NN08201, 2707

Stellingen

1. Pectine uit sojabonen blijkt uit alleen hoogvertakte segmenten (hairy regions) te bestaan, de onvertakte homogalacturonaan segmenten (smooth regions) die tot nu toe in celwanden van andere onderzochte plantenweefsels van dicotylen voorkomen ontbreken. (dit proefschrift)

2. Onderzoekers proberen de resultaten van onderzoek naar celwandpolysachariden altijd te vertalen naar universele modellen. De waarde van deze modellen is echter beperkt en geldt alleen voor dat specifieke weefsel van de onderzochte plantensoort. (dit proefschrift)

3. Het aantal suikersubstituenten per 100 xylose-eenheden in de hoofdketen is een betere maat voor de substitutiegraad van arabinoxylanen dan de verhouding tussen de hoeveelheid arabinose en xylose. (dit proefschrift)

4. De verzelfstandiging van scholieren dankzij het studiehuis leidt tot nieuwe aanpassingsproblemen in de steeds schoolser wordende universiteiten.

5. De zoektocht naar beschikbare verloskundigen is een zware bevalling.

6. Door het constante gebruik van de mobiele telefoon is de bereikbaarheid juist afgenomen.

7. Als namen binnen een organisatie sneller veranderen dan de productietijd van het briefpapier met logo is er sprake van een identiteitscrisis.

8. Als mannen ook verplicht waren verlof op te nemen na de bevalling zouden zij eraan gewend raken een groter deel van de zorg voor de kinderen op zich te nemen.

Stellingen behorende bij het proefschrift

Elucidation of the chemical fine structure of polysaccharides from soybean and maize kernel cell walls

M.M.H. Huisman

Wageningen, vrijdag 3 maart 2000

Elucidation of the chemical fine structure of polysaccharides from soybean and maize kernel cell walls

Promotor: Prof. dr. ir. A.G.J. Voragen
hoogleraar in de levensmiddelenchemie

Co-promotor: Dr. ing. H.A. Schols
universitair docent
departement Agrotechnologie en Voedingswetenschappen

2727

Elucidation of the chemical fine structure of polysaccharides from soybean and maize kernel cell walls

Miranda M.H. Huisman

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
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in het openbaar te verdedigen
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VOORWOORD

Dit proefschrift had nooit geschreven kunnen worden als niet een heleboel mensen daaraan een steentje hadden bijgedragen. Een aantal mensen wil ik graag met naam noemen.

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Alle collega's van lmc, jullie hebben gemaakt dat ik met veel plezier heb gewerkt. Niet alleen de sfeer op de werkvloer, maar ook de gezellige labuitjes, AIO-reisjes, congresbezoeken etc. hebben daaraan bijgedragen. Jean-Paul labgenoot van het eerste uur, jij hebt me laten zien hoe leuk onderzoek kan zijn. Ook alle andere labgenoten van 504 en 533 (Olga, Margot, Toos, Kavitha, Riet, Ahmed, etc.) bedankt voor alle gezelligheid. Lex mijn vraagbaak bij computerproblemen en mede-Disneyfan. Mijn kamergenoten, Willemiek en Esther, wij hadden (nog steeds trouwens) een geweldig theehok met z'n drieen. Er was altijd tijd voor het bediscussiëren van vragen of ideeën en zo kwamen we altijd wel tot (een) antwoord(en). Ook de hulp die andere mensen in het Biotechnion op een of andere manier hebben geboden in de afgelopen jaren werd zeer gewaardeerd.

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ABSTRACT

Huisman, M.M.H. Elucidation of the chemical fine structure of polysaccharides from soybean and maize kernel cell walls.

Ph.D. thesis. Wageningen University, Wageningen, The Netherlands, 2000

Key words: *Glycine max*, *Zea mays*, homogalacturonans, rhamnogalacturonans, xylogalacturonans, arabinogalactans, pectic substances, xyloglucans, glucuronoarabinoxylans

In soybean cell wall material, pectic substances are the major non-starch polysaccharide. These pectic substances distinguish themselves from pectic substances of cell wall material from other plants in the absence of homogalacturonan, the presence of fucose residues in the xylogalacturonan, and two uncommon structural features of the pectic arabinogalactan side chains, namely the presence of internal (1,5)-linked arabinofuranose and terminal arabinopyranose. Therefore, these pectic substances are rather resistant to degradation by both established (like polygalacturonase) and novel (like RG-hydrolase) pectic enzymes. The hemicellulosic polysaccharides in the soybean cell wall appeared to be predominantly xyloglucans, composed of XXXG-type building units like most legume xyloglucans.

In the cell wall material from maize kernels, glucuronoarabinoxylans are the major non-starch polysaccharides. The glycosidic linkage composition of the extracts and their resistance to endo-xylanase treatment indicated that the extracted glucuronoarabinoxylans were highly substituted. The same conclusion could be drawn from their degree of substitution (87%), defined as the number of sugar substituents per 100 xylose residues in the backbone. The glucuronoarabinoxylans from maize kernel cell walls appeared to be more complex than those from sorghum cell walls, which were the most complex glucuronoarabinoxylans described so far.

The uncommon structural features of soybean cell wall pectic substances and the complexity of maize kernel cell wall glucuronoarabinoxylans explain their resistance to degradation by enzymes generally used to degrade this kind of polymers, and indicates that a search for new enzymes is required to enable enzymatic modification of these polysaccharides.

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CHAPTER 1

INTRODUCTION

BACKGROUND

This thesis forms part of a larger research project on the elucidation of the chemical fine structure of polysaccharides from plant cell walls directed to their functional properties, funded by the Dutch technology Foundation (NWO/STW). The plant cell wall polysaccharides play important roles in the processing of the plant materials to food and feed. The determination of the structure of polysaccharides usually includes the isolation of the polysaccharides from their original source by removal of other constituents like protein, fat, and starch, without modifying or removing a part of these polysaccharides. Subsequently, they can be characterised by determination of their sugar composition, glycosidic linkage composition, and establishing the presence of substituents (*e.g.* acetyl, methoxyl, feruloyl, and coumaryl groups). NMR spectroscopy and mass spectrometry are used in the analysis of not too complex polysaccharides or polysaccharide fragments. Chemical and specific enzymatic degradation is used to obtain fragments that fit within the analytical range of NMR and MS analyses. This strategy is shown in Figure 1.1. NMR spectroscopy and mass spectrometry were performed at the Bijvoet Centre of the University of Utrecht. Based on the obtained knowledge of polysaccharide structures, it was tried to get a better understanding of the digestibility and the nutritional value of soybean meal and maize polysaccharides in feed applications (Department of Animal Nutrition, Wageningen University).

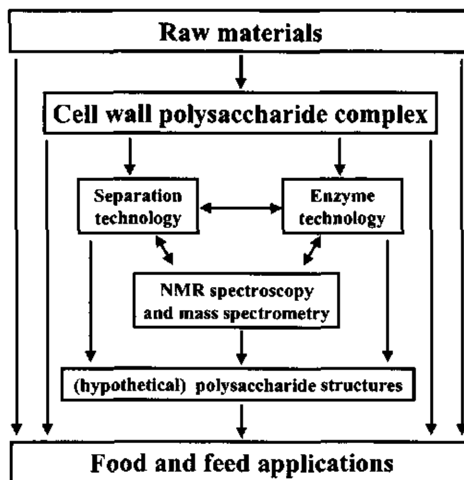

Figure 1.1. Scheme of techniques and data flow within the research project.

In this research project, attention is focussed on cell wall polysaccharides of two plant species, soy and maize. The two species investigated represent different taxonomic groups, soybean belonging to the dicotyledonous and maize to the monocotyledonous plants. Besides

containing two very important structures present in cell wall material, these raw materials are also of great importance in food and feed industry.

Soybean meal and maize by-products from the wet milling process are important agricultural by-products. These by-products are enriched in proteins and cell wall polysaccharides and are used in livestock feeds. At present, little information is available about the chemical structure of the individual cell wall polysaccharides in relation to their digestibility/fermentability and physiological action in the gastrointestinal tract. A number of effects of intact cell walls are the limited accessibility of nutrients within the cellular matrix of the plant, the limited degradation of the cell wall itself, and physical effects (particularly increased viscosity) of the cell walls in the small and large intestines[1]. Knowledge of the polysaccharide structure is needed for the selection of the appropriate tools for modification of feed ingredients. These pre-treatments can optimise the uptake of the feed and improve the well-being of the animals.

Soybean meal can also be used as a raw material for the production of soy protein isolate. Isolated soy protein is used in comminuted or emulsified meat products, in the baking industry, in (milk-free) infant formulas and food, and meat analogue products. In a number of cases, the purity of the protein product is desired to be above 90%. Knowledge of the polysaccharide structure is needed to select the enzymes able to remove polysaccharides from the protein fraction.

A third reason for the interest in the structure of the polysaccharides from soybean meal and maize kernels is their application as novel ingredients in foodstuffs, possibly after (enzymatic) modification.

In short, this research project focussed especially on the identification and characterisation of cell wall polysaccharides in relation to functional properties, enzymatic modifications to improve these properties, polysaccharide degradation by enzymes, detection of structural barriers limiting enzyme action and identification of enzyme activities with potential to overcome these limitations.

THE SUBSTRATES: SOYBEAN MEAL AND MAIZE KERNELS

Soybeans (*Glycine max*) belong to the pea family of the Leguminosae. They are the world's most important oilseed crop and a staple food of the Orient. World production in 1990 totalled 107 millions metric tons. Although indigenous to the Far East, it is now cultivated elsewhere – particularly in the United States[2].

The soybean seed consists of three basic parts: (1) the seed coat, which protects the embryo from fungi and bacteria before and after planting; (2) the embryo; and (3) the cotyledons, which account for most of the bulk and weight (90%) of the seed and contain

nearly all the oil and protein. Early in the maturity of the cotyledons, starch granules are predominant, but they decrease to less than 1% of weight as beans mature[2,3].

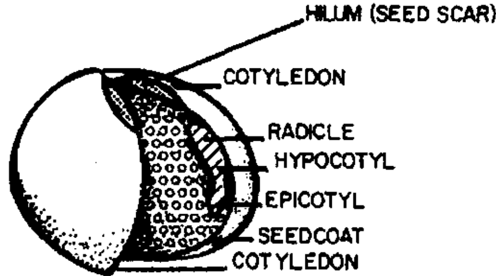

Figure 1.2. The soybean seed[2].

Soybeans are processed primarily to obtain (1) oil for use in shortenings, margarines, and salad dressings; (2) soybean protein products for direct human consumption; and (3) soybean meal for use as a protein supplement for livestock. The protein of soybean meal is of better quality than other protein-rich supplements of plant origin, because of its well-balanced amino acid profile[2].

As a first approximation soybeans contain 20% lipid, 40% protein, 35% carbohydrate, and 5% ash on a dry weight basis. Considerable variability exists in these numbers, depending on the cultivar and the growing conditions. The carbohydrates consist of soluble and insoluble carbohydrates. Insoluble carbohydrate includes pectin, cellulose and hemicellulose that largely form the cell walls in soybean cotyledons. This fibre component is more prevalent in soybean seed coats (hulls) than in cotyledons. The soluble carbohydrate in soybeans represents about 10% by weight of the dry bean and includes about 5% sucrose, about 4% stachyose, and about 1% raffinose[3].

Maize (*Zea mays*) is a plant of the tribe Maydea of the grass family of the Gramineae. Maize ranks as the second most widely produced cereal crop world-wide. Only wheat is produced in greater quantity[4]. North America has always been the centre for maize production. In 1990 the total world production was 475 millions metric tons[2].

The maize kernel is composed of four main parts: (1) the germ; (2) the endosperm, which forms the major portion (82%) of the kernel, and is comprised of a protein matrix encapsulating granules of starch; (3) the pericarp, which is composed entirely of dead empty cells, and is high in cellulose and hemicellulose; and (4) the tip cap, which was the point of attachment to the cob and the passageway for the movement of nutrients to the developing kernel[3].

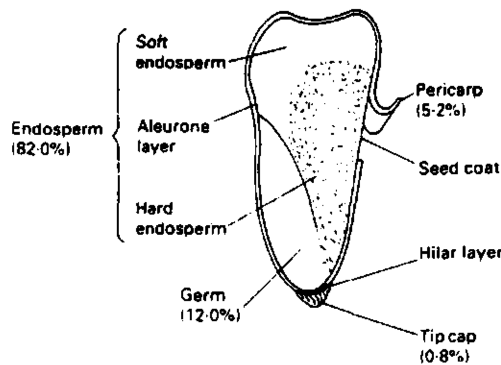

Figure 1.3. Mature maize kernel morphology[3].

Most of the corn crop goes directly into animal feed uses. Other direct uses of maize include sweet corn, popcorn, alkali-cooked corn to produce tortillas, breakfast cereals, and other foods made from whole maize or by traditional stone grinding. Other ways to utilise maize involve one or more levels of value-added processing. A large part of the maize production is wet milled, thus producing corn starch, corn sweeteners (sugar and syrup), and corn oil. The most valuable fractions are starch and germ. The by-products account for one-third of the mill output. Most of the by-products, except the oil recovered from the germ, are utilised as livestock feed[4].

As a first approximation maize kernels contain 4% lipid, 11% protein, 72% starch, 10% other carbohydrates, 2% crude fibre and 1% minerals on a dry weight basis[5].

THE CELL WALL OF MOST FLOWERING PLANTS

Cell walls are a major component of plant material. Although the primary wall has considerable mechanical and tensile strength, it is also flexible to accommodate turgor and to allow for cell elongation; it is permeable and yet an effective defence against micro-organisms. The cell wall is a fibre-composite material of skeletal cellulose microfibrils, which form the scaffolding framework of the wall, and so-called matrix polymers, which include xyloglucans, xylans, pectins, and proteins. Recent models of cell wall architecture have suggested that cell walls of dicots and non-graminaceous monocots are constructed from at least two independent but co-extensive and interactive networks, a cellulose/xyloglucan network and a pectin network, with a third interactive network of structural proteins in some cells (Figure 1.4)[6,7,8].

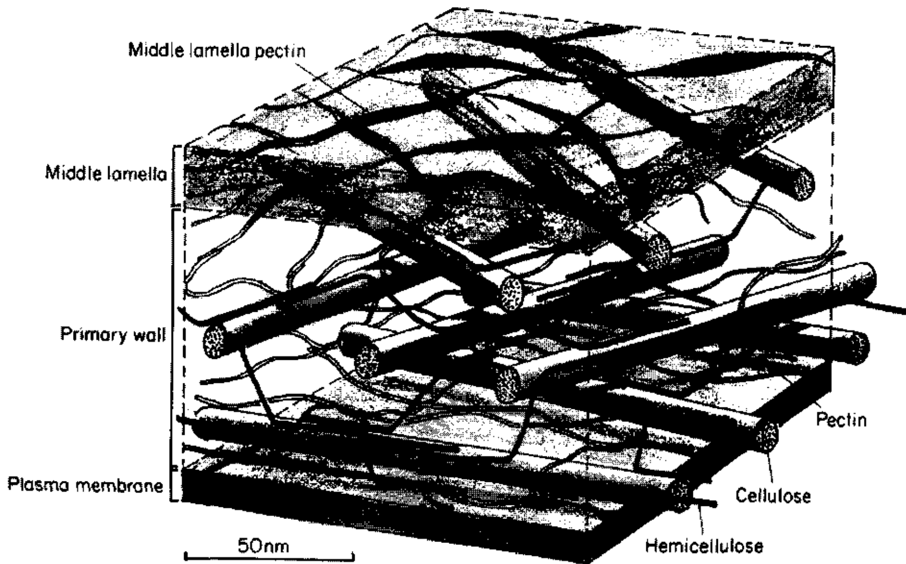

Figure 1.4. An extremely simplified and schematic representation of the onion parenchyma cell wall[7].

The cellulose/xyloglucan network

Cellulose ((1,4)-linked β-D-glucan) is the major component of the primary cell wall. The cellulose chains can associate into microfibrils by intermolecular hydrogen-bonding. The surfaces of these microfibrils are coated with hemicelluloses to prevent them from aggregating[9]. Two hemicelluloses – xyloglucan and arabinoxylan – are components of all primary cell walls, although the relative amounts of the two hemicelluloses vary from plant to plant. It is possible that a small amount of a third hemicellulose, a glucomannan or galactoglucomannan, is a component of primary cell walls[10].

In the cell wall of dicots, the principal hemicelluloses are xyloglucans[6]. The basic structure of this cell wall polymer consists of a backbone of β-(1,4)-linked-D-glucosyl residues, with D-xylosyl side chains α-linked to C6 of some of the glucosyl residues. Some of the xylosyl side chains are extended by the addition of a β-linked-D-galactose residue, or a α-L-fucosyl-(1,2)-β-D-galactose dimer to C2 of the xylosyl residues. Arabinosyl residues are occasionally linked to C2 of some of the xylosyl residues of some xyloglucans[11]. The degree of backbone branching appeared to be characteristic; most xyloglucans are composed of either XXXG-type or XXGG-type building units[12]. The letters "G" and "X" refer to an unbranched β-D-Glc*p* residue and an α-D-Xyl*p*-(1→6)-β-D-Glc*p* segment, respectively[13]. The poly-XXXG class includes xyloglucans from both gymnosperms and angiosperms, xyloglucans from solanaceous plants belong to the poly-XXGG group[12].

Xyloglucan is thought to form a tightly bound molecular monolayer on the surface of cellulose. A portion of a xyloglucan chain that is sterically prevented from binding to one cellulose microfibril will form, if possible, a multiple hydrogen-bond attachment to another cellulose microfibril, thereby cross-linking the microfibrils and creating a cellulose/ xyloglucan network[10].

The pectin network

The second polysaccharide network present in primary cell walls is composed of pectic polysaccharides. The organisation of pectic substances is a major control element in defining the sieving properties of the wall[14]. Other functions of plant cell wall pectins are determining cell wall porosity; providing charged surfaces that modulate wall pH and ion balance; and serving as recognition molecules that signal appropriate developmental responses to symbiotic organisms, pathogens, and insects[6].

The pectic substances comprise a family of acidic polymers like homogalacturonans, and rhamnogalacturonans with several neutral polymers like arabinans, galactans and arabinogalactans attached to it[15,16,17]. A model of apple pectin molecules is presented in Figure 1.5. The pectin consists of smooth galacturonan regions interrupted by blocks of ramified rhamnogalacturonan regions, so-called hairy regions. The branches are neutral sugar rich side chains[18]. The relative amounts of the different pectic sub-units vary from plant to plant.

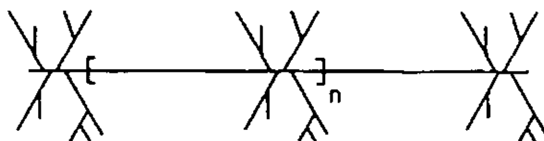

Figure 1.5. Schematic structure of apple pectin[19].

The hairy regions of apple appeared to be composed of three sub-units, and a structural model is shown in Figure 1.6. The pectic sub-units are described below.

Homogalacturonan is the most well known part of pectic substances (Figure 1.5). It consists of (1,4)-linked α-galacturonic acid residues. Methylesterification of the carboxyl groups is the most common modification of homogalacturonan. Another type of substitution is acetylation of homogalacturonan on C2, C3, or both C2 and C3 positions of the galacturonic acid in potato[20] and bamboo[21], and especially sugar beet homogalacturonan has a high acetyl content[16].

Xylogalacturonan (sub-unit I) is a relatively recently discovered sub-unit of pectic substances. The backbone consists of (1,4)-linked α-D-galacturonic acid residues. Xylose residues are β-(1,3)-linked to part of the galacturonic acid residues. A part of the galacturonic acid residues are methyl esterified, and the methyl esters are found to be equally distributed among the substituted and unsubstituted galacturonic acid residues. Xyloglacturonan is probably associated with rhamnogalacturonan regions[22,23].

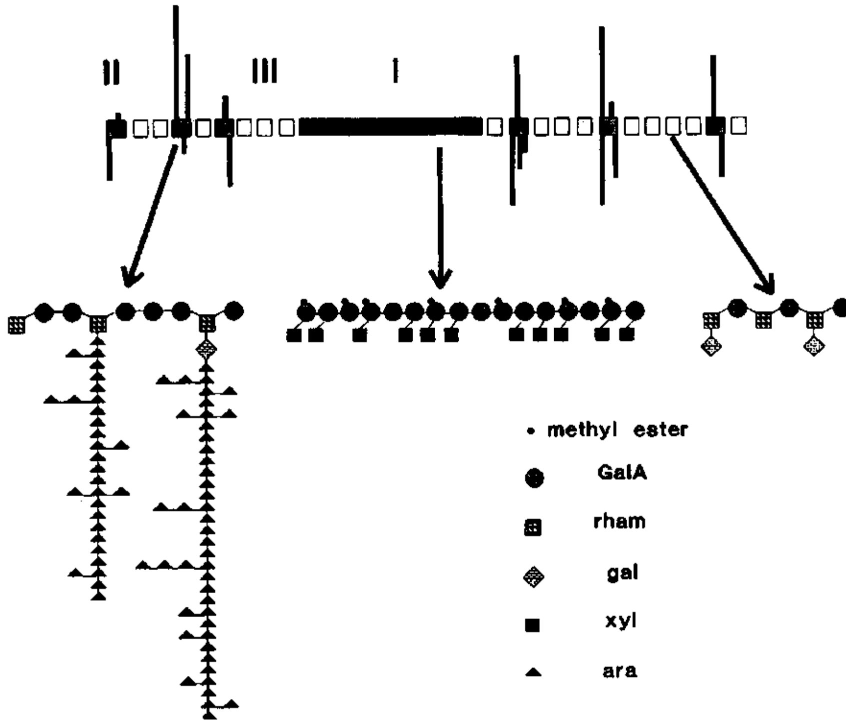

Figure 1.6. Hypothetical structure of apple pectin MHR (modified hairy regions). Sub-unit I, xylogalacturonan; sub-unit II, stubs of the backbone rich in arabinan side chains; sub-unit III, rhamnogalacturonan oligomers. The distribution of acetyl groups is not presented, but the major part is located within sub-unit III[24,25]. No information is available on the presence of methyl esters in sub-unit II[26].

Rhamnogalacturonan (sub-units II and III) is the second major type of pectic polysaccharide. It is the collective noun for pectic fragments having a rhamnose to galacturonic acid ratio, varying between 0.05 and 1[26]. Arabinosyl- and galactosyl-rich side chains are attached to C4 of the rhamnosyl residues, although the proportion of rhamnosyl residues with attached side chains varies from ~20% to ~80% depending on the source of the

polysaccharide[10]. The side chains can vary in size from a single glycosyl residue to 50 or more glycosyl residues[27,28]. Rhamnogalacturonans are typically highly acetylated[29] at positions C2 and C3 of the galacturonic acid residues[20,30]. The rhamnogalacturonans include the intensively studied pectic segments which have a strictly alternating sequence of rhamnose and galacturonic acid, named rhamnogalacturonan I[17].

Rhamnogalacturonan II is another common pectic sub-unit in fruit and vegetable cell walls[31,32,33]. It was, however, not detected in apple pectin MHR by Schols et al.[26] (due to their removal during ultrafiltration of the enzyme-treated apples), and therefore not present in Figure 1.6. This sub-unit has a highly conserved structure. The backbone is composed of about nine (1,4)-linked α-D-galacturonic acid residues. Four different, complex side chains are attached to C2 or C3 of four of the backbone residues. These side chains contain rhamnose and several rare characteristic monosaccharides such as apiose, 2-*O*-methyl-L-fucose, 2-*O*-methyl-D-xylose, aceric acid (3-*C*-carboxy-5-deoxy-L-xylose), KDO (3-deoxy-D-manno-octulosonic acid), and DHA (3-deoxy-D-lyxo-heptulosaric acid)[34].

THE CELL WALL OF GRASSES

In Gramineae, the chemical structure of the wall differs from that of all other flowering plant species. This type of cell wall is composed of cellulose microfibrils similar in structure to those of the cell walls of flowering plants. Instead of xyloglucan, the principal polymers that interlock the microfibrils are (glucurono)arabinoxylans. This type of cell wall is poor in pectin.

Arabinoxylans are linear chains of β-(1,4)-linked D-xylopyranosyl residues, to which α-L-arabinofuranosyl residues are attached as side chains to the C2, C3 or both C2 and C3 position[35]. Arabinoxylans can also be substituted with glucuronic acid or 4-*O*-methyl-glucuronic acid at the C2 of the xylosyl units[36,37,38,39], consequently designated glucuronoarabinoxylans. Additionally, branching with single unit side chains of xylose is suggested[40]. Besides these single unit substituents, a variety of di- and trimeric side chains have been identified as minor constituents of (glucurono)arabinoxylans. These side chains can be composed of arabinose only[39,41,42,43], or can include xylose and galactose residues[35,44,45,46,47]. Another feature of (glucurono)arabinoxylans is the presence of acetic, ferulic or coumaric acid as ester groups. Xylans can carry *O*-acetyl ester groups at position 2 of an arabinofuranose residue[48], ferulic and coumaric acid are covalently linked via an ester linkage to the C5 position of arabinofuranose residues[48,49,50,51]. In addition, *O*-acetyl ester groups can also be attached to C2 or C3 of the xylose residues in the backbone, but so far this was only found in hardwood xylans[52,53,54,55,56,57]. Arabinoxylans from various plants share the same basic chemical structure, the main differences are found in the ratio of arabinose to xylose, in the relative proportions and sequence of the various linkages between these two

sugars, and in the presence of other substituents[58]. As an example, the structural model of sorghum glucuronoarabinoxylan is shown in Figure 1.7. Sorghum glucuronoarabinoxylan is the most complex arabinoxylan described in literature. Neither rye, wheat endosperm, nor barley arabinoxylan contains glucuronic acid or short side chains, and this makes them more easily degradable by xylanases.

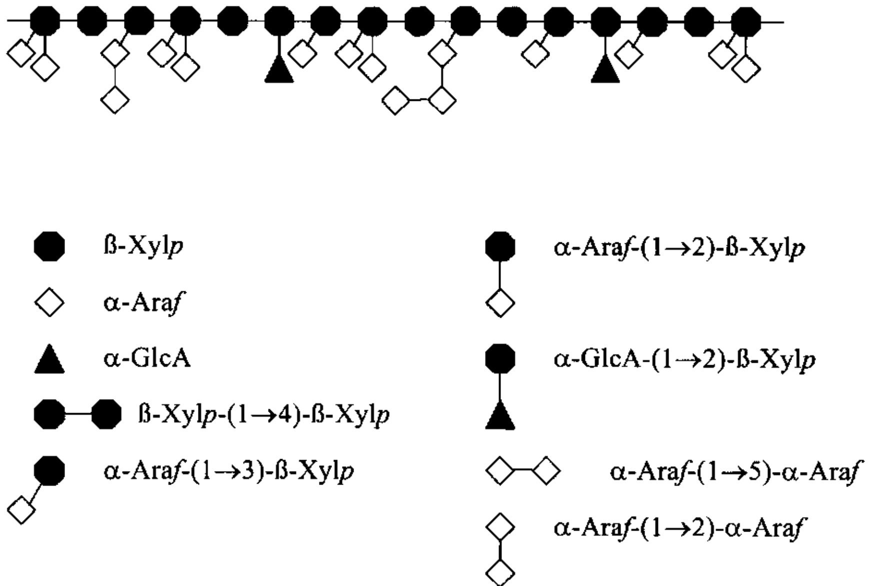

Figure 1.7. A structural model for sorghum glucuronoarabinoxylan[59].

Like xyloglucan, the unbrached (1,4)-linked xylans can hydrogen bond to cellulose or to each other[60]. The extend of hydrogen bond formation for glucuronoarabinoxylans is limited as a result of the presence of substituents[61]. Besides these interactions through hydrogen bonding, ferulic acid is capable of forming both ester and ether linkages and, therefore, it may participate in cross-linking reactions of cell wall macromolecules[62].

OUTLINE OF THE THESIS

The structures of cell wall pectic substances and xyloglucan from soybean meal and of (glucurono)arabinoxylan from maize kernels have been studied extensively. To study the cell wall polysaccharides, isolation procedures were performed in which non-cell wall components like fat, starch, and proteins are removed.

Specific extraction procedures were used to extract the different classes of pectins and hemicelluloses from soybean WUS. The obtained fractions were characterised by determination of their sugar composition and molecular weight distribution (chapter 2). It appeared that soybean WUS is hardly degraded by enzymes. It is however possible that no degradation can be achieved by enzymes even when the appropriate substrate is present. This might be caused by low cell wall porosity, adsorption of the enzyme to other cell wall polysaccharides, inhibition of the enzymes by cell wall components or hindrance by side chains, including both spatial hindrance and a high degree of substitution of the backbone. Although the CDTA-extractable pectic substances are no longer part of the cell wall matrix, they could still not be degraded by pectic enzymes, except for their arabinogalactan side chains (chapter 3). After incubation with pure and well-defined arabinogalactan-degrading enzymes an enzyme resistant pectic backbone remained. Significant degradation of this pectic polymer could only be achieved by acid hydrolysis. The resulting fragments of the pectic backbone were characterised, both chemically and enzymatically (chapter 4). The arabinogalactan fragments released after incubation of the CDTA-extractable pectin with the arabinogalactan-degrading enzymes were the subjects of research in chapter 5. The degradation products were separated by size-exclusion and anion-exchange chromatography, and subsequently characterised using mass spectrometry. Information on the structures of the various oligomers and mode of action of the enzymes led to more detailed knowledge on the structure of the intact cell wall polysaccharides.

The pectic substances form only one network of the plant cell wall. The other network is the cellulose/hemicellulose network. The hemicelluloses of the soybean cell wall were under investigation in chapter 6. Analyses of the oligomers formed after enzymatic degradation showed the formation of characteristic poly-XXXG xyloglucan oligomers.

The approach and methodology developed for the elucidation of polysaccharide structures in soybean meal was also successfully applied to maize kernel glucuronoarabinoxylan (chapter 7). These glucuronoarabinoxylans appeared to have a very complex structure, more complex and more resistant to enzymatic degradation than those of sorghum kernel cell walls.

In the concluding remarks (chapter 8), an overview of the thesis work is given and the results are discussed in the context of existing polysaccharide knowledge and research aims.

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CHAPTER 2

CELL WALL POLYSACCHARIDES FROM SOYBEAN (*GLYCINE MAX.*) MEAL

ISOLATION AND CHARACTERISATION

M.M.H. HUISMAN, H.A. SCHOLS & A.G.J. VORAGEN

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Cell wall material was isolated as Water-Unextractable Solids (WUS) from soybean meal. The isolation of WUS yields a fraction that contains 92% of the polysaccharides present in soybean meal and only few other components. Arabinose, galactose, uronic acids and glucose (cellulose) were the major constituent sugars. WUS was sequentially extracted with chelating agent (Chelating agent Soluble Solids, ChSS), dilute alkali (Dilute Alkali Soluble Solids, DASS), 1 M alkali (1 M Alkali Soluble Solids, 1 MASS) and 4 M alkali (4 M Alkali Soluble Solids, 4 MASS) to leave a cellulose-rich residue (RES). ChSS was the major extract, yielding 38% of the polysaccharides present in the WUS. All extracts and the residue were characterised by their sugar composition and their molecular weight distribution. The extracts ChSS and DASS were fractionated by anion exchange chromatography. They showed identical elution patterns: an unbound fraction, five bound fractions of which one fraction eluted only with alkali. Anion exchange chromatography was also performed after saponification of both pectin-rich extracts, again resulting in identical elution patterns.

INTRODUCTION

One of the basic products of the soybean is oil. The by-product of the industrial oil extraction, soybean meal, is enriched in proteins and cell wall polysaccharides. Soybean meal is used in livestock feeds and as a raw material for the production of soy protein isolates. The polysaccharides in soybean meal are badly utilised by monogastric animals. Partial degradation of these polysaccharides with enzymes could improve the utilisation by animals. Knowledge of the polysaccharide structure is needed to select enzymes, which are able to degrade the polysaccharides in a way that is optimal for the uptake by and well-being of the animals. In the production of soy protein isolate from dehulled and defatted soybean meal, the purity of the protein product is desired to be above 90%. Enzymes able to remove polysaccharides from the protein fraction could be of importance in protein isolation. Identification and selection of such enzymes requires knowledge of the polysaccharide structure in the soybean cell walls.

Some structures of soybean cell wall polysaccharides have already been partly elucidated during the sixties. Within this group of polysaccharides, the arabinogalactans have been studied most intensively[1,2,3,4], and showed to contain chains of (1,4)-linked β-D-galactopyranose residues in which some residues carry through C3 a side chain of ((1,5)-linked) L-arabinofuranose residue(s). However, large variation is present in the degree of branching and in the distribution of the substituents over the main chain. Aspinall and Cottrell[5] also isolated a highly branched arabinan, containing (1,3)- and (1,5)-linked arabinofuranose residues.

Within the group of acid polysaccharides, the sugar composition has been determined and a number of hydrolysis products have been characterised. The results indicate a main

chain consisting of D-galacturonic acid and L-rhamnose residues and side chains containing mainly galactose and arabinose residues. Two noteworthy observations were firstly the presence of oligosaccharides containing contiguous rhamnose residues, and secondly the presence of xylosyl-galacturonic acid dimers, indicative for the presence of xylogalacturonan[6]. The structure of the pectin molecules as a whole has not yet been investigated.

Until now no survey has been published in which fractions from soybean meal are characterised with respect to their (polysaccharide) composition. Moreover, isolated soybean polysaccharides have also not been investigated in detail, except for chelating agent extracted pectic substances by Brillouet and Carré[7]. The purpose of the present investigation was to isolate the intact cell wall polysaccharides from soybean meal and to characterise them in order to perform further structural investigations of the important fractions.

MATERIALS AND METHODS

PLANT MATERIAL

Solvent-extracted, untoasted soybean meal was obtained from Cargill BV (Amsterdam, The Netherlands).

ISOLATION OF WATER-UNEXTRACTABLE SOLIDS (WUS)

Dehulled, defatted, untoasted soybean meal was ground to pass a 0.5-mm sieve. This meal (800 g) was extracted with 3 l distilled water containing 0.05% NaN_3 during 2h at room temperature. The suspension was centrifuged (11000 g; 30 min). The pellet was resuspended, and this procedure was repeated four times. The combined supernatants were subjected to ultrafiltration using a tubular system (Cobe Nephross BV, Boxtel, The Netherlands), resulting in a filtrate (UFF) containing material smaller than 5000 Da and a retentate (UFR) containing polymers larger than 5000 Da.

Subsequently, the protein was extracted from the residue with 3 l 1.5% (w/v) sodium dodecylsulphate solution containing 10 mM 1,4-dithiothreitol, during 3h at room temperature. After centrifugation (11000 g; 30 min), this extraction was repeated three times. The final pellet was washed twice with distilled water. The combined supernatants were dialysed, concentrated and freeze-dried (SDSS).

The residue was then suspended in 1 l of distilled water (pH 5.0) at 85 °C, and starch gelatinisation was allowed to proceed for 1h. The residue obtained after centrifugation (11000 g; 30 min) was suspended in 1 l buffer solution (pH 6.5) containing 10 mM maleic acid, 10 mM NaCl, 1 mM $CaCl_2$ and 0.05% NaN_3. Porcine pancreatic α-amylase (2 mg; Merck art. 16312) was added and the mixture incubated at 30 °C for 19h. After centrifugation (11000 g; 30 min), the residue was washed

with 1 l hot distilled water (65 °C) and centrifuged again. The α-amylase digestion and hot water washing were repeated once. The combined supernatants were dialysed, concentrated and freeze-dried (HWS), and the remaining unextractable residue was resuspended in distilled water and freeze-dried (WUS).

SEQUENTIAL EXTRACTION OF WUS

Soybean WUS (20 g) was sequentially extracted, based on the procedure described by Redgwell & Selvendran[8], with 0.05 M 1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid (CDTA) and 0.05 M NH_4-oxalate in 0.05 M NaAc-buffer, pH 5.2 (8 times 600 ml) at 70 °C for 1h (Chelating agent Soluble Solids, ChSS); washed with distilled water (two times 600 ml) and these extracts were added to the ChSS fraction; extracted with 0.05 M NaOH (three times 600 ml) at 2 °C for 1h (Dilute Alkali Soluble Solids, DASS); 1.0 M KOH + 20 mM $NaBH_4$ (5 times 600 ml) at room temperature for 2h (1 M Alkali Soluble Solids, 1 MASS); 4 M KOH + 20 mM $NaBH_4$ (three times 600 ml) at room temperature for 2h (4 M Alkali Soluble Solids, 4 MASS). After each extraction, solubilised polymers were separated from the insoluble residue by centrifugation (19000 g; 30 min). All extracts were acidified to pH 5.2 (if necessary) by glacial acetic acid, concentrated, dialysed and freeze-dried. ChSS (including the two supernatants obtained after washing the residue from this extraction step) were dialysed against 0.1 M NH_4Ac buffer (pH 5.2) before dialysing against distilled water. The final residue (RES) was suspended in water, acidified to pH 5.2, dialysed and freeze-dried.

ION-EXCHANGE CHROMATOGRAPHY

Approximately 500 mg of ChSS, saponified ChSS (sChSS), DASS and saponified DASS (sDASS) were fractionated on a column (550 mm x 15 mm) of DEAE Sepharose Fast Flow, which was initially equilibrated in 0.005 M NaAc-buffer pH 5.0, using a Hiload System (Pharmacia).

The ChSS and DASS fractions were suspended in water, the insoluble residues were removed by centrifugation (5400 g; 5 min) and the supernatants were applied onto the column. Saponification of ChSS and DASS was performed by dissolving them in 0.1 M NaOH (0 °C, 16h) followed by neutralisation with 0.1 M HAc. These saponified samples were also centrifuged and the supernatants applied onto the column.

Elution was carried out sequentially with 400 ml of 0.005 M NaAc-buffer pH 5.0, a linear gradient from 0.005 to 0.5 M NaAc buffer pH 5.0 (1200 ml), a linear gradient 0.5-2 M NaAc-buffer pH 5.0 (1000 ml) and 400 ml 0.005 M NaAc-buffer pH 5.0. Residual bound polysaccharides were washed from the column with 400 ml of 0.5 M NaOH. The elution rate was 10 ml/min except for the first step, in which the sample was applied onto the column and the elution rate was 2.5 ml/min. Fractions (23 ml) were collected and assayed by automated methods for neutral sugar content[9] and uronic acid content[10]. The appropriate fractions were pooled, concentrated, dialysed, freeze-dried and analysed for neutral sugar composition and uronic acid content.

ANALYTICAL METHODS

Moisture content was determined by drying at 105 °C until no further decrease in weight was observed.

Starch content was determined enzymatically using a test kit (Boehringer, Mannheim, Germany).

Non-starch polysaccharide content of soybean meal and HWS was determined according to Englyst and Cummings[11]. The starch was enzymatically hydrolysed, the residue was dried and the sugar composition was determined.

Neutral sugar composition was determined by gas chromatography according to Englyst and Cummings[11], using inositol as an internal standard. The samples were pre-treated with 72% w/w H_2SO_4 (1h, 30 °C) followed by hydrolysis with 1 M H_2SO_4 for 3h at 100 °C and the constituent sugars were analysed as their alditol acetates. Cellulosic glucose was calculated as the difference between the content of glucose with and without prehydrolysis.

Uronic acid content was determined by the automated colorimetric *m*-hydroxydiphenyl assay[9,10,12] using an auto-analyser (Skalar Analytical BV, Breda, The Netherlands). Corrections were made for interference by neutral sugars present in the sample.

Protein content was determined by a semi-automated micro-Kjeldahl method[13]. The conversion factor used was 6.25.

Degree of acetylation and methylation was determined by HPLC after saponification with 0.4 M NaOH[14]. Quantification was performed using acetic acid and methanol standards.

High-Performance Size-Exclusion Chromatography (HPSEC) was performed on a SP8800 HPLC (Spectra Physics) equipped with three columns (each 300 x 7.5 mm) of Bio-Gel TSK in series (60XL, 40XL and 30XL; Bio-Rad Labs.) in combination with a TSK guard column (40 x 6 mm) and elution at 30 °C with 0.4 M NaAc buffer pH 3.0 at 0.8 ml/min. Calibration was performed using dextrans, ranging from 500 to 4 kDa. The eluate was monitored using a Shodex SE-61 Refractive Index detector.

High-Performance Anion-Exchange Chromatography (HPAEC) was performed on a Dionex Bio-LC system as described by Schols *et al.*[15]. The gradient was obtained by mixing solutions of 0.1 M NaOH and 1 M NaAc in 0.1 M NaOH.

For the determination of small neutral oligomers, fructose, sucrose, raffinose and stachyose, the (4 x 250 mm) CarboPac PA1 column was equilibrated with 0.016 M NaOH. Twenty µl of the

sample were injected, and a linear gradient to 0.1 M NaOH in 33 min was applied, followed by a linear gradient from 0 to 0.04 M NaAc in 0.1 M NaOH in 12 min. The column was washed for 5 min with 1 M NaAc in 0.1 M NaOH, then 5 min with 0.1 M NaOH and then equilibrated again for 12 min with 0.016 M NaOH. Calibration was performed with standard solutions of fructose, sucrose, raffinose and stachyose.

RESULTS AND DISCUSSION

YIELD AND COMPOSITION OF THE WUS

The yield and the composition of the fractions of soybean meal obtained during the isolation of WUS are shown in Table 2.1. The recovery of this fractionation is 94%. The major part of the material is water soluble (UFF and UFR), namely 59%. The yield of the WUS fraction is 16%.

Table 2.1. Yield and composition of soybean meal and fractions thereof (percentage dry weight).

	Soybean meal	UFF	UFR	SDSS	HWS	WUS	Recovery
Yield	100	19.5	39.6	18.5	0.4	15.7	93.7
Protein content	57.3	21.1	87.8	84.2	15.4	2.1	95.7
Starch content	1.0	0	0	0	8.5	0	3.4
NSP content	15.4[b]	50.2	13.7	3.0	43.3[a]	95.8	102.4[b]
Acetic acid groups	1.1	1.2	0.7	0.3	1.4	2.8	92
Methanol groups	0.3	0.1	0.2	t	1.1	1.1	92
Fructose	0.6	6.6	3.5	0	0	0	446
Sucrose	5.4	0	0	0	0	0	0
Raffinose	0.8	0.5	0.1	0	0	0	17
Stachyose	4.9	8.4	1.2	0	0	0	43

t = trace amount.

