a hydroxyl group of another polysaccharide chain (Lamport, 1970). Figure adapted from Hilz (2007)

Calcium-pectin complexes

Low methyl esterified pectins are thought to gel according to the egg box model (De Vries *et al.*, 1983), first suggested for alginates (Morris *et al.*, 1982). Sections of two pectic chains, which must be free of ester groups, are held together by a number of

calcium ions. It is reported that blocks of 7-20 free GalA residues are required for association with calcium (Braccini *et al.*, 1999; Kohn, 1975; Powell *et al.*, 1982).

Ferulic acid esters

Pectins originating from spinach and sugar beet contain ferulic acid residues in the arabinan side chains. In sugar beet, 45-50% of ferulic acid can be attached to the *O*-6 position of galactose residues in (1→4)-linked galactans (Guillon & Thibault, 1989; Guillon *et al.*, 1989; Ralet *et al.*, 1994; Rombouts & Thibault, 1986b). Ferulic acid dihydrodimers account for approximately 22% of the total ferulates in sugar beet pectin (Waldron *et al.*, 1997).

Rhamnogalacturonan II dimer formation

The demonstration that RGII exists in primary walls as a dimer that is covalently cross-linked by a borate diester (Ishii *et al.*, 1999) was a major advance in the understanding of the structure and function of this pectic polysaccharide. RGII is covalently linked to HG and as a consequence dimer formation results in the cross-linking of two HG chains, which could lead to the formation of a three-dimensional pectic network *in muro* (Ishii & Matsunaga, 2001). This network contributes to the mechanical properties of the primary wall and is required for normal plant growth and development. Changes in wall properties resulting from decreased borate cross-linking of pectin lead to many of the symptoms associated with boron deficiency in plants (Ishii & Matsunaga, 2001; Ishii *et al.*, 1999; O'Neill *et al.*, 2004).

Uronyl esters

Lamport (1970) suggested that HG could be linked to relatively non-polar putative alcohols by uronyl esters. In pectin originating from cultured spinach cells up to approximately 5% of the GalA residues could be cross-linked in this way (Brown & Fry, 1993). These observations have been revisited, with the additional hypothesis that (particular) pectin methylesterase(s) (PME) could catalyze a trans-esterification reaction (Gelineo-Albersheim *et al.*, 2001). The energy imparted in the methyl ester bond is used in the wall by PME to synthesize cross-links between HG chains; the methanol is released and the carboxylgroup of the galacturonosyl moiety is attached to a –OH group of a galacturonosyl moiety of another HG chain. Because HG is mainly deposited in the cell wall in a methylesterified form, it is evident that these molecules hold an enormous potential for cross-linking. Interestingly, within the Arabidopsis genome, about 60 PME genes have been found that await further characterization (Henrissat *et al.*, 2001). It is possible that PMEs specialized in catalyzing the formation of uronyl esters can be found

among these. More work is needed to further substantiate the abundance, formation, and role of this cross-link.

Pectin models

In 1934 pectin obtained from citrus fruits was recognized/visualized as a primarily linear polygalacturonic acid (Morell *et al.*, 1934). Although this idealized view has been used in handbooks till the nineties of the last century, pectin structural data collected over the last decades have drastically changed this view on pectin's structure. It has become clear that pectin is a very complex macromolecule and that it is a big challenge to accommodate all available information in a model structure. Some of the most cited hypothetical models are summarized below:.

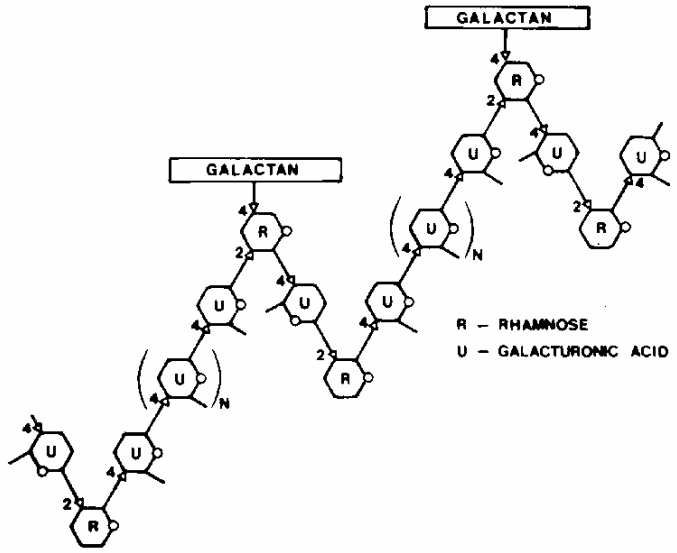

Figure 4: A proposed structure for the rhamnogalacturonan. The sugar residues in the figure are designated as R = rhamnose and U = galacturonic acid. N = an undetermined number, probably between 4 and 10. Reprinted from Talmadge et al. (1973). Copyright American Society of Plant Biologists (www.plantphysiol.org).

Rhamnogalacturonan model

The backbone of pectin isolated from sycamore cells was defined as RG, consisting out of chains of α-1,4-linked galacturonosyl residues interspersed with 2-linked Rha residues. The rhamnosyl residues were considered not to be randomly distributed in the chain, but probably to occur in sequences of the rhamnosyl -(1→4)-galacturonosyl-(1→2)-disaccharide. This sequence appeared to alternate with HG sequences, containing approximately eight residues of 4-linked GalA. About half of the rhamnosyl residues were found to be branched, having a substituent attached to *O*-4. This was considered to be the attachment site of the 4-linked galactan (Talmadge *et al.*, 1973). A schematic representation of this model is given in figure 4.

Smooth and hairy regions model

De Vries *et al.* (1981) demonstrated that the distribution of the neutral sugars in apple pectin is discontinues. By analysis of enzymatic digests it was found that all neutral sugars were located on 5% of the galacturonosyl residues, constituting the so called hairy regions. The degradable unsubstituted part was defined as the smooth region (homogalacturonan). The observed neutral sugar distribution curves, obtained by anion-exchange and size-exclusion chromatography, indicated a specific ratio of smooth versus hairy regions within the different eluted populations. Since hairy regions contain only 5% of the GalA residues, the molecular weight of pectin must be very high. In apple pectin three main types of pectin molecules were identified, having one, two, or three hairyregions, respectively.

A model was constructed (figure 5), placing the neutral sugar side chains in blocks at regular intervals and close to the chain ends, hereby providing an explanation for the inverse relationship between the neutral sugar content and the apparent molecular weight of some pectins (De Vries *et al.*, 1982; De Vries *et al.*, 1981).

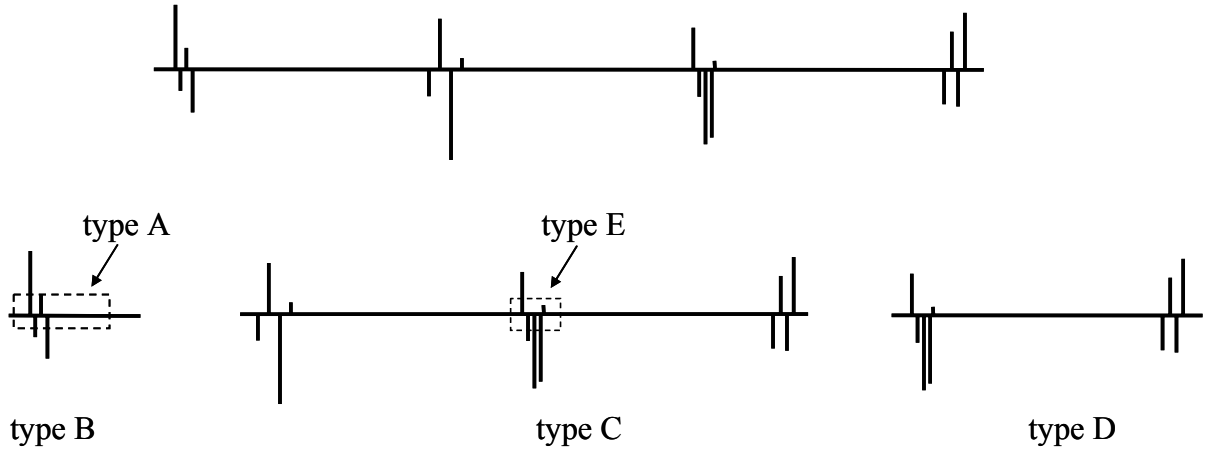

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Figure 5: Pectin model based upon the sugar composition and molecular weights of the different pectin extracts B, C, and D. Type A and Type E are considered to be degraded pectins. Horizontal lines: rhamnogalacturonan backbone of the pectin molecule. Branched areas: blocks of neutral sugar side chains (number and length are arbitrary). Reprinted from de Vries *et al.* (1982), with permission from Elsevier

Enzymatically updated smooth and hairy regions model

Discovery of the enzyme RGH (Schols *et al.*, 1990a) enabled fragmentation of hairy regions and a better identification of the building blocks. Based on degradation products of RGH, hairy regions are considered to consist of XGA segments (subunit I); rhamnogalacturonan stubs rich in arabinan side chains (subunit II), and of RGH oligosaccharides as released from the rhamnogalacturonan region (subunit III). The pectin model was refined (figure 6), using the relative amounts of the different subunits present in cell wall digests of various plant sources. Based on these findings, pectin is believed to be a block polymer composed of structural elements accommodated in hairy regions, interspersed by smooth (HG) regions containing 70-100 GalA residues (Schols & Voragen, 1996; Thibault *et al.*, 1993). The position of RGII is not addressed in this model, but this structural element is believed to be an integral part of some HG segments, which can crosslink two HG molecules (Albersheim *et al.*, 1996; Ishii & Matsunaga, 2001; Ishii *et al.*, 1999).

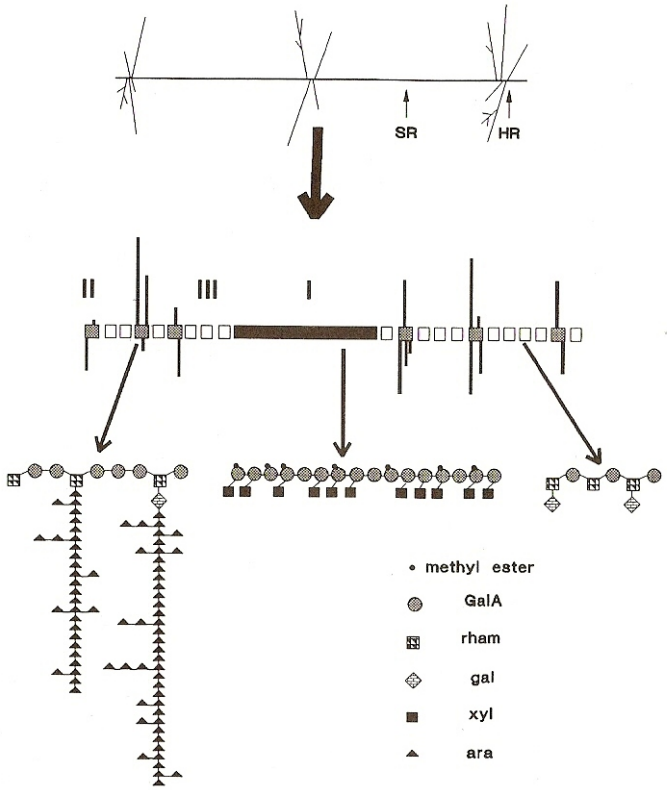

Figure 6: Hypothetical structure of apple pectin and of the prevailing population of MHR isolated here from. SR, smooth regions; HR, hairy regions. Subunit I, xylogalacturonan; subunit II, stubs of the backbone rich in arabinan side chains; subunit III, rhamnogalacturonan hydrolase oligosaccharides. Reprinted from Schols and Voragen (1996), with permission from Elsevier.

RGI backbone model

Vincken *et al.* (2003) listed a number of observations, which challenged the smooth and hairy regions model.

No evidence exists for the presence of single interspacing rhamnosyl moieties within the HG smooth regions (Zhan *et al.*, 1998). These linkages were incorporated in the smooth and hairy regions model to explain the observed length-periodicity (Thibault *et al.*, 1993) of HGs after treatment with dilute acid.

The release of substantial XGA type oligosaccharides by ExoPG (Beldman *et al.*, 1996) combined with a modest decrease in M_w by EndoXGH, makes an internal position of XGA unlikely. The most plausible explanation is that XGA is a side chain (Vincken *et al.*, 2003).

A detailed structural analysis of the rhamnogalacturonan segment, substituted with arabinan and arabinogalactan side chains, revealed that after removal of the arabinan side chains, EndoPG could release oligogalacturonides from these rhamnogalacturonan segments. The released fragments are not likely an integral part of the backbone, since only GalA oligosaccharides were detected. Together with the GalA:Rha ratio, which contains an excess of GalA, this indicates that part of the GalA residues are present in side chains of RGI or as remaining stubs on chain ends (McNeil *et al.*, 1980; Vincken *et al.*, 2003).

It is likely that many different synthetases are involved in the biosynthesis of the HG and RGI backbones, and that there is no single enzyme complex known to synthesize the "pectin backbone" (Vincken *et al.*, 2003).

A new model (figure 7) was thereafter introduced to incorporate the listed findings presented above (Vincken *et al.*, 2003). Homogalacturonan was located as a side chain of RGI, where the HG side chains can either be attached to the rhamnosyl residues of RGI, or to GalA residues of RGI (Vincken *et al.*, 2003). It does not seem unreasonable that HG is a side chain of RGI, because HG and XGA have the same backbone structure (Vincken *et al.*, 2003).

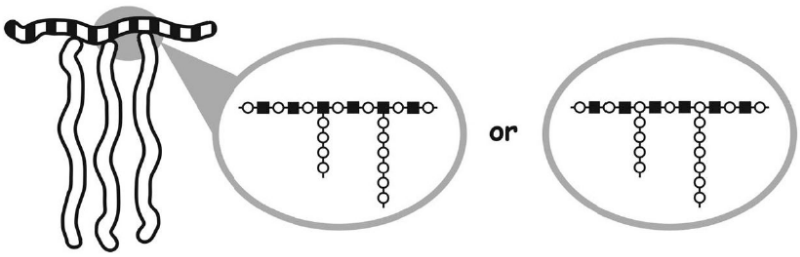

Figure 7: Schematic illustration of the pectin model as described by Vincken *et al.* (2003). RGI is decorated with HG side chains, where it is unknown where these side chains are attached. Reprinted with permission from Springer Science and Business Media.

Neutral sugar side chains

Literature suggests that RGI can differ in its "hairstyle" (Bush *et al.*, 2001; Bush & McCann, 1999; Oomen *et al.*, 2003; Willats *et al.*, 2001). In both the linear and branched model arabinan and arabinogalactan are depicted as side chains attached to a rhamnose moiety within RGI. Although, some older reports describe covalent linkage between arabinose to GalA (Aspinall *et al.*, 1968). The arrangement of the various structural elements remains speculative. RGH is a typical endo-acting enzyme, which is characterized by the generation of large products in the early stages of the reaction (Benen *et al.*, 2003; Benen *et al.*, 2002). The release of varying small oligosaccharides out of apple MHR by RGH indicates that regions of scarcely substituted alternating rhamnose and galacturonic acid sequences are not extremely long, and probably interrupted with other structures resistant to RGH (Schols & Voragen, 1996). The enzymatic *breakdown* might be hindered by several side chains, which are highly flexible and sufficiently long to be able to wrap around the RGI backbone (Willats *et al.*, 2001). Therefore, the distribution of the neutral sugars over the RGI backbone could influence the degradability of this structural element, further complicating the elucidation of pectin structure.

Pectins from different sources

Hairy regions isolated from plant material of other origin like leek, onion, carrot, pear, and potato principally consists of the same building blocks, although the arrangement of these blocks as well as the arabinose and xylose content may vary (Schols & Voragen, 1994). In all examined sources, RGH treatment resulted in MHR degradation, but the M_w distribution behaviour of remaining fragments varied significantly. Degradation by RGH of MHR fractions from different sources resulted in the same series of RGH oligosaccharides (Schols *et al.*, 1995b). For different extracts (*e.g.* hot buffer soluble solids vs. dilute alkali soluble solids) from one specific source, it is suggested that the released fragments are originating from the extremities of the molecules, whereas in another extract, they are thought to be distributed more randomly over the pectin molecule (Schols *et al.*, 1995b). Therefore, the ratio between the subunits I, II, and III (figure 5) may vary. Especially the presence of xylogalacturonan subunit seems to depend on the MHR origin (Schols & Voragen, 1994). Although the ratio between the different pools varied significantly, their size distribution was identical as well as the presence of different structural elements (De Vries *et al.*, 1981; McNeill *et al.*, 1979; Schols *et al.*, 1995b; Schols & Voragen, 1996), therefore an apple based pectin model might also be valid for pectin isolated from other plant sources. In table 1 the occurrence and proportions of the various pectin structural elements from different sources is described, demonstrating that the same structural elements occur in various quantities in different

plant sources. Homogalacturonan and XGA structural elements seem to be confined to specific species (Huisman *et al.*, 2003; Voragen *et al.*, 2001).

Uncharted areas

In apple, pear, carrot, leek, onion, and potato MHR, typical enzyme resistant polymers exist (Schols *et al.*, 1995a; Schols & Voragen, 1994). These polymers have a Rha:GalA ratio of roughly 1:2 and a mass of around 5.4 kDa (Schols *et al.*, 1990b). Even after de-esterification these structures cannot be degraded by HG nor by RGI degrading enzymes, hampering complete structural elucidation of pectin. The backbone structure of these enzyme resistant polymers may even deviate from a strictly alternating sequence, resulting in short chains of e.g. GalA (or Rha), which would confirm previous findings, such as a dimer of rhamnose-(1,2)-rhamnose (Gao *et al.*, 1990), Gal*p*A-(1,4)-Gal*p*A-(1,2)-Rha (Aspinall *et al.*, 1967), GalA(1,2)-Rha*p*-(1,2)-Rha*p* (Aspinall *et al.*, 1967). The presence of these polymer populations, which cannot be analyzed in detail, demonstrate the requirement for novel degradation and/or analysis techniques.

Table 1: Occurrence and proportions of the various structural elements in natural products.

	black currant[a]	bilberry[a]	grape[b]	soybean[c]	sugarbeet[c]	apple[c]
total polysaccharides [% of dry matter]	19	12	11	16	67	20
pectic substances [% of total PS]	61	33	56	59	40	42
structural element [% of pectic substances]						
homogalacturonan	68	65	65	0	29	36
xylogalacturonan	0	0	n.a.	21	<1	4
rhamnogalacturonan I	5	6	10	15	4	1
neutral side chains	24	27	23	60	48	47
rhamnogalacturonan II	3	2	2[c]	4	4	10

n.a. = not analysed

[a] from Hilz (2007)

[b] recalculated from Nunan, Sims *et al.* (1997)

[c] from Voragen, Beldman *et al.* (2001)

Release, fractionation, and identification of connection points

Why has the linkage between RGI and HG not been demonstrated until now? The scarcity of the HG-RGI linkage, in combination with the difficulty to find selective methods to enrich a particular fraction in the cross-link, are beyond doubt important reasons (Mort, 2002). In order to determine indisputably how the different structural elements are linked to each other, linkage points have to be isolated and their structures identified.

Chemical degradation

To be able to reveal its structure, pectin is commonly degraded in to smaller oligosaccharides, as the pectin molecule is too large and heterogeneous to analyze as a whole (Voragen *et al.*, 1992). Pectins can be rather selectively degraded through partial acid hydrolysis, where advantage is taken from the different hydrolysis rates of various glycosidic bonds (BeMiller, 1967; Mort *et al.*, 1989). ß-elimination is an alternative fractionation method for pectin oligosaccharides. This reaction occurs at neutral or even weakly acidic conditions and is competing with the de-esterification reaction (Albersheim *et al.*, 1960). Cold alkali treatment promotes de-esterification rather than the competitive ß-elimination reaction (Kravtchenko *et al.*, 1992; van Buren & Pitifer, 1992). This procedure, also known as saponification, results in simplified chromatograms and spectra due to the removal of acetyl and methyl esters (Saulnier & Thibault, 1999; Zhan *et al.*, 2001).

Enzymatic degradation

Next to the chemical degradation methods, enzymes are used in structure research because of their specificity and selectivity (De Vries, 1983). Pure enzymes have been used to hydrolyze complex carbohydrates, in order to reveal structural characteristics (Daas *et al.*, 1999; Schols *et al.*, 1990a). The complexity of pectin hampers enzymatic degradation. As a consequence, a lot of substitutions and structural organisations require treatment with several enzymes simultaneously or in a particular sequence for degradation (Searle-van Leeuwen *et al.*, 1996; Versteeg, 1979). Several pectin degrading enzymes have been demonstrated to act synergistically (Bonnin *et al.*, 2002). When pectin structures are not degradable by the available enzymes, a combination of chemical and enzymatic approaches can be applied. For instance, in the structural characterization of enzyme resistant highly branched RGI structures, partial side chain removal by chemical treatments enables subsequent enzymatic breakdown (An *et al.*, 1994; Azadi *et al.*, 1995; Mutter *et al.*, 1998c). Furthermore, the enzyme activity of EndoPG (Searle-van Leeuwen *et al.*, 1996), RGH (Kauppinen *et al.*, 1995), and XGH (van der Vlugt-Bergmans *et al.*, 2000) improved after removal of acetyl groups and/or methyl esters.

Analytical approaches

Before pectin can be characterized on a structural level, it has to be extracted out of the cell wall matrix, usually by sequential extraction steps with different buffers (Selvendran & Ryden, 1990; Voragen *et al.*, 1995). The molecular weight can be estimated with size exclusion chromatography. The sugar composition (De Ruiter *et al.*, 1992; Englyst & Cummings, 1984), the sugar linkage composition (Hakomori, 1964), and the degree of

methylation and acetylation (Voragen *et al.*, 1986) of the extracted pectin can be determined among several other possible analyses. These analyses, which are conducted on the whole molecule, are, however, not sufficient to give insight in the pectin structure. Therefore, pectin is often degraded by chemical or enzymatic approaches. The effect of this degradation is twofold; "pure" structural elements can be obtained after fractionation of the degradation products, and the resulting fragments are in the analytical range of a broad set of analytical techniques (Schols & Voragen, 2002), such as high performance anion exchange chromatography (HPAEC), capillary electrophoresis (CE) and mass spectrometry (MS). Using HPAEC, sugar oligosaccharides are separated based on their charge differences. The separation is performed at pH 12 to ensure that even neutral sugars are charged. The negatively charged sugars bind to the column material and elute through competitive binding with an increasing salt gradient (Lee, 1996). As an alternative, pectin oligosaccharides can be analyzed at pH 5 to retain information about methyl esters distribution over the backbone (Daas *et al.*, 1998). After elution sugars are often detected by a pulsed ampherometric detector (Lee, 1996). An alternative separation for pectin oligosaccharides can be obtained by CE, using the negative charge of pectin oligosaccharides at high pH, or introduced charges by coupling pectin oligosaccharides to a charged label (Mort & Chen, 1996; Zhang *et al.*, 1996). In both HPAEC and CE techniques, the eluting oligosaccharides are annotated based on their elution times relative to standards (Lee, 1996; Mort & Chen, 1996). However, for many complex oligosaccharides, standards are not available (Lee, 1996). To circumvent this shortcoming, the HPLC eluent can be fractionated and analyzed off line by mass spectrometric techniques (Kabel *et al.*, 2001). The combination of low sample quantity together with intrinsic difficulties for fractionation, make CE less suitable for sequential MS analysis. Therefore, HPAEC is the most frequently used technique to identify sugar oligosaccharides, sometimes in combination with off-line MS (Kabel *et al.*, 2001; Lee, 1996). Matrix assisted laser desorption/ionisation mass spectrometry is often used for off-line MS analysis, due to its tolerance to residual salts, the relative simple sample preparation, and high speed of analysis (Daas *et al.*, 1998). Using this technique, masses of oligosaccharides and their MS-fragmentation products can be determined (van Alebeek *et al.*, 2000). Iontrap MS is used as an alternative to gain more detailed structural information of a specific compound, through multiple MS analysis stages (Quemener *et al.*, 2003).

It is clear that the structure of pectin cannot be drawn based upon results from one single analytical method, but a combination of different analytical methods combined with several sample preparation procedures is needed.

Aim and outline of the thesis

The aim of the research described in this thesis was to develop new approaches to identify linkages which are involved in the connection of different structural pectin elements. Identification of these linkages would lead to an improved understanding of pectin structure. Better understanding of its structure will result in improved cell wall models, optimized use of pectin as a gelling and thickening agent, and better control of enzymatic degradation of pectin during industrial processing of fruits and vegetables.

Following the general introduction, chapter 2 describes a controlled acid hydrolysis of apple MHR, and the subsequent identification of selected structural elements by combined liquid chromatography-MS and Nuclear Magnetic Resonance (NMR) approaches. The connecting linkage between HG and RGI, was demonstrated by the oligosaccharides $GalA_3Rha_1$, $GalA_4Rha_2$ and $GalA_5Rha_3$. Furthermore, evidence for a covalent linkage between XGA and HG was given by the $GalA_6Rha_3Xyl_1$ oligomer. Single side chains obtained from RGI rich pectin structures from white cabbage, were isolated and characterized, in order to identify structures which trigger immune responses through the complement fixing system, which is an important part of innate immunity (chapter 3). To optimize pectin oligomer analysis, new CE-MS^n methods were developed, which enabled effective separation of differently substituted GalA containing oligosaccharides by low pH CE analysis. Moreover, with on-line MS/MS capabilities it was possible to localize sugar residues substitutions (chapter 4). In chapter 5 ß-eliminative breakdown of pectin is combined with of CE-MS^n. β-eliminative breakdown in combination with borate and high temperature results in the specific breakdown of the RGI backbone, which enables analysis of previously not accessible RGI hairy regions, leaving the neutral sugar side chains intact. In chapter 6 the implication of the results described in the previous chapters on the pectin model and pectin analysis for the enzyme and pectin industry are discussed.

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Chapter 2

Identification of the connecting linkage between homo- or xylogalacturonan and rhamnogalacturonan I

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Abstract

Pectin is of interest both as cell wall component and as food additive. The precise chemical structure of pectin remains under debate, although the structural elements of pectin are rather well described. In order to get more insight in the inter linkage between the various structural elements, apple pectin modified hairy regions were degraded by controlled acid hydrolysis. From the degradation products oligomeric fragments were selected which could represent interconnection points, and these oligosaccharides were characterized using LC-MS and NMR approaches.

It was shown that the oligosaccharides $GalA_3Rha_1$, $GalA_4Rha_2$, and $GalA_5Rha_3$ consisted out of a homogalacturonan and a rhamnogalacturonan I segment connected via a GalA*p* α-(1→2) Rha*p* linkage. In addition, a $GalA_6Rha_3Xyl_1$ oligosaccharide was identified, which consisted out of a xylogalacturonan and a rhamnogalacturonan I segment. These oligosaccharides indicated that in apple pectin both homogalacturonan and xylogalacturonan were covalently linked to rhamnogalacturonan I. With these new insights, currently used pectin models were refined.

Keywords

Homogalacturonan; xylogalacturonan; rhamnogalacturonan I; pectin model; covalent linkage; pectin structure

Introduction

Pectin is an important plant cell wall component, which is thought to influence the porosity and strength of the primary cell wall, and the growth mechanism of the plant cell (Bacic *et al.*, 1988). Furthermore, it is of great importance to the food industry as it is one of the main natural gelating additives. Next to that, pectin determines to a large extend food processing characteristics of fruits and vegetables e.g. juices, nectars, purees and preserves (De Vries, 2004; Schols & Voragen, 1996). To be able to improve our understanding of pectin, knowledge of its structure is essential in order to comprehend its biological function and to explain its gelling and stabilizing properties (Daas *et al.*, 2001; May, 2000; Voragen *et al.*, 1995). However, more than 180 years of pectin research (Braconnot, 1825) did not succeed in unambiguously unravel the structure of this plant polysaccharide partly due to the fact that its structure is affected by plant species (De Vries *et al.*, 1981), plant development stage (Huisman *et al.*, 1996), and cell location (Redgwell & Selvendran, 1986).

Pectin may consist out of different associated structural elements, such as homogalacturonan (HG), xylogalacturonan (XGA), rhamnogalacturonan I (RGI), rhamnogalacturonan II (RGII), arabinan, and arabinogalactan (Schols & Voragen, 2002). Homogalacturonan has intensively been studied, revealing characteristics such as distribution of methyl esters and acetyl groups over the galacturonan backbone, which contributes to the understanding of gelling behavior and ripening processes of fruits and vegetables (Albersheim *et al.*, 1996; Guillotin *et al.*, 2005). Xylogalacturonan also has a HG backbone, but 25-75% of the galacturonic acid (GalA) units are substituted with xylose (Le Goff *et al.*, 2001; Schols *et al.*, 1995a; Voragen *et al.*, 2001). Furthermore the GalA sugar residues comprising the XGA backbone can be methyl esterified (Schols et al., 1995a). Arabinan and (arabino)galactan (Beldman *et al.*, 1997; Carpita & Gibeaut, 1993; Mohnen, 1999; Ridley *et al.*, 2001; Schols *et al.*, 1990) are covalently linked to RGI (Albersheim *et al.*, 1996; Lau *et al.*, 1987; McNeil *et al.*, 1980), were RGII is thought to be covalently linked to HG (O'Neill *et al.*, 2001; O'Neill *et al.*, 2004; Ridley *et al.*, 2001; Vidal *et al.*, 2000). The placement of XGA in respect to RGI is indistinct, but regarded exchangeable with HG (Huisman *et al.*, 2001; Schols & Voragen, 1996; Vincken *et al.*, 2003b). When isolating these structural elements, using enzymes, a number of unidentified structures remain to be further characterized (Schols & Voragen, 1994).

Using this information, two types of models for pectin structure have been composed: 1) a model in which the pectin backbone consists out of (alternating) RGI and HG (De Vries *et al.*, 1981; Schols & Voragen, 1996; Voragen *et al.*, 1995); 2) a model in which the backbone consists out of RGI, with HG and XGA as side chains (Vincken *et al.*,

2003a). Arabinan and (arabino)galactan chains are covalently linked to RGI in the different models. Furthermore, both models assume that RGI and HG/XGA are covalently attached, although no linkage has ever been demonstrated (Ridley et al., 2001; Vincken et al., 2003a). The assumption of a covalent linkage between these structures is based upon co-elution (De Vries *et al.*, 1982), molecular weight shift after *endo*polygalacturonase or pectate lyase digestion (Schols *et al.*, 1995b), and the release of RGI, RGII, and oligogalacturonides from *endo*- and *exo*-polygalacturonase degraded sugar beet pectin (Ishii & Matsunaga, 2001).

It has been shown that HG elements are build up by 81-117 galacturonic acid (GalA) residues (Thibault *et al.*, 1993; Yapo *et al.*, 2007), although no firm evidence has been presented how these HG elements are coupled. Rees (1971) reported the presence of one single interspersing L-rhamnose unit interspacing HG elements, which would result in a kink in the molecule (Rees & Wight, 1971). Single interspersing rhamnose could, however, not be isolated from an *endo*-polygalacturonase digest of citrus pectin, indicating a scarcity or complete lack of interspersing single rhamnose residues (Zhan *et al.*, 1998), indicating that longer Rha-GalA sequences are involved in coupling HG elements.

Identification of the linkage types between two neighboring elements is needed to gain further insight in the structure of pectin. For this reason, we describe in this paper the mild acid hydrolysis of apple pectin hairy region, the enrichment of rhamnose and galacturonic acid and the subsequent isolation and identification of connection points. Finally, using the experimental data described in this paper, currently used pectin models were refined.

Experimental

Modified hairy regions

The starting material was prepared similarly as described by Schols (1990). Homogenized apple tissue was incubated with Rapidase Liq+ (DSM Food Specialities, The Netherlands). The resulting suspension was centrifuged (Pennwalt Sharpless P600 Decanter) and the supernatant was ultra-filtrated (60 KDa). The retentate was freeze dried and named apple MHR (Schols et al., 1990). The apple MHR was saponified in 0.05 M NaOH at 4°C during 24 h. at a concentration of 10 mg/mL. Subsequently the mixture was neutralized with 0.25 M HCl to pH 7 and used for further analysis.

Controlled acid hydrolysis

Neutralized saponified sample solution (10 mg/mL) was hydrolyzed for 48 h with 0.1 M HCl at 80°C. Thereafter, the hydrolysate was dialyzed (12 KDa) and lyophilized to remove liberated neutral sugars. A sequential hydrolysis step with 0.05 M trifluoroacetic

acid (TFA) at 100°C for 6 h (10 mg/mL) was conducted to initiate backbone degradation without extensively degrading the polymer. Hereafter, the sample was centrifuged at 18,500 x g. The supernatant was filtered through a P3 glass filter. The filtrate was concentrated with rotational film evaporation (40°C, 40 mbar) and subsequently freeze dried. The freeze dried hydrolysate (6 g.) was dissolved to a concentration of 400 mg/mL and 3 X 5 mL was applied onto a column (100x2.6 cm i.d.) of Biogel P2 (Bio-Rad, USA) at 60°C and eluted with distilled water (30 mL/h). The polymer population was collected and subsequently lyophilized.

Molecular weight distribution

Hydrolyzed polysaccharide was dissolved in distilled water (4 mg/mL) and analyzed by High Performance Size Exclusion Chromatography (HPSEC) performed on a SpectraSystem HPLC (Thermo Separation Products, USA) using three TosoHaas TSK-gel columns in series (4000-, 3000, 2500, PWxl (300x7.5mm; TosoH, Japan). preceded by a TSK PWxl guard column (40x6 mm; TosoH, Japan). The sample (20 µL) was injected and eluted at 30°C using 0.8 mL/min 0.2 M $NaNO_3$. Detection was performed using a Shodex RI 71 refractive index detector (Showa Denko K.K., Japan).

Sugar composition

The sugar composition was determined according to De Ruiter (1992). Samples were dried at 40°C under vacuum over P_2O_5 and hydrolyzed with 2 M HCl in dry methanol for 16 h at 80°C followed by 1 h of 2M TFA at 121°C. The monomeric sugars were analyzed by High Performance Anion Exchange Chromatography (HPAEC) using a SpectraSystem HPLC (Thermo Separation Products, USA), equipped with a CarboPac PA-1 column (4 mm ID X 250 mm; Dionex, USA) in combination with a CarboPac PA guard column (4 mm ID X 25 mm) and a ED40 PAD-detector (Dionex, USA) (De Ruiter *et al.*, 1992). A flow rate of 1 mL/min was used with the following gradient of distilled water, 0.1 M NaOH, and 1 M NaAc in 0.1 M NaOH: 0-15 min, 30 mM NaOH; 15-16 min, 30-100 mM NaOH; 16-55 min 0-500 mM NaAc in 0.1 M NaOH. Each run was followed by a 1 M NaAc in 0.1 M NaOH washing step for 14 min and an equilibration step of 100 mM NaOH of 5 min followed by 30 mM NaOH elution for 15 min.

Fractionation and desalting of oligosaccharides separated by anion exchange chromatography

Anion exchange chromatography was performed using a SpectraSystem HPLC (Thermo Separation Products, USA), equipped with a CarboPac PA-1 column (2 mm ID X 250 mm; Dionex, USA) in combination with a CarboPac PA guard column (2 mm ID X

25 mm) and a ED40 PAD-detector (Dionex, USA). A flow rate of 0.2 mL/min was used with the following gradient of NaOAc buffer (pH 5.0) in water: 0-8 min, 10 mM; 8-12 min, 175 mM; 12-108 min 175-860 mM. Each elution was preceded by a 15-min 10 mM NaOAc equilibration step and the run was completed with an 8-min 1 M NaOAc washing step. Oligomer detection was possible after post column addition of NaOH (1 M; 0.2 mL/min). The eluate of the anion exchange chromatography was desalted online by two desalting units, connected in-line after the PAD detector. First, the Anion Self-Regenerating Suppressor-Ultra 4 mm-unit (ASRS; Dionex, USA) was connected to exchange the sodium ions for hydronium ions (H_3O^+). Next, the Cation Self-Regenerating Suppressor-Ultra 4 mm-unit (CSRS; Dionex, USA) was installed to exchange the acetate ions for hydroxide (OH^-). Desalting the eluent was achieved by electrolysis of deionized water (8 mL/min) in both suppressors. Fractions (0.5 min/fraction; 200 µL) were collected in a 96-well Multiscreen-BV plate (Millipore, USA) filled with regenerated Dowex AG 50W-X8 (Bio-Rad Laboratories, USA) in the hydrogen form, using a FC-203B fraction collector (Gilson, USA) to remove the residual salts. After this desalting step, samples were collected in a 96-well plate by centrifugation (1000 x *g*) of the multiscreen plate. This step was repeated two times with multiscreen plates containing regenerated Dowex AG 50W-X8.

