Propositions

1. Each plant species has its own pectin.
   This thesis

2. The rheological behaviour of okra rhamnogalacturonan (RG) I with acetyl groups and alpha galactose attached to rhamnosyl residues is greatly determined by hydrophobic interaction caused by the acetylation pattern.
   This thesis

3. The use of renewable plant biomass as raw material for a bio-based economy is hampered by the lack of tailored pretreatments enabling full saccharification of carbohydrates present as major components of the biomass.

4. The growing acceptance of so-called traditional medicines based on scientific evidence strongly enhances the search for new sources of "novel" bioactive plant polysaccharides.
   B.S. Paulsen, Current Organic Chemistry, 2001, 5, 939-950

5. When discussing the transesterification products of oligosacharides by lipases in anhydrous organic solvent, Tsukamoto et al. overlook the possibility that not the enzyme, but the molecular sieve used to dry the solvent is responsible for the formation of double and triple substituted oligosaccharides.
   J. Tsukamoto, S. Haebel, G.P. Valença, M.G. Peter, T.T. Franco, Journal of Chemical Technology & Biotechnology 83 (2008) 1486-1492.

6. In view of the often low production costs, wide application range, satisfaction for a substantial part of users and high profits it is recommended to explore the feasibility to launch the drug "Placebo".

Propositions belonging to the doctoral thesis of Nipaporn Sengkhamparn entitled
"Chemical, physical and biological features of Okra pectin"
Wageningen University, The Netherlands, 2 December 2009.

Chemical, physical and biological features of Okra pectin

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Chemical, physical and biological features of Okra pectin

Nipaporn Sengkhamparn

Thesis

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Prof. dr. M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Doctorate Board
to be defended in public
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Abstract

Okra pods, *Abelmoschus esculentus* (L.) Moench, are used in Thailand as vegetable as well as traditional medicine. Both textural and healthy properties are suggested to originate from the high polysaccharide content of okra pods, although our knowledge concerning the precise chemical structure of the different okra polysaccharides is far from complete. Consequently, the first aim of the research described in this thesis was to characterize all polysaccharides present in okra cell walls with emphasis on pectic molecules, and in addition to establish their physical and biological properties.

Okra cell wall polysaccharides were fractionation by sequential extraction and the pectins were obtained in the three fractions. Different types of rhamnogalacturonan I structures were recognized to be present in the buffer soluble and the chelating agent soluble fraction. By using enzymatic degradation of the pectins using pure and well characterized enzymes, followed by identification of the oligomers released by NMR and mass spectrometry, it was found that the buffer soluble, okra typical RG I, contained a rather unique substitution of the rhamnosyl moieties present in the backbone with acetyl groups and alpha galactosyl residues. The chelating agent soluble RG I was linked to homogalacturonan structural elements and resembled more commonly found RG I with substitutions of short chains of beta-linked galactose including some arabinose decoration.

The rheological properties of the okra typical RG I differed from other pectins since diluted solutions gave rise to very high viscosities and a slimy appearance. It is suggested that the acetylation of the rhamnosyl residues greatly affect its rheological properties and plays an important role through hydrophobic associations. Okra pectins also showed a high anti-complementary activity using the complement-fixing activity assay. Moreover, cell line studies indicated its possible use as a coating material for medical devices as demonstrated before for more complex RG I segments from apple pectin.

The hemicellulose populations found in okra cell walls, extracted with concentrated alkali, were characterized by sugar (linkage) composition and enzymatic degradation studies to be a XXXG-type xyloglucan and a 4-O-methylglucuronoxylan.

The research described leads to the recognition of new pectic RG I structures being quite different from the ones described so far.

Contents

Chapter 1

General Introduction

The okra plant

The okra plant *Abelmoschus esculentus* (L.) Moench belongs to the family of Malvaceae and originates from Africa. Today, it is cultivated in basically warm climate areas such as Asia, Middle East and the southern states of the USA. It can grow up to 2.5 m in height and carries a yellow mallow-type blossoms and a greenish fruit (Fig. 1) (BeMiller et al., 1993). The okra fruits/pods have a slightly curved shape with seeds inside containing high amounts of oil (BeMiller et al., 1993). The young and gentle (5 – 10 cm in length) fresh okra pods are often used as a vegetable, used for cooking or preserved by freezing or canning (Woolfe et al., 1977; BeMiller et al., 1993). Moreover, the okra pods are also used as a thickening agent in soup or stew (BeMiller et al., 1993), used as a traditional medicine for many different purposes; e.g. as diuretic agent, for treatment of dental diseases and to reduce/prevent gastric irritations (Ndjouenkeu et al., 1996; Lengsfeld et al., 2004).

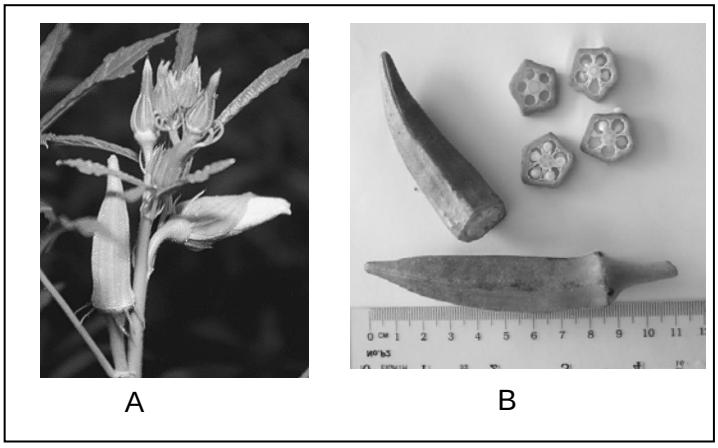

Figure 1 A) The okra plant (*A. esculentus* (L.) Moench) and
B) The okra pod

The plant cell wall

The plant cell is enclosed by a cell wall which consists of different macromolecules forming a complex network. One of the models of the cell wall which is currently the most popular is the "tethered network" model (Cosgrove, 2001) which is mainly based on the model of McCann and Roberts (1991) (Fig. 2). Generally, the primary walls from higher plant consists predominately of polysaccharides (cellulose, hemicelluloses and pectin) with a relatively small proportion of structural proteins, phenolic compounds, minerals and enzymes (Carpita and Gibeaut, 1993; O'Neill and York, 2003). The hemicellulose is linked to two or more cellulose microfibrils via hydrogen bonding (McCann and Roberts, 1991; Carpita and Gibeaut, 1993). Pectin and structural protein are imagined to act as a co-extensive network which is physically entangled with the cellulose-hemicellulose network (Hayashi, 1987; Fry, 1988; Cosgrove, 2001). The different polysaccharides present in plant cell wall materials can be extracted by sequential extraction with different aqueous extractants (Voragen et al., 1995). The structural elements of the various polysaccharides from plant cell wall material are shown in Figure 3 and will be further described below.

Celluloses

Cellulose is a homopolysaccharide consisting of a backbone of β (1, 4)-linked-D-glucosyl residues with wide molecular weight distribution (Åman and Westerlund, 1996). A cellulose molecule in a higher plant cell wall may contain 2000 – 6000 glucose building blocks (Delmer, 1987). The individual glucan chains can be aggregated and form regular crystallines by hydrogen bonding (Kroon-Batenburg and Kroon, 1997). In crystalline native cellulose, 2 different amorphous cellulose forms can be recognized: cellulose I_α (triclinic) and cellulose I_β (monoclinic) with a ratio of I_α/I_β which depend on the origin of the cellulose (VanderHart and Atalla, 1984). Cellulose is the major component which determines the strength properties of wood pulp fibers, cellulose I_β is the most thermodynamically stable form (Åkerholm et al., 2004). Many techniques have been reported to determine the composition of both amorphous cellulose I forms such as FT-IR

spectroscopy (Sassi et al., 2004), dynamic FT-IR spectroscopy (Åkerholm et al., 2004), NMR (VanderHart and Atalla, 1984; Yamamoto et al., 1996).

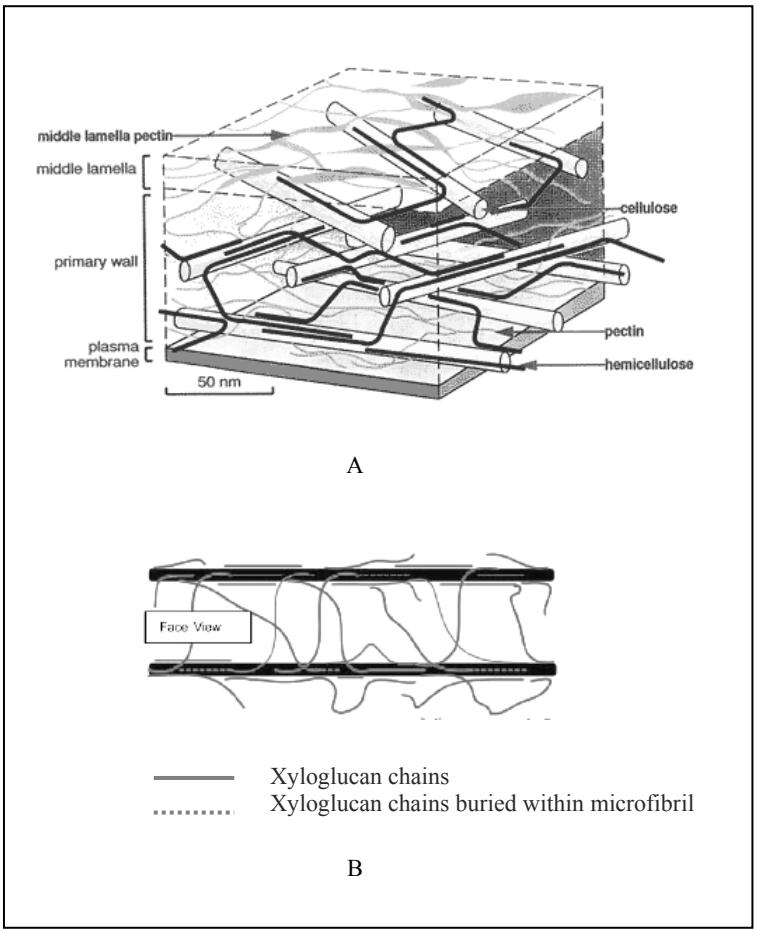

Figure 2A Simplified schematic view of the primary plant cell wall
(McCann and Roberts, 1991)
2B The "tethered network" (Cosgrove, 2001)

Hemicelluloses

Hemicelluloses are defined as polysaccharides in plant cell walls which are solubilized by aqueous alkali. Xyloglucans, xylans, mannans and arabinogalactans are the most abundant representatives (O'Neill and York, 2003).

Xyloglucans are the most abundant hemicellulose in dicotyledons. Their precise chemical structure strongly depends on their origin. Xyloglucan is generally composed of a backbone of β-(1,4)-linked-D-glucosyl residues with two or three out of four D-glucosyl residues being substituted at position O-6 with α-D-xylosyl residues (Vierhuis et al., 2001). Moreover, some of the D-xylosyl residues can be substituted with either monosaccharides (D-galactose or L-arabinose) or disaccharides (e.g. D-galactose-L-fucose) (Vierhuis et al., 2001). The xyloglucans are classified into 2 types, poly-XXXG type and poly-XXGG type depending on the degree of glucan backbone substitution with D-xylosyl residues (Fry et al., 1993). Recently, Hilz et al. (2007) found the α-D-xylosyl-β-(1,4)-D-xylosyl side chain of xyloglucan in bilberries.

Xylans represent an other types of hemicellulose present in the cell wall and have a backbone consisting of β-(1,4)-linked-D-xylosyl residues. The D-xylosyl residues in the backbone as present in monocotyles (cereals and grasses) can be substituted with L-arabinosyl residues, D-glucuronosyl residues (or its 4-O-methyl derivative) and acetyl groups (McNeil et al., 1984; Brett and Waldom, 1990; Åman and Westerlund, 1996). In dicotyles (hard woods and herbs) regularly highly acetylated acidic (O-methyl) glucuronoxylans almost without any arabinose substitution can be found (McNeil et al., 1984; Brett and Waldom, 1990).

Pectins

Pectin is an important cell wall component of plants and probably the most complex macromolecule in nature (Vincken et al., 2003). Pectin is composed of 17 different monosaccharides which are arranged in a number of structural elements forming the building blocks of the pectin network which is shown in Figure 3 (Mohnen, 1999; Vincken et al., 2003; O'Neill et al., 2004). The pectin backbone can be classified into three

classes based on the elements present; homogalacturonan, substituted galacturonan and rhamnogalacturonan.

Homogalacturonan (HG) is composed of a backbone of α-(1,4)-linked-D-galacturonosyl residues in which a variable part of the galacturonic acid is methyl esterified (Talmadge et al., 1973). The degree of methyl esterification (DM) in HG can classify pectin into 2 main types, high DM pectin (more than 50%) and low DM pectin and it strongly influences their functionality (Voragen et al., 1995). Parts of HG may be cross-linked and be involved in forming a three dimensional network, a pectin gel, which is important in controlling the porosity and mechanical properties of the cell wall and contributing to the maintenance of intercellular adhesion (Carpita and Gibeaut, 1993; Willats et al., 2006). The gelling property of pectins may be influenced by many different factors such as type and origin of the pectin and the level and distribution of methyl esters and acetyl groups. The methyl esters can be distributed either randomly or block-wise over the HG segment which strongly effect the calcium binding of pectin as will be discussed later. In addition, HG may be partially O-acetylated at position O-2 and/or O-3 of the D-galacturonosyl residues such as pectin from sugar beet where the presence of acetyl groups has a negative effect on the gelling behaviour (Rombouts and Thibault, 1986; Oosterveld et al., 2000).

HG can also be branched with a single unit of β-D-xylose residue or longer 1,2-linked or 1,4 linked β-D-xylose chains attached to O-2 and O-3 of the D-galacturonosyl residues which is called xylogalacturonan (XGA) (Schols et al., 1995; Albersheim et al., 1996; Le Goff et al., 2001; Nakamura et al., 2002; Zandleven et al., 2007). Like HG, part of D-galacturonosyl residues in XGA may be methyl esterified (Schols et al., 1995; Yu and Mort, 1996).

Rhamnogalacturonan (RG) I contains a backbone of alternating α-(1, 2)-linked-L-rhamnosyl and α-(1,4)-linked-D-galacturonosyl residues with the ratio of rhamnose to galacturonic acid of 1:1 (McNeil et al., 1980; Lau et al., 1985; Schols et al., 1990). Many RG I are partially O-acetylated at position O-2 and/or O-3 of the D-galacturonosyl residues but so far, no evidence has been reported about the presence of methyl esterified D-galacturonosyl residues within the RG I backbone (Ishii, 1997; Perrone et al., 2002). Approximately 20-80 % of all L-rhamnosyl residues are branched at position O-4 with β-

(1, 4)-linked galactan (Gur'janov et al., 2007), α-(1,5)-linked arabinan or branched arabinans (Ridley et al., 2001; Vincken et al., 2003) depending on origin and plant tissue (McNeil et al., 1982; Lau et al., 1987; Ishii et al., 1989). Besides, arabinogalactan type I (AG I) and type II (AG II) may be presented as side chains of RG I. AG I consists of a β-(1, 4)-linked galactan backbone with α-L-arabinofuranosyl residues attached to the O-3 position of D-galactosyl residues (Vincken et al., 2003). AG II is a branched polymer composed of a backbone of β-(1,3)-linked-D-galactosyl residues containing side chains of α-L-Ara*f*-(1→6)-[β-D-Gal*p*-(1→6)]n (n=1,2 or 3) (Mohnen, 1999; Ridley et al., 2001; Vincken et al., 2003).

Rhamnogalacturonan II is a special structural element of pectin, which has a highly conserved structure and is found in many different plants (O'Neill et al., 2004). It was found to be covalently linked to HG (O'Neill et al., 2001) and consists of 12 different sugars with some very peculiar sugar building blocks such as Apiose, Aceric Acid, 3-deoxy-D-lyxo-2-heptulosaric acid (DHA) and 2-keto-3-deoxy-D-manno-octulosonic acid (DKO) (O'Neill et al., 2004). RG II is composed of a backbone of α-(1,4)-linked-D-galacturonosyl residues to which four well conserved but different side chains are connected (O'Neill et al., 2004).

Enzymatic degradation

According to the specificity and selectivity of enzymes, analysis of digests obtained from incubation of complex polysaccharides with well characterized enzymes can provide valuable information of the structures of the polysaccharides (De Vries, 1982; Schols et al., 1990; Daas et al., 1999). The potential use of enzymatic degradation in the elucidation of the structure of polysaccharides depends on the purity of the enzyme used, and on the knowledge of their substrate specificity and pattern of their action (Voragen et al., 1993).

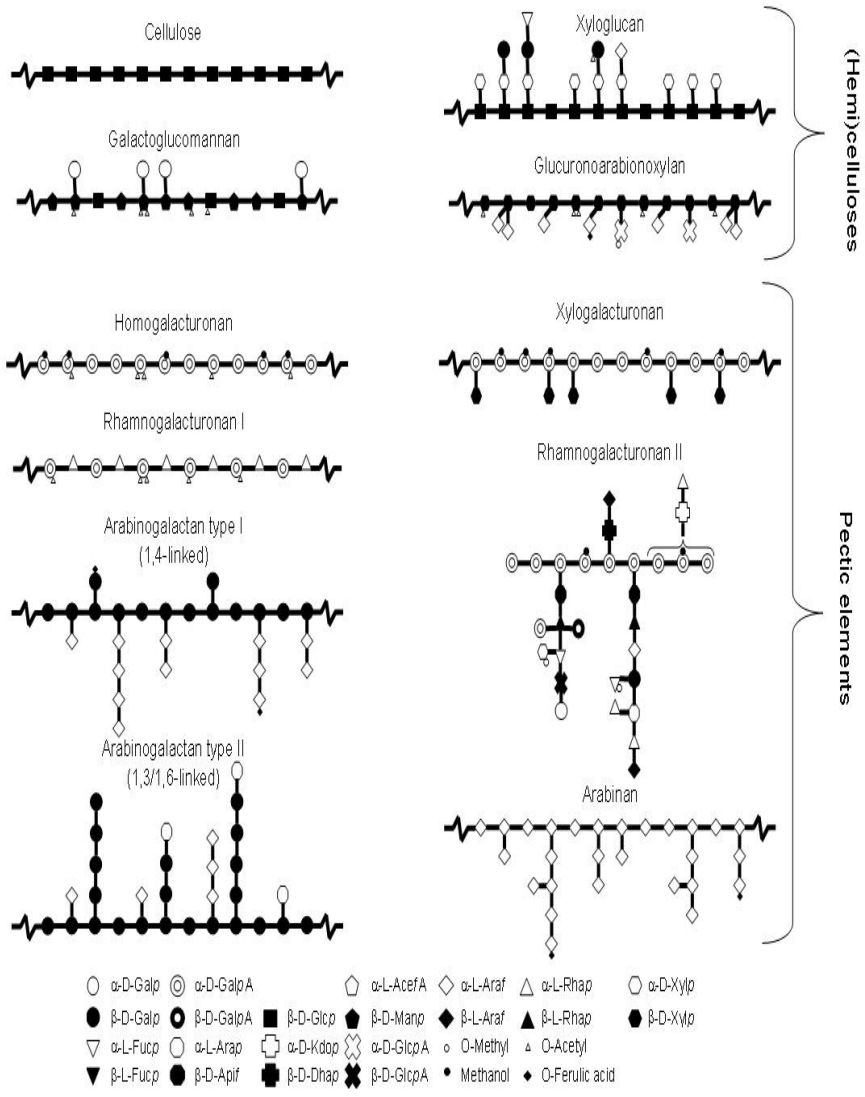

Figure 3 Schematic representation of plant cell wall polysaccharides (Hilz, 2007)

Hemicellulosic enzymes

Hemicelluloses, present in plant cell wall are xyloglucans, xylans, mannans and arabinogalactans (O'Neill and York, 2003). Xyloglucans can be degraded by xyloglucan specific endo-glucanase. This enzyme cleaves linkages between unsubstituted glucose and xylose-substituted glucosyl residues (Pauly et al., 1999). The released oligomers always contain an unbranched glucose moiety at the reducing end (Hilz et al., 2007) and analysis of all fragments may provide valuable information about the different building blocks present within the xyloglucan under investigation (Fry et al., 1993; Hilz et al., 2007).

More information about structure of xylans can be revealed by using endo-xylanases from different Glycosyl Hydrolase families (http://www.cazy.org/) having different modes of actions towards substituted xylans. In general family GH 10 xylanases may be able to cleave rather close to a substitution side releasing shorter (substituted) xylo-oligosaccharides, while family GH 11 xylanases are more hindered by substitution with different side groups (Biely et al., 1997; Rantanen et al., 2007).

Pectic enzymes

Pectin modifying enzymes can be devided into hydrolases and lyases both able to split within the pectin backbone, and esterases able to specifically remove either the methyl ester or the acetyl group from pectins. Specific groups of such enzymes have been recognized to act either on galacturonans or rhamnogalacturonans (Benen et al., 2003; Benen and Visser, 2003; Vincken et al, 2003).

Polygalacturonase (PG) cleaves the linkage between galacturonic acid by hydrolysis (Benen and Visser, 2003). The PGs can be divided into endo PGs and exo PGs. Endo PGs cleaves the HG backbone randomly and needs a number of adjacent non-methyl-esterified galacturonosyl residues (Benen and Visser, 2003; Vincken et al., 2003; Daas et al., 1999), while the exoPGs cleave mono- or di-mers from the non reducing end of a HG backbone and need a non esterified GalA residue at subsite -2, -1 and +1 of the enzyme (Benen and Visser, 2003). The PG action is hindered by the presence of methyl esters and acetyl groups (Vincken et al., 2003) and consequently, the activity of PG can be improved by simultaneously using pectin methyl esterase and/or pectin acetylesterase (Pilnik and

Voragen, 1993; Searle-Van Leeuwen et al., 1996; Benen et al., 2003). The released oligomers may provide valuable information concerning the ester distribution over the HG segment (Daas et al., 2000 and 2001; Guillotin et al., 2005). Pectate lyase (PAL) and pectin lyase (PL) cleave the linkage between galacturonic acid by β-elimination and form end products with a Δ4, 5 unsaturated bond at the non-reducing end (Benen and Visser, 2003). PL cleaves highly methyl esterified pectins and needs a methyl esterified GalA residue next to the cleaved linkage while PAL cleaves HG and low methyl esterified pectins (Benen and Visser, 2003). Pectin esterases (PEs) hydrolyze the esters linkage from the HG backbone which can be classified into 2 classes, pectin methyl esterase (PME), pectin acetyl esterase (PAE). The PME hydrolyze the methyl esters from GalA residues while PAE hydrolyzes the acetyl groups from the O-2 and/or O-3 position of the GalA residues in the HG backbone (Benen et al., 2003). Endogenous plant PME in general hydrolase methyl esters from HG in a blockwise manner while fungal PME hydrolyse methyl ester in a random manner (Benen et al., 2003).