[a] After enzymatic removal of starch.

[b] In this calculation, UFF and UFR are omitted.

The protein content of the soybean meal is very high, as expected (57%). The recovery of protein in this procedure is 96%. The major part of the protein present in soybean meal is recovered in the UFR fraction (61%); these are the water-soluble proteins. The SDS/DTT solution is able to extract another 27% of the proteins in the material. The amount of protein

recovered in the WUS fraction is 0.6% of the protein present in the soybean meal and represents only 2.1 % of the WUS.

The soybean meal contains 1% of starch. Starch molecules are degraded by the use of α-amylase in the extraction of HWS, and degradation products are removed during dialysis. Only 3.4% of the starch resists degradation with α-amylase, these are limit dextrins from amylopectin.

The polysaccharide content of the soybean meal (15.4%) and the HWS fraction (43.3%) is determined after removal of starch and oligomeric sugars. In the determination of the polysaccharide content of the other fractions (UFF, UFR, SDSS and WUS) this step was omitted, because none of these fractions contained starch anymore. Thus, the polysaccharide content of the soybean meal does not include the neutral oligomeric sugars, whereas the 'polysaccharide content' of the UFF and UFR fractions includes these small sugars. Therefore, the calculation of the recovery of polysaccharides (102%) only includes the polysaccharide contents of the SDSS, HWS and WUS fractions. Of the total polysaccharides in these fractions, 95% is recovered in the WUS fraction. The acetic acid and methanol groups are believed to be present as substituents of the uronic acid residues in the polysaccharides. They are expected to be recovered in the WUS fraction, which is confirmed by the results in Table 2.1, showing 40% of the acetic acid groups and 58% of the methanol groups are recovered in the WUS fraction. The water-soluble polysaccharides (in the UFF and UFR fractions) contain 45% of the acetic acid groups and 33% of the methanol groups.

The small sugars - fructose, sucrose, raffinose, and stachyose - are water-soluble and thus recovered in the UFF and UFR fraction. The soybean meal contains 0.6% fructose, 5.4% sucrose, 0.8% raffinose and 4.9% stachyose, which is in agreement with the figures of Sosulski *et al.*[16] who found 6.35% sucrose, 1.15% raffinose and 2.85% stachyose. The recoveries of sucrose (0%), raffinose (17%), and stachyose (43%) detected in the fractions are very low. For fructose, however, the recovery is unrealistically high (446%). A possible explanation for this observation is that the di-, tri-, and tetrasaccharide are degraded by endogenous enzymes, which would lead to the formation of glucose, fructose and galactose containing oligomers and monomers. If all oligomers that had not been recovered had been degraded, the recovery of fructose would have been 600%. The lacking 150% can be explained by the new unidentified oligomers appearing in the HPAEC patterns of the UFF and UFR fractions. This enzymatic degradation of oligosaccharides takes place during ultrafiltration of the cold water-soluble fraction, because so far, no heat treatment has been given to inactivate enzymes present in the soybean meal. Enzyme activities that might be present in the residue are inactivated by SDS and DTT during the next extraction step and will not degrade the polysaccharides present in the residue. Since 95% of the polysaccharides were recovered in the WUS fraction and no indications for polysaccharide degrading activities were found, it is believed that degradation of polysaccharides did not occur, and research directed towards the WUS was continued.

Table 2.2. Sugar composition of soybean meal and fractions thereof (mol%).

	Sugar composition								
Fraction	rha	fuc	ara	xyl	man	gal	glc	galA	Carbohydrate content[a]
Soybean meal[b]	2	3	19	8	3	28	21	18	14.5
UFF	0	0	2	1	6	29	58	3	40.7
UFR	1	t	4	1	13	26	49	6	12.4
SDSS	7	1	11	4	19	14	18	25	2.9
HWS[b]	1	1	23	4	2	33	10	26	40.7
WUS	2	3	19	8	2	29	21	17	89.3

t = trace amount.

[a] Expressed as % w/w.

[b] After enzymatic removal of starch.

The sugar composition of all fractions from soybean meal was determined and is shown in Table 2.2. The polysaccharides in the meal and in the WUS fraction consist mainly of galactose, glucose (mainly cellulose), arabinose, and uronic acids. The kind of uronic acid was not determined, but Aspinall *et al.* [3,6] have shown that the uronic acids present in soybean cotyledon meal are primarily galacturonic acids. The cellulose content of soybean meal and the WUS fraction is 2.7 and 17.7% w/w, respectively.

In the determination of the polysaccharide content of the UFF and UFR fractions, starch and oligomeric sugars were not removed prior to hydrolysis. Fructose, raffinose (gal-glc-fru) and stachyose (gal-gal-glc-fru) interfere with the analysis of the sugar composition of these fractions. In this analysis fructose is partly determined as mannose and glucose. The major sugars in both the UFF and the UFR fraction are glucose, galactose, and mannose. The amount of mannose residues in the UFR fraction (13%) is higher than the mannose content of the UFF fraction (6%), whereas the amount of fructose (present as monomer and in raffinose and stachyose) in the UFR fraction is slightly lower than in the UFF fraction. This is an indication of the presence of mannose containing polysaccharides. This can be a result of incomplete removal of the hulls from the soybean meal, since soybean hulls consist, to a large extend, of galactomannans, which are isolated by extraction with cold water[17,18].

The SDSS and HWS fraction are not of importance in our study, because the amounts of polysaccharides extracted in these two steps are very low. The WUS fraction, however, is really enriched in polysaccharides. The most important constituent sugars are galactose, glucose (cellulose), arabinose, and uronic acids. This is an indication of the presence of a considerable amount of pectins in the WUS. These results are in agreement with Brillouet and Carré[7], who also found galactose to be the major sugar constituent, followed by galacturonic acid, arabinose, and glucose in soybean cotyledon cell walls.

From the data in Table 2.2, it can be concluded that this isolation procedure yields a WUS fraction in which almost all cell wall polysaccharides are recovered and which is almost

free of other components. The sugar composition of the WUS fraction is very similar to that of the soybean meal, which indicates that no sugar residues were specifically removed during the isolation procedure. Surprisingly, over 90% of the uronic acids present in the soybean meal were recovered in the WUS fraction. In the isolation procedure of cell wall material from for instance onions[8], apples[19], and olives[20], a significant amount of the pectins is water- or buffer-soluble. This could be an indication of more complex pectic molecules or greater diversity within the architecture of the soybean cell walls compared with other plant cell walls.

SEQUENTIAL EXTRACTION OF THE WUS

CDTA and NH_4-oxalate are most generally used to abstract Ca^{2+} from the cell walls, and most of the pectic polysaccharides held in the walls by ionic cross-links will be solubilised. The ChSS fraction, which is the main fraction (38%), is rich in arabinose, galactose, and uronic acids. The remaining pectic polysaccharides are probably ester cross-linked within the wall matrix and are (partially) extracted with dilute alkali and recovered in the DASS fraction. The sugar composition of this extract is identical with the composition found for the ChSS fraction. The galactose:arabinose ratio found in both the ChSS and DASS fraction is 1.5:1, and the uronic acid:rhamnose ratio is 14:1. The sugar composition of the ChSS and DASS fractions is quite similar to that of the EDTA-soluble pectic substances extracted by Brillouet & Carré[7], their extract also contains pectic substances rich in galactose and arabinose with a molar ratio of 1.5:1 and has a uronic acid:rhamnose ratio of 13:1.

Further extraction of the residue with stronger alkali (1 and 4 M KOH) solubilises small amounts of additional pectic material along with the hemicelluloses. Besides arabinose, galactose, and uronic acids, the 1 MASS fraction contains also 11 mol% of xylose. Xylose and glucose are the predominant sugars in the 4 MASS fraction, which may indicate the presence of xyloglucan in this extract. The final α-cellulose residue (RES) still contains a small amount of uronic acids, representing 11% of the uronic acids present in the WUS. These uronic acids can be galacturonic acids as well as glucuronic acids. The galacturonic acids might be present as pectic molecules tightly bound to or firmly entangled in the cellulose/hemicellulose network. The glucuronic acids will probably be present as hemicellulose substituents. The yields and sugar compositions of the extracts from WUS are shown in Table 2.3.

The degree of acetylation and methylation in the WUS are, respectively, 49 and 36%. The ChSS fraction has a degree of methylation of 35% and a remarkably high degree of acetylation of 36%. The low recoveries of the acetyl groups and methyl esters after sequential extraction (30% and 41%, respectively) are caused by the fact that part of these groups are saponified during extraction with alkali, and as a result of that can not be determined. Although extraction of the DASS fraction was performed using NaOH, part of the methyl and

Table 2.3. Yield on sugar basis (%) and sugar composition of extracts from WUS expressed as mol%, and as a percentage of that particular sugar in the WUS (in parentheses).

		Sugar composition										
Extract	Yield	rha	fuc	ara	xyl	man	gal	glc	uronic acids	Carbohydrate content[a]	DA[b]	DM[c]
WUS	100	2	3	19	8	2	29	21	17	89.3	49	36
ChSS	38	2 (40)	3 (42)	25 (48)	6 (30)	1 (15)	37 (49)	1 (1)	26 (53)	52.7	36	35
DASS	7	2 (8)	3 (8)	24 (9)	6 (6)	1 (3)	38 (10)	1 (t)	25 (10)	73.9	22	29
1 MASS	16	2 (15)	3 (22)	23 (20)	11 (23)	1 (7)	35 (21)	5 (4)	20 (18)	74.2		
4 MASS	7	1 (4)	3 (9)	12 (4)	28 (24)	2 (8)	18 (5)	26 (9)	10 (4)	84.5		
RES	18	1 (8)	0 (0)	3 (3)	3 (6)	4 (41)	2 (1)	76 (65)	11 (11)	75.9		
Recovery	86%	75%	81%	84%	88%	74%	85%	80%	96%	86%	30%	41%

t = trace amount.

[a] Expressed as % w/w.

[b] Expressed as mol acetic acid/100 mol uronic acids.

[c] Expressed as mol methanol/100 mol uronic acids.

acetyl groups were still present (DA = 22% and DM = 29%). This phenomenon was also found by Ros *et al.*[21] extracting a diluted sodium hydroxide soluble pectin fraction from the albedo of lemons. Assuming that these substituents will be partially saponified during the extraction of DASS, the degree of acetylation and methylation of this extract will be underestimated.

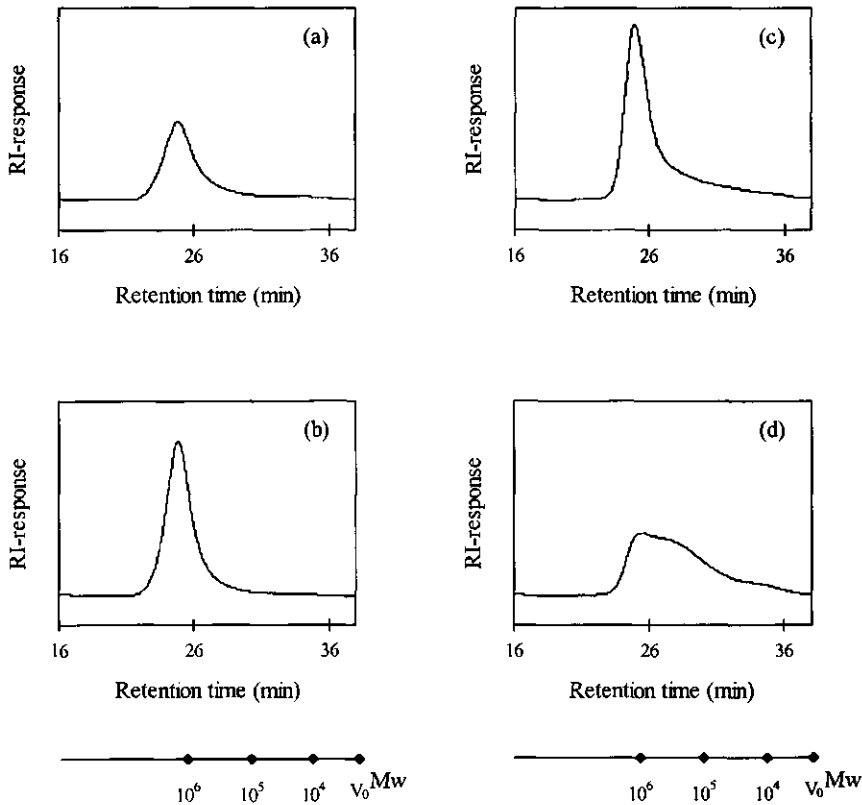

Figure 2.1. HPSEC elution patterns of (a) ChSS, (b) DASS, (c) 1 MASS, and (d) 4 MASS extracted from soybean WUS.

The extracts were further characterised by the determination of their molecular weight distributions, which are shown in Figure 2.1. The ChSS and DASS fractions show almost identical symmetrical peaks, with an average molecular weight of about 10^6 Da based on calibration with dextrans. Since uronide-containing polymers have a larger hydrodynamic volume than dextrans, due to intramolecular electrostatic repulsion by charge effects and therefore elute faster than expected on the basis of their molecular weight[22], the molecular weight of these two fractions containing pectin-rich polysaccharides, will be smaller than

mentioned above. The average molecular weight of the 1 MASS is similar to that of the first two fractions, but the distribution tails to lower molecular weights. The molecular weight distribution of the 4 MASS fraction is much broader, and the average molecular weight is lower than the preceding three extracts. For each extract, a standardised amount was solubilised as much as possible. The differences in the areas under the peaks are partly caused by differences in the sugar contents of the extracts, which depends on the amount of residual salt and water in the fractions. The ChSS fraction has the lowest sugar content (52.7%), due to the fact that the CDTA is difficult to dialyse away from pectins[23]. The differences in the solubility of the extracts are also partly responsible for the differences in the areas under the peaks. This mainly concerns the elution pattern of the 4 MASS fraction, because this fraction is not completely soluble, whereas the others are.

ANION-EXCHANGE CHROMATOGRAPHY OF ChSS AND DASS

The polysaccharides present in the pectin-rich extracts ChSS and DASS are very similar with respect to their sugar compositions and molecular weight distributions. For further characterisation, these polysaccharides were fractionated, based on their charge density. The soluble parts of these extracts, representing over 90% of the polysaccharides of the extracts, are applied onto the column. The elution pattern of ChSS and saponified ChSS (sChSS) are shown in Figure 2.2a and 2.2b, respectively. The residues contain less than 10% of the polysaccharides present in the different extracts, and are enriched in glucose.

The elution patterns of DASS and saponified DASS (sDASS) are identical with those of ChSS and sChSS, and therefore not shown here. The unbound fractions are pooled as pool I and the bound fractions are pooled to give pool II-V as indicated in Figure 2.2. The sugar composition and the uronic acid content of these pools, the residue remaining after centrifugation of the suspensions (residue), and the strongly bound polysaccharides washed from the column with 0.5 M NaOH (alkali wash), are presented in Table 2.4. The data obtained for DASS and sDASS are again analogous and are not shown.

The recovery of the fractionations is high. All the pools are rich in arabinose, galactose, and uronic acids. As can be seen from Figure 2.2, the relative uronic acid content of the fractions increased with increasing salt concentration of the eluent. This is confirmed by the ratio neutral sugars to uronic acids, which decreases with increasing retention time of the fraction. At the same time a shift from galactose to arabinose takes place, the arabinose:galactose ratio increased from 0.52 (pool II) to 0.70 (pool V) for ChSS, from 0.55 to 0.72 for sChSS, from 0.53 to 0.75 for DASS, and from 0.53 to 0.76 for sDASS.

Pool I consists of the unbound polysaccharides, and is therefore expected to have a low uronic acid content. However, this pool contains about 20 mol% uronic acids. A possible explanation for not binding is that the neutral sugar containing side chains prevent the interaction of the uronic acids with the column material, and the polysaccharides will be

eluted in the void. Alternatively, these unbound uronic acid-containing polysaccharides could represent methyl esterified (neutral) pectic material. This later possibility is in contradiction with the fact that this unbound fraction is also found in the elution pattern of the saponified extracts.

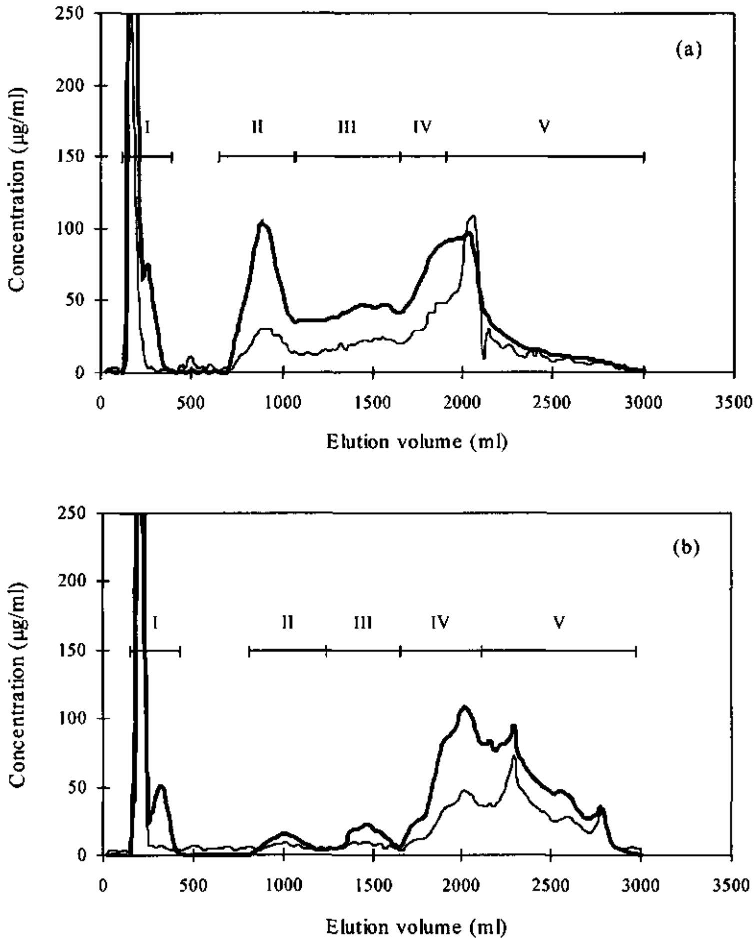

Figure 2.2. Elution profile of (a) ChSS and (b) sChSS on anion-exchange chromatography. Uronic acid content (—), neutral sugar content (—).

The bound polysaccharides from the saponified extract are slightly lower in their uronic acid content than those from the unsaponified extract, except for the alkali wash. Furthermore, a shift in the amount of polysaccharides from pool II and III to pool IV and V can be detected. By saponification of the methanol groups, the pectins will possess more charged groups and will therefore need a higher ionic strength of the buffer to be eluted.

Table 2.4. Yield on sugar basis (%) and sugar composition (mol%) of fractions obtained after anion-exchange chromatography of ChSS and saponified ChSS.

		Sugar composition							
	Yield	rha	fuc	ara	xyl	man	gal	glc	uronic acids
ChSS		2	3	25	6	1	37	1	26
Residue	8.2	2	2	21	8	t	34	9	24
Pool I	24.8	2	3	29	6	1	41	1	19
Pool II	14.7	2	3	24	6	t	45	1	19
Pool III	13.2	2	4	22	8	1	39	1	24
Pool IV	11.2	2	3	23	8	1	35	2	27
1 V	19.0	2	2	21	7	1	29	1	37
Alkali wash	7.4	2	2	21	7	2	32	2	32
Recovery	98.4	104	97	98	114	100	103	263	88
sChSS									
Residue	4.9	2	2	21	8	2	33	13	21
Pool I	27.8	2	3	27	6	1	44	1	18
Pool II	6.1	2	2	26	4	1	48	3	13
Pool III	5.0	1	2	24	5	1	47	4	16
Pool IV	21.2	2	3	24	6	1	44	1	19
Pool V	22.8	2	4	22	9	1	31	1	30
Alkali wash	8.0	2	2	21	7	2	25	3	40
Recovery	95.7	97	96	98	107	128	105	292	77

t = trace amount.

CONCLUSIONS

Isolation of the cell wall polysaccharides from soybean meal, which contains 57% of proteins, yields a fraction containing almost all polysaccharides present in the meal and few other components. A complete mass balance for both proteins and polysaccharides of the recovered fractions during the isolation of the WUS is given. The sugar compositions of the soybean meal and the isolated WUS fraction are quite similar, indicating that no polysaccharides were specifically removed during the isolation procedure.

Sequential extraction of the isolated cell walls with solutions, which selectively solubilise particular polysaccharides, results in two pectin-rich extracts (ChSS and DASS), an extract which contains pectins as well as hemicelluloses (1 MASS), an extract mainly containing hemicelluloses (4 MASS) and a cellulose-rich residue. The pectin-rich extracts have identical sugar compositions and contain predominantly galactose, arabinose, and uronic acids. The 1 MASS fraction contains xylose in addition to the former three sugars, and the hemicellulose-rich fraction contains mainly xylose and glucose.

Besides having identical sugar compositions, ChSS and DASS also exhibit similar molecular weight distributions and behaviour in anion-exchange chromatography. The sugar composition of the pools obtained by ion-exchange chromatography of ChSS and DASS were also the same. So far no indications have been found to state that ChSS and DASS are structurally different, although it is obvious that their arrangement in the cell wall was not identical because they were obtained with different extractants.

Further research will be directed towards more detailed characterisation of the extracts by methylation analysis and degradation with specific enzymes. Another item of interest is to obtain information about physical properties of the isolated polysaccharides. From the sugar composition of the ChSS and DASS extracts, and the pools thereof obtained by anion-exchange chromatography, the presence of xylogalacturonan in these extracts is expected. Isolation and characterisation of a xylogalacturonan-containing fraction will also be one of the objects of further research.

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CHAPTER 3

ENZYMATIC DEGRADATION OF CELL WALL POLYSACCHARIDES FROM SOYBEAN MEAL

M.M.H. HUISMAN, H.A. SCHOLS & A.G.J. VORAGEN

CARBOHYDRATE POLYMERS 1999, 38, 299-307

Soybean meal, soybean water-unextractable solids (WUS) and extracts thereof, which contain particular cell wall polysaccharides, were incubated with a number of cell wall degrading enzymes. The intact cell wall polysaccharides in the meal and WUS were hardly degradable, while the extracts from WUS were well degraded. The arabinogalactan side chains in the pectin-rich ChSS fraction (Chelating agent Soluble Solids) could to a large extent be removed from the pectins by the combined action of endo-galactanase, exo-galactanase, endo-arabinanase, and arabinofuranosidase B. The remaining polymer was isolated and represented 30% of the polysaccharides in the ChSS fraction. Determination of the sugar composition showed these polymers to be very highly substituted pectic structures. It still contained 5 mol% of arabinose and 12 mol% of galactose, representing 7% and 12% respectively, of the arabinose and galactose present in the ChSS fraction before degradation. Further, the presence of uronic acid (50 mol%) and of xylose (18 mol%) indicated the presence of a xylogalacturonan.

INTRODUCTION

The structure of the cell wall polysaccharides from soybean was the subject of a number of investigations in the sixties. A first determination of the constituent sugars of the fractionated soybean polysaccharides is described by Kawamura and Narasaki[1]. Morita and co-workers[2,3,4] determined the structure of an arabinogalactan in the "hot-water-extract" from defatted soybean flour. They concluded that the backbone of the polysaccharide is a β-(1,4)-linked polygalactopyranose chain with little branching. The arabinofuranosyl residues are attached as (1,5)-linked side chains, with an average length of two sugar units, to galactose residues in the (1,4)-linked main chain by (1,3)-linkages. Aspinall *et al.*[5] describe the same structure to be present in an extract from soybean meal, obtained with a 2% ethylene diaminetetra-acetic acid disodium salt solution.

A different picture is provided by Labavitch *et al.*[6]. A soybean fraction containing mainly arabinose and galactose was degraded by a purified endo-galactanase, and their results indicate that the arabinose in this soybean fraction is organised primarily in rather large oligo- or polyarabinosides.

The soybean arabinan-galactan described by Labavitch *et al.*[6] was obtained by alkaline extraction, by which neutral sugar containing side chains might be detached from branched pectic polysaccharides. Bacic *et al.*[7] and O'Neill *et al.*[8] already reported neutral polysaccharides like arabinans, galactans, and arabinogalactans to be attached to rhamnogalacturonan in pectic polysaccharides from other sources. In all the other previously mentioned investigations, the defatted soybean meal had also been in contact with a sodium hydroxide solution before extraction of polysaccharides. Aspinall *et al.*[5] themselves already raise the possibility that the neutral polysaccharide might have arisen as a degradation product

from the acidic polysaccharide, which is also present in the extract, by inadvertent cleavage of glycosidic bonds of uronyl residues by base-catalysed β-elimination.

To confirm these findings, we deproteinated soybean meal under milder conditions, to prevent the occurrence of β-elimination, by using SDS (sodium dodecylsulphate) and DTT ((1,4)-dithiothreitol). A CDTA (1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid) extract of soybean meal pre-treated this way contained 25 mol% of arabinose and 37 mol% of galactose. This extract showed only one symmetrical peak on size-exclusion chromatography, and no neutral fraction was obtained by anion-exchange chromatography[9]. This indicated that the arabinose and galactose residues present in this extract are part of the pectins.

Information about the structure of polysaccharides can be obtained by both chemical and enzymatic degradation of polysaccharide fractions, followed by the identification of the formed degradation products. However, chemical hydrolysis with dilute acid cleaves the glycosidic bonds in a rather unspecific way, which prevents the conversion of the obtained knowledge into a hypothetical structure of the polymer. Pure enzymes have high substrate specificity and form characteristic oligomers; therefore they are a valuable tool in structure elucidation[10]. We now report on the enzymatic degradation of soybean cell wall polysaccharides and the first steps in the structural elucidation of a pectin-rich extract from soybean cell wall polysaccharides.

MATERIALS AND METHODS

MATERIALS

Water-unextractable solids (WUS) were isolated from solvent-extracted, untoasted soybean meal and sequentially extracted as described by Huisman *et al.*[9].

ENZYMATIC DEGRADATION OF SOYBEAN POLYSACCHARIDES

Solutions (0.25% (w/w)) in 0.05 M sodium acetate buffer (pH 5.0) containing 0.01% NaN_3 were incubated with a number of enzymes or enzyme combinations at 30 °C rotating 'head over tail'. Soybean meal, WUS, ChSS, DASS (Dilute Alkali Soluble Solids), and 1 MASS (1 M Alkali Soluble Solids) were used as substrates in the degradation studies with cloned enzymes. Incubations with purified enzymes were performed with the ChSS extract. Between two subsequent enzyme incubations and at the end of all incubations the enzymes were inactivated by heating at 100 °C for 10 min. Polysaccharides degrading activities were determined by HPSEC and HPAEC analyses of the digests.
Endo-galactanase, endo-arabinanase, rhamnogalacturonan hydrolase (RG hydrolase), rhamnogalacturonan acetyl esterase (RGAE), and Polygalacturonase-1 (PG-1), an endo-polygalacturonase, were cloned from *Aspergillus aculeatus*[11] and were kindly provided by Novo

Nordisk A/S (Bagsvaerd, Denmark). The cloned microorganisms produce low amounts of their own enzymes in addition to the genetically introduced enzyme, so the cloned enzymes are not completely pure. Pectin methyl esterase (PE) was purified from a culture medium of *Aspergillus niger*[12].

The arabinogalactan degrading enzymes used to degrade the ChSS extracts were purified from culture filtrates of fungi. Endo-arabinanase originated from *Aspergillus aculeatus* and arabinofuranosidase B from *Aspergillus niger*[13]. Exo-galactanase was purified from *Aspergillus niger*[14]. The endo-galactanase is purified from Pectinex Ultra-SP-L; a technical enzyme preparation derived from *Aspergillus aculeatus* (Novo-Nordisk Ferment (Switzerland) Ltd., Dittingen, Switzerland)[15]. The amount of enzymes used was 0.13 μg protein/ml substrate solution for endo-arabinanase and arabinofuranosidase B, 0.5 μg protein/ml substrate solution for exo-galactanase and 0.05 μg protein/ml substrate solution for endo-galactanase.

ISOLATION OF THE POLYMERIC RESIDUE REMAINING AFTER ENZYMATIC DEGRADATION OF THE ARABINOGALACTAN SIDE CHAINS FROM SOYBEAN PECTINS

A 1% ChSS solution (250 mg/25 ml) in 0.05 M sodium acetate buffer (pH 5.0) containing 0.01% NaN_3 was incubated with endo-galactanase, exo-galactanase, endo-arabinanase, and arabinofuranosidase B at 30 °C for 48h, rotating 'head over tail'. After incubation the enzymes were inactivated (10 min, 100 °C) and the supernatant was applied onto a Sephacryl S-100 HR column, which was initially equilibrated in 0.05 M sodium acetate buffer pH 5.0, using a Hiload System (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Elution was carried out using the same buffer and the elution rate was 2.5 ml/min. Fractions (10 ml) were collected and assayed by automated methods for neutral sugar content[16] and uronic acid content[17]. The appropriate fractions were pooled, concentrated, dialysed, freeze-dried and analysed for neutral sugar composition and uronic acid content.

ANALYTICAL METHODS

Neutral sugar composition was determined by gas chromatography according to Englyst and Cummings[18], using inositol as an internal standard. The samples were pre-treated with 72% (w/w) H_2SO_4 (1h, 30 °C) followed by hydrolysis with 1 M H_2SO_4 for 3h at 100 °C and the constituent sugars were analysed as their alditol acetates.

Uronic acid content was determined by the automated colorimetric *m*-hydroxydiphenyl assay[17,19] using an auto-analyser (Skalar Analytical BV, Breda, The Netherlands). Corrections were made for interference by neutral sugars present in the sample as measured by the orcinol-sulfuric acid method[16].

High-Performance Size-Exclusion Chromatography (HPSEC) was performed on a SP8800 HPLC (Spectra Physics, San José, CA, USA) equipped with three columns (each 300 x 7.5 mm) of Bio-Gel TSK in series (60XL, 40XL and 30XL; Bio-Rad Labs., Richmond, CA USA) in combination with a

TSK guard column (40 x 6 mm) and elution at 30 °C with 0.4 M sodium acetate buffer pH 3.0 at 0.8 ml/min. Calibration was performed using dextrans. The eluate was monitored using a Shodex SE-61 Refractive Index detector (Showa Denko K.K., Tokyo, Japan).

High-Performance Anion-Exchange Chromatography (HPAEC) was performed on a Dionex Bio-LC system (Sunnyvale, CA, USA)[20]. The gradient was obtained by mixing solutions of 0.1 M NaOH and 1 M sodium acetate in 0.1 M NaOH.

For the determination of arabinogalactan oligomers, the (4 x 250 mm) CarboPac PA100 column (Dionex) was equilibrated with 0.1 M NaOH. Twenty μl of the sample was injected and a linear gradient to 0.4 M sodium acetate in 0.1 M NaOH in 40 min was applied. The column was washed for 5 min with 1 M sodium acetate in 0.1 M NaOH and equilibrated again for 15 min with 0.1 M NaOH. Calibration was performed with standard solutions of arabinose, galactose and a series of arabinan oligomers.

RESULTS AND DISCUSSION

DEGRADATION OF SOYBEAN POLYSACCHARIDES WITH CLONED ENZYMES

A first screening of the degradability of soybean meal, WUS and extracts thereof was performed with cloned enzymes, because purified enzymes are available in only very small amounts. Incubation is performed with endo-galactanase, endo-arabinanase, RG hydrolase, a combination of RG hydrolase and RGAE or a combination of PG-1 and PE. Since RG hydrolase is hindered by the presence of *O*-acetyl groups[21] RGAE was added to remove these groups. Similar reasons underlie the addition of PE to PG-1, to remove methylester groups. Hydrolysis by PG-1 preferably takes place next to a free carboxyl group and PE is able to remove the methoxyl groups from methylated galacturonic acid residues.

Table 3.1. Sugar composition of soybean polysaccharides fractions expressed as mol%.

	Sugar composition								
Fraction	rha	fuc	ara	xyl	man	gal	glc	galA	Carbohydrate content[a]
WUS	2	3	19	8	2	29	21	17	89.3
ChSS	2	3	25	6	1	37	1	26	52.7
Polymeric residue (P)	6	7	4	18	2	12	1	50	69.7

[a] Expressed as % w/w

The digests obtained from soybean meal and WUS contained some arabinose and galactose, probably because of side-activities in the enzyme preparations. Endo-arabinanase

was able to solubilise an amount of arabinan oligomers, which was relatively small considering the high arabinose content (Table 3.1). Although the sugar composition of soybean WUS indicates the presence of rhamnogalacturonan regions, RG hydrolase - whether combined with RGAE or not - did not release the characteristic RGase oligomers described by Schols *et al.*[20]. The combination of PG-1 and PE was able to release some galacturonic acid monomers in addition to the neutral sugar residues, but did not release galacturonan oligomers in spite of the high galacturonic acid content of the WUS. Analysis with HPSEC showed that none of these enzyme preparations were able to solubilise polysaccharides from both the soybean meal and WUS (elution patterns are not shown). So none of these enzymes were able to degrade the cell wall as present in soybean meal and soybean WUS, only some small neutral degradation products and some galacturonic acid residues were released. The network of the cell wall polysaccharides present in soybean appears to be too complex or too dense to be penetrated by the applied enzymes. Removal of the protein, which accounts for about 60% of the soybean meal, from the cell wall material did not increase the susceptibility of the polysaccharides for enzymatic degradation, as the degradability of the WUS is not improved compared to the degradability of the soybean meal.

Disruption of this cell wall polysaccharide network by sequential extraction can possibly increase the degradability by enzymes. Therefore, the degradability of the pectin-rich extracts - ChSS, DASS and 1 MASS - from soybean WUS was determined by incubation of the extracts with the cloned enzymes mentioned previously.

Analyses of the digests with HPSEC (only the digests of ChSS are shown in Figure 3.1) exhibit large decreases of the molecular weight of the polysaccharides in these extracts, except for the PG-1 and PE digests of ChSS and DASS. HPAEC analyses of the digests show the release of galactose and arabinose monomers in addition to small amounts of arabinan, galactan and arabinogalactan oligomers. RG hydrolase and PG-1 are also able to release galacturonic acid residues. The amounts and diversity of oligomers formed with the various enzyme preparations and from the three substrates are different.

Extraction of the pectic polysaccharides did indeed increase their degradability, although this mainly concerned the degradability of the (arabino)galactan side chains. All the enzymes used seem only to be able to degrade part of the (arabino)galactan side chains present in the pectin-rich extracts from soybean WUS. RG hydrolase and PG-1 preparations, which are theoretically able to degrade the pectin backbone, do not release their typical degradation products. The only degradation detected is that of the (arabino)galactan side chains because of side activities of the preparation. Even after enzymatic removal of *O*-acetyl and methoxyl groups from the uronic acid residues, degradation of the pectin backbone could not be determined.

These results indicate that soybean pectic polysaccharides differ from pectic polysaccharides from other sources in that they are not degraded by the enzymes as used in this experiment. The fact that the backbone could not be degraded indicates that the

galacturonic acid residues are presumably highly substituted or that they are not present in extended galacturonan chains.

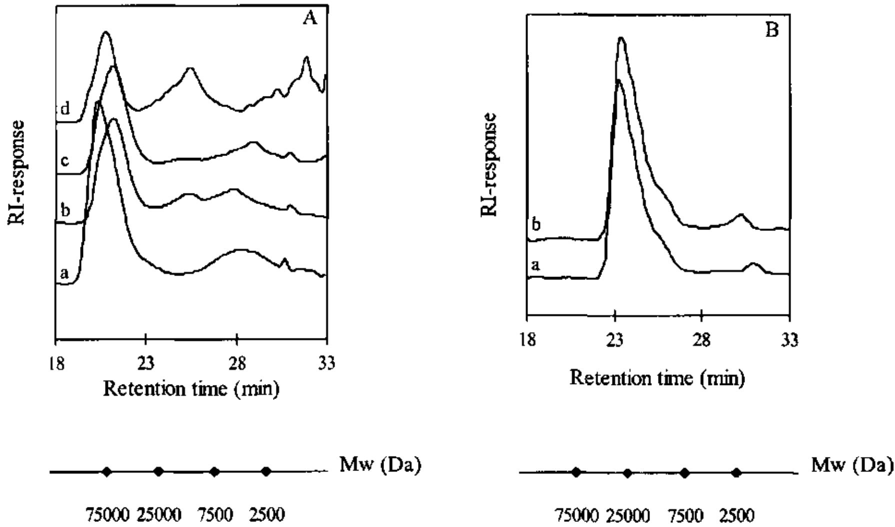

Figure 3.1. HPSEC elution patterns of the soybean ChSS fraction after incubation with cloned enzymes for 24h. A: (a) before; and (b) after incubation with endo-galactanase; (c) endo-arabinanase; (d) RG hydrolase and RGAE. B: (a) before; and (b) after incubation with PG-1 and PE.

Screening of the degradability of soybean polysaccharides as described previously was performed with cloned enzymes. However, these enzyme preparations still contain some side-activities. Therefore we continued our studies on the characterisation of the cell wall polysaccharides with highly purified enzymes. They allow better conclusions about which parts of the polysaccharides are degraded. The next step in the characterisation of the polysaccharides will use these purified enzymes to investigate which parts of the polysaccharides in the pectin-rich ChSS fraction are degraded, to determine which degradation products are formed, and to obtain the part of the polymers which is not degraded.

Degradation of the ChSS fraction with (combinations of) arabinan/galactan degrading enzymes

The galacturonic acid-rich ChSS fraction is rich in arabinose and galactose residues (Table 3.1). This indicates the presence of pectins with a considerable amount of arabinan, galactan or arabinogalactan side chains. Incubation with enzymes, able to (partially) degrade these side

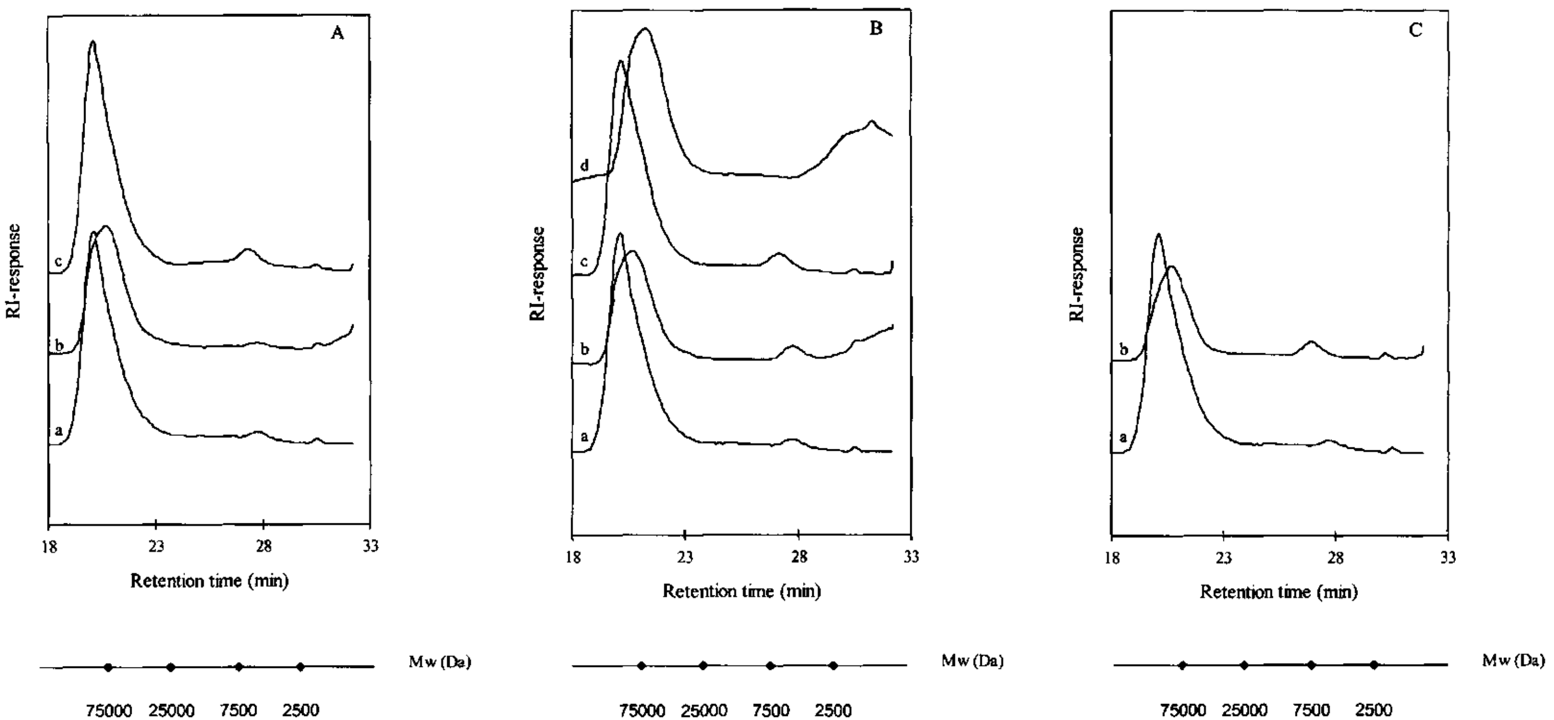

Figure 3.2. HPSEC elution patterns of the soybean ChSS fraction, A: (a) before; and (b) after incubation with endo-galactanase; (c) endo-arabinanase. B: (a) before; and (b) after incubation with endo-galactanase and exo-galactanase; (c) endo-arabinanase and arabinofuranosidase B; and (d) endo-galactanase and arabinofuranosidase B. C: (a) before; and (b) after incubation with a combination of endo-galactanase, exo-galactanase, endo-arabinanase, and arabinofuranosidase B. All digests were incubated with the previously mentioned enzymes for 24h.

chains, can provide information about their structure. Therefore incubations with endo-galactanase, exo-galactanase, endo-arabinanase, and arabinofuranosidase B are performed.

First, the ChSS fraction was incubated with all enzymes separately. The elution patterns of the endo-galactanase digest and of the endo-arabinanase digest (24h incubation) after HPSEC and HPAEC are shown in Figures 3.2(A) and 3.3(A). In addition, incubations with combinations of the enzymes were performed, both simultaneously and subsequently after inactivation of the first enzyme. Figures 3.2(B),(C) and 3.3(B),(C), show only the elution patterns of the most interesting digests, because a number of digests are (almost) identical.