Annotation of oligosaccharides using Maldi-tof mass spectrometry

For Matrix Assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry (Maldi-tof MS) analysis an Ultraflex workstation (Bruker Daltonics, Germany) equipped with a 337 nm laser was used. The mass spectrometer was operated in the positive mode and calibrated with a mixture of maltodextrins (mass range 300-3000 Da). After a delayed extraction time of 200 ns, the ions were accelerated with a 25 kV voltage. The ions were detected using the reflector mode.

Two microliter of each fraction was automatically transferred from the 96 well plate to the Maldi sample plate and mixed with 2 µl of matrix by using a Symbiot–I robot (PerSeptive Biosystems, USA) and dried under a stream of warm air. The matrix solution was prepared by dissolving 9 mg 2,5-dihydroxy benzoic acid in a ~1 mL acetonitril:water (300 µL:700µL) mixture. Mass to charge signals of the sodium adducts were translated in oligomeric structures (*e.g.* 685 m/z→$GalA_2Rha_2$), using an in-house software program and HPAEC data. Mass to charge signals, which could not be assigned unambiguously were determined with Post Source Decay/Maldi-tof MS, using the same settings as for Maldi-tof MS. The spectrum was divided into different independently measured segments, which were merged to one PSD spectrum by the FlexAnalysis Software (Bruker Daltonics, Germany)(Verhoef et al., 2005).

Partial structural elucidation with Nanospray mass spectrometry of ^{18}O labeled oligosaccharides

Detailed structural information was obtained after labeling the reducing end with ^{18}O. The samples were lyophilized and 0.5% formic acid in $H_2{}^{18}O$ (Campro Scientific, the Netherlands) was added, followed by 72 hours incubation at 40°C. Static nanospray MS (NSI-MS) of labeled samples was performed with a LTQ Ion Trap (Thermo Electron, USA). Ten micro liter sample was transferred into a capillary needle (New Objective, USA) and MS analysis was carried out in the positive mode using a capillary temperature of 200°C and a spray voltage of 1.8 kV. The automated tune function generated the remaining settings, using the sample material. Fragmentation was performed using a window of 2 m/z at 25% collision energy.

Enzymatic degradation of oligomeric fragments with exopolygalacturonase and rhamnogalacturonan galacturonohydrolase

Fractions containing connecting oligosaccharides were dialysed (100 Da; Spectrapore, USA), lyophilized and dissolved in 100 µL distilled water. Thereafter incubated with an overdose of *exo*-polygalacturonase (ExoPG) of *Aspergillus tubingensis* (Kester *et al.*, 1996). The resulting digest was sequentially digested with rhamnogalacturonan α-D-galacturonohydrolase (RGGH) from *Aspergillus aculeatus* (Mutter *et al.*, 1998a). Incubations were carried out at 37°C for 24 hours, and after each incubation samples were heated for 10 minutes at 100°C to inactivate the enzymes. Enzyme activity and -overdose were confirmed by incubating polygalacturonic acid and RGI oligosaccharide $GalA_3Rha_3$ standard solutions.

^1H- NMR spectroscopy of oligomeric fragments

Prior to NMR analysis, samples were reduced to their corresponding alditols. The reduced sugars were sequentially exchanged in 99.96% D_2O (Cambridge Isotope Laboratories, USA) and after freeze-drying dissolved in 99.996% D_2O (Cambridge Isotope Laboratories, USA) and inserted in NMR microtubes (Shigemi, USA). NMR spectra were recorded at a probe temperature of 25°C on a Bruker AMX-500 spectrometer (Bruker Biospin, Germany) located at the Wageningen NMR Center. Chemical shifts were expressed in parts per million relative to internal acetone: δ=2.225 ppm for ^1H.

The 1D ^1H proton spectra were recorded at 500.13 Mhz. using 1024 scans and a sweep width of 3000 Hz. Two dimensional homonuclear COSY and TOCSY spectra were recorded using standard pulse sequences delivered by Bruker using 512 experiments of 128 scans (Oosterveld *et al.*, 2004).

Results

Partial degradation of apple MHR to generate linkage point fragments

Different glycosidic linkages hydrolyze under different circumstances; therefore it is possible to rather specifically remove sugars. Linkages between two GalA sugars are more stable then aldobiuronic linkages (GalA-Rha) or pseudo-aldobiuronic (Rha-GalA) sugars. Linkages between neutral sugars are the most susceptible to acid hydrolysis; hence controlled acid hydrolysis is frequently used to remove neutral sugars (BeMiller, 1967; Guillon & Thibault, 1990; Thibault *et al.*, 1993). Acetyl and methyl esters were removed by saponification to reduce the number of signals/peaks in spectra and chromatograms. Saponified Apple MHR was partially degraded by controlled acid hydrolysis (hydrochloric acid and trifluoroacetic acid) to breakdown neutral sugar side chains and to release oligosaccharides, involved in the connection between HG and/or XGA to RGI. The degradation was monitored with HPSEC analysis (figure 1).

Saponification of the MHR resulted in a similar elution profile, with a small shift to the left of the 30.5 min. population, which would be attributed to aggregation of HG

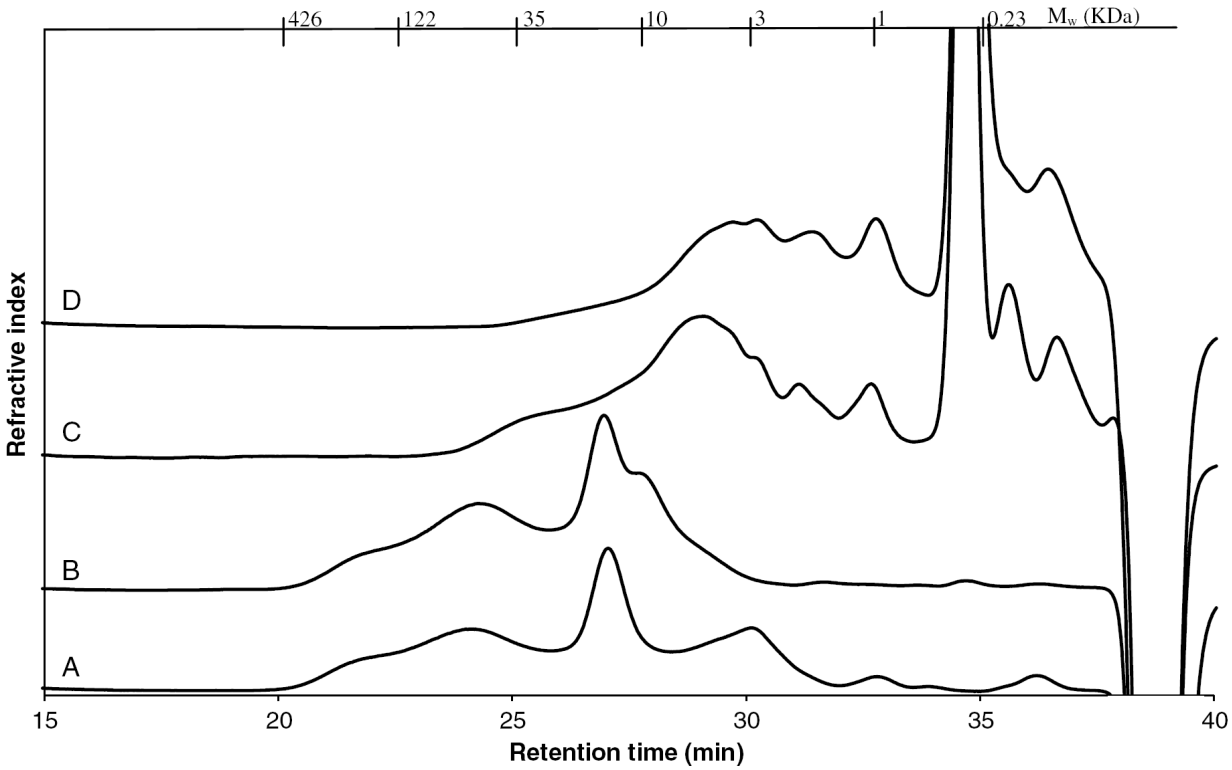

Figure 1: HPSEC molecular weight distribution profiles of (A) apple MHR, (B) Saponified apple MHR, (C) HCl hydrolyzed saponified apple MHR, and (D) TFA and HCl hydrolyzed saponified apple MHR (molecular weight annotations in top x-axis are based on pullulan standards).

chains (Kravtchenko *et al.*, 1992). The saponified sample was dialyzed and lyophilized, which explains the absence of low molecular weight peaks. Controlled acid hydrolysis degraded high molecular weight populations to a mixture of oligosaccharides. The first hydrolysis step is performed with HCl, this mild condition almost specifically hydrolyzes neutral sugar linkages. After hydrochloric acid hydrolysis, a peak remains present at 25 min, which is expected to consist primarily out of galacturonic acid residues, since the aldobiuronic galacturonic acid linkage is the most stable pectin linkage next to GalA-GalA (Thibault et al., 1993). TFA was used to partly hydrolyze the aldobiuronic linkages between the molecules constituting the population at 25 minutes, resulting in a sample containing mainly oligosaccharides and monosaccharides.

Sugar composition of apple MHR during different controlled acid hydrolysis steps

To monitor the relative enrichment of galacturonic acid and rhamnose in the polymer and oligosaccharide material during controlled acid hydrolysis, the sugar composition was determined (table 1). The sugar composition shows that the saponified sample has only minor differences with the original sample, showing that saponification indeed only removed esters. The 48 h 0.1 M HCl 80°C hydrolysis removed primarily arabinose and galactose. The sequential 6 h 0.05 M TFA 100°C hydrolysis resulted in a further decrease in the neutral sugars as well as the removal of fucose. By removing these neutral sugars the resulting material was relatively enriched in Rha and GalA content and slightly further degraded. Xylose resists hydrolysis better than arabinose and galactose, which is in accordance with the results of Thibault *et al.* (1993). We hypothesize that the presence of the carboxyl group in close vicinity could provide a protective effect on the ß-(1→3) xylose linkage stability. Sugar analysis showed that during different hydrolysis steps the mixture was enriched in galacturonic acid and rhamnose moieties as described before (Thibault et al., 1993). From the GalA:Rha ratio 2.2:1 it can be concluded that HG and RGI structures remain present in the sample after TFA and HCl hydrolysis. Enzymes

Table 1: Sugar composition (mol%) of the different MHR fractions.

	Sugar composition (mol%)								
Fraction	Fuc	Rha	Ara	Gal	Glc	Man	Xyl	GalA	GlcA
Apple MHR[a]	1	17	8	15	3	0	13	42	1
Saponified apple MHR [a]	2	20	6	18	2	0	11	39	2
HCl treated saponified apple MHR[a]	1	20	2	14	3	0	8	51	1
TFA&HCl treated saponified MHR[b]	0	27	1	4	2	0	6	59	1

[a]obtained after dialysis (12KDa)

[b]obtained by pooling the polymer peak after Biogel P2 separation (distilled water elution)

could not be used for enrichment of connection points out of the MHR, since MHR is the enzyme resistant product left after degrading apple pectin with a complex mixture of pectic enzymes.

Isolation of connecting fragments out of the apple MHR hydrolysate

In order to recognize oligomeric fragments involved in the connection between HG or XGA to RGI, the hydrolysate was fractionated using high performance anion-exchange chromatography (HPAEC; figure 2). The separation was performed at pH 5 to selectively separate galacturonic acid containing oligosaccharides, since neutral oligosaccharides will not bind to the column at these conditions. Galacturonic acid oligosaccharides are annotated using a PGA digest consisting out of mono-, di-, tri and tetragalacturonic acid. All other peaks were annotated by off line Maldi-tof MS of desalted fractions (Kabel *et al.*, 2001).

The HPAEC elution pattern shows the oligosaccharide composition of the sequential HCl and TFA hydrolyzed sample. Controlled acid hydrolysis of saponified apple MHR resulted in a complex mixture of more than 50 different peaks. The first peak represents neutral sugar oligosaccharides, which do not bind to the column. The gradient is optimized to separate oligosaccharides with at least two galacturonic acid residues; under these conditions mono GalA eluted at 15 minutes in a broad peak. Peaks are annotated, based on sugar composition of the injected sample, oligosaccharide elution behavior, and apparent Maldi-tof MS masses. In addition, salt signals, which are usually regarded

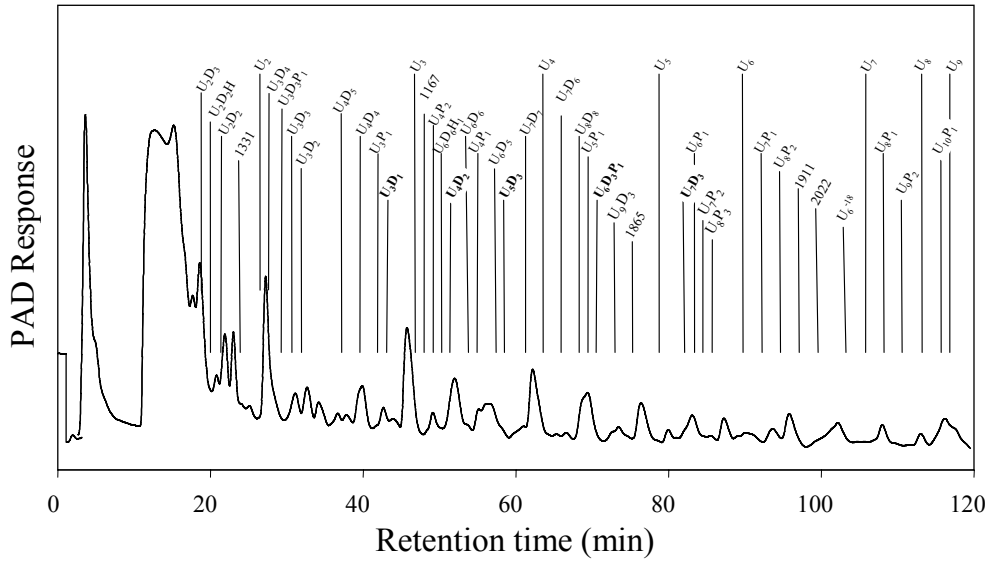

Figure 2: HPAEC elution profile of an oligosaccharide mixture obtained after mild acid treatment of apple MHR, including peak annotation based on off line Maldi-tof MS analysis of fractions collected (U=uronic acid: galacturonic acid; D=deoxyhexose: rhamnose; P=pentose: xylose).

undesirable because of the negative influence on signal intensity, were used to confirm hypothized structures. The presence of additional salt signals neighboring the sodium adduct of an oligosaccharide is caused by the exchange of a proton of one or more carboxyl group(s) with a sodium or potassium ion and is therefore indicative for the number of GalA moieties within an oligosaccharide. When more m/z signals are present in one fraction, the annotation is related to the signal intensity. RGI oligosaccharides are identified in the hydrolysate, so controlled acid hydrolysis succeeded in releasing "backbone" oligosaccharides from apple MHR. HG and XGA type oligosaccharides are recognized as well to be present in the hydrolysate.

Depending on their size, oligosaccharides with similar charges and differing in neutral sugars content may co-elute. RGI backbone oligosaccharides with either a single pentose or hexose substitution are identified, which are considered remnants of arabinan or galactan side chains (Gur'janov *et al.*, 2007; Schols *et al.*, 1994). Peaks which could not be annotated were shown by their m/z value. After fractionation it was possible to identify oligosaccharides using Maldi-tof MS, which could not be detected in the whole hydrolysate (data not shown). Maldi-tof MS was used for annotation in combination with a PGA digest, since most other structures are not available as standards.

The structures $GalA_3Rha_1$, $GalA_4Rha_2$, $GalA_5Rha_3$ and $GalA_6Rha_3Xyl_1$ (represented in bold) are of hybrid nature. Their galacturonic acid to rhamnose ratio, indicates the presence of a RGI segment and a HG segment within one oligosaccharide. Since the m/z ratios were annotated based on sugar composition of the injected sample and elution behavior, these annotations were confirmed with Post Source Decay (PSD)/Maldi-tof MS (data not shown). The first 3 oligosaccharides are expected to be involved in the HG to RGI linkage, the $GalA_6Rha_3Xyl_1$ oligosaccharide indicates a XGA to RGI linkage. The xylose annotation for the pentose rather than arabinose (which has an equal mass) in $GalA_6Rha_3Xyl_1$, was based upon the fact that almost no arabinose was present in the sample.

A GalA trimer is covalently linked to an alternating Rha-GalA segment

More detailed structural information was obtained by the analysis of connecting oligosaccharides with nano spray ionization mass spectrometry (NSI-MS) after labeling their reducing end with ^{18}O. Labeling was performed to establish if generated fragments included the reducing end of the oligosaccharide. The fragments are annotated according to the systematic nomenclature of Domon and Costello (1988). When analyzing the MS^1 spectrum of labeled oligosaccharide fractions, signals next to the labeled mother ion appeared to be present having an additional +2 Daltons increase (Data not shown). Galacturonic acid oligosaccharides and maltodextrin standards were analyzed to explain

this phenomenon (Data not shown). The mass increase of 2 Dalton occurred only with GalA oligosaccharides, which contain carboxylic acid groups. Furthermore, the number of peaks with an increment of 2 Dalton depends on the number of uronic acid residues within the oligosaccharide. No increment of 2 Dalton was found for the maltodextrin standard.

The relation with the uronic acid leads us to the "multiple ^{18}O label" hypothesis that there is an exchange between the carboxylic oxygen and ^{18}O.

It becomes evident from the signals at 569 m/z and 551 m/z (A), that the oligosaccharide with 717 m/z consists out of a galacturonic acid trimer connected to rhamnose. The position of the rhamnose is determined with the MS^3 spectrum of the 365 m/z (^{18}O labelled GalA-Rha) fragment (B), which indicates that rhamnose contains the reducing end (189 m/z). Both spectra represented in figure 3 show labeled uronic acids moieties, but this was recognized as the "multiple ^{18}O label" artifact. Additionally, primarily B and Y fragments are formed, which also annotate rhamnose as the reducing end, since in the positive mode the glycosidic oxygen is principally retained on the

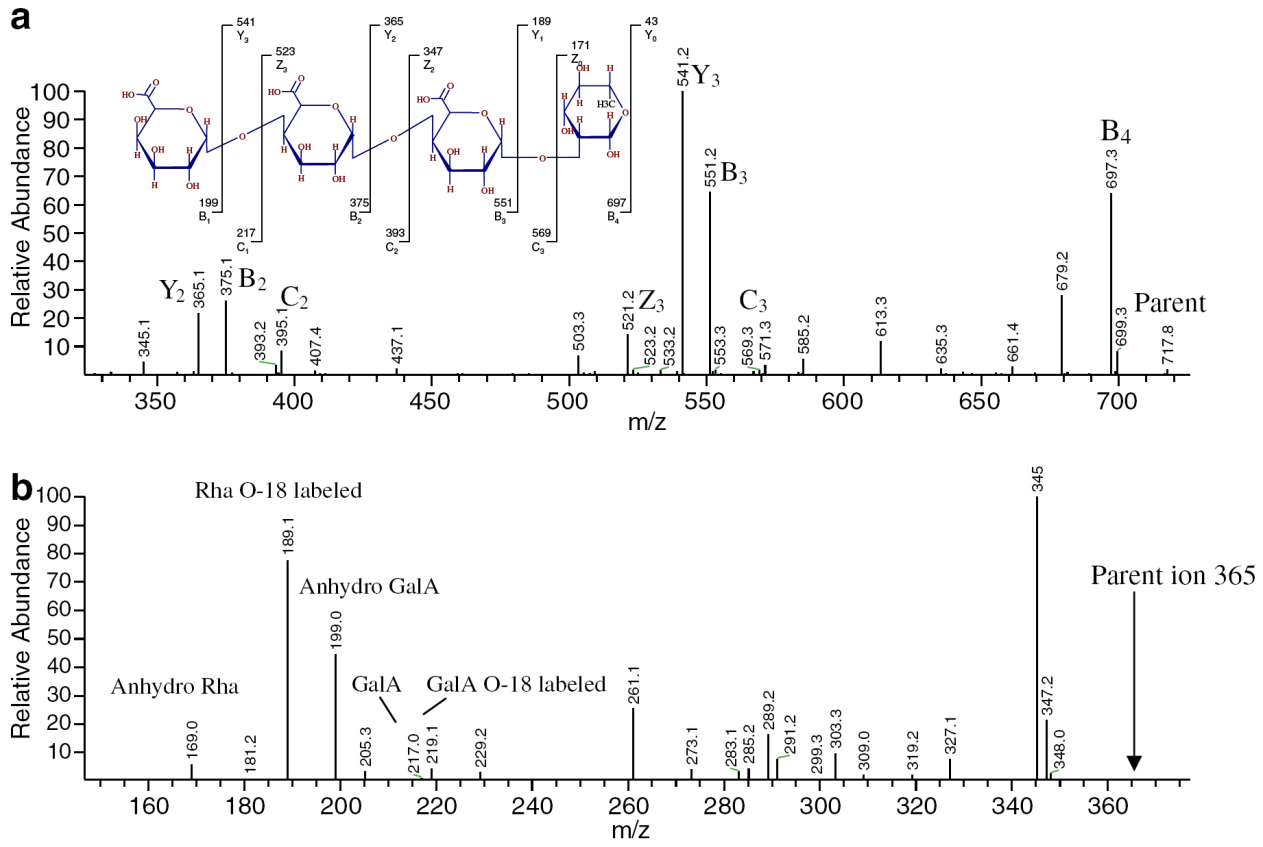

Figure 3: MS2 fragmentation pattern of ^{18}O labeled connecting oligosaccharide $GalA_3Rha$ (717 m/z) (A), and MS3 fragmentation pattern of 365 fragment (B) analyzed in de positive mode; a schematic representation of the oligosaccharide is given, with assumed linkages between the sugars.

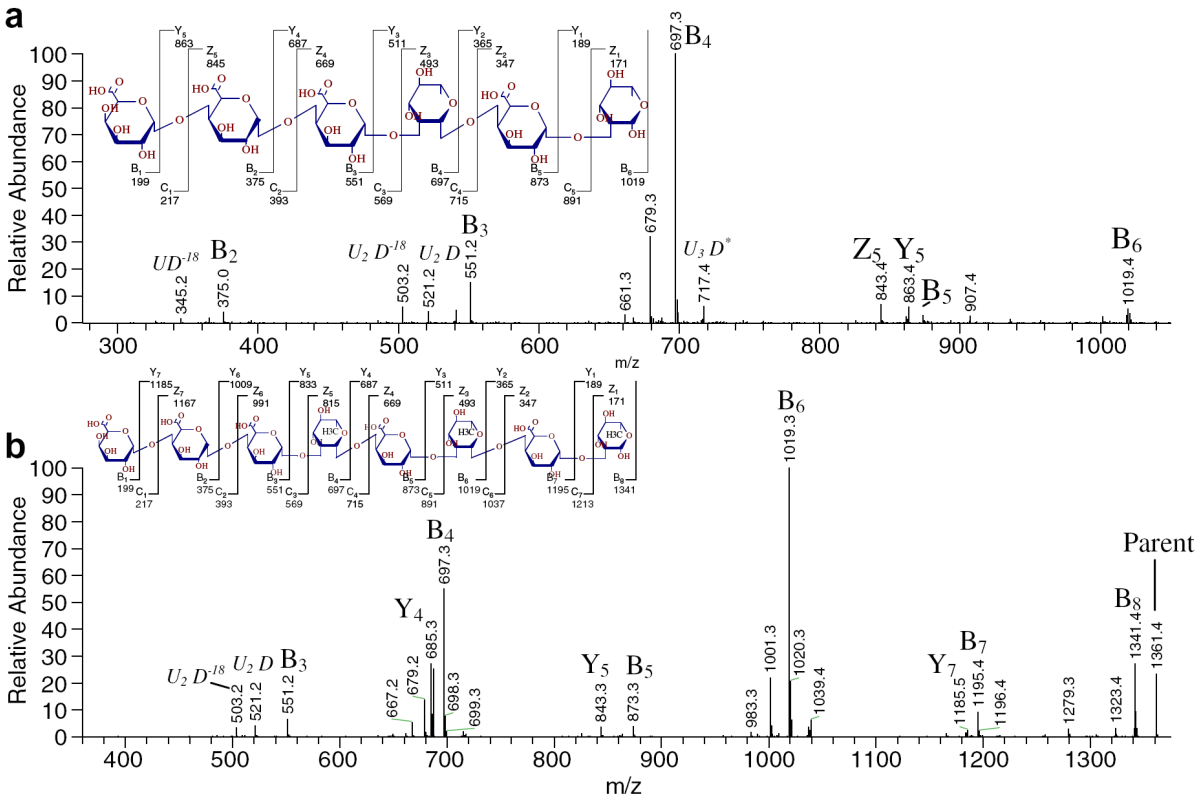

Figure 4: MS2 fragmentation patterns of ^{18}O labeled key oligomeric fragments $GalA_4Rha_2$ [1039 m/z] (A), and of $GalA_5Rha_3$ [1361 m/z](B) analyzed in de positive mode. Asterisk represents ^{18}O label, $^{-18}$ represents -18 m/z mass loss, presumably due to an-hydro sugar or structural rearrangement. In both spectra a schematic representation of the oligomer is given, with assumed linkages between the sugars.

fragment including the reducing end (van Alebeek *et al.*, 2000). Because the galacturonic trimer fragment does *not* contain the glycosidic oxygen, it is likely positioned on the non reducing end, leaving the reducing end as only possible location for the rhamnose. To corroborate the annotation of rhamnose as the reducing end, $GalA_5Rha_3$ was labeled by reduction through $NaBH_4$, which gives a specific mass label of +2 Dalton on the reducing end. This experiment (data not shown) unambiguously annotates rhamnose at the reducing end.

The spectrum indicates the presence of one dominant oligosaccharide with lower amounts of impurities. These impurities consist out the potassium adduct of $GalA_3Xyl$, like the 585 m/z ($galA_3$-K^+ adduct) oligosaccharide (Fig 2.3a). Other interference like the 571 m/z, 553 m/z, 395 m/z and 219 m/z signals come from $GalA_3Rha$ oligosaccharides, which have ^{18}O labeled (+2 m/z) galacturonic acid instead of a labeled reducing end. Combining these data results in the schematic structure represented in the inlay in figure 3a. The linkages are still hypothetical, but the corresponding fragment weights will be applicable for all possible glycosidic cleavages.

Next to the $GalA_3Rha$ oligosaccharide, $GalA_4Rha_2$ and $GalA_5Rha_3$ oligosaccharides were as well collected during the HPAEC run of the MHR hydrolysate (retention times respectively 51 and 58 minutes) and sequentially ^{18}O labeled. The spectra of these labeled oligosaccharides are shown in figure 4.

Both spectra (figure 2.4a and 2.4b) show a similar fragmentation pattern. The 551 m/z signal indicates a galacturonic acid trimer within these oligosaccharides. There is a loss of galacturonic acid-rhamnose blocks resulting in mainly B type fragments. This indicates that the galacturonic acid trimer fragment is coming from the non reducing end (van Alebeek et al., 2000). This is confirmed by ^{18}O labeling, which assigns rhamnose as the reducing end. In these spectra signals are as well present coming from "multiple ^{18}O labeling", as described for the $GalA_3Rha_1$ oligosaccharide.

Both spectra contain the B fragment 697 m/z, which is as well an apparent signal in figure 3. MS^3 experiments with the 697 m/z fragment for the different oligosaccharides $GalA_3Rha_1$, $GalA_4Rha_2$, $GalA_5Rha_3$, resulted in similar spectra (data not shown), indicating structural resemblance. In both MS^2 spectra (fig. 2.4a and 2.4b) fragments are observed like $GalA_2Rha$, which can only be generated by double cleavages in the oligosaccharide, which is as well observed before (Hilz *et al.*, 2006).

Both $GalA_4Rha_2$ and $GalA_5Rha_3$ oligosaccharides contain a GalA trimer (remaining HG segment) on the non reducing end and a rhamnose (remaining RGI segment) at the reducing end. The absence of connection points with the HG segment on the reducing end is remarkable, since in the Voragen model (Voragen et al., 1995), the pectin backbone consists out of alternating HG and RGI structural elements, which would yield oligosaccharides, with HG on the reducing end as well. The weaker linkage stability of pseudo-aldobiuronic linkage is a possible explanation for the specific formation of these oligosaccharides during hydrolysis (Thibault et al., 1993), but since RGI segments (up to $GalA_8Rha_8$) are isolated as well this explanation is not unequivocal.

A xylose substituted GalA trimer is covalently linked to an alternating Rha-GalA segment

The $GalA_6Rha_3Xyl_1$ oligosaccharide (fig. 2.5; retention time 71 minutes) was analyzed with nano-spray ionization mass spectrometry (NSI-MS) after ^{18}O labeling to obtain detailed structural information (figure 5).

The spectrum shows a dominant $GalA_6Rha_3Xyl_1$ oligosaccharide and a small $GalA_7Xyl_3$ signal. The impurity was attributed to the higher content of $GalA_7Xyl_3$ (XGA fragment; recognized by the loss of a second consecutive xylose [1405 m/z]) in the hydrolysate in relation to $GalA_6Rha_3Xyl_1$ (connection point), which resulted in co-elution of the connecting oligosaccharide and the end of the $GalA_7Xyl_3$ peak. In analogy to the

structures discussed before, rhamnose is located at the reducing end of this oligosaccharide. The 727 m/z fragment shows that a galacturonic acid tetramer is part of the molecule. Xylose is substituted to one of the uronic acids located at the non reducing end, which can be concluded from the 507 m/z fragment ($GalA_2Xyl$). The release of a single GalA fragment, indicated by the 1493 m/z signal, indicated that the second GalA sugar from the end terminus is substituted with this xylose. Combination of these results leads to an oligosaccharide consisting out of a XGA element, attached to a RGI element, as represented in the inlay. It was not possible to further characterize the structures on a linkage level with NSI-MS, nor by investigation of ring fragmentations as described by Quemener *et al.* (2006) and Zaia (2004). On an abundantly present $GalA_3Rha_3$ oligosaccharide, no recognizable cross ring cleavages could be detected, which led to discontinuation of this approach.

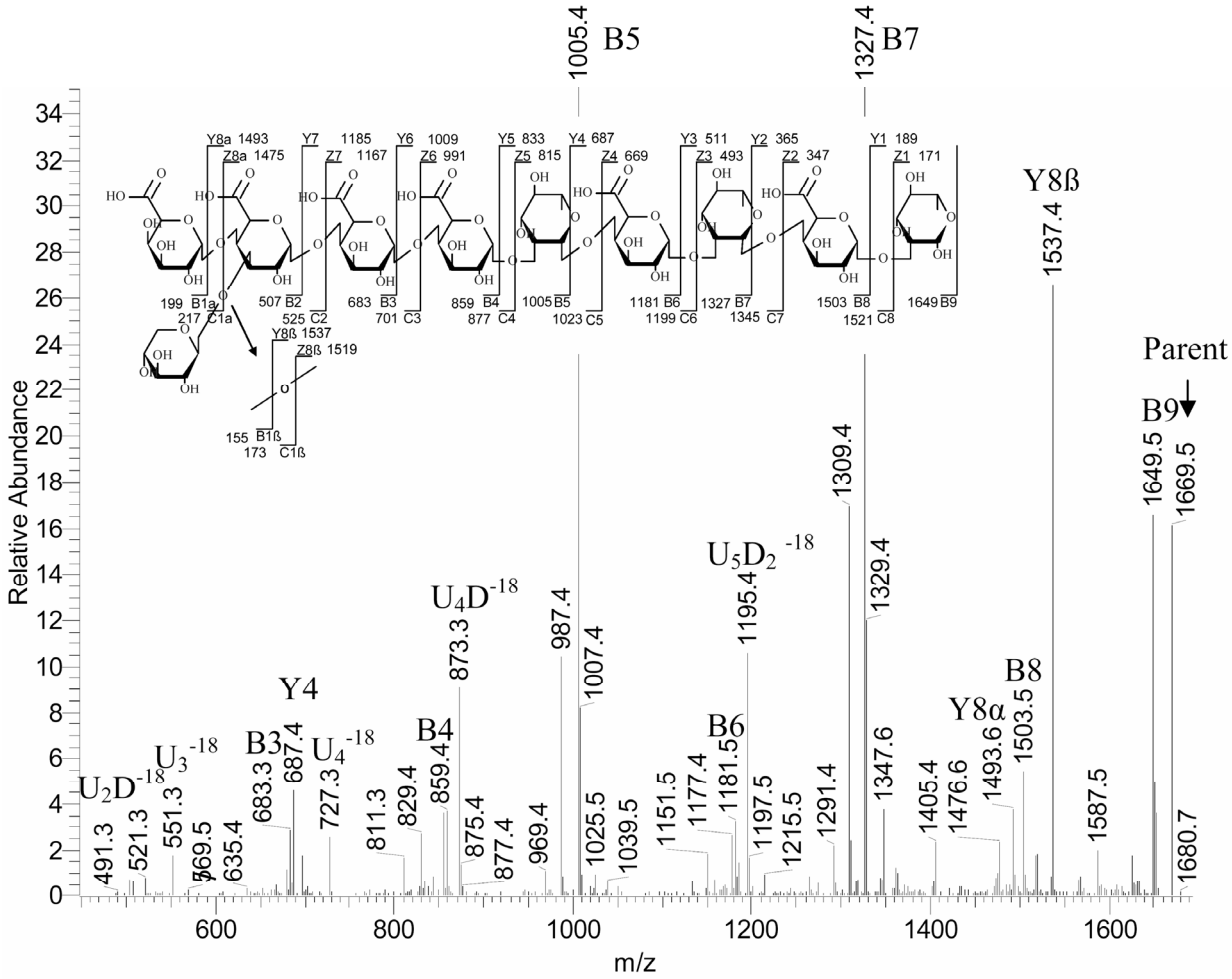

Figure 5: MS^2 fragmentation pattern of $H_2{}^{18}O$ labeled oligomer $GalA_6Rha_3Xyl_1$ [1669 m/z] (A). In the spectra a schematic representation of the oligomer is given, with assumed linkages between the sugars.

Indications for a α-(1→2) linkage between GalA trimer and RGI moiety within the oligosaccharides

The oligosaccharides $GalA_5Rha_3$ (1359 Da) and $GalA_6Rha_3Xyl_1$ (1667 Da) are enzymatically degraded with ExoPG and RGGH (figure 6) to confirm the given structure and to get more information about the linkage between the HG or XGA element to the RGI element. ExoPG attacks the substrate from the non reducing end and is able to remove terminally (1→)-linked galacturonic acid residue from HG chains, while it is also known that ExoPG is tolerant for a xylose substation, removing a GalA-Xyl dimer (Beldman *et al.*, 1996). RGGH is able to release a galacturonic acid connected to a rhamnose from the non reducing side of RGI chains and unable to liberate galacturonic acid from HG (Mutter et al., 1998a).

Both oligosaccharides $GalA_5Rha_3$ and $GalA_6Rha_3Xyl_1$ are stepwise enzymatically degraded to the RGI end-product with rhamnose on both sides, which indicates that HG is indeed linked to RGI, most probably α-(1→2). It was not possible to determine if the enzymes were absolutely specific for α-(1→4) (ExoPG) and α-(1→2) (RGGH), since side-activities for these enzymes were not investigated so far, due to the unavailability of suitable substrates (Beldman *et al.*, 1996; Benen *et al.*, 2002; Kester *et al.*, 1996; Mutter *et al.*, 1998a).