The endogenous pectic enzymes are initializing the textural change in fruits and vegetables during ripening, storage and processing (Pilnik and Voragen, 1991). Endogenous (endo) PME can protect and improve the texture and firmness of processed fruits and vegetables such as in apple slices (Wiley and Lee, 1970), carrot (Lee et al., 1979) and canned tomato (Hsu et al., 1965). Endo PME present in citrus fruit is the cause of cloud loss in orange juice (Pilnik and Voragen, 1991). The addition of exogenous pectic enzymes (PG or PL) can prevent the cloud loss in juice by the break down of the polymeric pectin, before calcium coagulation, to low molecular weight segments which are not calcium sensitive (Pilnik and Voragen, 1991). Moreover, the exogenous pectic enzymes are also used as processing aid such as a combination of endo PG and PME in juice liquefaction (Pilnik and Voragen, 1991) and in apple juice clarification (Endo, 1965).

The class of enzymes specific for the RG I backbone has been reported so far to consist of 4 types of enzymes: RG-hydrolase, RG-rhamnohydrolase, RG-galacturonohydrolase and RG-lyase. The RG-hydrolase is able to cleave the GalA-Rha linkage within RG I after removal of acetyl groups present (Searle-van Leeuwen et al., 1996; Mutter et al., 1996 and 1998b). The RG-rhamnohydrolase removes the terminal non-

reducing rhamnosyl residues (Mutter et al., 1994) while RG- galacturonohydrolase removes galacturonic acid from the RG segment (Mutter et al., 1998a). The RG-lyase cleaves the Rha-GalA linkage releasing fragments with an unsaturated GalA unit at the non-reducing end and a rhamnosyl residue at the reducing end of the fragments (Mutter et al., 1996). Rhamnogalacturonan acetyl esterase (RGAE) acts towards the RG I-backbone which hydrolyzes the acetyl groups attached to the GalA residues of the RG I backbone (Benen et al., 2003).

Okra polysaccharide structure

Previous investigations of the composition and properties of okra polysaccharides have been reviewed by BeMiller et al. (1993). Various workers have reported different compositions of the polysaccharides, most probably caused by the differences in the extraction procedure and the use of different okra varieties.

Whistler and Conrad (1954) reported that okra from southern USA contained an acidic polysaccharide which consisted of galactose, rhamnose and galacturonic acid as the main sugars. Recently, Lengsfeld et al. (2004) and Deters et al. (2005) mentioned that an okra polysaccharide from a commercial supplier in Germany also contained glucose and glucuronosyl residues. In addition, Agarwal et al. (2001) reported that the galacturonic acid in okra polysaccharides from India was in the L-configuration form.

Okra polysaccharides grown in Japan, extracted with cold water and purified using a 10 % chelating agent solution was shown to contain a repeating unit of α-(1,2)-linked rhamnosyl and α-(1,4)-linked galacturonosyl residues and dimeric β-(1,4)-linked galactan side chains which were substituted to O-4 of half of the rhamnosyl residues (Tomada et al., 1980). It was demonstrated that the pectin was acetylated (5.5 % w/w) and contained about 12% protein (Tomada et al., 1980). However, the precise position of the acetyl groups within the pectin was not mentioned.

A further fractionation of water-extracted pectic polysaccharides from de-seeded okra pods was done by Lengsfeld et al. (2004) using anion exchange chromatography. From the linkage composition data, it was concluded that some of the okra pectin

subpopulations consisted of a rather pure galacturonan structural element. Information concerning other polysaccharides like hemicelluloses is still missing.

Functional properties of pectins

Pectin are widely used as gelling, stabilizing or thickening agent in many food products such as jam, yoghurt drink, fruity milk drinks and ice cream (Laurent and Boulenguer, 2003).

The viscosity of a polysaccharide solution depends on many factors such as molecular mass, hydrodynamic volume, stiffness and charge of the molecule (Williams and Phillips, 2000). The charged polymers generally have a higher viscosity than non-ionic polymers at similar mass and chemical structure due to the intermolecular charge repulsion (Williams and Phillips, 2000). Although pectin carries free carboxyl groups on the backbone and it behaves as a polyelectrolyte (Voragen et al., 1995), pectin has not been used very frequently as a thickener because pectin solutions have relatively low viscosities when compared to other biopolymers at similar concentrations (Voragen et al., 1995). The viscosity of pectin solutions depend on chemical and physical characteristic of the pectins, on the ionic strength of the solution (Pals and Hermans, 1952) and on the presence of sugar (Chen and Joslyn, 1967; Michel et al., 1985).

The junction zones of hydrocolloid gels are normally formed via physical interaction such as hydrogen bonding, hydrophobic association and cation-mediated cross linking (Williams and Phillips, 2000). Previous studies have shown that the gel properties of pectin gels strongly depend on molecular properties of the polymer. The gelation properties of pectins are influenced by the molecular weight of the pectin, the length of the pectin side chains, the level and distribution of methyl esterification and the level of acetylation. For example, Schmelter et al. (2002) suggested that pectin with shorter side chains gave better gelation properties than pectin with longer side chains. Moreover, the pattern of esterification, block-wise or random, has a great impact on gel characteristics (Willats et al., 2001) the enzymatic removal of acetyl groups as present in sugar beet pectin led to an improved gelation and a much stiffer gel (Oosterveld et al., 2000).

Okra polysaccharide functional properties

Solutions of okra polysaccharides were found to exhibit pseudoplastic and viscoelastic behaviour (BeMiller et al., 1993). Baht and Tharathan (1987) found that the viscosity of the okra polysaccharides extracted with water showed a maximum viscosity in the pH range of 4-6. The viscosity of okra polysaccharides decreased by addition of glucose and sucrose (5-40%) and divalent salts (0.1-10% of $CaCl_2$ and $MgSO_4$) in contrast to a rather small increase found after addition of maltodextrin and the presence of 0.1-10% of monovalent salts (NaCl and KCl). Furthermore, the viscosity of an okra polysaccharide solution sharply increased with increasing concentration. Woolfe et al (1997) reported that heating okra polysaccharide solutions to 90 ^{o}C resulted in a decrease in viscosity while cooling the solution back to room temperature caused in increase in the viscosity.

Okra pectin was found to form a gel at a relatively low concentration (6 g/L) (Woolfe et al., 1977), while the formation of a stable gel also has been reported after heating okra polysaccharides at 60 ^{o}C for 30 min followed by a cooling step at 4 ^{o}C for 24 h (Baht and Tharathan, 1987). In addition, a synergistic effect was found when okra polysaccharide was mixed with xanthan gum; mixing with locust bean gum did not give any synergistic effect (Baht and Tharathan, 1987).

Okra polysaccharide was also shown to have unusual lubricity properties and to be able to form a tenacious coating on the skin which is difficult to remove by washing (BeMiller et al., 1993). The lubricity property is also an important property for a good food fat mimetic (Glicksman, 1991) and okra polysaccharides can be used as fat substitute in many products like chocolate bars and cookies. Many quality characteristics of such fat free cookies were comparable with those of full fat cookies (Romanchik-Cerpovicz et al., 2002). Likewise, okra polysaccharide has also been used as a milk-fat substitute in chocolate frozen dairy desserts where it could replace the milk-fat up to 70 % while the melting points of the products did not change, although the melting rate decreased slightly (Constantino & Romanchik-Cerpovicz, 2004). Okra polysaccharides also exhibit foam (Baht and Tharathan, 1987) and emulsion stabilizing properties (BeMiller et al., 1993). It behaves like egg white at higher concentrations which can form threads and stabilize

foams, therefore, okra polysaccharides is also use as a dried egg white substitute (Woolfe et al., 1977).

Biological properties of pectins

Pectins are gaining interest for their health promoting properties and many studies in this field have been reported. Citrus pectin, derived from extracted citrus pulp and peel, was shown to inhibit lung and bone metastasis in humans (Yamada, 2000; Glinskii et al., 2005). Citrus pectin with DM 90 exhibited anti-inflammatory properties by binding lipopolysaccharides (Chen et al., 2006) and, water extracted pectin from roots of *Bupleurum falcatum* exhibited anti-ulcer activity against hydrochloric acid-ethanol induced ulserogenensis in mice (Yamada et al., 1991).

One of the interesting health promoting functions of pectins is its property to influence the human immune system, making pectin belonging to the group of immunomodulators (Diallo et al., 2001). Many publications are indicating that the RG I regions may influence the complement system. A (1,6)–linked galactose containing side chains seem to be connected with such bioactivity (Yamada and Kiyohara, 1999). The presence of 3-O-methylgalactose in the polysaccharides from a Thai medicinal plant *Acanthus ebracteatus* was reported to play a role in its bioactivity (Hokputsa et al., 2004). Recently, Westereng et al., (2006) reported that Arabinogalactan type II and/or arabinan structural elements from cabbage pectin were of key importance for an in *vitro* anti-complementary activity.

During the last decade, modification of the surface of medical materials and devices with polysaccharides are a topic of great interest. Plant pectins are found to be a potential biomaterial for coating applications due to their anti-inflammatory properties and the ability to control their structure (Morra et al., 2004; Chen et al., 2006; Kokkonen et al., 2007). Many researches have investigated the potential of hairy regions of pectin for coating medical devices and materials, however the relationship between the structure of pectic hairy region and its direct/indirect biological effects has not been clearly elucidated.

Although no correlation has been made with the pectin present, also okra is considered to exhibit health promoting effects. Okra pods are used in folk medicine as a

diuretic agent, in treatment of dental disease (Ndjouenkeu et al., 1996) and in the treatment of gastric irritative and inflammative diseases (Lengsfeld et al., 2004). The okra polysaccharide showed anti-complementary and hypoglycaemic activity which were related to the RG I structure present in okra (Tomoda et al. 1989). In addition, okra polysaccharides may be able to lower the cholesterol level in blood and may prevent cancer, due to its capability to bind bile acids and, consequently prevent recirculation of the bile acids (Kahlon et al., 2007).

Aim of the thesis

The aim of this thesis is:

(i) to elucidate the chemical fine structure of polysaccharides as present in the cell wall of okra pods. Although some chemical characteristic of okra pectins have been described in the literature, the other polysaccharide such as hemicelluloses are still very poorly described. Chapter 2 describes the first characterization of all main cell wall polysaccharides present in okra cell wall. Okra cell wall material was sequentially extracted with different aqueous extractants to obtain different pectin-, hemicellulose- and cellulose-fractions. Two pectin fractions, one extracted by hot buffer and another one by chelating agent were further characterized and described in chapter 3. The structure of both pectins was elucidated by making use of pure and well characterized enzymes, followed by analyses of the oligomers released. Special attention has been directed to reveal the position of acetyl groups present and to establish the length of galactan side chains substituted to the pectin backbone.

(ii) to come to a better understanding of the relationship between chemical structure and functionality (chapter 4). The rheological properties of the two pectins obtained from sequential extraction were studied and related to their chemical characteristics.

(iii) to obtain a better understanding of the relation of structural and bioactive properties of okra pod polysaccharides. The complement- fixing activity was performed to check for bioactivity and to investigate the effect of molecular weight of RG I and the effect of the presence of acetyl groups (chapter 5). Chapter 5 also describes the

performance of okra RG I as compared to hairy regions of other pectic material in the surface modification for implant material.

Chapter 6 summaries all information about okra polysaccharide structures and some ideas about structure function relationships are given. Implications of our findings as a lesson for the structural investigation of pectins from other source are discussed.

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Chapter 2

Characterization of Cell Wall Polysaccharides from Okra (*Abelmoschus esculentus* (L.) Moench)

Sengkhamparn, N., Verhoef, R., Schols, H.A., Sajjaanantakul, T., & Voragen, A.G.J. Characterization of Cell Wall Polysaccharides from Okra (*Abelmoschus esculentus* (L.) Moench). *Carbohydr. Res*., 2009, 344, 1824-1832.

Abstract

Okra pods are commonly used in Asia as a vegetable, food ingredient, as well as a traditional medicine for many different purposes; for example, as diuretic agent, for treatment of dental diseases and to reduce/prevent gastric irritations. The healthy properties are suggested to originate from the high polysaccharide content of okra pods, resulting in a highly viscous solution with a slimy appearance when okra is extracted with water. In this study, we present a structural characterization of all major cell wall polysaccharides originating from okra pods. The sequential extraction of okra cell wall material yielded fractions of soluble solids extractable using hot buffer (HBSS), chelating agent (CHSS), dilute alkaline (DASS) and concentrated alkaline (CASS). The HBSS fraction was shown to be rich in galactose, rhamnose and galacturonic acid in the ratio 1.3:1:1.3. The degree of acetylation is relatively high (DA = 58) while the degree of methyl esterification is relatively low (DM = 24). The CHSS fraction contained much higher levels of methyl esterified galacturonosyl residues (63% galacturonic acid; DM = 48) in addition to minor amounts of rhamnose and galactose. The ratio of galactose to rhamnose to galacturonic acid was 1.3:1.0:1.3 and 4.5:1.0:1.2 for HBSS and CHSS, respectively. These results indicated that the HBSS and CHSS fractions contain rhamnogalacturonan type I next to homogalacturonan, while the latter is more prevailing in CHSS. Also the DASS fraction is characterised by high amounts of rhamnose, galactose, galacturonic acid and some arabinose, indicating that rhamnogalacturonan I elements with longer arabinose- and galactose-rich side chains were part of this fraction. Partial digestion of HBSS and CHSS by pectin methyl esterase and polygalacturonase resulted in a fraction with a lower Mw and lower viscosity in solution. These samples were subjected to NMR analysis, which indicated that, in contrast to known RG I structure, the acetyl groups in HBSS are not located on the galacturonosyl residues, while for CHSS only part of the acetyl groups are located on the RG I galacturonosyl residues. The CASS fraction consisted of XXXG-type xyloglucan and 4-methylglucuronoxylan as shown by their sugar (linkage) composition and enzymatic digestion.

KEY WORDS: Okra, Polysaccharides, Pectin, Xyloglucan, Xylan

Introduction

The okra plant, *Abelmoschus esculentus* (L.) Moench, a native plant from Africa, is now grown in many other areas such as Thailand, the Middle East and the southern states of the USA. The okra pod is often used as a vegetable. Its water extracts contain thick slimy polysaccharides and are used to thicken soups and stews (Woolfe et al., 1977; BeMiller et al., 1993). The immature fruit is also used in folk medicine as a diuretic agent and for treatment of dental disease (Ndjouenkeu et al., 1996). Okra polysaccharides are also used as egg white substitute (Costantino and Romanchick-Cerpoviez, 2004), fat substitute in chocolate bar cookies (Romanchik-Cerpovicz et al., 2002) and in chocolate frozen dairy dessert (Romanchik-Cerpovicz et al., 2006).

The okra polysaccharide was found firstly as an acidic polysaccharide consisting of galactose, rhamnose and galacturonic acid (Whistler and Conrad, 1954). Deters et al. (2005) confirmed the findings as mentioned by Lengsfeld et al. (2004) that okra polysaccharide consisted of the sugars rhamnose, galacturonic acid, galactose, glucose and glucuronic acid. Agarwal et al. (2001) suggested that galacturonic acid in the okra polysaccharide could be in the L-configuration. The main structural elements of okra polysaccharide was described by Tomada et al. (1980) who concluded that it contained a repeating unit of alternating α-(1→2)- linked rhamnosyl and α-(1→4)-linked galacturonosyl residues with a disaccharide side chain of β-(1→4)-linked galactosyl moieties attached to O-4 of about half the L-rhamnosyl residues. The acetyl content of the okra polysaccharide was determined to be 5.5% w/w while the precise position of the acetyl groups within the polysaccharides was not mentioned. Lengsfeld et al. (2004) suggested from linkage analysis data that okra polysaccharide sub-fractions, which were extracted by water and fractionated by anion-exchange chromatography, contained more galacturonan than rhamnogalacturonan as the main structural elements.

In contrast to the chemical characteristics of okra pectin, the information about other polysaccharides like hemicelluloses is still lacking. In this study, we present the characterization of all main cell wall polysaccharide in okra, which were extracted sequentially with different aqueous extractants of increasing strength, with emphasis on the detailed structures of the various pectin fractions.

Results and discussion

Specific parts of the okra pod

In order to have an impression on the proportion of individual parts of the fresh okra pods, the whole okra pod was separated into three parts; calyx, pulp and seed. The relative amounts of the different parts of fresh okra expressed as fresh weight, dry weight and alcohol-insoluble solids (AIS) are shown in Table 1. The pulp was the major part (~ca. 72 g/100 g) of fresh okra, whereas the calyx represented ~ca.15 g/100 g of fresh okra and the seed represented ~ca. 9 g/100 g of fresh okra. As can been seen from the figures, about 4% of the material was not recovered and this is probably due to some losses of the seed fraction. Since the okra pulp formed the major part of the okra pod, this part was subjected to further studies. The okra pulp yielded about 5.8 g/100 g fresh okra of alcohol-insoluble solid (AIS) representing cell wall materials.

Table 1 Relative amount of the different parts of fresh okra pods

Parts of okra pods	Fresh weight[a)]	Dry weight[a)]
calyx	14.6	1.4
pulp	71.9	7.4
seed	9.1	1.3

[a)] gram qualities per 100 g of fresh okra pods

Sugar composition and absolute configuration of okra AIS and okra AIS extracts

The sugar composition of okra AIS is shown in Table 2. Okra AIS consisted of mainly glucose (44 mol %), galactose (17 mol %) and galacturonic acid (16 mol %). In addition to polysaccharides the AIS also contained 15.8% of protein. The sugar composition suggests that okra AIS consisted of different types of polysaccharides including pectin, hemicelluloses such as xylan and xyloglucan, and cellulose. The sequential extraction of the cell wall material (AIS) provides information about the extractability of the different polysaccharides, namely pectins, hemicellulose and cellulose

(Voragen et al., 1995). The okra AIS was therefore sequentially extracted with different aqueous extractants. Table 2 shows the sugar composition of the fractions obtained from sequential extraction of okra AIS of which the HBSS and DASS fractions were the main fractions.

The HBSS fraction consisted of 35 mol % of galacturonic acids, in addition to high amounts of rhamnose (26 mol %) and galactose (34 mol %). Galactose was found as the main neutral sugar in the HBSS fraction, and about 30% of all galactose present in the AIS was recovered in this fraction. The ratio of the main sugars presented in the HBSS fraction was 1.3:1.0:1.3 for galactose:rhamnose: galacturonic acid, respectively, which was rather similar to that reported by Tomada et al. (1980). Lengsfeld et al. (2004) reported that this ratio was 0.9:1.0:0.6 for the okra polysaccharide water extract. Compared to this result, our HBSS fraction contained slightly more galactose. This was also true when compared with the data for water-extracted okra polysaccharides as described by Deters et al. (2005).

The CHSS fraction contained higher amounts of galacturonic acid content (63 mol %) and less galactose (17 mol %) and rhamnose (14 mol %) when compared to the HBSS fraction. The CHSS fraction contained also 3 mol % arabinose while this sugar was not found in the HBSS fraction. No xylose was found in the HBSS, neither in the CHSS fractions providing evidence that no xylose containing pectic polymer like xylogalacturonan was present in both fractions. In addition, the HBSS and CHSS fractions contained low levels of glucuronic acid (3 and 2 mol %, respectively) which was lower than the levels of glucuronic acid found in water-extracted okra polysaccharides (8.8 mol %) as described by Lengsfeld et al. (2004).

The HBSS fraction had a higher ratio of rhamnose to galacturonic acid (0.7) than the CHSS fraction (0.2). Generally, rhamnogalacturonan I (RG I) consists of alternating rhamnose and galacturonic acid residues as a backbone (Schols and Voragen, 2002). The ratio of rhamnose: galacturonic acid within a RG I backbone is 1:1. Consequently, the HBSS fraction was found to contain mainly RG I segments (85%) and much less homogalacturonan (HG) segments. The CHSS contained mainly HG segments (74%) and less RG I segments. The high amounts of RG I segments in the HBSS fraction is a bit uncommon since no water extract from other sources showed such a high amount of RG I

segments. For example, water soluble soybean polysaccharide contained 43% of RG I segments within the polysaccharides (Wang et al., 2005), water extracts from sugar beet pulp contained 22% of RG I segments within the polysaccharides (Oosterveld et al., 1996). The ratio of neutral sugars to rhamnose roughly indicates the length of the side chains. The ratio of (galactose and arabinose) to rhamnose was 1.3 and 1.4 for the HBSS and CHSS fractions, respectively. This suggests that the CHSS fraction contained slightly longer side chains than the HBSS fraction.

The absolute configuration of sugar moieties in the HBSS and the CHSS fractions were determined by using GC-FID after methanolysis and conversion to their corresponding butylglycosides. The results showed that all sugars as present in the HBSS and CHSS fractions were in D-configuration except for the rhamnosyl residues which were in the L-configuration. These results are in contrast with those reported by Agarwal et al. (2001) who stated that the okra gum contain L-galacturonic acid. The HBSS, okra rhamnogalacturonan I is also different from rhamnogalacturonan I of flax seed mucilage which contains L galactose as a neutral sugar (Naran et al., 2007).

The DASS fraction contained high amounts of galactose and galacturonic acid. The ratio of rhamnose to galacturonic acid (0.3) was higher than that found for the CHSS fraction (0.2). It can be calculated that the DASS fraction contained 43% of RG I segments and 57% of HG segments. Furthermore, the DASS fraction was relatively enriched in arabinose and galactose. The ratio of arabinose to rhamnose and the ratio of (arabinose and galactose) to rhamnose of the DASS fraction were 1.0 and 2.5, respectively, which was higher than that found for the CHSS fraction (0.2 and 1.4, respectively).