The molecular weight distributions shown in Figure 3.2(A) demonstrate that endo-galactanase is the only tested enzyme which is able to reduce the hydrodynamic volume of the polymers. Endo-galactanase causes a small shift of the molecular weight and a simultaneous decrease of the amount of polymeric material (70% of the original area of the high molecular weight peak remains after 90 min and 65% after 24h). A small peak of intermediate molecular weight with a retention time of 26 min is formed after 90 min of incubation (not shown). These rather large degradation products are further degraded after prolonged incubation (Figure 3.2(Ab)). Since endo-galactanase is only able to degrade the (arabino)galactan side chains of the pectins in the ChSS fraction, the release of the intermediate products indicates that these side chains can be of considerable length. Endo-arabinanase is not able to cause any change in the molecular weight distribution of the ChSS fraction. Van de Vis [22] also found that endo-arabinanases showed very little activity on soybean arabinogalactan. Exo-galactanase and arabinofuranosidase B (not shown), do not influence the HPSEC elution pattern. Although the exo-enzymes do release monomeric sugar residues from the polysaccharide, the effect of these enzymes on the hydrodynamic volume of heterogeneous polysaccharides is usually negligible.

To determine whether the presence of branches on the galactan chain influence the degradation by endo-galactanase, the ChSS fraction was incubated with combinations of endo- and exo-galactanase and of endo-galactanase and arabinofuranosidase B. Combined action of endo-galactanase and exo-galactanase, both simultaneously (Figure 3.2(Bb)) and subsequently (not shown), brings about a shift in the molecular weight distribution which is identical to the action of endo-galactanase alone. The amount of remaining polymeric material decreased further to 60% of the polymers in the ChSS blank, suggesting that exo-galactanase is able to release galactose from the polymers, and enhances endo-galactanase action. This additional degradation does not effect the hydrodynamic volume of the remaining polymers. Incubation of the ChSS fraction with the combination of endo-galactanase and arabinofuranosidase B causes a shift to lower molecular weight, which is slightly larger than the shift caused by endo-galactanase alone (Figure 3.2(Bd)). The amount of the remaining polymer in the digest is also a little smaller, approximately 60% of the polymers in the blank. The resulting elution pattern is quite similar to the profile of the digest obtained by endo- and exo-galactanase. The sequence in which the enzymes are added to the ChSS fraction does not

influence the HPSEC elution pattern, suggesting that arabinofuranosidase B is able to remove arabinofuranosyl groups from arabinogalactan side chains, but this does not increase the susceptibility of these chains for endo-galactanase.

Labavitch *et al.*[6] demonstrated that rather large arabinan chains are present in a fraction from soybean meal, which are expected to be degraded by endo-arabinanase. The absence of a shift in the molecular weight distribution in Figure 3.2(Ac) and the absence of large amounts of arabinan oligomers in Figure 3.3(Ab) shows that endo-arabinanase alone is not able to degrade the arabinan chains of the ChSS fraction. A linear (1,5)-α-L-arabinan is a better substrate for endo-arabinanase than a branched arabinan, and since the degree of branching of pectic arabinans from various plant tissues is found to be relatively high[23], it might be necessary to linearise the arabinan chain before degradation by endo-arabinanase can take place. To rule out the hindrance of arabinofuranosyl side chains, the ChSS fraction was incubated with endo-arabinanase and arabinofuranosidase B, simultaneously. However, this also did not result in a change of the molecular weight distribution of ChSS (Figure 3.2(Bc)).

The combination of endo-galactanase, exo-galactanase, endo-arabinanase, and arabinofuranosidase B is very effective in degrading the ChSS fraction. This digest shows the largest decrease of the hydrodynamic volume and the smallest peak of remaining polymeric material (Figure 3.2(Cb)). About 45% of polymeric material remained. Arabinose and galactose represent over 60 mol% of the polysaccharides in the ChSS fraction (Table 3.1). This indicates that this combination of enzymes can remove almost all arabinogalactan side chains and that the enzymes act in synergism, because the total effect of the combination of enzymes is greater than the sum of the individual effects.

The HPAEC elution patterns of the digests obtained after incubation of the ChSS fraction with endo-galactanase, endo-arabinanase and arabinofuranosidase B is shown in Figure 3.3(A). Degradation with endo-galactanase releases relatively large (arabino)galactan oligomers, and after incubation for 90 min, the main oligomer seems to be a tetraose (not shown). After prolonged incubation (24h), this series of oligomers is further degraded by the endo-galactanase to mainly mono, di and trimers (Figure 3.3(Aa)). Endo-arabinanase was not able to degrade the ChSS fraction (Figure 3.3(Ab)), the digest contains very few oligomeric degradation products. Exo-galactanase releases galactose monomers, as expected (not shown), although it could not be concluded whether the galactose residues were released from the non-reducing end of galactan chains or from galactose branches. Arabinofuranosidase B releases mainly arabinose monomers and some arabinan oligomers from the ChSS fraction (Figure 3.3(Ac)).

When endo-galactanase is combined with exo-galactanase (Figure 3.3(Ba)), the amount of galactose increases compared with the amount of galactose in the endo-galactanase digest.

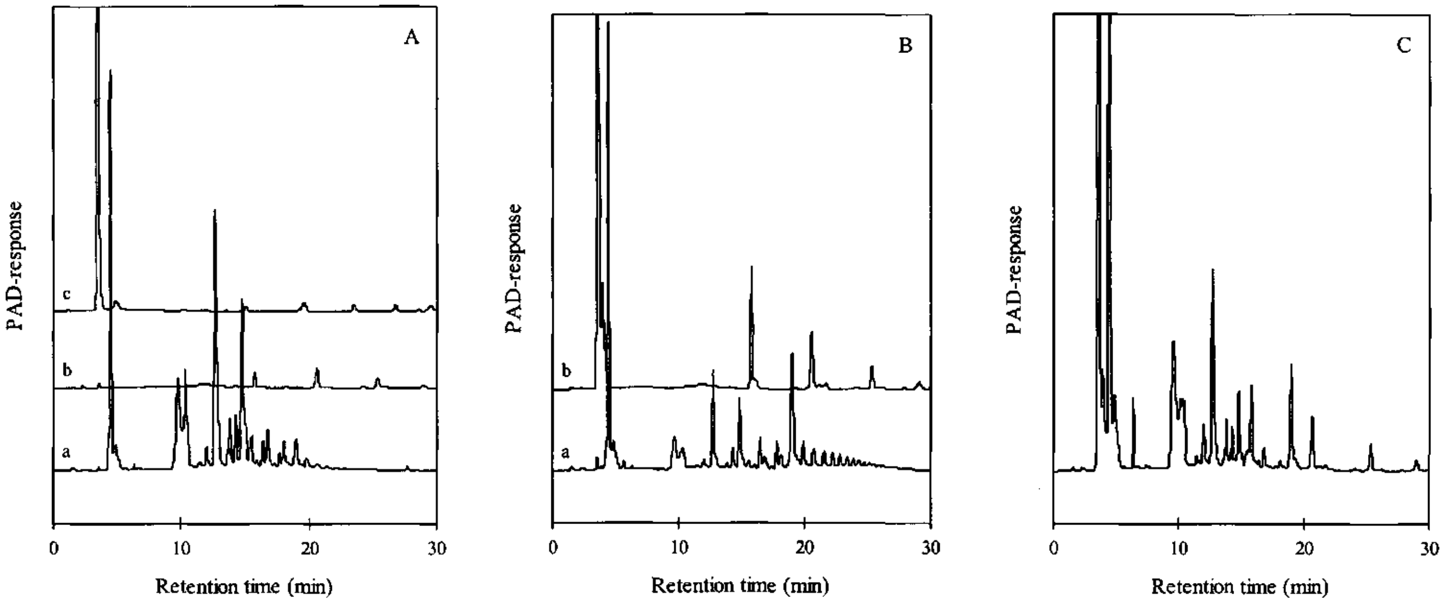

Figure 3.3. HPAEC elution patterns of the soybean ChSS fraction, A: (a) endo-galactanase; (b) endo-arabinanase; (c) arabinofuranosidase B. B: (a) endo-galactanase and exo-galactanase; (b) endo-arabinanase and arabinofuranosidase B. C: combination of endo-galactanase, exo-galactanase, endo-arabinanase, and arabinofuranosidase B. All digests were incubated with the previously mentioned enzymes for 24h.

The elution patterns show a decrease of the amount of galactan and/or arabinogalactan oligomers, although some larger oligomers seem still to be present in the digest. The oligomers formed by endo-galactanase are probably further degraded by the exo-galactanase activity. The endo-galactanase and arabinofuranosidase B digest of the ChSS fraction (not shown) contains the same oligomers as the endo-galactanase digest. The amount of arabinose monomers is much higher than in the endo-galactanase digest and identical to the amount of arabinose monomers in the arabinofuranosidase B digest. Thus, arabinofuranosidase B appears to be unable to further degrade the oligomers formed by endo-galactanase; the enzyme is only able to release arabinose residues from the arabinans present in the polymeric material.

Combined action of endo-arabinanase and arabinofuranosidase B releases a considerable amount of arabinose residues and small amounts of arabinose oligomers, oligomers with a degree of polymerisation up to 5 can be detected in Figure 3.3(Bb). Rombouts *et al.*[13] and Beldman *et al.*[23] showed that arabinofuranosidase B releases arabinose as the sole product, resulting in linearised arabinan chains, which are better substrates for the endo-enzyme. The release of larger arabinan oligomers may have taken place, but the arabinofuranosidase B present in the incubation mixture is able to degrade these oligomers. This synergism between endo-arabinanase and arabinofuranosidase B on soybean polysaccharides was also found by van de Vis[22].

The combination of endo-galactanase, exo-galactanase, endo-arabinanase, and arabinofuranosidase B, which showed the largest decrease of the molecular weight and the smallest peak of remaining polymeric material in the HPSEC elution pattern, shows the release of high amounts of arabinose and galactose residues and a large number of different oligosaccharides: arabinan oligomers, galactan oligomers as well as arabinogalactan oligomers (Figure 3.3(C)).

Enzymatic degradation of the neutral side chains of soybean pectin shows that these side chains can be of considerable length. Combined action of endo-galactanase, endo-arabinanase, exo-galactanase, and arabinofuranosidase B is required to remove the larger part of these side chains and this indicates that arabinogalactan side chains are present in addition to arabinan and galactan side chains.

CHARACTERISATION OF THE RESIDUAL POLYSACCHARIDE AFTER REMOVAL OF THE ARABINOGALACTAN SIDE CHAINS FROM SOYBEAN CHSS

The incubation of the ChSS fraction with endo-galactanase, exo-galactanase, endo-arabinanase, and arabinofuranosidase B is performed on larger scale to enable isolation and further characterisation of the remaining polymeric residue. The elution pattern of the Sephacryl S-100 HR column shows two peaks; the first peak originates from the remaining polymeric part of the pectin (P) and the second from the oligomeric degradation products

(Figure 3.4). This polymeric residue yields 30% of the polysaccharides present in the ChSS fraction, representing 12% of the polysaccharides present in soybean WUS.

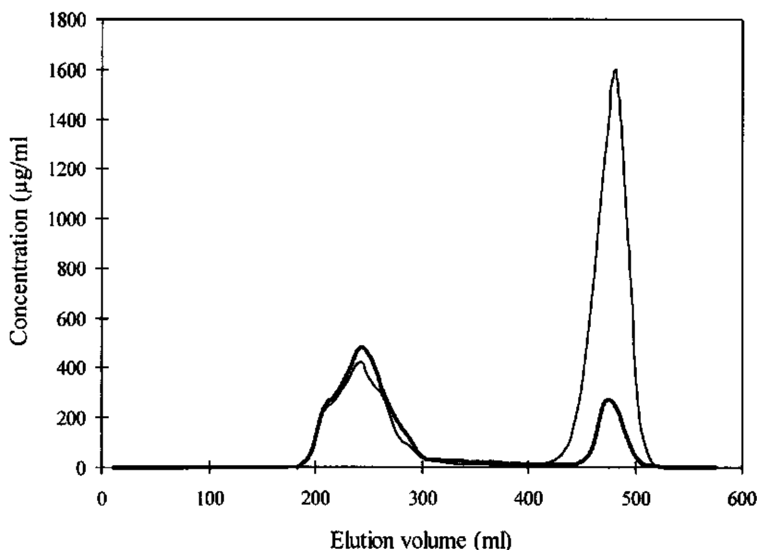

Figure 3.4. Elution profile of the digest of soybean ChSS, after incubation with endo-galactanase, exo-galactanase, endo-arabinanase, and arabinofuranosidase B, on Sephacryl S-100 HR. Uronic acid concentration (—), neutral sugar concentration (—).

Table 3.1 shows the sugar composition of the polymeric residue (P) and allows comparison with the sugar compositions of the WUS and the ChSS fraction from which it originates. The combined activities of the enzymes used were able to remove almost all arabinose, only 7% of the arabinose present in the ChSS fraction remained. The amount of remaining galactose residues was higher and represents 12% of the galactose present in the ChSS fraction before degradation. Additional incubation of a small sample of residue (P) with the same set of enzymes was performed to determine if more arabinose and galactose residues could be removed with an excess of enzymes. Both, HPSEC and HPAEC analysis of the digest, did not show any change of the molecular weight distribution or the release of any degradation product, so further removal of the arabinogalactan side chains was not possible using these enzymes.

The main constituent sugar of the polymeric residue P was uronic acid (50 mol%). Striking is the presence of 18 mol% xylose, which may indicate the presence of a xylogalacturonan. Preliminary results obtained in enzymatic degradation of this polymeric residue after acid hydrolysis with an exo-galacturonase[24] showed the release of the characteristic dimer of xylose and galacturonic acid, thus, confirming the presence of a xylogalacturonan. In addition, the polymer contains 6 mol% rhamnose, which is very likely to

be present in rhamnogalacturonan regions in the remaining pectin. In the ChSS fraction, on average 36% of the uronic acid residues carry an acetic acid group and 35% carries a methoxyl group. The degree of methylation and acetylation in the polymeric residue P could not be determined, because the available amount of this polymer was too small. However, the acetyl and methoxyl substituents are probably still present in the remaining polymer, because the incubation and isolation conditions are not likely to have removed any of these groups. Thus, the polymeric residue (P) of ChSS appears to be a highly substituted pectic structure.

CONCLUSIONS

It appears that rather pure cloned enzymes, having only one major activity, can not or hardly degrade the polysaccharides in the intact soybean cell wall. Previous research showed purified enzymes also to be rather limited in the degradation of soybean cell wall material[25]. Even incubation with very powerful commercial enzyme mixtures did not lead to the degradation of the intact cell wall polysaccharides. However, polysaccharides extracted from soybean WUS can be degraded by enzymes more easily. Extraction of part of the polysaccharides from the cell wall network might enlarge the pores present in this network and enable enzymes to penetrate and reach the available hydrolysis sites. This indicates that it is necessary to disrupt the network of the cell wall polysaccharides to enable the enzymes to degrade them. Still, it is not yet clear whether the proper enzymes are missing or that the inaccessibility of the substrate is the main reason.

The combination of endo-galactanase, exo-galactanase, endo-arabinanase, and arabinofuranosidase B is very effective in degrading the arabinogalactan side chains in the ChSS fraction. Analysis of the degradation products and the observed synergistic action of some enzyme combinations indicate that rather large branched arabinogalactan side chains are present in soybean pectic structures. About 30% of the sugars present in the ChSS fraction are recovered as the undegradable remaining polymer (P) in the digest after this incubation. This polymer still contains 4 mol% of arabinose and 12 mol% of galactose, which could not be removed by these enzymes. This polymer appears to be a very highly substituted pectic structure, containing rhamnogalacturonan regions and presumably xylogalacturonan. From an unfractionated soybean polysaccharide preparation, various acidic xylose-containing oligosaccharides were obtained by partial hydrolysis, by Aspinall *et al.*[26]. However, this is the first time that indications for the presence of *polymeric* xylogalacturonan regions in a pectic polysaccharide fraction from soybean meal were obtained. The presence of xylogalacturonan regions has been indicated in pectins extracted from pea hulls[27], apple[28], watermelon and cotton[29].

Further research will focus on the elucidation of the structure of both the remaining polymer (P) and the (arabino)galactan oligomers which are formed after degradation of the

soybean ChSS fraction with endo-galactanase, exo-galactanase, endo-arabinanase, and arabinofuranosidase B. This will include chromatography techniques, degradation with specific enzymes, and MS and NMR analyses. Knowledge of the structures of these oligosaccharides and of the mode of action of the enzymes can lead to the elucidation of the structure of the (arabino)galactan side chains from soybean cell wall pectin. The structure of the other cell wall polysaccharides of soybean will be another topic of investigation.

ACKNOWLEDGEMENT

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CHAPTER 4

DETERMINATION OF THE STRUCTURE OF THE BACKBONE FROM SOYBEAN PECTIC SUBSTANCES

M.M.H. HUISMAN, C.T.M. FRANSEN, J.P. KAMERLING, J.F.G. VLIEGENTHART, H.A. SCHOLS & A.G.J. VORAGEN

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Structural characteristics of pectic substances extracted from soybean meal WUS with a CDTA-containing buffer were studied. The arabinogalactans present as side chains to the rhamnogalacturonan backbone were largely removed by enzymatic hydrolysis using endo-galactanase, exo-galactanase, endo-arabinanase, and arabinofuranosidase B. The remaining pectic backbone appeared to be resistant to enzymatic degradation by pectolytic enzymes. After partial acid hydrolysis of the isolated pectic backbone (fraction P), one oligomeric and two polymeric populations were obtained by size-exclusion chromatography. Monosaccharide and linkage analyses, enzymatic degradation, and NMR spectroscopy of these populations showed that the pectic substances in the original extract contain both rhamnogalacturonan and xylogalacturonan regions, while homogalacturonan is absent.

INTRODUCTION

Pectic substances are the major group of polysaccharides in the primary cell wall of dicotyledonous plants. These pectic substances consist of a number of structurally different regions. Pectins from apple consist of highly methyl esterified linear homogalacturonan regions which alternate with "hairy" regions that comprise highly branched rhamnogalacturonan[1]. In addition, these hairy regions appeared to contain xylogalacturonan[2]. This concept of the appearance of pectic substances proved to be applicable to pectic substances from other sources, like for example cotton suspension-cultured cell walls, watermelon[3], and pea hulls[4,5]. However, differences in the relative amounts of the subunits may exist.

Another type of pectic polysaccharide in the plant cell wall is RG-II. RG-II is a small, structurally well defined, complex pectic polysaccharide. It consists of a homogalacturonan backbone to which side chains, containing rhamnose and several rare "diagnostic" monosaccharides, are attached[6].

Studies on the structure of soybean pectic substances go back to 1967. An acidic polysaccharide complex was extracted from soybean meal[7,8], which showed to possess a highly branched structure composed of galacturonic acid, galactose, arabinose, xylose, fucose, and rhamnose. The interior chains were found to comprise 4-substituted galacturonic acid and 2-substituted rhamnose residues, and exterior chains were composed mainly of neutral sugar residues. Some of the rhamnose residues were branched at C4, and some galacturonic acid residues were branched with xylose residues through C3. Most of the fucose and a substantial proportion of the xylose residues were present as non-reducing end groups. These results indicated that soybean pectic substances contain both rhamnogalacturonan and xylogalacturonan regions. However, only the formulation of partial structures for the soybean acidic polysaccharide complex was permitted, because the data were obtained after partial

acid hydrolysis. This is an a-specific way of hydrolysis, and resulted in rather small fragments.

In the preceding paper[9] we showed that the pectic backbone from CDTA-extractable pectins (CDTA, 1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid) could be isolated after enzymatic removal of the arabinogalactan side chains. In this study we report on both the enzymatic and acid hydrolysis of the remaining pectic backbone. Furthermore, structural analyses of this backbone and fractions thereof will be described.

Experimental

Materials

Water unextractable solids (WUS) were isolated from solvent-extracted, untoasted soybean meal and sequentially extracted[10]. The arabinogalactan side chains from the pectins in the chelating agent soluble solids (ChSS) extract were removed by enzymatic degradation and the remaining polymer was isolated and designated fraction P[9].

Weak acid hydrolysis of fraction P

A 8 mg/ml solution of fraction P was hydrolysed in 0.1 M HCl at 80 °C. During hydrolysis samples were taken after various time intervals, and the reaction was stopped by neutralisation of the solution with an equal amount of 0.1 M NaOH. The hydrolysates were analysed with HPSEC and HPAEC.

Enzymatic degradation

Solutions (0.25% (w/w)) of fraction P in 50 mM NaOAc buffer (pH 5.0) containing 0.01% NaN_3 were incubated with a number of purified enzymes at 30 °C rotating 'head over tail', during 24h. The purified enzymes used were endo-galactanase (0.34 μg protein/ml substrate solution), exo-galactanase (0.52 μg protein/ml substrate solution), PG (0.49 μg protein/ml substrate solution), PL (9.76 μg protein/ml substrate solution), RG hydrolase (0.53 μg protein/ml substrate solution), and exo-galacturonase (1.03 μg protein/ml substrate solution). Endo-galactanase was purified from *Aspergillus aculeatus*[11]. Exo-galactanase was purified from *Aspergillus niger*[12]. Polygalacturonase (PG; E.C. 3.2.1.15) was purified from *Kluyveromyces fragilis*[13]. Pectin lyase (PL; E.C. 4.2.2.10) was purified from *Aspergillus niger*[14]. Rhamnogalacturonan hydrolase (RG hydrolase) was purified from *Aspergillus aculeatus*[15,16]. Exo-galacturonase was purified from *Aspergillus aculeatus*[17].

Solutions (0.5% (w/w)) of fraction P were also incubated with technical multienzyme preparations under the conditions described above. The technical enzyme preparations used were Pectinex Ultra-SP-L derived from *Aspergillus aculeatus*, Viscozyme derived from *Aspergillus aculeatus* (Novo-

Nordisk Ferment (Switzerland) Ltd., Dittingen, Switzerland), Rapidase liq+ derived from *Aspergillus niger/Trichoderma sp.* (Gist-brocades, Delft, The Netherlands), and Driselase derived from *Irpex lacteus* (Sigma, St Louis, MO, USA). The enzyme preparations were dialysed against 50 mM NaOAc buffer (pH 5.0) and diluted 100 times. Fifty µl of the enzyme solution was added to 0.5 ml of the substrate solution.

Solutions (0.1% (w/w)) in 0.05 M NaOAc buffer (pH 5.0) containing 0.01% NaN_3 of the populations obtained after fractionation of the acid hydrolysate of fraction P (PI, PII and PIIIA) were incubated with PG (2.4 µg protein/ml substrate solution), RG hydrolase (0.18 µg protein/ml substrate solution), and exo-galacturonase (1.03 µg protein/ml substrate solution). In addition, these substrates were also incubated with xylogalacturonan hydrolase (XGH; 16 µg protein/ml substrate solution) purified from the culture filtrate of *Aspergillus tubigensis* cDNA library expression cloned in *Kluyveromyces lactis*[18] for 1h at 30 °C.

All enzymes were inactivated by heating at 100 °C for 10 min. Polysaccharide-degrading activities were determined by HPSEC and HPAEC analyses of the digests.

NEUTRAL SUGAR COMPOSITION

The neutral sugar composition was determined by methanolysis combined with TFA hydrolysis[19]. Samples were first dissolved in distilled water (1 mg/ml). An aliquot of 20 µl of this solution was dried by a stream of air followed by methanolysis with 0.5 ml anhydrous 2 M HCl in absolute methanol for 16h at 80 °C. After cooling, the liquid was evaporated by a stream of air and 0.5 ml of 2 M TFA solution was added and heated for 1h at 121 °C. The samples were dried and 100 µl of distilled water was added. Analysis of the liberated products was performed using HPAEC.

URONIC ACID CONTENT

The uronic acid content was determined by the automated colorimetric *m*-hydroxydiphenyl assay[20,21] using an auto-analyser (Skalar Analytical BV, Breda, The Netherlands). Corrections were made for interference by uronic acids present in the sample as measured by the orcinol-sulfuric acid method[22].

ABSOLUTE CONFIGURATION DETERMINATION

The absolute configuration of the monosaccharides was performed for ChSS only[23,24], since all other polymers were obtained from this material. The trimethylsilylated 2-butyl glycosides were analysed by GLC on a Chrompack CP9002 gas chromatograph, equipped with a CP-Sil 5 CB DFc.25 (Chrompack) capillary column (25 m x 0.32 mm), using a temperature program of 140-240 °C at 4 °C/min.

METHYLATION ANALYSIS

Methylation analysis was carried out essentially as described[25]. Briefly, freshly ground NaOH pellets (250 mg) were added to solutions of samples in Me_2SO (200 µl). After the material had dissolved, samples were cooled to 0 °C and MeI (0.5 ml) was added, followed by sonication at room temperature. The reaction was stopped after 45 min by adding aq $Na_2S_2O_3$ (1 ml, 100 mg/ml) and $CHCl_3$ (1 ml). The chloroform layer was washed with water (3 x 0.5 ml), then concentrated. After hydrolysis of the residues with 2 M TFA (0.3 ml; 120 °C, 1h), samples were dissolved in 0.5 M NH_4OH (250 µl) containing $NaBD_4$ (10 mg/ml) and kept for 1h, then neutralised with aq 99% HOAc and concentrated. Boric acid was removed by repetitive co-evaporation with 9:1 MeOH-HOAc and MeOH. After acetylation with Ac_2O (0.5 ml; 120 °C, 3h), quenching with water (0.5 ml), and neutralisation with $NaHCO_3$, the mixtures of partially methylated alditol acetates were extracted with CH_2Cl_2 (3 x 0.5 ml). The solutions were concentrated to about 20 µl (N_2), and analysed by GLC (see above) and GLC-EIMS. GLC-EIMS analyses were carried out on a Fisons MD800/8060 system (electron energy, 70 eV; carrier gas, He) equipped with a DB-1 fused-silica capillary column (30 m x 0.32 mm, J&W Scientific). Samples were injected using a split injector (split flow 1/10), and a temperature program of 140-240 °C at 4 °C/min.

For the determination of the substitution pattern of galacturonic acid the permethylated polysaccharide was reduced with superdeuteride (Aldrich Chemicals, 0.5 ml, 3 h). After quenching with 1:1 MeOH-H_2O, desalting (Dowex H^+, 2 ml)[26], and concentration, the sample was hydrolysed, reduced, acetylated and analysed as described above.

NMR SPECTROSCOPY

NMR spectroscopy was performed on samples which were deuterium exchanged twice in D_2O (99.9 atom % D, Cambridge Isotope Laboratories, USA) preceding NMR analyses and then dissolved in D_2O (99.96 atom % D, Isotec, USA). If necessary, the pD of the NMR sample was adjusted to 6.5-7.5. 1D/2D high resolution NMR spectra were recorded on a Bruker AMX-500 or a AMX-600 spectrometer (Bijvoet Center, Utrecht University), or a Bruker DRX-600 instrument (NSR Center, University of Nijmegen) at a probe temperature of 300, 333, or 353 K. Chemical shifts (δ) are expressed in ppm relative to external glucose (δ Glc*p* H1α 5.227 and C1α 92.9, in D_2O at all temperatures). 2D NMR experiments were performed essentially as described[27].

SIZE-EXCLUSION CHROMATOGRAPHY

The acid hydrolysate of fraction P (40 mg) was applied onto a Sephacryl S-500 HR column (110 × 0.5 cm), which was initially equilibrated in 0.05 M NaOAc buffer pH 5.0, using a Hiload System (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Elution was carried out using the same buffer and the elution rate was 0.4 ml/min. Fractions (1.2 ml) were collected and assayed by automated

methods for neutral sugar content[22] and uronic acid content[21]. The appropriate fractions were pooled, concentrated, dialysed, freeze dried and analysed for neutral sugar composition and uronic acid content.

The low molecular mass material (population PIII) was fractionated further on a Bio-Gel P-2 column (100 × 2.6 cm; Bio-Rad Labs., Richmond, CA, USA), which was initially equilibrated in water at 60 °C using a Hiload System. Elution was carried out using water and the elution rate was 0.5 ml/min. Fractions (7.5 ml) were collected and assayed as described above for the Sephacryl S-500 HR fractions.

High-Performance Size-Exclusion Chromatography (HPSEC)

HPSEC was performed on a SP8800 HPLC (ThermoQuest Corporation, San José, CA, USA) equipped with three columns (each 300 x 7.5 mm) of Bio-Gel TSK in series (40XL, 30XL and 20XL; Bio-Rad Labs., Richmond, CA USA) in combination with a TSK guard column (40 x 6 mm) and elution at 30 °C with 0.4 M NaOAc buffer pH 3.0 at 0.8 ml/min. Calibration was performed using pectins in the range 10-82 kDa. The eluate was monitored using a Shodex SE-61 Refractive Index detector (Showa Denko K.K., Tokyo, Japan).

High-Performance Anion-Exchange Chromatography (HPAEC)

HPAEC was performed on a Dionex Bio-LC system (Sunnyvale, CA, USA)[28] using a (4 x 250 mm) CarboPac PA1 column (Dionex). Twenty-µl aliquots were injected and the gradient was obtained by mixing solutions of 100 mM NaOH, 1 *M* NaOAc in 0.1 *M* NaOH, and distilled water, at a flow rate of 1 ml/min. Different gradients were used for the sugar composition after methanolysis combined with TFA hydrolysis, the release of monomers during acid hydrolysis[19], rhamnogalacturonan oligomers[29], and XGH digests[18].

For the determination of galacturonan oligomers, the column was equilibrated with 0.2 M NaOAc in 0.1 M NaOH. Elution was performed with a linear gradient to 0.6 M NaOAc in 0.1 M NaOH in 35 min, and a linear gradient to 1 M NaOAc in 0.1 M NaOH in 5 min. The column was washed for 5 min with 1 M NaOAc in 0.1 M NaOH, and equilibrated again for 15 min with 0.2 M NaOAc in 0.1 M NaOH.

Exo-galacturonase digests were analysed by equilibrating the column with 0.1 M NaOH. Elution was performed with a linear gradient to 0.31 M NaOAc in 0.1 M NaOH in 25 min, and a linear gradient to 1 M NaOAc in 0.1 M NaOH in 5 min. The column was washed for 5 min with 1 M NaOAc in 0.1 M NaOH, and equilibrated again for 15 min with 0.1 M NaOH.

RESULTS AND DISCUSSION

Fraction P is the polymeric residue, remaining after enzymatic removal of the arabinose and galactose-containing side chains from soybean ChSS. The sugar composition of both ChSS and fraction P are presented in Table 4.1. Determination of the absolute configuration of the starting material ChSS reveals the presence of L-arabinose, L-fucose, D-galactose, D-galacturonic acid, L-rhamnose, and D-xylose. Fraction P yields 30% of the polysaccharides present in the ChSS extract, and represents 12% of the polysaccharides present in soybean WUS. The residual amount of arabinose plus galactose in fraction P represents 8% of the amount in the ChSS extract. Thus, the side chains containing arabinose and galactose are largely removed. Based on the general structural features of pectic substances, the sugar composition of fraction P indicated that it contains very highly substituted pectic structures, amongst which are rhamnogalacturonan and presumably xylogalacturonan regions[9]. Further characterisation of fraction P is performed by enzymatic degradation studies.

Table 4.1. Monosaccharide composition of soybean polysaccharide fractions expressed as mol%.

Fraction	Rha	Fuc	Ara	Xyl	Gal	GalA
ChSS	4	4	25	7	40	21
P	11	9	7	18	12	43
PI	9	tr	2	26	6	58
PII	16	3	0	20	9	55
PIIIA	24	3	2	12	13	46

tr, trace amount

ENZYMATIC DEGRADATION OF FRACTION P

The galactose content in fraction P (12 mol%, Table 4.1) is high and represents 12% of the galactose present in the ChSS extract before degradation of the pectic arabinogalactan side chains. Re-incubation of fraction P with endo-galactanase and exo-galactanase did not show a further release of galactose.

Galacturonic acid represents 43 mol% of the constituent sugars of fraction P. Both PG and PL could not bring about changes in the molecular mass distribution of the polymers in this fraction and galacturonic acid oligomers could not be detected in the HPAEC elution patterns. Saponification of the methyl esters with 0.1 M NaOH did not enable the degradation by PG.

The presence of rhamnose residues (11 mol%) in fraction P suggests the presence of rhamnogalacturonan regions. The enzyme RG hydrolase is able to cleave

galactopyranosyluronic acid-rhamnopyranosyl linkages within the rhamnogalacturonan backbone when acetyl esters on C2 and/or C3 on the galacturonic acid residues are absent and the rhamnose residue is unbranched or substituted through C4 with a single galactose residue[28]. Only after saponification of fraction P, the HPAEC elution pattern of the digest shows the release of small amounts of rhamnogalacturonan oligomers. HPSEC however, does not show changes in the elution pattern in the polymeric region, irrespective of saponification. Thus, acetyl-containing rhamnogalacturonan regions occur in the extremities of fraction P and become susceptible for RG hydrolase after saponification.

Table 4.2. Linkage analysis of soybean polysaccharide fractions.[a]

Glycosyl residue [b]	ChSS	P	PI	PII	PIIIA
t-Ara*f*	13	4	17	-	tr
5-Ara*f*	11	6	-	-	-
2,5-Ara*f*	2	tr	-	-	-
3,5-Ara*f*	3	tr	-	-	-
t-Fuc*p*	4	12	tr	-	tr
t-Gal*p*	4	19	1	12	34
4-Gal*p*	49	7	5	6	17
4,6-Gal*p*	1	tr	-	-	-
2,3,4,6-Gal*p*	1	9	65	12	tr
t-Rha*p*	-	-	-	-	8
2-Rha*p*	1	10	1	32	8
2,4-Rha*p*	2	6	1	12	17
t-Xyl*p*	3	13	10	26	8
2-Xyl*p*	4	10	-	-	8
4-Xyl*p*	1	1	tr	-	-
2,3-Xyl*p*	1	3	-	-	-

[a] Expressed as relative peak areas of corresponding partially methylated alditol acetates;
Galacturonic acid is determined in separate experiments, which revealed the presence of t-Gal*p*A in ChSS, P, and PI, 4-Gal*p*A in every polysaccharide fraction, 3,4-Gal*p*A in ChSS, P, PI, and PII;
tr, trace amount; -, not detected.

[b] Numbers preceding residues indicate positions of attachment of other glycosyl residues in the intact polysaccharide (e.g. 5-Ara*f* = 5-substituted arabinofuranose);
t, nonreducing terminal residue.

The high xylose content (18 mol%) suggests the presence of xylogalacturonan regions in fraction P. Linkage analysis shows that part of the galacturonic acid residues is branched at C3, and that most of the xylose is non-reducing terminal xylose (Table 4.2). Single unit side chains of xylose do not hinder exo-galacturonase and this enzyme should be able to release galacturonic acid residues and xylosyl galacturonic acid dimers (β-Xyl*p*-(1→3)-Gal*p*A) from the polymer[17]. The HPAEC elution pattern shows the release of small amounts of galacturonic acid and the characteristic xylosyl galacturonic acid dimer by exo-galacturonase, particularly after alkaline saponification. However, the HPSEC elution pattern does not show changes. This is explained by the fact that exo-galacturonase is an exo-enzyme and therefore not able to change the hydrodynamic volume of polymers to a large extent. This is in agreement with Beldman et al.[17], who were also able to release the xylosyl galacturonic acid dimer (β-Xyl*p*-(1→3)-Gal*p*A) from a soluble pectic polysaccharide from soy (SPS)[30] using exo-galacturonase. Approximately 0.7% of the galacturonic acid present in fraction P is released from the non-reducing chain end by exo-galacturonase. After saponification, there was an increase of the amount of galacturonic acid released (x 4.5) to 3.6%.

None of the enzymes used in these experiments were able to cause a large shift in the molecular mass distribution of the pectic structures in fraction P. To check if there are any enzymes at all which are able to degrade fraction P, some technical multienzyme preparations were tested. Ultra SP-L and Viscozyme are able to remove an additional amount of galactose from fraction P, which was not removed by purified endo-galactanase and exo-galactanase. Presumably, these enzyme preparations contain a very specific galactose-releasing enzyme or accessory enzymes enabling other enzymes (such as endo-galactanase, exo-galactanase, or galactosidase) to act. However, none of the enzymes in Pectinex Ultra-SP-L, Viscozyme, Rapidase liq+, and Driselase was able to degrade the pectic backbone of CDTA-extractable soybean pectins.

WEAK ACID HYDROLYSIS OF FRACTION P

Since enzymatic degradation of fraction P was not possible with the available enzymes, the polymers were degraded by weak acid hydrolysis. During acid-catalysed hydrolysis of glycosides, linkages between two uronic acids or between an uronic acid and a neutral sugar are more stable than linkages between two neutral sugars[31]. These differences in susceptibility of the glycosidic linkages to acid hydrolysis were used to remove the (neutral) side chains as much as possible without seriously degrading the pectic backbone. Very weak hydrolysis conditions were used, 0.1 M HCl, 80 °C. Acid hydrolysis of demethylated pectins (apple, beet, and citrus) under these conditions showed that neutral side chains were rapidly split off[32]. The hydrolysis of fraction P with HCl was followed in time by analysis of the hydrolysates using HPSEC and HPAEC.

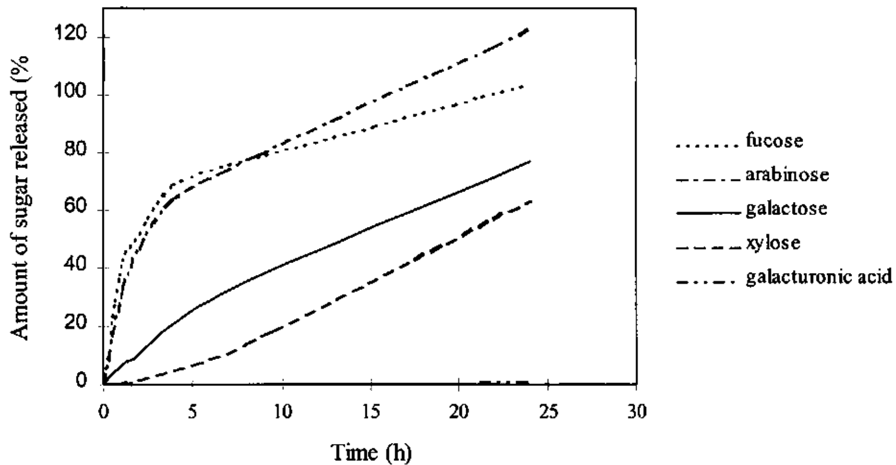


Figure 4.1. Release of sugar residues during acid hydrolysis of fraction P as followed with HPAEC analysis.

The HPAEC elution patterns show a rapid release of arabinose, fucose, galactose and xylose from fraction P during acid hydrolysis. In figure 4.1 the amounts of specific, monomeric sugar residues are shown as a percentage of the amount of this residue originally present in the polymer that is released during acid hydrolysis. The amount of arabinose released exceeds 100%. This unrealistic number is caused by comparing sugar compositions determined in two different ways, as the sugar composition of fraction P was determined by gas chromatography[33]. Galacturonic acid residues were not released during the first 8h of hydrolysis, and after 24h still only 1% of the galacturonic acid residues was released as monomers.

Line a in Figure 4.2 represents two populations containing high molecular mass polymers. The first population probably originates from aggregation, which results in accumulation of molecules in the void volume. These aggregates are not observed at low concentrations of P. The increase of the total RI-area after hydrolysis was caused by the solubilisation of polymers that were not completely soluble in 0.1 M HCl without heat treatment. The intensity of the peak of high molecular mass material decreases during acid hydrolysis and the formation of degradation products can be observed in the HPSEC elution patterns. The largest shift of the molecular mass distribution occurs between 8 and 24h of hydrolysis (Figure 4.2, lines e and f). Prolonged acid hydrolysis causes almost complete degradation of the high molecular mass polymers, including degradation of the pectic backbone (Figure 4.2, line f).

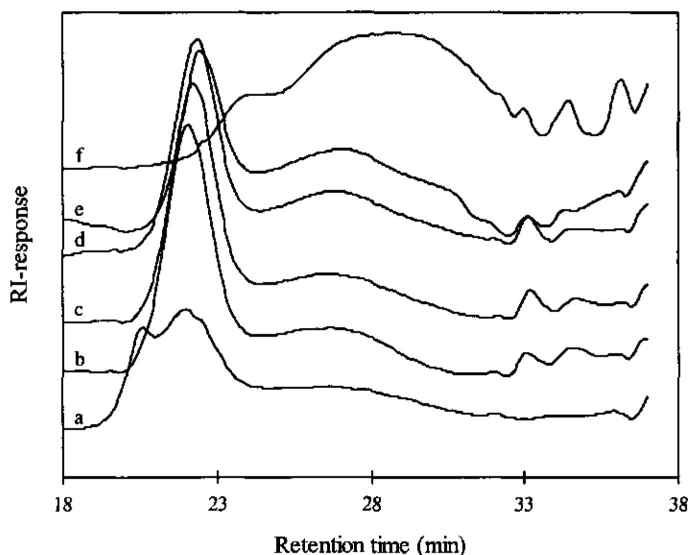


Figure 4.2. HPSEC elution patterns of fraction P during acid hydrolysis; (a) blank, after (b) 1h, (c) 2h, (d) 4h, (e) 8h, and (f) 24h of acid hydrolysis.

It appears that the pectic backbone is hardly affected during the first 8h of acid hydrolysis. The elution pattern of this hydrolysate still shows a peak with high molecular mass (retention time 22.5 min; molecular mass > 82 kDa), a peak representing material with an intermediate molecular mass (retention time 27.1 min; molecular mass ≈ 11 kDa) and some oligomers (Figure 4.2, line e). The ratio of the areas under these peaks is 4:6:1. Characterisation of the polymeric residue after 8h of hydrolysis can provide information about the structure of fraction P, and consequently about the structure of the CDTA-extractable pectins.

FRACTIONATION OF THE ACID HYDROLYSATE OF FRACTION P

Fraction P was hydrolysed with 0.1 M HCl for 8h on a large scale and the hydrolysate fractionated by size-exclusion chromatography. The elution profile (Figure 4.3) shows three populations. In the first two populations galacturonic acid prevails, whereas in the third population, containing low-molecular-mass degradation products, neutral sugars predominate. The recovery of the hydrolysate after fractionation on Sephacryl S-500 HR is almost 100%.