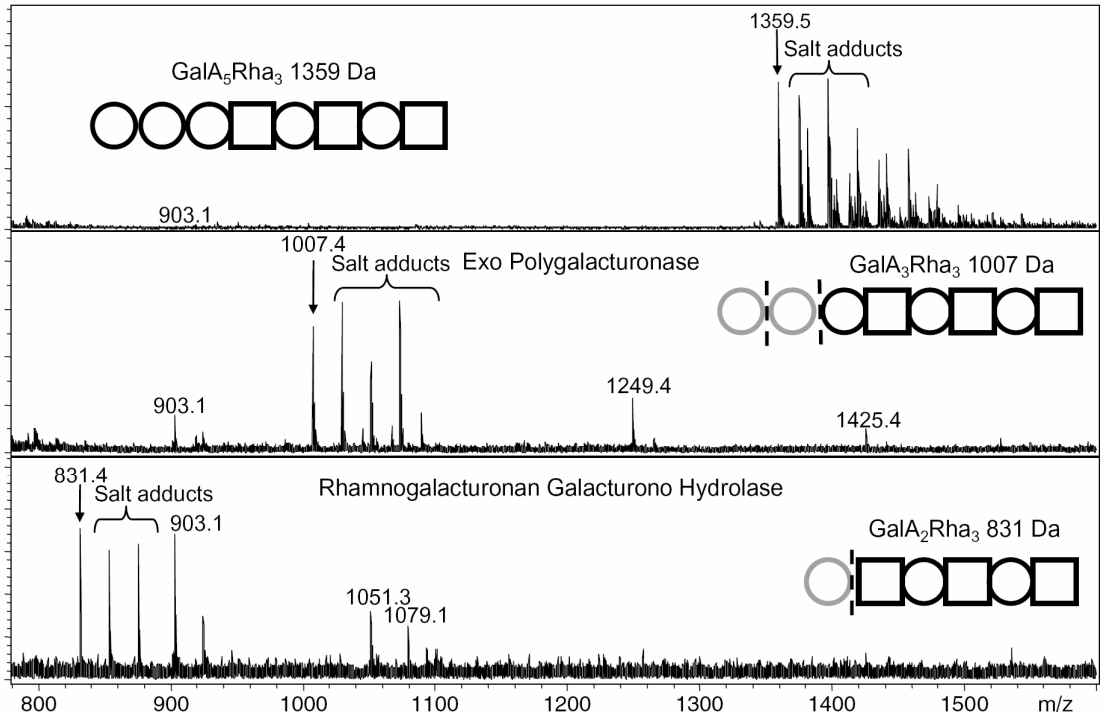

Figure 6: Maldi-tof mass spectra of the $GalA_5Rha_3$ oligosaccharide, before and after treatment with ExoPG and RGGH, with schematic representation in the right side. (○ =Galacturonic acid □ =Rhamnose).

GalA trimer is α-(1→2) linked to RGI moiety within connecting oligosaccharide $GalA_5Rha_3$

To substantiate the structural information about the linkages within the connecting oligosaccharides provided by the enzymatic digestions, ^1H chemical shifts were determined by NMR spectroscopy of the reduced $GalA_5Rha_3$ oligosaccharide (table 2).

The presence of only one anomeric signal for *internal* rhamnose points out that both rhamnose sugars present in the oligosaccharide are linked in the same way. The ^1H chemical shifts are in good agreement with those reported previously (Colquhoun *et al.*, 1990; Mutter *et al.*, 1998b), being specific for α-(1→2) linked rhamnose, within a RGI backbone. The α-D-$GalA_{HG}$ spin system belongs to HG (ppm anomeric signal) (Bushneva *et al.*, 2002; Habibi *et al.*, 2005; Kardosova *et al.*, 2004; Schols *et al.*, 1995b). The H^1 chemical shifts differ slightly from the literature value, but the remaining chemical shifts definitely point toward an internal α-D-$GalA_{HG.}$ The α-D-$GalA_{RG}$ signal is coming from the alternating Rha-GalA unit (ppm anomeric signal)(Habibi *et al.*, 2005; Mutter *et al.*, 1998b; Renard *et al.*, 1998; Schols *et al.*, 1990). The ^1H signal of the reduced rhamnose is outside the anomeric region, where it is overlapped by the other signals in the bulk region. This position was, however, already demonstrated by MS results. An α-L-Rha was identified as well, indicating incomplete reduction of the reducing end (Schols et al., 1990). However, the ß-L-Rha signal could not be detected, meaning that only a small proportion was not reduced. Chemical shifts distinctive for other structures were not present.

The →4)-α–D-Gal*p*A-(1→4)-α–D-Gal*p*A–(1→2)-α–L-Rha linkage has been suggested previously for an acid soluble pectin fraction of *Opuntia ficus-indica* (Habibi *et al.*, 2005). This suggestion was based on relative amounts of different linkages determined by NMR and GC-MS techniques. The →4)-α–D-Gal*p*A-(1→4)-α–D-Gal*p*A–(1→2)-α–L-Rha structure itself was, however, not identified. Since other isomer structures could be drawn up as well, the resulting structure remained ambiguous.

For the $GalA_6Rha_3Xyl_1$ oligosaccharide it was not possible to obtain a good NMR signal, possibly due to the low quantity. But looking to the homology with the $GalA_5Rha_3$

Table 2: ^1H chemical shifts (ppm) of the reduced $GalA_5Rha_3$ oligosaccharide determined at 500 Mhz.

Residue	H-1	H-2	H-3	H-4	H-5	H-6
α-L-Rha_{red}	5.22	4.06	3.96	3.46	3.85	1.23
α-L-Rha_{int}	5.26	4.12	3.88	3.41	3.78	1.25
α-D-$GalA_{RG}$	5.01	3.91	4.11	4.42	4.72-4.65	
α-D-$GalA_{HG}$	5.05[a]	3.95	4.06	4.41	4.80-4.65	

[a]Tentative assignment (weak signal)

oligosaccharide in the results described above, we postulate that the linkage between XGA and RGI will as well be α-(1→2).

Furthermore, results were in agreement with the enzymatic degradation patterns. Combining these findings, the structure for connection between RGI and HG is proposed (figure 7).

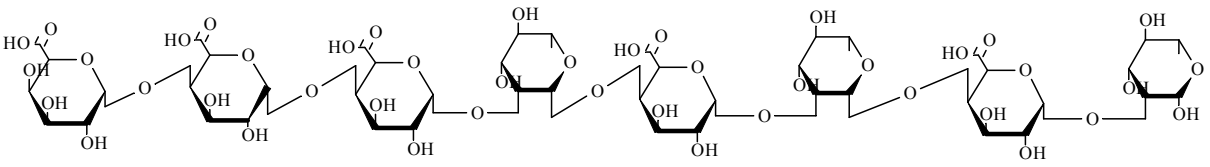

Figure 7: Representation of a connection between rhamonogalacturonan I and homogalacturonan.

Conclusion

By controlled acid hydrolysis of apple MHR a mixture of oligosaccharides was obtained. This mixture was enriched in galacturonic acid and rhamnose content during various hydrolysis and purification steps, as shown by sugar composition analysis. HPAEC at pH 5 in combination with Maldi-tof MS revealed that this MHR hydrolysate consisted out of RGI, HG, and XGA type oligosaccharides next to neutral sugars. Furthermore, oligosaccharide structures with features of HG as well as XGA and RGI were present which indicates that these are the linkage between HG/XGA and RGI structural elements. MS^n experiments with these labeled structures ($GalA_3Rha$, $GalA_4Rha_2$, $GalA_5Rha_3$, $GalA_6Rha_3$, and $GalA_6Rha_3Xyl_1$ oligosaccharides) showed that the position of the RGI block was at the reducing end of the oligosaccharide and the HG/XGA block was at the non-reducing end. Both blocks were α-(1→2) linked, as was indicated by enzymatic degradation with ExoPG and RGGH and NMR spectroscopy. To our knowledge this is the first time that the covalent linkage of a HG or a XGA structural element to RGI was demonstrated at oligosaccharide level. These results partly correspond with the model of Voragen (Voragen et al., 1995), were the backbone consisted out of consecutive HG and RGI structural elements. Since it could not be ruled out that we only identified part of the connecting points present in the pectin and not all unknown oligosaccharides could be identified so far, the model where HG is positioned as a RGI side chain (Vincken et al, 2003b) could not be excluded irrevocably. The absence of oligosaccharides which contain HG structures at the reducing end could indicate that the backbone consisted out of only out one HG and one RGI structural element. This hypothesis does, however, not account for the Mw of pectins (50-100 kDa), considering that the RGI element is 12 kDa and the HG element is 17.5 kDa (Prade *et al.*, 1999; Yapo *et al.*, 2007; Zhan *et al.*, 1998). The ratio Rha:GalA in apple (1:21), citrus peel (1:31), soy

(1:3.5), black currant (1:20), bilberries (1: 21), and sugar beet (1:8) pectins (Hilz *et al.*, 2005; Voragen *et al.*, 2001; Yapo *et al.*, 2007) would advocate for more, or longer, HG chains per RGI unit. Additional research is needed to determine the size of various pectin structural elements, in order to elucidate the macromolecular build up of this polymer in a more detailed manner.

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Chapter 3

CE-MS^n of complex pectin derived oligosaccharides

In press
Coenen, G.J., Kabel, M.A., Schols, H.A. and Voragen, A.G.J. (2008). CE-MS^n of complex pectin derived oligomers. Electrophoresis, elps-2007-00465.

Abstract

As pectin molecules are too large and heterogeneous to analyze as a whole, the polymer is usually degraded to smaller oligosaccharides, which are often analyzed by high performance anion exchange chromatography (HPAEC). However, the high salt concentration necessary to elute pectin oligosaccharides by HPAEC is incompatible with on line mass detection. To overcome such a disadvantage, a CE-IT-MS system was set up to further elucidate the fine structure of charged oligosaccharides.

An effective separation of differently substituted galacturonic acid containing oligosaccharides was obtained by low pH CE-Laser induced fluorescence (LIF) analysis. By adapting the buffer and capillary on line MS detection was enabled. Moreover, with MS/MS it was possible to localize sugar residues substitutions. With this combined CE-MS approach LIF electropherograms of xylogalacturonan and rhamnogalacturonan I digests could be annotated. The method was further exemplified by a complex oligosaccharide mixture of acid hydrolyzed apple pectin, which was separated and characterized by CE-MS^n. Oligomers present in low amounts could be localized by their corresponding m/z, as was demonstrated by selected mass range representation.

Keywords

APTS, galacturonic acid, MS, oligosaccharides, pectin.

Introduction

Pectin is a key constituent of plant raw materials in addition to an important ingredient in the food industry. Knowledge of its structure is essential to explain its gelling and stabilizing properties (Daas *et al.*, 2001; De Vries, 2004; May, 2000; Schols & Voragen, 1996; Voragen *et al.*, 1995). Pectin is also of interest for medical applications due to its health promoting effects and because of its ability to modify surfaces of medical materials. Elucidation of the structure of the polysaccharide as an active ingredient is, therefore, imperative (Becker *et al.*, 2006; Guggenbichler-J-P *et al.*, 1997; Kastner *et al.*, 2002; Lim *et al.*, 1997; Morra *et al.*, 2004; Torto, 1998; Yamada *et al.*, 2003).

Pectin is the collective noun for a group of plant cell wall polysaccharides which are rich in galacturonic acid (GalA) (Willats *et al.*, 2001). The polymer may be composed out of a number of different structural elements, such as homogalacturonan (HG), xylogalacturonan (XGA), rhamnogalacturonan I (RGI), rhamnogalacturonan II (RGII), arabinan and arabinogalactan. They appear to be connected, by both covalent and ionic cross-links (Schols & Voragen, 2002). Although the complex structure of RGII is highly conserved, the fine structure of HG and RGI is shown to be quite diverse (Willats *et al.*, 2001). Homogalacturonan consists of a linear chain of galacturonic acid, which can be decorated with methyl esters or acetyl groups (Albersheim *et al.*, 1996; O'Neill & York, 2003). Xylogalacturonan is also a homogalacturonan, which contains xylose residues attached to *O*-3 of the galacturonan backbone (Schols *et al.*, 1995). Rhamnogalacturonan oligosaccharides have an alternating GalA-Rha backbone, were rhamnose can be substituted with galactose or arabinose (Lau *et al.*, 1985; McNeil *et al.*, 1980; Schols & Voragen, 1994).

To be able to reveal its structure, pectin is commonly degraded into smaller oligosaccharides, as the pectin molecule is too large and heterogeneous (140 KDa-225 KDa for commercial pectin (Guillotin, 2005), until 1400 KDa for extracted sugar beet pectin (Oosterveld *et al.*, 2000)) to analyze as a whole (Voragen *et al.*, 1992). Identification of the resulting oligosaccharides in these complex degradation mixtures gains more insight in pectin structure (Coenen *et al.*, 2007) and enables the correlation to technical and biological functionalities (Samuelsen *et al.*, 2007).

Pectin oligosaccharides are often characterized by high performance anion exchange chromatography (HPAEC) (Lee, 1996; van der Hoeven *et al.*, 1992). Peak annotation by HPAEC is based on standards, and therefore depends on standard availability (Lee, 1996; van der Hoeven *et al.*, 1992). When standards are not available, peaks can be annotated by (off line) mass spectrometry (MS) (Coenen *et al.*, 2007; Kabel *et al.*, 2001). However, on-line mass annotation of HPAEC peaks is not always possible, due to high salt

concentration of the eluent (Packer *et al.*, 1998; van der Hoeven *et al.*, 1992). Effective desalting for on-line MS detection is demonstrated only until ~400 mM sodium concentration (Bruggink *et al.*, 2005; Guignard *et al.*, 2005). As a consequence, only acidic oligosaccharides which have an degree of polymerization (DP) < 3 can readily be characterized (Niessen *et al.*, 1996). Oligomers eluting at higher salt concentrations are analyzed off-line, after peak collection and desalting (Coenen *et al.*, 2007; Kabel *et al.*, 2001), which hampers progress in characterizing complex oligosaccharide mixtures (Packer *et al.*, 1998).

A powerful alternative for HPAEC-MS can be capillary electrophoresis (CE) (Arentoft *et al.*, 1993; Balaguer & Neusüss, 2006; Campa *et al.*, 2006; Naran *et al.*, 2007; Zemann *et al.*, 1997). CE evolved as a promising alternative in carbohydrate analysis with respect to fast and highly efficient separations (Klockow *et al.*, 1995). Commonly carbohydrates are analyzed underivatized, while derivatization of oligosaccharides enables high resolution CE (Campa *et al.*, 2006). The separation of 8-aminonaphthalene-1,3,6-trisulphonate (ANTS) labeled galacturonic acid oligosaccharides has already been demonstrated as an elegant way to study pectin enzyme activity using CE (Mort & Chen, 1996; Zhang *et al.*, 1996). The introduction of labels enhances detection considerably when compared to straightforward UV detection and marks the reducing end of an oligosaccharide (Chen *et al.*, 1998), hereby removing the need of additional ^{18}O labeling in case of MS identification. (Strom & Williams, 2004). Furthermore, the stoichiometry of labeling is one molecule of fluorophore per reducing end, enabling accurate mol-based quantification of unknown oligosaccharides (Chen *et al.*, 1998). The separation basis of carbohydrates is the increased Stokes radius (Mort & Chen, 1996) which allows the partial or almost complete separation of oligosaccharides having the same molecular weight, but different linkage types (Oefner & Chiesa, 1994). Finally, the time of analysis is reduced by a factor 3-4 compared to HPAEC analysis (Hilz *et al.*, 2006). Recently, the use of CE-MS in carbohydrates was extended from glycoproteins (Balaguer & Neusüss, 2006) to plant polysaccharides (Hilz *et al.*, 2006; Kabel *et al.*, 2006). Peak annotation of CE depends, just as HPAEC, on standard availability. However, coupling of CE to an mass spectrometer overcomes this drawback and in addition allows mass-annotation of unknown APTS-labeled oligosaccharides separated by CE (Hilz *et al.*, 2006; Kabel *et al.*, 2006). The MS/MS possibilities provided by an iontrap MS enables allocation of side chains and substitutions within an oligosaccharide. The APTS label at the reducing end complicates the mass spectrum, due to the 3 possible charge states, but helps identifying fragmentation products (Hilz *et al.*, 2006). Using low concentrations of volatile MS compatible salts as CE buffers, in combination with electrospray ionization (ESI) iontrap (IT) MS, oligosaccharides can be detected on line (Hilz *et al.*, 2006; Kabel *et al.*, 2006).

The efficiency and selectivity of the separation are slightly lower for CE-MS in comparison to CE-UV, as a result from the alternative buffer, higher dead volume and sheat liquid dilution (Gennaro *et al.*, 2002).

To demonstrate the potential of CE-MS as an alternative for analyzing pectin oligosaccharides, we describe the separation of complex mixtures of APTS labeled RGI, XGA and HG oligosaccharides by CE and their subsequent identification by on-line MS^n.

Materials and methods

Materials

Xylogalacturonan (XGA) was prepared from gum tragacanth with NaOH (0.05 M, 8 hours, 4°C) and trifluoro acetic acid (TFA; 0.1 M, 5 hours, 100°C) (Beldman *et al.*, 2003). Polygalacturonic acid (PGA; ICN, USA) and galacturonic acid mono-, di- and trimer (Sigma, USA) were purchased. The galacturonic acid tetramer and pentamer were purified from enzymatically degraded PGA (van Alebeek *et al.*, 2000). Modified hairy regions (MHR) were isolated from apple liquefaction juice and were sequentially saponified according to the method of Mutter (Mutter *et al.*, 1994). The maltodextrine standard was supplied with the CE analysis kit (Beckman Coulter, USA)

Release of oligosaccharides from pectin structural elements

XGA was degraded by xylogalacturonan hydrolase (XGH) from *Aspergillus tubingensis*, which was cloned and expressed in the *Aspergillus Niger* "PlugBug" of DSM Food Specialities (Delft, The Netherlands) (Zandleven *et al.*, 2005). XGA (5.2 mg) was dissolved in MilliQ water (196 µl) and had a final pH between 3-4, without additional buffering. The incubation with an XGH overdose was performed head over tail at 37°C for 16 hours.

PGA (40 ml, 20 mg/ml) was degraded by endo polygalacturonase (endoPG) from *Kluyveromyces fragilis* (5.7 U) (Guillotin *et al.*, 2005). Samples were incubated head-over tail, during 16 hours at 37°.

Apple MHR was degraded by rhamnogalacturonan hydrolase from *Aspergillus aculeatus* yielding RGI oligosaccharides (Schols *et al.*, 1990). A 10 µl enzyme overdose was added to 1 ml of apple MHR solution (22 mg/ml). Samples were incubated head-over-tail, during 16 hours at 37°C. After incubation all enzymes were inactivated by a heating the reaction mixture for 10 minutes at 100°C.

A complex oligosaccharide mixture was obtained by controlled acid hydrolysis (0.1 M HCl 48 h at 80°C, followed by 0.05M TFA at 100°C for 6 h) of apple MHR (Coenen *et al.*, 2007). This mixture consisted of oligosaccharides originating from homogalacturonan, rhamnogalacturonan I and xylogalacturonan oligosaccharides, among other structures.

Oligomer separation by capillary electrophoresis and Laser Induced Fluorescence detection.

Oligomers were labeled with 8-aminopyrene-1,3,6-trisulfonate (APTS) using the ProteomeLab™ Carbohydrate Labeling and Analysis Kit (Beckman Coulter, USA). The labeled oligosaccharides were separated on a polyvinyl alcohol (NCHO) coated capillary (50 µm id X 50,2 cm, detection window after 40 cm; Beckman Coulter, USA), using a ProteomeLab PA 800 capillary electrophoresis system, equipped with a laser induced fluorescence (LIF) detector, at an 488 nm excitation and an 520 nm emission wave length (Beckman Coulter, USA). The separation was carried out in reversed polarity at 30 kV, with a 25 mM sodium acetate buffer containing 0.4% polyethylene oxide and 0.3 % formic acid at pH 2.98 or with 25 mM sodium acetate buffer containing 0.4% polyethylene oxide at pH 4.75. The addition of polyethylene resulted in a lower velocity of the sugar derivatives (Chiesa & Horvath, 1993).The capillary was kept at 25°C. Maltose was added to the sample before derivatisation as internal standard.

Mass annotation of oligosaccharides using CE-UV-ESIMSn

For identification of the different oligosaccharides, separation was carried out on a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, USA) with a fused silica capillary (50 µm id x 80 cm) connected to an internal UV-detector (Beckman Coulter, USA) and a LTQ ESI Iontrap mass spectrometer (Thermo electron, USA) by a device made in our laboratory (Hilz *et al.*, 2006). The separation was carried out in reversed polarity at 28 kV in a 50 mM HAc solution, containing 0.5% formic acid at pH 2.45. A maximum of 5 sequential runs are performed for each buffer vial, to ensure reproducibility. The capillary was kept at 15°C. For ESI-MS^n a 75 % isopropanol in water sheat flow of 4 µL/min was used. UV detection was performed at 254 nm in a capillary window located 20 cm from the inlet. This wavelength was selected based upon the available filters in our laboratory in combination with the reasonable response of the APTS label. ESI MS^n was performed in negative mode, using a spray voltage of 1.9 kV and an ion transfer capillary temperature of 200°C.

Structural characterization of oligosaccharides by ESI-MS^N

MS^2 and MS^3 spectra were obtained using data dependent peak selection controlled by XCalibur 2.0 SR2 and LTQ Tune 2.2 Software (Thermo Electron, USA). Peaks were selected between 350 and 1200 m/z. A peak width of 4 was chosen to further fragment the 4 most abundant peaks in a MS^1 spectrum. Minimal counts were set to 100. The masses 352, 391, 451 and 461, were added to the mass exclusion list, since these signals were abundantly present in the eluent. The scanning time was set to 10 ms/scan for MS^1

spectra. MS^n spectra were recorded for 33 ms/scan and the results of 3 scans are summarized. Peaks were fragmented using a window of 2 m/z and relative collision energy of 30%.

Results and discussion

Influence of pH on the separation of pectin oligosaccharides with CE-LIF

Capillary electrophoresis (CE) with laser induced fluorescence (LIF) has been used for

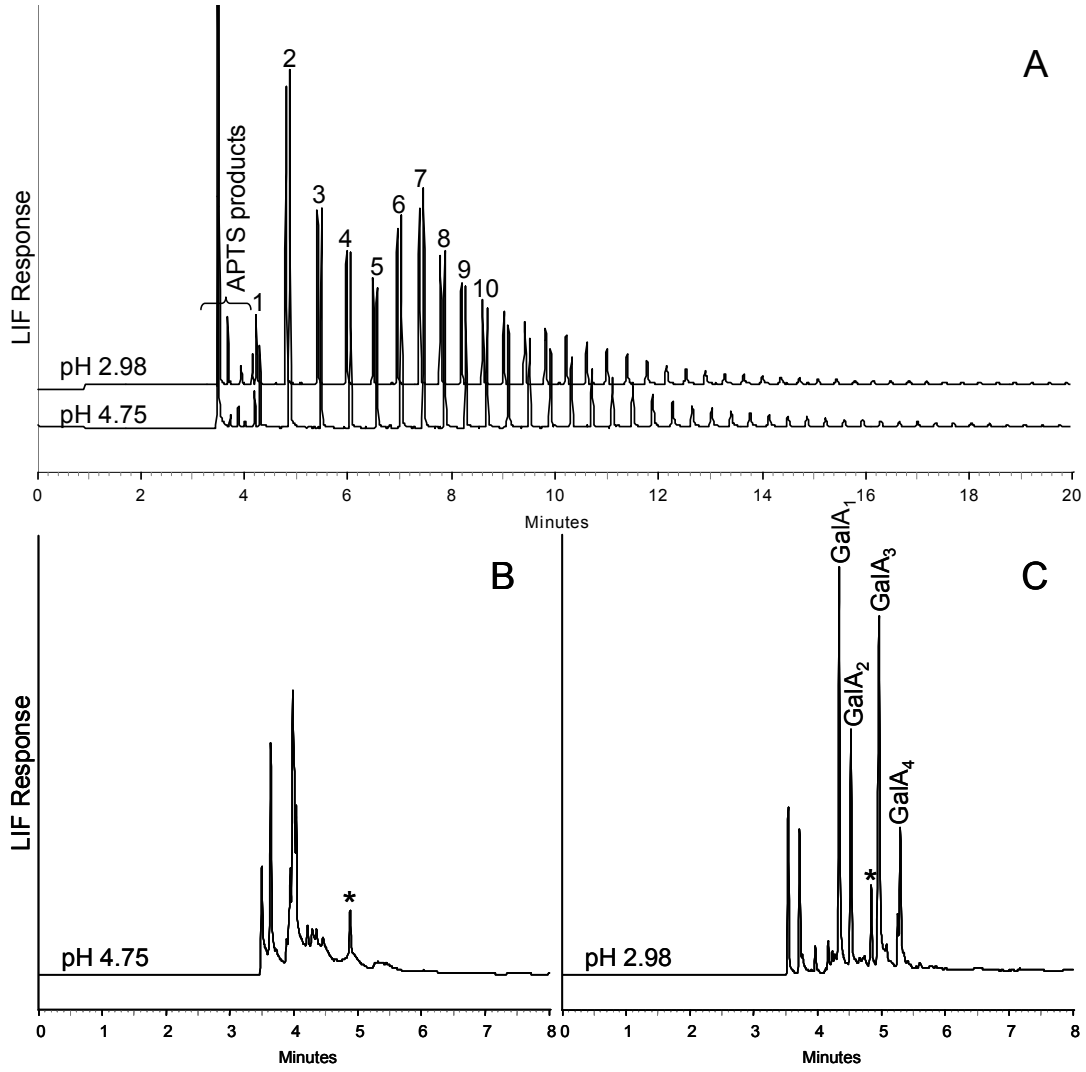


Figure 1: CE-LIF electropherograms of (A) APTS labeled maltodextrin oligosaccharides at pH 2.98 and pH 4.75; (B) APTS labeled PGA digest at pH 4.75, and (C) APTS labeled PGA digest at pH 2.98. * = maltose internal standard. Electropherograms were obtained with 25 mM sodium acetate buffer containing 0.4% polyethylene oxide and 0.3 % formic acid (pH 2.98) or with 25 mM sodium acetate buffer containing 0.4% polyethylene oxide (pH 4.75).

the study of labeled neutral sugar oligosaccharides (Chen *et al.*, 1998; Evangelista *et al.*, 1995; Hilz *et al.*, 2006; Kabel *et al.*, 2006; Oefner & Chiesa, 1994). Galacturonic acids have a pK_a of ~3.6 (Ralet *et al.*, 2001; Speiser *et al.*, 1945), therefore CE was performed at standard conditions of the Beckman CE kit (pH 4.75) and at conditions below the pK_a of GalA (pH 2.98), where the carboxyl group would be predominantly charged respectively mainly uncharged.

The pH of the buffer has only slight influence on the elution behavior of glucose oligosaccharides (figure 1a), as expected. The electropherograms of the PGA digest obtained at different pH values show clear differences. Using the buffer (pH 4.75) of the CE separation kit, the separation is poor (figure 1b) since all GalA oligosaccharides (DP 1-4) co-elute in one peak, just before the maltose internal standard. The migration time increased and peak separation improved when adjusting the buffer pH below the pK_a of galacturonic acid to 2.98 (figure 1c). Peaks eluting between 3.4 and 3.8 minutes are attributed to APTS reaction products as mentioned previously (Hilz *et al.*, 2006; Kabel *et al.*, 2006).

The effect of pH on the separation of neutral sugar oligosaccharides is negligible, which is attributed to the pK_a (~12-13) of the hydroxyl groups, and therefore remain uncharged in both buffers (Rendleman, 1973). Since maltodextrin oligosaccharides do not carry any charge except for the label and have a similar build-up, their separation should then be based on conformation and size, as was stated previously (Kabel *et al.*, 2006; Oefner & Chiesa, 1994).

The slightly different elution behavior of galacturonic acid oligosaccharides, when compared to glucose oligosaccharides of the same size (e.g. for glc_4 [6.0 min] and $GalA_4$ [5.3 min]) can be explained by the fact that at pH 2.98, still a small part of the carboxylic acid groups on galacturonic acid are dissociated (Ralet *et al.*, 2001; Speiser *et al.*, 1945). The average sum of these residual charges could explain the shorter migration times.

Separation of different complex pectin oligosaccharide mixtures by CE-LIF

Next to homologous series of oligosaccharides, complex pectin oligosaccharide mixtures, decorated by methyl esters or acetyl groups or composed out of heterogeneous sugar moieties, can be separated as well using CE. Three oligosaccharide mixtures originating from the pectin structural elements homogalacturonan, xylogalacturonan and rhamnogalacturonan I are analyzed on CE after labeling with APTS.

CE-LIF gives a good separation of the various GalA-containing oligosaccharides (figure 2). The relatively short analysis time of 10 minutes as compared to HPAEC (run time typically 35-60 min) together with the resolution as demonstrated in figure 2 underline the separating power. In every electropherogram the maltose internal standard

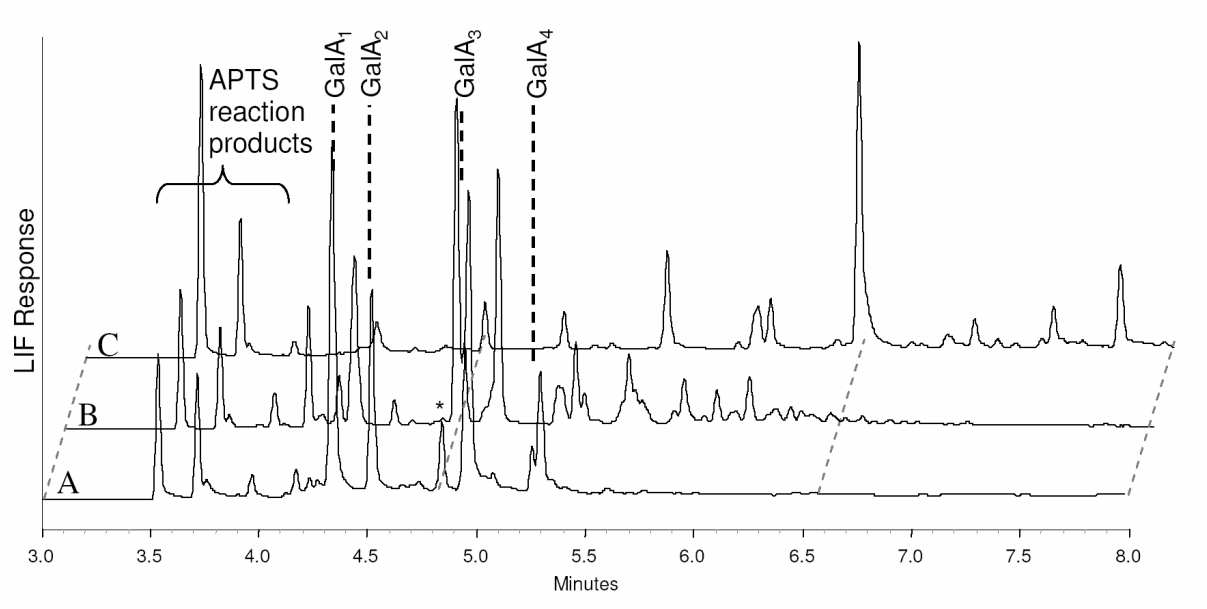


Figure 2: CE-LIF Electropherograms of APTS labeled (A) PGA digest, (B) XGA digest, and (C) RGI digest, obtained with 25 mM sodium acetate buffer containing 0.4% polyethylene oxide and 0.3 % formic acid (pH 2.98).

is eluting at 4.83 minute, emphasizing the stability of the CE system. Furthermore this maltose peak can be used for quantification, since all detected oligosaccharides are labeled with APTS in a 1:1 mol ratio. The efficiency of the labeling procedure is not dependent on DP or molecular identity, although the response could vary (<25%) depending on the sugar type (Chen *et al.*, 1998; Evangelista *et al.*, 1995).

EndoPG digestion of PGA yields primarily $GalA_1$ and $GalA_2$ as reaction products (Benen *et al.*, 1999), where $GalA_1Xyl_1$ and $GalA_2Xyl$ are the main degradation products after xylogalacturonan degradation by xylogalacturonan hydrolase (Zandleven *et al.*, 2005). Combining all this information, a tentative assignment as $GalA_1Xyl_1$ and $GalA_2Xyl_1$ can be made for the peaks at 4.8 and 5.0 min, taking into account their size and elution time relative to GalA oligosaccharides described in figure 1. It was not possible to assign RGI oligosaccharides due to structural differences and broad peak distribution compared to PGA and XGA oligosaccharides, resulting from the more heterogeneous starting material. The wide range of oligosaccharide distribution compared to the PGA and XGA digest, is attributed to the larger oligosaccharideic fragments after enzymatic degradation of RGI by RGH, compared to XGA degradation by XGH and PGA degradation with endo PG (Benen *et al.*, 1996; Mutter *et al.*, 1998a; Mutter *et al.*, 1998b; Pasculli *et al.*, 1991; Zandleven *et al.*, 2005). The smallest released degradation product is expected to be $GalA_2Rha_2$, possibly substituted with galactose(s) (Mutter *et al.*, 1998b).

Adapting capillary, buffer and detector to facilitate on-line MS peak annotation

It is not always possible to annotate peaks due to the absence of standards or insufficient knowledge of the samples (Lee, 1996; van der Hoeven *et al.*, 1992). To overcome this drawback CE is coupled to an iontrap MS, to enable on line peak annotation. The NCHO capillary is not commercially available at lengths necessary to perform a MS bridge, therefore a fused silica (FS) capillary is used. Furthermore an alternative buffer with volatile constituents is used, which is more compatible with the MS interface (Kabel *et al.*, 2006). Using the adapted set-up RGI digest was analyzed.

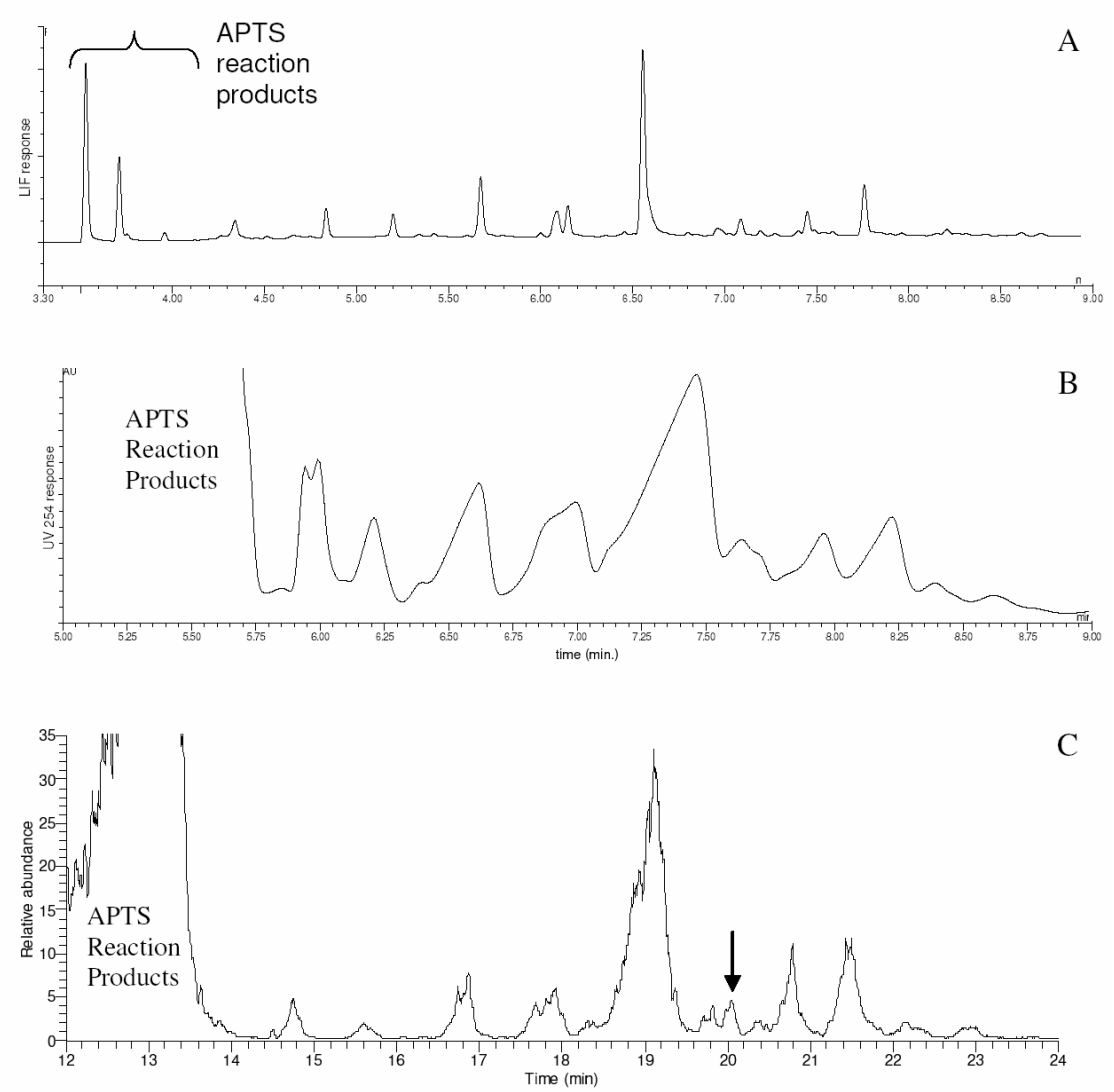


Figure 3: CE electropherograms of APTS labeled rhamnogalacturonan I digest with (A) LIF detection on a NCHO coated capillary, (B) UV254 detection on Fused Silica capillary, and (C) MS Base peak detection, on a Fused Silica capillary.