The CASS fraction contained glucose (52 mol %) and xylose (27 mol %) as the main neutral sugars, and only low amounts of glucuronic acid were present which were quite similar to other fractions. The presence of glucose xylose and glucuronic acid residues may indicate that next to xyloglucans, acidic xylans were also part of this fraction.

Table 2 Yield, composition and Degree of acetylation (DA) and methyl esterification (DM) of okra AIS and okra polysaccharide extracts and residues

	Yield (g/100gAIS)	Rha	Ara	Fuc	Xyl	Man	Gal	Glc	GalA	GlcA	DA(%)[a]	DM(%)[a]	Carbohydrate content[b]	Protein content[b]
							(mol%)							
AIS		3(2)[c]	5(2)	0	5(2)	3(2)	17(10)	44(25)	16(14)	7(1.7)	40	59	57.5	15.8
HBSS	11.2	26(2.5)	0	0	0	0	34(3.2)	1 (0.1)	35(4.0)	3(0.3)	58	24	90.0	3.5
CHSS	4.8	14(0.5)	3(0.1)	0	0	0	17(0.6)	1(0)	63(3.0)	2(0.1)	18	48	86.2	10.5
DASS	13.2	13(1.2)	13(1.0)	0	0	0	19(1.8)	4(0.4)	48(5.5)	2(0.2)	-[e]	-[e]	76.9	16.6
CASS	4.1	1(0.05)	3(0.1)	2(0.1)	27(0.8)	3(0.1)	8(0.3)	52(2.0)	3(0.1)	1(0.1)	-[e]	-[e]	86.8	13.2
RES	26.5	3(0.5)	5(0.8)	0	5(0.9)	tr(0)	7(1.3)	78(15.3)	0	1(0.2)	-[e]	-[e]	73.7	16.1

[a] moles acetyl or methanol per 100 moles of galacturonic acid

[b] gram qualities per 100 g of fraction

[c] values in brackets give g/ 100 g of AIS for the individual sugars

[e] Not determined

tr Trace amounts

In the extraction residue, the main sugar was glucose (78 mol %) representing approximately 50% of all glucose present in AIS. This glucose originates from cellulose and hemicellulose. The presence of xylosyl (5 mol %) and galactosyl (7 mol %) residues indicated that xyloglucan partly remained in the residues. This xyloglucan is either strongly embedded in the structure of the cellulose fibrils or so extensively hydrogen bonded to the cellulose fibrils that it resisted extraction by 6 M NaOH.

The residue fraction showed that the solvents used to extract the okra AIS were able to solubilize pectic material. However, only one-third of polysaccharides in the AIS could be extracted while 26% was recovered in the residue. This results in a recovery of 60%. In addition, respectively, 26%, 32% and 15% of all glucose, galactose and xylose residues in AIS were not covered by the analysis of all fractions. They most probably belong to the hemicellulosic material and were lost during the extraction step with concentrated alkali.

Degree of methyl esterification and acetylation

The degree of methyl esterification (DM) of the HBSS pectins (24%) was surprisingly low and much lower than the DM of the CHSS. The DASS fraction was not included in the analysis due to the removal of methyl esters and acetyl groups during the dilute alkaline extraction. According to the sugar composition (Table 2), 75% of all galacturonic acid present in the HBSS pectins originates from RG I for which there is no evidence that galacturonosyl residues in RG I segments are methyl esterified (O´Neill and York, 2003). Assuming that the methyl ester is only present within HG segments of HBSS pectin, the DM of this HG could be as high as 96%. The DM of the CHSS pectins (48%) was quite low since chelating agents are expected to extract calcium-sensitive pectins with low DM (Ralet et al., 2003) being present in the form of calcium pectate gels (Voragen et al., 1995).

The degree of acetylation (DA) was much higher in the HBSS fraction (58%) compared to the CHSS fraction (18%). So far there is only evidence for the presence of O-acetyl groups on O-2 and/or O-3 of galacturonosyl residues in HG segments and RG I segments (O´Neill and York, 2003; Rombouts and Thibault, 1986). In general, the DA is

high in the RG I segments (hairy regions) of pectin as illustrated by the DA of 60% found for modified hairy regions from apple (Schols et al., 1990). Therefore the HBSS fraction represents pectins having a unique structure which differs from other pectins for instance apple, sugar beet and soya pectin. Moreover, a pure RG I with high DA in water extraction has not frequently been reported for other plants, although recently an Arabidopsis seed mucilage was described by Deng et al., (2006) showing a water extractable linear rhamnogalacturonan I.

Glycosidic linkage composition

To obtain more information about the different cell wall polysaccharides present in the different extracts, the samples were subjected to linkage analysis by permethylation. In general the data obtained were more qualitative than quantitative. First of all the uronic acids were not reduced completely to their neutral sugar analogues and therefore they were not measured. Secondly an underestimation of the terminal pentose and 1,4-linked galactosyl residues could occur due to the evaporation of terminal pentose and complex formation of 1,4-linked galactosyl residues with borate during the acetylation procedure (Harris et al., 1984), respectively.

For the HBSS fraction, the sugar linkage composition results (Table 3-1 and 3-2) indicate the presence of highly branched RG I structures, since the majority (89%) of all 1,2-linked rhamnosyl residues were O-4 substituted. These rhamnosyl residues were substituted with short galactan side chains containing 1 or 2 galactosyl residues since 65% of all galactose was present as terminal residues and 23% as 1,4-linked units.

The CHSS fraction was found to represent a slightly less branched RG I structure as shown by lower levels of O-4 substituted rhamnosyl residues (66% of all rhamnose). Only 22% of all galactosyl residues were present terminally linked revealing the fact that slightly longer galactan side chains were present in the CHSS compared to the HBSS. Summarizing, it can be stated that the HBSS fraction contained RG I backbones with monomeric or dimeric galactan side chain, while the CHSS fraction contained RG I with slightly longer galactan side chains. This observation was also reported by Tomada et al. (1980) for water-extracted okra polysaccharides. Apart from the 1,4-linked galactosyl

residues found in rather high amounts, some 1,6-linked and 1,3,6-linked galactosyl residues were found to be present indicating the presence of arabinogalactan type II as side chain. The amounts of arabinose estimated in the linkage analysis procedure for the CHSS fraction were higher than found in the sugar composition analysis as reported in Table 1. About 42% of all arabinose were present as 1,5-linked which indicated the presence of linear arabinan side chains.

Compared to HBSS and CHSS, the DASS fraction contained less branched RG I as shown by the low levels of 1,4- and 1,2,4-linked rhamnosyl moieties. The majority of all arabinose present in the CHSS and DASS were present as 1,5-; 1,3,5- and 1,2,5-linked arabinosyl residues in the furanose form, which indicate the presence of 1,2 and 1,3 branched α-(1,5)-arabinans. The DASS fraction is relatively rich in branched arabinans in which the number of terminally linked arabinosyl residues fitted rather well with the number of branching points. Besides arabinan side chains, the DASS fraction contains a mixture of AG I and AG II structures as shown by the presence of both 1,4-linked and 1,3,6-linked galactosyl residues.

The CASS fraction is obviously rich in hemicelluloses such as xylan, mannan and glucan present as long linear 1,4-linked chains. Besides, xylose, galactose and fucose were also present as terminal residues and in combination with the presence of 1,4,6-linked glucosyl residues, this points to the presence of a xyloglucan (Vincken et al., 1994).

Molecular weight distribution

The Mw distribution of the polysaccharides in the different fractions obtained from okra AIS is shown in Figure 1. The Mw distribution pattern of HBSS showed only one population having a rather high Mw, while CHSS shows a much broader Mw distribution representing populations with molecular weights higher and lower than HBSS. Moreover, the Mw distribution pattern of the DASS is similar to the Mw distribution pattern of the CHSS. The CASS fraction that represented predominantly containing hemicellulosic polysaccharides showed one broad Mw distribution representing populations with lower Mw values than the other fractions. These trends are also reported for olives (Vierhuis et al., 2000), blue berries and black currents (Hilz et al., 2005). It should be

mentioned that the ultrafiltration step of the extract as was performed using a 30 kDa membrane did remove possibly present low Mw material.

Table 3-1 Sugar linkage composition of okra polysaccharide fractions

Glycosyl residue	HBSS	CHSS	DASS	CASS
		Mol%		
t-ara*f*	0.5	4.2	7.5	0.3
1,2-ara*f*		0.3	0.2	0.6
1,5-ara*f*	0.8	29.4	22.4	
1,3-ara*f*	0.6	0.2	0.6	
1,3,5-ara*f*		6.8	4.5	0.2
1,2,5-ara*f*		1.0	1.7	0.2
1,2,3,5-ara*f*	0.4			0.2
total ara	**2.3**	**41.9**	**36.9**	**1.5**
t-xyl*p*	0.1	0.3	0.1	0.8
1,4-xyl*p*	0.9		2.6	11.0
1,2-xyl*p*				1.7
1,3-xyl*p*	0.5			1.3
1,3,4-xyl*p*	0.4	0.2	0.4	
1,2,4-xyl*p*		1.7	1.8	0.7
1,2,3,4-xyl*p*		0.4		
total xyl	**1.9**	**2.6**	**4.9**	**15.5**
t-rha*p*	0.3			
1,2-rha*p*	3.3	1.4	2.7	
1,3-rha*p*	0.1	0.3		
1,4-rha*p*	0.5	1.1	0.2	
1,2,4-rha*p*	34.0	5.5	5.0	0.1
total rha	**38.1**	**8.3**	**7.9**	**0.1**
t-fuc*p*			0.03	1.2
total fuc			**0.03**	**1.2**

[a]Uronic acid were not reduced prior to methylation

Table 3-2 Sugar linkage composition of okra polysaccharide fractions

Glycosyl residue	HBSS	CHSS	DASS	CASS
		Mol%		
t-glc*p*	1.0	1.8	2.2	2.7
1,4-glc*p*			25.4	52.1
1,2,4-glc*p*				0.1
1,4,6-glc*p*	0.6	0.3	0.3	16.5
1,3,4-glc*p*	0.7	0.1	0.3	
1,2,3,4,6-glc*p*	0.8	0.3		
total glc	**3.1**	**2.5**	**28.2**	**71.4**
1,4-man*p*	0.7	2.1	0.4	2.2
1,2,3,4,6-man*p*	0.8	0.6	0.2	0.01
total man	**1.5**	**2.7**	**0.6**	**2.2**
t-gal*p*	34.5	9.4	6.1	5.3
1,6-gal*p*	1.9	3.5	2.3	0.1
1,4-gal*p*	12.2	12.5	4.8	0.6
1,2,6-gal*p*		0.6		0.2
1,2,4-gal*p*	0.4	0.5	0.3	0.8
1,4,6-gal*p*	1.5	3.7	2.2	0.7
1,3,4-gal*p*	0.2	0.7	0.2	
1,3,6-gal*p*	1.6	9.0	4.6	0.2
1,3,4,6-gal*p*		0.6		
1,2,3,4,6-gal*p*	0.8	1.4	1.0	0.2
total gal[a]	**53.0**	**42.0**	**21.5**	**8.1**
ratio t/b	**0.8**	**0.4**	**0.7**	**0.4**

[a]Uronic acid were not reduced prior to methylatio

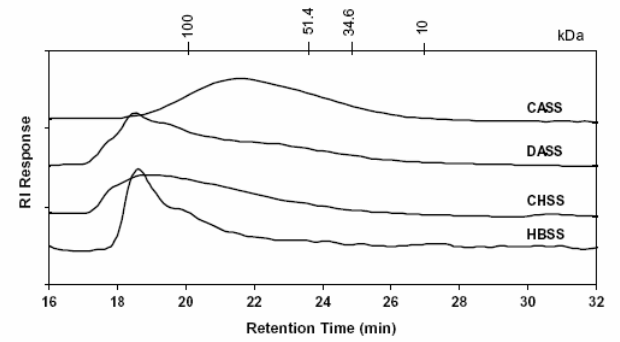

Figure 1 Molecular weigh distributions of okra polysaccharide fractions (the molecular weight indications are based on pectin standards).

Pectin- rich fractions

The sugar (linkage) composition of the HBSS fraction indicates that the HBSS mainly contains RG I segments next to some HG with high acetyl levels representing uncommon structure for water-extracted polysaccharides. Therefore, anion-exchange chromatography was performed to obtain information about the homogeneity of HBSS. The structural features of the sub-fractions were determined and compared to pectin structures present in the CHSS fraction.

Anion exchange chromatography

The homogeneity with respect to charge of the HBSS and CHSS fractions was determined using anion-exchange chromatography. The sample solution was applied to a DEAE Sepharose Fast Flow column, and the material which bound to the resin was eluted with a linear gradient of NaOAc buffer of pH 5. Figure 2 shows the DEAE anion-exchange chromatography patterns of the HBSS and CHSS samples. Almost all material eluted in one major peak at about 0.8 and 0.6 M NaOAc buffer for the HBSS and CHSS, respectively. The sugar composition of this major peak was determined after pooling the fraction and

was found to be comparable to the starting material. The co-elution of all sugars in one single peak having the same sugar composition as the starting material indicated that only 1 pectin population is present in both the HBSS and CHSS fractions.

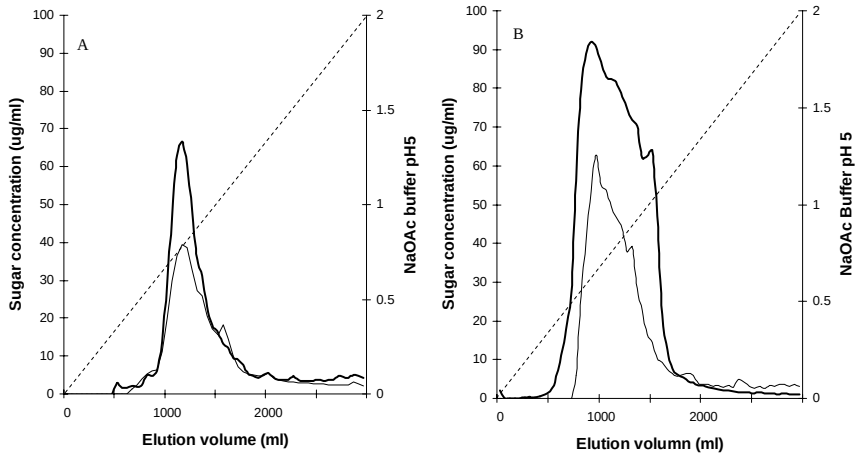

Figure 2 DEAE anion-exchange chromatography patterns of HBSS (A) and CHSS (B). Bold line: uronic acid, thin line: neutral sugars, dotted line: NaOAc gradient.

Nuclear Magnetic Resonance

The pectin fractions were found to consist mainly of two structural elements, HG and RG I. The sugar composition and degree of acetylation analysis exhibited that the HBSS fraction contained mainly RG I and a high substitution with acetyl groups while the CHSS fraction contained mainly HG and less acetyl groups. To reduce the viscosity of the pectins to improve the quality of the NMR spectra, HBSS was digested with polygalacturonase and pectin methyl esterase and CHSS was digested with polygalacturonase only. The digests were then dialyzed, freeze-dried, dissolved in D_2O and then proton NMR spectra were recorded.

The spectra are shown in Figure 3. From the data obtained for HBSS PG/PME, it is obvious that the sample contains both unbranched α-1,2-linked rhamnose (1.25 ppm) and branched α- 1,2,4-linked rhamnose (1.33 ppm); the chemical shifts typically found for H-6

of these rhamnosyl moieties and 1 or 2 anomeric signals between 5.22 and 5.25 ppm were assigned to rhamnosyl residues. The anomeric signals at 5.01 and 4.96 ppm in combination with signal at 4.42 ppm were assigned to H-1 and H-4 of galacturonic acid (Lerouge et al., 1993; Renard et al., 1997; ; Renard et al., 1999; Huisman et al., 2001; Habibi et al., 2004).

Similar to the HBSS sample, also for PG treated CHSS 1,2-linked-, 1,2,4-linked-rhamnosyl units were found. In addition, several signals (signal in boxes 1, 2 and 3 in Fig. 3) belonging to galacturonic acid were found that these were derived from HG and RG I, and suggests the presence of substitution of acetyl group on both structural entities (Colquhoun et al., 1990; Lerouge et al., 1993; Renard et al., 1997; Needs et al., 1998; Renard and Jarvis, 1999; Renard et al., 1999; Perrone et al., 2002).

Furthermore, the presence of several anomeric signals in region between 4.45 and 4.65 ppm of both samples indicated the presence of terminal galactose and 1,4-linked galactose within samples. These indicate that both samples contain short galactan side chains and are in agreement with the sugar composition and linkage analysis. Both samples also showed a signal between 2.10 and 2.20 ppm typically for an O-acetyl substituent. Lerouge et al. (1993) found that when an O-acetyl group is attached to galacturonic acid at position O-2 or O-3, two extra signals appear within the anomeric region at 5.1 and 5.4 ppm. These signals are not present in the HBSS spectrum. The CHSS NMR spectra showed different galacturonic acid residues in combination with two major signals for O-acetyl at 2.10 and 2.07 ppm. This indicates that acetyl groups are linked to different positions of the galacturonic acid present in HG and RG I. Most reasonably for HBSS the acetyl should be linked to either a rhamnose or a galactose building block. However, it cannot be ruled out that also within CHSS other sugars are acetylated.

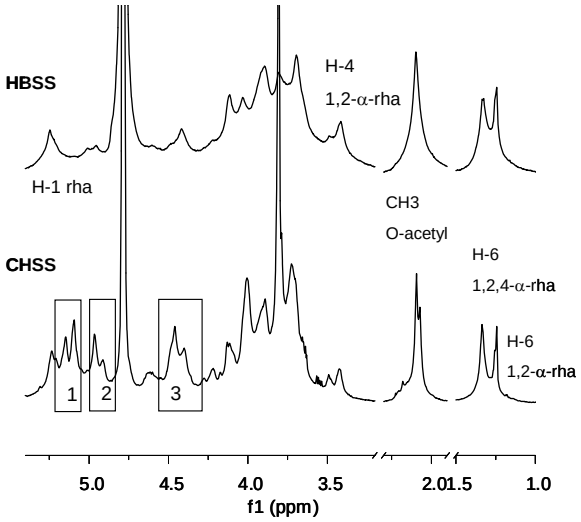

Figure 3 [1]H NMR spectra of CHSS PG and HBSS PG/PME; 1: galacturonic acid derived from HG or O-acetylated galacturonic acid; 2: galacturonic acid derived from RG I and non substituted; 3: galacturonic acid H-4

Hemicellulose –rich fractions

The sugar linkage composition demonstrated that the CASS fraction obviously consists of hemicelluloses. Generally, the hemicelluloses in plant cell wall are xyloglucans, xylans, mannans and/ or arabinogalactans (O´Neill and York, 2003). Therefore, to obtain more information about the structure of hemicellulose, the okra CASS fraction was incubated with specific enzyme and the digest subsequently analyzed for the oligomers released.

Xyloglucan specific endo-glucanase (XEG) degradation of okra CASS fraction

Xyloglucans represent a major hemicellulosic polysaccharide in the primary cell wall of many plants. In general, xyloglucan can be classified into three types, XXXG-type, XXGG-type and XXXX-type, which can be distinguished by the variation in the backbone substitution with xylosyl residues (Fry et al. 1993; Vierhuis et al., 2001). The high amounts of glucose and xylose found in okra CASS indicate the presence of xyloglucan. To obtain

more information about this xyloglucan, this fraction was incubated with a xyloglucan-specific glucanase, and fragments released were analysed. This specific xyloglucanase (Pauly et al., 1999) cleaves xyloglucans between the xylose-substituted glucosyl residues and the unsubstituted glucosyl residues releasing oligosaccharide building blocks from the xyloglucan (Fry et al., 1993). Oligomeric building blocks were identified using xyloglucanase digests from potato and tamarind seeds (Vincken et al., 1996), and confirmed by MALDI-TOF mass spectrometry (results not shown). The HPAEC pattern (Fig. 4) of the digest shows the release of three main (XXXG, XXFG and XLFG) and three minor (XLXG, XXLG and XLLG) xyloglucan oligomers. These patterns are rather similar to the xyloglucan oligomers obtained from many plant cell wall (Hoffman et al., 2005), and were also recently found for black currents (Hilz et al., 2006). These results indicated that the okra CASS fraction contained poly-XXXG type xyloglucan carrying both galactose and fucose substitutions which is generally found in dicotyledonous plants (Hilz et al., 2006).

Endo-Xylanase degradtion of okra CASS fraction

Besides xyloglucan, xylose could be presented as a constituent of another hemicellulosic polysaccharide existing in cell walls, for example, xylan. Xylans are present in monocotyledonous plants that are usually of the arabinoxylan type, while in the dicotyledonous plants often an acidic xylan almost without any arabinose substitution is found (McNeil et al., 1984; Brett and Waldom, 1990). The okra CASS fraction was incubated with endo-Xylanase from *Aspergillus awamori* CMI 142717 (Xyl III belonging to GH family 11) (Vierhuis et al., 2001) to obtain more information about the xylan structure present.

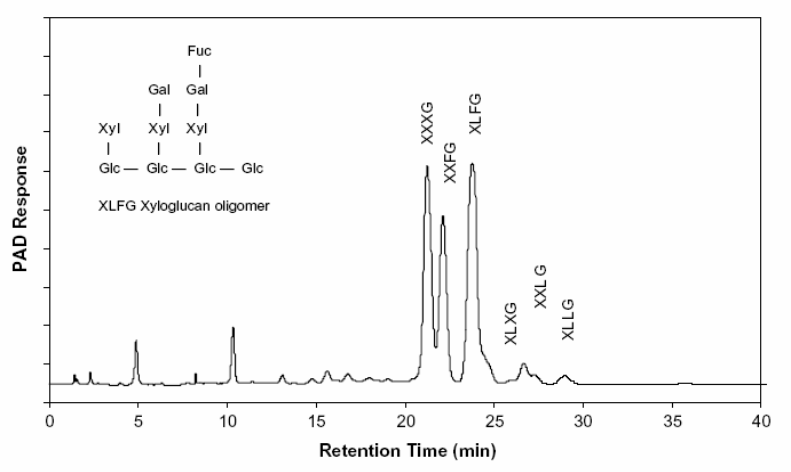

Figure 4 HPAEC pattern of okra CASS fraction after incubation with xyloglucan-specific *endo*-glucanase.