The desalting and further fractionation of the third population (PIII) was performed by Bio-Gel P-2 chromatography. The elution profile (Figure 4.4) shows a peak eluting in the

void volume of the column (PIIIA), containing oligomeric pectic material with a ratio of neutral sugars to galacturonic acid of 1:2.1. The second population (PIIIB) contains neutral sugars only.

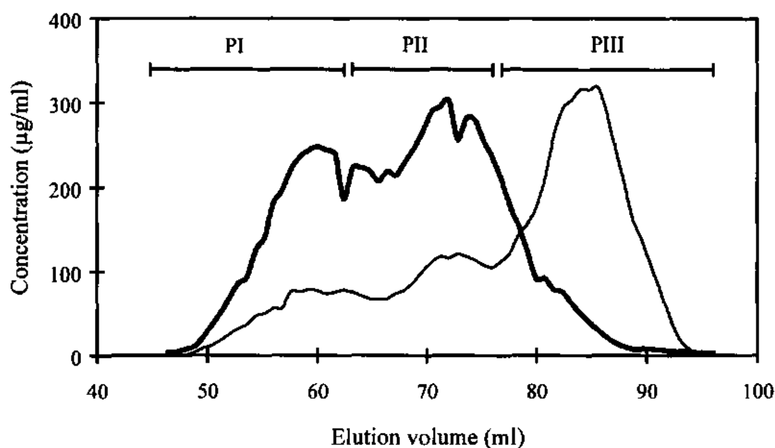


Figure 4.3. Elution profile of the acid hydrolysate of fraction P on Sephacryl S-500 HR. Uronic acid concentration (——), neutral sugar concentration (——).

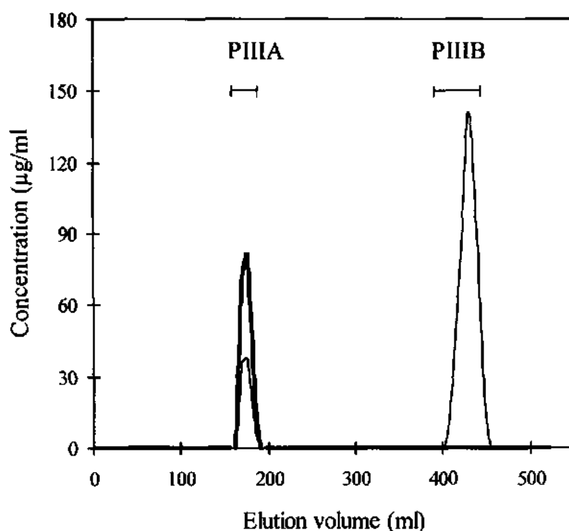


Figure 4.4. Elution profile of fraction PIII on Bio-Gel P-2. Uronic acid concentration (——), neutral sugar concentration (——).

CHARACTERISATION OF THE POPULATIONS OF THE ACID HYDROLYSATE

The sugar compositions of the parental fraction and the populations obtained after enzymatic and acid hydrolysis are shown in Table 4.1. The ratio neutral sugars to galacturonic acid in the ChSS extract is relatively high (3.8:1), because all the arabinogalactan side chains of the pectins are still present. Fraction P still contains a large amount of neutral sugars (ratio is 1.4:1), although the major part of the arabinose and galactose residues was removed by enzymatic digestion. Acid hydrolysis causes a further decrease of the relative amounts of neutral sugars in PI, PII and PIIIA.

The high xylose content in fraction PI (26 mol%), which is terminally-linked (Table 4.2), suggests the presence of xylogalacturonan regions. The PI population has the highest ratio of xylose to galacturonic acid (1:2.2), similar to the value determined in fraction P (1:2.3), in spite of the fact that part of the xylose is removed during acid hydrolysis. Rhamnogalacturonan regions are indicated by the presence of rhamnose (9 mol%), which is both 2- and 2,4-linked (Table 4.2). The presence of these two structural units is also suggested in the PII population by the high xylose and rhamnose contents (20 and 16 mol%, respectively), and the occurrence of the same linkages (Table 4.2) as in the PI population.

Population PIIIA contains only small pectic fragments, as can be concluded from the elution behaviour on size-exclusion chromatography. The high rhamnose content in fraction PIIIA (24 mol%) and the galacturonic acid content (46 mol%) suggest that this population is very rich in rhamnogalacturonan structures, substituted with (arabino)galactan side chains. These structures exceed 63% of this population. The ratio of arabinose plus galactose to rhamnose is 1:1.6; therefore not all the rhamnose residues are substituted with neutral sugar residues. This population may contain some xylogalacturonan sequences, as indicated by the presence of 12 mol% xylose.

Generally, NMR analysis of small fragments is easier than of polymers. Due to the presence of a dominating non-carbohydrate contaminant in the minor fraction PIIIA, being available in low amounts only, this fraction was not analysed by NMR spectroscopy. As the signals in the 1D ^{1}H NMR spectrum of PI are broader than in that of PII, the latter will be described first. Fraction PII was converted into its acidic form by treatment with Dowex H^{+} yielding fraction PII-H. Inspection of the 1D ^{1}H NMR spectra of PII and PII-H (data not shown) showed a better resolution for PII-H. The absence of a methyl-ester signal of galacturonic acid (^{1}H, δ 3.85/3.83; ^{13}C, δ 53.4) in the HSQC spectrum of PII-H (Figure 4.5C) indicated de-esterification during the Dowex H^{+} treatment. In view of the quality of the NMR spectra, fraction PII-H was chosen for a detailed structural analysis.

The anomeric region (δ 4.5-5.5) in the 1D ^{1}H NMR spectrum of PII-H (Figure 4.5A) shows several H1α and H1β signals. Using TOCSY (Figure 4.5B) measurements and HSQC (Figure 4.5C) and HMBC experiments, most of the ^{1}H and ^{13}C signals could be

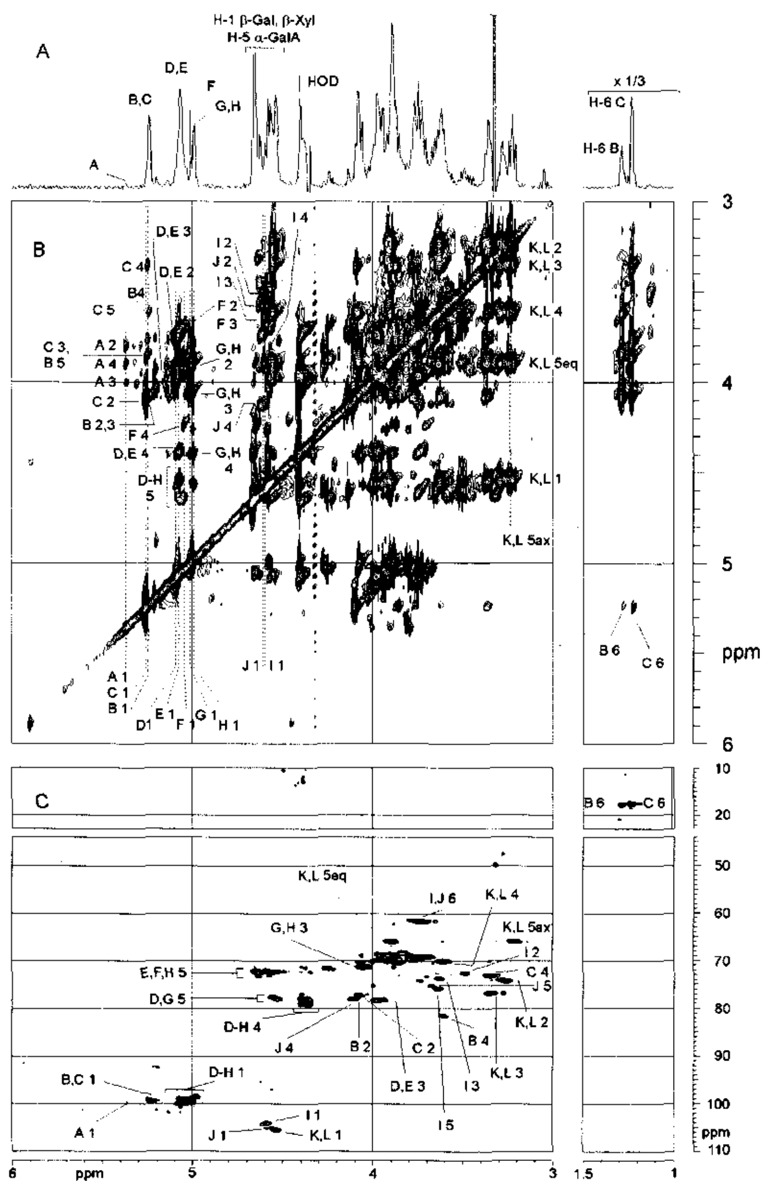


Figure 4.5. 1D ^{1}H NMR spectrum (A), TOCSY spectrum, mixing time 124 ms (B), and HSQC spectrum (C) of PII-H, recorded at 600 MHz and 333 K. Peak labels in A represent different residues, which are explained in the text. For an overview, see Tables 4.5 and 4.6. B 2 means a cross-peak between H2 and C2 of residue B. Xylose H5 equatorial and axial are indicated with eq and ax, respectively. * Not assigned to a methyl ester as the chemical shift is too low (δ 50 instead of 53.4).

Table 4.3. ^{1}H Chemical shifts of PII-H, recorded at 333 K

Residue		Type	H1	H2	H3	H4	H5(ax)	H5eq/H6a/H6b
Fuc	A	α-Fuc-(1→	5.37	3.80 [a]	4.00 [a]	3.90 [a]	- [b]	-
Rha	B	→2,4)-α-Rha-(1→	5.235	4.10	4.06	3.62	3.84	1.276
	C	→2)-α-Rha-(1→	5.24	4.08	3.86	3.36	3.77	1.217
GalA	D	→3,4)-α-GalA-(1→	5.07	3.70	3.98	4.38	4.54	
	E	→3,4)-α-GalA-(1→	5.06	3.73	3.98	4.38	4.65	
	F		5.02	3.67	3.85	4.24	4.64	
	G	→4)-α-GalA-(1→	5.01	3.89-3.87	4.06	4.38	4.56	
	H	→4)-α-GalA-(1→	4.99	3.89-3.87	4.03	4.38	4.56	
Gal	I	β-Gal-(1→	4.59	3.49	3.63	3.89	3.65	3.76/3.71
	J	→4)-β-Gal-(1→	4.60/4.	3.53/3.57	3.72/3.	4.13	3.68	3.76/3.71
Xyl	K	β-Xyl-(1→	4.54	3.27	3.35	3.61	3.22	3.92
	L	β-Xyl-(1→	4.54	3.27	3.35	3.61	3.22	3.90

[a] Values may have to be interchanged.

[b] -, not determined.

assigned (Tables 4.3 and 4.4, respectively). The low-intensity down-field H1 signal for residue A was assigned to α-fucose H1; its TOCSY track (Figure 4.5B) showed a clear α-fucose H1,2,3,4 spin system[34].

The H1 TOCSY tracks for residues B and C show complete spin systems up to signals in the methyl region at δ 1.276 and 1.217, respectively, thereby indicating that B and C are 6-deoxysugars. Based on the monosaccharide analysis data and the typical NMR positions for the B/C H2 protons (δ ~ 4.10, O2 substituted α-*manno*-hexoses[35]) both residues were assigned as 2-substituted α-rhamnose residues. Moreover, inspection of the two sets suggests an additional substitution at O4 of rhamnose B, whereby the ratio of 2- to 2,4-substituted rhamnose as deduced from the H6 signals in the 1D ^{1}H NMR spectrum is 2.6:1. This is confirmed by linkage analysis data (Table 4.2), although linkage analysis results in qualitative rather than quantitative information. It should be noted that a α-rhamnose residue usually does not give a TOCSY signal between H1 and H2, as a result of the equatorial H atom at C2. Therefore, the observed transfer of magnetisation is probably caused by spin diffusion. A further support for the 2- or 2,4-substitution of rhamnose are the δ-values of the rhamnose B/C C2 and C4 atoms in the ^{13}C NMR spectra (Table 4.4; methyl α-L-rhamnopyranoside: δ_{C2},

71.0; δ_{C4}, 73.1[36]). The $^{1}J_{C1,H1}$ coupling constant value of 173 Hz for both rhamnose residues point to a α-configuration[37].

The H1 signals of the residues D-H in the α-anomeric region were all correlated with α-galacturonic acid residues ($^{1}J_{C1,H1}$ ~ 173 Hz). Making use of the HSQC and HMBC spectra, in nearly all cases the typical spin systems H1,2,3,4,5 and C1,2,3,4,5 for a *galacto*-hexose could be derived (Tables 4.3 and 4.4, respectively). The downfield chemical shift value of H5 (δ ~ 4.5-4.6) is indicative for the differentiation between galacturonic acid and galactose. Presumably, spin diffusion is responsible for the observation of the H5 cross-peaks on the H1 TOCSY tracks. According to their ^{1}H and ^{13}C chemical shifts, at least two different substitution patterns for α-galacturonic acid exist. Residues G and H represent 4-substituted galacturonic acid residues and D, E and F 3,4-substituted ones[2] (α-D-galactopyranosyluronic acid: δ_{C3}, 69.5; δ_{C4}, 70.9[36]); the assignment of residue F as 3,4-substituted galacturonic acid is ambiguous. The deduced substitution patterns fit the linkage analysis data.

Table 4.4. ^{13}C Chemical shifts of PII-H, recorded at 333 K

Residue		Type	C1	C2	C3	C4	C5	C6
Fuc	A	α-Fuc-(1→	99.7	68.8 [a]	75.4 [a]	69	- [b]	20.8
Rha	B	→2,4-α-Rha-(1→	99.4	77.9	70.3	81.57	68.4	17.45
	C	→2-α-Rha-(1→	99.2	77.4	70.47	73.1	69.7	17.69
GalA	D	→3,4)-α-GalA-(1→	98.97	69.14	78.3	77.0/77.7 [c]	78.1	176.3 [d]
	E	→3,4)-α-GalA-(1→	99.94	69.14	78.3	77.9 [c]	72.3	176.1 [d]
	F		99.46	-	-	78.5 [c]	72.5	175.9 [d]
	G	→4)-α-GalA-(1→	98.9	68.84	71.28	79.0 [c]	77.8	175.4 [d]
	H	→4)-α-GalA-(1→	98.5	68.84	71.28	79.4 [c]	72.3	
Gal	I	β-Gal-(1→	104.2	72.6	73.7	69.6	76.0	61.7
	J	→4)-β-Gal-(1→	104.3/105.1	72.9/74.2	73.3/74.2	78.1	75.4	61.4
Xyl	K/L	β-Xyl-(1→	105.53	74.22	76.92	70.11	65.89	

[a] Values may have to be interchanged.

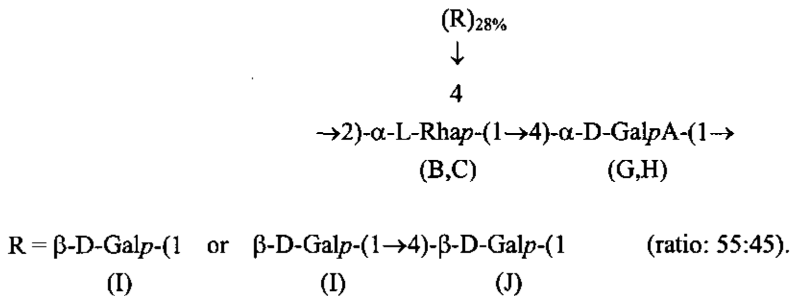
[b] -, not determined.

[c] Values for GalA C4 may have to be interchanged.

[d] Values for GalA C6 may have to be interchanged.

Monosaccharide and methylation analysis combined with NMR spectroscopy demonstrated the presence of terminal galactopyranose (I) and 4-substituted galactopyranose (J) in the molar ratio 2.2:1. Both TOCSY H1β (δ 4.59 and 4.60/4.61, respectively; Table 4.3) tracks show the typical cross-peak pattern of H1,2,3,4 of a *galacto*-hexose. Downfield shifts of H4 and C4 of residue J prove a 4-substitution. The β-configurations of both galactose residues are supported by $^{1}J_{C1,H1}$ values of 163 Hz, as determined from a HSQC experiment (Figure 4.5C).

NOE cross-peaks were observed for galacturonic acid G/H H1,rhamnose B/C H2 (strong), galacturonic acid G/H H1,rhamnose B/C H1 (strong), galacturonic acid G/H H1,rhamnose B/C H3 (weak), rhamnose B/C H1,galacturonic acid G/H H4 (strong), and rhamnose B/C H1,galacturonic acid G/H H5 (weak). These data imply the presence of rhamnogalacturonan regions. The NMR spectra do not give any indication for an irregular distribution of 2- (residue C) and 2,4-substituted (residue B) rhamnose. Therefore, it was concluded that these residues are distributed regularly in the rhamnogalacturonan chain. An additional cross-peak between galactose I/J H1 and rhamnose B H4 connects residues I/J with the rhamnogalacturonan backbone. Elongation of the galactose residue J at O4 is evidenced by a cross-peak between galactose I H1 and galactose J H4. In summary this building block can be depicted as follows:



Clear NMR indications for xylose were found in the HSQC spectrum of PII-H (Figure 4.5C), showing cross-peaks between C5 and H5ax and between C5 and H5eq. Further assignments followed from TOCSY and COSY experiments, as well as from literature NMR data for terminal β-xylose residues[2,38] ($^{1}J_{C1,H1}$ 163 Hz). Based on the presence of two different δ-values for xylose H5eq (δ 3.92 and 3.90), two xylose residues, K and L, were established.

As the NOESY spectrum shows a weak interresidual xylose K/L H1,galacturonic acid D/E H3 cross-peak, a β-Xyl*p*-(1→3)-α-Gal*p*A D/E element is indicated. For galacturonic acid D/E H1 several NOE cross-peaks are observed, namely with galacturonic acid D/E H2,3,4,5. Taking into account the (1→3) linkage between xylose and galacturonic acid, the NOE cross-peak galacturonic acid D/E H1,galacturonic acid D/E H4 can be interpreted as an interresidual

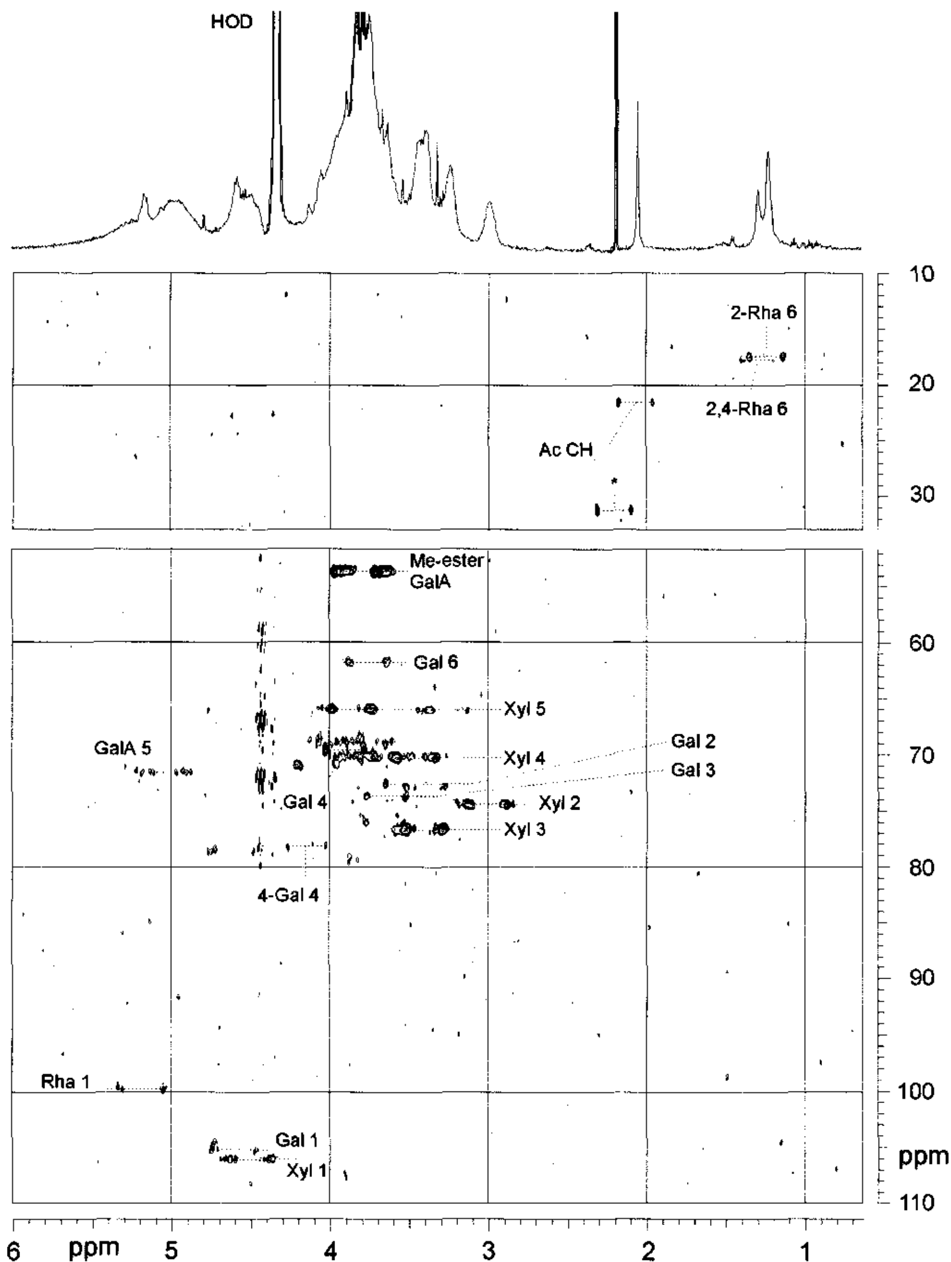
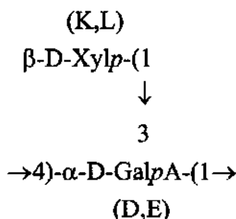


Figure 4.6. HSQC spectrum of PI, recorded at 600 MHz and 333 K. Xyl 2 refers to a cross-peak between H2 and C2 of Xyl. * Not assigned to an acetyl group as the ^{13}C chemical shift is too high (δ 30.8 instead of 21.1).

cross-peak reflecting 1→4 linkages between neighbouring galacturonic acid D/E residues. In this reasoning the cross-peak galacturonic acid D/E H1,galacturonic acid D/E H3 can be explained as a second interresidual and/or an intraresidual (spin diffusion) cross-peak. It should be noted that the cross-peaks galacturonic acid G/H H1,galacturonic acid G/H H2,3 (rhamnogalacturonan backbone, see above) can only be interpreted as intraresidual cross-peaks, whereby the galacturonic acid G/H H1,galacturonic acid G/H H3 cross-peak is probably caused by spin diffusion, which stops at H3. In summary this building block can be depicted as follows:



For the assignment of the NMR spectra of PII, the same rationale was used as described for PII-H. Similar ^{1}H and ^{13}C chemical shifts were found for the different residues in fraction PII. Galacturonic acid appeared to be methyl-esterified (δ 3.85/3.83 and 35.4 for ^{1}H and ^{13}C, respectively). In the xylogalacturonan part of PII approximately 85% of the galacturonic acid residues is methyl-esterified. This was concluded from the intensities of the xylose H1,H2 cross-peaks in a COSY spectrum of PII, as the chemical shift of xylose H2 strongly depends on galacturonic acid being methyl-esterified or not (xylose H2, δ 3.056/3.038 and 3.280/3.272 for methyl-esterified and non-esterified galacturonic acid, respectively)[2].

Like for PII-H, several spin systems were identified for fraction PI using TOCSY and HSQC (Figure 4.6) spectra (Table 4.5 and 4.6). The presence of xylogalacturonan was indicated by the chemical shift values of xylose, assigned in a similar way as described for fraction PII-H. Rhamnogalacturonan was identified by the spin system of rhamnose (Figure 4.6). The ratio of (1,2)-linked rhamnose to (1,2,4)-linked rhamnose in fraction PI is 1.4:1 (H6 rhamnose δ 1.24 and 1.30, respectively). The HSQC spectrum (Figure 4.6) contains a clear signal for a methyl ester (^{1}H, 3.84-3.76; ^{13}C, 53.4), indicating methyl-esterification of galacturonic acid, and acetyl signals (^{1}H, 2.06; ^{13}C, 21.2), presumable belonging to *O*-acetyl groups linked to galacturonic acid.

All three populations in the acid hydrolysate from fraction P appear to contain xylogalacturonan, rhamnogalacturonan, and some remaining (arabino)galactan side chains. Assuming that the rhamnose to galacturonic acid ratio in the rhamnogalacturonan regions can vary from 1:1 to 1:20[39], the amount of rhamnogalacturonan (including arabinogalactan substituents) is estimated to exceed 26% of the PI population and 41% of PII. Combination of the results described above shows that the average length of the remaining arabinogalactan

side chains in the PI population is 2.1 residues. The average length of the remaining galactan side chains in fraction PII is 1.4 residues. Based on the contents of xylose and galacturonic acid (26 and 58 mol%, respectively) and a xylose to galacturonic acid ratio in xylogalacturonan varying between 1:1 and 1:2, xylogalacturonan accounts for 52 to 78% of the PI fraction, and for 40 to 60% of the PII fraction. In addition, the polymeric populations (PI and PII) still contain methyl-esterified galacturonic acid residues. Acetylation of galacturonic acid residues occurs at position 2 or 3. The degree of acetylation decreases from 47% in fraction P to 16% in fraction PI, as determined by NMR spectroscopy. Removal of acetyl groups must have occurred during acid hydrolysis. Labile groups such as ester-linked components are likely to be removed under acidic conditions[40].

Table 4.5. ^{1}H Chemical shifts of PI, recorded at 353 K.

Residue	Type	Me/Ac	H1	H2	H3	H4	H5(ax)	H5eq/H6a/H6b
Fuc	α-Fuc-(1→		-[a]	-	-	-	3.77	1.46
Rha	→2,4)-α-Rha-(1→		5.26	-	4.00	3.60	3.81	1.30
	→2)-α-Rha-(1→		5.26	-	3.82	3.36	3.74	1.24
GalA			-	-	-	-	5.05-5.00	
GalA6Me		3.84-3.76						
Acetyl		2.06						
Gal	β-Gal-(1→		4.57	3.50	3.63	3.96	-	3.75
	→4)-β-Gal-(1→		4.59	3.56	3.73	4.13	-	3.75
			4.58	3.31	3.74	-	-	-
Xyl	β-Xyl-(1→		4.45	2.99	3.39	3.44	3.24	3.84
	β-Xyl-(1→		4.49	3.01	3.39	3.44	3.24	3.84

[a] -, not determined.

The presence of homogalacturonan in populations PI and PII is not necessary to explain the high galacturonic acid content; it can be accounted for by the rhamnogalacturonan and xylogalacturonan regions in these populations. The amount of galacturonic acid in the rhamnogalacturonan regions in populations PI and PII will exceed 9 and 16 mol%, respectively. In addition, at most 52 mol% of galacturonic acid in population PI and 40 mol% of galacturonic acid in population PII can be present in xylogalacturonan regions. The NMR

spectra of PII-H confirms the absence of a (α1→4)-linked homogalacturonan. The absence of homogalacturonan regions in pectic substances has never been reported before.

Table 4.6. ^{13}C Chemical shifts of PI recorded at 333 K.

Residue	Type	Me/Ac	C1	C2	C3	C4	C5	C6
Fuc	α-Fuc-(1→		- [a]	-	-	-	-	-
Rha	→2,4)-α-Rha-(1→		99.6	-	-	-	-	17.5
	→2)-α-Rha-(1→		99.6	-	-	-	-	17.3
GalA			-	-	-	-	71.4	-
GalA6Me		53.4						
Acetyl		21.2						
Gal	β-Gal-(1→		105.0	72	73.4	69.4	-	61.5
	→4)-β-Gal-(1→		105.0	72		78.1	-	61.5
Xyl	β-Xyl-(1→		105.8	74.3	76.4	70.0	65.7	

[a] -, not determined.

ENZYMATIC DEGRADATION OF THE POPULATIONS FROM THE ACID HYDROLYSATE OF FRACTION P

To obtain additional information about the structure of the pectic backbone in soybean meal, the populations PI, PII, and PIIIA were incubated with PG, RG hydrolase, exo-galacturonase, and XGH. The digests were analysed with both HPSEC (Figure 4.7) and HPAEC (Figure 4.8).

Although PI is obtained after fractionation on Sephacryl S-500 HR, it contains two polymeric populations on HPSEC analysis (Figure 4.7Aa). Incubation with PG (Figure 4.7Ab) results in a small shift of the maximum of the first peak and the minimum between these two peaks is less deep than in the blank. PG is not able to release galacturonic acid oligomers from PI (Figure 4.8Aa). RG hydrolase does not change the molecular mass of the first peak, and the molecular mass of the second peak decreases only slightly (Figure 4.7Ac). The amount of polymeric material in both populations decreases, while a shoulder with lower molecular mass arises (as indicated by the arrow). Analysis of the digest on HPAEC (Figure

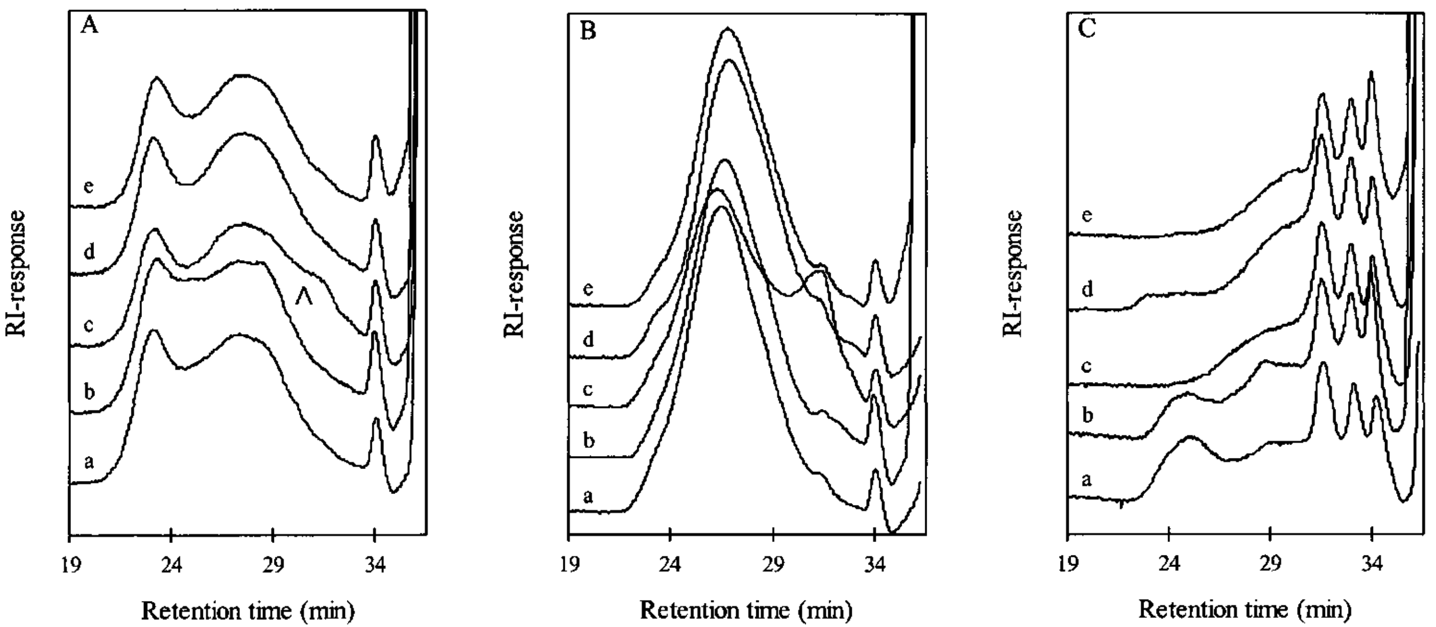


Figure 4.7. HPSEC elution patterns of the digests of PI (A), PII (B), and PIIIA (C) before (a) and after enzymatic degradation with PG (b), RGase (c), XGH (d), and exo-galacturonase (e).

4.8Ba) shows the release of very small amounts of characteristic RG hydrolase oligomers[28]. XGH and exo-galacturonase have less effect than PG, both cause only slight changes in the elution pattern of PI (Figure 4.7Ad and e). The HPAEC elution pattern of the XGH digest (Figure 4.8Ca) does not show the release of oligomeric degradation products. XGH was expected to cause degradation, because this polygalacturonase is specific for xylose-substituted galacturonan and the PI is thought to be rich in xylogalacturonan. The HPAEC elution pattern of the exo-galacturonase digest (Figure 4.8Da) shows the release of both galacturonic acid and xylosyl galacturonic acid dimer. The PII population shows one polymeric population on HPSEC analysis (Figure 4.7Ba). HPSEC (Figure 4.7Bb) and HPAEC analyses (Figure 4.8Ab) show that PG is not able to degrade this fraction. Removal of the methyl-ester groups from PII (PII-H) did not increase the susceptibility for PG. RG hydrolase does degrade fraction PII very well (Figure 4.7Bc). The shape of the HPSEC elution pattern changes, it now shows two populations of which the retention time of the first population is lower than in the blank. This indicates that the broad peak in the blank represents two populations: one with a high molecular mass which is not degraded and one with lower molecular mass which is degraded by RG hydrolase. The oligomers released by RG hydrolase (Figure 4.8Bb) can be assigned by comparison with the rhamnogalacturonan oligomers from apple MHR[28]. These oligomers have a backbone of alternating rhamnose and galacturonic acid residues, partly substituted with galactose residues to C4 of the rhamnose moiety. XGH hardly affects the molecular mass distribution of PII (Figure 4.7Bd). In figure 4.8Cb the release of small amounts of xylosyl galacturonic acid dimer is shown in addition to very small amounts of unknown oligomers. The exo-galacturonase digest (Figure 4.7Be) shows the same small shift of the peak as the XGH digest, but now the amount of polymeric material also decreases slightly. The amounts of galacturonic acid and the xylosyl galacturonic acid dimer released from PII (Figure 4.8Db) are higher than the amounts released from PI. The amount of monomeric galacturonic acid in the PI digest is approximately 29 μg/ml and in the PII digest 47 μg/ml, which means that 8.0% of the galacturonic acids present in PI and 10.5% of the galacturonic acids present in PII is released by exo-galacturonase. From Figure 4.8D, it can be seen that the xylosyl galacturonic acid dimer content in the PII digest is slightly higher than in the PI digest. The content of the dimer can not be quantified properly, because a standard is absent. When the response factor of galacturonic acid is used to quantify the amount of dimer in the digests, the content in the digest of PI is 23 μg/ml and the content in the digest of PII is 24 μg/ml. This means that 3.8% of the galacturonic acid present in PI and 3.2% of the galacturonic acid present in PII is released as the xylosyl galacturonic acid dimer. This indicates that the degree of substitution of galacturonic acid with xylose (remaining after weak acid hydrolysis) in the extremities of the pectic substances in PI is higher than in PII. The total amount of material released from PII is higher than from PI, which can be explained by the number of potential degradation sites for exo-galacturonase. The molecular mass of the pectins in population PII is lower than in population

PI. Starting with the same substrate concentration, the number of molecules (and accordingly the number of non-reducing chain ends) in the PII solution is higher than in the PI solution.

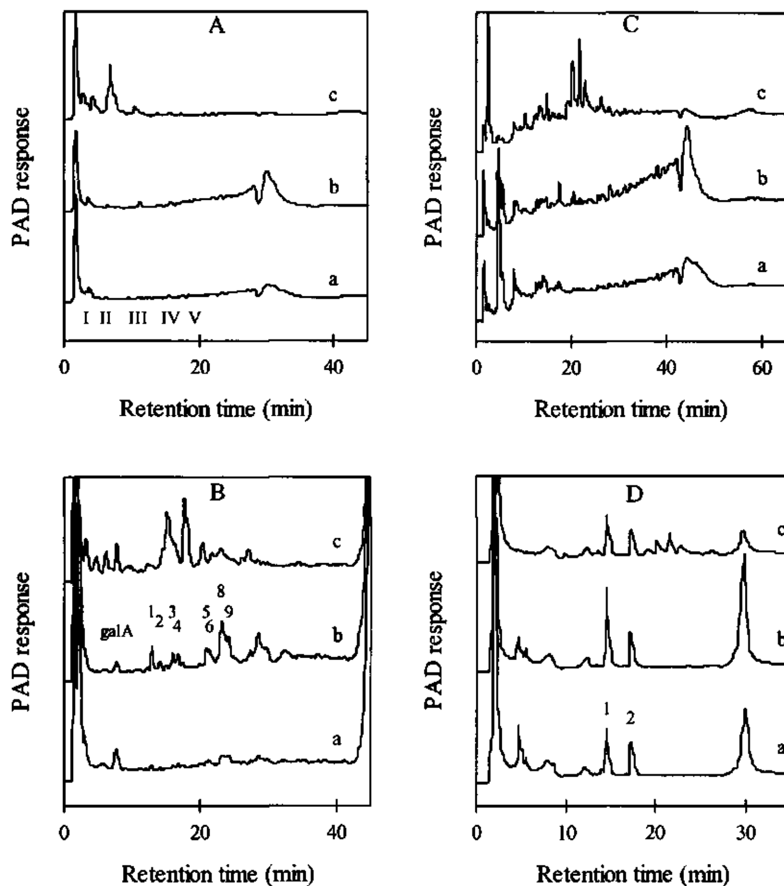


Figure 4.8. HPAEC elution patterns of the digests of PI (a), PII (b), and PIIIA (c) after enzymatic degradation with PG (A), RGase (B), XGH (C), and exo-galacturonase (D). In figure A, the elution times of galacturonic acid and galacturonan oligomers are marked with a roman number corresponding to their degree of polymerisation. In figure B, the elution times of galacturonic acid and structures 1, 2, 3, 4, 5, 6, 8, and 9 as described by Schols et al.[28] are marked. In figure D, the elution times of galacturonic acid and the xylosyl galacturonic acid dimer are marked with 1 and 2.

The PIIIA blank contains predominantly oligomeric material in addition to a low amount of polymeric material (Figure 4.7Ca). PG is not able to degrade fraction PIIIA. The HPSEC elution pattern shows a change in the relative amounts of the peaks of the oligomers

(Figure 4.7Cb). However, the HPAEC elution pattern does not show changes with regard to untreated PIIIA (Figure 4.8Ac). RG hydrolase is also not able to degrade the oligomers in this fraction, but it is able to degrade the small peak of polymeric material. Release of RG hydrolase oligomers can not be detected using HPAEC (Figure 4.8Bc). XGH degrades a large part of the polymeric material (Figure 4.7Cd). However, this does not result in a change of the HPAEC elution pattern when compared to the elution pattern of the blank (Figure 4.8Cc). Exo-galacturonase degrades the peak of polymeric material, and it changes the relative amounts of the oligomers present in this fraction (Figure 4.7Ce). This is confirmed by the elution pattern on HPAEC, which shows the release of monomeric galacturonic acid and the xylosyl galacturonic acid dimer (Figure 4.8Dc).

The high galacturonic acid content of PI and PII could give the impression that a homogalacturonan is present in these populations. However, enzymatic degradation with PG confirms our earlier statement that none of the fractions contains homogalacturonan.

Most of the rhamnogalacturonan oligomers released from apple MHR[28] can also be released from PI and PII, but not from PIIIA. The PII fraction appears to contain a relatively large amount of rhamnogalacturonan. Judging from the ratio of rhamnose to galacturonic acid in fraction PIIIA, which is relatively high (0.53), RG hydrolase treatment is expected to result in some degradation. It has been demonstrated before that a suitable ratio of rhamnose to galacturonic acid is no guarantee for degradation by RG hydrolase[41]. The structures present in PIIIA already have low molecular masses, which can explain the inactivity of RG hydrolase on PIIIA. The smallest oligomer that can be cleaved by RG hydrolase is a rhamnogalacturonan nonamer, while the possible influence of galactose side chains is neglected[42]. Thus PIIIA presumably contains some rhamnogalacturonan oligomers, which are too small or contain too many substituents to be further degraded.

Xylogalacturonan appears to be present in all populations from fraction P as shown by the release of galacturonic acid and the xylosyl galacturonic acid dimer and changes in the HPSEC elution pattern. However, exo-galacturonase is much more effective in the degradation of soybean xylogalacturonan than XGH.

CONCLUSIONS

A large part of the arabinogalactan side chains can be removed from CDTA-soluble pectins from soybean meal by the combined action of endo-galactanase, exo-galactanase, endo-arabinanase, and arabinofuranosidase B. It appears that the remaining polymeric structure (fraction P) can not be degraded by the purified enzymes tested here. Moreover, even crude commercial multienzyme preparations – containing a wide range of pectin-degrading enzymes - were not able to degrade the pectic backbone present in fraction P. Therefore, resort had to be taken to weak acid hydrolysis, which is less specific. Monitoring the release

of sugar residues and the molecular mass distribution in time showed that the pectic backbone was hardly affected during the first 8h of hydrolysis. Prolonged incubation resulted in the release of galacturonic acid residues and disappearance of the high molecular mass material.

The acid hydrolysate of fraction P was fractionated into two polymeric populations (PI and PII), one oligomeric fraction (PIIIA), and monomeric sugars (PIIIB). The oligomeric fraction (PIIIA) can not be degraded further and probably contains rhamnogalacturonan oligomers and some xylogalacturonan oligomers. This study showed that PI and PII contain xylogalacturonan and rhamnogalacturonan regions, and all analyses agreed on the absence of homogalacturonan regions. The NMR analyses of PII-H clearly demonstrate that this technique is suitable for the characterisation of complex plant polysaccharides.

The absence of homogalacturonan in population PI and PII indicates the absence of homogalacturonan in fraction P, because it is unlikely that these regions are degraded by acid hydrolysis. This is in agreement with the undegradability of fraction P by PG (after saponification) and PL. Fraction P was obtained from the ChSS fraction by enzymatic removal of a large part of the arabinogalactan side chains, not changing the pectic backbone. So, the CDTA-extractable pectin from soybean is composed of both xylogalacturonan and rhamnogalacturonan (hairy regions), and homogalacturonan is absent. It is remarkable that these pectic substances are extracted by CDTA, because it has been suggested that pectins extractable with hot chelating agents originate from the middle lamella, where they are presumed to be present in the form of calcium pectate gels[43]. This gelation is due to the formation of intermolecular junction zones between homogalacturonan regions of different chains. Since homogalacturonan appears to be absent in the ChSS extract from soybean meal, the presence of a calcium pectate gel must be excluded. It was verified that only a small part of these CDTA-extractable pectic substances could be extracted from the WUS with a (hot) buffer solution. Probably a specific effect of CDTA, other than the chelating effects, can solubilise pectic substances. Renard & Thibault[44] suggested this earlier.