Comparing the LIF signal (figure 3a) to the mass spectrogram obtained with a fused silica capillary (fig 3c) the resolution decreased when coupling the CE to the iontrap MS, using an alternative capillary and buffer. The use of a fused silica capillary resulted in rather similar separations compared to the coated capillary when using the (acidified) buffer recommended by the supplier. However, lowering the pH and removing polyethylene oxide (giving unacceptable MS background signals) resulted in a decreased separation power. For our purposes, the separation was sufficiently maintained to detect a clear association between the peaks of the different electropherograms. Figure 3b represents the UV254 signal which was an assist in setting up the system, to confirm elution of components before analyzing the MS signal. Due to the MS bridge, the capillary length and consequently the elution time increased. The APTS reagent peaks give higher responses using UV254 detection with FS capillary compared to LIF detection with NCHO capillary. The FS capillary analysis time is comparable with the NCHO capillary, which is remarkable due to the factor 2 longer distance (40 cm LIF detection vs. 20 cm UV detection) between inlet and detection window. Apparently the use of a different capillary and/or buffer compensates for the increased capillary length, since voltage, current, and internal capillary diameter are similar in both cases.

On line MS detection of oligosaccharides eluting from CE

The base peak chromatogram (figure 3c) of the MS detector is used for comparison with the electropherogram. It is conventional to call the most abundant ion in each MS detector scan [Y-axis] versus time [X-axis] the base peak chromatogram (Anonymous, 1997). The MS spectra are used to mass annotate the corresponding peaks in the base peak chromatogram, and accordingly all peaks present in the LIF pattern. To demonstrate the sensitivity of our setup the small peak eluting at 20.15 minutes (~5 nmol oligosaccharide) indicated in figure 3c (7.1 min; figure 3a), is further characterized by MS. The MS^1 spectrum of this small peak (figure 4a) shows two abundant signals present at 528.33 and 792.33 m/z. These signals are coming from the same component and are caused by different charge states of the APTS label. The charge state (z) of a component generating a MS signal can be determined in two ways. Firstly, the distance between different isotopes equals 1/z (insert figure 4a), hence an isotope difference of 0.33 m/z corresponds to a z –value of 3. Secondly, the ratio between masses of different signals, which is ~1.5 between the z=2 and z=3 charge. Both approaches come to the same conclusion; the 528.33 m/z signal corresponds to z=3 and 792.33 m/z signal corresponds to z=2. All major components can be mass identified within a single run, as demonstrated by the peak annotations in the LIF chromatogram (figure 4b). For the peak at 4.83 and 5.20 min only the z=2 is given, since the z=3 signal falls outside the set mass detection range (350-1200

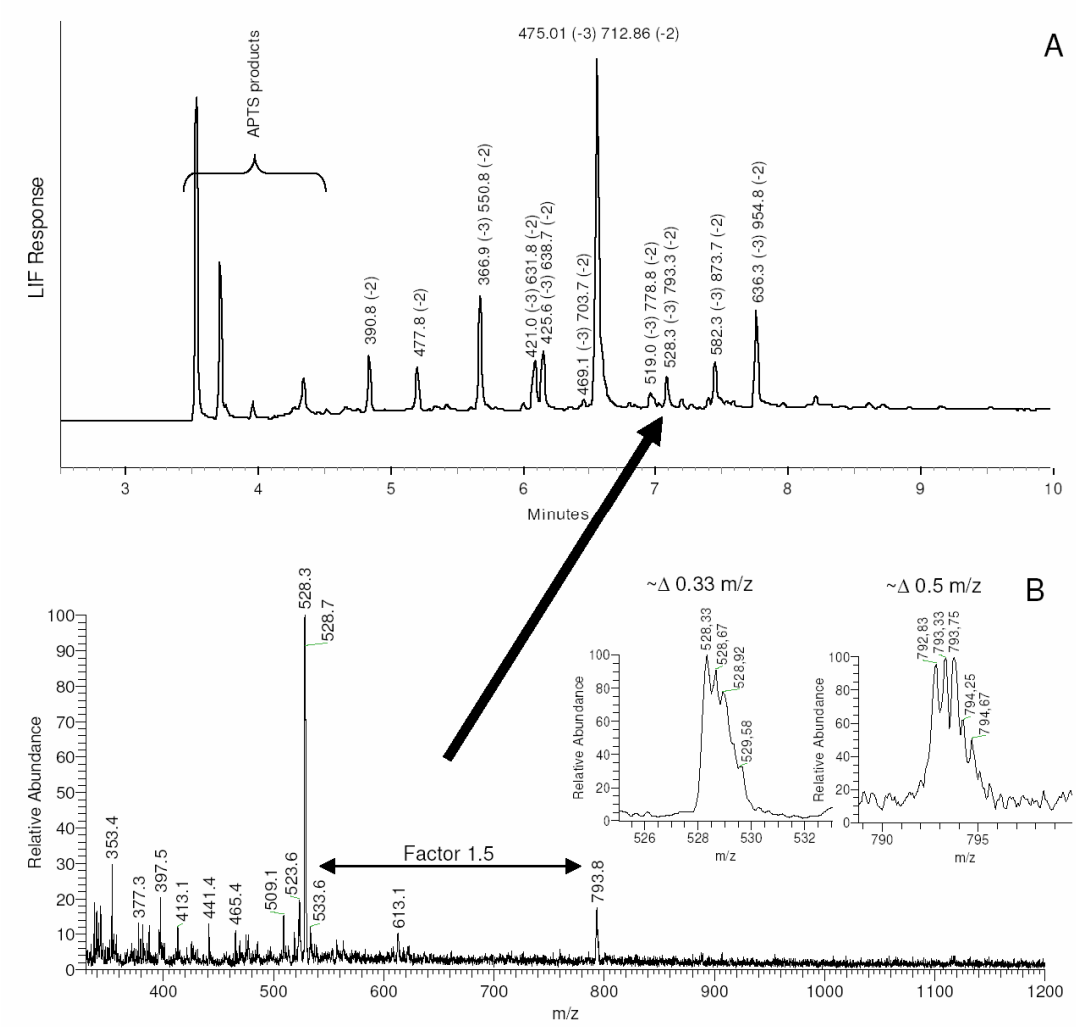


Figure 4: (A) Mass annotation of peaks in the CE electropherogram of the APTS labeled rhamnogalacturonan I digest enabled by coupling (B) the MS^1 spectrum of the CE-MS run to the corresponding LIF signal of the separation performed on the NCHO capillary. The insert in figure 4b represents a zoom of two peaks within the spectrum. This enables the annotation of (-2) and (-3) charge states to peaks constituting the same component.

m/z). The z=1 signal is not detected at all, which can by explained by the fact that aromatic sulfonic acids remain ionized at pH values as low as 2.5 (Oefner & Chiesa, 1994). CE indeed separates on the basis of size, which can be concluded from the increasing mass of all components, at later elution times, as was reported before (Mort & Chen, 1996; Oefner & Chiesa, 1994). The molecular weight of the oligosaccharide constituting the peak can then be calculated by the following formula.

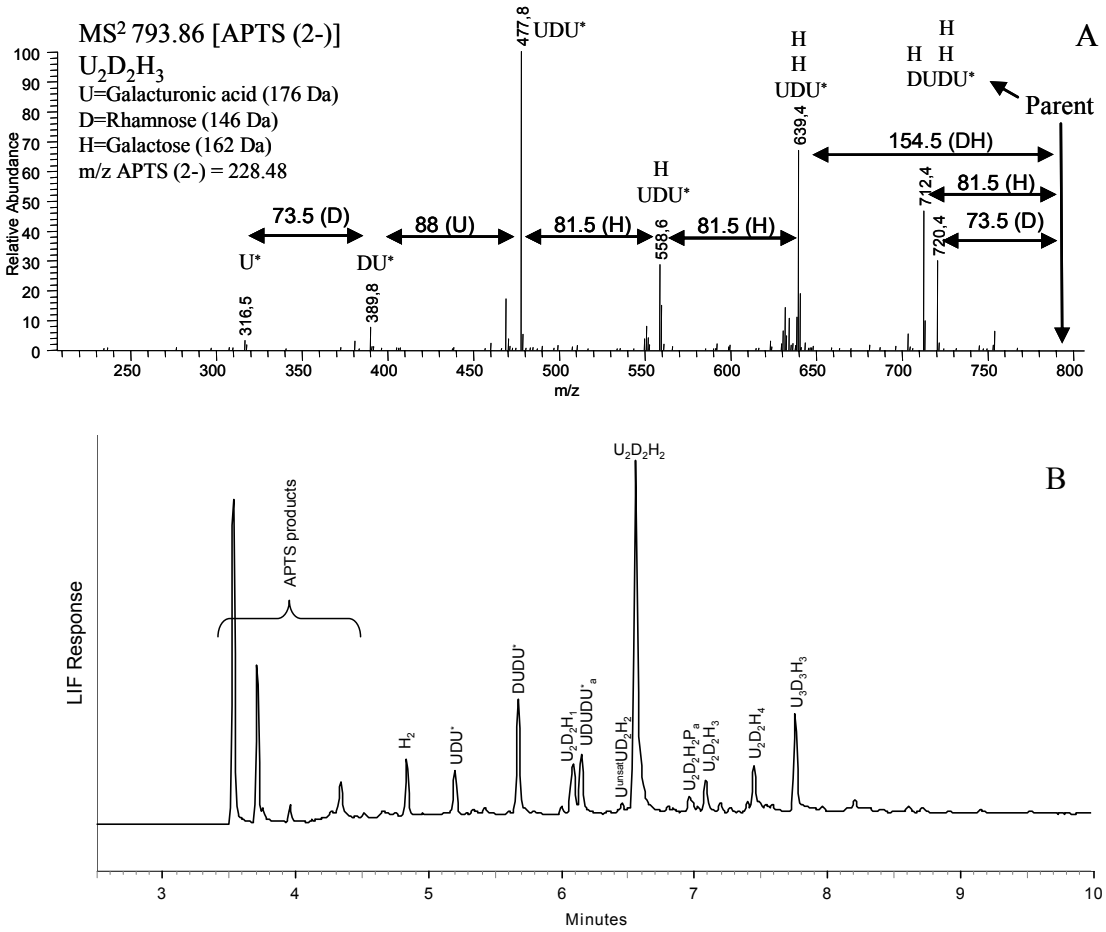
$$Mass\ oligosaccharide\ (Da) = [(m/z \times z) - (457.45 - z)] + 18$$

The 528.33 and 792.83 m/z signals in figure 4a correspond to an oligosaccharide mass of 1148.33 Da, which has 5 solutions ($H_2D_1P_5$; $U_1H_1P_6$; $U_2H_3D_2$; $U_3H_2D_1P_1$; $U_4H_1P_2$,

U=Uronic acid, D=deoxyhexose, H=Hexose and P=Pentose) for a structural annotation. These 5 solutions were composed taking into account that the sample was saponified (removing methoxyl, acetyl and feruloyl groups) and disregarding the possibility of having potassium adducts and unsaturated galacturonic acids.

Structural annotation of CE-LIF peaks with corresponding MS^n spectra

When having defined samples with specific treatments (for instance a maltodextrin ladder obtained by an amylase digest of amylose) MS^1 spectra are often sufficient to assign structural annotations to different peaks. When analyzing samples with unknown composition it is necessary to perform additional MS experiments to annotate peaks. The



[a] Annotation based on mass difference with neighbouring peak

Figure 5: Mass spectrum corresponding to the peak at 7.09 min. in the LIF chromatogram (19.8 min. in the base peak chromatogram; figure 3c) of the RGI digest; (A) MS2 of 792.9 m/z, enabling the annotation of the $GalA_2Rha_2Gal_3$ oligosaccharide (B) Mass annotation of all major peaks in LIF electropherogram of RGI digest.

data dependent ion mode of the MS detector allows generation of up to MS^3 spectra of different peaks, enabling the annotation of unknown sugar oligosaccharides.

The MS^2 spectrum (figure 5a) of the small peak eluting at 20.15 minutes in figure 3c leads to the assignment $GalA_2Rha_2Gal_3$. Because the separated mixture is the result of an RGH degradation of an RGI rich sample, the sugar moieties which build up this oligosaccharide can be identified based on their corresponding masses. The reducing end is carried by the galacturonic acid moiety in the 792.86 m/z APTS labeled oligosaccharide, which can be concluded from the 316 m/z signal (m/z $APTS^{2-}$ + m/z $GalA^{2-}$). The structure was constructed, by adding the different Δm/z to the U-APTS mass. The galactose was located as a rhamnose substitution, since rhamnosyl residues can carry neutral sugar substituents on *O*-4 within RGI (Lau *et al.*, 1987; McNeil *et al.*, 1980). RGH is tolerant for (residual) neutral sugar substituents attached to the rhamnose moiety within RGI (Mutter *et al.*, 1998b). The second galactose, derived from the mass difference between the 639.4 and 558.6 m/z signals, is attached to the other galactose sugar, since galacturonic acid moieties are commonly unsubstituted within RGI (Lau *et al.*, 1987; McNeil *et al.*, 1980). Finally the rhamnose-

galactose fragment is placed at the non-reducing end. The tentative structure $GalA_2Rha_2Gal_3$ is given for the oligosaccharide represented in figure 5a. The indication of a solitary rhamnose release provided by peak 720 m/z is not in agreement with the proposed structure and could indicate a mixture of components with mass/charge ratio of 792.3 m/z. Likewise signals like 477.8 m/z are difficult to agree with 639.4 m/z (both result from a single fragmentation step), giving again an indication for a mixture of $GalA_2Rha_2Gal_3$. Finally the 550.7 m/z signal corresponds with $GalA_2Rha_2$ fragment, indicating the loss of a Gal_3 side chain. The dimer and trimer galactose side chains were not previously described for apple pectin. However, these structures were previously described in bast fibre peels of developing flax (Gur'janov *et al.*, 2007). Due to the tentative nature of the location of branched structures, in the LIF pattern only the sugar composition is annotated. All oligosaccharides in the RGI digest, as identified by MS^2, contain GalA as the reducing end, which is in accordance with cleavage pattern of rhamnogalacturonan hydrolase (Mutter *et al.*, 1998b).

Separation and annotation of APTS labeled xylogalacturonan oligosaccharides, by CE-MS

To demonstrate the wide applicability of the CE-MS system for pectic structural oligosaccharides, a xylogalacturonan (XGA) digest was labeled by APTS, analyzed by CE-LIF, and peaks were annotated by CE-MS (figure 6).

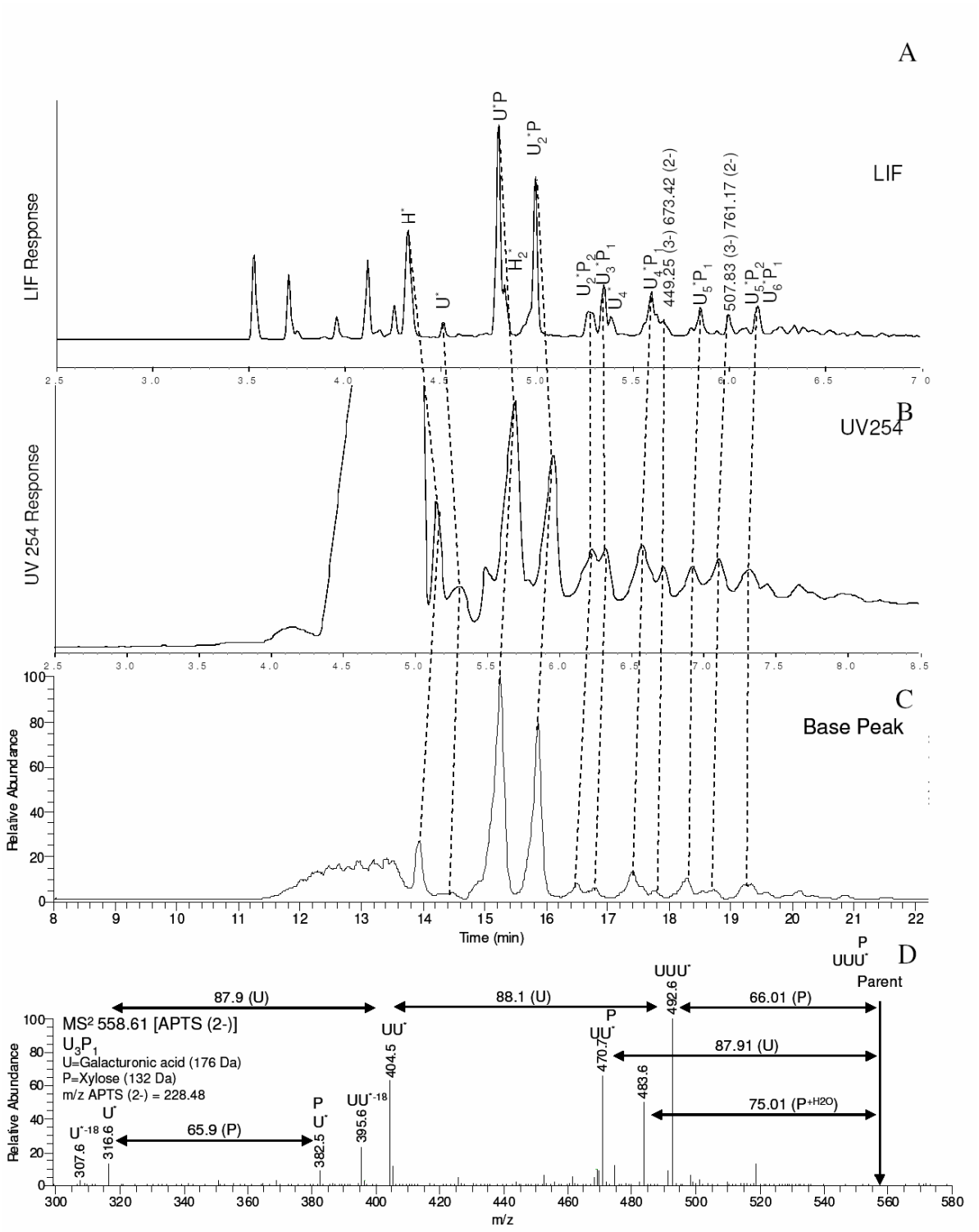


Figure 6: Structural characterization of APTS labeled xylogalacturonan digest by combination of the following analyses (A) CE-LIF on NCHO capillary, (B) UV254 on FS capillary, (C) Base peak detection mass spectrum, and (D) MS^2 spectrum generated by automated MS^n (Example: 558.6 m/z 16.79 min in figure 6c).

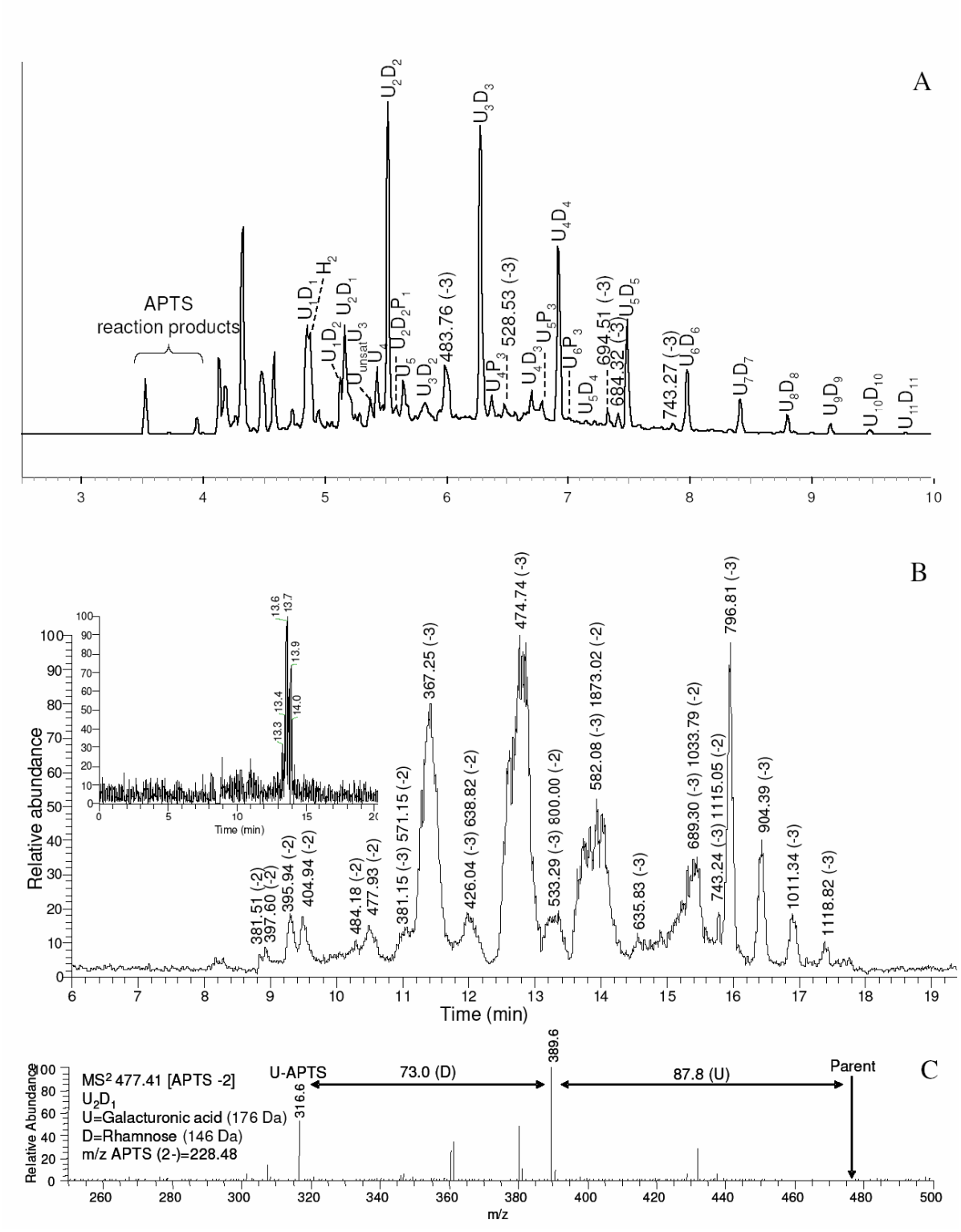


Figure 7: Mass annotated CE-LIF electropherogram of an acid hydrolysate of APTS labeled apple MHR (A) based on recorded base peak spectrum (B) and individual mass spectra (C). The example in figure 7c is based recorded at 10.45 min of figure 7b. The inlay in figure 7b illustrates the sensitivity of the MS, by locating the minor component U_5D_3 using a selected mass range.

The LIF signal of the XGA digest (figure 6a), correlates well with the UV254 signal (figure 6b), which was used as an assist in setting up the system. Both LIF and UV254 signals correspond to the base peak chromatogram (figure 6c), allowing mass peak annotation in the LIF chromatogram. Figure 6d exemplifies the annotation of the U_3P peak. The reducing end is carried by a galacturonic acid as deduced from the 316.6 m/z signal. From the consecutive Δ 88 m/z between signals 316.6, 404.5,and 493.6 m/z a $GalA_3$ fragment is identified. The xylose is substituted on the galacturonic acid, carrying the reducing end, as can be concluded from peak 382.5 m/z. This annotation is tentative, since peaks which would indicate xylose substitution to a galacturonic acid present in a fragment not containing the reducing end, would not be detected due to the absence of a charge (which is linked to the terminal uronic acid). Typical XGA degradation products are identified, for instance a single xylose substitution on galacturonic acid mono- until hexamers. In figure 6a the main products are $GalA_1Xyl_1$ and $GalA_2Xyl_1$ which are as well observed by Zandleven *et al*. (Zandleven *et al.*, 2005). All identified oligosaccharides have as well been described for an XGH digest of gum tragacanth (Zandleven *et al.*, 2005). The main difference between these results and the results of Zandleven *et al*. is the analytical system. The CE-LIF/MS was able to characterize this sample in 2 runs, where Zandleven *et al*. performed several laborious fractionation and desalting steps, before off-line MS analysis.

Characterization of an acid hydrolysate of Apple MHR

Enzyme digests of structural elements results in oligosaccharides with structural conformity, originating from the enzyme specificity. Acid hydrolysates potentially yield a wider diversity of oligosaccharides, since their break down is not limited by active sites and substrate accessibility. An acid hydrolysate of Apple MHR exemplifies the use of CE-MS for acid hydrolysates (figure 7).

Comparing the base peak signal with the LIF signal, there is a clear resemblance between the latter parts (base peak: 15.6-18 min and LIF: 7.9-9.5 min) of both electropherograms (figure 7). The resolution in the first half of the base peak signal is rather poor, since the sample concentration was over the top of the iontrap MS detection range. However, the advantage of the quantity injected was the largely improved recognition and identification of components eluting >16 min present at low levels. The peak masses of the base peak signal were translated to structural annotations by their corresponding MS^2 and MS^3 spectra. Figure 8c illustrates the structural annotation of 477.42 m/z [APTS 2-] to U_2D via the MS^2 spectrum of 477.41 m/z. The signal at 316.6 m/z recognizes uronic acid to be present at the reducing end. The Δ73.0 m/z corresponds with rhamnose and Δ87.8 m/z identifies galacturonic acid. The absence of a signal of

404.4 m/z, which would be caused by two linked galacturonic acid moieties, corresponds with the expectance of an alternating GalA-Rha-GalA sequence (Lau *et al.*, 1985; McNeil *et al.*, 1980; Schols & Voragen, 1994). LIF annotation could still be performed, with the elution times of maltose, $GalA_2Rha_2$ and $GalA_3Rha_3$, being used as verification points. There is a clear series of RGI oligosaccharides present in this sample, which were also recognized previously (Coenen *et al.*, 2007), as well as some (xylosidated) galacturonic acid oligosaccharides. Localization of specific oligosaccharides, which cannot be detected in the base peak signal, can be performed with the selected mass range chromatogram. As an example the $GalA_5Rha_3$ oligosaccharide is given, which is known to be present in low amounts in acid treated apple MHR (Coenen *et al.*, 2007). The insert in figure 7b clearly localizes this oligosaccharide, eluting simultaneously at 6.9 min (13.7 min base peak) with the U_4D_4 peak front.

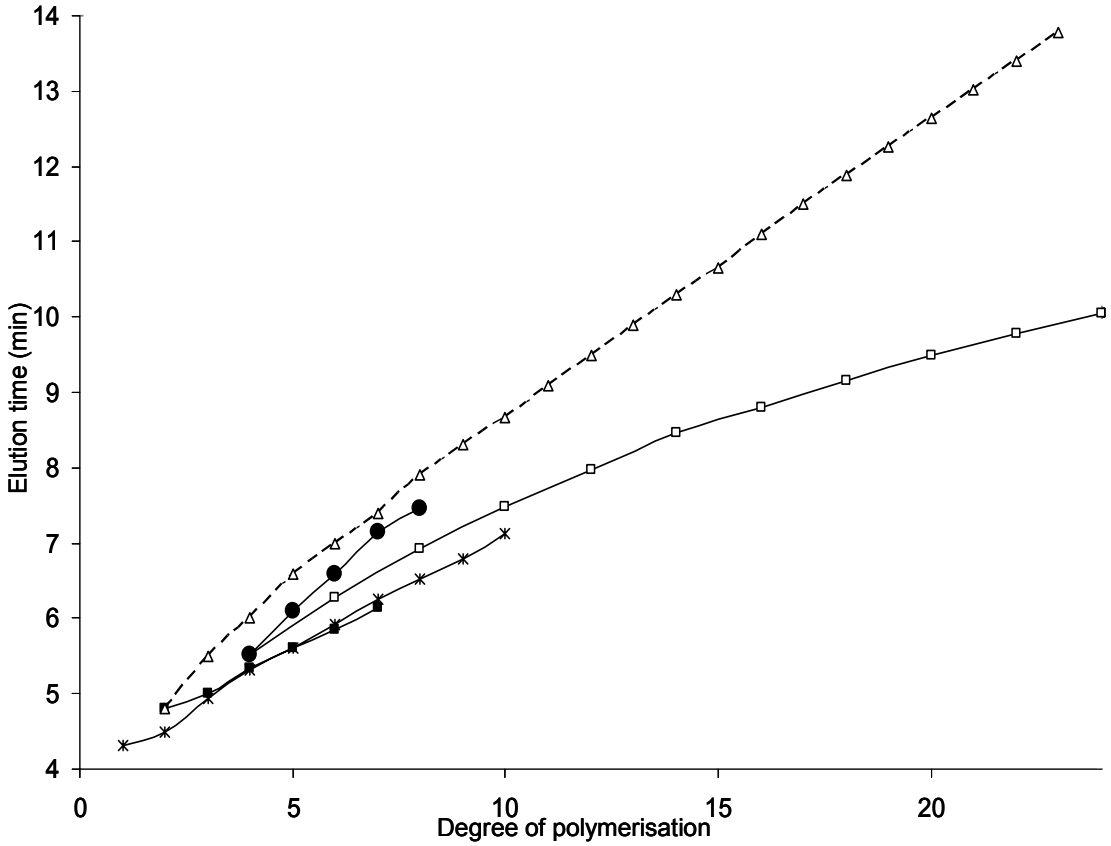


Figure 8: Migration behavior of APTS labelled oligosaccharides, with CE-LIF on NCHO coated capillary and acidified carbohydrate separation buffer (pH 2.98). * α -1,4 Galacturonic $acid_n$ ■ Galacturonic $acid_nXyl_1$ □ $(Rhamnose\text{-}Galacturonic\ Acid)_n$ ● $Rhamnose_2Galacturonic\ Acid_2\ Galactose_n$ Δ α 1-4 $Glucose_n$.

Elution behavior of APTS labeled oligosaccharides

To gain insight in the elution behavior of APTS labeled pectin oligosaccharides with CE-LIF, their elution times are plotted against their degree of polymerization (DP) in figure 8.

APTS labeled pectin derived oligosaccharides have apparent linear elution behavior between 0 to 8 minutes, while the elution time difference decreases slightly at higher DP. The extent of this decrease varies between oligosaccharide types, as can been derived from the different lines of maltodextrins (Δ) and RGI oligosaccharides (□;figure 8). Unknown samples are however not possible to annotate, due to the rather similar elution times of the different oligosaccharides, as shown in figure 8 at 5.5 minutes. At this elution time 4 different oligosaccharides migrate, of which three oligosaccharides have a different DP. Galacturonic acid oligosaccharides elute earlier than glucose oligosaccharides, which can be explained by the negative charge of the carboxyl group. Another explanation is the sterical conformation of galacturonic acid. It is interesting to observe that the $U_2D_2H_n$ series elutes later than the U_nD_n series. It could be that the negative charge of additional GalA (e.g. $U_2D_2H_2$ vs. U_3D_3) pulls the oligosaccharide faster to the anode. An alternative explanation is that the side chain orientation of the galactose(s) increases the hydrodynamic volume. The crossing of the α-1,4 Galacturonic $acid_n$ (*) and α-Galacturonic $acid_nXyl_1$ (■) series cannot be explained at this moment. It was expected that GalA oligosaccharides eluted faster for each DP, since they carry more negative charge and do not contain substituents, which would increase the hydrodynamic volume.

The overlapping of peaks together with the incomplete understanding of the CE separation, further underlines the necessity for on-line MS detection of unknown samples. Alternatively, the CE-MS coupling will lead to a better understanding of CE migration behavior of a wide range of (novel) compounds.

Concluding remarks

In conclusion this paper presents structural MS annotation of complex pectin derived oligosaccharides. This annotation was realized by adapting the buffer and capillary from a commercial CE analysis kit to enable on-line coupling between CE and iontrap MS. It was demonstrated that APTS labeled complex mixtures of sugar oligosaccharides could be separated and identified. Using this approach even new structures for apple pectin were identified, which will be the subject for further research. A large set of structural data was obtained in a single analysis in a relative short time compared to more standard HPAEC-MS methods, used in carbohydrate chemistry. In a following paper the buffer-, label and capillary effect on the elution behavior will be further investigated together with the possibilities of quantification introduced by the APTS label.

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Chapter 4

Release and characterization of single side chains of white cabbage pectin and their complement fixing activity

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Abstract

Single side chains from white cabbage pectin, ranging from degree of polymerization 1 to 30 were isolated by applying mild chemical conditions promoting β-elimination. The pectin fragments were characterized by their molecular weight distribution, sugar composition, and by ^{13}C NMR, and Maldi-tof MS analysis. These analysis revealed that large oligosaccharides resulting from β-eliminative treatment contained primarily arabinose and galactose, which had the structural features of -1,5 linked arabinosyl residues with 2 and 3 linked α-arabinosyl side groups and β-1,4 linked galactosyl groups. Fractions were measured for complement-fixing activity in order to determine their ability to trigger immune reactions. These tests strongly indicated that there was a minimal unit size responsible for the complement activation. Neutral chains (~8 kDa) obtained from β-elimination were inactive, despite the fact that they contained a sugar composition previously shown to be highly active. Larger chains (~17 kDa) isolated from β-eliminative treatment retained some activity, but much lower than polymers containing RGI from the same source reported earlier. This implied that structural elements containing multiple side chains expressed higher complement fixing activity.

Keywords

Pectin, white cabbage, *Brassica oleracea* var. *Capitata*; biological activity, complement-fixing activity, β-elimination.

Introduction

White cabbage is an important vegetable in Scandinavian households (Wennberg *et al.*, 2002), and it is used for nutrition as well as in traditional medicine to treat bedsores (Wicklund, 1996) and inflammation in skin and joints (Utting & Currall, 2003). The mechanism governing the wound healing property of plant material in not clear, but might be partly due to pectic substances triggering the immune system (Hart *et al.*, 1988; Samuelsen *et al.*, 1995).

Pectin is one of three main polysaccharide groups constituting the cell wall of dicotyledons next to cellulose and hemi-celluloses. It consists of a diverse set of structural elements. Homogalacturonan (HG) is mainly composed of partly methyl esterified stretches of α-D-1,4-linked Gal*p*A and has been suggested to consist of unique repeats of 80-120 α-D-Gal*p*A units (Yapo *et al.*, 2007). Additionally, the *O*-2 and/or *O*-3 position(s) of Gal*p*A may be acetylated to some degree (Perrone *et al.*, 2002). Some part of HG is named xylogalacturonan (XGA) (de Vries *et al.*, 1983; Schols *et al.*, 1995) since it contains monomeric β-D-Xyl*p* linked to *O*-3 of the Gal*p*A residue. Rhamnogalacturonan I (RGI) is referred to as regions with 30-40 repeats of Gal*p*A and Rha*p* pairs (Prade *et al.*, 1999; Yapo *et al.*, 2007; Zhan *et al.*, 1998) with varying numbers of Rha*p* residues (20-80%) branched with neutral side chains at *O*-4. These side chains consist mainly of Ara*f* and Gal*p* residues linked in various manners, constituting polymers known as arabinogalactan I (AGI) (Pérez *et al.*, 2000) and arabinogalactan II (AGII) (Darvill *et al.*, 1978). AGI is composed of a 1,4-linked β-D-Gal*p* backbone with *O*-3 substitutions of α-L-Ara*f* (Ridley *et al.*, 2001) and the Gal*p* backbone can have interspacing α-L-1,5-Ara*f* units (Huisman *et al.*, 2001). AGII consists of highly ramified galactan with predominantly interior 1,3-linked β-D-Gal*p* with substitutions of short 1,6-linked chains exteriorly. The latter has further attachments of 1,3- and/or 1,5-linked α-L-Ara*f* (Darvill *et al.*, 1978). Additionally, the highly complex, largely conserved oligosaccharide rhamnogalacturonan II (RGII) is present in minor amounts (Albersheim *et al.*, 1996). RGII exists as a 5 kDa monomer or a 10 kDa dimer where the units may cross link with a borate diester (Ishii *et al.*, 1999).

Pectin has the ability to activate the complement system (Michaelsen *et al.*, 2000), which plays an important role in human innate immunity and consists of more than 20 serum proteins, which take part in a cascade mechanism (Sakurai *et al.*, 1999; Yamada & Kiyohara, 2007). Protein ligand complexes, which bind cascade initiator complement proteins, interact in different manners thus activating complement through the classical, alternative (Zheng *et al.*, 2006), and the lectin pathway (Ikeda *et al.*, 2005). Pectin has been suggested to mainly activate the complement system via the classical pathway and

alternative pathway (Leung *et al.*, 2006; Michaelsen *et al.*, 2000) The classical activation pathway is initiated through the complement protein 1 subunit q (C1q), which has the shape of a bouquet of flowers with six globular heads with a range of ligand specificities (Gaboriaud *et al.*, 2003).