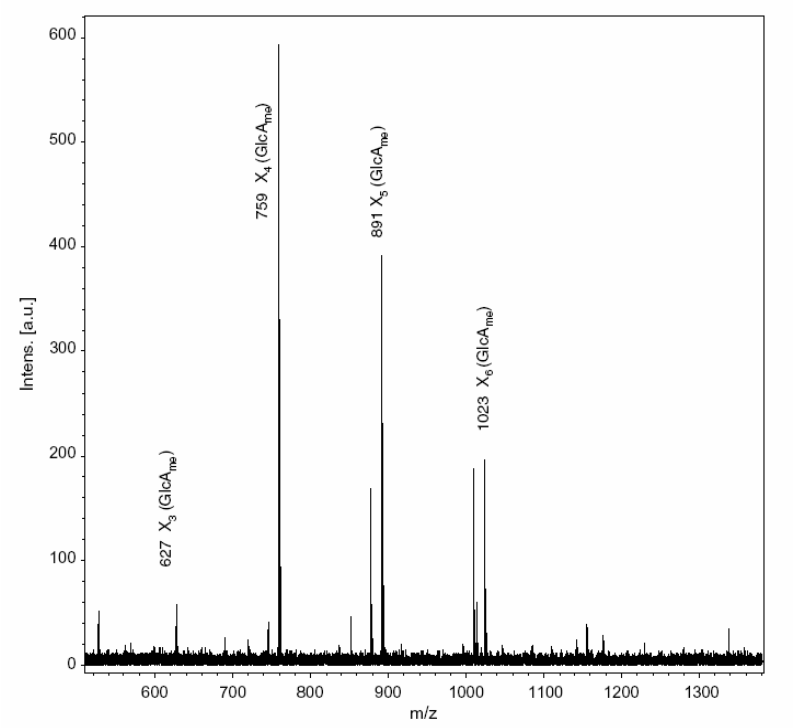

Figure 5 MALDI-TOF mass spectrum of the okra CASS fraction digest with Xyl III.

After 24 h incubation of the okra CASS fraction with Xyl III, the products released were analysed by HPAEC (data not shown), and compared with data found before for, for example, olive xylans (Vierhuis et al., 2001). From the HPAEC pattern, mainly xylose, xylobiose and xylotriose were found to be present, while arabinose-substituted xylan oligomers were only found as minor product in addition to some compounds which were retained longer by the anion-exchange. For additional information the digests were also analyzed with MALDI-TOF MS. The spectrum (Fig. 5) shows the presence of series of xylo-oligomers substituted with one 4-O-methylglucuronic acid [$X_{3\text{-}7}(GlcA_{me})$]. The major MS signals were m/z of 759 and 891 corresponding to $X_4(GlcA_{me})$ and $X_5(GlcA_{me})$, respectively. The formation of these oligomers can be explained by the substrate specificity of the enzyme used since Xyl III is hindered by substitution of the xylan backbone, and can only cleave glycosidic linkages between two unsubstituted xylosyl residues which are not adjacent to singly or doubly substituted xylose residue (Kormelink et al, 1993; Vierhuis et al., 2000). Therefore, the xylan presented in okra CASS fraction is a 4-O-methylgluconoxylan which is generally found in the dicotyledonous plants such as olive (Vierhuis et al., 2000) and also in hard wood such as eucalyptus wood (Kabel et al., 2002). Furthermore, the 4-O-methyl glucuronic acid groups seem to be distributed rather randomly over the xylan backbone.

Conclusions

In summary, sequential extraction of okra AIS yielded different polysaccharide populations. The sugar (linkage) composition showed that the galactose-rich pectic molecules are found in the three different extracts (HBSS, CHSS and DASS) which have different chemical structures. The HBSS fraction is a homogenous charged polymer and contains a regular structure which consists of mainly highly branched RG I with very short galactan side chains (1-2 galactose residues on average). The HBSS further contains high level of acetyl groups which, unexpectedly, as NMR analysis revealed that the acetyl groups are not attached to galacturonic acid units.

The CHSS fraction is also a homogeneously charged polymer and contains mainly HG and some RG I with galactan side chains consisting of on average 2 to 3 galactosyl residues. Also more heterogeneous side chains of arabinan and AG II are present. Both HBSS and CHSS showed one population on anion-exchange chromatography indicating different ratios of homogalacturonan and rhamnogalacturonan in HBSS and CHSS. The NMR studies of PG treated HBSS and CHSS showed the presence of acetyl groups that are linked to other sugar residues present like rhamnose and/or galactose than galacturonic acid. Moreover, the DASS fraction contains even more complex pectins with arabinans, AG I and AG II, as neutral side chains.

Besides pectic material, the okra AIS also contains hemicellulosic polysaccharides which were mainly present in the CASS fraction. The hemicellulosic material in this fraction was found to consist of mainly XXXG-type xyloglucan and 4-methylglucuronoxylan.

To obtain more information about the position of the acetyl groups and the length of galactan side chains, more enzyme degradation studies of the polymer and NMR and MS characterizations of oligomers will be performed.

Material and methods

Material

The soft and mature of okra pods, *A. esculentus* (L.) Moench, (5 – 10 cm in length) were grown and collected at local market in June 2005, Thailand.

Isolation of alcohol-insoluble solid

After removal of the seeds, the okra pods were sliced and homogenized 2 times with 70 % (v/v) aqueous ethanol at room temperature. After filtration, the insoluble residues were combined and washed with two volumes of chloroform/methanol (1/1, v/v) with gentle stirring for 30 min to remove low molecular weight (colored) compounds (Monpien, 2005). After filtration, the filtrates were washed with acetone and air dried (Alcohol Insoluble Solid, AIS).

Sequential extraction of okra AIS

Okra AIS (20 g) were sequentially extracted according to Vierhuis et al. (2000) with 600 mL of the following extractants; 0.05 M sodium acetate buffer at pH 5.2 and 70 ^{o}C (Hot Buffer Soluble Solids, HBSS), 0.05 M EDTA and 0.05 M sodium acetate in 0.05 M sodium oxalate at pH 5.2 and 70 ^{o}C (CHelating agent Soluble Solids, CHSS), 0.05 M sodium hydroxide at 0 ^{o}C and 20 mM $NaBH_4$ (Diluted Alkali Soluble Solids, DASS), and 6 M sodium hydroxide at 0 ^{o}C and 20 mM $NaBH_4$ (Concentrated Alkali Soluble Solids, CASS). The extraction was continually performed until the total sugar content of the last supernatant was lower than 40 µg/mL, which was determined by phenol–sulfuric acid assay (Dubois et al., 1956). After each extraction, solubilized polymer was separated from the insoluble residue by centrifugation (19,000g for 25 min). The supernatants were ultra filtrated through a 30 kDa membrane (A/G Technology Corporation) and freeze-dried. The final residues were dialyzed and freeze-dried.

Analytical methods

Total Neutral sugar and uronic acid content

The total neutral sugar content and uronic acid content were determined colorimetrically by automated orcinol/sulfuric acid assay (Tollier and Robin, 1979) and m-hydroxydiphenyl assay (Ahmed and Labavitch, 1977; Thibault, 1979; Kintner and van Buren, 1982), respectively, using an auto-analyser (Skalar Analytical BV, Breda, The Netherlands). Galactose and galacturonic acid were used as a standard. Corrections were made for the interference of uronic acid in the samples. Distinction between galacturonic acid and glucuronic acid was made by high performance anion-exchange chromatography (HPAEC, Dionex, USA) after methanolysis according to De Ruiter et al. (1992)

Sugar composition

The neutral sugar composition of okra AIS was determined by gas chromatography (Englyst and Cummngm, 1984) using inositol as internal standard. The samples were submitted to a prehydrolysis treatment with 72% w/w sulfuric acid at 30 ^{o}C

for 1 h followed by a hydrolyses step using 1 M sulfuric acid at 100 ^{o}C for 3 h. The sugars were converted to their alditol acetates and analyzed by GC according to Hilz et al. (2005)

For the obtained fractions, after drying at 40 ^{o}C under vacuum over P_2O_5, the samples were hydrolyzed with 2 M HCl in dry methanol at 80 ^{o}C for 16 h and followed by 2 M TFA at 121 ^{o}C for 1 h. The monomers were analyzed by using high performance anion-exchange chromatography (HPAEC, Dionex, USA) equipped with the (2 x 250 mm) CarboPac PA 1 column (Dionex, USA) and post column (Dionex, USA) addition. Millipore water, 0.1 M NaOH and 1 M NaOAc in 0.1 M NaOH with a flow of 0.3 mL/min at 20 ^{o}C were used as eluent, and 0.5 M NaOH with a flow of 0.1 mL/min at 20 ^{o}C was added to post column for allowance of the pulsed amperometric detection.

The following gradient was applied using a flow of 0.3 mL/min at 20 ^{o}C of NaOH: 0–30.0 min, 0 mM; 30.0–30.1 min, 0–100 mM; 30.1–50.0 min, 100 mM. The simultaneous gradient of NaOAc was 0.0–30.1 min, 0 mM; 30.1–45.0 min min, 0–400 mM; 45.1–50.0, 1000 mM. Millipore water was used from 0.0 to 30.0 min. For the post column, 0.5 M NaOH with a flow of 0.1 mL/min was used during 0.0–30.1 min. After each run the column was washed for 5 min with 1 M NaOAc in 0.1 M NaOH, for 8 min with 0.1 M NaOH and subsequently equilibrated with water for 15 min (Westphal, Y. *Unpublished data*).

Absolute configuration

The configuration of all sugars present was analyzed by gas chromatography and methyl-α-D-galactopyranoside was used as internal standard. The sample was methanolyzed with 1M HCl in dry methanol. After conversion to their corresponding – (-) 2- butyl glycosides and trimethylsilation, the trimethylsilated (–) - 2 – butyl glycosides were analyzed by GC-FID (Gerwig et al., 1978).

Degree of acetylation and methyl esterification

The degree of acetylation and methyl esterification of samples were determined after saponification with 0.4 N sodium hydroxide in isopropanol/water (50/50 v/v) by using Thermo Finnigan (USA) High Performance Liquid Chromatography equipped with Aminex HPX 87H column (Bio-Rad, USA) (Voragen et al., 1986). The elution took place at 40 ^{o}C

with 0.01 N H_2SO_4 at a flow rate of 0.6 mL/min. The degree of acetylation and methyl esterification were calculated as moles of acetic acids and methanol per 100 moles of galacturonic acid, respectively.

Protein content

Protein content was determined by the combustion method (Dumas, 1983) with a Thermo Quest NA 2100 Nitrogen and Protein Analyzer (Interscience, The Netherlands). D-Methionine was used as external standard. The protein content was calculated using 6.25 as nitrogen to protein conversion factor.

High Performance Size Exclusion Chromatography (HPSEC)

High performance size exclusion chromatography (HPSEC) was performed on a Thermo Separation Products (USA) HPLC, equipped with three TosoH Biosep-TSK-Gel G columns (Methyacrylate resin) in series (7.8 mm x 30 cm, $4000PW_{XL}$-$3000PW_{XL}$-$2500PW_{XL}$) in combination with a PW_{XL} – guard column (TosoH, Japan). Samples (5 mg/mL) were eluted 30 ^{o}C with 0.2 M sodium nitrate at a flow rate of 0.8 mL/min (Chen et al., 2004). The column effluent was monitored using a reflective index detector (Shodex SE-61, Showa Denko K.K., Japan). Calibration was done using pectins having known molecular weight (Schols et al., 1990).

Glycosidic linkage analysis

The glycosidic linkage composition of each sample was analyzed as described by Hakomori (1964) and modified by Verhoef et al (2002). The partially methylated alditol acetates were analyzed GC-FID and GC–MS according to Verhoef et al (2002).

Enzymatic degradation

Pectin degradation

The HBSS fraction was dissolved (3 mg/mL) in 50 mM NaOAc buffer, at pH 5, and incubated with 0.016 units of polygalacturonase from *A. aculeatus* and 1.08 units of pectin methyl esterase from *A. niger* (Schols et al., 1990). The CHSS fraction (4 mg/mL) was incubated with 0.024 units of polygalacturonase. The incubations were performed at 40 ^{o}C for 24 h and the digests were subsequently heated for 5 min at 100 ^{o}C to inactivate the enzyme. The digests were dialyzed by Centricon centrifugal filter devises with 3 kDa cut off at 1500g for 30 min and then, freeze dried.

Xyloglucan specific endo-glucanase (XEG) degradation

The CASS fraction was dissolved (5 mg/mL) in 50 mM NaOAc buffer, at pH 5, and incubated with 2.3 units of xyloglucan specific *endo*-glucanase from *A. aculeatus* (Pauly et al., 1999) at 40 ^{o}C for 16 h. The digest was heated at 100 ^{o}C for 5 min to inactivate the enzyme.

endo- Xylanase III degradation

The CASS fraction was dissolve (10 mg/mL) in 150 mM NaOAc buffer, at pH 5, and incubated at 40 ^{o}C for 24 h with 0.09 units of *endo*-xylanase III from *A. awamori* (Vierhuis et al., 2001). The enzyme was inactivated by heating the digest for 5 min at 100 ^{o}C.

HPAEC of oligosaccharides

For the determination of xyloglucan oligomer were analysed by using a 4 x 250mm CarboPac PA 100 column (Dionex, USA) with pulsed amperometric detection. Gradients of NaOH and NaOAc with a flow of 1 mL/min were use to elute the oligomer according to Vincken et al (1996).

For the determination of digest from endo-xylanase III, the (2 x 250 mm) CarboPac PA 1 column (Dionex, USA) with pulse amperometric detection was equilibrate with 100 mM NaOH. Gradients of NaOH and NaOAc were used simultaneously to elute the oligomers according to Vierhuis et al (2001).

Matrix –Assisted Laser Desorption Ionization-Time Of Flight Mass Spectrometry (MALDI-TOF MS) analysis

MALDI-TOF MS analysis was performed on Ultra flex instrument (Bruker Daltonics, Germany) with a Nitrogen 337 nm laser beam. The data were collected from average of 200 shots with the lowest laser energy (35%). The digest sample was desalted with resin (AG 50W-X8 Resin, H^+ form; Bio – Rad, USA). A small spatula of Dowex resin was added to 10 μL of sample solution, mixed and centrifuge for 5 min at 13,000g. One μL of matrix solution ,10 mg/mL of 2,5-dihydroxybenzoic acid in millipore water (Milli-Q Gradient A-10, USA), was placed on a MALDI-TOF plate together with 1 μL of sample solution and dried under a constant of warm air.

Anion exchange chromatography

Anion-exchange chromatography was performed on a DEAE Sepharose Fast Flow column (50 x 2.6 cm, Amersham Bioscience, Sweden) using an Akta explorer system (Amersham Biosciences, Sweden). The sample (280 mL, 0.5 mg/mL) was loaded to the column (10 mL/min). After loading with sample the column was washed with water for 1 column volume (250 mL, 50 mL/min) and eluted successively with a linear gradient of 0-2 M of NaOAc buffer, pH 5, within 10 column volumes. Finally the column was washed with 2 column volumes of 1 N NaOH. During elution with NaOAc buffer, fractions of 50 mL were collected as well as the column washed and analyzed for neutral sugar and uronic acid content as described. The alkaline fractions were neutralized directly by adding acetic acid. After pooling appropriates fractions, pools were dialyzed and freeze dried.

^{13}C and ^{1}H Nuclear Magnetic Resonance (NMR)

Prior to NMR analyses, the sample was dissolved in 5 mM NaOAc, pH 5, to set the pD = pH and freeze dried. Then the sample were dissolved in 99.96% D_2O (Cambridge Isotope Laboratories, USA) and after freeze-drying dissolved again in 99.996% D_2O (Cambridge Isotope Laboratories, USA). NMR spectra were recorded at a probe temperature of 25°C on a Bruker AV-600 spectrometer equipped with a cryoprobe located at the Biqualys, Wageningen. Chemical shifts were expressed in ppm relative to internal acetone: δ=2.225 ppm for ^{1}H. The 1D ^{1}H proton spectra were recorded at 600.13 MHz using 64 scans of 8192 data points and a sweep width of 3000 Hz.

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Chapter 3

Okra pectin contains an unusual substitution of its rhamnosyl residues with acetyl and alpha –linked galactosyl groups

Sengkhamparn, N., Verhoef, R., Bakx, E.J., Schols, H.A., Sajjaanantakul, T. & Voragen, A.G.J. Okra pectin contains an unusual substitution of its rhamnosyl residues with acetyl and alpha –linked galactosyl groups. *Carbohydr. Res*., 2009, 334, 1842-1851.

Abstract

The okra plant, *Abelmoschus esculentus* (L.) Moench, a native plant from Africa, is now cultivated in many other areas such as Asia, Africa, Middle East, and the southern states of the USA. Okra pods are used as vegetables and as traditional medicines. Sequential extraction showed that the Hot Buffer Soluble Solids (HBSS) extract of okra consists of highly branched rhamnogalacturonan (RG) I containing high levels of acetyl groups and short galactose side chains. In contrast, the CHelating agent Soluble Solids (CHSS) extract contained pectin with less RG I regions and slightly longer galactose side chains. Both pectic populations were incubated with homogeneous and well characterized rhamnogalacturonan hydrolase (RGH), endo-polygalacturonase (PG), and endo-galactanase (endo-Gal), monitoring both high and low molecular weight fragments. RGH is able to degrade saponified HBSS and, to some extent, also nonsaponified HBSS, while PG and endo-Gal are hardly able to degrade either HBSS or saponified HBSS. In contrast, PG is successful in degrading CHSS, while RGH and endo-Gal are hardly able to degrade the CHSS structure. These results point to a much higher homogalacturonan (HG) ratio for CHSS when compared to HBSS. In addition, the CHSS contained slightly longer galactan side chains within its RG I region than HBSS. Matrix-assisted laser desorption ionization-time of flight mass spectrometry indicated the presence of acetylated RG oligomers in the HBSS and CHSS enzyme digests and electron spray ionizationion trap-mass spectrum showed that not only galacturonosyl residues but also rhamnosyl residues in RG I oligomers were O-acetylated. NMR spectroscopy showed that all rhamnosyl residues in a 20 kDa HBSS population were O-acetylated at position O-3. Surprisingly, the NMR data also showed that terminal α-linked galactosyl groups were present as neutral side chain substituents. Taken together, these results demonstrate that okra contained RG I structures which have not been reported before for pectic RG I.

KEY WORDS: Rhamnogalacturonan, Acetylated rhamnose, Enzymatic degradation, Alpha-Galactose, NMR, ESI-IT-MS^n

Introduction

The okra plant, *Abelmoschus esculentus* (L.) Moench, family Malvaceae, is cultivated for its immature pods. The immature pod contains a thick and slimy mucilage. The okra pod is used as a vegetable and as a thickening agent for soups and stews (BeMiller and Whistler, 1993). In addition, it is used in traditional medicine as a dietary meal in the treatment of gastric irritations (Lengsfeld et al., 2004) and dental diseases (Ndjouenkeu et al., 1996) due to its high content of polysaccharides. Physiological studies showed that the okra polysaccharides (OKP) had hypoglycaemic properties and lower plasma cholesterol levels in rats (Lengsfeld et al., 2004). In food applications, the OKP was a suitable egg-white substitute (Costantino and Romanchick-Cerpoviez, 2004) and a fat substitute in cookies and in chocolate frozen dairy dessert (Romanchik-Cerpovicz et al., 2002; Romanchik-Cerpovicz et al., 2006).

The OKP was an acidic polysaccharide which consists of galactose, rhamnose, and galacturonic acid (Whistler and Conrad, 1954). The OKP has been reported to have a backbone repeating units of -4-α-Gal*p*A-(1,2)-α-L–Rha*p*-1- dimers and, on average, dimeric side chains of β–Gal*p*–(1,4)–β–Gal*p*-1- (Tomada et al., 1980). The acetyl content was about 5.5% w/w (Tomada et al., 1980). Sequential extraction of okra cell wall material showed that okra contained different types of polysaccharides, that is, pectins, xyloglucans, xylans, and celluloses (Sengkhamparn et al., 2009). The Hot Buffer Soluble Solids (HBSS) fraction was the main fraction which contained mainly rhamnogalacturonan (RG) I with short galactose-containing side chains. The CHelating agent Soluble Solids (CHSS) fraction mainly contained homogalacturonan (HG) and slightly longer galactose-containing side chains connected to the RG I segments. In addition, the degree of acetylation of the galacturonic acid moieties for HBSS was relatively high. About 58 moles of acetyl groups were present for every 100 galacturonic acid moieties. NMR studies of HBSS polymer after incubation with polygalacturonase (PG) and pectin methyl esterase(PME) showed that the majority of the acetyl groups were not linked to galacturonosyl residues and substitution to other sugars such as rhamnosyl residues had to be considered (Sengkhamparn et al., 2009). In this study, we provide further structural information of the HBSS and CHSS fractions by degradation studies using homogeneous and well characterized enzymes. Furthermore,

NMR spectroscopy and mass spectrometry were used to indicate the position of the acetyl groups and details concerning the galactose side chains in both samples.

Results and discussion

The okra AIS was sequentially extracted with hot buffer and chelating agent. The sugar composition (Table 1) showed that the HBSS contained mainly rhamnogalacturonan (RG) I (85%) with short galactose-containing side chains and hardly any homogalacturonan (HG). The CHSS contained mainly HG and some RG I (24%) with more galactose and arabinose present in side chains (Sengkhamparn et al., 2009). Moreover, the configuration of all sugar compositions present in HBSS and CHSS was in D-configuration except of rhamnose, which was in the L-configuration (Sengkhamparn et al., 2009). The degree of acetylation (DA) was quite high in HBSS (58%) which is in agreement with levels found, example for apple RG I (Schols et al., 1990). However, NMR studies of HBSS polymer showed that no acetyl groups substitution was present on the galacturonosyl residues of RG I backbone which is normally the case (Sengkhamparn et al., 2009). The DA of CHSS was relatively low and NMR studies of CHSS polymer showed that acetyl may be linked to galacturonic acid as well as to some other sugar residues.