The enzymatic degradation of fraction P showed that CDTA-extractable pectic polysaccharides from soybean are different from pectic polysaccharides extracted from other sources, like apple[29,45,46], carrot[47], kiwifruit[48,49], onion[50], pea[5], pear[51], potato[52,53], suspension-cultured sycamore cells[26,54,55] and sugar beet[56]. All these pectins contain homogalacturonan and rhamnogalacturonan regions, which can be degraded (possibly after saponification) with polygalacturonase and rhamnogalacturonase, respectively. CDTA-extractable pectins from soybean meal could not be degraded by these enzymes. Acid hydrolysis improves the susceptibility of the remaining polymers for RG hydrolase and exo-galacturonase.

Further studies will focus on the structural reasons for the resistance of the rhamnogalacturonan and xylogalacturonan regions in fraction P to degradation. Furthermore, attempts will be made to elucidate the unresolved pectic structure in CDTA-soluble pectin from soybean meal. In addition, the residual pectic substances present in the 1 MASS[10] extract will be studied and characterised.

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CHAPTER 5

THE OCCURRENCE OF INTERNAL (1,5)-LINKED ARABINOFURANOSE AND ARABINOPYRANOSE RESIDUES IN ARABINOGALACTAN SIDE CHAINS FROM SOYBEAN PECTIC SUBSTANCES

M.M.H. HUISMAN, L.P. BRÜLL, J.E. THOMAS-OATES, J. HAVERKAMP,

H.A. SCHOLS & A.G.J. VORAGEN

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CDTA-extractable soybean pectic substances were subjected to enzymatic digestion with arabinogalactan degrading enzymes that yielded a resistant polymeric pectic backbone and arabino-, galacto-, and arabinogalacto-oligomers. The complex digest was fractionated using size-exclusion chromatography. Monosaccharide composition analysis, HPAEC fractionation and MALDI-TOF MS analysis of the resulting fractions showed that each contains a mixture of oligosaccharides of essentially the same degree of polymerisation, composed of only arabinose and galactose. MALDI-TOF MS analysis was used for molecular mass screening of oligosaccharides in underivatised HPAEC fractions. The monosaccharide sequence and the branching pattern of oligosaccharides (degree of polymerisation from 4 to 8) were determined using linkage analysis and ES CID tandem MS analysis of the per-*O*-methylated oligosaccharides in each of the HPAEC fractions. These analyses indicated the presence of common linear (1,4)-linked galacto-oligosaccharides, and both linear and branched arabino-oligosaccharides. In addition, the results unambiguously showed the presence of oligosaccharides containing (1,4)-linked galactose residues bearing an arabino*pyranose* residue as the non-reducing terminal residue, and a mixture of linear oligosaccharides constructed of (1,4)-linked galactose residues interspersed with an *internal* (1,5)-linked arabino*furanose* residue. The consequences of these two new structural features of pectic arabinogalactan side chains are discussed.

INTRODUCTION

Arabinose- or galactose- containing homoglycans are known to occur in nature, but heteropolysaccharides containing both types of monosaccharide residue are much more abundant. Arabinogalactans are often linked covalently to protein, or to pectic substances[1]. They can be subdivided into two main structural types: the arabino-4-galactans (type I), and the heavily branched arabino-3,6-galactans (type II).

Type I pectic L-arabino-D-galactans are arabinose-substituted derivatives of linear (1,4)-linked β-D-galactan. Ara*f* and Gal*p* groups form stubs linked via C3 along the main chains. No association with protein has been reported for this group[1,2,3].

The second group of arabinogalactans, the type II L-arabinosyl-substituted branched 3,6-D-galactans, are widespread in plant tissues, tissue cultures, and exudate gums. They comprise a highly branched polysaccharide with ramified chains of β-D-Gal*p* residues joined by (1,3)- and (1,6)-linkages, the former predominantly in the interior and the latter in the exterior chains. β-D-Gal*p* residues terminate the bulk of the exterior chains containing L-Ara*f*, with, to a lesser extent, L-Ara*p* residues terminating some of the chains. In addition to Ara and Gal, type II arabino-3,6-galactans contain a range of other monosaccharides, including D-GlcA*p* and its 4-*O*-methyl ether and D-GalA*p*[1,2,3,4]. The type II arabinogalactans can also occur as pectic side chains, consisting of a (1,3)-linked galactan backbone to which (1,6)-

linked galactosyl side chains are attached to carbon 6 of the backbone. These side chains possess *Araf* side chains attached to carbon 3[5].

The pectic substances present in the soybean cell wall contain arabinan, galactan, and/or arabinogalactan side chains. The study of the fine chemical structure of the neutral pectic side chains requires their isolation. Cell wall material was isolated from soybean meal and sequentially extracted. The chelating agent soluble solids (ChSS fraction) contained the major part of the cell wall pectic substances[6]. The neutral side chains of these pectic substances may be successfully degraded by the combined activity of endo-galactanase, exo-galactanase, endo-arabinanase and arabinofuranosidase B[7]. In the literature, the arabinogalactan is described as a β-(1,4)-linked Gal*p* chain with little branching. *Araf* residues are present as (1,5)-linked side chains with an average length of two monosaccharide units attached to C3 of the Gal residues[8,9,10,11]. However, research carried out by Labavitch and co-workers[12] indicates that the Ara residues are primarily present in large oligo- or polyarabinosides.

Structural details of the neutral side chains of soybean pectic substances have not yet been determined. Therefore, the present study of oligomers released during enzymatic degradation of soybean pectic arabinogalactan side chains has been performed. Arabino-, galacto-, and arabinogalacto-oligosaccharides were isolated by size-exclusion and anion-exchange chromatography and analysed by monosaccharide and linkage analyses, mass spectrometry, and enzymatic degradation studies.

MATERIALS AND METHODS

ENZYMATIC DEGRADATION OF PECTIC ARABINOGALACTAN SIDE CHAINS FROM SOYBEAN

A solution of soybean CDTA-extractable pectic substances (250 mg) in 0.05 M sodium acetate buffer (25 ml) was digested with a combination of endo-galactanase, exo-galactanase, endo-arabinanase and arabinofuranosidase B for 10h at 30 °C, continuously mixed 'head over tail'. The incubation was stopped by heating for 10 minutes at 100 °C[7].

SIZE-EXCLUSION CHROMATOGRAPHY

The arabinogalacto-oligomers were separated from the polymeric pectic residue by fractionation on a Sephacryl S-100 HR column using a Hiload System (Amersham Pharmacia Biotech AB, Uppsala, Sweden)[7]. The oligomers were pooled and concentrated, and further fractionated based on their size on a column (100 x 2.6 cm) of Bio-Gel P-2 (200-400 mesh, Bio-Rad, Richmond, CA, USA) using a Hiload System. Components were eluted with distilled water at 60 °C (flow rate was 0.5 ml/min) and

monitored by refractive index detection using a Shodex RI-72 detector. Fractions (7.5 ml) were collected and fractions arising from individual peaks were pooled.

OFF-LINE HPAEC-MALDI-TOF MS

Bio-Gel pools 4 to 8 were further fractionated by HPAEC performed on a Dionex Bio-LC system[13]. The gradients were obtained by mixing solutions of 0.1 M NaOH and 1 M sodium acetate in 0.1 M NaOH. The gradient was optimised for each pool (Table 5.1). The (4 x 250 mm) CarboPac PA1 column was always equilibrated for 15 minutes before 20 microlitres of the sample were injected. After a run the column was washed for 5 minutes with 1 M NaAc in 0.1 M NaOH.

Table 5.1. Gradients used for fractionation of pools 4 to 8 by HPAEC.

Time (min)	Conc. NaAc (M)	Conc. NaOH (M)
	dp 4 and 5	
0	0	0.1
40	0.4	0.1
45	1	0.1
	dp 6	
0	0.07	0.1
5	0.07	0.1
40	0.14	0.1
45	1	0.1
	dp 7 and 8	
0	0.07	0.1
5	0.07	0.1
40	0.11	0.1
50	1	0.1

After passing a Dionex PED detector operated in the pulsed amperometric detection (PAD) mode, the eluate of the CarboPac PA1 column was desalted on-line using a self-regenerating anion suppressor 4 mm-unit (Dionex ASRS-ULTRA), and fractions (167 µl) were collected. The HPAEC fractions were then directly analysed using MALDI-TOF MS.

ENZYMATIC DEGRADATION

Bio-Gel P-2 pool 6 was incubated with endo-galactanase. The incubations were performed in 50 mM NaAc buffer (pH 5.0) containing 0.01% NaN_3 at 30 °C for 7h. Carbohydrate-degrading activities were

determined by HPAEC analysis of the digest, using the optimised gradient for pools 6 (as described above).

PER-*O*-METHYLATION OF OLIGOSACCHARIDES

Per-*O*-methylation[14] of the lyophilised oligosaccharides was performed by adding freshly-ground sodium hydroxide to the lyophilised oligosaccharide fractions dissolved in 200 µl dimethyl sulfoxide. Aliquots of 250 µl methyl iodide were added after 0, 10, and 30 minutes. The reaction was stopped 20 minutes after the final addition of methyl iodide by adding 1 ml sodium thiosulphate solution (100 mg/ml) and 1 ml chloroform. The chloroform layer was washed six times with water, after which the organic layer was evaporated to dryness under nitrogen.

ANALYTICAL METHODS

The neutral monosaccharide composition of the Bio-Gel P-2 pools was determined following release of the monosaccharides using methanolysis combined with TFA hydrolysis[15]. Samples were first dissolved in distilled water (1 mg/ml). An aliquot of 20 µl of this solution was dried under a stream of air. The dried sample was then submitted to methanolysis in 0.5 ml anhydrous 2 M HCl in absolute methanol for 16h at 80 °C. After cooling, the liquid was evaporated under a stream of air and 0.5 ml of 2 M TFA solution was added and heated for 1h at 121 °C. The samples were dried and 100 µl of distilled water was added. Analysis of the liberated monosaccharides was performed using HPAEC fractionation and PAD detection[15].

Glycosidic linkage analysis was performed following hydrolysis, reduction and *O*-acetylation of the per-*O*-methylated oligosaccharides[16]. GC/MS analyses were performed using a Fisons MD800 mass spectrometer fitted with a Carlo Erba GC8060 gas chromatograph and an on-column injector and using helium as the carrier gas. Monosaccharide derivatives were separated on a DB-5MS column (30m x 0.32 mm i.d.; J&W Scientific). Partially methylated alditol acetates (PMAAs) were injected in solution in dichloromethane (1 µl injected) and separated using the following temperature program: 50 °C for 2 min, 50→130 °C at 40 °C/min, held for 2 min, 130→230 °C at 4 °C/min and 230 °C isothermal for 15 min. Mass spectra were recorded under electron impact conditions in the positive ion mode with an electron energy of 70 eV and were recorded using linear scanning from *m/z* 55 to 400 over 0.9 s.

High-Performance Anion-Exchange Chromatography (HPAEC) was performed on a Dionex Bio-LC system (as described above). Different gradients were used for determination of the monosaccharide composition after methanolysis combined with TFA hydrolysis[15], and for the elution of arabinogalacto-oligomers.

For the determination of the arabinogalacto-oligomers, the CarboPac PA1 column was equilibrated with 0.1 M NaOH. Twenty microlitres of the sample were injected, and a linear gradient from 0 to 0.4 M NaAc in 40 minutes was applied. The column was washed for 5 minutes with 1 M NaAc, and then equilibrated again for 15 minutes with 0.1 M NaOH. Calibration was performed with series of Gal- and Ara-oligomers, obtained on enzymatic degradation of linear galactan by endo-galactanase and enzymically-debranched arabinan[17] by endo-arabinanase.

Matrix-Assisted Laser-Desorption/Ionisation Time-of-Flight Mass Spectrometry (MALDI-TOF MS). The matrix solution was prepared by dissolving 9 mg 2,5-dihydroxybenzoic acid and 3 mg 1-hydroxy isoquinoline in 700 μl distilled water and 300 μl acetonitrile. A 1 μl volume of this solution was placed on the sample plate together with 1 μl of the sample solution and allowed to dry at room temperature. The sample plate was then placed in the instrument.

MALDI-TOF mass spectra were recorded on a Voyager-DE RP Biospectrometry Workstation (PerSeptive Biosystems, Inc., Framingham, MA, USA) (Department of Food Technology and Nutritional Sciences, Wageningen University) equipped with a nitrogen laser operating at 337 nm (3-ns pulse duration), a single stage reflector, and delayed extraction. The accelerating voltage used was 12 kV and the delay time setting was 200 ns. Each spectrum was produced by accumulating data from 100-256 laser shots. Mass spectra were calibrated with an external standard containing GalA-oligomers (degree of polymerisation 2-9).

Tandem Mass Spectrometry. Collision induced dissociation (CID) tandem mass (MS-MS) spectra were obtained using a Micromass Q-TOF hybrid tandem mass spectrometer (Department of Mass Spectrometry, Utrecht University) equipped with a Z-Spray sample introduction system and gold coated glass capillaries in a nanoelectrospray ionisation source. Argon was used as collision gas and a collision energy of 60 eV was employed. Cone voltage and skimmer off-set were set at approximately 75 V and 5 V respectively with a capillary voltage of 2100 V. Ten percent of the native sample was used for methylation. The native and per-*O*-methylated products were dissolved in 100 μl methanol:water (1:1) and 1 μl of the sample was introduced into the glass capillary. Spectra were acquired with the TOF analyser over a mass range that is dependent on the molecular mass of the analyte, data were integrated every 2.3 s, and processed using the MassLynx software, version 3.0.

RESULTS AND DISCUSSION

ENZYMATIC DEGRADATION OF PECTIC ARABINOGALACTAN SIDE CHAINS FROM SOYBEAN

Soybean pectic substances obtained by CDTA extraction of water-unextractable cell wall material (ChSS fraction), were digested with endo-galactanase, exo-galactanase, endo-arabinanase and arabinofuranosidase B, as described by Huisman *et al.*[7]. However, in the

current experiments, the objective of the incubation was to obtain oligosaccharides large enough to provide information about the structure of the arabinogalactan side chains. This was achieved by incubating for only 10h. It should be pointed out that the oligosaccharides in the digest are not necessarily limit-digest products. The first step in isolating the oligosaccharides was the removal of the remaining polymeric material by fractionation on a Sephacryl S-100 HR column. The oligosaccharide-containing pool, on fractionation using HPAEC with PAD detection yielded a very complex chromatogram, indicating that this pool contains a wide variety of different oligomers (Figure 5.1).

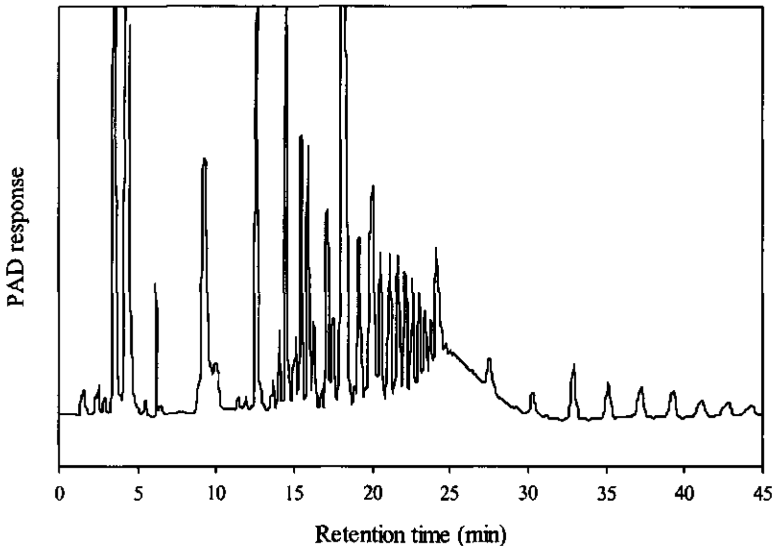

Figure 5.1. HPAE chromatogram for the oligosaccharide-containing pool from the ChSS digest obtained after incubation of the soybean ChSS fraction with endo-galactanase, exo-galactanase, endo-arabinanase, and arabinofuranosidase B.

The oligomers in the oligosaccharide pool fraction were fractionated by Bio-Gel P-2 chromatography (Figure 5.2A). The peaks corresponding to oligomers with a degree of polymerisation exceeding 4 were not resolved in the chromatogram because they are masked by the enormous signal caused by eluting salt. Consequently the fractions corresponding to these oligomers were desalted and re-applied to the Bio-Gel P-2 column (Figure 5.2B). Pool 12 eluted in the void volume and contained oligomers with a degree of polymerisation of 12 and higher. The numbers of the pools (1 to 11) correspond to the degree of polymerisation of

the oligosaccharides, as determined by MALDI-TOF MS analysis of the oligosaccharides (see below).

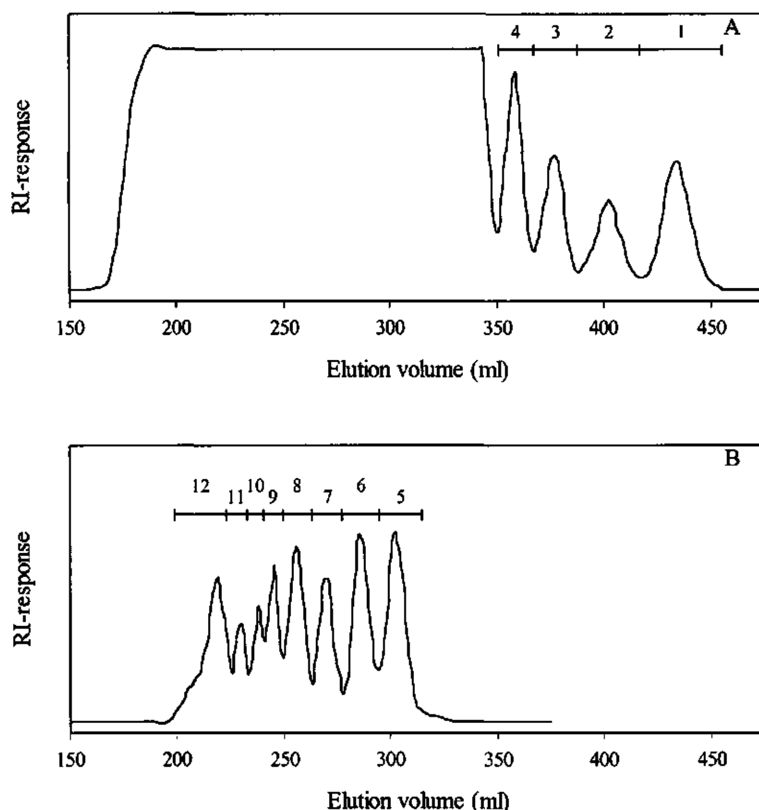

Figure 5.2. Chromatogram of (A) the polymeric fraction from the ChSS digest and (B) the desalted oligosaccharides with a degree of polymerisation exceeding four on Bio-Gel P-2.

CHARACTERISATION OF THE BIO-GEL P-2 POOLS

The Bio-Gel P-2 pools were subjected to HPAEC and MALDI-TOF MS analyses. Both techniques showed that the pools contain mixtures of oligosaccharides. The MALDI-TOF mass spectra indicated that the oligosaccharides have essentially the same degree of polymerisation (n). In addition, as n increases the presence of homologues with a degree of polymerisation of n+1 and n-1 is also detected. This is a direct result of the decreasing resolution of the Bio-Gel P-2 column with increasing n, and of the differences in hydrodynamic volume of Ara and Gal residues.

The HPAE chromatograms obtained from the Bio-Gel P-2 pools are shown in Figure 5.3. Pool 1 contains Ara and Gal monomers only (trace a). The presence of four compounds is suggested by the HPAE chromatogram obtained from pool 2 (trace b). The first eluting compound represents the Gal monomer, the second eluting compound represents the dimer(s) Ara_1Gal_1, the third eluting compound represents Gal_2, and the last eluting compound represents Ara_2. This was concluded from the retention times of the compounds in the HPAE chromatogram which were identical to the retention times of the series of standard Ara- and Gal-oligosaccharides used to calibrate the column.

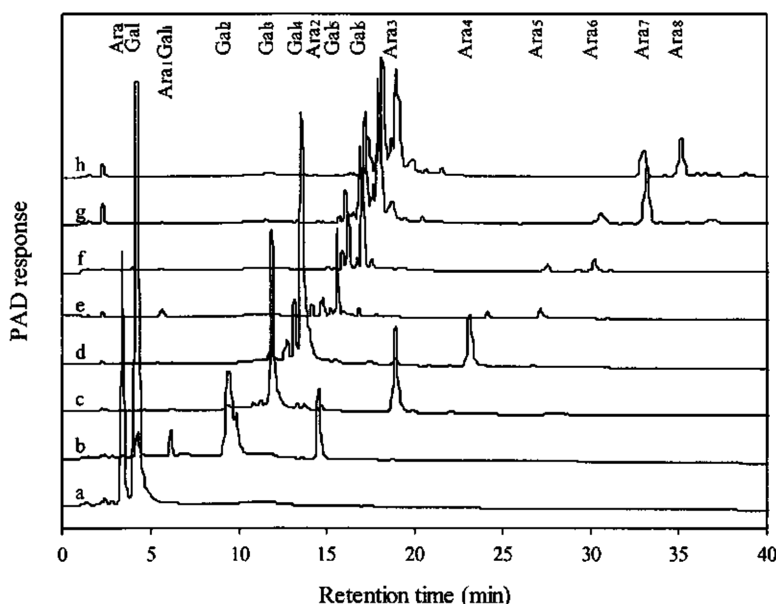

Figure 5.3. HPAE chromatogram for arabinogalacto-oligomers in Bio-Gel P-2 pools 1 to 8 (traces a to h, respectively).

As the degree of polymerisation of the pool increases, the number of oligomers in the pool increases and the chromatogram becomes more complex (Figure 5.3). Traces c to h in Figure 5.3 show first a cluster of components eluting within 20 minutes, co-eluting with Gal_n-oligomers, followed by one or two components co-eluting with Ara_n-oligosaccharides. Monosaccharide composition analyses of pools 3 to 8 revealed that Ara and Gal are the only neutral monosaccharide residues present in these pools.

The HPAE chromatograms suggest a large diversity of oligomers within the pools, but do not give information about their composition. The molecular masses of the compounds in the pools analysed by MALDI-TOF MS are indicative of the compositions of the different oligomers with respect to the number of Ara and Gal residues present. As an example, the

MALDI-TOF mass spectrum of the arabinogalacto-hexamers is shown in Figure 5.4. The sodium-cationised $[M + Na]^+$ ions are the dominant species observed in the spectra of the Bio-Gel P-2 pools, although some ions are accompanied by the potassium-cationised $[M + K]^+$ species. MALDI-MS analysis of Bio-Gel P-2 pool 6 yielded a spectrum containing ions for all possible Gal_xAra_y hexasaccharide compositions. The most abundant ion in the MALDI-TOF mass spectrum obtained from Bio-Gel P-2 pool 6 is observed at *m/z* 1013 and corresponds to sodium-cationised Gal_6. In addition to compounds with n=6, the spectrum shows the presence of two different pentamers (Gal_5 and Gal_4Ara) and one heptamer (Ara_7). The occurrence of the pentasaccharides in Bio-Gel P-2 pool 6 is a result of the reduced resolution of the Bio-Gel P-2 column in this mass range. The presence of Ara_7 in pool 6 is a logical consequence of its molecular mass, which is similar to the molecular masses of most of the arabinogalacto-hexasaccharides.

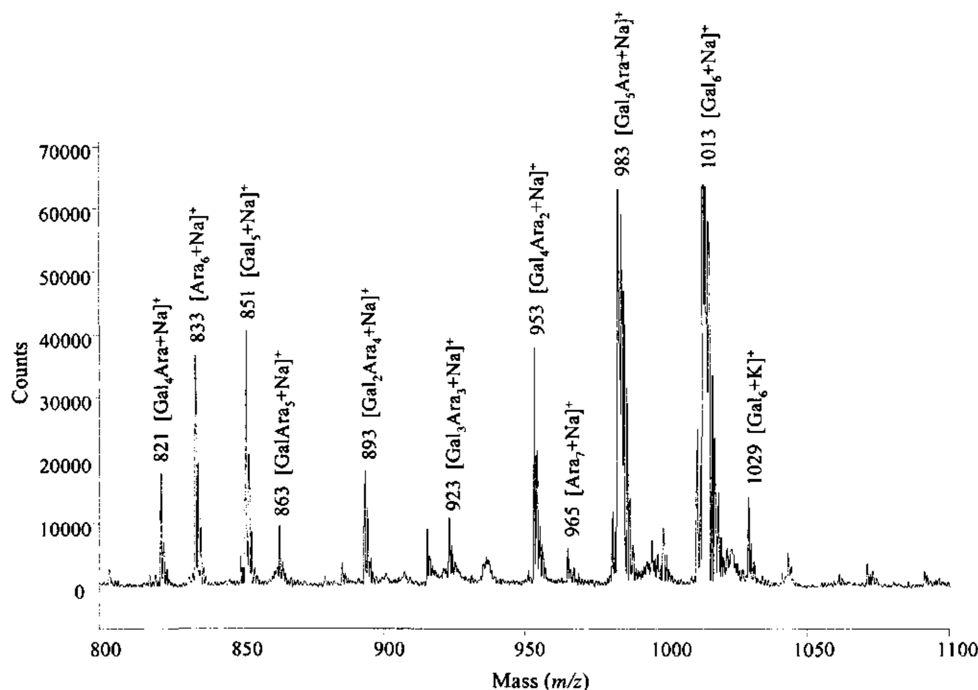

Figure 5.4. MALDI-TOF mass spectrum of Bio-Gel P-2 pool 6 containing oligomers released from soybean pectic substances by arabinogalactan-degrading enzymes.

The other Bio-Gel P-2 pools were also analysed by MALDI-TOF MS. Pools 3 to 5 also contain the whole range of possible Gal_xAra_y compositions for that particular degree of polymerisation. The pools containing oligomers with higher values for n do not contain the whole range of possible oligomer compositions, but only those in which the majority of monosaccharide residues is Gal. As stated before in this publication, the results of MALDI-

TOF analysis show the reduced resolution of the Bio-Gel P-2 column in the higher mass range, i.e. as the relative amounts of (n ± 1)-oligomers increase.

CHARACTERISATION OF THE ARABINOGALACTO-OLIGOMERS BY OFF-LINE HPAEC MALDI-TOF MS ANALYSIS

HPAEC separation and MALDI-TOF MS analysis of the Bio-Gel P-2 pools showed that these pools contain mixtures of oligosaccharides. The elution order of the oligosaccharides from HPAEC can not be predicted, and little structural information can therefore be deduced from these experiments. Pools 4 to 8 were fractionated using an analytical Carbopac PA1 column to allow structural studies of the oligosaccharides to be carried out. The high sodium concentration in the mobile phase eluting from the column was reduced on-line using a self-regenerating anion suppressor 4 mm-unit (Dionex ASRS-ULTRA). The 'desalted' HPAEC fractions were then directly analysed using MALDI-TOF MS.

As an example the HPAE chromatogram obtained from Bio-Gel P-2 pool 6 using an optimised gradient for this mixture is shown in Figure 5.5. The most abundant component in the HPAE chromatogram (fraction 6.4; retention time 12.5 min) yielded an ion at *m/z* 1013 on MALDI-TOF MS analysis, corresponding to sodium-cationised Gal_6. This is consisitent with its elution behaviour, which suggested a β-(1,4)-linked Gal_6 based on its co-elution with the linear β-(1,4)-linked galacto-hexasaccharide in the standard. Fraction 6.1 contains Gal_5 (*m/z* 851); fractions 6.2 and 6.3 contain hexasaccharides composed of one Ara and five Gal residues (*m/z* 983). The MALDI-TOF mass spectrum of fraction 6.5 is identical to the spectrum of fraction 6.4, indicating Gal_6. Fractions 6.6 and 6.7 both show ions at m/z 833, corresponding to sodium-cationised Ara_6. Their retention times are consistent with the elution behaviour of a linear α-(1,5)-linked Ara_6 standard.

The HPAE chromatograms of the other Bio-Gel P-2 pools resemble that of pool 6 in the order of elution of analogous compounds, but the retention times increase with increasing degree of polymerisation. The general elution order is: Gal_{n-1}, $Gal_{n-1}Ara$, Gal_n, followed by the arabino-oligosaccharides. With increasing degree of polymerisation of the pool, the number of peaks in the HPAE chromatogram increases and the chromatogram becomes more complex. The HPAE chromatogram of Bio-Gel P-2 pool 4 thus has only four peaks corresponding to compounds with n=4: one Gal_3Ara, two Gal_4 isomers and one Ara_4[18]. The HPAE chromatogram of Bio-Gel P-2 pool 8, in contrast, contains ten peaks corresponding to compounds with n=8 (not shown): three Gal_7Ara, two Gal_8, and five Ara_8 isomers.

In the MALDI-TOF mass spectra of the Bio-Gel P-2 pools (dp=n) every possible oligosaccharide composition from Ara_0Gal_n up to Gal_0Ara_n was present (Figure 5.4). On HPAEC fractionation, however, only Gal_n, $Gal_{n-1}Ara$, Gal_{n-1}, and Ara_n were detected. This can be explained by the fact that the peaks from the higher Ara-containing species in the MALDI-TOF mass spectrum are less intense than those from Gal_n, $Gal_{n-1}Ara$, Gal_{n-1}, and Ara_n, and

MALDI-TOF MS is much more sensitive than the pulsed amperometric detector. The possibility that the ions assigned as corresponding to the higher Ara-containing species could actually correspond to fragment ions derived from the higher mass species (i.e. those with more Gal in them) crossring-cleavage can be ruled out, since ions arising by crossring-cleavage would then also be expected in the MALDI-TOF mass spectra of the isolated components (HPAEC fractions) and they are not.

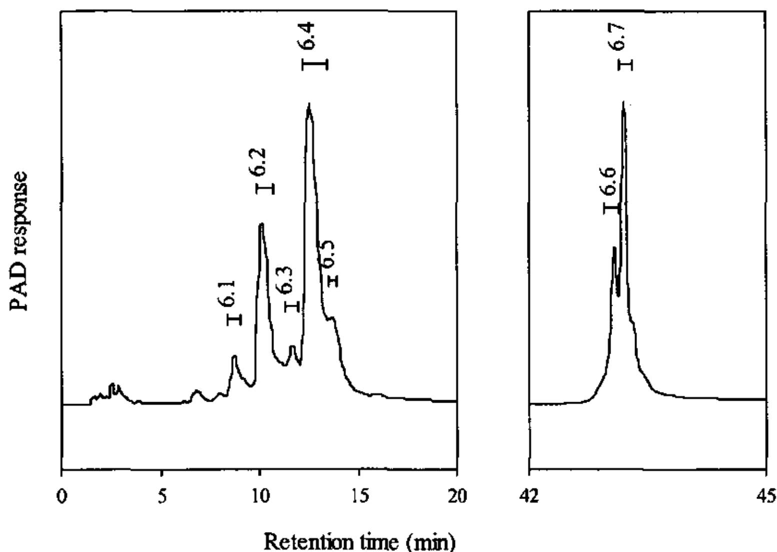

Figure 5.5. HPAE chromatogram for Bio-Gel P-2 pool 6 from the soybean ChSS digest.

The different behaviour of oligosaccharides of identical composition on HPAEC indicates that their structures are not identical; they might differ in their reducing terminal residue, in the type of glycosidic linkages, or in branching pattern[19]. To determine the structural details of these oligosaccharides, additional types of analysis are required, such as linkage analysis, tandem mass spectrometry, NMR spectroscopy, and digestion of the oligosaccharides by specific enzymes. Fractions 6.2 to 6.7 were purified, but the amounts available were insufficient for analysis by NMR spectroscopy. The results of linkage analysis and tandem mass spectrometry are described below.

LINKAGE ANALYSIS

Analysis of the partially methylated alditol acetates (PMAAs) from fractions 6.4 and 6.5 resulted in derivatives indicative of terminal and (1,4)-substituted galactose residues, indicating the presence of linear (1,4)-linked Gal_6 in both fractions. To explain their different

elution behaviours on HPAEC, additional structural information about these galacto-hexasaccharides is required.

Linkage analysis of permethylated fraction 6.2 ($AraGal_5$) indicates the presence of (1,4)-substituted and terminal Gal, and (1,5)-substituted Ara*f*. A small peak that was observed corresponding to a PMAA derived from (1,4,6)-substituted galactose could be indicative of the presence of branched galactose residues, but is more likely to be the result of undermethylation, since tandem mass spectrometry of fraction 6.2 shows the presence of linear oligosaccharides only (see below).

Fraction 6.3, having the same monosaccharide composition as fraction 6.2, yielded different linkage analysis data. It gave PMAA derivatives indicative of (1,4)-substituted Gal, terminal Gal, terminal arabinopyranose, and (1,5)-substituted arabinofuranose residues. Since only Gal_5Ara was demonstrated in this fraction, it must contain a mixture of at least two compounds.

TANDEM MASS SPECTROMETRY

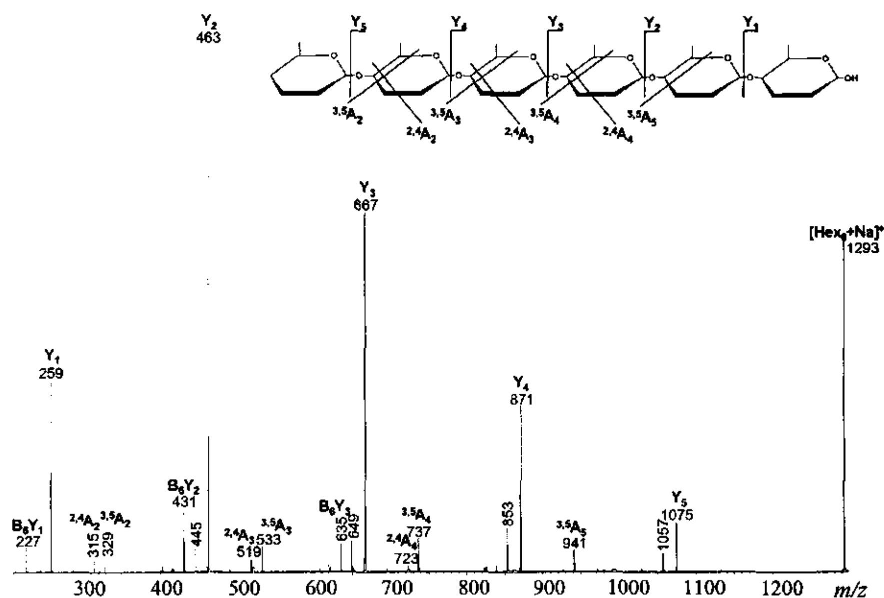

Figure 5.6. Positive ES CID tandem mass spectrum of per-*O*-methylated $[Gal_6+Na]^+$ from HPAEC fraction 6.4, released from soybean pectic substances by arabinogalactan-degrading enzymes.

Per-*O*-methylated fraction 6.4 was analysed using tandem mass spectrometry. In the tandem mass spectrum obtained from the sodium-cationised pseudomolecular ion of Hex_6, at

m/z 1293 [Hex_6 + Na]$^+$ (Figure 5.6), a complete series of Y_n ions and the absence of Y_n-14 ions are indicative of a linear hexasaccharide. If the oligosaccharide were branched, one os more Y_n ions would be absent, depending on where the oligosaccharide is branched and one or more ions at *m/z* Y_n-14 would be expected to be observed. Neither the absence of Y_n ions nor the presence of additional Y_n-14 ions is observed. It is theoretically possible that a mixture of differently branched isomeric structures could together yield a complete series of Y_n ions, however, such a mixture would also yield Y_n-14 ions from each of the branch points and these are not observed.

Ions at *m/z* 227, 431 and 635 are the result of double cleavage events. The Z_n ions at *m/z* 445, 649, 853 and 1057 are the result of the loss of water from the Y_n ions, which according to the nomenclature of Domon and Costello[20] are Z_n ions. A series of cross-ring cleavage ions, observed at *m/z* 315, 329, 519, 533, 723, 737, and 941, is indicative of (1,4)-substitution of the monosaccharide residues. Since the only hexose present in pool 6 is galactose, the tandem mass spectrum of the per-*O*-methylated [Hex_6 + Na]$^+$ indicates a linear (1,4)-linked galacto-hexasaccharide, which is consistent with the results from linkage composition analysis of fraction 6.4.

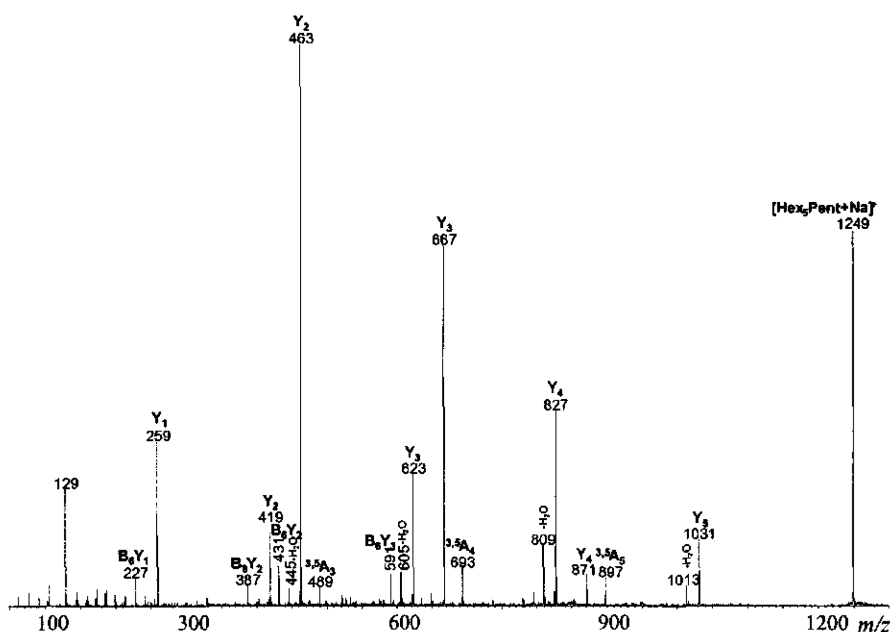

Figure 5.7. Positive ES CID tandem mass spectrum of per-*O*-methylated [Hex_5Pent + Na]$^+$ from fraction HPAEC 6.2, released from soybean pectic substances by arabinogalactan-degrading enzymes.

The tandem mass spectrum obtained from the [Hex_6 + Na]$^+$ ion in fraction 6.5 (not shown) does not significantly differ from the spectrum obtained from fraction 6.4. This

indicates that fraction 6.5 also contains a linear (1,4)-linked galacto-hexasaccharide, as was already suggested by its linkage composition. Separation on HPAEC suggests the presence of different anomers[19].

In the CID tandem mass spectrum of per-*O*-methylated $[Hex_5Pent + Na]^+$ in fraction 6.2, an intense series of Y_n ions is observed at *m/z* 259, 463, 667, 827, and 1031 (Figure 5.7). These ions are indicative of a linear oligosaccharide corresponding to structure II (Figure 5.8). The ion observed at *m/z* 827 is a Y_4 ion indicative of a structure containing three hexose and one pentose residues. If this ion derives from a sodiated Hex_3Pent structure with a core of hexoses and a terminal pentose, then either a Y_1 ion at *m/z* 215 indicative of a reducing pentose or a Y_5 ion at *m/z* 1075 indicative of a non-reducing terminal pentose might be expected. Neither of these ions is present so that the compound that yields this Y_4 ion must therefore have an internal pentose. In the tandem mass spectrum of $[Hex_5Pent + Na]^+$ further Y_n ions for structures that bear a pentose are also present: at *m/z* 419 (PentHex) and 623 ($PentHex_2$) and 1031 ($PentHex_4$). The absence of the Y_n ions at *m/z* 215 and 1075, in combination with the pentose-bearing Y_n ions *m/z* 1031, 827, 623 and 419, means that none of the oligosaccharide structures I to IV (Figure 5.8) can be ruled out as being present in fraction 6.2.

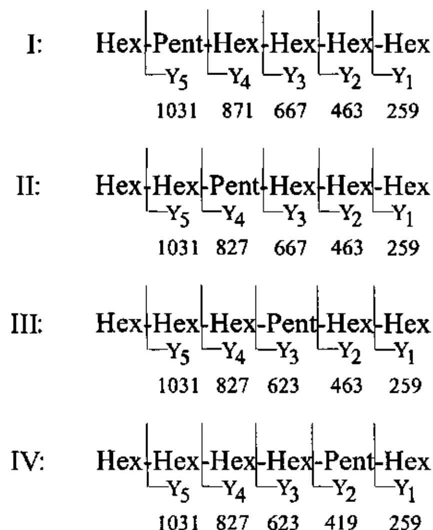

Figure 5.8. Possible structures for Hex_5Pent isomers with an internal pentose present in fraction 6.2, based on results from tandem mass spectrometry.

Other ion series as indicated in the spectrum (Figure 5.7) result from double cleavage or cross-ring cleavage events and corroborate the interpretation given above. They indicate the presence of either (1,4) or (1,6) linkages. Since the ion observed at *m/z* 489 is composed

of a Hex, a Pent, and the remainder of the next Hex residue the internal Pent is substituted to the C6 or C4 of the cleaved Hex.

From these data, taken together with the monosaccharide composition of pool 6 and the linkage analysis results for fraction 6.2, it can be deduced that fraction 6.2 contains a mixture of (1,4)-linked galacto-hexasaccharide isomers with an internal (1,5)-linked arabinofuranose residue.

The tandem mass spectrum of per-*O*-methylated $[Hex_5Pent + Na]^+$ in fraction 6.3 (Figure 5.9) is significantly different from that obtained from the ion at the same *m/z* in fraction 6.2 (Figure 5.7). The most noticeable difference between the spectra is found in the presence of an ion at *m/z* 1075 in the spectrum obtained from fraction 6.3. This ion corresponds to a Y_5 ion composed of five hexose residues. Since this Y_5 ion can only result from the loss of a pentose from the parent ion and since 'internal residue loss' has not been observed from sodium-cationised oligosaccharides[21] the Pent residue in this oligosaccharide has to be in a terminal position.