It has been suggested that the structure responsible for complement-fixing activity contains 1,3- and 1,3,6-linked galactans (Kiyohara *et al.*, 1989; Sakurai *et al.*, 1999). The smallest carbohydrate reported to activate the complement system to our knowledge is an arabinogalactan with an estimated size of 5 kDa (Sakurai *et al.*, 1999). Since it is unclear which specific polysaccharide structure is responsible for activating the complement system, research is needed to characterize the specific architecture of the pectic neutral sugar side chains.

However, it has proven difficult to isolate single side chains from pectin, both by applying enzymatic and chemical approaches. The use of enzymes has been preferred to tailor the pectin structure, due to their ability to specifically degrade pectin at mild conditions. Structure analysis based on enzyme treatment has in many cases proven difficult due to structural variations limiting enzyme susceptibility. For instance, enzymes degrading RGI backbone structures only release oligosaccharides containing short side chains or none at all (Schols *et al.*, 1994) implying the inability to degrade RGI structures with longer side chains. Even after treating pectin with a mix of enzymes, enzyme-resistant material remains (Schols & Voragen, 1994).

Chemical treatment of pectin with the purpose to degrade the rhamnogalacturonan backbone structure has been of great interest in order to isolate single side chains. In this way, a more thorough structure annotation could be explored. The HG and RG backbone have successfully been degraded by the use of lithium in ethylenediamine to specifically cleave internal α-D-Gal*p*A linkages (Mort & Bauer, 1982), regardless whether they are methyl esterified or not (Lau *et al.*, 1987). This method, however, results in undesired side chain deterioration in addition to the intended backbone cleavage. Recently, a method has been described utilizing sodium tetraborate at neutral pH and elevated temperature, which induces efficient β-elimination of pre-methyl esterified pectic samples (Deng *et al.*, 2006). However, the authors did not report whether the method could be used for the release of longer more complex side chains, which are common in native pectins. This would be of interest for structure elucidation, as well to identify functional properties of such unique structures.

This study exploits chemical treatments in order to obtain fractions containing single side chains from RGI with complement-fixing activity. To our knowledge, this is the first time that the potential complement activity of neutral sugar side chains without a RGI backbone has been examined.

Materials and Methods

Isolation of 'prep scale' pectin from white cabbage (*Brassica oleracea*)

White cabbage (*Brassica oleracea* var. Capitata, Bartolo cultivar) was cultivated at the Vollebekk testfield (Aas, Akershus-Norway), and kept one week post harvest at 0°C. 200 kg of cabbage was de-stemmed and cut in 3-5 mm slices, immediately put into boiling water and kept for 3 hours with continuous stirring. The treatment was set up to obtain a pectic product which would resemble the pectin content of blanching water used in vegetable processing. The resulting 200 L liquid 'broth' was poured into 20 L buckets and cooled to 50°C. Efficient removal of protein was obtained by adding Neutrase (0.1 g/L; Novozymes, Bagsvaerd, Denmark) to the cabbage broth. The protease efficiency was followed by applying samples to SDS-PAGE gels followed by subsequent silver staining (Blum *et al.*, 1987). The Neutrase did not show any glycosyl hydrolase side activities. Following the Neutrase treatment, plant material solids were removed from the broth by sieving. Low molecular weight sugars and color pigments were removed by ultra-filtration (cut-off 10 kDa, Abcor membranes, Koch, USA), followed by volume reduction by vacuum evaporation (end volume 8 L). Samples were ultra-filtered against MilliQ water with Pellicon 2B 10kDa cutoff tangential flow membranes (Millipore). Ultrafiltration was stopped when the conductivity of the permeate was ≤ 1 μS. The concentrated solution was filtered (GF/C and F with Celite, Whatman, Kent, England) as filter aid, prior to overnight polysaccharide precipitation at 4°C in 60% isopropanol. The precipitation was followed by two subsequent steps of washing with 60% ethanol, and finally one step of washing with pure ethanol. Centrifugation (15 min. at 4000 x g) after each step pelletized the pectic material. Ethanol washed material was dried at 40°C and the resulting 280 gram *Brassica* pectic material (BP) was finely grinded with a blender.

Selective chemical degradation of RGI

BP (5 g) was first methyl esterified by treatment of tetrabutylammonium fluoride (TBAF, 10 g) and iodomethane (MeI, 5 mL) in wet DMSO (8% water) at 25°C. The reaction mixture was poured into three volumes of cold water (0 °C) and centrifuged (4500 x g) to remove iodine. The supernatant was desalted by ultrafiltration (cut-off 10 kDa), against MilliQ water which was subsequently exchanged by 0.2 M sodium borate buffer pH 7.3 (0.2 M boric acid pH adjusted by 50 mM sodium tetraborate) to a final sample concentration of 5 mg/mL. β-elimination was carried out in sealed tubes at 125°C for 2.5 hours and the reaction was terminated by immersing the sample containers in cold water. The reaction mixture from β-eliminative treatment was desalted on a Sephadex G25 column (26x100mm, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) with 4

mL/min MilliQ as eluent coupled to a RID10A refractive index detector (Shimadzu , Tokyo, Japan) and a SuperFrac (GE Healthcare Bio-Sciences AB). Samples (~ 200 mg) were applied with a 50 mL superloop (GE Healthcare Bio-Sciences AB). This is an adapted method according to the Deng protocol (Deng et al., 2006).

Fractionating neutral sugar side chains

A 5 mg/mL solution of desalted material was separated by Q-Sepharose (GE Healthcare Bio-Sciences AB). HiLoadTM Q Sepharose 26/10 fast flow was operated at 5 mL/min by a BioLC system (Dionex, Sunnyvale, USA) with post column addition of 1M NaOH (1 mL/min). The eluent was split using a custom made 1/20 splitter, before pulsed amperometric detection (PAD). Gradient used: water MilliQ quality (A), 1 M NaCl (B); 0-10 min 100% A, 10-20 min an exponential gradient with eluent 0-100 % B; 20-25 min 100 % B; 25-30 min 100-0 % B, and 30-35 min 0-100 % A. The eluent was fractionated into two fractions (BP-neutral and BP-charged; figure 1).

Preparative size exclusion chromatography of the BP-neutral fraction

BP-neutral was remethoxylated (RME) and β-elimination was carried out a second time as described above, followed by desalting/separation on Superdex 30 columns (GE Healthcare Bio-Sciences AB). Preparative SEC was conducted with 4 mL/min 100 mM ammonium acetate eluent to a Sephacryl 400 column (5x100cm, GE Healthcare) coupled to a Shimadzu RID10A refractive index detector and a SuperFrac (GE Healthcare). Samples (1 g) were applied with a 50 mL superloop (GE Healthcare). Two fractions were collected, one of high molecular weight (BP-neutral-A) and one of lower molecular weight (BP-neutral-B).

Analytical size exclusion chromatography (SEC)

BP, BP-charged, BP-neutral-A, and BP-neutral-B were subjected to high performance size exclusion chromatography (HPSEC) with three PL Aquagel-OH 40-50-60 columns (Polymer Laboratories, Shropshire, UK) coupled in series and eluted at 40°C with 50 mM Na_2SO_4 (0.8 mL/min). Detection was carried out with a Shimadzu RID6A refractive index detector. Pullulans from Polymer Laboratories LTD were used as standards. Weight-average molar mass (M_w) was calculated by the WinGPC software package (PSS, Polymer Standards Service).

Monosaccharide composition

Methanolysis and GC analysis was conducted by a modification of the method of Chambers and Clamp (1971) as described by Samuelsen *et al*. (1995). Polysaccharides

were methanolyzed in 3 M methanolic HCl (Supelco) at 80°C for 24 hours, dried under N2 and derivatized with TMS. TMS-derivatives were separated on a DB-5 fused silica capillary column (30m x 0.32 mm i.d; J&W Scientific, Folsom, USA). Samples were analyzed in triplicates, manitol was used as internal standard.

^{13}C-NMR

Samples were prepared according to Westereng *et al.* (2006). ^{13}C-NMR spectra were recorded on a Varian 300 MHz instrument (spectrometer frequency 75.44 MHz) at 40°C applying 2 sec pulse delay, 1.64 sec acquisition time, 85° pulse angle, a sweep width of 15974 Hz, and collection of approximately 60000 data points. Chemical shifts are given relative to TMS (δ 0) via DMSO δ 39.6).

Sugar linkage analysis

Prior to linkage analysis a reduction step was performed twice according to the method described by Kim and Carpita (1992), including sodium borodeuteride to discriminate between Gal*p* and Gal*p*A with MS. The samples were methylated corresponding to the method of McConville and coworkers (1990) and further hydrolyzed by 2.5 M TFA for two hours at 100°C. The samples were reduced with sodium borodeuteride prior to acetylation. The partially methylated alditolacetates were extracted with dichloromethane, dried, dissolved in dry methanol and identified by GC-MS with a Varian Factor Four VF column (30 m x 0.25 mm i.d.) and flame ionization detection. Quantitative results in the linkage analysis were based on the relative distribution of the differently linked monomers. The distribution of each monomer from methanolysis was subsequently used to give the total amount of each monomer linkage. The diagnostic fragments; terminal-Gal*p*/Gal*p*A (205/207 fragments) and (1,4-Gal*p*/Gal*p*A 233/235 fragments) were used to discriminate between Gal*p* and Gal*p*A which both are represented in the Galactose peaks.

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (Maldi-tof MS) analyses of oligosaccharides

Two μL of a 9 mg/mL mixture of 2,5-dihydroxy-benzoic acid (DHB; Bruker Daltonics, Bremen, Germany) in 30% acetonitrile was applied to a MTP 384 target plate ground steel T F (Bruker Daltonics). 1 μL sample (0.1 mg/mL) was then mixed into the DHB droplet and dried under a stream of air (Verhoef *et al.*, 2005). The samples were analyzed with an Ultraflex instrument (Bruker Daltonics) with a Nitrogen 337 nm laser beam operated in positive acquisition mode. The data were collected from averaging 250 laser shots, with the lowest laser energy necessary to obtain sufficient spectra intensity. 10 μL of a 10 mg/mL solution of BP-charged, BP-neutral-A, and BP-neutral-B were added to a small

spatula of Dowex 50 particles (H+-form), mixed, and centrifuged (13000x *g*, 5 min). Additionally, one part of each fraction (10 µL) was enriched with 1 µL 20 mM NaCl prior to spotting on the MALDI plate to generate primarily sodium adducts.

Complement-fixing activity assay

Samples were subjected to an assay, which determined their ability to interfere with the complement system as described by Michaelsen *et al.* (2000). Human serum, stored in aliquots at -70 oC was used as complement source. For the analysis, a dilution of human serum was chosen, which resulted in 50% lysis of a 1% suspension of sheep erythrocytes sensitized with rabbit anti-sheep antibodies (Hemolysin, Virion Ltd, Ruschlikon, Switzerland). Human serum was incubated with polysaccharide samples, which either activate or inhibit complement proteins to various degrees. In both situations less complement activity remains to lyse the added sensitized sheep erythrocytes. The resulting lysis was measured by absorbance at 405 nm. The assay was run in an isotonic buffer system (veronal buffer) containing 0.2 mM Ca^{2}+ and 0.8 mM Mg2+ required for complement activation via the classical pathway. PMII, a well characterized pectin fraction derived from *Plantago major* L (Samuelsen *et al.*, 1996) was used as a positive control. Samples were run in triplicate. The activity was measured as ICH50: the lowest concentration showing 50 % inhibition of hemolysis in the test system (Michaelsen *et al.*, 2000). All samples were standardized based on the amounts of sugars obtained by sugar composition analysis. Diagrams were obtained by the use of Sigmaplot 2001 software package.

Results and discussion

To get a more detailed picture of the structural properties of *Brassica* pectin (BP) and its side chains it was attempted to chemically release single side chains, by means of a selective β-eliminative treatment. The glycosyl residue sequence of the fragmented side chains was studied by various analytical techniques. Since differently treated samples are discussed throughout this manuscript, figure 1 gives a schematic overview of the samples and their preparation.

BP was degraded according to the method of Deng *et al.* (2006) and the reaction products were applied to ion-exchange chromatography (IEC) resulting in one neutral (BP-neutral) and one charged (BP-charged) fraction (figure 1). Since the neutral fraction presumably contained both intact RGI connected to the neutral sugar side chains of interest, BP-neutral was retreated with the β-eliminative treatment, and fractionated by

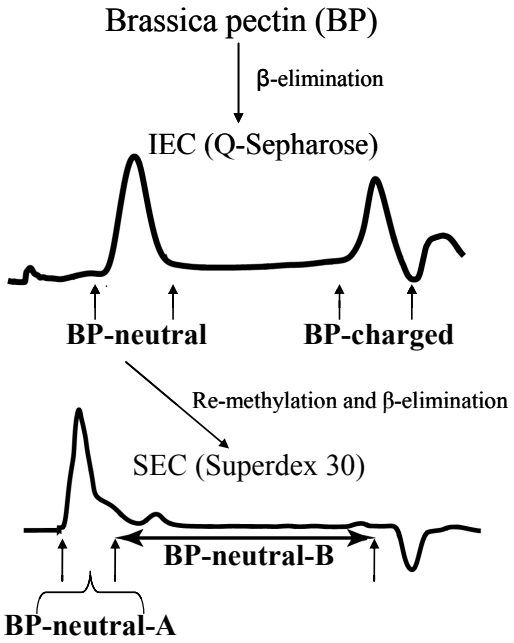

Figure 1: Flowchart describing the preparation of white cabbage pectin fractions, obtained after selective chemical degradation of *Brassica* pectin (BP; BP-charged, BP-neutral-A and BP-neutral-B).

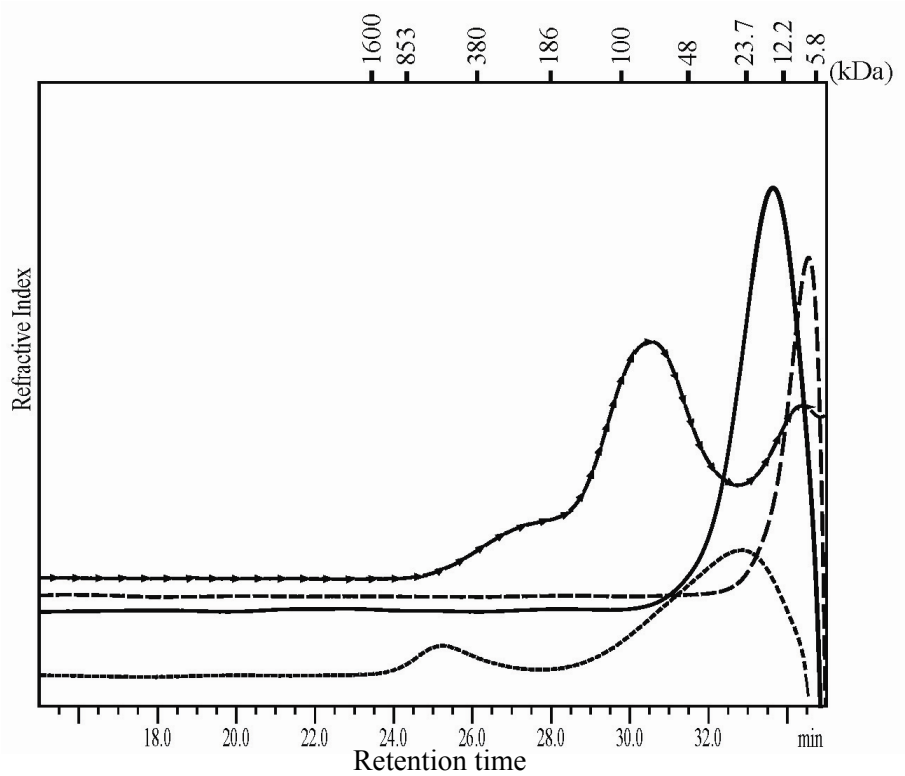

Figure 2: HPSEC molecular weight distribution profiles of BP (▪▪▪▪), BP-neutral-A (——), BP-neutral-B (— —), and BP-charged (→→).

size exclusion chromatography (SEC) producing two fractions containing large oligosaccharides: BP-neutral-A, and BP-neutral-B.

Molecular weight distribution of *Brassica* pectin and its β-eliminative degradation products

To get insight in the molecular weight of the obtained samples, these are further characterized by size exclusion chromatography (SEC; figure 2)

The *Brassica* pectin starting material was relatively polydisperse (figure 2) containing two well separated molecular fractions with one high M_w average (630 kDa) and one low M_w average (40 kDa). The late eluting peak observed contained putatively fragments of the high molecular weight population as a result of β-elimination during the extraction process (3 hours, 100°C) (Westereng *et al.*, 2007). The low M_w average determined for BP-neutral-A (~17 kDa) and BP-neutral-B (~8 kDa) indicated that long side chains were released from the RG-backbone through the β-eliminative treatment, since 17 kDa would correspond with ~130 arabinose or ~100 galactose residues.

Sugar composition of *Brassica* pectin and its β-eliminative degradation products

To determine the nature of the isolated polymer fractions of the different BP samples, the sugar composition was determined (table 1).

Both neutral fractions contained small, but clearly detectable amounts of Rha*p* and Gal*p*A, putatively originating from degraded RGI backbone. The high arabinose content, observed in all fractions, indicated that the major neutral sugar (NS) substitutions in RGI of white cabbage pectin are arabinan side chains. However, a large amount of galactose was observed as well in the BP-charged and BP-neutral-B fractions, which was probably present in galactan side chains. The lower galacturonic acid content of all chemically treated fractions in comparison to the BP pectin, was explained by the desalting step, removing Gal*p*A oligosaccharides formed during the β-elimination reaction. The IEC

Table 1: Sugar composition (mol %) of *Brassica* pectin *(*BP) and preparative scale fractions obtained from BP after β-eliminative treatment(s).

	Ara	Rha	Fuc	Xyl	Gal	Glc	GalA	M_w(kDa)
BP	25	9	≤1	3	12	≤1	51	630+40
BP-charged	39	11	≤1	3	18	≤1	29	90
BP-neutral-A	87	≤1	nd	nd	7	nd	6	17
BP-neutral-B	70	3	nd	nd	22	nd	6	8

nd=not detected

separation step was successful in separating the NS side chains from the charged oligosaccharides, as could be concluded from the high arabinose and galactose content in the neutral fractions in relation to the Gal*p*A content in the charged fraction.

Characterization the neutral sugar side chains in *Brassica* pectin by ^{13}C-NMR experiments

The elucidation of the overall pectin structure was obtained by ^{13}C-NMR experiments. The ^{13}C NMR spectra (figure 3) revealed that treatment of BP caused large structural changes and that the samples contained a variety of pectin structural elements. Anomer signals for backbone residues of α-L-Rha*p* (98.3-99.1 ppm), α-D-Gal*p*A (99.8-100.7 ppm) and putative side chain elements of α-L-Ara*f* (107.0-108.1 ppm) (Dong & Fang, 2001; Fry *et al.*, 1993) and β-D-Gal*p* (103.2-105.0 ppm) (Cartier *et al.*, 1987) were identified. The ^{13}C NMR spectra of the BP-neutral fractions revealed that side chains had a predominant Ara*f* part with a smaller degree of Gal*p* as seen from the anomeric signals, which corresponds to the sugar composition analysis (table 1). This indicated that arabinan was the main side chain polymer in both neutral BP fractions. BP-charged contained RGI rich regions with arabinan and galactan side chains, which were of a much more intricate nature than BP-neutral-A and BP-neutral-B, as can be inferred from the more complex NMR spectrum. The presence of low levels of Gal*p* indicated that in BP-neutral-B also AGI side chains polymers were present. Additional analysis of the NMR data revealed two methyl carbon peaks at 17.3 and 17.5 ppm (figure 3) representing *C*-6 of α-L-1,2-Rha*p* and α-L-1,2,4-linked Rha*p*, respectively. The ratio between these methyl carbon peaks in BP indicated that mainly unsubstituted Rha*p* was present in BP, where in BP-charged ~ 40% of the rhamnose residues seems to be branched.

Characterising *Brassica* pectin arabinogalactan by sugar linkage analysis

The sugar linkage analysis (data not shown) was more qualitative then quantitative, but clearly revealed that the overall structure of the BP material consisted of 1,4- and some 1,3,4-linked Gal*p*A with the interspacing α-L-Rha*p* having 1,2- and 1,2,4-linkages within the backbone structures. The side chain elements consisted predominantly of α-L-Ara*f* and β-D-Gal*p*. Most α-L-Ara*f* were terminally- and 1,5-linked with lesser amounts of 1,2,5- and 1,3,5- branching points, whereas β-D-Gal*p* was present as t- and 1,4- linked residues in addition to minor amounts of 1,3-, 1,6- and 1,3,6-linked residues. The relative high amount of t-ara, together with substantial amounts of 1,2,5- and 1,3,5- branching points indicates an highly branched arabinan structure. The sugar linkage composition demonstrates that (highly) branched arabinan is the main side chain polymer in both neutral BP fractions. Additionally in BP-neutral-B also arabinogalactan I side chains

polymers are present. Linkages indicative for arabinogalactan II are only present in trace levels.

Determination of the individual chain composition

The individual chain composition of the BP-neutral-A and BP-neutral-B side chains was investigated by Maldi-tof MS.

From the mass spectrum, homologous series of oligosaccharides were recognized. The ion at *m/z* 683 was interpreted to be the sodium adduct of uGal*p*A-Rha*p*-Gal*p*A (uGal*p*A

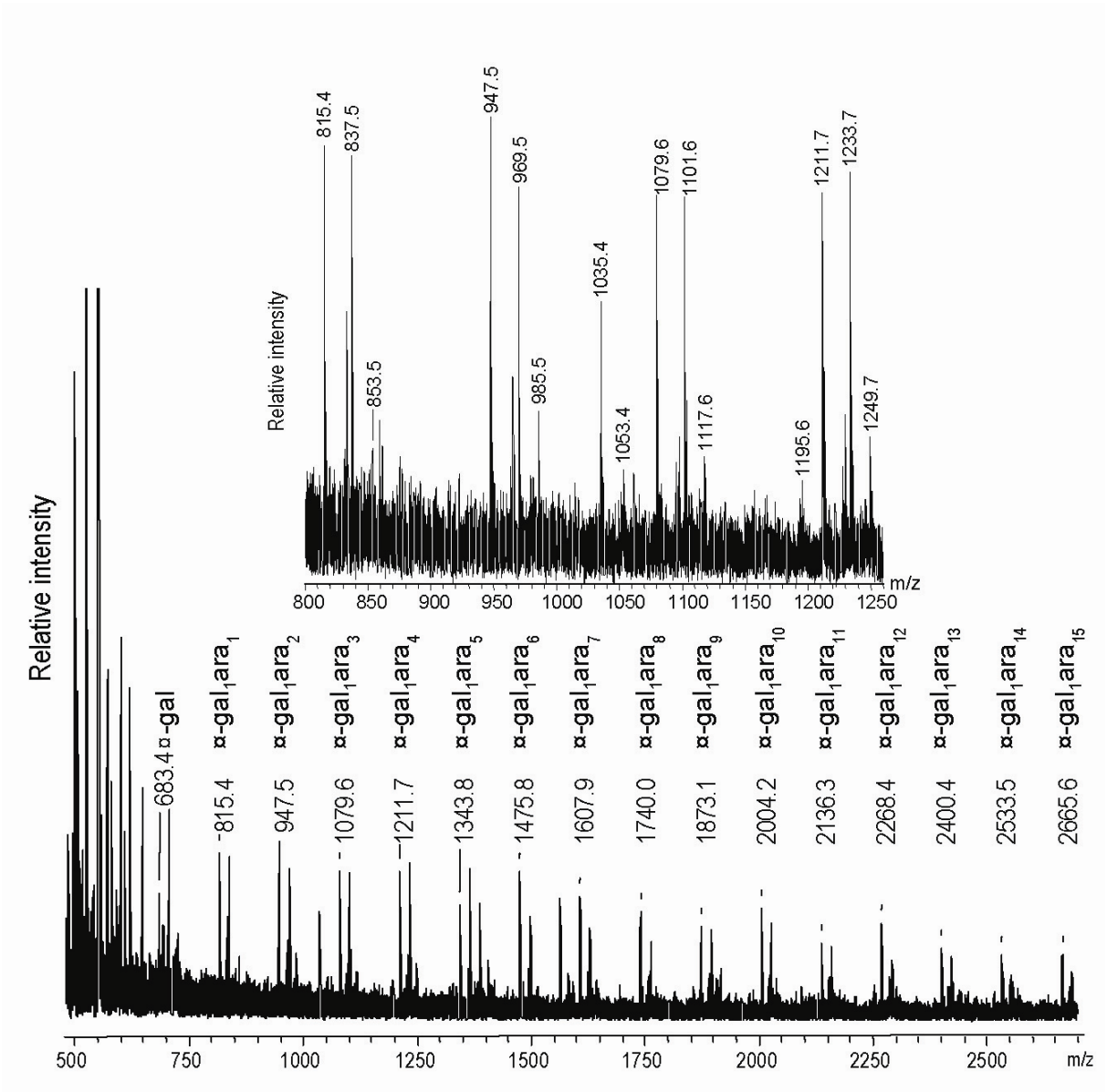

Figure 4: Maldi-tof mass spectra of BP-neutral-A with a zoom view of the 800-1250 *m/z* windows (¤ = uGalA-Rha-GalA^{Na+}). Based on the sugar composition, putative sugars were annotated.

= unsaturated Gal*p*A), with Gal*p* substituted Rha*p* (denoted as ¤-gal in figure 4). As shown in the inserted zoomed spectrum, the peak clusters were resulting from sodium (+22 m/z) and potassium (+38 m/z) salts of the sodium adduct. The 837 (815+22) m/z is caused by the presence of a sodium salt of a GalA within the sodium adduct, where the 853.3 (815+38) m/z peak is produced by the potassium salt within the sodium adduct (Coenen *et al.*, 2007). Furthermore, the peak at 1035.4 m/z corresponded to a Gal_1Ara_3 side chain segment with an uGal*p*A-Rha*p* backbone fragment.

The Maldi-tof MS analysis of BP-neutral-A (figure 4) suggested that the sample contained a pool of short backbone parts with attachments of single side chains indicated by the homologues series of signals at *m/z* 683, 815, and 947; subsequent additions of 132 Da up to 4800 *m/z* (not shown) corresponded to a backbone fragment (*m/z* 683) with (Ara*f*)n additions (n = 1-30). The predominance of pentose was in accordance with the sugar composition analysis (table 1) and the NMR spectra (figure 3).

The identified oligosaccharides containing arabinan side chains of varying length support the hypothesis that large oligosaccharide side chains could be isolated after β-

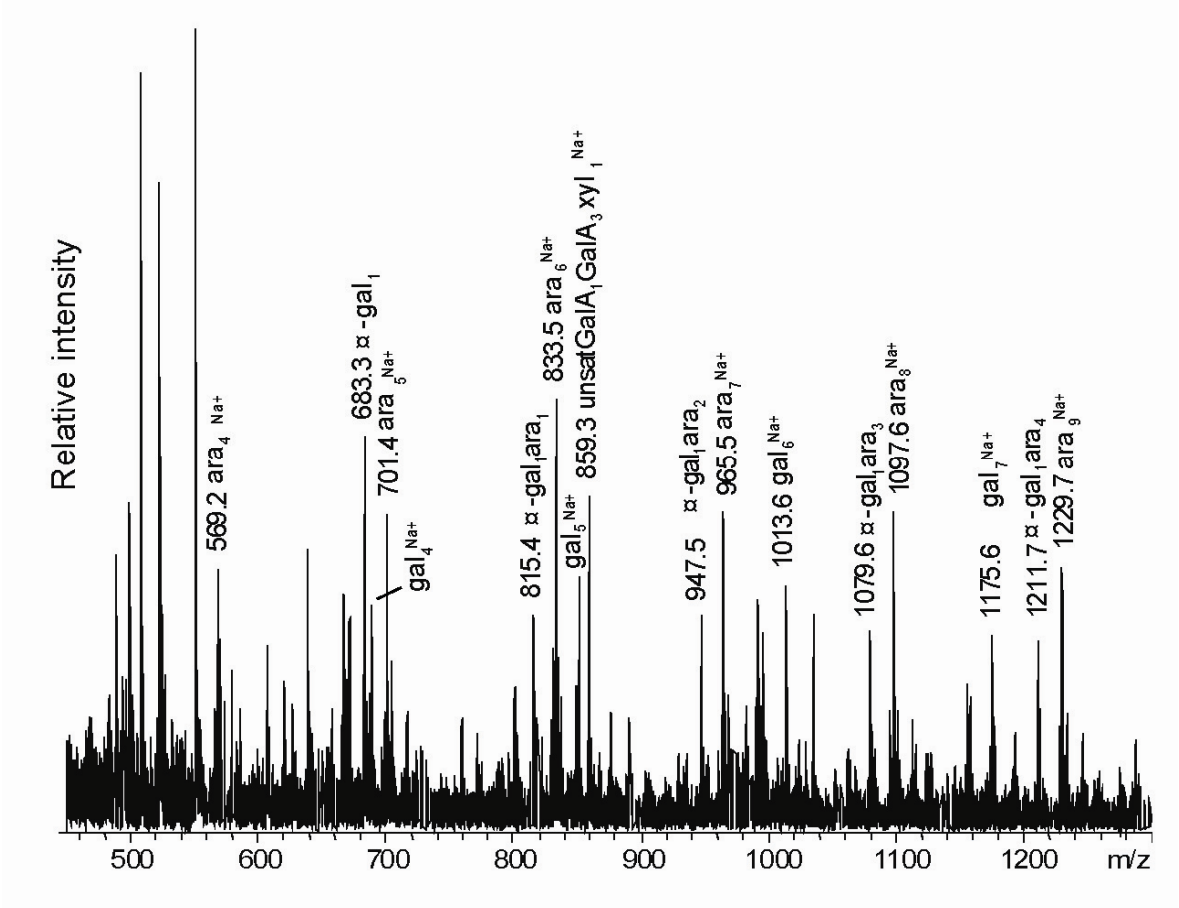

Figure 5: Maldi-tof mass spectra of BP-neutral-B with sodium adducts (H=Hexose=Galactose; P=Pentose= Arabinose; ¤ = UGalA-Rha-GalA; Na^+ = sodium adduct).

eliminative treatment of the RGI part of pectin. Additionally, the fragments at *m/z* 1035, 1563, and 2092, weighing 44 m/z less than the corresponding fragments at *m/z* 1079, 1607 and 2136, could not be annotated. The latter fragments corresponded to uGal*p*A-Rha*p*-Gal*p*A (¤) backbone fragments substituted with Gal_1Ara_n side chains.

ESI-MS^n experiments were conducted on the following ions *m/z* 683, 815, and 947 to get conclusive evidence of the above-mentioned reasoning. Unfortunately, we were unable to obtain MS-MS spectra confirming the uGal*p*A-Rha*p*-Gal*p*A (¤) backbone fragment.

The BP-neutral-B contained a more complex mixture of compounds than BP-neutral-A as indicated by the Maldi-tof mass spectrum (figure 5). A stretch of ions at *m/z* 689-2797 with subsequent additions of 162 Da was annotated as hexose (β-D-Gal*p*) tetra-heptadeca-mers. Furthermore, a homologous series of signals from *m/z* 569-1229 with 132 Da repeats was annotated as tetra-nonamers of Ara*f*. These series were as well present in small amounts in β-eliminative degraded potato galactans and sugar beet arabinans, used as controls (data not shown). Possibly, there are linkages present within the neutral sugar side chains, which are labile for β-eliminative treatment. Alternatively, these oligosaccharides could originate from autolysis, caused by the high temperature during the β-eliminative treatment. In addition, some fragments with repeats of 132 Da from *m/z* 683-1475 corresponded with the fragment ¤-Gal*p*1 $Ara f_n$ additions as observed in BP-neutral-A.

In both neutral fractions, NS chains were identified up to degree of polymerization (DP) 30 (BP-neutral A) and DP 17 (BP-neutral-B). The presence of both galactan and arabinan side chains in BP-neutral-B, was in agreement with the sugar composition of this pool, as well as the observed difference with BP-neutral-A (table 1).

The 859.3 m/z signal ($unsatGalA_1GalA_3Xyl_1$) indicates the presence of XGA in white cabbage, which was confirmed by additional analysis, which will be described in another manuscript (Westereng *et al*, personal communication).

Maldi-tof MS analysis of BP-charged (90 kDa, figure 2 and table 1), which was not subjected to a second β-elimination treatment (spectrum not shown), revealed similar series of pentose and hexose oligosaccharides as observed for BP-neutral-A and B. However, neutral sugar side chains connected to rudimentary backbone elements could not be identified, except for the low intensity ion at *m/z* 815 (¤-gal-ara). Additionally, ions at *m/z* 551, 727, 903, and 1079 in the ratio 10:4:2:1 were observed (data not shown). These masses point to α-D-Gal*p*$A_{3\text{-}6}$ fragments where the non-reducing end Gal*p*A was 4-deoxy-β-L-threo-hex-4-enepyranosyluronic acid (uGal*p*A). The occurring Δ 22 m/z representing sodium counter ions gave further evidence for the presence of uronic acids. We suggest based on the fragments observed, that the late eluting peaks in the HPSEC run

of BP-charged (figure 2) were uronic acid containing oligosaccharides. The early eluting molecules in the predominant HPSEC peak (90 kDa) were not observed by Maldi-tof MS and putatively held the abundant content of Ara*f* and Gal*p* (table 1).

The observed effects of the β-eliminative treatment indicated that this treatment promoted more reactions and involved more products than previously acknowledged. It was not possible to confirm the backbone fragment, which contained a putative Gal*p*A residue at the reducing end. However, ongoing experiments have proven successful in the elucidation of such oligosaccharides by utilizing improved methods for fractionation. The mechanism of the formation of these degradation products is at present not understood. This is currently under investigation in our laboratory. It appeared that the highly ramified RGI was quite resistant towards the β-eliminative process. A possible cause could be the spatial size of TBAF (planar surface of 10 Å) which may obstruct it from acting as a phase transfer catalyst. Hence, this sterical hindrance in the bulky hairy regions may lead to inefficient methyl esterifications at GalA *C*-6 in this region.

It should be noted that sodium tetraborate used for the β-elimination process generated strong signals in Maldi-tof MS analysis with an alternating sequence of 162 and 176 Da mass differences (data not shown). If care is not taken to remove this salt, misinterpretation of Gal*p* and Gal*p*A may occur as these have equivalent masses. Hence, a complete removal of tetraborate is needed.

Complement-fixing activity

Based on the fact that arabinans demonstrated complement fixing activity (Alban *et al.*, 2002; Yamada & Kiyohara, 1999), combined with the knowledge that small size polysaccharides have previously been reported to contain complement fixing activity (Sakurai *et al.*, 1999) it was of interest to compare the complement fixing activity of BP with the BP-neutral fractions, which were shown to consist out of several large arabinan containing oligosaccharides.

To screen for the presence of a complement fixing activity, the BP and BP-neutral side chain fractions were subjected to a complement fixing assay (figure 6). The PM II control has the highest complement fixing activity. The BP fraction has as well a very high inhibition at low (20 μmol/mL) concentrations. The complement fixing activity of BP-neutral-A is lower compared to the PMII control and BP, but still considerable. BP-neutral-B possesses no detectable inhibition activity. From the figure it can be calculated that the bioactivity of BP-neutral-A decreases with 33% compared to the BP fraction, which indicated that there seemed to be a minimal structure required for accommodating complement fixing activity. The requirement for a minimal structure was further underlined by BP-neutral-B, which was expected to be an active molecule based on its

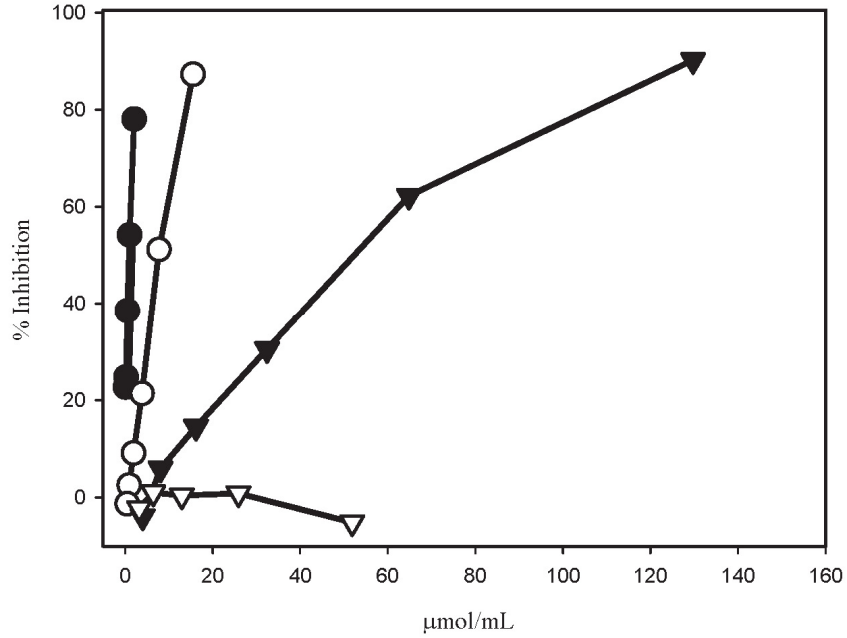

Figure 6: Complement-fixing activity of crude extracts based on mole; BP (o); BP-neutral-A (▼); BP-neutral-B (Δ) and control (PM II) (●). Samples were run in triplicates.

sugar composition containing Gal*p* in addition to being rich in arabinose residues, (Hirano *et al.*, 1994; Sakurai *et al.*, 1999; Samuelsen *et al.*, 1996; Wang *et al.*, 2005). Still, BP-neutral-B was not active, putatively due to its low molecular size (8 kDa).