Table 1 Sugar composition (mol %) of HBSS and CHSS fraction obtain from okra AIS (Sengkhamparn et al., 2009)

	Rha	Ara	Gal	Glu	GalA	GlcA	DM[a] (%)	DA[a] (%)	Total sugar[b]
HBSS	26	0	34	1	35	3	24	58	90
CHSS	14	3	17	1	63	2	48	18	86

[a] moles methanol or acetyl per 100 moles of galacturonic acid

[b] gram qualities per 100 g of fraction

Enzymatic degradation of okra pectins

Polygalacturonase treatment

To characterize the HG segments within the extract sample, the samples were incubated with endo-polygalacturonase (PG) from *Aspergillus aculeatus*. This enzyme can cleave the α-1,4-D-galacturonosyl linkages of the HG-backbone by hydrolysis although PG action is hindered by the presence of methyl esters and acetyl groups (Vincken et al., 2003).

HPSEC of the PG digests of HBSS and saponified HBSS (sHBSS) (data not shown) showed a slight shift of the high Mw pectin population to lower Mw values. Some minor quantities of oligomers were released as well. Analyses by HPAEC showed the presence of monomers, dimers, and trimers of galacturonic acid (GalA) accounting only for about 0.1% (HBSS) and 3% (sHBSS) of all GalA residues present.

The HPSEC patterns of PG-treated CHSS (Fig. 1A) indicated that approximately 37% of CHSS remained as high Mw material, and 52% and 11% were found as intermediate and low Mw fragments, respectively. For sCHSS, about 50% of the polymer was present as low Mw fragments and no intermediate fragments were observed (Fig. 1B). The (limited) action of PG toward HBSS and CHSS confirms the relative abundance of HG segments in the samples and also reflects the methyl esterification in the CHSS sample.

Treatment with galactose releasing enzymes

To obtain more information about the length of the galactose containing side chains, the samples were incubated with endo-Galactanase (endo-Gal) from *A. aculeatus*. This enzyme cleaves the 1,4 linkage between β-linked galactosyl residues within galactan chains and releases mono-galactose and galactose oligomers (Ralet et al., 2002).

The HPSEC patterns of HBSS and sHBSS after treatment with endo-Gal showed no shift of the polymer to low Mw material and no oligomers were released as indicated by HPAEC (results not shown). Only 5% of all galactose present was released by endo-Gal in HBSS and sHBSS. For CHSS and sCHSS, endo-Gal digest showed a minor shift to lower Mw values and HPAEC analysis showed a release of mono- and dimeric galactose representing about 10% (CHSS) and 15% (sCHSS) of all galactosyl residues present. These

observations confirm our earlier indications for the presence of short galactan side chains within HBSS and CHSS (Sengkhamparn et al., 2009), mostly resistant against enzyme action.

Since endo-Gal showed only a limited action toward the HBSS fraction, we also studied the activity of α and β galactosidases for their ability to release galactose. Both enzymes were hardly able to remove galactosyl residues from the HBSS fraction.

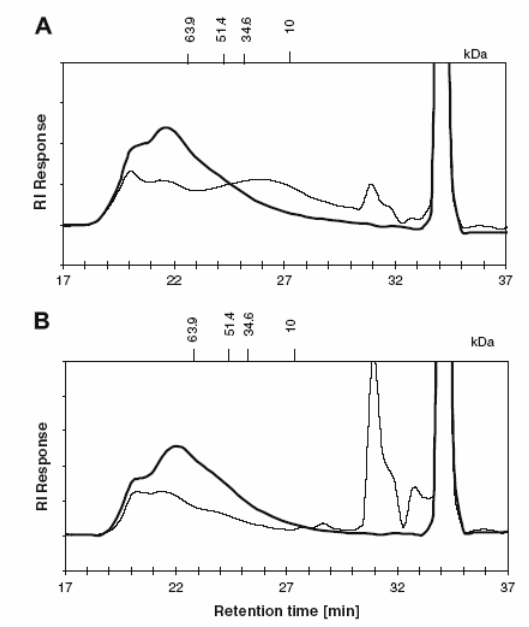

Figure 1 HPSEC elution patterns of CHSS (A) and sCHSS (B). Bold line: before incubation, thin line: after incubation with PG (the molecular weight indication is based on pectin standards)

Rhamnogalacturonan hydrolase (RGH) treatment

To gain information about the RG I structure, the samples were incubated with RGH from *A. aculeatus*. This enzyme acts exclusively on RG I (Vincken et al., 2003) and is unable to split the RG I backbone in case of substitution with long galactose side chains and when acetyl groups are present in the backbone (Mutter et al., 1998).

The HPSEC patterns of the HBSS (Fig. 2) after incubation with RGH showed that about 20% of the HBSS polymer remained as high Mw material and 80% of the HBSS polymer was shifted to medium or low Mw material. The HPSEC pattern of sHBSS digests showed only 2 populations of which the <10 kDa Mw fraction was most dominant (66% of the sHBSS digest). The enzymatic degradation of sHBSS by RGH from sHBSS confirmed that the HBSS was indeed built up by RG I segments with rather short side chains and that acetyl groups were present partly inhibiting enzyme action toward HBSS (Searle-Van Leeuwen et al., 1996). This indicated that acetyl free regions were sufficiently present in RG backbones of HBSS to allow RGH to act. Another option we considered was that (some of) the acetyl groups may not be substituted to the galacturonosyl residues in the RG I backbone as indicated by the NMR results and so may not hinder the enzyme (Sengkhamparn et al., 2009).

Comparison of the HPAEC elution patterns of the HBSS RGH digest (Fig. 3) with those obtained for RGH-degraded RG I from apple (Schols et al., 1994) confirmed that HBSS digest indeed contained the typical RG oligomers. The RGH digest from HBSS differed slightly from the sHBSS digest in which less large oligomers were observed. The HPAEC pattern showed that RGH can release $Rha_2GalA_2Gal_2$ (34 min) and $Rha_3GalA_3Gal_2$ (40 min) and these oligomers represented 40% and 80% of released oligomers for the HBSS and sHBSS, respectively. These results demonstrate that sHBSS has much more RGH cleavable sites probably due to the hindering effect of acetyl groups in HBSS.

MALDI-TOF MS was employed to obtain mass and composition of the oligomers and established the presence and site of substitution of acetyl groups. The MALDI-TOF mass spectra of the HBSS and sHBSS digests are shown in Figure 4. The major RG oligomers released within the HBSS mass spectrum correspond to $(RhaGalA)_{2\text{-}3}$ and Rha_2GalA_3 oligomers, while the major fragments found in the sHBSS mass spectrum were $(RhaGalA)_2Gal_{2\text{-}3}$. The presence of Rha_2GalA_3 in RGH-HBSS digests was probably due to some contamination of RGH with RG-rhamnohydrolase which removed rhamnose from the non-reducing end of the RG segments (Mutter et al., 1994; Schols et al., 1994).

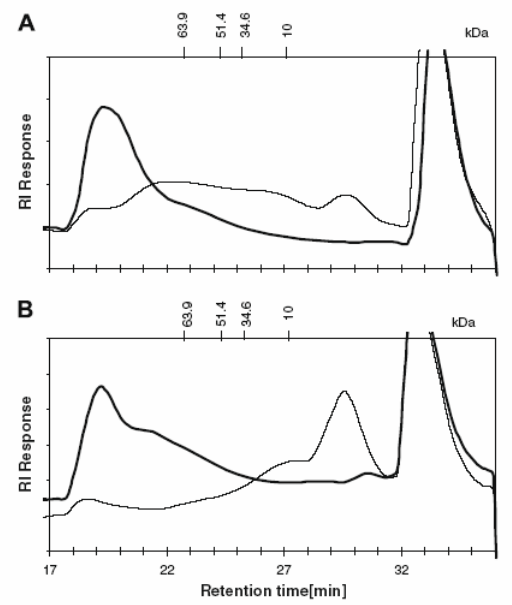

Figure 2 HPSEC – elution patterns of HBSS (A) and saponified HBSS (B) before (bold line) and after (thin line) incubation with RGH (the molecular weight indication is based on pectin standards)

The mass spectrum of the HBSS digest showed clearly that the acetylated oligomer fragments $Rha_1GalA_1Gal_1Ac_3$, and $Rha_2GalA_2Ac_1$ were predominantly present while $Rha_2GalA_2Gal_2Ac_{1\text{-}2}$ and $Rha_2GalA_2Gal_3Ac_3$ were only minor products. So far it has never been reported that RGH is able to degrade acetylated RG I (Mutter et al., 1998). This information together with the NMR data of the HBSS polymeric fragments gave rise to doubt about the precise location of acetyl groups within okra RG I (Sengkhamparn et al., 2009).

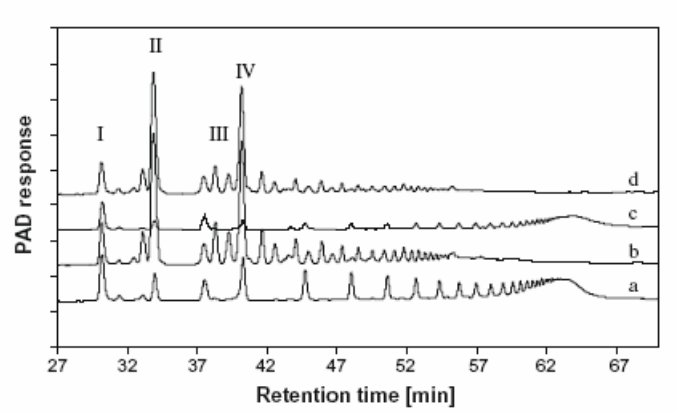

Figure 3 HPAEC elution patterns of sample after incubation with RGH: (a) HBSS, (b) saponified HBSS, (c) non-saponified CHSS, and (d) saponified CHSS (I: Rha_2GalA_2; II: $Rha_2GalA_2Gal_2$; III: Rha_3GalA_3; IV: $Rha_3GalA_3Gal_2$).

The HPSEC patterns of RGH treated CHSS and sCHSS showed that about 54% of the sCHSS polymer shifted to low Mw fragments while in the non-alkali treated CHSS polymer only a small part of the molecules shifted to low Mw (data not shown). This finding revealed that RGH hardly degrades CHSS in contrast to the rather good degradation of sCHSS. Moreover, the HPAEC patterns of both samples (Fig. 3) showed that the released RG oligomers correspond to $Rha_2GalA_2Gal_2$ and $Rha_3GalA_3Gal_2$, although the level of oligomers released is quite different for CHSS and sCHSS. The MALDI–TOF mass spectra showed the presence of Rha_2GalA_2 and $Rha_2GalA_2Gal_2$ in CHSS and sCHSS digests, next to the minor fragments $Rha_2GalA_2Gal_4$ and $Rha_3GalA_3Gal_{4\text{-}5}$ in sCHSS (data not shown). In conclusion, also the RG I in sCHSS consists of the typical Rha-GalA repeats. However, the preference of RGH to act on sCHSS rather than on CHSS pointed to the presence of acetylation on the galacturonosyl residues within RG I. The presence of small amounts of acetylated oligomers indicate that only a minor part of the acetyl groups may be located on other sugar residues as is the case in HBSS RG I.

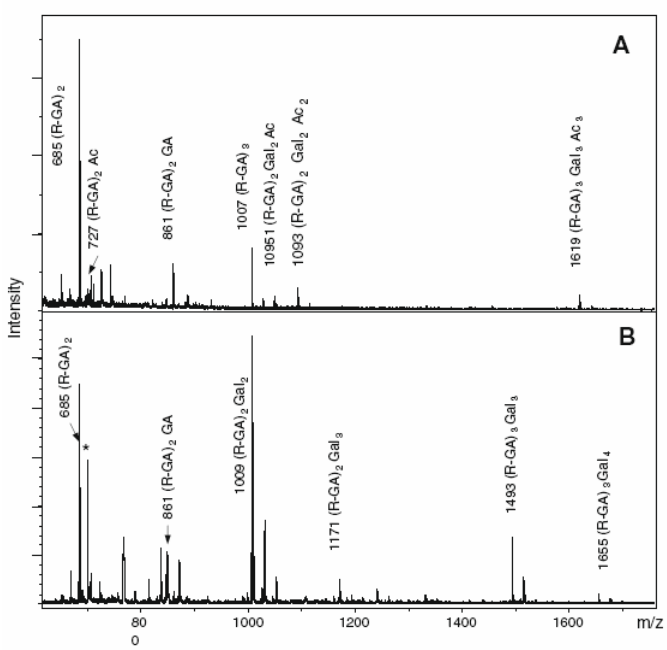

Figure 4 MALDI-TOF mass spectra of non-saponified HBSS (A) and saponified HBSS (B) after treatment with RGH (all ions are present as Na adduct except *: present as K adduct).

The position of acetyl groups in the RG I backbone

The possible hindrance of RGH by acetyl groups was verified by using rhamnogalacturonan acetyl esterase (RGAE) known to remove the acetyl groups from RG I extracted from apple which contained similar amount of acetyl groups. RGAE was unable to release acetyl groups from HBSS (data not show) but was active toward CHSS confirming our conclusions stated above.

Electron spray ionization ion trap mass spectrometry

Electron spray ionization ion trap mass spectrometry (ESI-ITMS) was performed to get an insight in the structure of RG oligomers obtained after treatment of sHBSS with RGH. The MS^2 mass spectrum of the ion with mass to charge ratio (m/z) of 1171, corresponding to a $Rha_2GalA_2Gal_3$ oligomer, is shown in Figure 5A. The parent ion peak

m/z = 1171 was fragmented to m/z = 863 (releasing of Rha-Gal fragment) and 699 (releasing of Gal-Rha-GalA fragment), indicating that one galactosyl residue was attached to one rhamnosyl residue. In addition, the removal of Gal_2Rha_1 fragment (m/z = 701) and $Gal_2Rha_1GalA_1$ fragment (m/z = 507) in the MS^2 spectrum of m/z = 1171 pointed to two galactosyl residues linked to one rhamnosyl residue. The possible structure of oligomers with m/z = 1171 is shown in the right top corner of Figure 5A. However, it was not possible to make a difference between a side chain of two galactoses or two galactoses, both attached to the rhamnose at positions O-3 and O-4.

Also, the presence of fragment of m/z = 863 (leaving of a Gal_2Rha_1fragment) and 699 (leaving of a $Gal_2Rha_1GalA_1$ fragment) in the MS^2 spectrum of m/z = 1333 representing $Rha_2GalA_2Gal_4$ (Fig. 5B) confirmed that two galactosyl residues are substituted to one single rhamnosyl residue. Fragments representing the removal of Rha_1Gal_1 (m/z 1025) and Rha_1Gal_3 (m/z 863) were only found as minor fragments in the MS^2 spectrum of m/z = 1333. The possible structure of main oligomers with m/z = 1133 is shown in the right top corner of Figure 5B. Therefore, the main RG oligomers released from sHBSS by RGH consist of a Rha-GalA repeat with galactose dimers linked to the rhamnose moiety, although rhamnoses without, with one or with three galactose units are also present in minor amounts. Linkage analysis already pointed out that galactoses may be linked to each other (Sengkhamparn et al., 2009).

To locate the acetyl groups within the HBSS RGH oligomers, ESI-IT-MS^n was performed. The ESI-IT-MS^2 spectrum of ions peak 729 (^{18}O labeled $Rha_2GalA_2Ac_1$) is shown in Figure 6A and the most apparent fragments were m/z = 583 and 533 which were formed after removal of rhamnose and ^{18}O-labeled GalA, respectively. The MS^3 spectrum of m/z = 583 showed the leaving of $GalA_1Ac_1$ (m/z = 327 and 345) pointing out that an acetyl group was located on the galacturonosyl residue. Unexpectedly, the MS^3 spectrum of m/z = 533 ($Rha_2GalA_1Ac_1$) showed the release of Rha_1Ac_1 (m/z = 327 and 345) suggesting that an acetyl group was linked to a rhamnosyl residue. From this data, it is concluded that the oligomers with a m/z of 727/729 represent a mixture of different isomers with an acetyl group either on the GalA or Rha moieties. This finding was confirmed for ion peak m/z 1093 (^{18}O labeled $Rha_2GalA_2Gal_2Ac_2$) also indicating that acetyl could be linked to either

the GalA or Rha residues. Fragments with an additional loss of one water (-18) refer to different O-glycosyl fragmentation (Doman and Costello, 1988) while the loss of a second water molecule refers to a rearrangement of the galacturonosyl residues.

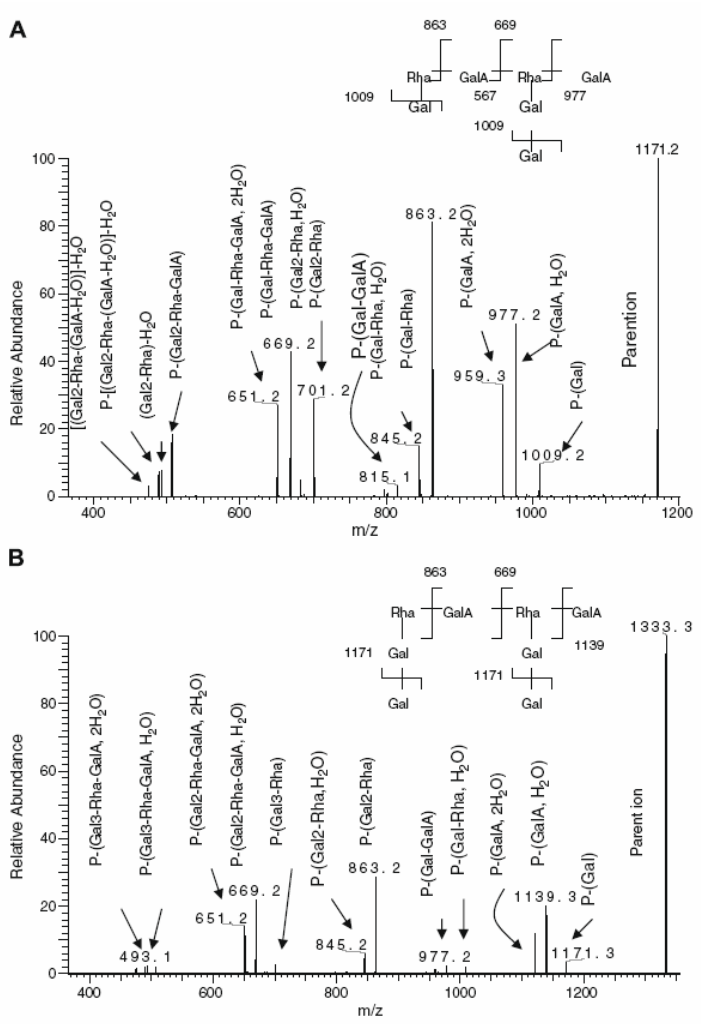

Figure 5 The MS^2 spectrum of $Rha_2GalA_2Gal_3$ (P: m/z=1171) and $Rha_2GalA_2Gal_4$ (P: m/z=1333) for sHBSS digest with RGH. All ions are present as Na adducts (possible structures of the oligomers, m/z= 1171 and 1333, are shown in the right top corner).

From the MS^2 and MS^3 spectra of HBSS RGH oligomers, it can be derived that rhamnosyl residues were O-acetylated. These findings might explain the activity of RGH and RGAE toward HBSS as well as the fact that the acetyl groups attached to the rhamnosyl residues cannot be removed by RGAE but do not hinder RGH either. Some acetyl groups were, however, located on galacturonosyl residues hindering RGH, explaining the different digestion of HBSS and sHBSS. However, MS is not a very reliable technique to quantify the acetylated oligomer since esters are known to have a positive effect on fragmentation efficiency.

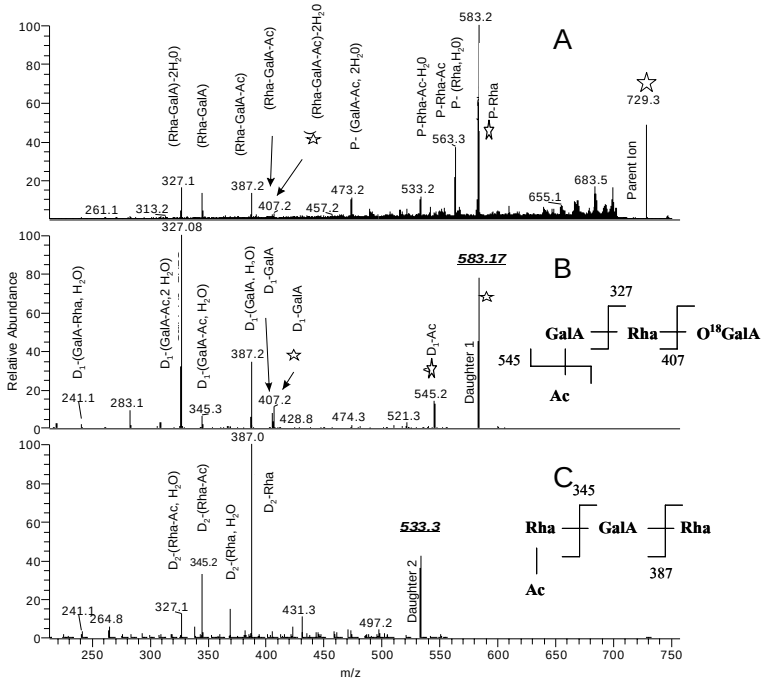

Figure 6 ESI MS^2 (A) of ^{18}O labeled Rha_2GalA_2Ac (Parent, P: m/z = 727) and MS^3 of ion peak Daughter D_1: m/z =583 (B) and D_2: m/z = 533 (C) obtained from the HBSS digest with RGH (the fragments having a star are ^{18}O-labeled). All ions are present as Na adducts (possible structures of the fragments, m/z= 583 and 533, are shown in the right top corner)

Nuclear magnetic resonance of okra HBSS

To confirm the position of the acetyl groups within the HBSS on both the rhamnose and GalA moieties and to enable some quantitation, NMR was performed on a 20 kDa RGH HBSS fragment obtained by preparative SEC over Sephacryl 500 and Sepharcryl 300 (Fig. 7) after RGH digestion in order to overcome the high viscosity of the intact polymer.