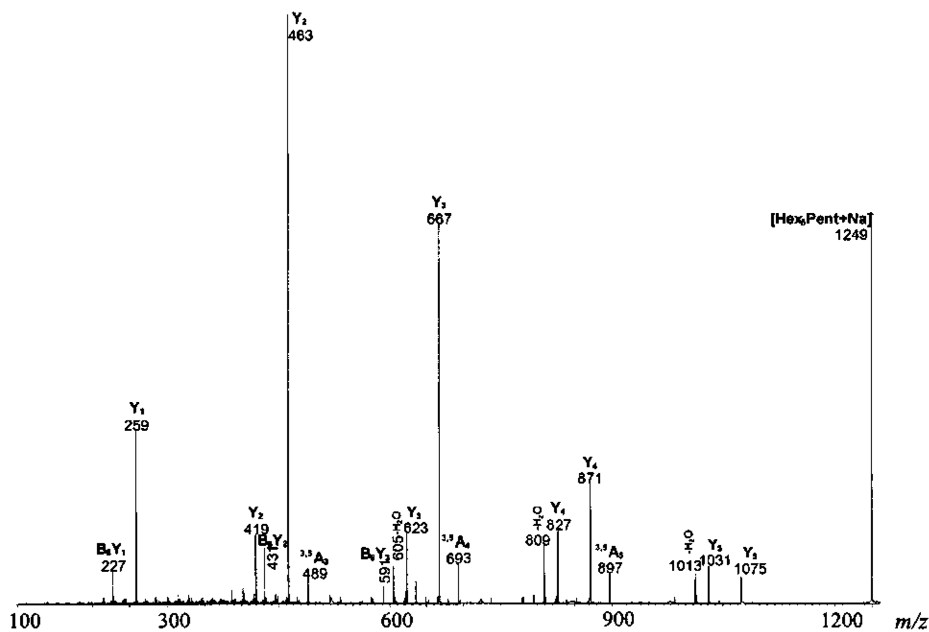

Figure 5.9. Positive ES CID tandem mass spectrum of per-*O*-methylated $[Hex_5Pent + Na]^+$ from fraction HPAEC 6.3, released from soybean pectic substances by arabinogalactan-degrading enzymes.

The Y_n ion series in the spectrum obtained from $[Hex_5Pent + Na]^+$ from HPAEC fraction 6.3 present at *m/z* 259, 463, 667, 871, and 1075 is not accompanied by corresponding Y_n-14 fragment ions, indicating linear oligosaccharides. The most plausible structural explanation for the ion at *m/z* 1249 in fraction 6.3 is a linear hexasaccharide bearing the

pentose on the non-reducing terminus. The remainder of the spectrum is very similar to that from fraction 6.2, consistent with fraction 6.3 also containing a mixture of isomeric structures with an internal pentose. This is consistent with the results of linkage analysis, showing both a terminal pentopyranose and (1,5)-linked pentofuranose. The hexoses are present as either (1,4)-linked or as terminal hexoses, as in fraction 6.2.

The presence of isomers with an internal arabinose residue is most probably caused by the fact that fractions 6.2 and 6.3 are not fully resolved, and the relative amount of fraction 6.2 in the mixture is much larger than of fraction 6.3.

In the linear arabinogalacto-oligosaccharide bearing the arabinopyranose residue on the non-reducing terminus, this arabinose is most probably not the remainder of a side chain, because side chains are usually linked to C3 along the main chain and not to C4. This oligomer can also not originate from a galactan chain interspersed with an arabinopyranose residue, because none of the enzymes used in the degradation of soybean ChSS is able to hydrolyse the glycosidic linkage between a galactose and an arabinopyranose residue, and oligomers with an internal arabinopyranose residue would also have been present in the digest. This terminal arabinopyranose was probably present as a terminal arabinose in the polymer. Other arabinofuranose residues might have been attached to it, but it is uncertain whether endo-(1,5)-α-L-arabinanase or arabinofuranosidase B are able to hydrolyse the glycosidic linkage between an arabinofuranose and an arabinopyranose.

The mass spectra obtained from fractions 6.6 and 6.7 (not shown), contain ions at *m/z* 1029, which correspond to per-*O*-methylated sodium-cationised $Pent_6$. In the tandem mass spectra obtained from *m/z* 1029 from both fractions (not shown), a complete series of Y_n ions is present (*m/z* 215, 375, 535, 695, and 885), indicative of a linear $Pent_6$ oligosaccharide. Ions observed at *m/z* 361, 521, and 681 can be described as Y_n-14 ions, which are the result of two glycosidic bond cleavages both accompanied by proton transfer and which are indicative of cleavage at a branched residue. The ion observed at *m/z* 507 is indicative of a triply substituted residue. So these fractions contain mixtures of linear and various branched arabinohexasaccharides. The characterisation of two co-eluting branched arabinotetrasaccharides was described previously by Brüll *et al.*[18].

Similar fractionation and tandem mass spectrometric analyses of the fractions from pools 4 to 8 were performed. All these pools were shown to contain linear (1,4)-linked galacto-oligosaccharides, (1,4)-linked galacto-oligosaccharides bearing an arabino*pyranose* residue at the non-reducing terminus, a mixture of linear oligosaccharides constructed of (1,4)-linked galactose residues interspersed with one *internal* (1,5)-linked arabino*furanose* residue, and both linear and branched arabino-oligosaccharides. This indicates that the length of the galactan chain between two internal arabinose residues can vary. The presence of linear β-(1,4)-linked galacto-oligosaccharides is consistent with published structures of soybean (arabino)galactan[11]. Arabinose-containing side chains might have been present, but could have been removed during enzymatic degradation by arabinofuranosidase B. Prior to this

analysis it had been anticipated that arabinogalacto-oligosaccharides with a galactan main chain and (residual) arabinofuranose residues as side chains would be isolated. The presence of arabino-oligosaccharides (which can be degraded further by arabinofuranosidase B) demonstrates that arabinofuranosidase B had not digested the mixture to completion. Therefore, it is remarkable that all arabinose-containing side chains appear to have been removed from the galactan main chain. A possible explanation is that arabinose-containing side chains were not present in the original arabinogalactan pectic side chains in the ChSS extract. Another possible explanation, that arabinose substituents attached to a galactan main chain might somehow be more accessible to the enzyme than arabinose substituents attached to an arabinan main chain, is not very likely.

Labavitch *et al.*[12] have described the presence of large arabinan chains in soybean cell walls. The present research extends the knowledge of the structures of the arabinan side chains as being heavily branched. However the presence of an internal arabinofuranose residue and a terminal arabinopyranose residue in a pectic galactan chain of cell wall polysaccharides has not been reported previously, either in soybean, or in other fruit or vegetable cell walls.

FURTHER ENZYMATIC DEGRADATION OF THE ARABINOGALACTO-OLIGOSACCHARIDES

Confirmation of the structures of the oligosaccharides identified by ES tandem MS, using enzymatic digestion is difficult. The main reason is that suitable pure enzymes are not available. In theory, arabinofuranosidase A and B should only be able to degrade the oligomers in the pools that consist only of arabinose. These enzymes are unable to release the internal arabinofuranose from the compounds in fraction 6.2 and are also unable to release the terminal arabino*pyranose* residue from the oligosaccharides in fraction 6.3. Incubation of pool 6 with arabinofuranosidase B, indeed showed the further degradation of the compounds in 6.6 and 6.7. The enzyme that is required for the degradation of compound 6.3 would be an arabinopyranosidase. Such an enzyme, β-L-arabinopyranosidase, is poorly described in the literature[22,23]. The enzyme described by Dey is a true β-L-arabinopyranosidase isolated from *Cajanus indicus* seeds, and is unable to hydrolyse *p*-nitrophenyl α-D-galactoside, *p*-nitrophenyl α-D-fucoside or *p*-nitrophenyl β-D-galactoside[22,23]. This enzyme would probably be able to release the terminal arabinopyranose from the Ara*p*-(1→4)-Galp_n oligomers.

Further characterisation of the arabinogalacto-oligosaccharides using endo-galactanase, exo-galactanase or β-galactosidase is complicated. A major complication is the presence of mixtures of compounds in the Bio-Gel P-2 pools and of different isomers in the HPAEC fractions (particularly fraction 6.2). Due to the incomplete resolution of fractions 6.2 and 6.3 on HPAEC it is impossible to obtain the arabinopyranose-containing oligosaccharides in a pure form. A further difficulty is that the degree of polymerisation of the oligosaccharides influences the ability of the enzymes to hydrolyse the glycosidic linkages, so it would be

difficult to distinguish whether the reason for the inability of the enzyme to hydrolyse the structure is its degree of polymerisation or the structure of the oligomers.

To illustrate the difficulty of carrying out enzymatic degradation studies on these mixtures, Bio-Gel P-2 Pool 6 was incubated with endo-galactanase. The resulting HPAE chromatogram obtained from the digest was indeed very complex. Peaks corresponding to fractions 6.3, 6.4 and 6.5 were completely and very rapidly lost, whereas peak 6.2 was removed more slowly. This may indicate that the enzyme has different affinities for the isomers in the mixture or that all these isomers are slowly degraded because of the presence of the internal Ara*f*. The reaction products are very diverse and it is impossible to determine from which oligosaccharide in the pool a specific product peak is derived. It is therefore not possible to draw any conclusions concerning the structures of the components giving rise to the product peaks or to identify from which parental oligosaccharides they were produced.

CONCLUSIONS

The pectic arabinogalactan side chains present in soybean pectic substances have been shown to be more complex than had been suggested by previous studies[8,9,10,11]. Prior to this study, the structures of the neutral arabinogalactan side chains of soybean pectin had been described as β-(1,4)-linked polygalactopyranose chains with Ara*f* residues present as (1,5)-linked side chains with an average length of two monosaccharide units attached to Gal residues in the main chain by (1,3)-linkages. In this study we have shown that arabinose residues are not only present as external substituents of the galactan main chain, but that they also occur as internal residues in the main chain. Internal arabinose has been described once before in arabinogalactan type II from larch, where arabinose was shown to be present in the side chains as well as in the backbone of the molecule[2,24,25]. The presence of an internal arabinofuranose residue in a pectic arabinogalactan chain in cell wall polysaccharides has not been reported previously, either in soybean, or in other fruit or vegetable cell walls.

Another feature that attracts attention is that the linkage analysis of the arabinogalacto-oligomer showed the presence of arabinose residues in both the furanose and pyranose ring form, the arabinofuranose residues being (1,5)-linked and the arabinopyranoses terminal residues. The ring forms of these terminal arabinoses explains their resistance to the applied enzymes, since arabino*furanosidase* B was used. Both ring forms of arabinose also occur in arabinogalactan type II, in which L-arabinofuranosyl, and to a lesser extent L-arabinopyranosyl residues, terminate some of the side chains[4]. However, in soybean arabinogalactan we are dealing with linear (1,4)-linked β-D-galactan isolated from the pectic substances, which is type I arabinogalactan. The existence of arabinopyranose residues in pectic arabinogalactan is uncommon, since the presence of only arabinofuranose residues is generally reported. Only pectic substances isolated from the roots of *Angelica acutiloba* Kitagawa were shown to contain a small proportion of arabinopyranose[26].

In spite of the use of size-exclusion and anion-exchange chromatography most fractions remained mixtures of isomeric compounds. This is a handicap in the analysis of the constituent oligosaccharides and further fractionation is needed for determination of all detailed structures. However, options for further fractionation are very limited. A possibility would be immobilised lectin affinity chromatography, if lectins interacting with arabinopyranose residues were available. Further fractionation of the arabinogalacto-isomers could alternatively be attempted using normal or reversed phase HPLC, possibly after derivatisation of the oligosaccharide mixture. A drawback of derivatisation would be that the isolated oligomers are modified.

The difference in elution behaviour of the two linear galacto-hexasaccharides (also true for galacto-oligosaccharides with a degree of polymerisation of 4 to 8) is intriguing. Using both linkage analysis and tandem mass spectrometry, we were unable to differentiate between these two compounds. The difference in elution behaviour, however, may well point to differences in their anomeric linkages.

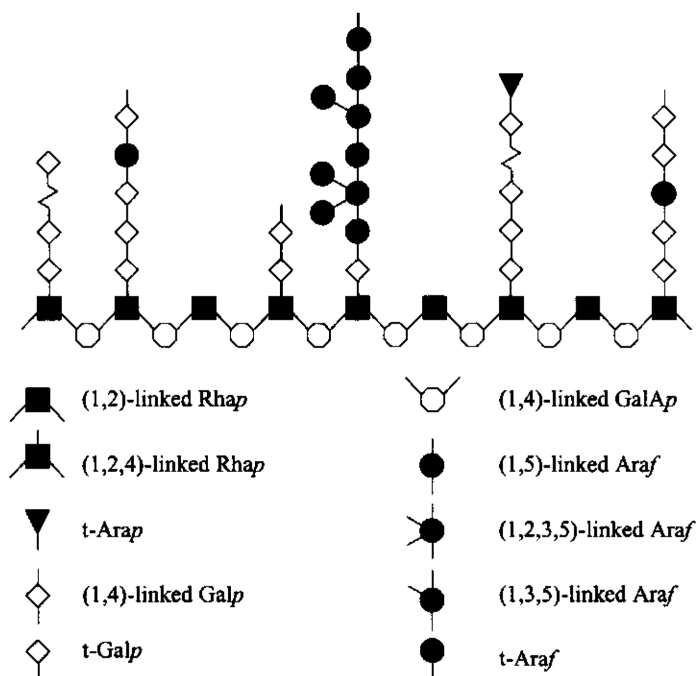

Figure 5.10. Hypothetical structure of the rhamnogalacturonan regions of CDTA-extractable soybean pectic substances. The distribution of the acetyl groups is not shown. A strictly alternating sequence of Rha and GalA, termed RG-I[27], is presented here, but the structure of the rhamnogalacturonan backbone is as yet unknown. Only short (arabino)galactan side chains are presented in this figure, but the true average length of the side chains is 45 to 50 residues.

Based on our results, a hypothetical structure of the pectic arabinogalactan side chains is proposed (Figure 5.10). The ratio of (1,2)- to (1,2,4)-linked Rha in the ChSS extract is 1:2[28]. From the monosaccharide composition of the ChSS fraction[6], it can be concluded that the average length of the (arabino)galactan side chains is in the range of 45 to 50 residues. This was confirmed by the length of the arabinan/galactan/arabinogalactan oligomers released from the ChSS extract on brief incubation with endo-galactanase, exo-galactanase, endo-arabinanase, and arabinofuranosidase B. HPAEC analysis of this digest showed the presence of galactan and arabinogalactan oligomers with a degree of polymerisation up to about 15 and arabinan oligomers with a degree of polymerisation up to 30-35. It is not clear how the arabinan side chains were attached to the rhamnogalacturonan backbone. Although side chains commencing with an arabinosyl residue might be present in soybean, all side chains in Figure 5.10 start with a galactosyl residue. This is mainly based on the results of previous studies, in which NMR analysis showed that terminal or (1,4)-linked Gal was attached to the rhamnose residues after enzymatic and chemical removal of large stretches of the chains leaving a short stub behind[28].

In this study, enzymes were used to degrade pectic arabinan, galactan, and arabinogalactan side chains in the ChSS extract to fragments, which fit very readily within the mass range of HPAEC and mass spectrometric methods. However, these enzymes failed to be helpful in the elucidation of the structures of these fragments, as can easily be understood from Figure 5.10. This study shows the need to isolate enzymes able to hydrolyse the glycosidic linkage between a (terminal or (1,4)-linked) galactose residue and a (1,5)-linked arabinose residue, to hydrolyse the glycosidic linkage between a (terminal or (1,5)-linked) arabinose residue and a (1,4)-linked galactose residue, together with the need for arabinopyranosidases. These enzymes could be helpful in the determination of the structure of the purified oligosaccharides, and they could be helpful in the elucidation of the structure of the *polymeric* pectic arabinogalactan side chains from soybean meal.

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CHAPTER 6

XYLOGLUCAN FROM SOYBEAN (*GLYCINE MAX.*) MEAL IS COMPOSED OF XXXG-TYPE BUILDING UNITS

M.M.H. HUISMAN, K.G.C. WEEL, H.A. SCHOLS & A.G.J. VORAGEN

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Soybean cell wall material was depectinated by extraction with hot chelating agent and cold dilute alkali. The hemicelluloses were solubilised from the residue with 1 and 4 M KOH solutions, resulting in a 1 MASS (1 M Alkali Soluble Solids) and a 4 MASS (4 M Alkali Soluble Solids) fraction. The polysaccharides extracted with 1 M KOH were fractionated by ion-exchange chromatography, yielding a neutral and a pectic population. The sugar composition of the neutral population indicated the presence of xyloglucans and possibly xylans. Enzymatic degradation with endo-xylanases and endo-glucanases showed the presence of xyloglucans only. Analysis of the digest formed after incubation of the neutral population with endo-glucanase V using both HPAEC and MALDI-TOF MS showed the formation of the characteristic poly-XXXG xyloglucan oligomers (XXG, XXXG, XXFG, XLXG, and XLFG).

INTRODUCTION

Soybeans (*Glycine max*) belongs to the pea family of the Leguminosae. Their primary cell wall is built up of skeletal cellulose microfibrils and so-called matrix polymers, which include xyloglucans, xylans, pectins, and proteins. The cell walls of dicots consist of two main interpenetrating networks, one of cellulose and hemicellulose and one of pectin[1].

Hemicelluloses are non-cellulosic wall polysaccharides other than pectins[1], which can be extracted from the walls with alkaline solutions, typically 1-4 M. The requirement for relatively strong alkali for their extraction from the wall is due to strong hydrogen bonding between the hemicellulose and cellulose microfibrils. The hemicelluloses vary greatly in different cell types and in different species. In most cell types, one hemicellulose predominates, with others present in smaller amounts[2].

In the cell walls of most Dicotyledonae, the principal hemicelluloses are xyloglucans. Other hemicelluloses, such as gluco- and galactoglucomannans, galactomannans, (1→3)-β-D-glucans, and glucuronoarabinoxylans are found in much lower amounts[3]. Xyloglucans are linear chains of (1→4)-β-D-glucan with xylosyl residues added at regular sites to the O-6 position of the glucosyl units. Additional sugar residues, like galactose, fucose, and arabinose, are added to the O-2 of some xylosyl residues[3,4,5,6]. The galactose residues can be O-acetylated[7].

In previous publications, the structure of the pectic substances in the pectin network of the cell wall has been described extensively[8,9,10]. In the current study we will describe the structural elucidation of the most prominent hemicellulose in soybean cell wall material.

MATERIALS AND METHODS

MATERIAL

Polysaccharides have been sequentially extracted from water unextractable solids (WUS) isolated from solvent-extracted, untoasted soybean meal[8]. The 1 MASS and 4 MASS fraction were obtained by extraction with 1 and 4 M KOH, respectively.

ION-EXCHANGE CHROMATOGRAPHY

Approximately 250 mg of 1 MASS was fractionated on a column (100 x 2.6 cm) of DEAE Sepharose Fast Flow, which was initially equilibrated in 0.005 M NaAc-buffer pH 5.0, using a Hiload System (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

Elution was carried out sequentially with 530 ml of 0.005 M NaAc-buffer pH 5.0, a linear gradient from 0.005 to 1 M NaAc buffer pH 5.0 (1060 ml), a linear gradient 1-2 M NaAc-buffer pH 5.0 (530 ml) and 265 ml 2 M NaAc-buffer pH 5.0. Residual bound polysaccharides were washed from the column with 530 ml of 0.5 M NaOH. The elution rate was 10 ml/min except for the first step, in which the sample was applied onto the column and the elution rate was 5 ml/min. Fractions (20 ml) were collected and assayed by automated methods for neutral sugar content[11] and uronic acid content[12]. The appropriate fractions were pooled, concentrated, dialysed, freeze dried and analysed for neutral sugar composition and uronic acid content.

ENZYMATIC DEGRADATION

The unbound, neutral population from anion-exchange chromatography (1 MASS Neutral) was treated with pure and well-defined enzymes, endo-xylananse I[13,14] and endo-glucanase I and V[15]. Solutions (0.25% w/w)) of 1 MASS Neutral in 50 mM NaAc buffer (pH 5.0) containing 0.01% NaN_3 were incubated at 30 °C rotating 'head over tail', during 24h. The enzyme concentration used in these experiments was 1 µg/ml. The enzymes were inactivated by heating at 100 °C for 10 minutes. The digests were analysed by HPSEC and HPAEC.

ANALYTICAL METHODS

Neutral sugar composition was determined by gas chromatography according to Englyst & Cummings[16], using inositol as an internal standard. The samples were pre-treated with 72% w/w H_2SO_4 (1h, 30 °C) followed by hydrolysis with 1 M H_2SO_4 for 3 h at 100 °C and the constituent sugars were analysed as their alditol acetates.

Uronic acid content was determined by the automated colorimetric *m*-hydroxydiphenyl assay[12,16,17] using an auto-analyser (Skalar Analytical BV, Breda, The Netherlands). Corrections were made for interference by neutral sugars present in the sample.

High-Performance Size-Exclusion Chromatography (HPSEC) was performed on a SP8800 HPLC (Spectra Physics) equipped with three columns (each 300 x 7.5 mm) of Bio-Gel TSK in series (40XL, 30XL and 20XL; Bio-Rad Labs.) in combination with a TSK guard column (40 x 6 mm) and elution at 30 °C with 0.4 M NaAc buffer pH 3.0 at 0.8 ml/min. Calibration was performed using dextrans, ranging from 500 to 4 kDa. The eluate was monitored using a Shodex SE-61 Refractive Index detector.

High-Performance Anion-Exchange Chromatography (HPAEC) was performed on a Dionex Bio-LC system[18]. The gradient was obtained by mixing solutions of 0.1 M NaOH and 1 M NaAc in 0.1 M NaOH.

For the determination of xylan oligomers the gradient described by Verbruggen et al.[19] was used. Calibration was performed with a standard xylan digest. For the determination of xyloglucan oligomers the gradient described by Vincken et al.[20] for the CarboPac PA-100 column was used. Xyloglucan oligomers prepared from apple cell wall polysaccharides[20] were used as standards.

MALDI-TOF MS. The matrix solution was prepared by dissolving 9 mg 2,5-dihydroxybenzoic acid and 3 mg 1-hydroxy isoquinoline in 700 µl distilled water and 300 µl acetonitril. A 1 µl volume of this solution was placed on the sample plate together with 1 µl of the sample solution and allowed to dry at room temperature. The sample plate was then placed in the instrument.
MALDI-TOF mass spectra were recorded with a Voyager-DE RP Biospectrometry Workstation (PerSeptive Biosystems, Inc., Framingham, MA, USA) equipped with a nitrogen laser operating at 337 nm (3-ns pulse duration), a single stage reflector, and delayed extraction. The accelerating voltage used was 12 kV and the delay time setting was 200 ns. Each spectrum was produced by accumulating data from 100-256 laser shots. Mass spectra were calibrated with an external standard containing galacturonic acid oligomers (degree of polymerisation 2-9).

Results and discussion

Fractionation of the 1 MASS extract

In a previous paper we described the sequential extraction of soybean meal WUS[8]. The WUS were first extracted with CDTA and 0.05 M NaOH, to remove the pectic substances. The residue was extracted with 1 M KOH to yield the 1 MASS extract. This extract contains 16% of the polysaccharides in the WUS. The sugar composition (Table 6.1) indicates the presence

of both pectic substances and hemicelluloses, and these hemicelluloses might be xyloglucans and xylans.

Table 6.1. Sugar composition of soybean meal fractions as mol%.

Fraction	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	Uronic acids
WUS	2	3	19	8	2	29	21	17
1 MASS	2	3	23	11	1	35	5	20
1 MASS Neutral	2	4	8	24	1	19	40	3
1 MASS Charged	2	3	22	10	tr	34	6	25

tr = trace amount.

The 1 MASS extract was fractionated using anion-exchange chromatography. The elution profile (Figure 6.1) shows that the 1 MASS extract contains both a neutral (1 MASS Neutral) and a charged (1 MASS Charged) population. The sugar compositions of both populations are shown in Table 6.1. The recovery per individual sugar residue is satisfactory, varying between 90 and 114%. Except for glucose, which has a recovery of 190%. This is caused by a pollution of the column with glucose-rich material, which was washed from the column with 0.5 M NaOH. This is of no concern during this research, because this late-eluting material was not further studied.

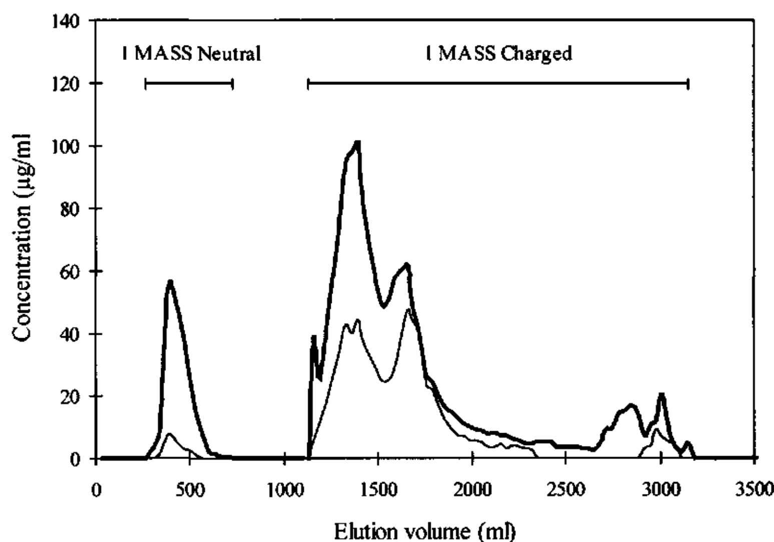

Figure. 6.1. Elution profile of soybean 1 MASS on anion-exchange chromatography. Uronic acid concentration (—), neutral sugar concentration (—).

The neutral population yields 10.2% of the recovered polysaccharide material. The high contents of xylose and glucose are an indication for the presence of xyloglucans, and the presence of fucose suggests that the xyloglucans are fucosylated. Both, galactose (19 mol%) and arabinose (8 mol%) can also occur in xyloglucans. In addition, the arabinose residues might also be present as substituents of xylans in arabinoxylans.

ENZYMATIC DEGRADATION OF THE HEMICELLULOSES FROM 1 MASS

The specificity of enzymes can be used to show the presence of particular polysaccharides[21]. In this study endo-glucanase I and V, and endo-xylanase I are used demonstrate the presence of (xylo)glucans and arabinoxylans, respectively. Endo-glucanase I is known to have a high activity towards glucans and a much lower activity towards xyloglucan, and endo-glucanase V has a high activity towards xyloglucan[22].

The elution profile of the endo-xylanase I digest on HPSEC is similar to the elution pattern of the blank 1 MASS Neutral (Figure 6.2, lines d, and a). Also, the elution profile on HPAEC does not show the release of any (arabino)xylan oligomers (Figure 6.3A). So, endo-xylanase I appears to be unable to degrade any polysaccharides in this fraction, demonstrating that the presence of arabinoxylans in the 1 MASS Neutral fraction is not very likely. Although the presence of a heavily branched arabinoxylan, which is resistant to degradation with endo-xylanase I, can not be ruled out completely.

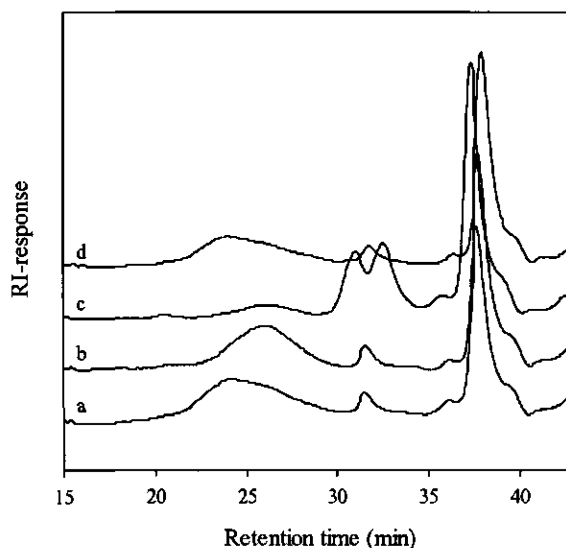

Figure. 6.2. HPSEC elution profiles of (a) 1 MASS Neutral, (b) endo-glucanase I digest, (c) endo-glucanase V digest, and (d) endo-xylanase I digest.

Endo-glucanases (I and V), on the other hand, are able to degrade the neutral polymers. Incubation of the 1 MASS Neutral fraction with endo-glucanase I results in a small decrease of the molecular masses of the polysaccharides. Endo-glucanase V causes almost complete degradation of the polymers in the 1 MASS Neutral fraction (Figure 6.2, line c) to products that elute at 31 and 32.5 minutes. So the neutral population of the 1 MASS fraction most probably contains xyloglucans, and no linear glucans.

The HPAEC profiles of these two digests (Figure 6.3B) confirm these results. Endo-glucanase I is not able to release oligomeric degradation products (line b). Endo-glucanase V, on the other hand, releases large amounts of oligomeric degradation products (line c). The retention times of the oligomers in HPAEC analysis are identical to those formed from apple[20] and sugar beet xyloglucan[23] by endo-glucanase V. The structures of the oligomers in the apple xyloglucan endo-glucanase V digest are known, and it can be assumed in all probability that the structure of the oligomers in the digest from soybean xyloglucan are identical. Small changes in the substitution pattern of the xyloglucan oligomers result in considerable changes in their retention time[24], and their occurrence in this digest could consequently be ruled out.

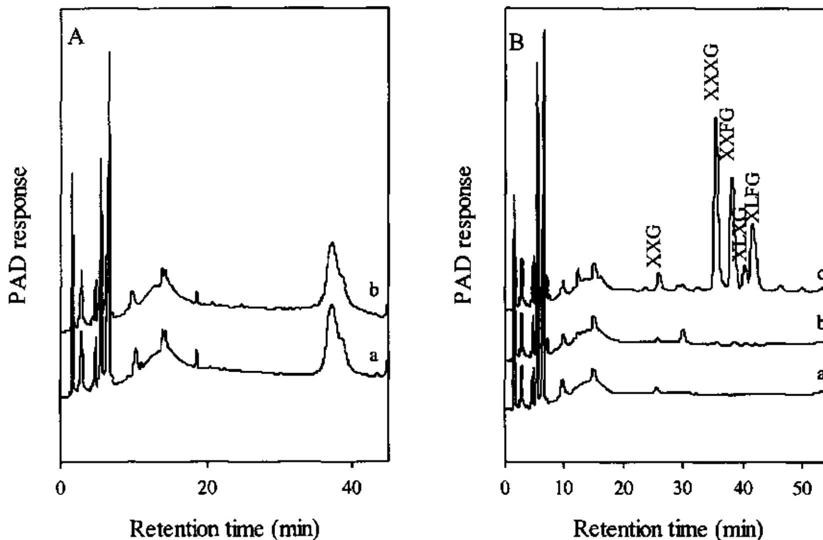

Figure 6.3. HPAEC elution profiles of (Aa) 1 MASS Neutral, (Ab) endo-xylanase I digest, obtained with a gradient for xylan oligomers[19], and of (Ba) 1 MASS Neutral, (Bb) endo-glucanase I digest, and (Bc) endo-glucanase V digest obtained with a gradient for xyloglucan oligomers[20].

The digest is also analysed by MALDI-TOF MS (Figure 6.4). The signal with mass 1085 is caused by a sodium-cationised oligomer composed of four hexoses and three pentoses. The signal with mass 1101 is caused by the potassium-cationised oligomer

composed of the same residues. Signals 1247 and 1263 corresponds to the sodium- and potassium-cationised oligomer with an additional hexose, hexose$_5$pentose$_3$. The signal 1393 (and 1409) is indicative for hexose$_5$pentose$_3$deoxyhexose. The last two signals, 1555 and 1571, correspond to a decamer containing an additional hexose.

From both HPAEC and MALDI-TOF MS it can be concluded that the oligomers obtained after incubation with endo-glucanase V were; XXG, XXXG, XXFG, XLXG, and XLFG, named according to the nomenclature of Fry et al.[25], and shown in Table 6.2.

The presence of these (already known) xyloglucan oligomers in the digest shows that three out of four glucose residues carry a side chain; it is composed of XXXG-type building units[6]. This was also seen for xyloglucans from many species such as apple, sycamore, tamarind and sugar beet[5,20,23]. This is in contrast with xyloglucans from potato and tomato, both belonging to the Solanaceae, for which the presence of two adjacent unbranched glucose residues is characteristic[24]. The presence of XXFG and XLFG also indicates that the xyloglucans from soybean are fucosylated, similar to xyloglucans from other sources[5,26]. The fucose residue is substituted to the O-2 of a galactosyl unit, resulting in a trisaccharide side chain attached to the glucan backbone.

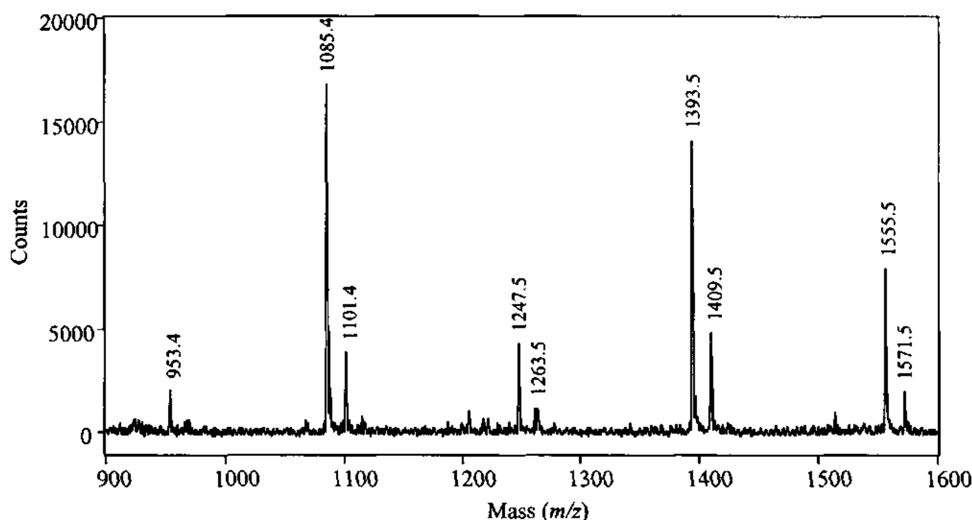

Figure. 6.4. MALDI-TOF mass spectrum of the endo-glucanase V digest of 1 MASS Neutral.

Information about the structure of the xyloglucan from soybean *seeds* was not published before, unlike that of the hypocotyl. The xyloglucan from suspension-cultured soybean cells, started from callus tissue derived from a hypocotyl of a seedling of soybean, was mainly constructed of two kinds of oligosaccharide repeating units, a heptasaccharide (XXXG) and a nonasaccharide (XXFG)[27]. These structures also correspond with a xyloglucan of the poly-XXXG type.

Table 6.2. The structure of oligomers obtained after incubation of 1 MASS Neutral by endo-glucanase V.

Code	Structure	$[M+Na]^+$
XXXG	Glc-Glc-Glc-Glc | | | Xyl Xyl Xyl	1085
XXFG	Glc-Glc-Glc-Glc | | | Xyl Xyl Xyl | Gal | Fuc	1393
XLFG	Glc-Glc-Glc-Glc | | | Xyl Xyl Xyl | | Gal Gal | Fuc	1555
XLXG	Glc-Glc-Glc-Glc | | | Xyl Xyl Xyl | Gal	1247

The sugar composition of the 4 MASS fraction from soybean WUS also indicates the presence of xyloglucan. Degradation of the 4 MASS fraction with endo-glucanase I and V showed that this fraction contains a small amount of glucan and that the major constituent is xyloglucan. HPAEC and MALDI-TOF MS of the digests showed that these xyloglucans are also composed of XXXG-type building units, resembling the structure of the xyloglucans extracted by the 1 M KOH solution. They distinguish themselves from the xyloglucans in the 1 MASS fraction only in the effort needed to extract them from the cell wall material, which is expressed in the higher concentration of alkali used. The xyloglucans in the 4 MASS fraction are probably tighter hydrogen-bonded to the cellulosic microfibrils or enclosed in these microfibrils and were released due to the swelling of cellulose caused by the high concentration of alkali[28].

CONCLUSIONS

The polysaccharides in the 1 MASS extract from soybean WUS contain both pectic substances and neutral polysaccharides, which can be separated by ion-exchange chromatography. The neutral population represents 1.6% of the polysaccharides of soybean WUS. The sugar composition and the formation of specific xyloglucan oligomers after degradation of the neutral polysaccharides in the 1 MASS extract with endo-glucanase V proves that this fraction consists of xyloglucans. The structure of the xyloglucan oligomers was determined by comparison of their elution behaviour on anion-exchange chromatography (HPAEC) with that of well-known references, and by molecular mass analysis by MALDI-TOF MS. It can be concluded that the xyloglucans are fucosylated and are composed of XXXG-type building units, similar to xyloglucans from many other plants. The structure of xyloglucan from soybean seeds has not been described before.

Combination of the specificity of enzymatic degradation with HPAEC and mass spectrometry proves to be very efficient in characterising polysaccharide isolated from any plant material. This applies to among others xyloglucans, as was shown in this research.

The sugar composition of the 1 MASS Charged fraction obtained after anion-exchange chromatography indicates that it is rich in pectic substances. Further research will focus on the structure of these pectins, and comparison of their structure with the pectic substances in the ChSS and DASS fractions from soybean meal.

ACKNOWLEDGEMENT

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Chapter 7

Glucuronoarabinoxylans from Maize Kernel Cell Walls are more complex than those from Sorghum Kernel Cell Walls

M.M.H. Huisman, H.A. Schols & A.G.J. Voragen

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Water-Unextractable Solids (WUS) were isolated from maize kernels. They contained 7% of protein, 8% of starch and 57% of non-starch polysaccharides (NSP). These NSP were composed mainly of glucose, xylose, arabinose, and glucuronic acid. Sequential extractions with a saturated $Ba(OH)_2$-solution (BE1 extract), and distilled water (BE2 extract) were used to solubilise glucuronoarabinoxylans from maize WUS. Cellulose remained in the insoluble residue. The glycosidic linkage composition of the extracts and their resistance to endo-xylanase treatment indicated that the extracted glucuronoarabinoxylans were highly substituted. In the maize BE1 extract 25% of the xylose was unsubstituted, 38% was monosubstituted and 15% was disubstituted. A new measure for the degree of substitution is defined. The resulting degree of substitution for maize BE1 arabinoxylan (87%) is higher than for sorghum BE1 arabinoxylan (70%). The glucuronoarabinoxylans in maize BE1 can be degraded by a sub-fraction of Ultraflo, a commercial enzyme preparation from *Humicola insolens*. The digest contains a number of series of oligomers: $pentose_n$, $pentose_n$GlcA, $pentose_n$hexose, and $pentose_n$$GlcA_2$.

INTRODUCTION

Cell walls of Gramineae consist predominantly of (glucurono)arabinoxylans and (1,3),(1,4)-β-D-glucans, with smaller amounts of cellulose, heteromannans, protein, and esterified phenolic acids[1]. The glucuronoarabinoxylans consist of a β-D-(1,4)-linked xylopyranoside backbone and can be substituted with α-L-arabinofuranose on C2 and/or C3, α-D-glucopyranosyl uronic acid, or its 4-*O*-methyl derivative on C2, acetyl on C2 or C3 of some xylose residues[2,3,4]. Ferulic acid and *p*-coumarica acid can occur esterified to the C5 of arabinosyl units of (glucurono)arabinoxylans[5,6]. The degree and pattern of substitution of the (glucurono)arabinoxylans appears to vary with the source from which they are extracted. These differences are reflected in the ratio of arabinose to xylose, in the relative amounts of the various linkage types of arabinose and xylose, in the presence of other substituents such as (4-*O*-methyl-)glucuronic acid, and the presence of small side chains such as xylopyranosyl-arabinose[7] and dimeric side-chains of arabinose[8].

The arabinoxylans from maize kernels have a highly branched structure, as was shown by linkage analysis data[9,10]. They were shown to meet a lot of the structural characteristics which were described above, like substitution by single units of arabinose or glucuronic acid. In addition, they comprise side-chains containing arabinose, xylose and galactose residues[11,12,13]. Some feruloylated oligosaccharides, obtained after acid hydrolysis, were

identified[13] and are suggested to be present as side-chain constituents of the heteroxylans in maize bran.

Most of the information about the structure of maize kernel glucuronoarabinoxylan described so far, is obtained by identification of oligosaccharides formed after acid hydrolysis of the heteroxylans. A schematic structure, which takes into account the monosaccharide composition, the linkage profile and the structures of some oligosaccharides obtained by acid hydrolysis, is presented by Saulnier *et al.*[14]. However, due to the low specificity of acid hydrolysis, much of the structural information of the polymer is of limited value. The use of specific enzymes can overcome this problem and provide more information about the structure of the polymer, in particular about the distribution of the substituents over the main chain. In this study, we describe the isolation of water-unextractable solids from maize kernels. The WUS is characterised and extracted with bariumhydroxide to obtain rather pure glucuronoarabinoxylans[15]. The extracts and their digests obtained after enzymatic degradation are chemically characterised to obtain information about the structure of the maize glucuronoarabinoxylans.

MATERIALS AND METHODS

MATERIALS

In this research whole maize kernels (*Zea mays* L.) harvested in the Alsace region (France) were used for the isolation of maize WUS. Sorghum WUS was isolated by Verbruggen et al.[16]

ISOLATION OF THE WATER-UNEXTRACTABLE SOLIDS (WUS)

The isolation of water-unextractable cell wall material from maize kernels was based on the procedure described by Verbruggen et al.[16]. Maize kernels were ground to pass a 0.5-mm sieve. This meal (500 g) was defatted by Soxhlet extraction with 3 l of petroleumether, refluxing for 6h. The air-dried residue was extracted with 2 l distilled water containing 0.05% NaN_3 during 2h at room temperature. The suspension was centrifuged (11000 g; 30 min). The pellet was resuspended and this procedure was repeated three times.

Subsequently, the protein was extracted from the residue with 1.5 l of 1.5% (w/v) sodium dodecylsulphate solution containing 10 mM 1,4-dithiothreitol, during 3h at room temperature. After

centrifugation (11000 g; 30 min) this extraction was repeated twice. The final pellet was washed twice with distilled water.