BP-neutral-A and BP-neutral-B had a similar structural composition, as demonstrated by the sugar composition and ^{13}C-NMR, but varied in size (17 and 8 kDa, respectively; figure 2, table 1). Complement activity is triggered by binding of the recognition subcomponent C1q to antibodies complexed to an antigen on the bacterial surface (Arlaud *et al.*, 2002). If C1q has the ability of direct interaction with carbohydrates through C1qs globular heads previously suggested by Matsushita *et al.*, (2004), there is enough space to accommodate binding of 8-10 sugar residues on the 45 Å long surface. Taken into account the inactivity of the approximately 30 residue BP-neutral-B, it possibly needed interaction of at least two globular heads to initiate the C1 activation. If there is a minimal unit size responsible for activating the complement, the oligosaccharides in BP-neutral-B might be too small, whereas the size of the oligosaccharides in BP-neutral-A was sufficient to retain some, but very low activity (ICH_{50} = 50 µmol/mL). This means about six times higher concentration of BP-neutral-A was needed to express the same activity as the untreated BP (ICH_{50} = 7.5 µmol/mL) and about 60 times higher concentration than the positive control PMII (ICH_{50} = 0.8 µmol/mL).

Conclusions

Structural findings

This study presented evidence for the successful isolation of single side chains from RGI rich pectic structures from white cabbage. β-Eliminative degradation of RGI with side chains was achieved. The degradation pattern of the β-eliminative treatment was most certain more complex than expected and the apparent degradation of side chains is currently further studied. The oligosaccharides obtained were almost pure arabinans or galactans as evidenced by Maldi-tof MS. No evidence was obtained for side chains composed of a combination of arabinose with several galactose moieties and hence, no AGII/AGI structures could be positively identified. This study indicated that arabinan was present in separate clusters on RGI, but unambiguous evidence is still needed.

Biological activity of fractions

The results of complement fixing activity strongly indicated that a minimal unit size is necessary for complement activation. The BP-neutral-B oligosaccharides were probably too small for expressing activity, although these sugars possessed structures previously shown to be highly active (Alban *et al.*, 2002; Yamada & Kiyohara, 1999). BP-neutral-A exhibited complement fixing activity, but this activity was weaker compared to BP, probably due the small size of polymers present in the BP-neutral-A fraction. The sugar composition of BP-neutral-A indicated arabinan as the complement fixing polymer.

Acknowledgments

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Chapter 5

Characterizing the chemical structure of complex pectins using β-eliminative degradation in combination with CE-MS^n

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Abstract

Enzymatic degradation is the most favorable approach to depolymerize pectin in structure analysis, since this approach enables tailored breakdown. However, this approach can only be used when appropriate enzymes are available. Recently, a method has been described to selectively degrade the rhamnogalacturonan backbone. This method is based on the methyl esterification of galacturonosyl residues in the backbone followed by β-eliminative degradation of the complete methyl-esterified rhamnogalacturonan. To obtain more detailed information on the structure of the degradation products, this paper describes the application of a method in which β-eliminative breakdown was combined with CE-MS^n analysis, which was shown to be able to separate complex oligosaccharide mixtures.

Enzyme resistant soy pectin polymers were specifically degraded by β-elimination and fragments were subsequently separated and pooled by size exclusion chromatography with a volatile buffer. Using CE-LIF and Maldi-tof MS, a broad range of complex oligosaccharides (DP 3 to 12) was observed. In addition, CE-MS^n analysis identified a number of oligosaccharides consisting out of an unsatGalA-Rha "backbone" fragment, where rhamnose was substituted with sugar moieties. Using these methods in combination, detailed information on the neutral sugar side chain structure and distribution is obtained of some enzyme resistant structural elements of pectin. Thus it was demonstrated that soy RGI contains stretches with single galactose substitution.

Keywords

Pectin, soy, enzyme, CE-LIF, CE-MS^n, APTS, β-elimination

Introduction

Pectin is the most complicated polysaccharide in the plant cell wall (Mohnen, 2002) and it comprises a set of heterogeneous polysaccharides, such as rhamnogalacturonan I (RGI), homogalacturonan (HG), xylogalacturonan (XGA), rhamnogalacturonan II (RGII), arabinan, and arabinogalactan (Knox, 2002; Schols & Voragen, 2002). RGI is composed of [→2)-α-L-Rha*p*-(1→4)-α-D-Gal*p*A-(1→] repeats (Albersheim *et al.*, 1996; McNeil *et al.*, 1980), in which the rhamnosyl residues can be substituted at *O*-4 with neutral sugars (NS) side chains (Colquhoun *et al.*, 1990; Lau *et al.*, 1987; McNeil *et al.*, 1980). These side chains are mainly composed out of galactosyl and/or arabinosyl residues (Lau *et al.*, 1987; Lerouge *et al.*, 1993).

In general the pectin molecule is too large and heterogeneous to analyze as a whole. Therefore, the molecule is often fragmented and subsequently analyzed by various analytical techniques (Lau *et al.*, 1987; Voragen *et al.*, 1992). There are different strategies to degrade pectin (Voragen *et al.*, 1992) of which enzymatic degradation is the most favorable technique, since enzymes cleave sugar linkages rather specifically, enabling tailored breakdown of the polymer (Daas *et al.*, 1999; De Vries *et al.*, 1982; Rouau & Thibault, 1984; Schols & Voragen, 2002; Voragen *et al.*, 1992). In spite of the fact that a large array of pectin degrading enzymes is available, there still remain enzyme resistant structures. Furthermore, steric hindrance and low enzyme catalytic activities attribute to low pectin degradability. In studies toward the structure of pectins of several fruits and vegetables Schols and Voragen (1994) always remained with three main fractions after exhaustive degradation of the pectin fractions with commercial enzyme mixtures. Although removal of ester-type substituents may enhance enzymatic degradation, these native pectin fractions are only partly degradable by a variety of purified pectolytic, hemicellolytic, and cellulytic enzymes (Schols & Voragen, 1994). Most of the resulting fragments are in the high M_w range, making further structural analysis rather complex.

Chemical depolymerization methods have been used as an alternative for enzymatic degradation of pectin. Controlled acid hydrolysis and lithium degradation are chemical methods often used in structure elucidation studies. Depolymerization via controlled acid hydrolysis is based on the difference in susceptibility of sugar linkages for acid conditions (BeMiller, 1967; Guillon & Thibault, 1990; Thibault *et al.*, 1993). Controlled acid hydrolysis can be conducted with hydrofluoric acid (HF), which has a high cleaving specificity for various sugar linkages (Mort, 1983; Mort & Bauer, 1982). Unfortunately, this method is laborious, requires extensive safety precautions and is therefore not practical. As an alternative, hydrochloric acid (HCl) or trifluoroacetic acid (TFA) is often

used, both having the disadvantage of a lower specificity compared to HF, resulting in simultaneous hydrolysis of different sugar linkages (Thibault *et al.*, 1993). Furthermore, acid hydrolysis is not suitable for studying the neutral sugar build-up or distribution of side chains over the RGI pectin backbone, since neutral sugar linkages are more susceptible to acid hydrolysis compared to aldobiuronic and pseudo-aldobiuronic linkages (BeMiller, 1967; Guillon & Thibault, 1990; Thibault *et al.*, 1993).

Chemical degradation of RGI using lithium in ethylenediamine was introduced by Lau *et al*. (1987). By this treatment the galactosyluronic acid residues within complex carbohydrates are degraded, leaving intact the neutral glycosyl residues and their glycosidic linkages. Unfortunately, it could not be excluded that this degradation method is restricted only to the cleavage of galactosyluronic acid residues (Lau *et al.*, 1987). Furthermore, only low rhamnose levels could be recovered due to the volatile nature of rhamnitol after the reduction step (Lau *et al.*, 1987). Deng *et al*. (2006) described a more selective chemical depolymerization method of rhamnogalacturonans. Using unbranched RGI isolated from *Arabidopsis thaliana*, GalA residues were methyl-esterified followed by β-eliminative cleavage to selectively degrade the polymer. This method could be a suitable tool to investigate the appearance of NS side chains in rhamnogalacturonans that are enzyme resistant.

Soybean soluble polysaccharide (SSPS) is a pectin which is rather enzyme resistant (Huisman *et al.*, 1999) and, furthermore, contains high levels of NS side chains (Nakamura *et al.*, 2001). This commercially available polymer is extracted from soybean cotedylons, after removal of protein and fats, and it is primarily used as a food stabilizer (Nakamura *et al.*, 2001; Nakamura *et al.*, 2002b). SSPS was used to explore the potential of this method, to obtain more information of the mutual proportions of the β-eliminative generated complex fragments. Subsequently detailed structural information was gained about the characteristics of the NS side chains and their distribution over the SSPS backbone. In this paper we describe the combination of selective chemical depolymerization as described by Deng *et al*. (2006) with CE-MS^n for uronic acid oligosaccharides (Coenen *et al.*, 2008).

Materials and methods

Materials

Soybean soluble polysaccharide (SSPS; Soyafibe-S-DA100) was obtained from Fuji Oil Co. Ltd (Osaka, Japan) and prepared from the residue of soy protein extraction, as described previously (Nakamura *et al.*, 2001; Nakamura *et al.*, 2002b). SSPS was saponified in 0.05 M NaOH at 4°C during 24 hours at a concentration of 10 mg/mL.

Thereafter, the mixture was neutralized with 0.25 M HCl to pH 7, dialyzed, subsequently lyophilized, named sSSPS and used for further analysis.

Neutral sugar side chain shaving of sSSPS

Neutral sugars were partly removed from sSSPS by enzymatic digestion (Huisman *et al.*, 1999). A 15 ml (5 mg/ml; pH 6) solution was incubated with an overdose of the following enzymes: endo-galactanase (EndoGal; *Aspergillus aculeatus; 3.2.1.89*; Novozymes, Bagsvaerd, Denmark), β-galactosidase (β-gal; *Aspergillus niger*; *3.2.1.23*; Megazyme, Wicklow, Ireland), exo-galactanase (ExoGal; *Aspergillus niger; 3.2.1.22*), and arabinofuranosidase (*Aspergillus niger;* 3.2.1.55), where the latter two were purified in our laboratory. After 16 h head-over-tail incubation at 37°C, the enzymes were inactivated by a 10 min 100°C heating step. Finally the digested sample was dialyzed and lyophilized, named sSSPS-NS and used for further analysis.

Incubation of sSSPS-NS with pectin degrading enzymes

sSSPS-NS was separately incubated with the following enzymes: *endo* polygalacturonase (EndoPG; *Aspergillus* aculeatus; Novozymes), xylogalacturonan hydrolase (XGH; *Aspergillus niger*; DSM, Delft, the Netherlands), rhamnogalacturonan hydrolase (RGH; *Aspergillus aculeatus*; Novozymes), and rhamnogalacturonan lyase (RGL; *A. aculeatus*; Novozymes) as described by Huisman *et al*. (1999). The XGH enzyme was used as described by Zandleven *et al*. (2005). Incubations were performed for 16 hours, head-over-tail at 37°C. An enzyme overdose was used for every incubation. After incubation, the enzymes were inactivated by a 100°C, 10-min heating step.

Analytical size exclusion chromatography

Sample material was dissolved in distilled water (4 mg/mL) and analyzed by High Performance Size Exclusion Chromatography (HPSEC) performed on a SpectraSystem High Performance Liquid Chromatography system (HPLC; Thermo Separation Products, San Jose, USA) using three TosoHaas TSK-gel columns in series (4000, 3000, and 2500 PWxl; 300x7.5mm; TosoH, Tokyo, Japan) preceded by a TSK PWxl guard column (40x6 mm; TosoH). The sample (20 µL) was injected and eluted at 30°C using 0.8 mL/min 0.2 M $NaNO_3$. Detection was performed using a Shodex RI 71 refractive index detector (Showa Denko K.K., Tokyo, Japan).

Preparative size exclusion chromatography

An Äkta explorer system (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was used for the separation of different pools on preparative scale. Sample (40 mg) was dissolved in 1 ml and applied onto a Sephacryl S200 HR column (XK26, 100 cm; GE

Healthcare Bio-Sciences AB), which was eluted with 50 mM NaH_2PO_4 buffer at pH 7 at 2.65 mL/min. The collected fractions (10 mL) were re-injected on a analytical HPSEC system for M_w analysis, and pooled based on the observed M_w distribution profile. Subsequently, the different pools (ranging from 60-80 mL) were dialyzed and lyophilized.

Sugar composition

The sugar composition was determined according to De Ruiter *et al.* (1992). Samples were dried at 40°C under vacuum over P_2O_5 and hydrolyzed with 2 M HCl in dry methanol for 16 hours at 80°C followed by 1 hour of 2M TFA at 121°C. The monomeric sugars were analyzed by High Performance Anion Exchange Chromatography (HPAEC) using an ISC3000 HPLC system (Dionex, Sunnyvale, USA), equipped with a CarboPac PA-1 column (2 mm ID X 250 mm; Dionex) in combination with a CarboPac PA guard column (2 mm ID X 25 mm; Dionex) and an ISC3000 ED PAD-detector (Dionex). A flow rate of 0.3 mL/min was used with the following gradient of distilled water: 0.1 M NaOH, and 1 M NaAc in 0.1 M NaOH: 0-15 min, 30 mM NaOH; 15-16 min, 30-100 mM NaOH; 16-55 min 0-500 mM NaAc in 0.1 M NaOH. Each run was followed by a 1 M NaAc in 0.1 M NaOH washing step for 14 min and a 5-min equilibration step with 100 mM NaOH followed by 30 mM NaOH elution for 15 min.

Selective chemical depolymerisation

The selective chemical depolymersation of the sample material was adapted from the method described by Deng *et al*. (2006). The carboxylgroups of the GalA residues in the samples were selectively methyl-esterified using tetrabutylammonium fluoride and iodomethane in dimethyl sulfoxide (DMSO) containing 8% water (Deng *et al.*, 2006). Alternatively from using NMR spectroscopy, the degree of methyl esterification was monitored using Fourier Transformed Infrared spectroscopy (FTIR), as methyl esters and free carboxyl groups exhibit different wave numbers at pH 6 (Guillotin *et al.*, 2007). Spectra were obtained using a Bio-Rad FTS 6000 spectrometer (Cambridge, USA), with the MCT/DTGS detector set at 4 cm^{-1} resolution and interferograms were collected to obtain a high signal-to-noise ratio. Sample solutions were spread over a Fourier crystal and dried carefully with a flush of air. The ratio between the peak area of methyl esters (absorption band at 1742 cm^{-1}) and the peak area of free carboxyl groups (absorption band at 1611 cm^{-1}) was used to calculate the degree of methyl esterification (Guillotin *et al.*, 2007). When more than 80% (arbitrary) carboxyl groups were methyl-esterified, a solution of the methyl-esterified samples (10 mg) was degraded by β-elimination in 5 mL 0.2 M sodium borate, pH 7.3 at 125°C for 150 min. Subsequently, methanol was added to remove the borate as methylborate by evaporation in dry air.

Fractionation and desalting of oligosaccharides separated by size exclusion chromatography

The depolymerized products were fractionated by size exclusion chromatography (SEC) on a Superdex-Peptide 10/300 GL column (Amersham, Piscataway, USA), and monitored by UV absorbance at 235 nm and RI detection. The column was eluted at 0.5 ml/min with a 200 mM ammoniumformiate buffer at pH 5. The collected fractions were lyophilized twice to remove the volatile buffer salts completely.

Annotation of oligosaccharides using Maldi-tof MS mass spectrometry

For Matrix Assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry (Maldi-tof MS) analysis an Ultraflex workstation (Bruker Daltonics, Bremen, Germany) equipped with a 337 nm laser was used. The mass spectrometer was operated in the positive mode and calibrated with a mixture of maltodextrins (mass range 500-2500 Da). After a delayed extraction time of 200 ns, the ions were accelerated with a 25 kV voltage and subsequently detected using the reflector mode.

One microliter of sample solution was mixed with 1 µl of matrix and dried under a stream of warm air. The matrix solution was prepared by dissolving 9 mg 2,5-dihydroxybenzoic acid in ~1 mL of a acetonitril:water (300 µL:700µL) mixture. Mass to charge signals of the sodium adducts were translated in oligomeric structures (*e.g.* 685 m/z→$GalA_2Rha_2$), using an in-house software program (Verhoef et al., 2005).

Oligosaccharide separation by capillary electrophoresis and Laser Induced Fluorescence detection

Oligosaccharides were labeled with 8-aminopyrene-1,3,6-trisulfonate (APTS) using the ProteomeLab™ Carbohydrate Labeling and Analysis Kit (Beckman Coulter, Fullerton, USA). The labelled oligosaccharides were separated on a polyvinyl alcohol (NCHO) coated capillary (50 µm id X 50.2 cm, detection window after 40 cm; Beckman Coulter), using a ProteomeLab PA 800 capillary electrophoresis system, equipped with a laser induced fluorescence (LIF) detector using a 488 nm excitation and a 520 nm emission wave length (Beckman Coulter). The separation was carried out in reversed polarity at 30 kV, with a 25 mM sodium acetate buffer containing 0.4% polyethylene oxide and 0.3 % formic acid at pH 2.98. The capillary was kept at 25°C. As internal standard, maltose was added to the sample before derivatization (Coenen *et al.*, 2008).

Mass annotation of oligosaccharides using CE-UV-ESI-MS^n

For identification of the different oligosaccharides, separation was carried out on a P/ACE MDQ capillary electrophoresis system (Beckman Coulter) with a fused silica

capillary (50 µm id x 80 cm) connected to an internal UV-detector (Beckman Coulter) and a LTQ electrospray ionization (ESI) Iontrap mass spectrometer (Thermo Electron, San Jose, USA) by a device made in our laboratory (Hilz *et al.*, 2006). The separation was carried out in reversed polarity at 28 kV in a 50 mM HAc solution, containing 0.5% formic acid at pH 2.45. A maximum of 5 sequential runs were performed using the same buffer vial to ensure reproducibility. The capillary was kept at 15°C. For ESI-MS^n a 75 % isopropanol in water sheat flow (4 µL/min) was used. UV detection was performed at 254 nm in a capillary window located 20 cm from the inlet. ESI-MS^n was performed in negative mode, using a spray voltage of 1.9 kV and an ion transfer capillary temperature of 200°C (Coenen *et al.*, 2008). MS^2 and MS^3 spectra were obtained using data dependent peak selection controlled by XCalibur 2.0 SR2 and LTQ Tune 2.2 Software (Thermo Electron). Peaks were selected between 350 and 1200 m/z. A peak width of 4 was chosen to further fragment the four most abundant peaks in a MS^1 spectrum. Minimal counts were set to 100. The masses 352, 391, 451 and 461 were added to the mass exclusion list, since these signals were abundantly present in the eluent. The scanning time was set to 10 ms/scan for MS^1 spectra. MS^n spectra were recorded for 33 ms/scan and the results of three scans were summarized. Peaks were fragmented using a window of 2 m/z and relative collision energy of 30% (Coenen *et al.*, 2008).

Results and discussion

Enzymatic degradation of SSPS

To generate an enzyme resistant polymer, SSPS was degraded as far as possible with neutral sugar removing enzymes. In order to enhance enzyme digestion, the substrate was first saponified to remove acetyl groups and methyl esters, known to inhibit enzymatic breakdown, yielding sSSPS. The effect of saponification and enzymatic degradation of SSPS with a NS degrading enzyme mix on the sugar composition is shown in table 1.

The galactose content decreased to approximately 30% of the original content in the enzyme-degraded sample (sSSPS-NS), resulting in a relative increase of the remaining sugars. Based on the specificity of the EndoGal, ExoGal and β-gal enzymes, these sugar moieties originate from β-1,4 linked galactan chains, which are presumed to be attached to RGI (Nakamura *et al.*, 2001). Furthermore, it was calculated that around 23% of arabinose was released by the enzyme mixture. A part of the residual neutral sugars could not be degraded by the enzyme mixture, possibly due to sterical hindrance, or by the absence of the required enzyme specificity.

From the ratio Rha:GalA in the sugar composition, it could be concluded that the fractions do not have a strict alternating Rha/GalA backbone but that sequences of adjacent GalA residues are present. The xylose content revealed that this could (partly) be

Table 1: Sugar composition of different SSPS fractions

Fraction	Sugar composition (mol%)							
	fuc	rha	ara	gal	glc	xyl	galA	glcA
SSPS	4	5	25	39	3	7	17	0
sSSPS	4	5	25	38	2	8	18	0
sSSPS-NS	6	9	26	15	1	14	29	0
Pool I	17	2	4	8	1	24	43	1
Pool II	12	11	6	9	2	19	40	1
Pool III	1	17	30	25	2	1	23	1

a xylogalacturonan. Huisman *et al.* (2001; 2003) demonstrated that a large part, if not all, of the galacturonan in soy is present as XGA.

Since sSSPS-NS contained different structural elements such as (substituted) RGI, XGA, and possibly HG, the sample was further degraded using "backbone degrading" enzymes in order to get a separation between the different structural elements. However, both EndoPG as well as XGH treatment did not result in any breakdown (data not shown). Probably EndoPG could not hydrolyze the substrate due to absence of unsubstituted galacturonan regions, as is indicated by the high Xyl:GalA ratio. The inactivity of XGH remained unexplained; however, it has been observed that XGH is inactive to some substrates, which are known to contain XGA. Beldman *et al*. (2003) released only minute amounts of XGA oligosaccharides out of pea hull XGA, which was attributed to the distribution of xylose over the galacturonan backbone or to the presence of longer side chains than single units of xylose. Furthermore, Nakamura *et al.* (2002a) demonstrated the presence of (β-D-Xyl)$_7$ side chains *O*-3 substituted to 1,4 linked (α-D-GalA)$_4$ regions present in a SSPS fraction, which structure is probably hindering XGH action as well.

Both RGH and RGL treatment resulted in a downward shift in molecular weight distribution profiles. This means that unsubstituted RGI regions were present in the sSSPS-NS polymer backbone, liable to RGH and RGL attack. Furthermore, the increase in UV 235 nm absorbance confirms RGL degradation and all reaction products were found to elute between 26 and 34 minutes. In order to further investigate the different populations, the RGL digest (sSSPS-NS-RGL) was fractionated by SEC and pooled into three pools as indicated in figure 1.

The sugar composition of these three pools (table 1) revealed that pool I and pool II both contained only a small proportion RGI, and are rich in XGA (xylose to galacturonic acid ratio 1:2). The main difference between pool I and II is in the arabinose content. The fucose (Fuc) content of both pool I and II was rather high compared to pool III, but similar Fuc levels have previously been described for SSPS, which was degraded by a combination of several hemicellulases and separated by SEC (Nakamura *et al.*, 2001). In

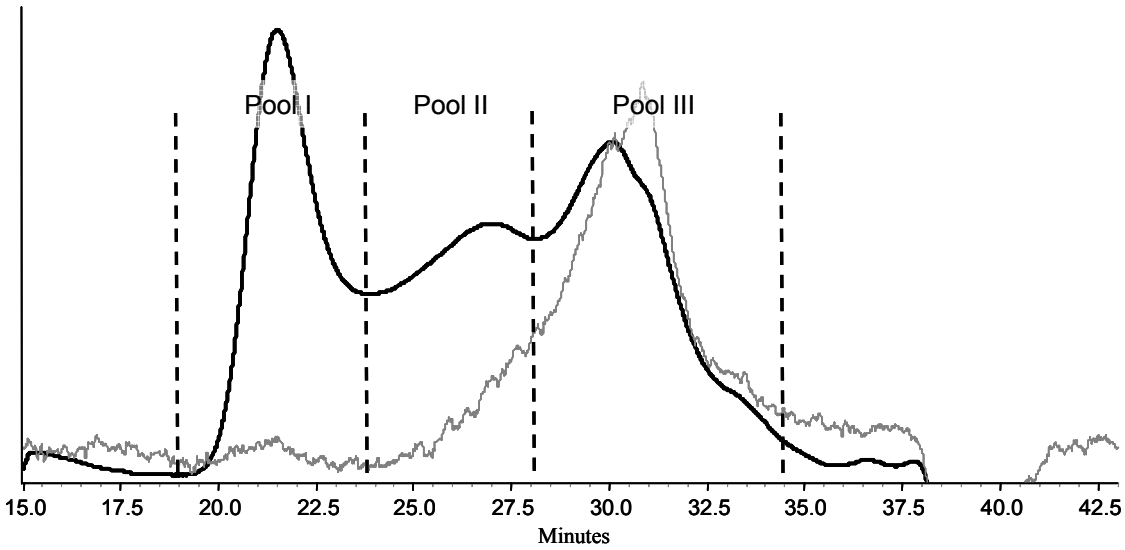

Figure 1: HPSEC molecular weight distribution profile of dialyzed sSSPS-NS-RGL: (—) RI response, (—) UV 235 nm signal. Vertical dashed lines indicated how the fractions of the preparative SEC were pooled.

addition, pool III differed from pool I and II in its high Rha content, with a Rha to GalA ratio of 0.74 to 1. The galactose content of pool III was the highest of all three pools. Finally, similar sugar compositions, molecular weight ranges and enzyme resistances were found in RGH degraded modified hairy regions (MHR) originating from different sources as described by Schols and Voragen (1994), which could indicate a structural similarity.

. Selective chemical depolymerisation

To enable analysis of the pools, while maintaining their NS substitution pattern, these pools were selectively methyl-esterified in order to make them liable to degradation by β-elimination. In this study, the level of methyl esterification was determined by FTIR. The results obtained by this method were comparable to the NMR method used by Deng *et al.* (2006), which required longer sample preparation- and analysis time. From comparing the area of the signal at 1742 cm^{-1} to the area at 1611 cm^{-1} it was demonstrated that at the start the three enzyme-digested sSSPS-NS-RGL pools indeed showed almost no methyl esterification (saponified). Around 80% of all carboxyl groups were found to be methyl-esterified after the tetrabutylammonium fluoride in iodomethane treatment (data not shown). It was decided that this 80% methyl esterification was sufficient to continue with the β-elimination. This high level of methyl-esterification was attributed to the excellent solubility of the sSSPS-NS-RGL pools in DMSO.

Following the methyl esterification step, the pools were subjected to β-eliminative degradation. To evaluate the β-eliminative treatment, samples were desalted via Superdex-Peptide SEC, and the masses of the components present were measured by Maldi-tof MS after partial removal of sodium borate. From earlier experience within our laboratory it

was known that the high molecular weight fractions eluting before 24 min. and the low molecular weight fraction eluting after 37 min did not attribute to good MS analysis, therefore only fractions within this time span were pooled and analyzed by Maldi-tof MS. Series of oligosaccharides were observed with a degree of polymerization (DP) between 3 and 12. These oligosaccharides were mainly B-type (unsatGalA-Rha-GalA-Rha) fragments, indicating that within the degraded regions, either the methyl- esterification or the β-elimination was not complete. Based on sugar composition and sample knowledge the mass differences were annotated by the putative sugar residues (e.g. Δ176 m/z corresponds to GalA). Primarily galactan side chains were determined, with a few arabinogalactan oligosaccharides in the higher (1500-2000) M_w region. The amount of fragments formed was the highest in pool III and the lowest in pool I, as could been seen from the signal intensity. The ratio between the observed masses differed as well between

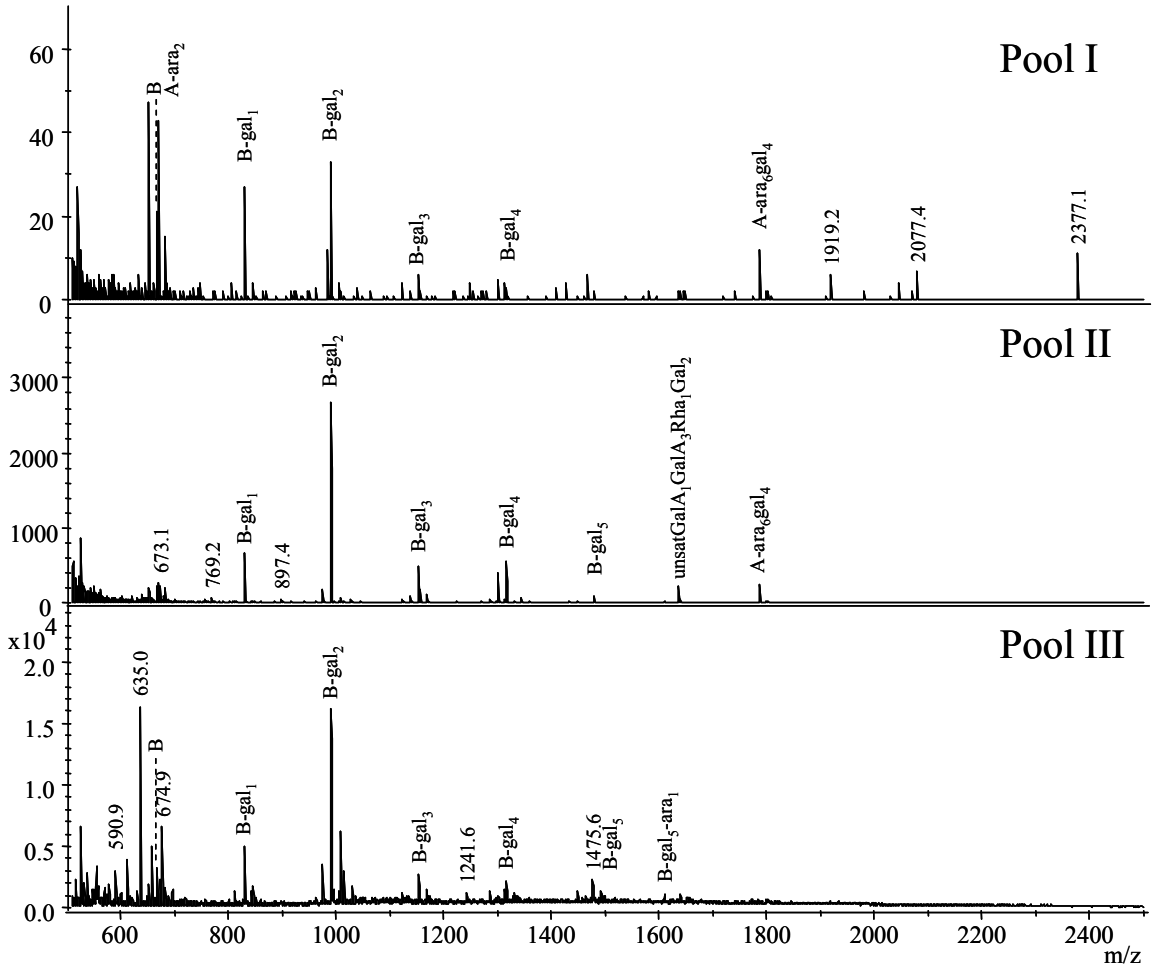

Figure 2: Maldi-tof MS mass spectra of the β-eliminative degraded sSSPS-NS-RGL pools I, II, and III. The annotations A (unsatGalA-Rha) and B (unsatGalA-Rha-GalA-Rha) are used to describe the different backbone fragments.

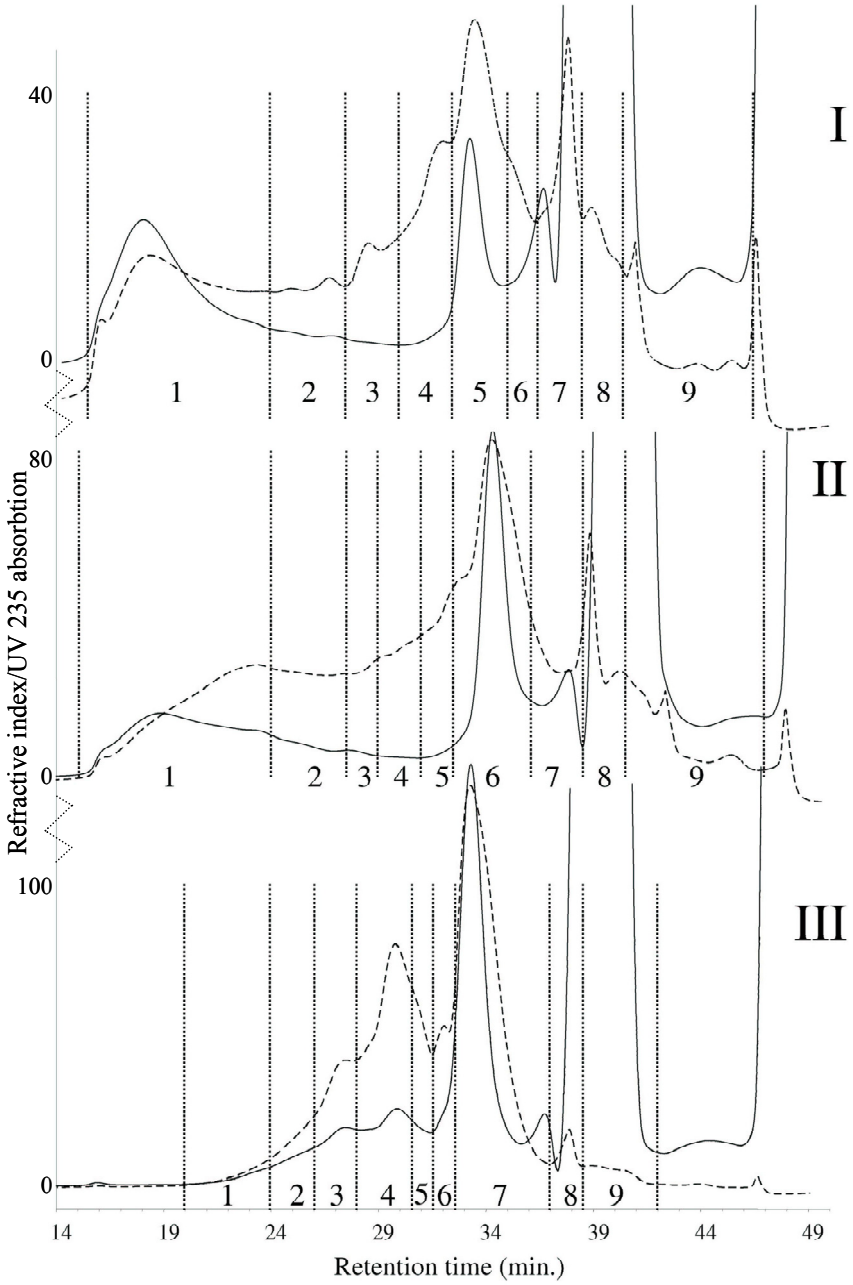

Figure 3: Size exclusion chromatography profiles obtained by Superdex-Peptide column separation of the products obtained upon β-eliminative degradation of the methyl-esterified sSSPS-NS-RGL pools I, II, and III. The responses of the RI (solid line) and UV 235 nm (dashed line) are shown, where the tick mark labels on the y-axis correspond to the RI response. Vertical dashed lines indicate the different Superdex-Peptide pools.

the different pools, Pool I contained relatively higher mass peaks than Pool II and III. The 991.2 m/z signal, corresponding to unsat$GalA_1GalA_1Rha_2Gal_2$, indicated the presence of stretches of scarcely substituted RGI present in SSPS. These finding were further corroborated by the putative annotation of unsat$GalA_1GalA_3Rha_4Gal_2$ (1635 m/z), which represents a RGI backbone octamer, substituted with two galactose sugar moieties. Since the oligosaccharide with the mass/charge ratio of 991.2 occurs abundantly in non enzyme degraded SSPS (data not shown), this structure was present in the original pectin. Therefore, the presence of the corresponding unsat$GalA_1GalA_1Rha_2Gal_2$ oligosaccharide was not attributed to enzymatic trimming of the galactan side chain and therefore present in "native pectin". Furthermore, pool I and II did not exhibit an UV 235 nm signal (figure 1) prior to β-eliminative degradation, hence the formation of the 991 m/z signal as a result of lyase-enzyme activity is most unlikely. The tentative annotated degradation products in these three pools indicate mainly oligosaccharides containing galactose substitutions. Based on the sugar composition (table 1) oligosaccharides containing a higher content of xylose, arabinose or galacturonic acid were anticipated. However, they were not present and this could imply that only a part of the polymers were degraded to oligosaccharides.