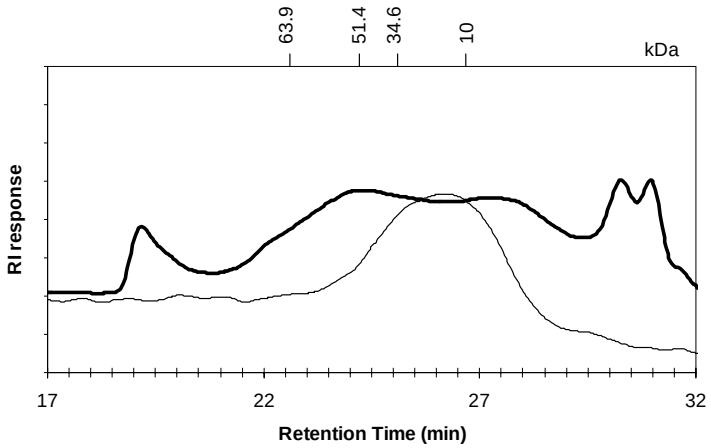

Figure 7 Molecular weigh distributions of HBSS RGH digest (bold line) and a 20 kDa fragment obtained after fractionation of Sepharcryl S500 pool 3 over Sepharcryl S300 (thin line). (The molecular weight indications are based on pectin standards)

Within the anomeric region of the ^{1}H NMR spectrum of recorded five major sugar moieties of a 20 kDa RGH HBSS fragment, 1,2,4-linked α-Rha*p* (A), 1,4-linked α-Gal*p*A (RG I) (B) (Colquhoun et al., 1990; Mutter et al., 1994; Renard et al., 1999; Deng et al., 2006), t-α-Gal*p* (C) (Gronberg et al., 1994; Strecker et al., 1995) , 1,4-linked Gal*p* (D) and t-β-Gal*p* (E) (Colquhoun et al., 1990; Huisman et al., 2001) could be observed (Table 2). In general, the spectra underline the regular structure of okra RG I being a highly branched rhamnogalacturonan with only one or two galactose units as side chains. A signal at 2.106 ppm typically belonging to an O-acetyl substituent was observed. In general, the O-acetyl units present on the RG I backbone are expected to be linked to O-3 and/or O-2 of the GalA

units present in the backbone (Lerouge et al., 1993; Ishii, 1997; Komalavilas and Mort, 1998). However, the spin systems found for the GalA units (3.95 and 4.11 ppm) did not show the expected significant downfield shift for proton 2 and/or 3 (5.10 and 5.40 ppm) (Lerouge et al., 1993; Ishii, 1997). Surprisingly, the O-acetyl substituent was thus found to be attached to O-3 of the rhamnosyl moiety as indicated by the significant down field shift of H-2 and H-3, 4.226 and 5.254 ppm, respectively. Furthermore, no indications for methyl esterification or other O-acetylation sites were found. O-acetylation of rhamnose within RG I had never been reported before. The chemical shift of the O-acetyl group attached rhamnose seems to overlap with the chemical shift of an acetyl group at position 3 of a GalA in RG I, as observed around 2.10 ppm by Peronne et al (2002). in spinach pectin and by Lerouge et al.(1993) in RG I from suspension cultured sycamore cells

Using a 2D homonuclear ROESY experiment the connectivity between the different residues observed could be established. The presence of a -2)-α-Rha(1 → 4)α-GalA-(1- dimeric repeat, representing the RG I backbone, could be established by the presence of ROESY cross-peaks between B H-1 and A H-1, B H-4 and A H-1, and A H-2 and B H-1 as indicated in Figure 8a and b. Furthermore, the integral of both anomeric signals, 1,2,4-linked rhamnose and 1,4-linked galacturonic acid showed a ratio of 1:1 again proving that all rhamnoses and galacturonic acid within this sample were derived from the RG I backbone. All rhamnosyl moieties within the samples were substituted at O-4 with either 1 or 2 galactosyl residues as proven by the proton chemical shifts (Table 2), indicative for a 1,2,4-linked rhamnose (Colquhoun et al., 1990; Huisman et al., 2001; Habibi et al., 2004) which is further substantiated by the substitution of acetyl at O-3 and by the chemical shift of H-6.

The anomeric signals D and E in the ROESY spectrum were β-galactosyl residues and could rather be well established using reference data from literature (Colquhoun et al., 1990; Huisman et al., 2001; Habibi et al., 2004; Deng et al., 2006). The chemical shift values of anomeric signal C, however, did not fit with any of the data observed for RG I or RG I fragments measured by proton NMR. However, according to the sugar composition of the RG I fragments measured, this anomeric signal C should be a galactose. Therefore, the proton chemical shift of anomeric signal C was inserted in the SweetDB database provided

by the German Cancer Research Center Heidelberg, Central Spectroscopic Division, Germany (http:// www.glycosciences.de/sweetdb/). The database search provided a close hit (ca. 98%) with a t-α-Gal linked to O-4 of a β-1,4-linked Gal (Gronberg et al., 1994;

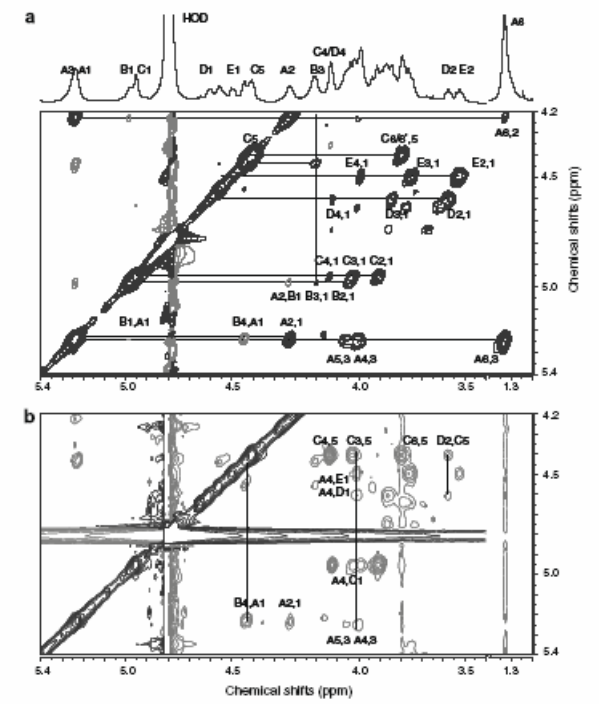

Figure 8 (a) Segment of 2D homonuclear TOCSY NMR spectrum with annotated cross-peaks; A1 = H-1 residue A, B3,1 = cross-peak between B H-1 and B H-3 etc. Gray cross peaks are ROESY peaks appearing negative in the spectrum they are annotated A4, B1 = inter residual coupling between A H-4 and B H-1 etc. (b) Segment of 2D homonuclear ROESY NMR spectrum with annotated cross-peaks; A1 = H-1 residue A, B3,1 = cross-peak between B H-1 and B H-3 etc. and A4, B1 = inter residual coupling between A H-4 and B H-1 etc. (A:1,2,4-linked α-Rha*p*, B: 1,4-linked α-Gal*p*A (RG I), C: t-α-Gal*p*,D: 1,4-linked Gal*p* and E: t-β-Gal*p*).

Strecker et al., 1995). The presence of an α-(1,4)-linked galactose dimer is proven by the D H-2, C H-5 ROESY cross-peak between the β(1,4)-linked galactosyl (D) and the t-α-galactosyl unit (C). The presence of a t-α-galactose at the terminus of RG I side chains had not been observed before in any other pectins. Moreover, the integral of t-α-galactose compared to 1,4-β-galactose was about 2:1 (Table 2).

To obtain additional proof for the linkage between and the sequence of the sugar moieties found, a 2D heteronuclear HMBC spectrum was obtained. A fully resolved spectrum seemed impossible due to the viscosity and heterogeneity of the samples. The OAc substituent could be used to determine the carbon chemical shifts of the more flexible moieties, for example, for rhamnose C-6. These chemical shifts are represented between brackets in Table 2. From the data that could be obtained, the presence of a t-α-galactose could also be confirmed based on the carbon chemical shifts. Furthermore, the only inter residual scalar coupling observed was between rhamnose C-4 and H-1 of the t-α-galactose proving the direct linkage between this moiety and the RG I backbone.

By using 20 kDa HBSS RGH population as a reference, the structure of intact RG I from HBSS could be revealed. A COSY, TOCSY, ROESY, and HMBC spectra of intact samples were recorded. Figure 9 shows the ^{1}H NMR spectrum of intact RG I in comparison to the 20 kDa RGH HBSS fragment. Obviously, intact structures showed the same structural elements as found within the 20 kDa RGH HBSS fragment. From the data obtained for intact RG I HBSS, it is obvious that the sample resembles the 20 kDa RGH HBSS fragment except for the presence of unbranched α-1,2-linked rhamnose in the intact RG I HBSS. The unbranched rhamnose has been removed from the 20 kDa RGH HBSS fragment by the treatment with RG hydrolase as already indicated by MALDI-TOF MS. Furthermore, the chemical shifts found for the non-substituted rhamnose correlated well with the chemical shifts found in literature (Gronberg et al., 1994; Renard et al., 1997; Renard et al., 1999; Habibi et al., 2004). This indicates that the unbranched rhamnose is not acetylated at position O-3 like the branched rhamnosyl residues. The relative amount of the different residues observed was calculated from the integrated signals present within the anomeric region of both the acetyl groups and rhamnosyl substituents with galatosyl units (Table 2). The ratios indicate that within HBSS, 62% of the rhamnosyl units were

Table 2 ^{1}H chemical shifts in ppm of HBSS RGH, intact RG I HBSS and intact RG I CHSS PG/PME

HBSS RGH	**H-1**	**H-2**	**H-3**	**H-4**	**H-5**	**H-6**	**RATIO**[2)]
(A) 1,2,4-α-L-Rha[1)]	5.238	4.227	5.249	3.903	3.965	1.332	1
				(78.11)	(69.33)	(18.19)	
(B) 1,4-α-D-GalA (RGI)	4.984	3.947	4.109	4.441	4.555		0.92
(C) t-α-D-Gal	4.952	3.808	3.923	4.039	4.399	3.697	1
	(101.57)[3)]	(70.55)	(70.29)		(72.06)	(61.84)	
(D) 1,4-β-D-Gal	4.603	3.477	3.743	4.025	-[4)]	-	0.66
(E) t-β-D-Gal	4.492	3.428	3.647	3.891	-	-	0.69
	(104.98)						
CH_3 (*O*-Acetyl)	2.106						0.98
	(22.09)						
COO	174.91						
RG I HBSS							
1,2-α-L-rha	5.257	4.12	3.889	3.415	3.765	1.252	0.55
1,2,4-α-L-rha	5.234	4.224	5.251	3.905	3.951	1.333	0.87
t-α-L-rha	5.229	4.059	3.797	3.353	-	1.231	
1,4-α-D-galA (RGI)	5.012	3.909	4.119	4.417	-		1.00
t-α-D-gal	4.955	3.802	3.935	4.035	4.404	3.696	0.97
1,4-β-D-gal	4.602	3.491	3.756	-	-		0.84
t-β-D-gal	4.492	3.425	3.65	3.888	-		1.11
unknown	4.556	3.563	3.836	4.334	4.563		
CH_3	2.099						1.18
RG I CHSS							
1,2-α-L-rha	5.243	4.116	3.879	3.409	3.745	1.245	0.15
1,2,4-α-L-rha (OAc)	5.199	4.221	5.243	-	3.911	1.328	0.38
1,2,4-α-L-rha	5.163	4.137	3.966	-	3.811	1.313	0.26
1,4-α-D-galA (RGI)	5.007	3.905	4.114	4.427	-		1.00
	4.907	3.719	4.003	4.456	-		
	4.965	3.719	4.003	4.456	-		
	4.951	3.798	3.924	-	-		
t-α-D-gal	4.951	3.789	3.923	4.027	4.392	3.96	0.53
1,4-β-D-gal	4.6	3.482	3.76	3.956	-	-	0.36
t-β-D-gal	4.483	3.421	3.557	3.882	-	-	0.34
1,4-α-D-galA (*O*-acetylated)							
	5.093	4.135	-	-	-		1.66
	5.165	3.976	-	-	-		
	5.216	3.988	-	-	-		
	5.309	4.002	4.416	-	-		
CH_3	2.094						0.31
CH_3	2.071						0.21
CH_3	2.181						0.03
1,4-α-D-galA (HG)							
E	5.105	3.731	4.014	4.466	-		1.01
F	5.143	3.748	4.044	-	-		

1) Arabic letters correspond to annotation in figure 8; 2) Ratio was calculated from the integrals of individual proton signals; 3) ^{13}C chemical shift between brackets; 4) - = not determined; values are expressed against internal acetone at 2.225 and 31.55 ppm

substituted. The remaining rhamnosyl moieties were not substituted. According to the integral of GalA (0.92) that was lesser than integral of Rha (1.0), it could be suggested that part of the galacturonosyl units are O-acetylated resulting in a down-field shift of the anomeric signals which are not fully recognized in the spectrum. From these NMR data, it also becomes clear that RGH is able to cleave the linkage between acetylated rhamnosyl and galacturonosyl moieties within RG I backbone, although the enzyme is much more active against non-acetylated structures. In addition, RGH is also tolerant for both α and β galactosyl residues present as short side chains positioned at rhamnose O-3.

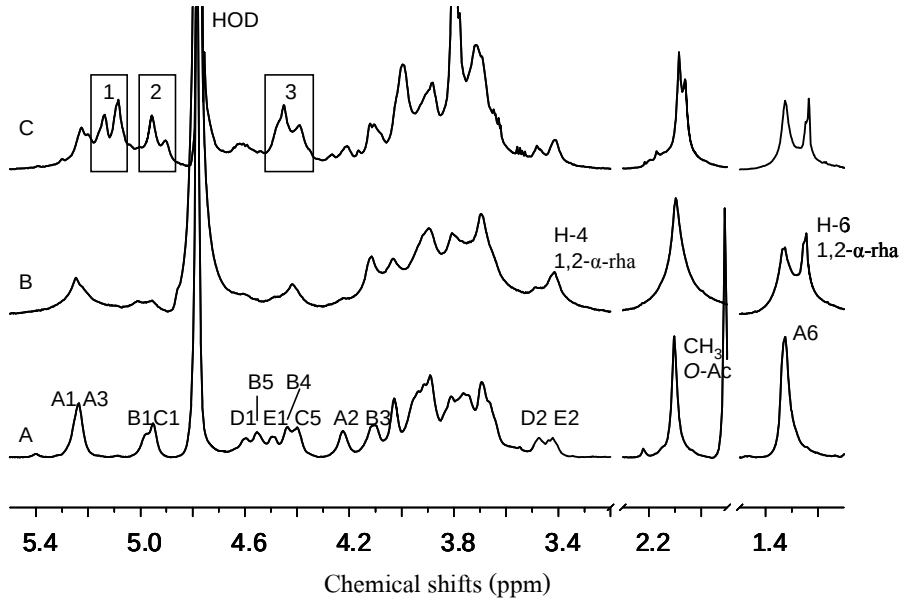

Figure 9 ^{1}H proton NMR spectra of A: 20 kDa HBSS RGH population; B: Intact RG I HBSS; C: Intact RG I CHSS, A = 3-O-acetylated-1,2,4-α-L-rha; B: 1,4-α-D-galA; C: t-α-D-gal; D: 1,4-β-D-gal; E: t- β-D-gal; 1: galA derived from HG or O-acetylated GalA 2: galA derived from RGI and non substituted; 3: galA H-4.

Nuclear magnetic resonance of okra CHSS

Converse to HBSS, RGAE is able to remove the acetyl groups from CHSS. To obtain more structural information of RG I within CHSS compared to RG I in HBSS, the structure of intact RG I from CHSS was revealed by using the 20 kDa RGH HBSS fragment. The ^{1}H NMR spectrum of CHSS intact RG I in comparison to the 20 kDa RGH HBSS fragment is shown in Figure 9. Since the PG treatment could not completely remove the HG part, possible acetylation of HG next to acetylation of the RG could not be ruled out. Therefore, the assignment of the different spin systems present is quite complex and the chemical shifts of the different galacturonosyl residues present could only be partially resolved. Similar to the intact RG I HBSS sample, intact RG I CHSS contained the t-α-galactosyl residues and O-acetylated rhamnosyl residues as observed in 20 kDa RGH HBSS fragment. Either 1,2-linked- and 1,2,4-linked rhamnosyl units, O-acetylated at O-3, could be found within the sample. The GalA interconnecting the 3-O-acetylated rhamnosyl units could be assigned completely except for the proton 5 and had similar chemical shifts as the 20 kDa RGH HBSS fragment. Furthermore, three non-acetylated GalA residue signals (RG I) could be observed as proven by the chemical shifts (Colquhoun et al., 1990; Renard et al., 1997; Habibi et al., 2004;). Moreover, two anomeric signals of GalA residues, with proton 2 chemical shift of 3.731 and 3.748 ppm, respectively, suggest that these signals are non-acetylated and belong to HG. These chemical shifts of GalA belonging to HG have been shown to appear at lower field than GalA belonging to RG I (Needs et al., 1998; Renard and Jarvis, 1999; Perrone et al., 2002). The other galacturonosyl moieties detected are probably derived from O-acetylated GalA since none of the residues showed a COSY cross-peak from the anomeric region to a proton with a chemical shift between 3.75 and 3.80 ppm typical for a non-substituted GalA. This reasoning is further substantiated by finding three significant signals at 2.094, 2.071, and 2.181 ppm, all belonging to an O-acetyl group attached to different positions.

Also for intact RG I CHSS, an estimate of the abundance of the different sugar moieties was made by integrating the proton signals (Table 2). However, due to extreme overlap of the signals sometimes, the values should only be seen as a rough estimate and can rather be under- or over-estimated.

Conclusions

In summary, HBSS contains predominantly RG I structures with monomeric and dimeric galactan side chains, although HG is part of the same molecule to a small extent. The methyl esters present in HBSS seem to be present on the HG part of the molecule. The acetyl groups are predominantly located at position O-3 of the rhamnosyl moiety. Another novelty of Okra RG-I as found in the HBSS fraction is the presence of α-galactose substitution at O-4 of the backbone rhamnosyl residue. In contrast to HBSS RG-I, the pectic material extracted by chelating agent and being homogeneous with respect to size and charge contains much more HG which is relatively highly methyl esterified. Minor amounts of the rhamnose-acetylated RG-I containing α-galactose substitutions are found. In addition, also more common RG-I structural elements are present consisting of a galacturonic acid-acetylated backbone, without α-linked galactosyl residues and with longer arabinose and galactose-containing side chains.

Material and methods

Sequential extraction of okra AIS

Soft and mature okra pods (5–10 cm in length) were collected at local market in June 2005, Thailand. Okra AIS was prepared and then extracted with 0.05 M sodium acetate buffer (Hot Buffer Soluble Solids, HBSS), followed by 0.05 M EDTA and 0.05 M sodium oxalate in 0.05 M sodium acetate (CHelating agent Soluble Solids, CHSS) according to Sengkhamparn et al (2009).

Intact RG I from HBSS and CHSS were prepared by incubation with endo-polygalacturonase (PG) and pectin methyl esterase (PME) according to Sengkhamparn et al (2009).

Analytical methods

Total neutral sugar content and uronic acid content

The total neutral sugar and uronic acid contents were determined by the automated colorimetric orcinol/sulfuric acid method (Tollier and Robin, 1979) and by the automated colorimetric m-hydroxydiphenyl method (Ahmed and Labavitch, 1977; Kintner and van Buren, 1982; Thibault, 1979), respectively. Galactose and galacturonic acid were used as a standard.

Enzyme degradation

A saponification step was performed for removing methyl ester and acetyl groups before incubation with enzymes. The HBSS and CHSS (3–4 mg) were dissolved in 300 µL millipore water and were saponified by adding 300 µL of 0.1 N NaOH. After storage overnight at 4 ^{o}C, the solutions were neutralized with 300 µL of 0.1 M acetic acid and the volume was adjusted to 1 mL with 0.2 M NaOAc buffer of pH 5.

The non-saponified (final concentration of 3–4 mg/mL in 50 mM NaOAc buffer of pH 5) and saponified solutions of HBSS and CHSS were incubated with the following enzymes individually: 0.547 µg protein/mg substrate of rhamnogalacturonan hydrolase (RGH) from *A. aculeatus*, 0.016 units of endo-polygalacturonase (PG) from *A. niger* (Schols et al., 1990), 2.5 units of endo-galactanase(endo-Gal) from *Aspergillus niger* (Guŕjanov et al., 2007), 0.04 units of β-galactosidase from *Kluyveromyces lactis*, 0.04 units of α-galactosidase from Guar seed (Megazyme) and *A.niger*. The incubations were performed at 40 ^{o}C for 24 h and were stopped by heating at 100 ^{o}C for 5 min for inactivating enzymes. The decrease in molecular weight (Mw) and the release of oligomeric products were analyzed by high performance size exclusion chromatography (HPSEC) and high performance anion-exchange chromatography (HPAEC), respectively.

High performance size exclusion chromatography (HPSEC)

The high performance size-exclusion chromatography (Thermo Separation Products, USA) equipped with three TosoH Biosep-TSKGel G columns in series

($4000PW_{XL}$-$3000PW_{XL}$-$2500PW_{XL}$) in combination with a guard PW_{XL} column (TosoH, Japan) was used to observe the change in Mw distribution of samples. The samples were eluted with 0.2 M sodium nitrate at 30 ^{o}C and with a flow rate of 0.8 mL/min (Chen et al., 2004). The eluent was monitored using RI detector (Shodex SE-61, Showa Denko K.K., Japan).

High performace anion-exchange chromatography (HPAEC)

High performance anion-exchange chromatography was performed on a Dionex ISO 3000 system with PAD detector system (USA). For the analysis of enzyme digests, a (2 x 250 mm) CarboPac PA 1 column (Dionex, USA) was equilibrated with 100 mM NaOH. Gradients of NaOH and NaOAc were used simultaneously to elute the oligomers with a flow of 0.3 mL/min. The results are in the following gradients of NaOH: 0.0–15.0 min, 0–16 mM; 15.0–15.1 min, 16–100 mM NaOH; 15.1–85.0 min, 100 mM. The simultaneous gradient of NaOAc was 0.0–15.0 min, 0 mM NaOAc; 15.0–15.1, 0–100 mM NaOAc; 15.1–55.0 min, 100–400 mM NaOAc; 55.0–80.0 min, 400–700 mM NaOAc; 80.0–85.0 min, 700–1000 mM NaOAc. The column was washed for 5 min with 1 M NaOAc in 0.1 M NaOH and equilibrated for 15 min with 16 mM NaOH.