The residue was filtered over a 45 µm sieve by washing with distilled water (6.5 l). The residue, which retained on the sieve, was suspended in 1 l distilled water (pH 5.0) at 85 °C and starch gelatinisation was allowed to proceed for 1 hour. The residue obtained after centrifugation (11000 g; 30 min) was suspended in 1 l buffer solution (pH 6.5) containing 10 mM maleic acid, 10 mM NaCl, 1 mM $CaCl_2$ and 0.05% NaN_3. Porcine pancreatic α-amylase (2 mg; Merck art 16312) was added and the mixture incubated at 30 °C for 18h. After centrifugation (11000 g; 30 min) the residue was washed with 1 l hot distilled water (65 °C) and centrifuged again. The α-amylase digestion and hot water washing were repeated once. The remaining unextractable residue was resuspended in distilled water and freeze-dried (WUS).

EXTRACTION OF WUS

Glucuronoarabinoxylans were obtained by the procedure described by Gruppen et al.[15]. Maize WUS (3 g) was extracted with saturated $Ba(OH)_2$ solution containing 260 mM $NaBH_4$ (500 ml for 16 h; 250 ml for 1 h) at room temperature with continuous stirring (BE1). The residue was acidified to pH 5 (acetic acid) and subsequently extracted with distilled water (4x300 ml; 1 h) (BE2). After each extraction, solubilised polymers were separated from the insoluble residue by centrifugation (18900 g; 45 min), and the pH of the extracts was adjusted to 5 with glacial acetic acid before dialysis.

The final residue from the water extraction step was freeze dried (RES). All extracts were kept at -18 °C and aliquots were thawed or freeze dried as needed.

ENZYMATIC DEGRADATION

Solutions (1.6 mg/ml) of maize BE1 were incubated with a number of purified enzymes. The purified enzymes used were endo-(1,4)-β-xylanase (E.C. 3.2.1.8, endo-xylanase I; 0.1 µg protein/ml substrate solution), AXH-m (0.05 µg protein/ml substrate solution), and AXH-d3 (an excess amount of enzyme). Endo-xylanase I[17] and AXH-m[18] were purified from *Aspergillus awamori*. AXH-d3 was purified from *Bifidobacterium adolescentis*[19].

A solution (1.5 mg/ml) of maize BE1 was also incubated with Ultraflo, a commercial enzyme preparation derived from *Humicola insolens* (Novo Nordisk, Bagsvaerd, Denmark). Before adding to the substrate solution, the enzyme preparation was dialysed against a 50 mM NaAc buffer (pH 5.0) containing 0.01% NaN_3 at 4 °C during 2h. Solutions (1.4 mg/ml) of maize BE1 were incubated with 'Ultraflo-arabinoxylan degrader', a sub-fraction of Ultraflo obtained during the fractionation of the preparation[20].

The incubations were performed in 50 mM NaAc buffer (pH 5.0) containing 0.01% NaN_3 at 30 °C rotating 'head over tail'. Incubations with AXH-d3 were performed in 25 mM $NaPO_4$ buffer (pH 6.5) containing 0.01% NaN_3. All enzymes were inactivated by heating at 100 °C for 10 minutes. Polysaccharide-degrading activities were determined by HPSEC and HPAEC analyses of the digests.

ION-EXCHANGE CHROMATOGRAPHY

An amount of the extracts which contains 20 mg of polysaccharides was applied on a column (400 mm x 16 mm) of DEAE Sepharose Fast Flow, which was initially equilibrated in 0.005 M NaAc buffer pH 5.0, using a Hiload System (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

Elution was carried out sequentially with 80 ml of 0.005 M NaAc-buffer pH 5.0, a linear gradient from 0.005 M to 1 M NaAc-buffer pH 5.0 (160 ml), a linear gradient from 1 M to 2 M NaAc-buffer pH 5.0 (80 ml) and 40 ml 2 M NaAc-buffer pH 5.0. Residual bound polysaccharides were washed from the column with 80 ml of 0.5 M NaOH. The elution rate was 5 ml/min except for the first step, in which the sample was applied onto the column using an elution rate of 1 ml/min. Fractions (5 ml) were collected and assayed by automated methods for uronic acid[21] and neutral sugar content[22].

ANALYTICAL METHODS

Neutral sugar composition was determined by gas chromatography according to Englyst & Cummings[23] (1984), using inositol as an internal standard. The samples were pre-treated with 72% w/w H_2SO_4 (1h, 30 °C) followed by hydrolysis with 1 M H_2SO_4 for 3h at 100 °C and the constituent sugars were analysed as their alditol acetates.

Uronic acid content was determined by the automated colorimetric *m*-hydroxydiphenyl assay[21,24] using an auto-analyser (Skalar Analytical BV, Breda, The Netherlands). Corrections were made for interference by neutral sugars present in the sample as measured by the orcinol-sulfuric acid method[22].

Starch was determined enzymatically using a test kit (Boehringer).

Glycosidic linkage analysis Carboxyl groups in all fractions were reduced according to Taylor and Conrad[25] using $NaBH_4$, and the reduced fractions were subsequently methylated using a modification of the Hakomori method[26], dialysed and dried in a stream of air. The reduction and methylation steps were repeated in order to improve the extent of both reactions. Partially methylated alditol acetates were prepared and analysed as described by Verbruggen et al.[27]. The derivatives were quantified according to their effective carbon response (ECR) factors[28]. The identity of the compounds was

confirmed by gas chromatography-mass spectrometry (GC-MS, Hewlett Packard, USA) using a mass selective detector (MSD) 5973 coupled to a HP 6890 gas chromatograph equipped with a fused silica column (CPSIL 19CB, 25m x 0.25mm; 0.2 μm; Chrompack). The temperature program was 160→185 °C at 0.5 °C/min, 185→230 °C at 10 °C/min and 230 °C isothermal for 5.5 min.

High-Performance Size-Exclusion Chromatography (HPSEC) was performed on a SP8800 HPLC (Spectra Physics) equipped with three columns (each 300 x 7.5 mm) of Bio-Gel TSK in series (60XL, 40XL and 30XL; Bio-Rad Labs., Richmond, CA, USA) in combination with a TSK guard column (40 x 6 mm) and elution at 30 °C with 0.4 M sodium acetate buffer pH 3.0 at 0.8 ml/min. The eluate was monitored using a combined RI detector and viscometer (model 250, Viscotek Corporation, Houston, Texas, USA), and a Right Angle Laser Light-Scattering detector (RALLS, Viscotek, LD 600). Molecular masses were calculated using the light scattering and universal calibration modules of the Trisec software (Viscotek). Calibration was performed using pullulans, ranging from 6 to 788 kDa (Polymer Laboratories, Amherst, MA, USA).

High-Performance Anion-Exchange Chromatography (HPAEC) was performed on a Dionex Bio-LC system as described by Schols et al.[29]. The gradient was obtained by mixing solutions of 0.1 M NaOH and 1 M sodium acetate in 0.1 M NaOH.

For the determination of arabinoxylan oligomers, the (4 x 250 mm) CarboPac PA-1 column was equilibrated with 0.1 M NaOH. Twenty μl of the sample was injected and a linear gradient to 0.2 M sodium acetate in 0.1 M NaOH in 30 minutes, and a linear gradient from 0.2 to 0.6 M sodium acetate in 0.1 M NaOH in the next 10 minutes was applied. The column was washed for 5 minutes with 1 M sodium acetate in 0.1 M NaOH and equilibrated again for 15 minutes with 0.1 M NaOH. Calibration was performed with standard solutions of arabinose, xylose, glucuronic acid, and a series of xylan oligomers.

Matrix-Assisted Laser-Desorption/Ionisation Time-of-Flight Mass Spectrometry (MALDI-TOF MS). The matrix solution was prepared by dissolving 9 mg 2,5-dihydroxybenzoic acid and 3 mg 1-hydroxy isoquinoline in 700 μl distilled water and 300 μl acetonitril. A 1 μl volume of this solution was placed on the sample plate together with 1 μl of the sample solution and allowed to dry at room temperature. The sample plate was then placed in the instrument.

MALDI-TOF mass spectra were recorded on a Voyager-DE RP Biospectrometry Workstation (PerSeptive Biosystems, Inc., Framingham, MA, USA) equipped with a nitrogen laser operating at 337 nm (3-ns pulse duration), a single stage reflector, and delayed extraction. The accelerating voltage used was 12 kV and the delay time setting was 200 ns. Each spectrum was produced by accumulating

data from 100-256 laser shots. Mass spectra were calibrated with an external standard containing galacturonic acid oligomers (degree of polymerisation 2-9).

RESULTS AND DISCUSSION

YIELD AND COMPOSITION OF THE WUS

The first step in the isolation of maize WUS from whole maize meal was the removal of lipids. Extraction with petroleumether removed 8.1% of the original material. Table 7.1 presents the composition of defatted whole maize meal and isolated maize WUS.

Table 7.1. Composition of whole maize meal and maize WUS (percentage dry weight).

	Whole defatted maize meal	Maize WUS
Yield	100	8.7
Protein content	10.8	7.0
Starch content	62.3	8.1
NSP content	5.5	56.5

The protein content of the defatted meal (10.8%) agrees well with results published by Watson[30], who found the protein content to vary from 8 to 12%. The starch content of the meal (62.3%) is lower than the average value found by Watson[30] (71.7%). The defatted whole maize meal contained 5.5% non-starch polysaccharides. The analyses performed here account for only 78% of the maize meal. Another component, which is very likely to be present, is lignin. The lignin content is not determined, but lignin is a common component of secondary thickening in the pericarp of all cereal grains[1]. In addition, maize kernels also contain phytate, tannins, mineral elements, vitamins, and other chemical compounds in low concentrations[1,30].

The WUS fraction still contained 7% of protein, this might be present as structural proteins, glycoproteins or intracellular proteins[31]. Only 5.6% of the protein originally present in the meal was found in the WUS fraction. Starch could not be removed completely. The WUS fraction contained 8% of starch, representing approximately 1% of the starch in the meal. This might be due to certain physical changes in the starch component introduced by

the isolation procedure which may cause resistance to degradation by α-amylases[32] or due to the presence of resistant starch which can not be degraded by α-amylase[33]. The WUS contains 57% of NSP, representing 89% of the NSP originally present in the WUS fraction. A small part of the residual 28% consists of acetyl, ferulic acid, and coumaric acid groups, the remainder can not be explained.

A substantial proportion of the polysaccharides in the cell walls of maize kernels consists of arabinoxylans and cellulose, as can be concluded from the high contents of arabinose, xylose and glucose in maize WUS (Table 7.2). In addition, phenolic acids were determined in the maize WUS; coumaric acid (0.1% w/w) and ferulic acid (1.6% w/w) may be involved in oxidative cross-linking of polysaccharides and other cell wall components. Acetyl groups have also been determined in maize WUS (4.9% w/w), indicating the presence of 36 acetyl groups per 100 xylose residues.

EXTRACTION OF GLUCURONOARABINOXYLANS FROM MAIZE WUS

Glucuronoarabinoxylans can be selectively extracted from cell wall material with saturated barium hydroxide solutions[15,34]. Extraction of maize and sorghum WUS with a $Ba(OH)_2$ solution resulted in two fractions for each starting-material, BE1 and BE2, both containing hemicellulosic material. Verbruggen et al.[27] also extracted glucuronoarabinoxylan from sorghum WUS, but they used a more extensive extraction procedure. Gruppen *et al.*[15] followed the same extraction procedure for wheat flour WUS. The BE1 fraction was released from the WUS directly, being extractable in $Ba(OH)_2$. The BE2 fraction was only released from the WUS-residue after lowering the pH and washing with distilled water. A cellulose-enriched residue (RES) remains. The sugar composition of the fractions is shown in Table 7.2. Saturated $Ba(OH)_2$ extracted 37% of the arabinose and xylose from maize WUS. In the event of wheat flour WUS, saturated $Ba(OH)_2$ extracted the bulk (80%) of the arabinose and xylose[15], while only 50% of the arabinose and xylose could be extracted from sorghum WUS (this research). BE1 from maize, sorghum, and wheat flour contained a rather pure (glucurono)arabinoxylan population, only minor amounts of other neutral sugars were present in these extracts.

Table 7.2. Molar sugar compositions of fractions from maize WUS and sorghum WUS.

Sample	Yield (% WUS)	Sugar composition						Carbohydrate content (% w/w)
		ara	xyl	man	gal	glc	glcA	
Maize WUS	100	23.0	30.3	0.9	4.5	32.9	8.5	82.6
Maize BE1	21.1	38.4	48.3	0.1	4.3	0.7	8.3	84.3
Maize BE2	18.1	34.3	43.5	0.3	7.6	6.2	8.3	86.2
Maize RES	46.3	16.4	22.9	1.6	3.7	47.6	7.8	92.9
recovery	85.5							
Sorghum WUS	100	25.2	23.9	1.6	2.3	38.2	9.0	72.2
Sorghum BE1	26.0	45.8	40.9	0.2	1.8	1.7	9.8	75.2
Sorghum BE2	14.5	29.3	23.1	1.0	2.9	31.1	12.0	80.1
Sorghum RES	48.7	9.6	28.2	1.4	1.2	54.1	5.7	74.7
recovery	89.2							

The structures of the arabinoxylans in both maize and sorghum are much more complex than those found in other cereals. The degree of substitution in maize and sorghum (molar arabinose: xylose ratio is 0.80 and 1.12, respectively) is higher than in wheat flour (0.54)[35], barley (0.72)[36], and wheat bran (0.71)[34] arabinoxylans. Moreover the arabinoxylans in maize and sorghum are additionally substituted with uronic acid (8.3 mol% and 9.8 mol%, respectively). HPAEC of H_2SO_4 hydrolysed samples of both maize and sorghum showed that the only uronic acid present in the samples was glucuronic acid[27]. Uronic acids are almost absent in wheat flour[35] and barley[36] arabinoxylans, wheat bran arabinoxylan contains 2.6 mol% uronic acid[34].

The BE2 extracts of maize and sorghum also contained predominantly arabinoxylan, but glucose-containing polysaccharides were co-extracted, especially in sorghum BE2. This co-extracted glucose in sorghum and wheat flour BE2 was present mainly in the form of (1,3),(1,4)-β-D-glucans[27,35]. The yields of these fractions for maize, sorghum and wheat flour, (18.1%, 14.5%, and 10.7%, respectively) were lower than the yield of the BE1 extracts. Glucose was by far the most important sugar in the residues, presumably present as cellulose and (1,3),(1,4)-β-D-glucans.

GLYCOSIDIC LINKAGE COMPOSITION

The results of linkage analysis of the extracted polysaccharides from maize and sorghum are presented in Table 7.3. The barium hydroxide extracts of maize appeared to comprise highly substituted arabinoxylans. Assuming that the backbone consists of only (1,4)-, (1,2,4)-, (1,3,4)-, and (1,2,3,4)-linked xylopyranosyl residues, 32% of the xylopyranosyl residues in the (1,4)-xylan backbone in maize BE1 was unsubstituted, and 49% was monosubstituted, with branch points mainly at the C3 position. In addition, the xylan backbone contained a large amount (19%) of C2,C3-disubstituted xylopyranosyl residues. The amount of unsubstituted xylose in maize BE1 (32%) is lower than in sorghum BE1 (40%) and wheat flour BE1 (63%)[35].

The degree of substitution of the xylan backbone can be expressed as the number of sugar substituents per 100 xylose residues in the backbone, by calculating the ratio of the number of branches attached to xylose residues to the total number of xylose residues in the backbone. It turns out that the degree of substitution in maize BE1 is much higher (87%) than in sorghum (70%) and wheat flour BE1 (56%). This is a better measure for the degree of substitution than the arabinose:xylose ratio, which is highest for sorghum BE1. This can be

explained by terminally-linked xylose residues being present in side chains[11,13,37], and the existence of oligomeric side chains of arabinose[8,38]. However, this measure does not give any information about the distribution of the substituents over the main chain.

Table 7.3. Linkage composition (mol%) of $NaBH_4$ reduced barium hydroxide extracts of maize and sorghum WUS.

Component	Linkage type	maize BE1	maize BE2	sorghum BE1	sorghum BE2
2,3,5-Me_3-Ara	(Ara*f*)1→	24.7	24.3	32.9	24.2
3,5-Me_2-Ara	→2(Ara*f*)1→	3.9	4.8	2.7	1.9
2,3-Me_2-Ara	→5(Ara*f*)1→	3.9	4.3	5.2	5.6
2,3,4-Me_3-Xyl	(Xyl*p*)1→	9.6	11.2	1.6	1.2
2,3-Me_2-Xyl	→4(Xyl*p*)1→	15.4	7.6	21.1	12.0
2,4-Me_2-Xyl	→3(Xyl*p*)1→	3.6	4.1		3.0
2-Me-Xyl	→4(Xyl*p*)1→ 3↑	18.6	17.0	19.2	14.0
3-Me-Xyl	→4(Xyl*p*)1→ ↑2	5.2	4.0	6.4	4.7
Xyl	→4(Xyl*p*)1→ 3↑ ↑2	9.5	9.9	6.0	4.6
2,3,4,6-Me_4-Glc	(Glc*p*)1→	2.0[a]	2.9[a]	2.0[a]	3.6[a]
2,3,6-Me_3-Glc	→4(Glc*p*)1→			0.6	20.6
2,4,6-Me_3-Glc	→3(Glc*p*)1→				3.4
2,3,4,6-Me_4-Gal	(Gal*p*)1→	3.1	5.6	1.1	1.4
2,3,6-Me_3-Gal	→4(Gal*p*)1→	0.5	4.2	1.0	
ratio terminal/branching		0.92	1.08	1.00	1.09

[a] (Partially) originating from terminally-linked glucuronic acid

In addition to terminally-linked arabinofuranosyl residues, the arabinoxylans in maize BE1and BE2 also contain (1,2)-, (1,5)-linked arabinofuranosyl residues, indicating

complicated structural features of its side chains. Short side chains containing these sugar linkages have been isolated from enzymatic and acid hydrolysates of some arabinoxylans[4,8,11,13,37,38]. The above results indicate that similar structural units are also present in maize BE1.

In these studies, uronic acids were reduced with $NaBH_4$ prior to methylation, hydrolysis and derivatisation, and therefore, were determined as glucose. The originally extracted polysaccharides (not reduced with $NaBH_4$) from maize did not contain any terminal glucose residues, the content of terminal glucose residues in the originally extracted sorghum polysaccharides was very low. This indicates the presence of terminal glucuronic acid residues in the original polysaccharide. Previously isolated and identified glucuronic acid-containing oligomers from glucuronoarabinoxylan hydrolysates showed that glucuronic acid is linked to the (1,4)-xylan backbone at the C2 position of xylose[8,39]. The linkage composition of the glucose in the sorghum BE2 extract confirms the presence of a (1,3),(1,4)-β-D-glucan, as shown before[27]. Comparison of the linkage analysis data with the sugar composition shows that the amounts of terminal glucose (after reduction) were lower than the amounts of uronic acids determined directly in the extracts. This discrepancy between sugar and linkage composition suggest an incomplete reduction of the uronic acids resulting in a substantial underestimation of the glucose originating from glucuronic acid content in the linkage composition.

Comparison of the linkage compositions of the BE2 to those of the BE1 extracts shows that the glucuronoarabinoxylans in BE2 have a higher degree of substitution than those in BE1 for both maize and sorghum. The portion of unsubstituted xylose in the backbone of the BE2 extracts of maize and sorghum (20% and 34%, respectively) is lower than that in the backbone of the BE 1 extracts (32% and 40%, respectively). The degree of substitution (calculated as described above) is 107% for maize BE2 and 79% for sorghum BE2.

HOMOGENEITY OF THE BARIUM HYDROXIDE EXTRACTS

The homogeneity of the extracts was studied by high-performance size-exclusion chromatography (HPSEC). As can be seen from the elution patterns in Figure 7.1, all extracts contained polysaccharides varying widely in hydrodynamic volume. The weight-averaged molecular masses of the extracts of maize are slightly higher than those of the extracts of sorghum, as is shown in Table 7.4. The RI-elution patterns of all extracts showed no distinct peaks, although the extracted polymers were clearly polydispers. This was confirmed by their

M_w to M_n ratio (Table 7.4), which is a measure for the heterodispersity. The BE1 fraction of both sorghum and maize is more homogeneous than the BE2 fraction, which was already found by Verbruggen et al.[27] for the sorghum extracts. The maize BE1 fraction is the most homogeneous extract. The intrinsic viscosity of all extracts, from both maize and sorghum, are in the same order of magnitude, varying between 2.33 and 2.61 dl/g.

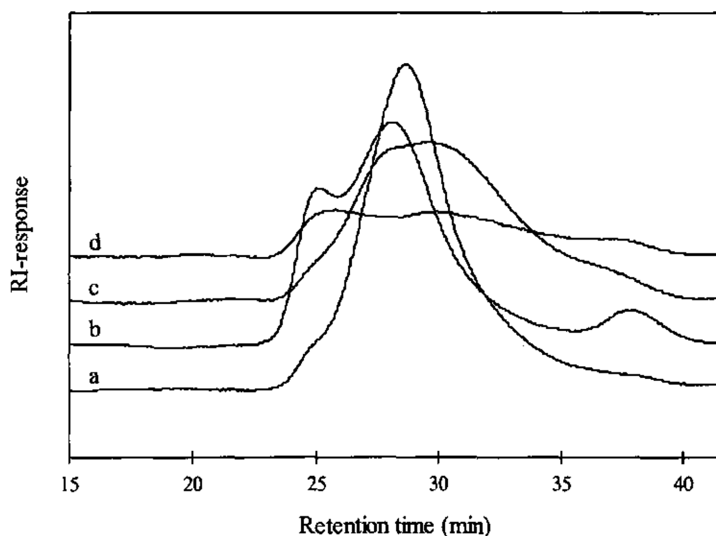

Figure 7.1. HPSEC elution profiles of (a) maize BE1, (b) maize BE2, (c) sorghum BE1, and (d) sorghum BE2.

Table 7.4. Average molecular weight, polydispersity, and intrinsic viscosity of the barium hydroxide extracts of maize and sorghum WUS.

Extract	M_w (kDa)	Polydispersity (M_w/M_n)	[η] (dl/g)
maize BE1	171	1.41	2.33
maize BE2	400	4.23	2.61
sorghum BE1	132	2.13	2.36
sorghum BE2	326	3.89	2.34

The extracts from both maize WUS and sorghum WUS were further studied for homogeneity based on their charge density. The results of anion-exchange chromatography (not shown) suggest that all extracts are very homogeneous. Almost all material was bound to

the column, due to the presence of glucuronic acids in all the arabinoxylan molecules. The glucuronic acids are probably evenly distributed over the arabinoxylan molecules, since the material eluted in one peak with similar uronic acid:neutral sugar ratios throughout the whole peak. Only a small proportion (2% for sorghum BE1, 7% for sorghum BE2, <1% for maize BE1 and 2% for maize BE2) of the recovered material was not bound. Another small proportion (3% for sorghum BE1, 10% for sorghum BE2, 1% for maize BE1 and 5% for maize BE2) was eluted with 0.5 M NaOH. The high homogeneity of the sorghum BE1 fraction was in good agreement with the results found by Verbruggen et al.[27]. Chanliaud et al.[10] also found that a major fraction of alkali extracted heteroxylans from maize bran, containing all the uronic acid, was bound to the column and was eluted in a gradient with sodium acetate buffer.

ENZYMATIC DEGRADATION OF ARABINOXYLANS FROM MAIZE

The BE1 fraction of maize was digested with pure and well-defined enzymes, and compared with the degradation of sorghum glucuronoarabinoxylan[8]. Degradation of maize BE1 by endo-xylanase I only, resulted in a small shift in molecular weight distribution on HPSEC (results not shown). Supplementation of the reaction mixture with AXH-m did not change the molecular weight distribution of the maize arabinoxylans in fraction BE1. However, degradation of sorghum arabinoxylan with endo-xylanase I and AXH-m showed a reasonable shift in the molecular weight distribution of the sorghum arabinoxylans, as was shown by Verbruggen et al.[8]. The arabinoxylans in the maize BE1 fraction appear to be more resistant to enzymatic degradation than those in the sorghum BE1 extract, probably due to the relatively low amount of unsubstituted xylose residues and the relatively high amount of disubstituted xylose residues, which are expressed in their higher degree of substitution (87% for maize BE1 and 70% for sorghum BE1). In addition, the *distribution* of the substituents can also play an important role in the resistance to enzymatic degradation.

Since the combined action of endo-xylanase I and AXH-m was not able to degrade maize BE1 to oligomeric degradation products, and the relatively high amount of disubstituted xylose is mentioned as a possible cause, incubation of maize BE1 with an arabinofuranohydrolase (AXH-d3[19,40]) was performed. It was expected that the removal of some arabinofuranosyl groups could increase the susceptibility of the substrate for AXH-m and endo-xylanase I. However, this enzyme showed no activity towards the maize BE1 extract.

The search for more powerful arabinoxylan degrading enzymes leads to a commercial enzyme preparation derived from the thermophilic fungus *H. insolens*. The degradation of the maize BE1 extract by Ultraflo goes fast and is almost complete. The main degradation products are arabinose, xylose and glucuronic acid. The amounts of arabinose and xylose *monomers* released after 20h of incubation are about 85% and 100% of the arabinose and xylose present in the substrate. As is shown in Figure 7.2, the larger part of the arabinose is released within the first hour of the incubation. The release of xylose is more moderate; indicating that arabinose has to be removed first before xylose can be released.

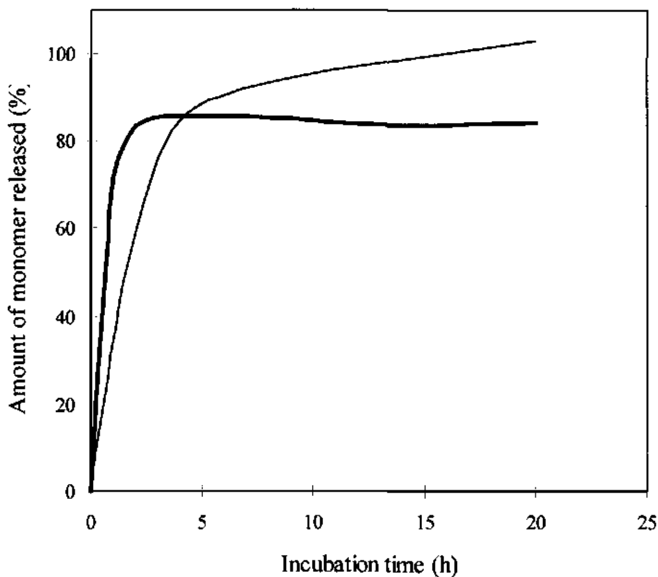

Figure 7.2. Release of arabinose (**—**) and xylose (—) monomers during incubation of maize BE1 with Ultraflo.

Probably because of the large variety of enzymes present in Ultraflo, the maize BE1 extract is almost completely degraded to monomers, by which all the information about the structure of the substrate is lost. Therefore, maize BE1 was incubated with a number of sub-fractions of Ultraflo, obtained during the isolation of two xylanases by Düsterhöft et al.[20]. One of these sub-fractions, designated 'Ultraflo-arabinoxylan degrader' here, is able to degrade maize BE1 and form a large variety of oligomeric degradation products. Although the

'Ultraflo-arabinoxylan degrader' is still a mixture of enzymes, it can be used as a tool for the production of oligosaccharides.

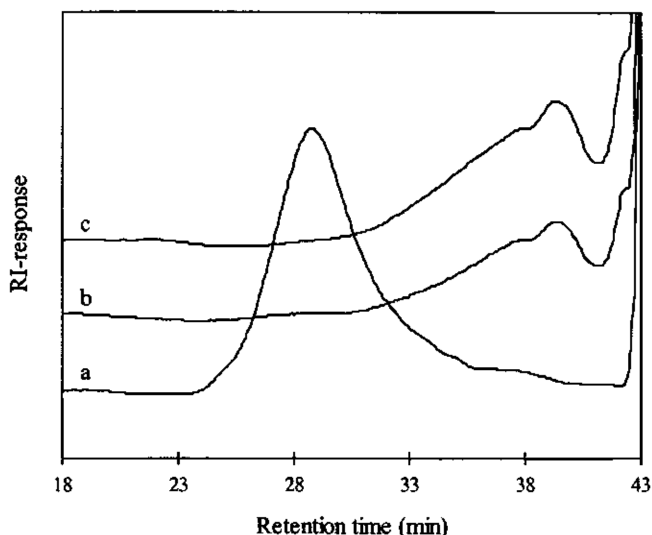

Figure 7.3. HPSEC elution profiles of (a) maize BE1 blank, (b) digest of maize BE1 after incubation with 'Ultraflo-arabinoxylan degrader' for 24h, and (c) digest of maize BE1 after sequential degradation with 'Ultraflo-arabinoxylan degrader' for 24h and endo-xylanase I for 24h.

Incubation of maize BE1 with 'Ultraflo-arabinoxylan degrader' degrades all polymeric material in the extract (Figure 7.3). Incubation of this digest with endo-xylanase I did not result in further degradation of the digest. After incubation, arabinose is released as the main degradation product (Figure 7.4). After 20h of incubation, about 40% of the arabinose, about 3% of the xylose, and only about 7% of the glucuronic acid originally present in the substrate is released as monomeric degradation products. Furthermore, a peak of xylobiose, xylotriose, and a lot of peaks representing unknown compound can also be seen in the elution profile of the digest.

In an earlier study, it was determined that this sub-fraction was able to degrade both wheat arabinoxylan and the disubstituted oligomer β-Xyl*p*-(1→4)[α-Ara*f*-(1→2)][α-Ara*f*-(1→3)] β-Xyl*p*-(1→4) β-Xyl*p*-(1→4) Xyl*p*. So it contains an arabinofuranohydrolase which can release arabinose from disubstituted xylose residues (Düsterhöft; unpublished results). It

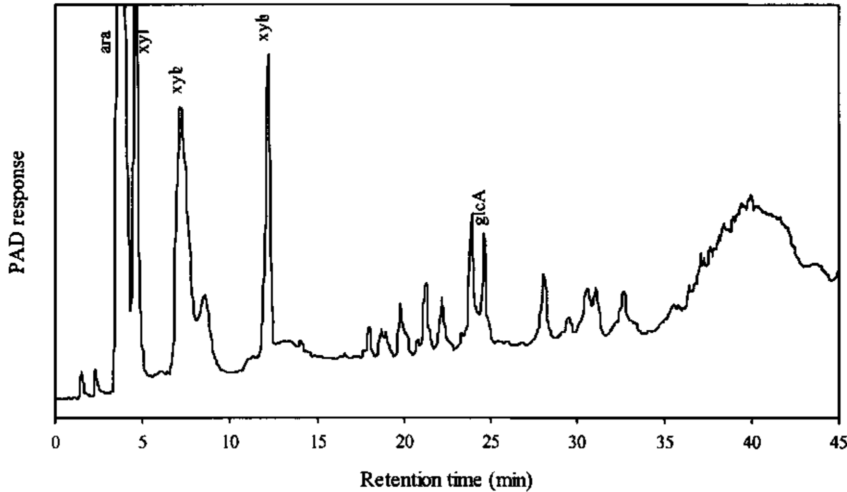

Figure 7.4. HPAEC elution profile of the digest of maize BE1 after incubation with 'Ultraflo-arabinoxylan degrader' for 20h.

is not sure whether this enzyme resembles the AXH-d3[19,40]. AXH-d3 was unable to release arabinose from maize BE1, possibly because it is hindered by the (4-*O*-methyl)glucuronic acid substituents or the high degree of branching[40]. The presence of α-glucuronidase (glcAase)[41], exo-glucuronase or other accessory enzymes in the 'arabinoxylan degrader' could possibly enable AXH-d3 to work. About 7% of the glucuronic acid was released as a monomeric degradation product, thus this possibility can not be ruled out. Another possibility is the existence of for example an AXH-d2, which would enable other enzymes to work. In addition to this, the presence of β-xylosidases, endo-xylanases and α-L-arabinofuranosidases able to remove arabinose substituents at C2 and/or C3 in the arabinoxylan degrader is demonstrated (Düsterhöft; unpublished results).

To obtain more information about the composition of the oligomers formed after incubation with 'Ultraflo-arabinoxylan degrader', the digest was analysed by MALDI-TOF MS. Although the spectrum, shown in Figure 7.5, appears to be rather complex, a few series of analogous oligomers can be distinguished. Firstly, oligomers containing only pentoses (xylose or arabinose; indicated with #) are present with a degree of polymerisation up to 11. Secondly, oligomers containing one hexuronic acid (glucuronic acid) in addition to pentoses (signals marked with +). The number of pentoses in these oligomers ranges from 3 to 10. In

the MALDI-TOF mass spectrum a signal of equivalent oligomers containing an additional methoxyl group was also shown (also marked with +). This methoxyl group is linked to C4 of the glucuronic acid residue. Thirdly, a series of oligomers containing pentoses and *two* glucuronic acids was present (signals marked with $). The number of pentose residues in these oligomers ranges from 5 to 8. A number of these oligomers are also present in which one or both glucuronic acid residues contain an additional methoxyl group. Finally, a series of oligomers can be detected, which consist of one hexose in addition to the pentoses (signals marked with *). The number of pentoses in these oligomers ranges from 2 to 6.

The presence of oligosaccharides containing pentoses of which the non-reducing terminal xylose unit is substituted with α-D-glucuronopyranosyl at C2 was previously shown in a sorghum GAX digest[8], and a hexose-containing pentosan oligomer (Gal*p*-(1→4) Xyl*p*-(1→2) [5-*O*-(*trans*-feruloyl)] ara*f*) was already found in an acid hydrolysate of maize bran[13]. Further analysis of the larger oligomers in this series could show whether this oligomer is present as a side-chain directly attached to the xylan backbone as suggested by Saulnier et al.[13] or not. However, we will only be able to detect the oligomer without the ferulic acid substituent, because the ester linkage was saponified during the extraction of the arabinoxylans.

This is the first time that the presence of arabinoxylan oligomers containing two glucuronic acids is indicated. The presence of these oligomers, but also of oligomers containing one glucuronic acid shows that the glucuronic acid substituents were evenly distributed over the xylan backbone. This is in agreement with the fact that fractionation based on differences in charge density was not possible. Blockwise distribution of the glucuronic acid substituents over the xylan backbone and the fact that only 7% of the glucuronic acid was removed during the enzymatic degradation, would give a large amount of oligomers containing pentose only and probably some remaining glucuronic acid-rich polymeric material. In addition, it is shown that MALDI-TOF MS is an adequate tool to show the presence of methoxyl ethers of glucuronic acid residues. HPAEC analysis of a digest cannot directly show the presence of these methoxyl groups, since it is not known how the retention time of a component is affected by the presence of an additional methoxyl group, and the elution pattern of an enzymatically obtained digest is very complex.

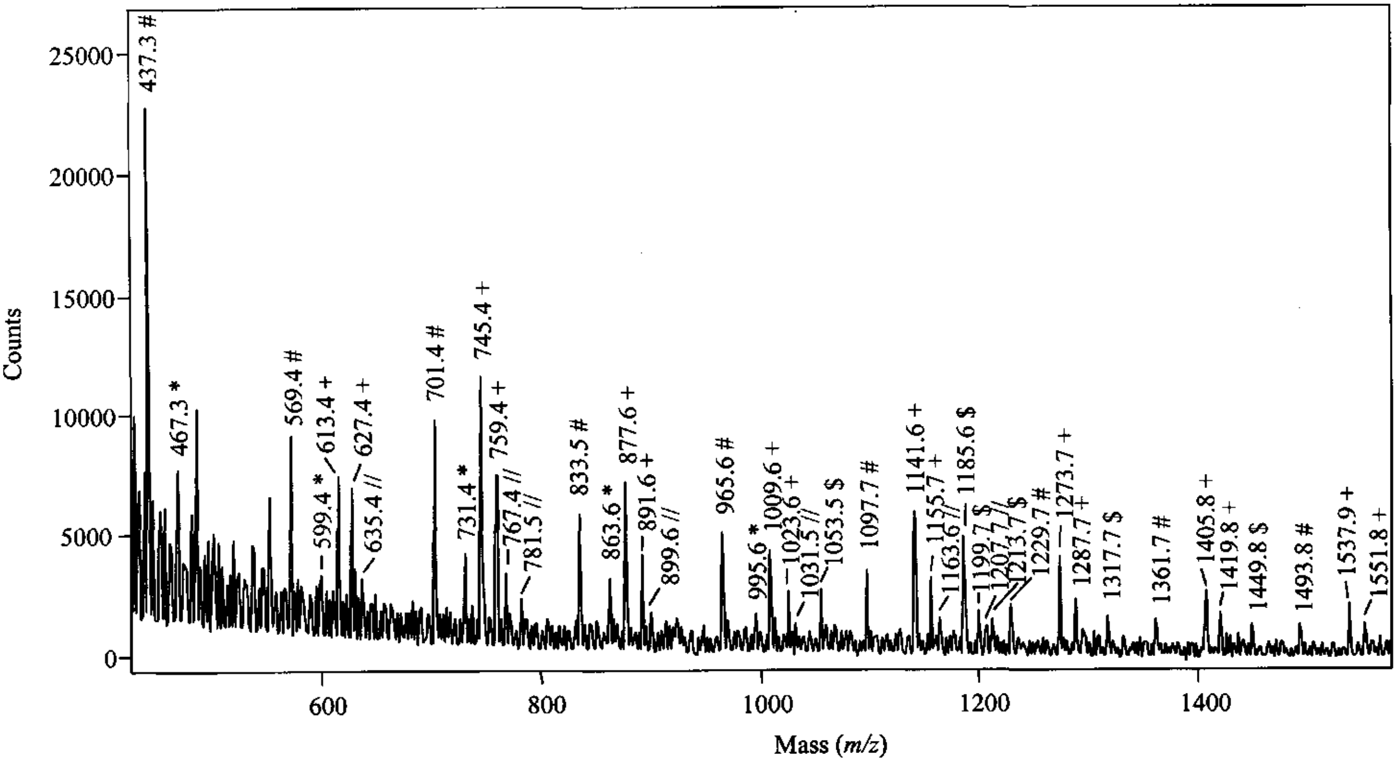

Figure 7.5. MALDI-TOF mass spectrum of the digest of maize BE1 after incubation with 'Ultraflo-arabinoxylan degrader' for 20h. Signals belonging to the same homologues series of sodium-cationised oligosaccharides; # = $pent_n$, * = $pent_n hex$, + = $pent_n Ome_m hexA$ (m = 0 or 1), $ = $pent_n Ome_m hexA_2$ (m = 0, 1, or 2), and // indicates doubly sodiated oligomers.

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CHAPTER 8

CONCLUDING REMARKS

RESEARCH MOTIVES

Plant cell wall polysaccharides are widely studied for various reasons. Cell walls form the skeleton of plant tissues, control the growth of plant cells, and form a structural barrier to invading pathogens. Plant cell walls represent the bulk of all plant biomass and they are exploited in a number of ways, as textiles, live-stock feed, paper, and food ingredients. The plant cell wall can also end up in large amounts of by-products and wastes enriched in fibres, e.g. soybean meal and maize by-products from the wet milling process.

The objective of the project was the elucidation of the chemical structure of polysaccharides directed to their functional properties. In the elucidation of the structure, the use of modern spectrometric techniques was emphasised. The use of these techniques in polysaccharide analysis is optimised by using these methods in combination with (enzymatic) degradation of the polysaccharides to obtain fragments fitting within the analytical range of these methods. The structure-function relationship was exemplified in the utilisation of the polysaccharides in livestock feed. The polysaccharides in livestock feed can be represented by the polysaccharides from soybean meal (pectic substances and xyloglucans) and maize kernels (glucuronoarabinoxylans). Accordingly, the aims of the investigations were to determine the chemical structure of the cell wall polysaccharides present in these plant materials in view of their processing and food quality functions in general, and their microbial/enzymatic degradability in particular.

This thesis adds information about the primary structure of both pectic substances, including their arabinogalactan side chains, and xyloglucans from soybean, and of glucuronoarabinoxylans from maize kernels to the data present in the literature. The exact structural features of these different classes of cell wall polysaccharides vary from plant to plant. Both soybean and maize kernel cell wall polysaccharides distinguish themselves in a number of respects from other plant cell walls polysaccharides. These differences will be discussed in this chapter.

SOYBEAN XYLOGLYCAN

In the cell wall of most Dicotyledonae, the principal hemicelluloses are xyloglucans. They can be extracted from the cell wall with alkaline solutions, typically 1-4 M. Xyloglucans are linear chains of (1,4)-β-D-glucan with xylosyl residues added at regular sites to the O-6 position of the glucosyl units. Additional sugar residues, like galactose, fucose, and arabinose, are added to the O-2 of some xylosyl residues[1,2,3,4]. The galactose residues can be O-acetylated[5]. An important characteristic of xyloglucans is the degree of backbone branching. Most xyloglucans are composed of either XXXG-type or XXGG-type building units (Figure 8.1).

In chapter 6, it is demonstrated that soybean xyloglucans extracted with 1 and 4 M alkali are composed of XXXG-type building units, similar to xyloglucans from many other plants. Some of the xylose residues are substituted at C2 with β-D-galactopyranose residues. The xyloglucans from soybean cell walls are fucosylated. In oligosaccharides XXFG and XLFG, position Q is substituted with an α-L-Fuc*p*-(1→2)- residue. During extraction of the soybean xyloglucans with alkali, possibly present acetyl groups will be lost and therefor no conclusions can be drawn with respect to their presence or ablsence. Xyloglucans isolated from various other legumes (*e.g.* pea and common bean) do bear acetyl substituents[4]. The structure of the soybean xyloglucan comes up to our expectations, since up to now xyloglucans isolated from other legume seeds also belong to the poly-XXXG type xyloglucans[6,7].