Separation of the β-eliminative degraded SSPS pools by SEC

In order to further investigate the molecular weight distribution profile of the degradation products in the different SSPS pools, the samples were fractionated on a Superdex-Peptide column, using NH_4COOH as buffer (figure 3).

The molecular weight distribution profile, obtained with the Superdex-Peptide SEC analysis, indicated a broad distribution of degradation fragments. Based on the UV 235 nm signals the different fractions were pooled (pool 1-9). At least 60% of the polysaccharide was fragmented into low-M_w (<1500 Da) oligosaccharides (pools 3-9). Although this SEC separation was performed on another SEC column compared to the M_w analysis of the sSSPS-NS-RGL pools, it can be concluded that the elution behavior of the remaining high M_w fractions in the three sSSPS-NS-RGL molecular weight distribution profiles demonstrated some similarities to the original pool size distribution (figure 1), which indicated that backbone segments were not homogenous distributed, since this would result in a decrease of M_w upon the formation of degradation products. Furthermore, the low intensity of the RI response of sSSPS-NS-RGL pool I compared to sSSPS-RGL Pool II and Pool III was in agreement with the intensity of the Maldi-tof MS mass spectra, although in the latter the response decrease in pool I is more prominent.

Additionally, the peaks at 39 and 47 min, which are respectively DMSO and residual borate signals, demonstrate that this size exclusion separation is as well an effective desalting step.

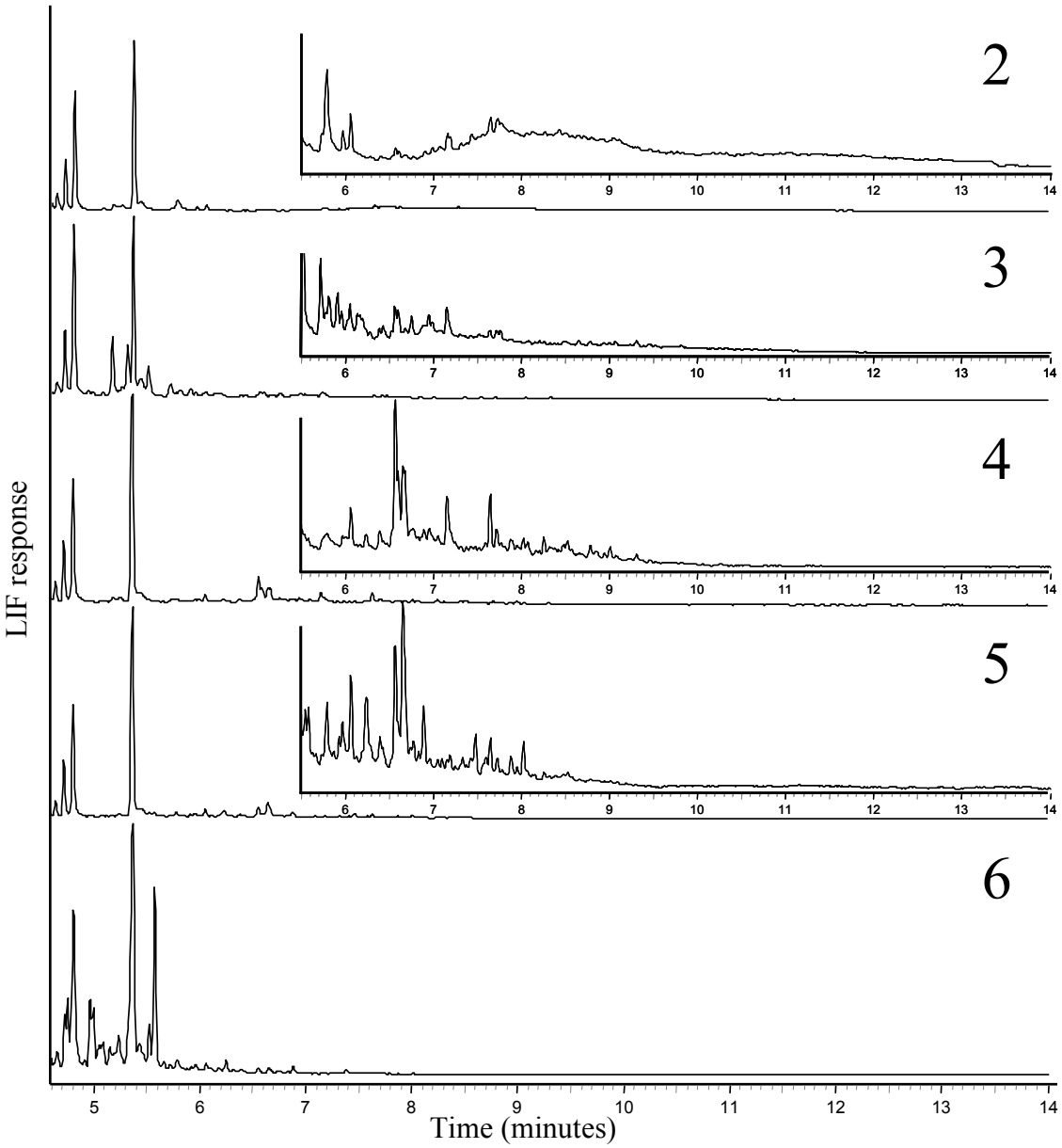

Figure 4: CE-LIF electropherograms of APTS labeled Superdex-Peptide pools (indicated by arabic indexes) of sSSPS-NS-RGL pool II.

Oligosaccharide separation by capillary electrophoresis with Laser Induced Fluorescence detection

The Superdex-Peptide pools of fractionated sSSPS-NS-RGL were further investigated in order to obtain insight in the number and properties of oligosaccharides present in the different Superdex-Peptide pools. Therefore, these samples were labeled with APTS and subsequently separated by CE with LIF detection.

The Superdex-Peptide pools of the β-eliminated sSSPS-NS-RGL pool I did not exhibit a large number of degradation products, which was in accordance with the low signal intensity of the Maldi-TOF mass spectra. Therefore, only CE-LIF electropherograms of pool II and pool III are discussed.

β-Eliminative treated sSSPS-NS-RGL pool II contained a variety of different oligosaccharides, as can be observed from the number of peaks in the different electropherograms obtained of the different pools. The inserted enlarged electropherograms show an elution time decrease, when the pool number increased. The peaks in the beginning of the electropherograms corresponded to APTS reaction products (4.66, 4.77, and 4.83 min; system peaks present in the blank) and the maltose internal standard (5.37 min). In pool 6 early eluting peaks (between 4.6-5.37 min) were present, which are located in the monomer region.

In sSSPS-NS-RGL pool III, the amount of β-eliminative material was higher as compared to pool I and II. Especially in Superdex-Peptide pools 4, 5, and 6 substantially more material was present. This corresponds with the relative high intensity observed in the Maldi-Tof mass spectrum of this pool. The peak eluting at 6.69 min was present in the electropherograms of all sSSPS-NS-RGL pools, and a similar elution behavior for rather similar RGI fragments has been discribed (Coenen *et al.*, 2008). Therefore, this LIF-signal may correspond to mass peak 991 m/z, which was also present in all three sSSPS-NS-RGL pools, as shown by Maldi-tof MS (figure 3). In the Superdex-Peptide pools 2-4 there was a wide range of oligosaccharides present, while in the pools 5-7 there were less, but larger peaks presents. The smaller size of the oligosaccharides present in pools 5-7 gave less possibilities for the structure annotation.

The CE-LIF chromatograms (figure 4 and 5) showed that the pooling borders of the Superdex-Peptide pools (figure 3) generated distinctively different populations, which were easily separated, giving information about the poly-dispersity of the oligosaccharides present in the different Superdex-Peptide pools. As shown in Figure 4 and 5, Superdex-Peptide pools are composed of a large number of different oligosaccharides (>10/pool). In the electropherograms the abundance of oligosaccharides increased when the pool number

increased, which corresponded with a higher abundance (in moles) of components in these pools. Simultaniously, the number of peaks decreases with increasing pool numbers.

Mass characterization of oligosaccharides present in Superdex-Peptide pools

In order to retrieve more information about the chemical structure of the various components present in the Superdex-Peptide pools, these pools were analyzed using CE-MS^n. This combined analytical technique enables oligosaccharide separation and subsequent identification, and localisation of sugar substituents based on their specific mass spectra.

In figure 6a the MS^1, MS^2, and MS^3 spectra are shown for the smaller oligosaccharide (461.6 m/z). The mass of 382.7 m/z indicated a substitution on the sugar, containing the APTS label. With the MS^3 spectrum this fragment was identified to consist out of a galactose substitution upon a rhamnose residue positioned at the reducing end of the oligosaccharide (figure 6a). The component described in figure 6b was larger, but had a similar build-up/fragmentation pattern. In the MS^1 spectrum two peaks could be observed at 468.9 and 704.7 m/z representing the -2 and -3 charged APTS-labelled oligosaccharide. The $[APTS]^{3-}$ peak was not visible in figure 6a, since its m/z value (307.7) fell outside the set MS detection range (350-2000 m/z). For interpretation purposes, the MS^2 spectrum of the $[APTS]^{2-}$ was more convenient to analyze compared to the $[APTS]^{3-}$ charged oligosaccharide. The MS^2 spectrum of peak 703.9 m/z revealed that this oligosaccharide was composed of unsatGalA, GalA, 2 galactose and 2 rhamnose sugar moieties. The reducing end was carried by the rhamnose, as indicated by the signal at 301.8 m/z. Furthermore this Rha was connected to a Gal (382.6 m/z) *and* to GalA (389.7 m/z), based on previous knowledge these are putative Gal (1→4) Rha and GalA (1→2) Rha linkages. The virtual absence of a dimeric galactose loss, together with an observed preference to split-off the second galactose first (data not shown), allowed drawing a putative structure of this component, where both rhamnoses carry one Gal substitution. Both MS^2 fragmentation spectra indicate that in both oligosaccharides unsaturated GalA was released first. This was also observed in all other examined oligosaccharides containing monomeric hexose substitutions (data not shown).

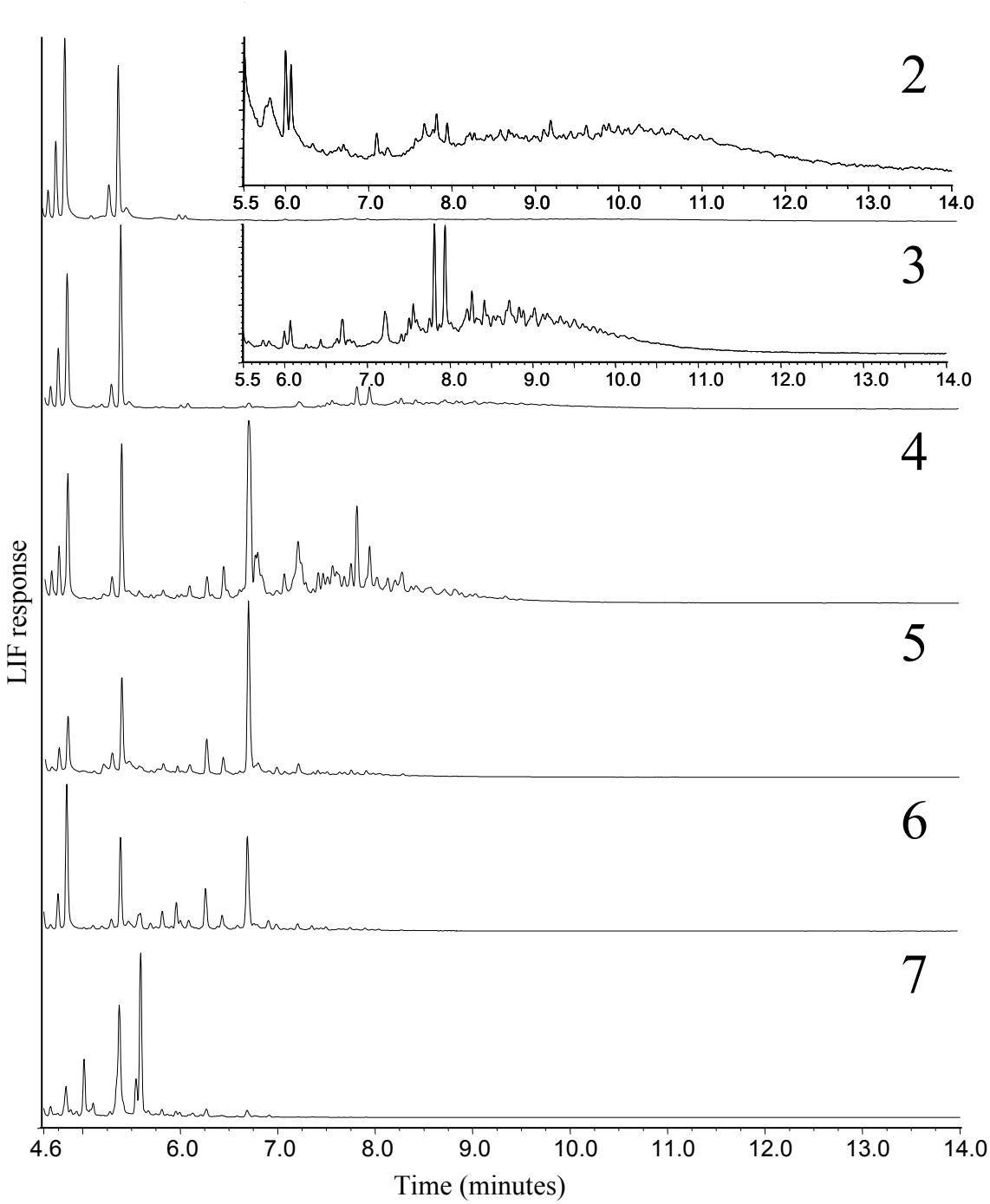

Figure 5: CE-LIF electropherograms of APTS labeled Superdex-Peptide pools (indicated by arabic indexes) of β-eliminated sSSPS-NS-RGL pool III.

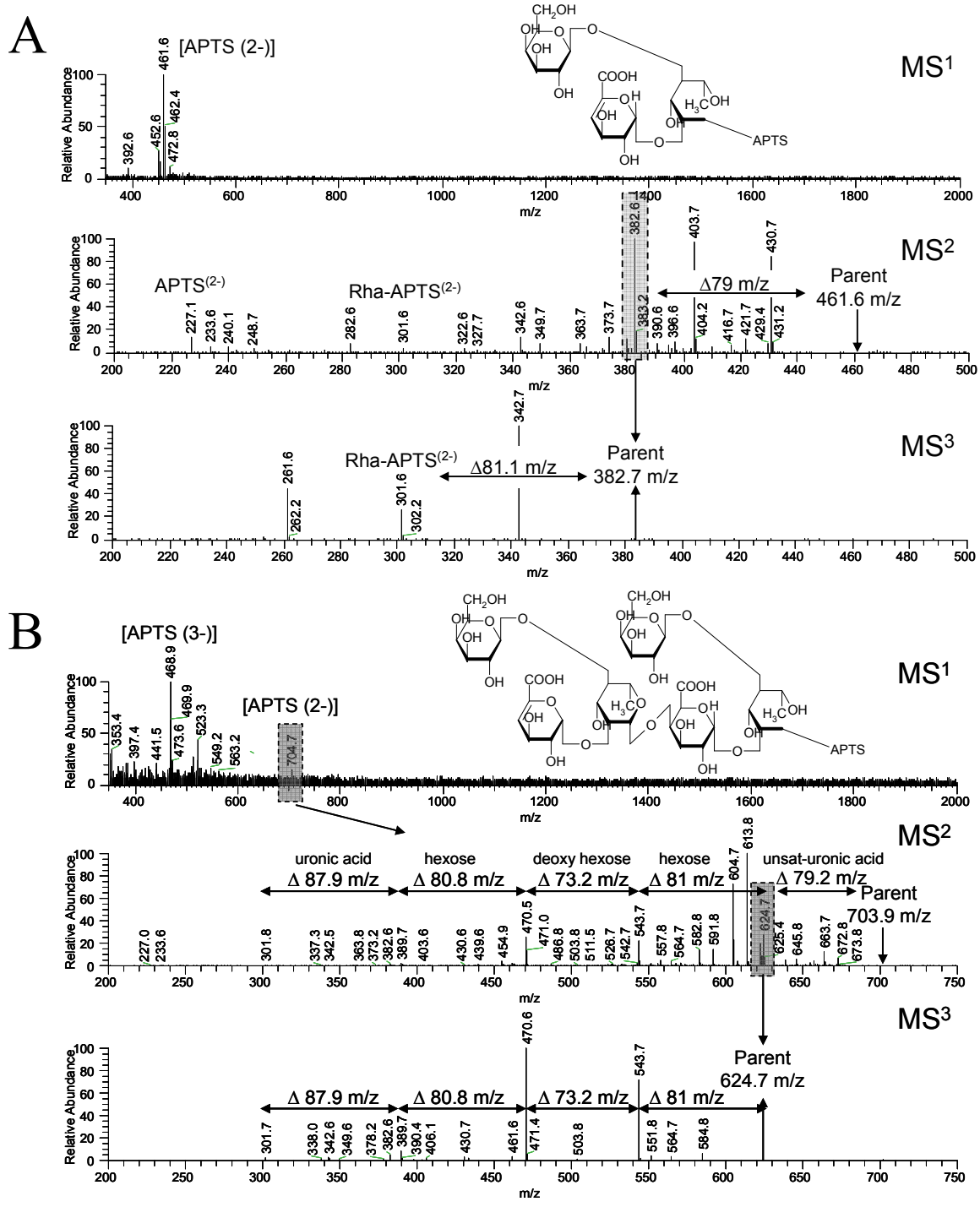

Figure 6: Structural characterization of APTS labelled oligosaccharides, originating from different Superdex-Peptide pools, obtained by CE-MS^n after selective degradation by β-elimination of sSSPS-NS-RGL pool III. (A) MS^1, MS^2, and MS^3 of the abundant 461.6 peak in Superdex-Peptide pool 7; (B) MS$^{1-3}$ of the 704.7 m/z oligosaccharide identified in Superdex-Peptide pool 4.

Conclusions

Using CE-MS^n with APTS labeling it was possible to mass-characterize degradation products obtained after selectively degrading sSSPS-NS-RGL pools by β-elimination. This information allowed detailing the structure of the neutral sugar side chains and the distribution of these side chains over the backbone of some enzyme resistant RGI elements. Selective methylation was monitored via FTIR, which proved an efficient alternative for NMR. Performing Maldi-tof MS on fragments obtained by β-eliminative degradation, oligosaccharide series were identified consisting out of a backbone fragment, with (arabino)galactan side chains. However, most of the identified fragments contained short side chains. The High M_w fraction indicated that not all side chains are released, possibly caused by incomplete methylation and consequently poor β-elimination. Until now, no residual side chains obtained via β-elimination were mass characterized via APTS labeling directly after separation. This approach provided a new strategy for structure elucidation of pectin fractions, which were previously inaccessible, and opens the way for identification of all peaks after β-elimination. In a following paper, the quantification and distribution of neutral sugar side chains over the RGI backbone of various fruits and vegetables will be presented, using this approach.

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Chapter 6

General discussion

Research motive

Pectin is an important component of the cell wall of many plant tissues. As such it plays an important role during ripening, storage and processing of plant raw materials for foods. It is also extracted from suitable plant sources and as such widely used in food industry because of its gelling, stabilizing, and thickening properties (Laurent & Boulenguer, 2003). Recently, pectin has gained interest as a functional food, because of possible health promoting effects (Yamada *et al.*, 2003). In all of these applications, the chemical fine structure of pectin strongly determines its functionality.

Even after more than 180 years of pectin research, the structure of pectin is still under debate (Braconnot, 1825; Vincken *et al.*, 2003a; Vincken *et al.*, 2003b). At this moment, there are two favorite models for describing the pectin structure: the smooth and hairy region model (Schols & Voragen, 1996), and the rhamnogalacturonan (RG) backbone model (figure 1)(Vincken *et al.*, 2003a).

In the first model, pectin is composed of hairy regions, consisting of rhamnogalacturonan I (RGI) decorated with neutral sugar side-chains, interspersed with smooth regions of (methyl esterified) homogalacturonan (HG) (Schols & Voragen, 1996). The second model positions HG as a side chain of RGI, similar to the neutral sugar side chains (Vincken *et al.*, 2003a). The placement of xylogalacturonan (XGA) in respect to RGI is indistinct, but regarded exchangeable with HG (Huisman *et al.*, 2001; Schols & Voragen, 1996; Vincken *et al.*, 2003b).

As the main difference between these models is formed by the connection of HG to RGI, we focused on the identification and characterization of oligosaccharides, which constitute these connection points and contain information about the linkages between different structure elements. In order to be able to analyze these components the analytical toolbox had to be extended. By combining different analytical techniques we were able to

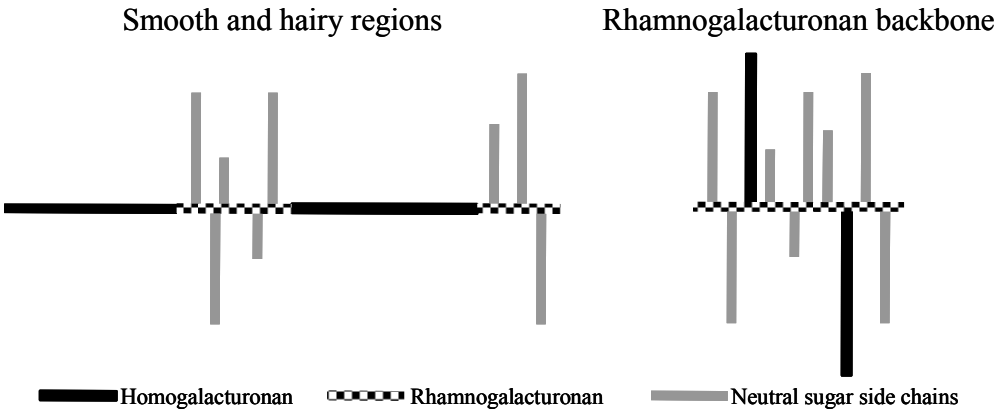

Figure 1: Schematic representation of two different models describing the hypothetical pectin structure, adapted from Vincken *et al.* (2003a).

develop new methodology and to identify some of these connecting oligosaccharides, which provided us with new insight in the macromolecular build up of the pectin polymer.

Outline

To identify the connecting fragments in an oligosaccharide mixture the off-line HPAEC-Maldi-tof MS method (Kabel *et al.*, 2001) was further improved, in order to enable MS analysis of galacturonic acid (GalA) containing oligosaccharides. For the first time, oligosaccharides of hybrid nature, consisting out of both HG and RGI sequences together with an oligosaccharide composed out of both XGA and RGI sequences were isolated out of an apple modified hairy regions (MHR) hydrolysate and characterized using chromatography and mass spectrometry (chapter 2). For identification of the linkages between HG/XGA and RGI the enzymes *exo* polygalacturonase (ExoPG) and rhamnogalacturonan galacturono hydrolase (RGGH) were used as diagnostic tools. This allowed refinement of the currently used pectin models, with emphasis on the linkage between the different structure elements.

The second part of our research focused on the improvement of methods used in polysaccharide characterization. An effective separation of differently substituted GalA residues was obtained by low pH capillary electrophoresis-laser induced fluorescence (CE-LIF) analysis (chapter 3). By adapting the buffer pH and capillary used, on-line MS^n was enabled. With this combined CE-MS approach peaks present in the LIF electropherograms of XGA and RGI digests could be annotated.

In chapter 5 the CE-LIF-MS^n method was used to identify products obtained after chemical degradation of pectins. The application of mild chemical conditions promoting β-eliminative breakdown (Deng *et al.*, 2006) allowed specific hydrolysis of the (enzyme resistant) RGI backbone while preserving the various neutral sugar side chains.

Another structure characterization study was conducted on white cabbage pectin, which was found to activate the human complement and therefore contain a physiological activity (chapter 4). The complement system plays an important role in the human body as a primary defense system against bacterial and viral infections (Helmy *et al.*, 2006). Due to its important physiological role, complement modulation, either inhibition or activation, is related to the cure of various diseases and therefore considered an interesting target for drug development (Alban *et al.*, 2002). After β-eliminative degradation, purification and identification of pectin fragments, it was found that short pectin side chains had no bioactivity, while larger side chain fragments contained a (much) lower activity than polymers containing intact RGI with more arabinan side chains, which implied that structural elements containing multiple side chains expressed a higher complement fixing activity.

Finally, in this chapter, the newly obtained information about pectin structure is summarized and the implications of these new findings for the pectin models are discussed. The importance of having detailed structure information for the study of pectin in physiological applications is presented. Thereafter, the possible applications of CE-MS within carbohydrate research are discussed.

Pectin structure

Over the years numerous publications dealing with the structure of pectin have been published. Chapter 1 provides a literature review about the structure of pectin elements until the start of this thesis project (2002). The findings recently published by Yapo *et al.*, (2007), were of specific importance for the interpretation of the results obtained in this study, and are therefore summarized here. In their study it was found that citrus peel pectin consisted of HG segments with a DP in the range of 81-117 GalA units, irrespective of their extraction method. In the same study, Yapo *et al.* (2007) hydrolysed pectins in such way that that they were able to recover around 90% of all RGI segments as chains having an average length of 30-40 residues.

Identification of homo- or xylogalacturonan segments attached to RGI

In a structure characterization study, using controlled acid hydrolysis, three different hybrid oligosaccharides were identified in apple MHR, composed out of part of a HG segment and part of a RGI segment. For one hybrid oligosaccharide the connection between the different structural elements was confirmed to be an GalA*p*α-(1,2)Rha*p* linkage (chapter 2). In addition, a $GalA_6Rha_3Xyl_1$ oligosaccharide was identified, which consisted out of both a XGA and a RGI sequence, connected via an GalA*p*α-(1,2)Rha*p* linkage. The xylose substitution was putatively located on the second GalA moiety on the non-reducing end. These oligosaccharides indicated that in apple pectin both HG and XGA were covalently linked to RGI.

The length of the GalA sequences, within the different HG-RGI hybrid oligosaccharides, was three GalA residues. Possibly, this reoccurring length is the result of *Endo*-polygalacturonase (EndoPG) and *Exo*-Polygalacturonase (ExoPG) enzyme activity during the MHR preparation due to sterical hindrance of neighboring sugar moieties or the requirement for at least three unsubstituted GalA residues on the reducing end of the enzyme' active site. On the other hand, the controlled acid hydrolysis treatment used to generate these elements out of apple MHR is expected to cleave some of the backbone linkages, hereby directly influencing the remaining pectin structure. Nevertheless, the identification of three different oligosaccharides, containing 3 galacturonic acid residues on the non-reducing end was remarkable.

The limited HG chain length of the hybrid HG-RGI oligosaccharides gives room for the hypotheses that these fragments are resulting from a linkage between XGA and RGI, as demonstrated in the fourth characterized oligosaccharide $GalA_6Rha_3Xyl_1$. It is possible that the GalA sequences (which consist of three GalA sugar moieties) are originating from XGA as well. It is not unconceivable that a plant has a fixed building pattern for the arrangement of the different pectin structure elements. Therefore, it could be possible that all of the characterized hybrid oligosaccharides are resulting from a XGA-RGI linkage (figure 2). Hypothetically, HG would than be connected on the terminal end of the rather homogeneous XGA (figure 2A), or the degree of xylosidation would decrease when coming closer to the pure HG segment at the non-reducing end (figure 2B).

Such a substitution pattern could be caused by a xylosidase, releasing xylose residues from XGA, which would be (sterically) hindered when approaching the RGI backbone. Xylogalacturonan is considered to be synthesized analogous to apiogalacturonan, where the apiose residue is likely to be incorporated during the synthesis of the galacturonan backbone (Mohnen, 2002). This could imply that highly substituted galacturonans are incorporated into pectin and are further modified by putative xylosidases.

This would explain the release of XGA when hairy regions polysaccharides were treated by RGH (Mutter *et al.*, 1996), which indicates a covalent connection of XGA and RGI in the cell wall (Albersheim *et al.*, 1996). Schols *et al*. (1995) observed that after HG removal by EndoPG, XGA was retained in the high M_w region, indicating as well that XGA is located close, or is part of, the RGI backbone. In addition, such observations suggest that if there is a direct linkage between XGA and HG, this linkage will only be located at one side, since no XGA segments are liberated by this EndoPG treatment. Furthermore, the increased degradability of XGA by XGH after removal of HG by

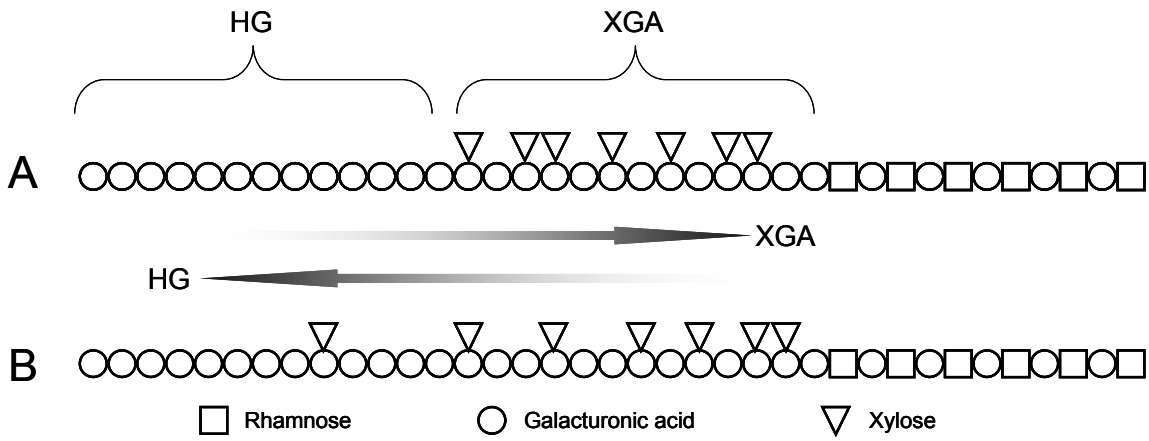

Figure 2: Two possibilities of linking HG via XGA structure to RGI. A) a clear and distinctive border between HG and XGA B) a gradual increase in xylosidation of the galacturonan backbone, when approaching the RGI segment.

EndoPG, indicates that these elements are located in each other vicinity within the pectin molecule (Zandleven, 2006).

The absence of xylose substitutions on the hybrid HG-RGI oligosaccharides, could indicate that also HG structural elements are attached to the non-reducing end of RGI. It has been hypothized that the presence of XGA could be restricted to certain reproductive tissues (Albersheim *et al.*, 1996). The low level of xylose (XGA) in citrus pectin (Ros *et al.*, 1998) indicates that the statement that XGA is present in reproductive tissues is not valid. On the other hand, Zandleven *et al*. (2007) reported the presence of XGA in different plant tissues of *Arabidopsis thaliana*, which indicates that XGA is ubiquitous present in other dicotyledonous plant cell walls in contrast to what was believed previously (Albersheim *et al.*, 1996), albeit sometimes in small amounts. The suggestion that XGA is present on a wider scale in plant cell walls is further substantiated for e.g. white cabbage (Westereng, personal communication).

Positioning of structure elements in relation to RGI

In our structure characterization studies (chapter 2) no sugar fragment was identified that contained a (xylo)galacturonan segment located on the reducing end of the RGI polymer. This could indicate that there are no alternating HG-RGI-HG-RGI segments present in the pectin backbone, as proposed by De Vries *et al.* (1982) and Schols and Voragen (1996). During controlled acid hydrolysis the more stable linkages were partly degraded, which resulted in the release of XGA, HG and RGI type oligosaccharides. The presence of RGI oligosaccharides demonstrates that controlled acid hydrolysis was mild enough to retain a part of the Rha-1,4-GalA in the backbone linkages, which are the most susceptible for acid hydrolysis. Therefore it is expected that putative hybrid RGI-HG oligosaccharide structures would be liberated containing the HG/XGA at the “reducing end” of RGI, if such structures would exist. However, the absence of such hybrid oligosaccharides could be caused by the different susceptibilities of the structure elements for acid hydrolysis. The weaker linkage stability of rhamnose (Rha)-1,4-GalA compared to GalA-1,4-GalA and GalA-1,2- Rha, would result in a lower abundance of oligosaccharides with the RGI segment located on the non-reducing side, which could inhibit detection.

It has been reported that a RGI element is approximately 12 kDa, which is a chain of about 40 Rha-GalA moieties. In addition, a HG element equals about 17.5 kDa, which approximates 100 GalA units (Prade *et al.*, 1999; Yapo *et al.*, 2007; Zhan *et al.*, 1998). Consequently, a RGI-HG element has a Rha:GalA ratio of 1:3.7 However, the published Rha:GalA ratios of pectin from different fruit and vegetable sources are rather different: citrus peel (1:31), apple (1:21), bilberries (1:21), black currant (1:20), sugar beet (1:8),

and soy (1:3.5) (Hilz *et al.*, 2005; Voragen *et al.*, 2001; Yapo *et al.*, 2007). Comparing both the reported ratios with the calculated ratio, it becomes clear that in general an excess of GalA exists (chapter 2; (Yapo *et al.*, 2007)). As a consequence, more HG chains per RGI unit should be present in pectin. Even when applying the alternating model, there would only be a minor increase in possible HG allocation sites. In the following paragraphs it will be discussed how this HG surplus may be allocated in the pectin molecule.

RGII as an alternative accommodation for the HG surplus

Pectin contains, next to HG, XGA, and RGI, another important structural element: rhamnogalacturonan II (RGII). It is known that RGII is allocated within HG elements of the pectin molecule (Ishii & Matsunaga, 2001). Furthermore, two RGII elements can be linked together via a boron ester linkage (O'Neill *et al.*, 2004). To explain the surplus of HG compared to RGI, a pectin structure as schematically represented in figure 3 could be suggested. In this pectin structure, different HG elements are linked together via RGII boron ester linkages resulting in large pectin molecules.

However, for the existence of these HG-RGII-boron ester linkages it is required that a HG element contains two RGII regions (figure 3); in other words the RGII to HG ratio should equal 2:1. In addition, a number of HG segments should be present, without a linkage to RGI. To quantify the level of RGII segments in a pectin mixture, the 2-O-methyl fucose (2-O-Fuc) content is measured, as the RGII monomer contains only one residue of this rare sugar (Darvill *et al.*, 1978; Hilz *et al.*, 2006). In literature, ratios of 1:142 for 2-O-Fuc to GalA have been published for black current pectin (Hilz *et al.*, 2006). As the length of HG elements of various pectins (apple, citrus or beet) described in literature varied between 81-117 GalA residues (M_w 17.5 kDa)(Thibault *et al.*, 1993; Yapo *et al.*, 2007), the 2-O-Fuc to GalA ratio in black current does not equal the 2:117 ratio, which would equal to two RGII units on each HG chain. Although the 2-O-Fuc:GalA ratio of only one pectin source is compared to the HG chain length of other pectin sources, this large discrepancy makes it unlikely that the excess of HG can fit in the proposed HG-RGII-boron crosslinking model. In addition, the release of pectins with a

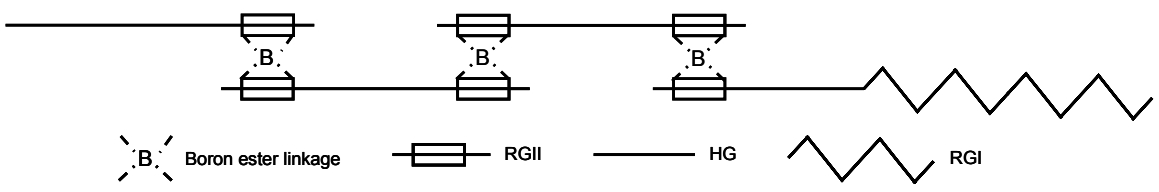

Figure 3: A pectin molecule backbone model in which homogalacturonan (HG) elements are linked to other HG segments or to HG-RGI (rhamnogalacturonan I) segments via rhamnogalacturonan II (RGII) boron ester linkages.

high degree of methyl esterification, thus rather calcium insensitive pectin, with chelating agent may partly be attributed to hydrolysis of the boron diester of RGII that is linked to homogalacturonan with a high degree of methylation (Hilz *et al.*, 2005). However, this chelating agent extraction of pectin does not result in a large decrease in M_w (Hilz *et al.*, 2005), which indicates that the RGII linkage is not involved in connecting relatively small backbone structures. Furthermore, the relatively high molecular weight of dilute alkali extracted pectin (Hilz *et al.*, 2005) indicates that uronyl esters are not involved in the interconnection of the surplus HG/XGA to the RGI backbone.