Matrix –assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis

A small spatula of AG 50W-X8 Resin (H^+ form; Bio-Rad, USA) was added to 10 µl of digest sample for desalting, followed by mixing and centrifugation for 5 min at 13,000g. One microliter of sample solution was placed on a MALDI-TOF plate together with 1 µL of matrix solution, 10 mg/mL of 2,5-dihydroxybenzoic acid (Bruker Daltonic's, Germany) in water, and dried under a constant flow of warm air. The oligomers were determined by using MALDI-TOF MS (Ultra flex instrument, Bruker Daltonics, Germany) with a lowest laser energy (35%) of Nitrogen 337 nm laser beam to correcting data from average of 200 shots. A mixture of maltodextrins, mass range of 300–3000 Da, was used for calibration.

Electron spray ionization ion trap mass spectrometry (ESI-IT-MS)

For ESI-IT-MS^n analysis, HBSS was dissolved in water and incubated with 0.547 µg/mg substrate of RGH at 40 ^oC for 24 hr. The digest sample obtained after freeze drying were dissolved in 50% acetonitril/ 49.9% water/ 0.1 % formic acid in H_2O (0.5 mg/mL). For labeling the reducing end with ^{18}O, the sample were dissolved in 50% acetonitril/ 49.9% water/ 0.1 % formic acid in $H_2{}^{18}O$ (0.5 mg/mL) and incubated for 72 hours at 40 ^oC. After centrifugation (15 minutes, 24.000*g* at 20°C) to remove solid impurities, the samples were applied on a LTQ Ion Trap Mass Spectrometer (Thermo Electron, USA) using direct infusion (3 µL/min) with the use of electron spray ionization and detection in positive mode. The capillary voltage was between 4 and 4.9 kV, and the capillary temperature was 225°C. Before use the instrument was tuned with the peak at *m/z* 1009 that is present in HBSS-Sap-RGH sample. The instrument was controlled by XCalibur 2.2 software (Thermo Electron, USA). The scan range was set on *m/z* 300-2000. MS^2 – MS^n was performed with a mass window of *m/z* 2 and the collision energy was optimized for every compound. Mass Frontier 5.0 (Thermo Electron, USA) was used for identifying unknown fragments.

Size exclusion chromatography (SEC)

In order to perform NMR analysis of the rather viscous HBSS sample, the viscosity was lowered by treating with RGH. HBSS (600 mg in 100 mL water) was incubated with RGH from *A.aculeatus* (0.547 µg protein/mg substrate) for 24 h at 40 ^oC and the enzyme was then inactivated at 100 ^oC for 5 min. An Akta explorer system (Amersham Biosciences, Sweden) was used for fractionation of the digest on preparative scale. The preparative SEC was performed on Sephacryl S500 column (560 x 160 mm ID.; Amersham Biosciences, Sweden) and Sephacryl S300 column (900 x 50 mm ID., Amersham Biosciences, Sweden). The sample was loaded and eluted with 20 mL/min of 0.1 M ammonium acetate buffer of pH 5 on the Sephacryl S500 column. Fractions of 250 mL were collected (28 fractions) and determined for the uronic acid and neutral sugars content as described above. After pooling appropriate fractions, the pool with the smallest Mw fragments from the Sephacryl S500 was fractionated using a Sephacryl S300 column.

The sample was eluted with 4 mL/min 0.1 M of ammonium acetate buffer of pH 5. Fractions of 12.5 mL were collected (54 fractions) and analyzed for uronic acid and neutral sugar content as described above. After pooling, all pools were desalted by dialysis through 0.1 kDa membrane and freeze dried.

^{13}C and ^{1}H Nuclear magnetic resonance (NMR)

Prior to NMR analyses, the sample was dissolved in 0.5 mM NaOAc buffer of pH 5, freeze dried, and then exchanged twice with 99.96% D_2O (Cambridge Isotope Laboratories). A Bruker AV-600 cryoprobe NMR spectrometer located at Biqualys, Wageningen, was used to record NMR spectra at a probe temperature of 25°C. The 1 D ^{1}H proton was recorded at 600.13 MHz as described by Sengkhamparn et al (2009). The 2D COSY spectrum was acquired using the double quantum filtered (DQF) method with a standard pulse sequence delivered by Bruker. 2D TOCSY spectra were acquired using standard Bruker pulse sequences with 110 ms mixing time. For all homonuclear 2D spectra, 512 experiments of 2048 data points were recorded using 16-64 scans per increment. Chemical shifts were expressed in part per million relative to internal acetone: δ=2.225 ppm for ^{1}H and δ=31.55ppm for ^{13}C.

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Chapter 4

Physicochemical properties of pectins from Okra (*Abelmoschus esculentus* (L.) Moench

Sengkhamparn, N., Sagis, L.M.C., de Vries, R., Schols, H.A., Sajjaanantakul, T. & Voragen, A.G.J. Physicochemical properties of pectins from Okra (*Abelmoschus esculentus* (L.) Moench. Food Hydrocolloid., 2010, 24, 35-41.

Abstract

Okra pectin obtained by hot buffer extraction (HBSS) consists of an unusual pectic rhamnogalacturonan I structure in which acetyl groups and alpha galactosyl residues are substituted on rhamnosyl residues within the backbone. The okra Chelating agent Soluble Solids (CHSS) pectin consists of slightly different structures since relatively more homogalacturonan is present within the macromolecule and the rhamnogalacturonan I segments carry slightly longer side chains. The rheological properties of both okra pectins were examined under various conditions in order to understand the unusual slimy behaviour of okra pectins. The viscosity of the okra HBSS pectin was 5–8 times higher than the viscosity of the okra CHSS pectin. The okra HBSS pectin showed an elastic behaviour ($G' > G''$) over a wide range of frequencies (10^{-1}–10 Hz), at a strain of 10%, while okra CHSS and saponified okra HBSS/CHSS pectin showed predominantly viscous responses ($G' < G''$) over the same frequency range. The results suggest that the structural variation within the okra pectins greatly affect their rheological behaviour and it is suggested that acetylation of the pectin plays an important role through hydrophobic associations. Dynamic light scattering was used to study the association behaviour of both okra pectins at low concentration (0.001–0.1% w/w). Results showed that the saponified okra pectins did not exhibit a tendency to aggregate in the concentration range studied, whereas both non saponified samples showed a substantial degree of association. These results suggest that the unusual slimy behaviour of the non saponified samples may be related to the tendency of these pectins to associate, driven by hydrophobic interactions.

KEY WORDS: Okra, Pectin, Rhamnogalacturonan I, Rheological properties, Dynamic light scattering, Hydrophobic association

Introduction

The okra plant, *Abelmoschus esculentus* (L.) Moench, is a native plant from Africa and now grown in many areas such as Asia, Middle East and the southern states of the USA. The Okra pod is used for many purposes; for example as a vegetable, as a thickening ingredient for soup and stews (BeMiller et al., 1993; Woolfe et al., 1977), and okra is also used in folk medicine as a diuretic agent and for treatment of dental diseases (Ndjouenkeu et al., 1996). Recently, it is also claimed that water extracted polysaccharides from okra could be used as egg- white substitute (Costantino and Romanchick-Cerpoviez, 2004) and as fat substitute in chocolate bar cookies (Romanchik-Cerpovicz et al., 2002) and in chocolate frozen dairy dessert (Romanchik-Cerpovicz et al., 2006).

Okra polysaccharides extracted with water can be characterized as an acidic polysaccharide consisting of galactose, rhamnose and galacturonic acid (Whistler and Conrad, 1954). The major structural element of okra pectic polysaccharide contains a repeating unit of alternating rhamnosyl and galacturonosyl residues and carries disaccharide side chains composed of galactose attached to O-4 of half the rhamnosyl residues (Tomada et al., 1980). The acetyl content of the okra polysaccharide was determined to be 5.5% (w/w) (Tomada et al., 1980). A detailed study by Sengkhamparn, et al. (2009a) recently described the different pectic and hemicellulose (xylan, xyloglucan) structures present in okra. The pectins present in the Hot Buffer Soluble Solids (HBSS) and Chelating agent Soluble Solids (CHSS) were studied after enzymatic degradation and subsequent elucidation of the structure of the fragments by mass spectrometry and NMR spectroscopy. It was indicated that the HBSS fraction consisted almost exclusively of an acetylated rhamnogalacturonan (RG) I with very short side chains (1–2 galactosyl residues on average) while CHSS consisted mainly of homogalacturonan (HG) (70%) next to 30% RG I segments which have somewhat longer galactosyl containing side chains (2–3 residues on average) when compared to HBSS RG I while also arabinan and arabinogalactan type II neutral sugar side chains are present (Sengkhamparn et al., 2009a). It was shown that acetyl groups present in HBSS were linked to the rhamnosyl residues, in contrast to RG I from other plant origin where the galacturonosyl moieties carries the acetyl substitution. The Degree of Methyl esterification of HBSS pectin (moles methanol per 100 mol of

galacturonic acid) was found to be 24%. However, following the literature stating that no evidence exists for methyl esterification of RG I, a DM of the HG segment of okra HBSS pectin was calculated to be about 80%. It was also found that 78% of all rhamnosyl residues present in RG I caries an acetyl group. In addition, an a linked galactosyl residue was found as a terminal sugar residue of the side chains of HBSS (Sengkhamparn et al., 2009b). The DM of the CHSS pectin was 48% indicating that the CHSS HG part carry quite some negative charges. Acetyl groups present in CHSS pectin were mainly located on the galacturonosyl residues, although there was some acetylation of the rhamnose moieties, as mentioned for the HBSS pectin. The HBSS pectin and CHSS pectins both showed only one population of molecules, each having a molecular weight of more than 100 kDa (Sengkhamparn et al., 2009a and 2009b).

Okra polysaccharide solutions were found to be pseudo-plastic and viscoelastic (BeMiller et al., 1993). Okra polysaccharides in solution with a fixed ionic concentration (0.1 M NaCl) gave intrinsic viscosity of 7.6 dl g^{-1} and showed shear thinning behaviour and strong tendency to self-association (Ndjouenkeu et al., 1996). Baht and Tharathan (1987) found that the viscosity of aqueous extracts of okra polysaccharide (BSP)was highest in the pH range of 4–6 and decreased on addition of glucose and/or sucrose (5–40%). The viscosity of BSP dispersions were only slightly affected by the presence of 0.1–10% of monovalent salts (NaCl and KCl), but were strongly affected by adding 0.1–10% of divalent salts ($CaCl_2$ and $MgSO_4$). BSP also exhibited foam stabilizing and gelling properties. An aqueous solution of BSP could form a gel by either keeping a 1% solution at 60 ^oC for 30 min and then cooling it at 4 ^oC for 24 h or by blending the BSP solution with xanthan gum (Baht and Tharathan, 1987).

It was observed that during the isolation of okra pectin from okra pods unusual viscous solutions were obtained which however, could readily be ultrafiltrated due to a shear thinning behaviour. In this study, we try to explain the typical behaviour of okra pectins describing some rheological properties of different okra pectins measured under various conditions and trying to related these properties to the chemical structure of these pectins.

Results and discussion

Flow behaviour measurements

Based on the chemical structure information, it can be concluded that the okra HBSS extract contains large molecules consisting exclusively of linearly branched RG I, and is a negatively charged polymer that has both slightly hydrophilic (neutral sugar side chains) as well as slightly hydrophobic sites (acetyl groups). Okra CHSS pectins are large polymers containing mainly linear HG chains and are negatively charged polymers with only minor amounts of branched RG I polymeric segments. The branched polymers within okra CHSS pectin carry slightly longer side chains than okra HBSS pectin. In contrast to HBSS where acetyl substitution is present on the rhamnose, most of the acetyl groups present in CHSS is present on the galacturonic acid moiety.

Table 1 Sugar composition (mol%) of HBSS and CHSS pectins obtain from Okra AIS. (Sengkhamparn et al., 2009a and 2009b)

	Rha	Ara	Gal	Glu	GalA	GlcA	DM[a] (%)	DA[a] (%)	Total sugar[b]
HBSS	26	0	34	1	35	3	24	58	90
CHSS	14	3	17	1	63	2	48	18	86

[a] moles acetic acid or methanol per 100 mole of galacturonic acid

[b] gram qualities per 100 g of fraction

An unusual slimy appearance of the okra HBSS pectin during extraction was observed, although the viscous solution was rather easily ultrafiltered. Therefore, a rheological characterization of okra HBSS and CHSS pectin in water was performed and the results were compared to de-esterified okra HBSS and CHSS pectins obtained after saponification (sHBSS and sCHSS) in order to gain insight in the relation between the chemical structure and the rheological behaviour in the native form and de-esterified form.

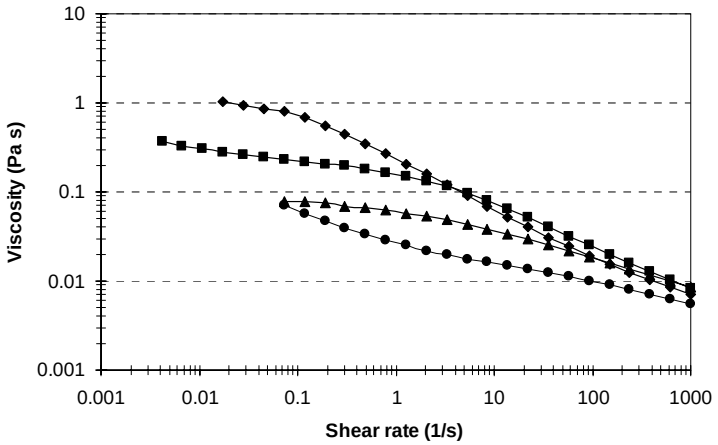

Figure 1 Flow curves (20°C) of okra pectins: HBSS (♦), sHBSS (■), CHSS (▲) and sCHSS (●) solutions at 0.1 % w/w concentration.

The shear rate sweeps (0.01–100 s^{-1}) of the viscosity for all samples at 0.1% w/w concentration in water (Fig. 1) show that the viscosity of a 0.1% w/w solution of okra HBSS pectin was 5–8 times higher than the viscosity of okra CHSS pectin. The viscosity of a polysaccharide solution depends on many factors such as molecular mass, stiffness and charge of the molecule (Williams and Phillips, 2000). Therefore, our results might be the result of differences in the molecular structure of the two pectins where okra HBSS RG I contains mainly acetylated rhamnosyl residues while the okra CHSS pectin contains relatively much more homogalacturonan. Our results suggest that the hydrophobic acetylated RG I molecules in HBSS, which may be clustered by hydrophobic associations through acetylated rhamnosyl residues, increased the viscosity to a much higher level than acetylated galA residues present in CHSS. Despite the fact that an increased level of total negative charge and/or higher charge densities (as in CHSS) in general will lead to an increased viscosity (Williams and Phillips, 2000), it seems that this effect has been exceeded by acetylated rhamnosyl moieties within the RG I backbone.

The effect of methyl esters and acetyl groups on physical properties of the okra pectins can be determined after chemical removal of these substitutions and comparison of the physical properties of the resulting pectins with those of the original sample is made. Chemical saponification at low temperature was used to remove the acetyl groups and methyl esters from the pectin, with only a slight decrease in the molecular weight distribution as was established by size exclusion chromatography (Fig. 2). Due to the removal of the methyl esters, the saponified sample contained more charged carboxyl groups than the original sample. Since the pH of the sample solutions was above the pKa of a galacturonic acid carboxyl group, it could be expected that the saponified sample would show a higher viscosity than the non saponified sample, since the increased charge will results in an increase in coil dimensions. However, our results show that at low shear rates okra sHBSS pectin had a (much) lower viscosity than the original okra HBSS pectin, confirming the importance of the acetylated backbone for the viscosity. Surprisingly, like okra sHBSS pectin, okra sCHSS pectin also showed a lower viscosity than the original CHSS although the net charge of the saponified molecules is quite high (63% Galacturonic acid, no esters present). However, the difference in viscosity between okra CHSS and sCHSS pectins was relatively small (about a factor of 2). From these results, it can be concluded that the acetylation of RG I plays an important role in the viscosity properties of okra pectins, probably by promoting hydrophobic associations. In addition, the precise location of the acetylation seems to play a major role where acetylated rhamnosyl moieties seems to lead to higher viscosities.

The specific viscosity is defined as $\eta_{sp} = (\eta-\eta_s)/\eta_s$, where η_s is the viscosity of the solvent (1 mPa s for water at 20 ^{o}C) (Morris et al., 1981). At a 0.1% w/w concentration the η_{sp} of okra HBSS pectinwas 1095, which was much higher than the η_{sp} (estimated from $[\eta]$ C, where $[\eta]$ is the intrinsic viscosity and C is the concentration = 0.1% w/w) of lime pectins (0.41, Koubala et al.,2008), amberella pectins (0.48, Koubala et al., 2008), apple pectins (0.31, Hwang and Kokini, 1992) and even higher than found for okra gum (0.76) as reported by Ndjouenkeu et al. (1996). The specific viscosities of these samples were determined in the presence of 50–100 mM salt, which affects the coil dimensions by screening the electrostatic repulsion between the segments. Our samples were measured in

pure water, which may account for the huge difference in specific viscosities between our samples and those reported in the literature.

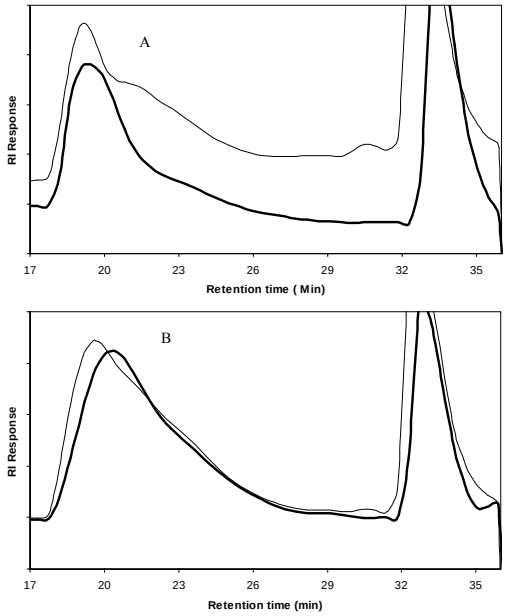

Figure 2 HPSEC elution patterns of HBSS (A) and CHSS (B) before (bold line) and after (thin line) saponification.

The flow curve of all okra pectin samples, HBSS, sHBSS, CHSS and sCHSS at 0.1% w/w concentration (Fig. 1) shows that all solutions revealed a shear thinning behaviour, as the viscosity decreased with increasing shear rates. For okra HBSS, sHBSS and CHSS pectins the zero shear plateau is clearly visible. Other pectins also showed shear thinning behaviour but at higher concentrations than for okra pectins such as pectins from pumpkin at 3% w/w concentration (Evageliou et al., 2005) and mango at 2% w/w concentration (Iagher et al., 2002).

The large differences in viscosity of solutions of okra HBSS and sHBSS pectins (5 times lower in okra sHBSS pectin) triggered us to investigate the effect of concentration on the viscosity of the solution. The zero shear viscosity (η_0) of okra HBSS and sHBSS pectins was plotted versus the concentration in Figure 3. In typical viscosity-concentration curves

of entangled polymer solutions a change of slope is observed at the entanglement concentration c*. At concentrations below c* the zero shear viscosity scales as $\eta_0 \alpha c^{1.3}$, and at concentrations above c* it scales as $\eta_0 \alpha c^4$ (Rubinstein and Semenov, 2001). The zero shear viscosities of our okra HBSS and sHBSS pectin solutions plotted in Figure 3, scale as $\eta_0 \alpha c^{2.0}$ and $\eta_0 \alpha c^{1.7}$, respectively, and no change in the slope of the curves was found in the range of concentrations of 0.1–0.5% w/w for either polymer. Ndjouenkeu et al. (1996) reported a value for c* for okra gum of about 0.15% w/w. For our samples c* is apparently lower than 0.1% w/w. This difference may be due to a difference in okra variety and extraction procedure (BeMiller et al., 1993). In addition, Ndjouenkeu et al. (1996) added 0.1 M NaCl to their samples, which would reduce the coil dimensions of the okra polymers, by screening the electrostatic repulsion between the polymer segments. The results plotted in Figure 3 suggest that the okra HBSS pectin molecules are able to entangle at a low concentration (<0.1% w/w), which is much lower than the entanglement concentrations mentioned for citrus pectins (0.6% w/w) (Kjoniksen et al., 2005). The scaling of the zero shear viscosity with concentration we have observed is close to that found by Ndjouenkeu et al. (1996), at concentrations above c*, and below c** (the start of the concentrated regime). In this regime they observed a scaling exponent of 2.1.

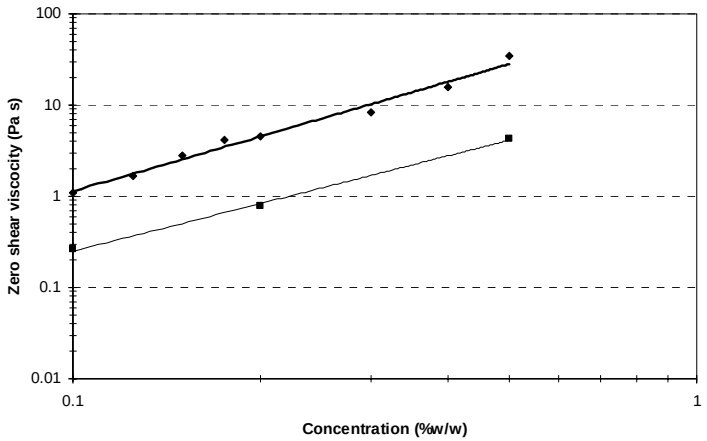

Figure 3 Dependence of zero shear viscosity (η_0) on the concentration of okra HBSS (♦) and sHBSS pectins (■).

The shear thinning region of the samples in each concentration was fitted with the Ostward-DeWaele equation, $\eta = m\,\gamma^{n-1}$, where *m* is the consistency index and *n* is the flow behaviour index. The *m* and *n* values of okra HBSS and sHBSS pectins are shown in Table 2 and it can be seen that the *n* values are lower than the *n* values found for apple pectins (0.83, Hwang and Kokini, 1992) which suggests that the okra HBSS pectin is more pseudo-plastic than the apple pectins due to entanglement of the okra pectins molecules. This can be explained by the nature of the substituents of the pectin backbone: it has already been mentioned by Hwang and Kokini (1992) that more side chains and more branched side chains may result in lower *n* values. Moreover, the okra HBSS pectins contained mostly RG I segments when compared to most other pectins such as citrus, apple pectins which contain mainly HG segments. The trend of a decrease of the n-values with increasing concentration of okra HBSS and sHBSS pectins does suggest that the polymers aggregate (Yanes et al., 2002). Moreover, all samples show Cox–Merz superposition (Cox & Merz, 1958) of the complex viscosity $\eta^*(\omega) = \sqrt{G'^2(\omega) + G''^2(\omega)}\,/\,\omega$ and the steady shear viscosity $\eta(\dot{\gamma})$ evaluated at $\dot{\gamma} = \omega$, which implies that the aggregation does not lead to the formation of a weak associative network, as observed in for example xanthan solutions.