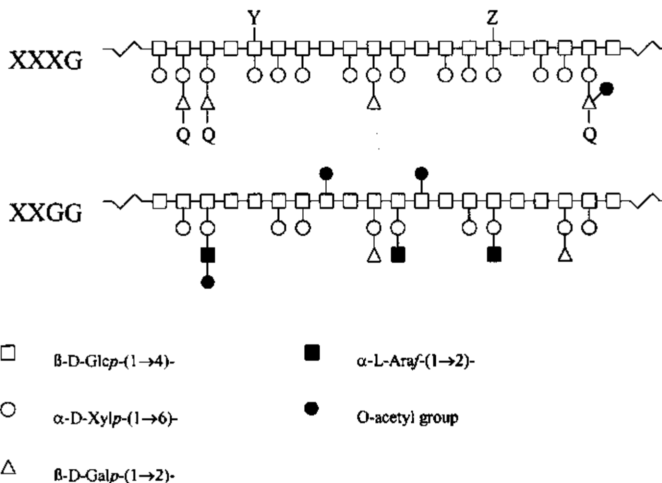

Figure 8.1. An overview of different branching patterns of xyloglucan. Position Q can be substituted with an α-L-Fuc*p*-(1→2)-residue or an α-L-Gal*p*-(1→2)-. Position Y can be substituted with a β-D-Xyl*p*-(1→2)-, an α-L-Ara*f*-(1→2)-, or an α-L-Ara*f*-(1→3)-β-D-Xyl*p*-(1→2)- side chain. Z can be substituted with β-D-Xyl*p*-(1→2)-residue[4].

SOYBEAN PECTIC SUBSTANCES

In general, pectic polysaccharides are major components of the primary cell wall of dicotyledons (e.g. sycamore, citrus and legumes) and gymnosperms (e.g. Douglas fir)[8]. This is also true for soybean. The ChSS fraction (38% of the WUS), the DASS fraction (7% of the WUS), and about 90% of the 1 MASS fraction (16% of the WUS) consist of pectic substances. This implies that at least 60% of soybean WUS consist of pectic polysaccharides.

If we let go of the classification of pectic polysaccharides in smooth and hairy regions as suggested by Schols *et al.*[9], and choose for a classification based on the structure of the backbone, we end up with two categories of pectic polysaccharides.

The first group contains those parts of the pectin with exclusively (1,4)-linked galacturonic acid in the backbone. According to this definition, homogalacturonan, rhamnogalacturonan II, and xylogalacturonan belong to this group. The biosynthesis of the backbone of this category of pectic polymers requires a polygalacturonate 4-α-galacturonosyltransferase capable of transferring UDP-galacturonic acid residue to the non-reducing end of a pre-existing chain of galacturonic acid residues. The homogalacturonan is first synthesised as an unesterified chain and subsequently at least partially methylesterified by an enzyme that transfers methyl groups to the carboxyl groups of polygalacturonic acid[10,11,12]. It seems likely that other substituents (*e.g.* acetyl esters and neutral sugar residues, like xylose) can be attached to the backbone in like manner.

The second group contains those parts of the pectin with a rhamnogalacturonan backbone. The biosynthesis of the backbone of this category of pectic polymers requires two glycosyl transferases, a galacturonic acid transferase capable of adding galacturonic acid residues to rhamnose and a rhamnose transferase capable of adding rhamnose residues to galacturonic acid in a rhamnogalacturonan chain[10]. Possibly followed by the attachment of arabinan, galactan, or arabinogalactan side chains. A rhamnogalacturonan I galactosyltransferase able to transfer a galactose residue to short pectic (1,4)-β-D-galactan chains was recently isolated[13].

Homogalacturonan

Homogalacturonans are chains of (1,4)-linked α-galacturonic acid, which may be partly methyl esterified. So far, pectic substances from plant cell walls were always found to consist of smooth galacturonan regions interrupted by blocks of ramified rhamnogalacturonan regions, so-called hairy regions (Figure 1.4). These smooth galacturonan regions were predominantly present in the fractions extracted with aqueous solvents, often containing chelating agents. It is shown in chapter 4 that CDTA-extractable pectins from soybean (38% of the WUS) do not contain homogalacturonans.

In an additional experiment, monoclonal antibodies to low ester pectin (JIM5[14]), high ester pectin (JIM7[14]), and de-esterified homogalacturonan (PAM1[15]) were used to characterise the CDTA-extractable pectic substances. In immuno-dot assays, JIM5 was found to bind to both native and saponified pectic substances in the ChSS extract. JIM 7 is only able to bind to the native pectins in the ChSS extract, being specific for high ester pectin. The epitopes recognised by these two anti-homogalacturonan monoclonal antibodies are however not defined[15]. The epitopes recognised by PAM1 are, however, well defined. PAM1 binds specifically to de-esterified and unsubstituted homogalacturonan, optimal binding requires in

the region of 30 de-esterified galacturonic acid residues[15]. PAM1 does not bind to saponified ChSS, again indicating that blocks of pectic homogalacturonan are absent. The absence of homogalacturonan explains the resistance of (saponified) CDTA-extractable pectic substances towards degradation with polygalacturonase, but makes their extraction by CDTA remarkable.

The absence of homogalacturonan in CDTA extractable pectic substances has a number of consequences. A first step in the elucidation of the structure of the pectic substances usually is the solibilisation of the pectic polysaccharides in the cell wall by using highly purified polygalacturonase[16,17,18,19], or a fractionation of isolated pectic substances after digestion with highly purified homogalacturonan-degrading enzymes[20,21,22]. Rhamnogalacturonan I and II are the major polysaccharides solubilised from various plant tissues using this approach. Due to the absence of homogalacturonan, this first step is not applicable. This implies that other routes had to be followed in this research, as is described in chapters 3 and 4.

A second consequence of the absence of homogalacturonan is the inability of soybean pectic substances to participate in calcium cross-linking and gel formation. This is not only important in the cell wall itself, but also indicates that extraction of soybean meal yields pectins unable to form the typical gels as known for other pectins (*e.g.* from apples and citrus). Jam and jelly manufacture is one of the main uses of industrially extracted pectins[23] and soybean pectic substances are most likely unsuitable for this purpose.

Another consequence is that the absence of homogalacturonan effects the physiology of the cell wall, since pectins are mediators of wall porosity. A mild treatment with pectinase can enlarge the channels (for macromolecular transport across plant cell walls), without adversely affecting cell viability, enabling significantly larger molecules to pass through the wall[24].

Rhamnogalacturonan II

Rhamnogalacturonan II is structurally very different from the previously discussed rhamnogalacturonans. It is covalently linked in the primary cell wall through homogalacturonans. It consists of approximately 60 glycosyl residues, many of which are very unusual and are described in chapter 1. The functions of rhamnogalacturonan II are not known. However, the discovery that the rhamnogalacturonan II occurs as dimers covalently cross-linked by borate diesters[25,26] suggest that these parts of the pectin are of importance in the interconnection of the pectic polysaccharides[27]. It is suggested that these boron cross-links are the "load-bearing", acid-labile linkages that are hydrolysed by a decrease in cell wall pH during auxin-induced cell expansion[28].

The ChSS fraction was analysed for its apiose content, a glycosyl residue diagnostic of rhamnogalacturonan II. The CDTA-extractable pectic substances appeared to be free of apiose, and consequently of rhamnogalacturonan II.

Xylogalacturonan

The presence of xylogalacturonan regions, in which terminal xylose is linked directly to the galacturonosyl residues, has been suggested before in soybean[20,29]. Xylogalacturonans also occur in apple[30], cotton suspension-cultured cell walls[31], watermelon[31], and pea hulls[32,33]. The xylogalacturonans take up 12 to 18% of the soybean CDTA-extractable pectic substances. The xylogalacturonans from different sources vary in the degree of xylose substitution, the degree of methyl esterification, and the distribution of the substituents over the backbone[9].

So far, two enzymes are known, which are able to hydrolyse xylogalacturonan. Firstly, an exo-galacturonase, which is not hindered in its action by single branches of xylose. It releases galacturonic acid and the β-Xyl*p*-(1,3)-GalA*p* dimer from apple hairy regions[34] and from soybean pectic substances (chapter 4). Secondly, xylogalacturonan hydrolase (XGH), a polygalacturonase which is specific for xylose-substituted galacturonan[35]. This enzyme is able to degrade xylogalacturonan derived from acid-hydrolysed gum tragacanth and apple hairy regions. Soybean pectic substances are resistant to degradation by XGH even after acid hydrolysis. The structure of soybean xylogalacturonan is obviously not identical to apple and gum tragacanth xylogalacturonan. The resistance to XGH can be caused by a different degree of xylose substitution or by an altered distribution of the substituents over the backbone. A third possibility is further substitution of the xylose substituents, as suggested by the presence of (1,2)-, (1,4)-, and (1,2,3)-linked xylose (chapter 4).

The most obvious possibility is further substitution of the xylose residues with fucose. The fucose content of the P1 fraction (chapter 4) is relatively high (7 mol%), compared to pectic substances originating from other sources, and has not been reported before except by Beldman *et al.*[34] a soluble pectic polysaccharide also from soy (11 mol% of fucose). In addition, the disaccharide Fuc*p*-(1,2)-Xyl was found to be present in the digest obtained after partial hydrolysis of soybean cotyledon polysaccharides[29]. This dimer cannot originate from other polysaccharides, because in the only hemicellulose containing both xylose and fucose (xyloglucans) they are not covalently linked to each other, but occur in the trimeric side chain α-L-Fuc*p*-(1→2)-α-L-Gal*p*-(1→2)-α-D-Xyl*p*-(1→. However, this disaccharide might be present as a side chain of polygalacturonic acid, since the linkage analysis composition of the ChSS fraction and fraction P (chapter 4) shows the presence of (1,2)-linked xylopyranose residues. The NMR analyses described in chapter 4 do not provide any information about the sugar residue to which the terminal fucose residue is attached.

To investigate the substitution of xylose residues with fucose, (saponified) soybean fraction P was incubated with α-fucosidase[36]. This enzyme released 5.5% of the total amount

of fucose in the fraction, and this increased the susceptibility of fraction P for exo-galacturonase, releasing3.6% of the galacturonic acid residues from saponified fraction P and 6.4% after pre-incubation with α-fucosidase. Due to the lack of a proper standard, the content of the characteristic dimer (Xyl-GalA) could not be quantified properly, however, it is clear that the amount released from saponified fraction P increased after pre-incubation with α-fucosidase. This proves that a part of the xylose substituents in the soybean xylogalacturonan are substituted with fucose residues, which would explain the resistance of this xylogalacturonan to XGH. However, XGH is still unable to degrade the saponified fraction P after pre-incubation with α-fucosidase. Substitution of the xylose residues with other sugar residues, like xylose[33], galactose or arabinose cannot be excluded. The structure of soybean xylogalacturonan is more complex than just a polygalacturonic acid chain substituted with single terminal xylose residues.

The α-fucosidase releases only 5.5% of the fucose from saponified fraction P. The amount of α-fucosidase added to the incubation mixture was abundant, so the remaining fucose can not be released by this enzyme. These fucose residues might be unavailable for the enzymes due to (1) small alterations in the structure of the fucosyl-containing side chain, (2) steric hindrance of other parts of the molecule, or (3) the fucose residues might be present in the rhamnogalacturonan regions, as in sycamore RG-I[8,37]. It was not determined if the α-fucosidase would be able to release these fucose residues.

The presence of xylogalacturonan could not be confirmed by the immuno-dot assay using antibodies to xylogalacturonan. Two xylogalacturonan monoclonal antibodies (7G3 and 5A10), developed using a pea xylogalacturonan, were used. The antibodies are likely to recognise more densely xylose-substituted homogalacturonan. No defined oligosaccharide inhibitor of antibody binding has been found (personal communication Dr. Bill Willats). Neither of the antibodies is able to bind to the native or saponified pectins in the ChSS extract and the P fraction. Based on the release of galacturonic acid and the characteristic dimer (Xyl-GalA) by exo-galacturonase we are convinced of the presence of xylogalacturonan in the CDTA-extractable soybean pectic substances. It is plausible that the distribution of the xylose residues over the galacturonan chain or the presence of fucose residues in the xylogalacturonan hinders binding of the antibodies.

Rhamnogalacturonan

The rhamnogalacturonan regions are substituted with neutral polymers like arabinans, galactans, and arabinogalactans[23,38,39]. The rhamnose to galacturonic acid ratio of these pectic substances varies between 0.05 and 1[9]. All the arabinose and galactose present in CDTA- and dilute alkali-extractable pectin is present in rhamnogalacturonan side chains (chapter 2). The rhamnogalacturonans (including the (arabino)galactan side chains) represent over 60% of the CDTA-extractable pectic substances. The arabinosyl- and galactosyl-rich side chains are

attached to C4 of the rhamnosyl residues. The ratio of (1,2)- to (1,2,4)-linked rhamnose in the ChSS extract is 1:2 (chapter 4). It can be concluded that the average length of the (arabino)galactan side chains would be in the range of 45 to 50 residues. This was confirmed by the length of the arabinan/galactan/arabinogalactan oligomers released from the ChSS extract by short incubation with endo-galactanase, exo-galactanase, endo-arabinanase, and arabinofuranosidase B. HPAEC analysis of this digest showed the presence of galactan and arabinogalactan oligomers with a degree of polymerisation up to about 15 and arabinan oligomers with a degree of polymerisation up to 30-35. The length of the (arabino)galactan side chains in soybean pectic substances exceeds the length of those in sycamore RG-I, ranging in size from one to fourteen glycosyl residues[37].

RG hydrolase is unable to degrade the rhamnogalacturonans in the ChSS extract. The enzyme is probably hindered by these long (arabino)galactan side chains. RG hydrolase is only able to hydrolyse the glycosidic linkage between a galacturonic acid and a rhamnose if the rhamnose residue is unbranched or substituted through C4 with only a single galactose residue[40]. Only few unbranched rhamnose residue are available in the ChSS extract (chapter 4) and based on the high arabinose and galactose content it is expected that none or only very few of the (1,2,4)-linked rhamnose residues will carry a single galactose residue. Besides, the long neutral side chains will sterically prevent the enzyme to approach the potential glycosidic bond to be hydrolysed. Enzymatic removal of a large number of the neutral side chains and reduction of the length of the remaining side chains increased the susceptibility of the rhamnogalacturonan for RG hydrolase, but only after saponification (chapter 4).

After enzymatic removal of a large part of the arabinogalactan side chains, NMR analysis showed that terminal and 4-linked galactose were attached to the rhamnose residues in the rhamnogalacturonan of the PI and PII population. In sycamore RG-I, terminal and 4-linked galactose were also found to be attached to rhamnose. In addition, terminal arabinosyl and 3-, 6-, 2,6-, and 3,6-linked galactosyl residues were attached to C4 of the 2-linked rhamnosyl residues in sycamore[41]. These results showed that at least seven different side chains, most commencing with a galactosyl residue, were attached to the RG-I backbone in sycamore. In the soybean ChSS fraction, 3-, 6-, 2,6- and 3,6-linked galactosyl residues do not occur (chapter 4). However, side chains beginning with an arabinosyl residue might be present in soybean. NMR analysis of PI and PII (chapter 4) did not show the presence of such side chains, but they might have been removed during acid hydrolysis. In sycamore, arabinosyl residues glycosidically linked to rhamnose were observed, and always further substituted with arabinosyl residues. Maize RG-I also contains arabinogalactan side chains, which consist of a single arabinosyl residue attached to a rhamnosyl residue, which is substituted with one or more galactosyl residues[42].

So far, soybean pectic side chains were described as galactan backbones with arabinose side chains of one or two residues. The results of our studies show that soybean pectin contains rather long arabinan side chains in addition to (arabino)galactan side chains.

The arabinan side chains are presumably of considerable length (up to dp 30-35). And more important arabinose was found to occur interspersed in the galactan chain as an *internal* (1,5)-linked arabinofuranose residue or as a terminal arabino*pyranose* residue (chapter 5). In this view it distinguishes itself from other arabinogalactans, described so far. Internal arabinose was detected once before, but from a very different source, namely arabinogalactan type II from larch[43,44,45]. The presence of an internal arabinofuranose residue in a pectic arabinogalactan chain in cell wall polysaccharides has not been reported previously, not in soybean, nor in other fruit or vegetable cell walls. Both ring forms of arabinose also occur in arabinogalactan type II, in which L-arabinofuranosyl, and to a lesser extent L-arabinopyranosyl residues, terminate some of the side chains[46]. However, in soybean arabinogalactan we are dealing with linear (1,4)-linked β-D-galactan, which is type I arabinogalactan. The existence of arabinopyranose residues in pectic arabinogalactan is rarely reported. Only pectic substances isolated from the roots of *Angelica acutiloba* Kitagawa were shown to contain a small proportion of arabinopyranose[47].

The presence of internal (1,5)-linked arabinofuranose and terminal arabinopyranose residues in the galactan chains has far-reaching consequences for amongst others the enzymatic degradation of these arabinogalactan side chains. It (partly) explains their resistance to enzymes like endo-galactanase. It is shown in chapter 3 that a combination of endo-galactanase, exo-galactanase, endo-arabinanase, and arabinofuranosidase B is required for optimal removal of the (arabino)galactan side chains. However, after incubation with this combination of enzymes 8% of the arabinose and galactose residues present in the ChSS extract still remained attached to the pectin, probably due to the complexity of the remaining part of the side chains. Arabinofuranosidase B is unable to release the non-reducing terminal arabinopyranose residue due to its ring form; an arabinopyranosidase would bring help here. An enzyme able to hydrolyse the glycosidic linkage between a (terminal or (1,4)-linked) galactose residue and a (1,5)-linked arabinofuranose residue is yet unknown. It is also unknown how the presence of an interspersed arabinose residue in a galactan chain effects the activity of the endo-galactanase. This could not be deduced from the structure of the degradation products, because complete degradation was not established and it is not known whether the resulting arabinogalacto-oligosaccharides can be degraded further by endo-galactanase. However, it is likely that the endo-galactanase is unable to degrade arabinogalactan chains in the vicinity of an internal arabinose residue.

So far, the incomplete removal of the arabinogalactan side chains from pectic polymers by enzymes remained unexplained. Now we have some potential causes in the presence of (1,5)-linked arabinofuranose and terminal arabinopyranose. With this knowledge and the constantly improving techniques we can focus further research on the complete removal of the pectic arabinogalactan side chains.

MAIZE KERNEL ARABINOXYLAN

The extract obtained with a saturated $Ba(OH)_2$-solution (BE1) of maize WUS contains predominantly glucuronoarabinoxylans (chapter 7). The BE1 extract from other cereals usually contains a considerable amount of (1,3),(1,4)-β-D-glucan. This extract from maize, however, contained only 0.7% of glucose. The glucuronoarabinoxylans were highly substituted with mainly terminal arabinose residues. A new measure for the degree of substitution was defined, and turned out to be higher for maize (87%) than for sorghum (70%). So, although sorghum glucuronoarabinoxylans were described as being very complex, those from maize kernels appeared to be even more complex. Not only the degree of substitution is higher, but the glucuronoarabinoxylan from maize also contains more complex side chains[48,49,50].

This causes a high resistance towards enzymatic degradation. Endo-xylanase I was not able to degrade the glucuronoarabinoxylans in the maize BE1 extract, also not when different arabinofuranohydrolases were added to introduce cleaving sites by removing arabinose residues. A sub-fraction of the commercial enzyme preparation Ultraflo from *Humicola insolens* was required for degradation of the polysaccharides in maize BE1. Analysis of the digest showed a wide diversity of degradation products to be present. One series of arabinoxylan oligomers had not been isolated or described before, and was distinctive in that they contained two glucuronic acid residues. MALDI-TOF MS of the digest showed the presence of both glucuronic acid and 4-*O*-methyl glucuronic acid residues. From the size of these oligomers (dp 7 to 10), it could be deduced that the glucuronic acids can be very close to each other in the xylan polymer, but are not distributed blockwise.

A tentative model for maize glucuronoarabinoxylan is shown in Figure 8.2. This model is based on the sugar and linkage composition, the degradation products after incubation with "Ultraflo arabinoxylan-degrader", the resistance to endo-xylanase I (chapter 7), and knowledge about the composition of the oligomeric side chains from literature[48,49,50,51]. Some structural features are, however, still unknown. In this model, disubstituted xylose residues containing two single-unit arabinose residues are present. It might be possible that these xylose residues carry a glucuronic acid residue at the C2 position and an arabinose residue or a short side chain at the C3 position, as suggested in the schematic structure of the sugar moiety of heteroxylans from maize bran by Saulnier *et al.*[52], but there is no proof for the existence of these structures yet. Other differences between this model (Figure 8.2) and the model for maize bran arabinoxylan[52] are the distribution of the glucuronic acid residues over the xylan chain, the degree of substitution of the schematic structure (0.94 versus 1.19), and the presence of dimeric side chains of arabinose in our model.

The linkage composition of maize BE1 shows the presence of equal amounts of (1,5)- and (1,2)-linked arabinose residues (chapter 7). The heterogeneous side chains (gal-xyl-ara)

described by Saulnier *et al.*[50] contain (1,2)-linked arabinose. The amount of (1,2)-linked arabinose corresponds to the amount of terminally-linked galactose, analysed in the maize BE1 fraction. The short arabinose side chains are consequently made of only (1,5)-linked arabinose residues. This is in agreement with the results of Nishitani and Nevins[53], who show that α-L-arabinofuranosyl-[(1→5)-*O*-α-L-arabinofuranosyl]$_n$-side chains are present in the arabinoxylans from maize coleoptile cell walls. The short side chains in sorghum, however, contain (1,2)-linked arabinose residues[51].

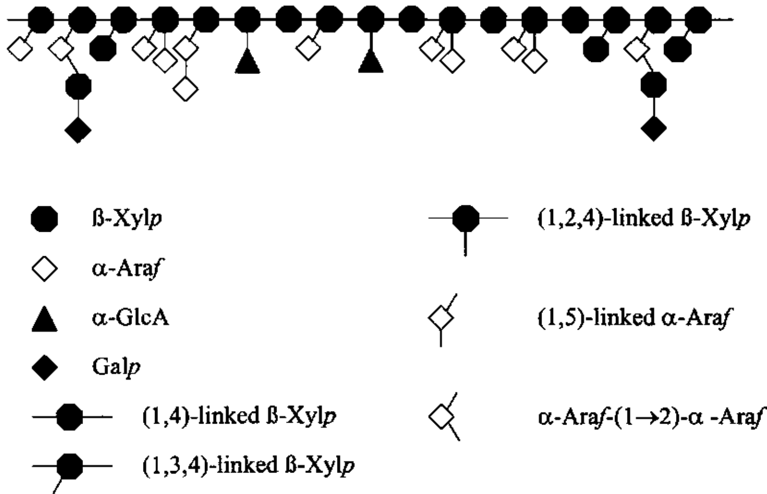

Figure 8.2. A tentative model for $Ba(OH)_2$-extractable glucuronoarabinoxylan from the maize kernel WUS based on their sugar and linkage composition, their resistance to degradation by endo-xylanase I, and the occurrence of small side chains described in literature[50,53].

The model for $Ba(OH)_2$-extractable glucuronoarabinoxylan from maize kernel WUS differs from the structural model for glucuronoarabinoxylan[54] from sorghum in a number of respects. Firstly, the model for sorghum describes the more substituted, endo-xylanase I-resistant 4 M KOH-extactable glucuronoarabinoxylans. Secondly, in sorghum glucuronoarabinoxylan, short heterogeneous side chains do not occur. Another important difference is the occurrence of xylose in monomeric or oligomeric side groups in glucuronoarabinoxylan from maize kernels, this was not detected in sorghum glucuronoarabinoxylan.

The degree of substitution of the xylan backbone determines to a considerable degree the solubility of the xylan and its ability to bind to cellulose[55]. The glucuronoarabinoxylans from maize have a high degree of side-chain substitution and will therefore be more soluble, will bind less tightly to cellulose, and are more resistant to enzymatic digestion.

CONCLUSIONS

From the discussion in this chapter, it can be concluded that many of the problems raised in chapter 1 are still unanswered. However, we did gain much knowledge about the different polysaccharides in the soybean and maize kernel cell wall. We now know that the pectins from soybean differ significantly from all pectins described so far. Not only the absence of homogalacturonan, but also the presence of internal (1,5)-linked arabinofuranose and terminal arabinopyranose in the pectic arabinogalactan side chains has never before been described for parenchymatous tissues of plants. The $Ba(OH)_2$-extractable glucuronoarabinoxylan from maize kernel cell walls appeared to be very highly substituted, more complex than any other glucuronoarabinoxylan described so far.

Soybean pectic substances and maize kernel glucuronoarabinoxylans appeared to be rather resistant to degradation by enzymes, which are generally used in our group to degrade this kind of polymers. Their structural features, as we know them now, explain this phenomenon. Their complexity appeared, however, to be of no concern during their degradation in the gastrointestinal tract of livestock[56,57]. So it is very likely that enzymes able to degrade these polysaccharides do exist. For maize kernel glucuronoarabinoxylan such an enzyme preparation was already found during this research. Due to the large number of enzymes present in this preparation some information about the structure of the glucuronoarabinoxylan was lost during digestion. Preservation of this information would require further fractionation and isolation of the enzymes in the commercial preparation.

Our approach to investigation of the chemical structure of cell wall polysaccharides proved to be successful. Extraction of the polysaccharides from their original source always has to be the first step in their characterisation. Chemical characterisation and enzymatic degradation studies already reveal the main lines of their chemical structure. The use of MALDI-TOF MS, MS/MS, and NMR spectroscopy provided valuable and detailed information on the structure of the polysaccharide fragments obtained after enzymatic or chemical degradation. Although a lot of pure and well-characterised enzymes are available nowadays, this research showed the need for additional enzymes, like for example arabinopyranosidase, RG hydrolases active on side chain-containing rhamnogalacturonan, *etc.*

In general, carbohydrate researchers try to translate their findings into universal models. Our investigations show that such models are limited in their value, knowledge about the structure of plant cell wall polysaccharides is only valid for that particular tissue of that plant species.

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SUMMARY

The subject of this thesis was the elucidation of the chemical fine structure of polysaccharides from cell walls of soybean and maize kernel. The two species investigated represent different taxonomic groups, soybean belonging to the dicotyledonous and maize to the monocotyledonous plants. Besides representing the most important structures present in cell wall material, these raw materials are of great importance in food and feed industry.

The characterisation of the soybean cell wall polysaccharides started with the isolation of the cell wall material as Water-Unextractable Solids (WUS) from soybean meal (chapter 2). The isolation procedure yielded a WUS fraction containing almost all polysaccharides present in the meal and only few other components. WUS was sequentially extracted with chelating agent (Chelating agent Soluble Solids, ChSS), dilute alkali (Dilute Alkali Soluble Solids, DASS), 1 M alkali (1 M Alkali Soluble Solids, 1 MASS) and 4 M alkali (4 M Alkali Soluble Solids, 4 MASS) to leave a cellulose-rich residue (RES). The pectin-rich extracts (ChSS and DASS) were found to have identical sugar compositions and contained predominantly galactose, arabinose, and uronic acid residues. The 1 MASS fraction contained xylose in addition to the former three sugars. The hemicellulose-rich fraction (4 MASS) contained mainly xylose and glucose. No indications were found that ChSS and DASS were structurally different, although it is obvious that their arrangement in the cell wall was not identical.

The intact cell wall polysaccharides in the meal and WUS were hardly degradable by enzymes. Once extracted, the polysaccharides from WUS were degraded more easily (chapter 3). The arabinogalactan side chains in the pectin-rich ChSS fraction could to a large extent be removed by the combined action of endo-galactanase, exo-galactanase, endo-arabinanase, and arabinofuranosidase B. The remaining polymer (fraction P) was isolated and represented 30% of the polysaccharides in the ChSS fraction (12% of the polysaccharides in the WUS). This polymer still contained some remaining arabinose and galactose residues, which could not be removed by the enzyme mixture used.

The pectic backbone (fraction P) appeared to be resistant to enzymatic degradation by both established (like polygalacturonase) and novel pectic enzymes (like RG-hydrolase). After partial acid hydrolysis of the isolated pectic backbone, one oligomeric and two polymeric populations were obtained by size-exclusion chromatography. Monosaccharide and linkage analyses, enzymatic degradation, and NMR spectroscopy of these two polymeric populations showed that the pectic substances in the original extract (ChSS) contained both rhamnogalacturonan and xylogalacturonan regions, while homogalacturonan was absent (chapter 4). The absence of homogalacturonan distinguishes the pectic substances from soybean from pectic polysaccharides extracted from other sources, which contain homogalacturonan and rhamnogalacturonan regions and can be degraded with

polygalacturonase and RG-hydrolase, respectively. Acid hydrolysis of fraction P improves the susceptibility of the remaining polymers for RG hydrolase and exo-galacturonase.

The xylogalacturonan present in the ChSS fraction distinguishes itself from xylogalacturonan from other sources known so far. A part of the xylose residues in the xylogalacturonan is substituted with fucose and the xylogalacturonan is resistant to degradation with XGH.

The arabinogalactan side chains, which were removed from the ChSS fraction to obtain fraction P, were the subjects of investigation in chapter 5. Fractionation, monosaccharide and linkage analyses, enzymatic degradation, and mass spectrometry of the oligosaccharides in the digest of ChSS after enzymatic digestion with arabinogalactan degrading enzymes indicated the presence of common linear (1,4)-linked galacto-oligosaccharides, and both linear and branched arabino-oligosaccharides. In addition, the results unambiguously showed the presence of oligosaccharides containing (1,4)-linked galactose residues bearing an arabino*pyranose* residue at the non-reducing terminus, and a mixture of linear oligosaccharides constructed of (1,4)-linked galactose residues interspersed with one *internal* (1,5)-linked arabinofuranose residue. The presence of an internal arabinofuranose residue in a pectic arabinogalactan chain in cell wall polysacchairdes has not been reported previously, not in soybean, nor in other fruit or vegetable cell walls. Another uncommon feature is the presence of arabinopyranose residues in pectic arabinogalactan.

The pectic substances form only one network of the plant cell wall, the other is the cellulose/hemicellulose network. The hemicelluloses were solubilised from the residue with 1 and 4 M KOH solutions (chapter 6). The polysaccharides extracted with 1 M KOH were fractionated by ion-exchange chromatography, yielding a neutral and a pectic population. The sugar composition of the neutral population indicated the presence of xyloglucans and possibly xylans. Enzymatic degradation with endo-xylanases and endo-glucanases showed the presence of xyloglucan fragments only. Analysis of the digest formed after incubation of the neutral population with endo-glucanase V showed the formation of the characteristic poly-XXXG xyloglucan oligomers (XXG, XXXG, XXFG, XLXG, and XLFG), so three out of four glucose residues carry a side chain.

In chapter 7, the structural features of glucuronoarabinoxylans from maize kernels are described. First of all, maize kernel cell wall material was isolated as Water-Unextractable Solids (WUS). As expected the non-starch polysaccharides (NSP) had concentrated in the WUS (57%). These NSP were composed mainly of glucose, xylose, arabinose, and glucuronic acid. Sequential extractions with a saturated $Ba(OH)_2$-solution (BE1 extract), and distilled water (BE2 extract) were used to solubilise glucuronoarabinoxylans from maize WUS. The glycosidic linkage composition of the extracts and their resistance to endo-xylanase treatment indicated that the extracted glucuronoarabinoxylans were highly substituted. In the maize BE1 extract 25% of the xylose was unsubstituted, 38% was monosubstituted and 15% was disubstituted. The glucuronoarabinoxylans in maize BE1 appeared to be resistant to endo-

xylanase treatment, but could be degraded by a sub-fraction of Ultraflo, a commercial enzyme preparation from *Humicola insolens*. The digest contained a number of series of oligomers: pentose$_n$, pentose$_n$GlcA, pentose$_n$hexose, and pentose$_n$GlcA$_2$. The presence of these glucuronic acid-containing series of oligomers showed that the glucuronic acids in the glucuronoarabinoxylancan can be very close to each other, but are not distributed blockwise. Finally, a new measure for the degree of substitution of glucuronoarabinoxylans was defined. It turned out that the degree of substitution in maize BE1 is much higher (87%) than in sorghum (70%) and wheat flour BE1 (56%). This indicates that the glucuronoarabinoxylans in maize BE1 are more complex than those in sorghum BE1 and explains their resistance to endo-xylanase treatment.

From this research, it can be concluded that both soybean and maize kernel cell wall polysaccharides distinguish themselves in a number of respects from other plant cell walls polysaccharides. The absence of homogalacturonan, but also the presence of internal (1,5)-linked arabinofuranose and terminal arabinopyranose in the pectic arabinogalactan side chains from soybean cell walls and the complexity of the glucuronoarabinoxylan from maize kernel cell walls are discussed in chapter 8. In addition, it was shown that techniques like mass spectrometry and NMR spectroscopy are powerfull techniques to be used after (enzymatic) fragmentation, for chemical characterisation of the original polysaccharides.

SAMENVATTING

Het in dit proefschrift beschreven onderzoek betrof de opheldering van de chemische structuur van polysachariden uit de celwanden van sojabonen en maïskorrels. De twee onderzochte soorten vertegenwoordigen verschillende taxonomische groepen, sojabonen behoren tot de dicotyle en maïskorrels behoren tot de monocotyle planten. Naast het vertegenwoordigen van de belangrijkste structuren in de celwand, zijn deze twee grondstoffen van groot belang in zowel de levensmiddelen- als de veevoederindustrie.

De karakterisering van de celwandpolysachariden uit sojabonen ving aan met de isolatie van het celwandmateriaal (WUS) uit sojameel (hoofdstuk 2). De isolatiemethode leverde een WUS fractie op die bijna alle polysachariden en nauwelijks andere bestanddelen uit het meel bevatte. De WUS werd sequentieel geëxtraheerd met CDTA (Chelating agent Soluble Solids, ChSS), verdunde loog (Dilute Alkali Soluble Solids, DASS), 1 M loog (1 M Alkali Soluble Solids, 1 MASS) and 4 M loog (4 M Alkali Soluble Solids, 4 MASS) en uiteindelijk resteerde een celluloserijke fractie (RES). De pectinerijke fracties (ChSS en DASS) bleken dezelfde suikersamenstelling te hebben, ze bevatten voornamelijk galactose, arabinose en uronzuur residuen. De 1 MASS fractie bevatte naast deze drie suikers ook nog xylose. De hemicelluloserijke fractie (4 MASS) bevatte vooral xylose en glucose. Niets duidde erop dat er verschillen waren in de chemische structuur van ChSS en DASS, maar het is duidelijk dat de manier waarop ze in de celwand hebben gezeten wel anders was.

De intacte celwandpolysachariden in het meel en in de WUS konden nauwelijks afgebroken worden met enzymen. Wanneer ze eenmaal waren geëxtraheerd werden de polysachariden uit de WUS makkelijker gedegradeerd (hoofdstuk 3). De arabinogalactaanzijketens in de pectinerijke ChSS fractie konden voor een groot deel verwijderd worden door incubatie met endo-galactanase, exo-galactanase, endo-arabinanase en arabinofuranosidase B. Het overblijvende polymeer (fractie P) werd geïsoleerd en vertegenwoordigde 30% van de polysachariden in de ChSS fractie (12% van de polysachariden in de WUS). Dit polymeer bevatte nog steeds wat arbinose en galactose residuen die niet door de gebruikte enzymmengsels verwijderd konden worden.

De hoofdketen van de pectines (fractie P) bleek resistent te zijn tegen enzymatische degradatie door zowel bekende (zoals polygalacturonase) als relatief nieuwe pectolytische enzymen (zolas RG-hydrolase). Na zure hydrolyse van de geïsoleerde hoofdketen van de pectines werden een oligomere en twee polymere populaties verkregen na gelpermeatie chromatografie. Bepaling van de suiker- en bindingstype-samenstelling, enzymatische degradatie en NMR spectroscopie van de twee polymere populaties toonde aan dat de pectines in het originele ChSS extract zowel rhamnogalacturonaan als xylogalacturonaan bevatte en dat homogalacturonaan afwezig was (hoofdstuk 4). De afwezigheid van homogalacturonaan onderscheidt de pectines uit sojabonen van pectines uit andere bronnen. De meeste pectines bevatten zowel homogalacturonaan en rhamnogalacturonaan en kunnen

gedegradeerd worden met respectievelijk polygalacturonase en RG-hydrolase. Zure hydrolyse van fractie P verhoogt de gevoeligheid van de resterende polymeren voor RG hydrolase en exo-galacturonase.

Het xylogalacturonaan aanwezig in de ChSS fractie onderscheid zich van tot dusver bekend xylogalacturonaan uit andere bronnen. Een deel van de xylose residuen in het xylogalacturonaan is gesubstitueerd met fucose en het xylogalacturonaan is resistent tegen degradatie met XGH.

De arabinogalactaanzijketens, die werden verwijderd uit de ChSS fractie om zo fractie P te verkrijgen, werden onderzocht in hoofdstuk 5. Fractionering, bepaling van de suiker- en bindingstype-samnestelling, enzymatische degradatie en massa spectrometrie van de oligosachariden in het digest van ChSS na incubatie met arabinogalactaan-afbrekende enzymen, indiceerde de aanwezigheid van de veelvoorkomende lineaire (1,4)-gebonden galacto-oligosachariden en zowel lineaire als vertakte arabino-oligosachariden. Daarnaast lieten de resultaten zeer duidelijk de aanwezigheid zien van oligosachariden bestaande uit (1,4)-gebonden galactose residuen met een arabino*pyranose* residu aan het niet-reducerende einde en van een mengsel van lineaire oligosachariden bestaande uit (1,4)-gebonden galactose residuen met een *intern* (1,5)-gebonden arabinofuranose residu. Het bestaan van een intern arabinofuranose residu in een arabinogalactaanzijketen van een pectine uit de celwand werd niet eerder gerapporteerd, niet in sojabonen, maar ook niet in andere celwanden uit groente of fruit. Een ander ongewoon fenomeen is de aanwezigheid van arabinopyranose residuen in arabinogalactanen uit pectine.

De pectines vormen één netwerk van de plantencelwand, het andere is het cellulose/hemicellulose netwerk. De hemicelluloses werden uit de WUS geëxtraheerd met 1 en 4 M KOH oplossingen (hoofdstuk 6). De polysachariden die werden geëxtraheerd met 1 M KOH konden verder gefractioneerd worden met ionenwisselingschromatografie, resulterend in een neutrale en een geladen populatie (pectine). De suikersamenstelling van de neutrale populatie toont dat xyloglucanen en mogelijk ook xylanen aanwezig zijn. Enzymatische degradatie met endo-xylanases en endo-glucanases bevestigt alleen de aanwezigheid van xyloglucaan fragmenten. Analyse van het digest gevormd na incubatie van de neutrale populatie met endo-glucanase V toont de vorming van karakteristieke poly-XXXG xyloglucaan oligomeren (XXG, XXXG, XXFG, XLXG, and XLFG), dus drie van de vier glucose residuen dragen een zijketen.

In hoofdstuk 7 wordt de structuur van de glucuronoarabinoxylanen uit maïskorrels beschreven. Allereerst werd het celwandmateriaal uit de maïskorrels geïsoleerd (WUS). Zoals verwacht hadden de niet-zetmeel polysachariden (NSP) zich in de WUS geconcentreerd (57%). Deze NSP bestonden voornamelijk uit glucose, xylose, arabinose en glucuronzuur. Sequentiële extracties met een verzadigde $Ba(OH)_2$-oplossing (BE1 extract) en gedestilleerd water (BE2 extract) werden gebruikt om de glucuronoarabinoxylanen uit de maïs WUS in oplossing te brengen. De bindingstype-samenstelling van de extracten en hun resistentie tegen

degradatie met endo-xylanase geeft aanwijzingen voor het feit dat de geëxtraheerde glucuronoarabinoxylanen hoog vertakt zijn. In het maïs BE1 extract was 25% van het xylose ongesubstitueerd, 38% was enkel gesubstitueerd en 15% was dubbel gesubstitueerd. De glucuronoarabinoxylanen in maïs BE1 waren resistent tegen behandeling met endo-xylanase, maar konden wel afgebroken worden met een sub-fractie van Ultraflo, een commercieel enzympreparaat van *Humicola insolens*. Het digest bevatte een aantal series van oligomeren: $pentose_n$, $pentose_nGlcA$, $pentose_nhexose$, and $pentose_nGlcA_2$. De aanwezigheid van deze glucuronzuurbevattende series laat zien dat de glucuronzuren in het glucuronoarabinoxylaan zeer dicht bij elkaar kunnen zitten maar niet bloksgewijs over de keten verdeeld zijn. Een nieuwe maat voor de substitutiegraad van glucuronoarabinoxylanen werd gedefinieerd. Hieruit bleek dat de substitutiegraad in maïs BE1 veel hoger(87%) is dan in sorghum (70%) and tarwemeel BE1 (56%). Dit toont nog eens dat de glucuronoarabinoxylanen in maïs complexer zijn dan die in BE1 en verklaart hun resistentie tegen endo-xylanase.

Uit dit onderzoek kan worden geconcludeerd dat celwandpolysachariden uit zowel sojabonen als maïskorrels zich in een aantal aspecten onderscheiden van celwandpolysachariden uit andere planten. De afwezigheid van homogalacturonaan, maar ook de aanwezigheid van intern (1,5)-gebonden arabinofuranose en terminaal arabinopyranose in de arabinogalactaanzijketens van pectine en de complexheid van de glucuronoarabinoxylanen uit de celwanden van maïskorrels worden bediscussieerd in hoofdstuk 8. Verder laten de resultaten in dit proefschrift zien dat massa spectrometrie en NMR spectroscopie krachtige technieken zijn die na (enzymatische) fragmentatie kunnen worden gebruikt voor de chemische karakterisatie van de originele celwandpolysachariden.

CURRICULUM VITAE

Miranda Maryska Helena Huisman werd geboren op 4 maart 1969 te Leiden. In 1987 behaalde zij het VWO-diploma aan het Bonaventura Collega te Leiden. In datzelfde jaar begon zij met de studie levensmiddelentechnologie aan de Landbouwuniversiteit Wageningen. In het kader van deze studie deed zij afstudeervakken bij de leerstoelen Levensmiddelenchemie en Organische Chemie en bij het KVL Centre for Food Research in Kopenhagen, Denemarken. De studie rondde zij af met een stage bij Quest International te Naarden. In juni 1993 studeerde zij af.

Van oktober 1993 tot december 1994 werkte zij als toegevoegd onderzoeker aan een Europees onderzoeksproject. Van december 1994 tot juni 1999 werkte zij als Onderzoeker in Opleiding aan de Wageningen Universiteit bij het departement Agrotechnologie en Voedingswetenschappen, Laboratorium voor levensmiddelenchemie, onder begeleiding van dr. ing. H.A. Schols en prof. dr. ir. A.G.J. Voragen. Het onderzoek uitgevoerd in deze periode staat beschreven in dit proefschrift. Vanaf november 1999 is zij bij bovengenoemd Laboratorium in dienst als postdoc.

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