Both sequential and branched galacturonan substitutions to RGI

In chapter 2 four oligosaccharides were identified of hybrid nature, which contained both a HG and a RGI segment, where the HG segment was located at the non-reducing end. HG was only located as a backbone element at the non-reducing end of Rha, due to the absence of oligosaccharides, carrying the HG segment at the reducing end. But even when HG was located on both sides of RGI, there was a surplus of HG chains, which would advocate for more or longer HG chains per RGI unit (chapter 2, (Yapo *et al.*, 2007)). Another possibility to explain the HG surplus is the presence of HG as side chains attached to RGI of the HG-RGI pectin backbone. Using Nuclear magnetic resonance (NMR) spectrometry approach Mort and Vasu (2007) reported findings supporting this hypothesis. Starting from enzyme digested saponified apple pectin, the presence of an oligosaccharide is reported, which indicates that galacturonan is attached in line to the non-reducing end of RGI, rather similar to the results reported in chapter 2. Furthermore, using the ratio of the linkages as indicated by 1 dimensional proton (^{1}H)-NMR and heteronuclear correlation (HMQC) NMR approaches, the presence of a relative high amount of terminal GalA and some terminal GalA with xylose (Xyl) attached through O-3 is reported (Mort & Vasu, 2007). Since the number of galactose and arabinose moieties is not sufficient to provide for all branched rhamnoses, these terminal (substituted) GalA sugar moieties are assumed to be connected to Rha within the RGI backbone. The number of xylosidated terminal GalA sugars indicates that in apple pectin XGA is positioned close to the RGI backbone, giving further support of the hypothesis that XGA is the galacturonan connected to RGI, where HG could than be linked to XGA.

These findings indicate that pectin consists out of a RGI backbone with XGA segments linked to the RGI non-reducing end *and* to a number of the Rha moieties within RGI. The allocation of HG elements as side chains of the pectin backbone has also been proposed previously by Round *et al*. (1997; Round *et al.*, 2001) who used atomic force microscopy to investigate the nature of long branches attached to pectin.

Combining the observed excess of galacturonan segments and the identification of a linear connection between HG/XGA and RGI (chapter 2), with the results as presented by Mort & Vasu (2007), leads to a pectin backbone model structure as presented in figure 4.

Possible explanation for the presence of linear HG/XGA to RGI linkage

It is not yet known whether pectin is synthesized as one polymer or whether it is assembled from the individual structural elements that become interconnected during or following insertion in the plant cell wall (Mohnen, 2002). The working hypothesis is that the structural elements are synthesized as independent polymers, which are sublocalized in the Golgi apparatus, by different glycosyl transferases (Mohnen, 2002; Staehelin & Moore, 1995).

The identification of xyloglucan transferases in barley, which catalyzes the formation of linkages between xyloglucan and several heterologous receptor substrates (Hrmova *et al.*, 2007a), triggered the possibility that also transferases may exist able to HG/XGA to RGI. Such a transferase fusion activity would most likely involve a polygalacturonase hydrolyzing enzyme, which would join HG to RGI, of which the terminal sugar moiety acceptor would be a GalA, since this would more likely fit in the active cleft of the

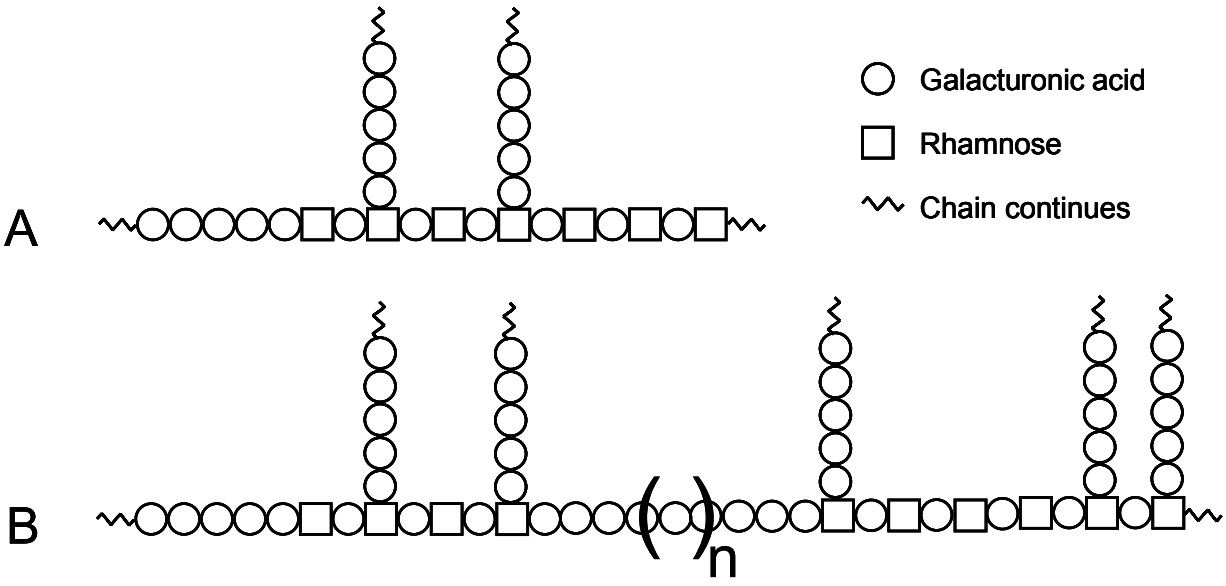

Figure 4: Putative pectin backbone models based on the observed excess of galacturonan segments and the identification of a linear connection between HG/XGA and RGI segments (chapter 2), combined with the data provided by Mort & Vasu (2007). Xylose residues are not included, but it is expected that these are substituted to the GalA sugar moieties close to the RGI backbone to give XGA segments. A) RGI with HG side chains, connected to Rha residues. Furthermore HG is in line attached to the non-reducing end of RGI. B) Sequential backbone of alternating RGI/HG segments with additional HG side chains substituted on Rha residues. The distribution of the HG chains over the RGI segment is not taken into account.

transferase/hydrolase (figure 5). The number of 52 identified open reading frames in *Arabidopsis thaliana* encoding for potential polygalacturonase genes (Benen & Visser, 2003), renders a polygalacturonase variant with a high transferase activity conceivable.

In addition, based on the hypothesis that galacturonans exist as a side chain of RGI, transferase activities are expected, which realize these linkages. Consequently the linkage of galacturonan to the terminal end of RGI might be a side reaction of such putative transferase activity.

Putative transferase activities would explain the presence of galacturonan segments attached in a linear manner towards RGI. For the putative branches transferase or synthesizing enzymes remain to be identified. On the other hand, the homogeneous DP of HG as described by Yapo *et al.* (2007) does not correspond with such putative transferase activity, which is expected to result in a more heterogeneous HG chain length.

The difference between acceptor concentration within the cell wall and in most laboratory conditions would complicate the identification of such synthesizing/transferase enzyme activity. It would be quite a challenge to test different endoPG's at high polysaccharide density for a transferase activity. RGI oligosaccharides could be labeled with a fluorescent probe and incubated with endoPG's in the presence of different HG segments/oligosaccharides, to detect putative hybrid structures emerging with an higher M_w, according to the method of Vincken *et al.* (1998).

Influence of the starting material on the results

The heterogeneity of pectin due to variations in species variations and growth and climate influences is a known factor hampering pectin analysis. The unraveling of pectin' structure is further hold back by the fact that the polymer is too large to analyze as a

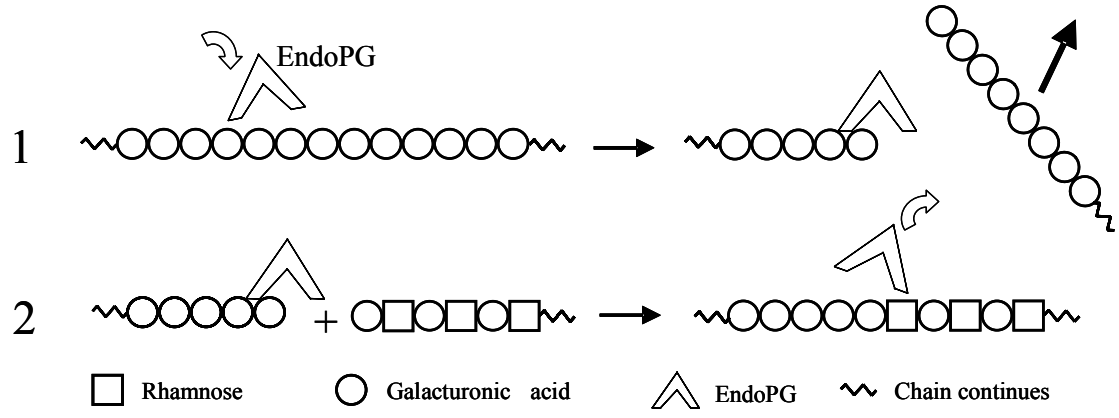

Figure 5: Schematic representation of the effect of a putative transferase activity of EndoPG, analogous to the activity of xyloglucan xyloglucosyl transferase (Hrmova *et al.*, 2007a; Hrmova *et al.*, 2007b). 1) Enzyme hydrolyses HG and retains a part of the polymer; 2) Enzyme transfers retained oligosaccharide onto RGI acting as an acceptor substrate.

whole. To overcome this analytical challenge, pectin is enzymatically degraded in order to fit the degradation products within the M_w range of state-of-the-art techniques, like MS and NMR. However, there remains always a part of the pectin molecule which cannot be specifically degraded. Such enzyme resistant populations were identified in apple, carrot, onion, leek, potato fibre, and soy pectin (Schols *et al.*, 1990)(chapter 4). The relevance of such populations for the pectin structure remains a question mark. Potentially, unknown structures, not accounted for, can attribute to false assumptions about the build-up of pectin structure, or withhold information which could possible explain other observations. The presence of Rha-(1→2)-Rha linkages, as reported by Gao *et al.* (1990) could for instance be related to the ratio GalA:Rha 1:0,76 in soy pectin.

Until now, a model based on only part of the sample material has been effective in describing pectin behavior. A high M_w pool obtained after RGH degradation of apple MHR was used to construct this pectin model described by Schols and Voragen (1996). Therefore, one could argue that the pectin model does not cover a structure of true pectin. On the other hand, the sugar linkage composition, molecular weight profiles and enzymatic degradability of pectins from different sources often show similar trends. Unusual results can commonly be explained by the absence of particular structure elements in one source, or by an alternative distribution of the different substitutions or structural elements.

Since characterization of the low M_w undegradable pectin population it is not feasible without having novel enzymes, it is (almost) impossible to make a model suitable for all types of (un)degradable pectin. Therefore, there is no other option, but to consider that we are not looking at the entire pectin molecule. Consequently, new enzymatic and chemical methods should be developed, which enable the investigation of undegradable pectin structures. These methods are expected to generate complex mixtures of fragments. Therefore, new approaches are needed to efficiently analyze these complex mixtures.

Possibilities and limitations of CE-LIF-MS^n

Chapter 4 describes the effective separation of differently substituted galacturonic acid containing oligosaccharides by CE-LIF analysis. By adapting this approach on line MS detection was attained, which enabled the localization of (non) sugar substituents, using nmol/mL sample quantities. Although this method was successful in the annotation of oligosaccharides present in complex RGI and XGA digests, as well as in an acid hydrolysate of Apple MHR, the observed resolution was unsatisfactory when analyzing enzyme digests or hydrolysates, which included methyl esterified GalA oligosaccharides.

In order to get an enhanced separation of different sub groups of acidic oligosaccharides, other CE-LIF-MS^n approaches were used then described in chapter 3.

First different labels were used, to be able to differentiate in the contribution of the moderate negatively charged carboxyl group of GalA on the separation, which was mainly based on the three SO_3 groups in the APTS label. The alternative labels contained 0, 1, and 2 sulfonic acid groups, in combination with an amidated conjugated system, which enabled UV detection as well as coupling the label to the reducing end of the oligosaccharide. The CE-UV detection was successful for the 1^- and especially for the 2^- charged label, which is as well fluorescent. However, when performing CE-MS^n it was not possible to obtain a stable ionspray and ion current. Nevertheless, when using standards this CE-UV approach could be useful for quantitative analysis.

As an alternative it is possible to vary in pH, buffer composition, buffer concentration, and capillary coating, since oligosaccharides will react differently to these changes, which possibly enhance their separation. Using this approach oligosaccharides could be separated, which were co-eluting performing CE-UV-MS^n as described in chapter 3. The method could be further improved by implementing LIF-detection within the CE-MS setup of APTS labeled oligosaccharides in order to perform CE-LIF-MS^n, since this gives sharper peak shapes and improved detection. The capillary coating influences the electro-osmotic flow (EOF), and is therefore of influence on the separation. Reducing the EOF by using coated fused silica capillary instead of regular fused silica could further improve this method. Possibly this could result in a stable current and ion spray, enabling CE-MS for alternative labels. Furthermore, CE-LIF-MS^n, enables quantitative (iontrap-MS) analysis of various components independent of dialysis, digestion, or (bio)synthesis procedures.

Selective chemical β-eliminative degradation

Although the β-eliminative method of Deng *et al.* (2006) was successful in liberating different neutral sugar side chains, which were previously not accessible, the method has also some drawbacks. Using this approach a small amount of galactose and arabinose series were visible in some Maldi-tof mass spectra. These series were as well present in small amounts in β-eliminative degraded potato galactan and sugar beet arabinan, used as controls. The results from the sugar composition revealed that these neutral sugars were present in low amounts in the low M_w populations, but abundantly in the Maldi-tof spectra of these samples. There are two possible explanations for this phenomenon; firstly, these oligosaccharides could originate from autolysis, caused by the high temperature during the β-eliminative treatment; secondly, there are linkages present within the neutral sugar side chains, which are labile for β-eliminative treatment.

After β-eliminative degradation of enzyme resistant polysaccharide pools, still a substantial part (~40%) is not degraded to low molecular weight fragments, being

accessible for analysis by HPAEC, CE-MS, ESI-MS^n or Maldi-tof MS. The sugar composition of this resistant part reveals that this population contains a relative large proportion of neutral sugars. Sterical hindrance, caused by these neutral sugar side chains could be a possible explanation for the resistance of these populations towards β-elimination. Alternatively, the low GalA content of these enzyme resistant polymers in relation to the neutral sugar side chain length could as well explain this high M_w population. An interesting follow-up experiment would be periodate oxidation, which specifically retains (1-3)-linked hexopyranosyl residues and produces specific degradation products for different linkage types (Perlin & Derek, 2006). Alternatively, the arabinose could be specifically removed via hydrolysis by 50-100 mM oxalic acid at 80-100 ^oC for 1 hour (Chambat *et al.*, 1984; Chanda *et al.*, 1950).

Bioactivity

Polysaccharides such as pectins, have been found to contain an immunomodulating activity (Yamada & Kiyohara, 2007). The primary function of the immune system is to protect the host from infectious microbes in its environment (Chaplin, 2006; Yamada & Kiyohara, 2007). The innate immune system includes all defense mechanisms comprising soluble proteins and bioactive small molecules, like complement proteins (Hancock *et al.*, 1995; Steinke & Borish, 2006; Yamada & Kiyohara, 2007). The complement system is composed of a group of structurally distinct pro-enzymes present in the blood plasma in an inactive form (Yamada & Kiyohara, 2007). The complement proteins can be activated through three cascade pathways, the classical, alternative and the lectin pathway (Ikeda *et al.*, 2005; Zheng *et al.*, 2006). Pectin has been suggested to mainly activate the complement system via the classical pathway and alternative pathway (Leung *et al.*, 2006; Michaelsen *et al.*, 2000). The classical pathway is presumed to be activated through C1q, a protein which has globular heads with the ability to interact with carbohydrates (Gaboriaud *et al.*, 2003; Matsushita *et al.*, 2004). Alternatively, *Bupleunum falcatum* pectin has interaction with IgG antibodies. IgG depleted serum gives reduced activity compared to normal serum, which indicates that there is not necessarily direct interaction between pectin and C1q (Kiyohara *et al.*, 2006).

Especially pectic polymers containing AG-II structural units with 1,3,6-branched or 6-linked gal residues exhibit complement fixing activity. There are, however, exceptions of other active carbohydrates like e.g. arabinans. Furthermore, not all polysaccharides containing an AG-II structure have revealed effects on the complement system (Alban *et al.*, 2002; Westereng, 2007; Yamada & Kiyohara, 1999, , 2007).

Chapter 4 demonstrated the successful isolation of single side chains obtained from RGI rich pectic structures from white cabbage. Arabinan, attached to a rudimentary RGI

fragment, was indicated as the complement fixing polysaccharide structure in Brassica pectin. The results of the complement fixing activity strongly indicated that a minimal unit size is necessary for complement activation, which is in agreement with literature, where a large dependence on pectin source and structure is reported in order to possess a complement stimulating or suppressing activity (Yamada & Kiyohara, 2007). Kweon *et al.* (2003) and Suzuki *et al.* (2003) obtained results that next to the polymer size also the three dimensional structure might be of importance for bioactivity.

Future perspectives

Pectin is an important ingredient in the food industry as well as a key constituent of the plant cell wall. (Daas *et al.*, 2001; De Vries, 2004; May, 2000; Schols & Voragen, 1996; Voragen *et al.*, 1995). Pectin is also of interest for the pharmaceutical industry due to its health promoting effects and because of its ability to modify surfaces of medical materials. (Becker *et al.*, 2006; Guggenbichler-J-P *et al.*, 1997; Kastner *et al.*, 2002; Lim *et al.*, 1997; Morra *et al.*, 2004; Torto, 1998; Yamada *et al.*, 2003).

One thing is certain, in all of these applications the structure is responsible for the observed effect. The identification of hybrid oligosaccharides, which locate galacturonan segments on the non-reducing end of RGI combined with the recognition of the surplus of HG, and its consequent allocation as side chain attached to Rha residues as suggested by the results of Mort and Vasu (2007) brings us one step closer for describing the correct pectin backbone structure.

The development of CE-LIF-MS^n for charged oligosaccharides introduces a new tool for the structure elucidation. The combination of this method, with the selective degradation of RGI enables the characterization of structures, which were previously not accessible for analysis. Consequently, the methods described in this thesis improve the possibilities for the structure elucidation of a broad range of polysaccharides, and therefore a better understanding of its different applications.

In order to gain more structural knowledge on pectin and other polysaccharides, it would be useful to “combine the combined approaches” described in this thesis, with other chemical and novel enzymatic degradation methods and by implementing these methods more quantitatively.

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Summary

Samenvatting

Summary

Pectin is one of the major plant cell wall components and probably the most complex macromolecule in nature, as it can be composed out of as many as 17 different monosaccharides containing more than 20 different linkage types. Pectin is used in foods mainly as gelling, stabilizing, or thickening agent in products such as jam, yoghurt drinks, fruity milk drinks, and ice cream. Furthermore, pectin increasingly gains interest as a possible health promoting polysaccharide. Knowledge on the precise structure of pectin would attribute to the understanding of its physiological function in the plant and to the further optimization of the industrial and medical applications of the polymer. The aim of the research described in this thesis was to develop new approaches to identify linkages which are involved in the connection of different structural pectin elements. Identification of these linkages would lead to an improved understanding of pectin structure.

In order to get more insight in the interlinkage between the various structural elements of pectin, apple pectin modified hairy regions (MHR) were degraded by controlled acid hydrolysis and a mixture of oligomers was obtained. Due to the selective removal of neutral sugars during these treatments, this oligosaccharide mixture was enriched in galacturonic acid (GalA) and rhamnose (Rha) content. High performance anion exchange chromatography (HPAEC) at pH 5 in combination with matrix assisted laser desorption/ionization – time of flight mass spectrometry (Maldi-tof MS) revealed that this controlled acid hydrolysate consisted out of rhamnogalacturonan I (RGI), homogalacturonan (HG), and xylogalacturonan (XGA) type oligomers next to neutral sugars. Furthermore, hybrid oligomer structures consisting out of HG/XGA and RGI segments were identified, which indicated that these are the linkages between HG/XGA and RGI structural elements. After sequencing these structures ($GalA_3Rha$, $GalA_4Rha_2$, $GalA_5Rha_3$, $GalA_6Rha_3$, and $GalA_6Rha_3Xyl_1$) using mass spectrometric techniques, it was shown that the position of the RGI block was always at the reducing end of these oligomers. Both HG and XGA segments were α-(1→2) linked to RGI, as was indicated by enzymatic degradation using pure and well characterized enzymes and NMR spectroscopy.

As HPAEC is incompatible with on-line mass spectrometric detection and off-line MS characterization of collected HPAEC fractions was quit laborious, a new method was explored which coupled capillary electrophoresis (CE) to iontrap MS to further elucidate the fine structure of charged oligosaccharides. An effective separation of differently substituted GalA containing oligomers was obtained by labeling oligomers present in complex mixtures, followed by low pH CE-Laser induced fluorescence (LIF) analysis. The label used was 8-aminopyrene-1,3,6-trisulfonate (APTS) which introduced a charge

needed for CE separation and marked the reducing end of each oligomer. By adapting the buffer and capillary of the original CE method on-line MS detection was enabled. Moreover, with MS^n it was possible to localize sugar residues substitutions. With this combined CE-MS approach signals in electropherograms of complex XGA and RGI digests could be annotated.

Recently, pectin gained interest as a health promoting polymer. In order to understand this health promoting effect, the structure containing the physiological activity of pectin needs to be elucidated. It has been suggested that the structure responsible for physiological activity contains 1,3- and 1,3,6-linked galactans. The smallest physiological active carbohydrate reported to our knowledge is an arabinogalactan with an estimated size of 5 kDa. Since it is unclear which specific polysaccharide structure is responsible for the bio activity, research is needed to characterize the specific architecture of the pectic neutral sugar side chains. Therefore, single side chains from white cabbage pectin, ranging from degree of polymerization (DP) 1 to 30, were isolated by applying mild chemical conditions promoting β-elimination. The pectin fragments were characterized by their molecular weight, sugar composition, and by ^{13}C NMR and Maldi-tof MS analysis, which revealed that these side chains were almost pure arabinans or galactans. No evidence was obtained for side chains composed of a combination of galactose and arabinose moieties (arabinogalactans; AG) and hence, no AGI/AGII structures could be positively identified. Fractions were measured for complement-fixing activity in order to determine their ability to trigger immune reactions. These tests strongly indicated that there was a minimal unit size responsible for complement activation. Neutral chains (~8 kDa) obtained from β-elimination were inactive, despite the fact that they contained a sugar composition previously shown to be highly active. Larger chains (~17 kDa) isolated from β-eliminative treatment retained some activity, but much lower than polymers containing intact RGI. This implied that structural elements containing multiple side chains expressed higher complement fixing activity. Furthermore, the results indicated arabinan, possibly connected connected to a rudimentary RGI backbone, to be the complement fixing polymer in *Brassica* pectin.

Using $CE\text{-}MS^n$, it was possible to mass-characterize degradation products of enzyme resistant soy pectin polymers obtained after beta elimination and separation using size exclusion chromatography. Using CE-LIF and Maldi-tof MS, a broad range of complex oligomers (DP 3 to 12) was shown to be present. In addition, $CE\text{-}MS^n$ analysis identified a number of oligomers consisting out of an unsatGalA-Rha “backbone” fragment, where Rha was substituted with short side chains. The high molecular weight fraction indicated that not all side chains were released, possibly due to sterical hindrance by these chains during the methylation reaction. Using this combined method, detailed information on the

neutral sugar side chain structure and distribution was obtained of some enzyme resistant structural elements of pectin, demonstrating RGI stretches with single Gal substitution.

The new approaches described in this thesis allowed characterization of oligosaccharides involved in the connection between different structural pectin elements. Based on a combination of the obtained results with literature data, an adapted pectin model was proposed to explain the surplus of HG chains, which would advocate for more or longer HG chains per RGI unit. To allocate these side chains HG/XGA structural elements both were positioned as a side chain of the Rha within RGI as well as on the non-reducing end of RGI.

The methods developed, together with the ideas we have now obtained up to now on the pectins architecture, can now be used to investigate complex pectin structures responsible for both techno- and bio-functional properties not understood so far.

Samenvatting

Pectine is een van de belangrijkste planten celwandcomponenten en waarschijnlijk het meest complexe macromolecuul in de natuur, aangezien het uit maar liefst 17 verschillende monosacchariden is opgebouwd die op meer dan 20 verschillende manieren verbonden kunnen zijn. Pectine wordt in levensmiddelen vooral gebruikt als geleermiddel, stabilisator of verdikkingsmiddel in producten zoals jam, yoghurtdranken, vruchten-zuiveldranken en ijs. Daarnaast is er in toenemende mate interresse in het mogelijk gezondheidbevorderend effect van dit polysaccharide. Kennis van de exacte structuur zal bijdragen aan het begrip van de fysiologische functie van pectine in de plant en aan een verdere optimalisering van industrieële en medische toepassingen van dit polymeer. Het doel van het in dit proefschrift beschreven onderzoek is het ontwikkelen van nieuwe benaderingen om bindingen te identificeren die betrokken zijn in de koppeling tussen verschillende structuurelementen van pectine. Identificatie van deze bindingen zal leiden tot een groter inzicht in de pectinestructuur.

Om meer inzicht te krijgen in de koppelingen tussen verschillende structuurelementen van pectine werden gemodificeerde “hairy regions” (MHR) uit appelpectine met behulp van gecontroleerde zure hydrolyse afgebroken. Door deze hydrolyse werden selectief neutrale suikers verwijderd, waardoor een oligosaccharide mengsel verrijkt in galacturonzuur (GalA) en rhamnose (Rha) werd verkregen. High performance anion exchange chromatography (HPAEC) bij pH 5 gecombineerd met Matrix assisted laser desorption/ionisation – time of flight mass spectrometry (Maldi-tof MS) toonde aan dat dit hydrolysaat bestond uit rhamnogalacturonaan I (RGI), homogalacturonaan (HG) en xylogalacturonaan (XGA) type oligomeren naast neutrale suikermonomeren. Bovendien werden hybride oligomeren geïndentificeerd die bestonden uit zowel HG/XGA als RGI segmenten, wat erop wijst dat HG/XGA en RGI structuurelementen aan elkaar gekoppeld zijn. Van verschillende GalA-Rha oligosacchariden uit het mengsel ($GalA_3Rha$, Gal_4Rha_2, $GalA_5Rha_3$, $GalA_6Rha_3$ en $GalA_6Rha_3Xyl_1$) werd met MS de exacte structuur bepaald, waaruit bleek dat het RGI-blok zich aan het reducerende eind van deze oligomeren bevond. Ook werd met behulp van enzymatische afbraak door zuivere en goed gekarakteriseerde enzymen in combinatie met NMR spectroscopie aangetoond dat HG en XGA segmenten beiden α-(1→2) gekoppeld zijn aan RGI.

Aangezien HPAEC niet direct gekoppeld kan worden aan de massaspectrometer en indirecte karakterisering van opgevangen HPAEC fracties vrij bewerkelijk is, werd een nieuwe methode onderzocht waarbij capillaire electrophorese (CE) gekoppeld werd aan iontrap MS, zodat de fijnstructuur van geladen oligosacchariden kon worden opgehelderd.

Galacturonzuur bevattende oligomeren met verschillende substituties werden effectief uit complexe oligomeermengsels gescheiden door middel van CE-laser geïnduceerde fluorescentie (LIF) bij lage pH. Voor deze CE-analyse werden de oligomeren gelabelled met 8-aminopyreen-1,3,6-trisulfonaat (APTS) wat een lading introduceerde, nodig voor de CE-analyse, aan het reducerende eind van elk oligomeer. Directe MS-detectie bleek mogelijk na aanpassing van de originele CE-methode met betrekking tot buffer en capillair. Met deze gecombineerde CE-MS-methode konden pieken in electroferogrammen van complexe XGA/RGI oligomeermengsels worden benoemd. Bovendien was het mogelijk met MS^n suikerresidue substituties te lokaliseren.

Momenteel is er een toenemende belangstelling voor pectine als gezondheidbevorderend polymeer, maar de kennis over het mechanisme achter deze fysiologische activiteit is beperkt. Gesuggereerd wordt dat 1,3- en 1,3,6-verbonden galactanen verantwoordelijk zijn voor deze activiteit. Het kleinste beschreven fysiologisch actieve pectine is, zover wij weten, een circa 5 kDa groot arabinogalactaan. Aangezien het onduidelijk is welke polysaccharidestructuur specifiek verantwoordelijk is voor de bio-activiteit, moet meer onderzoek worden verricht naar de specifieke opbouw van de neutrale suikerketens in pectine. Daartoe werden op witte kool (*Brassica*)-pectine milde chemische condities toegepast die β-eliminatieve ketenafbraak bevorderen, waardoor enkelvoudige zijketens van het pectine werden afgesplitst die variëreerden in polymerisatiegraad (DP) van 1 tot 30. Deze pectinefragmenten werden gekarakteriseerd op molecuulgewicht en suikersamenstelling en tevens met behulp van ^{13}C NMR en Maldi-tof MS geanalyseerd. Hieruit bleek dat deze zijketens uit bijna zuivere arabinanen en galactanen bestonden, terwijl zijketens bestaande uit een combinatie van galactose en arabinose (arabinogalactanen; AG) niet aanwezig leken. Vervolgens werden de verschillende fracties getest op hun vermogen om immuunreacties te initiëren, de zogenaamde complement fixerende activiteit. Een sterke indicatie werd gevonden dat een minimale molecuulgrootte is vereist voor deze activatiteit. Verder bleken neutrale zijketens (~8 kDa) geen activiteit te vertonen, ondanks hun overeenkomstige suikersamenstelling met structuren die in het verleden wel een grote activiteit vertoonden. Grotere zijketens (~17 kDa) toonden wel enige activiteit, maar deze was veel lager dan de gevonden activiteit van intacte RGI polymeren. Dit duidt erop dat structuurelementen die bestaan uit meerdere zijketens een hogere complement fixerende activiteit vertonen. Bovendien geven de resultaten aan dat verschillende arabinaan zijketens, verbonden via RGI stukken, verantwoordelijk zijn voor (een gedeelte van) de immunologische activiteit die aanwezig is in *Brassica* pectine.

Met $CE\text{-}MS^n$ konden β-eliminatieve afbraakproducten van enzym-resistente sojapectine polymeren na chromatografische scheiding op molecuulgrootte

gekarakteriseerd worden. De afbraakproducten bestonden onder andere uit complexe oligomeren (DP 3-12), wat door middel van CE-LIF en Maldi-tof MS werd aangetoond. Daarnaast werd met CE-MS^n een onverzadigd GalA-Rha hoofdketenfragment aangetoond, waarbij de Rha gesubstitueerd was met korte zijketens. De aanwezigheid van een hoog-moleculaire fractie duidde erop dat niet alle zijketens waren afgesplits wat waarschijnlijk werd veroorzaakt door sterische hindering door deze zijketens tijdens de methyleringsreactie. Door gebruik te maken van verschillende gecombineerde technieken werd gedetailleerde informatie verkregen over de structuur en opbouw van neutrale suikers van een aantal enzym-resistente structuurelementen van pectine en werd de aanwezigheid van RGI reeksen met enkelvoudige GalA-substitutie aangetoond.

De in dit proefschrift beschreven nieuwe analytische aanpak stellen ons in staat om oligosacchariden te karakteriseren die betrokken zijn bij de verbinding tussen verschillende structuurelementen van pectine. Op basis van de nieuw verkregen inzichten, in combinatie met literatuurgegevens stellen wij aanpassing van het huidige pectinemodel voor, waarbij de hoeveelheid HG-ketens per RGI segment wordt vergroot. In dit aangepaste model kunnen HG/XGA structuurelementen zowel als zijketen van Rha worden gepositioneerd als ook linear in de hoofdketen, verbonden via het niet-reducerende einde van RGI.

De ontwikkelde methoden, gecombineerd met de tot dusver opgedane inzichten over de opbouw van pectine, kunnen worden toegepast in onderzoek gericht op de opheldering van techno- en biofunctionele eigenschappen van complexe pectinestructuren.

Nawoord

Zeg nooit *nooit*. Na het afronden van mijn 2e afstudeervak wist ik toch wel héél erg zeker dat ik genoeg had gezien van de universiteit. Toen ik werd benaderd door Jean-Paul Vincken voor een AIO positie, wees ik dit aanbod resoluut van de hand. Het zaadje was echter geplant en zoals je hebt gemerkt is het uitgegroeid tot het proefschrift wat je in je hand houdt. Tijdens het verloop van mijn promotieonderzoek heb ik veel geleerd van en plezier beleefd met *al* mijn collega's. Een aantal mensen wil ik in het bijzonder noemen.

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In gezelschap van StephG, Joris, Yvonne,Taj en talloze studenten werden op Lab 526 het leeuwendeel van de experimenten uitgevoerd. Zonder de standaarden, enzymen en trucs die onder elkaar werden uitgewisseld, zou het promoveren niet zo voorspoedig zijn verlopen.

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Gerd-Jan

Curriculum vitae

Gerardus Johannes (Gerd-Jan) Coenen werd geboren op 23 maart 1978 te Horst. In 1997 behaalde hij het VWO-diploma aan het Dendron college te Horst, waarna hij in datzelfde jaar begon met de studie levenmiddelentechnologie aan de toenmalige Landbouwuniversiteit Wageningen. Hij specialiseerde zich in de richtingen Levensmiddelenmicrobiologie en Levensmiddelenchemie. In 2001 liep hij stage aan de University of Arkansas (Fayetteville, Arkansas, U.S.A.) en het jaar erna bij de research and developement afdeling van H.J. Heinz in Elst. In augustus 2002 behaalde hij zijn ingenieursdiploma aan Wageningen Universiteit. Van oktober 2002 tot augustus 2007 voerde hij een promotieonderzoek uit bij de leerstoelgroep Levensmiddelenchemie van Wageningen Universiteit; de resultaten hiervan zijn beschreven in dit proefschrift. Sinds oktober 2007 is hij werkzaam als postdoc in dienst bij de leerstoelgroep Levensmiddelenchemie van Wageningen Universiteit.

List of publications

Full papers

Oosterveld, A., Coenen, G. J., Vermeulen, N. C. B., Voragen, A. G. J. & Schols, H. A. (2004). Structural features of acetylated galactomannans from green Coffea arabica beans. *Carbohydrate Polymers, 58* (4), 427-434.

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Book Chapter

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Coenen, G.J., Schols, H.A.. & Voragen, A.G.J. (2004). Unraveling the coupling of structural pectin elements. In *X Cell Wall Meeting*. p.107. Sorrento, Italy.

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Overview of completed training activities

Discipline specific activities

Courses

Glycosciences Summer School (Wageningen, June 2004)

Advanced Food Enzymology (Wageningen, February 2005)

Meetings

CRC-W Meetings (Wageningen, 2002-2007)

Food Chemistry Symposium (Hamburg, Germany, 2004)

X Cell Wall Meeting (Sorrento, Italy, 2004)

Plant Cell Wall Polysaccharides (Leuven, Belgium, October 2004)

Food Chemistry Symposium (Osaka, Japan, December 2004)

XI Cell Wall Meeting (Copenhagen, Denmark, August 2007)

General Courses

Scientific writing (Wageningen, 2002)

Vlag PhD Introduction Days (Bilthoven, The Netherlands, 2002)

Course on preparation for Performance and Development Evaluation (Wageningen, 2004)

LCQ Operations Course (Hemel Hempstead, United Kingdom, June 2004)

Waters purification seminar (Almere, 2005)

Career Assessment (Wageningen, 2006)

Food Chemistry Colloquia (2002-2007)

Food Chemistry Seminar (Wageningen, 2002-2007)

Additional activities

Preparation of the PhD research proposal

Food Chemistry PhD excursion to Japan, Member of Organizing Committee, Dec. 2004

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Structural characterization of native pectins

Gerd-Jan Coenen

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