Oscillatory shear measurements

A strain sweep was carried out with deformation values of 10 –10^3% (Fig. 4) to determine the linear viscoelastic region of the pectin. A deformation value of 10 % for every concentration of the samples was in the linear viscoelastic region, and was used to determine the modulus G' and G''. At a frequency of 0.1 s^{-1} the okra HBSS pectin has a significantly higher storage modulus G' than the other samples. For the latter samples G'' is larger than G' for the entire range of strain values. For the okra HBSS pectin the G′ and G'' values were roughly equal for all strains (i.e. tan $\delta \sim 1$).

Table 2 The consistency index (m) and the flow behaviour index (n) of okra HBSS and sHBSS pectins in the power region of η versus γ ($\eta = m\,\gamma^{n-1}$) as a function of concentration.

Concentration (%w/w)	HBSS		sHBSS	
	consistency index (m)	flow behaviour index (n)	consistency index (m)	flow behaviour index (n)
0.100	0.22	0.47	0.11	0.53
0.125	0.31	0.45		
0.150	0.44	0.42		
0.175	0.52	0.41		
0.200	0.64	0.40	0.43	0.48
0.300	1.07	0.37		
0.400	1.71	0.33		
0.500	3.00	0.30	1.59	0.37

To determine the dependence of G' and G'' on the frequency, frequency sweeps were performed over a range of 10^{-1} to 10 Hz at strain of 10% as shown in Figure 5. The modulus G' was lower than the modulus G'' only at low frequency values. The crossover between viscous and elastic behaviour of the okra HBSS pectin was at a frequency of about 0.1 s^{-1} (Fig. 5). For the other okra pectin samples (sHBSS, CHSS and sCHSS) the crossover occurred at much higher frequencies ($f > 1$ s^{-1}). The crossover of G' and G'' values provides a good indication of viscoelastic behaviour of the material (Iagher et al., 2002) and defines the beginning of the elastic behaviour or approaching gel state (Norziah et al., 2001). The okra HBSS pectin solution presented a crossover point at frequency values lower than the okra sHBSS and CHSS pectins, probably due to the fact that the okra HBSS pectin structure can more easily form elastically active zones than okra sHBSS pectin. Although the presence of acetyl groups has a negative effect on the gel formation of sugar beet pectins (Oosterveld et al., 2000), our results show that the removal of acetyl group in okra HBSS pectin weakens the tendency to network formation. These results may be due to the

position of acetyl groups on the sugar residues of the backbone, which is located on O-2 or O-3 of galacturonic acid in HG segments for sugar beet pectin and which prevents the formation of calcium–pectate gels, while the acetyl group is linked to rhamnosyl residues on position O-3 in RG I segments for okra HBSS pectin, giving rise to hydrophobic interactions. The removal of acetyl groups results in decreasing the viscosity of the solution and changing the solution behaviour from an elastic behaviour ($G' > G''$) into a more viscous behaviour ($G' < G''$), in the frequency range from 10^{-1} to 10 Hz.

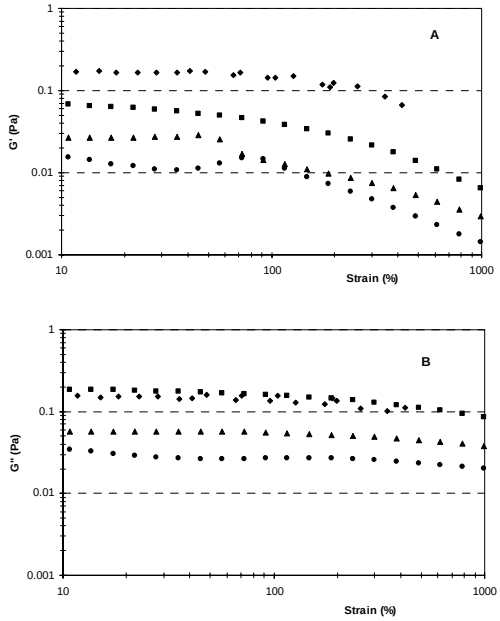

Figure 4 Strain sweeps at 0.1 s^{-1} frequency of modulus G' (A) and G'' (B) for okra pectins: HBSS (♦), sHBSS (■), CHSS (▲) and sCHSS (●) solutions at 0.1 % w/w concentration (20°C).

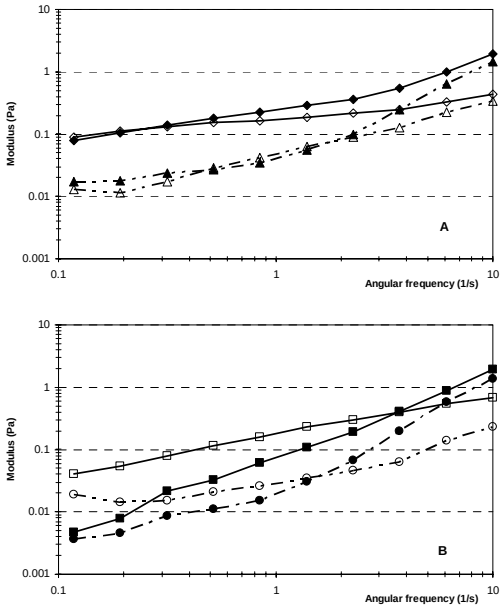

Figure 5 (A) Frequency sweeps at 10 % stain of modulus *G′* (filled symbols) and *G″*(opened symbols) for okra pectins solutions : HBSS (♦) and CHSS (▲). (B) Frequency sweeps at 10 % stain of modulus *G′* (filled symbols) and *G″*(opened symbols) for saponified okra pectin solutions: sHBSS (■) and sCHSS (●) at 0.1 % w/w concentration (20^{o}C)

The removal of acetyl groups and methyl esters influence the behaviour of okra HBSS pectin polymers more than is the case for okra CHSS pectin. Therefore, to understand the effect of acetyl groups on pectin behaviour, the concentration of HBSS and sHBSS pectin was varied and the behaviour of the solutions was investigated. Frequency sweeps (Fig. 6) of okra HBSS pectin at 0.2–0.5% w/w concentration showed that a decrease in concentration caused a shift of the crossover point to higher frequency values which is also found in okra sHBSS pectin (data not shown). This can be explained by the decrease in elastically active zones at lower pectin concentrations. The elastic modulus *G′* of okra HBSS and sHBSS pectin (Fig. 7) increases with increasing concentration probably due to the increase in the number of elastically active zones (Norziah et al., 2001). The

concentration dependency of G' can be expressed by the power law of $G' \alpha c^{\beta}$, with β = 1.7 and 1.5 for okra HBSS and sHBSS pectin. A dependence of $G' \alpha c^{2}$ is commonly found well above c* for normal polysaccharide gels (Clark and Ross-Murphy, 1987).

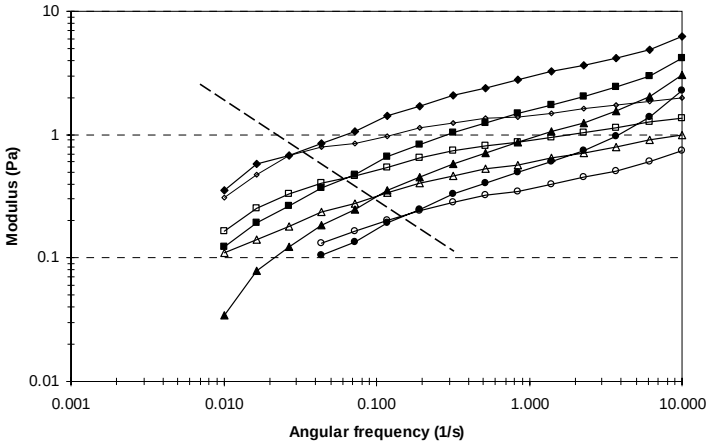

Figure 6 Frequency sweeps at 10 % stain of modulus G' (filled symbols) and G''(opened symbols) for okra HBSS solutions at 0.5 % w/w (♦), 0.4% w/w (■), 0.3% w/w(▲) and 0.2% w/w (●) concentration (20°C).

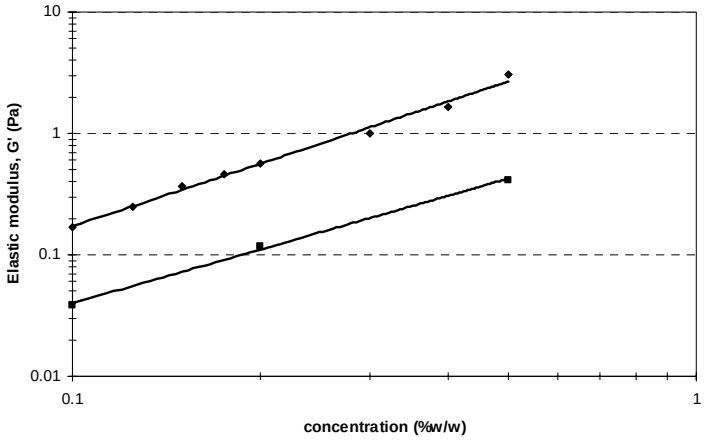

Figure 7 Dependence of elastic modulus (G') on concentration for okra HBSS (♦) and sHBSS (■) pectins at 11% strain and 0.1 s^{-1} frequency.

The frequency dependency of G' can also be expressed by the power law $G' \alpha \omega^p$, in which is $p \sim 1$ for a sample behaving like a viscous solution and $p \sim 0$ for samples which show an elastic behaviour (De Brito et al., 2005). The p values of okra HBSS pectin (Table 3) at 0.1% w/w concentration were lower than the other samples at the same concentration, which may suggest that the okra HBSS pectin solution was a more elastic system. The p values decreased with increasing concentrations, indicating a shift to a more elastic behaviour of the solution due to an increase in entanglements with increasing concentration. As mentioned before, the hydrophobic association of acetylated rhamnose seems to be the main factor to explain the viscoelastic behaviour of okra pectin when dissolved in water. This specific structure of okra pectin makes it different from all known other pectins.

Table 3 The power law p values of okra HBSS and sHBSS pectins in the power region of G' versus ω ($G' \alpha \omega^p$) as a function of concentration and power law p of okra CHSS and sCHSS pectins at 0.1 % w/w concentration.

Concentration (%w/w)	HBSS	sHBSS	CHSS	sCHSS
0.100	0.72	1.32	1.11	1.44
0.125	0.66			
0.150	0.58			
0.175	0.53			
0.200	0.52	0.86		
0.300	0.48			
0.400	0.42			
0.500	0.34	0.81		

[nd] Not determined

Dynamic light scattering

To confirm that our non saponified samples are indeed associating in solution, as a result of hydrophobic interactions, dynamic light scattering was performed at low concentrations (0.001–0.1% w/w). To increase reproducibility of the measurements all okra pectin samples were dissolved in a low ionic strength (10 mM) filtered sodium acetate buffer with a pH of 5.

The intensity of scattered light for sufficiently small objects, at sufficiently low concentrations is proportional to both the molecular weight M and the concentration C:

$$I \propto MC$$

At higher concentrations and for larger object sizes (roughly above 100 nm), this proportionality is no longer strictly valid (Finsy, 1994). The ratio *I/C* is therefore only roughly proportional to the molecular weight of the scattering objects. Figure 8 shows the dependency of *I/C* on the concentration for the different pectin samples. Evidently, the objects (aggregates/polymer coils) formed by the saponified okra pectins (sHBSS and sCHSS) have lower scattering intensity than non saponified okra pectins and the size of the aggregates/polymer molecules was not dependent on the concentration. This is in contrast to the concentration dependent sizes observed for the non saponified okra pectins (HBSS and CHSS). The higher scattering intensity of the CHSS and HBSS indicates a stronger tendency to aggregate, and aggregation is strongly concentration dependent. In conclusion, the saponified okra pectins have lower tendency to aggregate compared to non saponified okra pectins. Comparing okra HBSS to okra CHSS pectin, the scattering intensity of okra HBSS pectin was lower and less concentration dependent than for okra CHSS pectin indicating that at low concentrations and in acetate buffer okra HBSS pectin has less tendency to aggregate than okra CHSS pectin.

Sizes of the scattering objects can be deduced from a dynamic light scattering experiment in which the fluctuations of the scattered light are analyzed that are a consequence of the Brownian motion of the scattering objects. Analysis of the correlation function of the intensity of scattered light as a function of time, using the Malvern DTS software, version 5.0 showed that in general two types of particles were present, one slowly moving (pectin aggregates) and one fast moving (pectin coils). Estimated hydrodynamic

diameters of the pectin aggregates and pectin coils are given in Table 4. The size of the fast moving objects, presumably the pectin coils, was around 20–30 nm for all pectin samples and at all concentrations; however the sizes of the slow moving objects, pectin aggregates, were highly variable. The aggregate sizes of saponified pectin molecules were concentration independent, which is consistent with the scattering intensities, and aggregate sizes were found to be around 210 and 175 nm for sHBSS and sCHSS, respectively. The aggregate sizes of non saponified okra pectin were highly concentration dependent, especially for okra CHSS pectin in accordance with the static scattering intensity. The concentration dependencies of the aggregate diameters as found by analysis of the dynamic light scattering are shown in Figure 9.

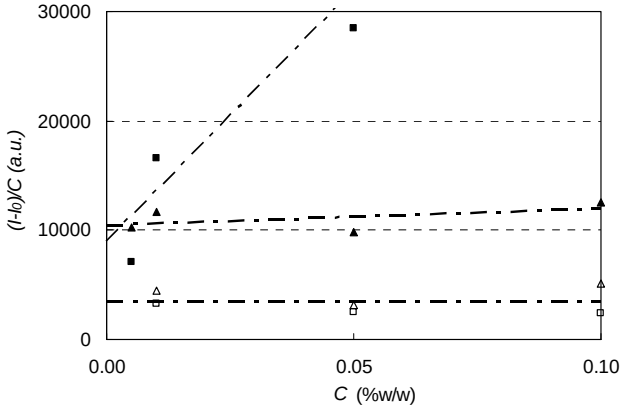

Figure 8 Intensity of light scattered from okra pectin solutions, as a function of pectin concentration. Solution conditions: 10 mM sodium acetate buffer pH at 5. Plotted is the scattered intensity (I), corrected for the scattering by the solvent (I_0), and scaled by the pectin concentration C (in weight %): HBSS (▲), sHBSS (Δ), CHSS (■) and sCHSS (□).

The observed lower tendency to aggregate for okra HBSS pectin compared to okra CHSS pectin seems to contradict our findings from the rheological measurements, where the higher viscosities for the HBSS samples suggested that this material was more

associated than CHSS. But we must keep in mind that the rheological measurements were performed at higher concentrations than the light scattering experiments. Unfortunately, due to the occurrence of multiple scattering, light scattering experiments could not be performed in the same concentration range used for the rheological experiments. The use of an acetate buffer in the light scattering experiments may also have affected the association, since it partially screens the electrostatic repulsion between the molecules. HBSS has a lower charge density than CHSS and its association will be less affected by the addition of salt than for CHSS. These two factors make it difficult to give a one-to-one comparison of the two experiments. But the light scattering experiments do show a big difference in association between the saponified (and therefore less hydrophobic) samples, and the non saponified samples, indicating that association driven by hydrophobic interactions is an important factor in the rheological behaviour of these pectins. For both saponified pectins (sHBSS and sCHSS), the additional charge introduced by the removal of methyl esters resulted in more electrostatic repulsion between the polymer, molecules yielding a lower tendency to aggregate and a non-concentration dependency of aggregate size.

Table 4 Estimated hydrodynamic diameters of okra pectin aggregates and pectin coil in 10 mM NaOAc buffer pH at 5 as determined using dynamic light scattering.

Samples	Pectin aggregates diameter (nm)	pectin coil diameter (nm)	Scattering by aggregates (%)
HBSS	-[a]	30 ± 5	85
sHBSS	210 ± 20	25 ± 5	60
CHSS	-[a]	30 ± 5	70
sCHSS	175 ± 30	20 ± 5	60

[a] Concentration dependence

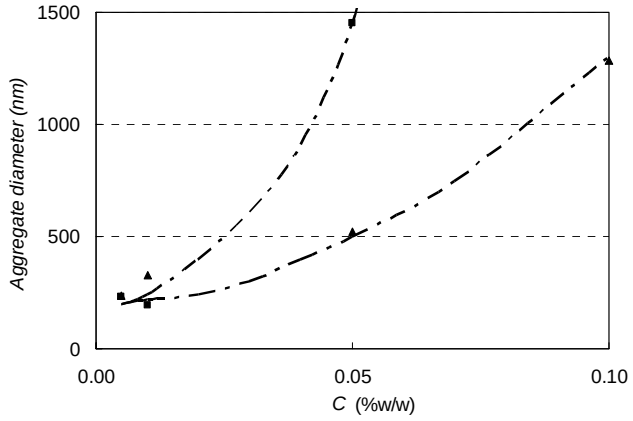

Figure 9 Concentration-dependent sizes of HBSS (▲) and CHSS (■) aggregates in 10 mM NaOAc buffer pH at 5, as determined using dynamic light scattering.

Conclusions

In this research we showed that some physical properties of okra HBSS and CHSS pectins can be related to their structures. In the absence of salts and at a pH above the pKa of the COOH groups of galacturonic acid, okra HBSS pectin solutions have a high viscosity and show shear thinning behaviour. The removal of acetyl groups and methyl esters of okra HBSS pectin decreases the viscosity of the solution and changes the behaviour of the solution from a predominantly elastic response ($G' > G''$) for okra HBSS pectin to a predominantly viscous response ($G' < G''$) for okra sHBSS pectin (in the frequency range from 10^{-1} to 10 Hz). Light scattering experiments show a decrease in association of the pectin molecules when the HBSS is saponified, indicating that association of pectin molecules driven by hydrophobic interactions (resulting from the acetylated rhamnosyl residues in RG I) play an important role in the rheology of these samples. The okra CHSS pectin solutions in water have a lower viscosity than HBSS solutions at the same concentration, and also show shear thinning behaviour. Saponified CHSS solutions had a lower viscosity than non saponified CHSS, and light scattering experiments again suggest

this decrease in viscosity is a result of a decrease in the degree of association of the molecules. At low concentrations and in the presence of salt the okra CHSS pectin shows a higher tendency to aggregate than the HBSS pectin. This may be a result of a difference in charge density of the molecules. The unique chemical fine structure of okra HBSS pectin really makes this pectin quite different in rheological and physical behaviour when compared to the more common homogalacturonan-rich pectins from apple and citrus

Material and methods

Sequential extraction of okra AIS.

The soft and mature okra pods, *A. esculentus* (L.) Moench, (5–10 cm in length) were grown in Thailand and were collected at a local market in June 2005. The alcohol insoluble solids from okra pods (Okra AIS) were prepared and were sequentially extracted with 0.05 M sodium acetate buffer at pH 5.2 and 70 ^{o}C ("Hot Buffer Soluble Solids", HBSS), 0.05 M EDTA in 0.05 M sodium acetate and 0.05 M sodium oxalate at pH 5.2 and 70 ^{o}C ("CHelating agent Soluble Solids", CHSS) (Sengkhamparn et al., 2009a).

Part of the HBSS and CHSS extract were saponified for 16 h at 4 ^{o}C by adding 50% NaOH to a final concentration of 0.05 M NaOH to completely remove acetyl groups and methyl esters. The solutions were then neutralized and ultrafiltrated through a membrane with molecular weight cut-off of 10 kDa and freeze-dried. The molecular weight distribution of HBSS, CHSS and saponified sample were determined by high performance size exclusion chromatography (HPSEC) (Sengkhamparn et al., 2009a).

The pectin samples obtained have been characterized by their sugar composition (Table 1; Sengkhamparn et al., 2009a and 2009b).

Rheological Measurements

HBSS, CHSS, saponified HBSS (sHBSS) and saponified CHSS (sCHSS) samples were dissolved in millipore water. The viscosity of the samples was determined using a Physica MCR 301 (Anton Paar, Austria) stress controlled rheometer with concentric double

gap cylinder geometry (DG 26.8). Flow curves with increasing shear rate (0.01–100 s^{-1}) were measured at 20 ^{o}C.

Oscillatory measurements were used to determine the storage modulus (G') and loss modulus (G'') of the samples. The linear viscoelastic region of the samples was determined by a strain sweep (10–10^{3}% at 1 Hz). Frequency sweeps (10^{-1}–10 Hz at 10% stain) were applied to study the frequency dependence of G' and G''.

Light Scattering

HBSS, CHSS, saponified HBSS (sHBSS) and saponified CHSS (sCHSS) samples were dissolved in 10 mM NaOAc Buffer pH at 5. The sample solutions were centrifuged at 18,500 x g, 10 ^{o}C for 30 min and 20 µL of samples were analyzed by light scattering using Helma quartz cuvettes with light path 3 mm and centre 9.65 mm (Type No. 105.251.005-QS). Light scattering was measured at 25 ^{o}C using a Malvern NanoS (UK), operating at a wavelength of 633 nm and a scattering angle of 173^{o}. The effective hydrodynamic radii reported are the peak positions of a distribution fit of the intensity autocorrelation function, as reported by the Malvern DTS software, version 5.0.

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Chapter 5

Complement-fixing activity and influence on cell behaviour of okra (*Abelmoschus esculentus* (L.) Moench) pectins

Sengkhamparn, N., Samuelsen, A.B., Michaelsen, T.E., Nagel, M-D, Schols, H.A. & Voragen, A.G.J.

Abstract

Okra pods are used as a vegetable in Asia, and as a traditional medicine for gastric irritation treatment and hypoglycaemic activity. The main structure of okra pectins obtained by hot buffer extraction (HBSS) consists of rhamnogalacturonan (RG) I backbone to which acetyl groups and α-galactosyl residues are attached to rhamnosyl residues. Preliminary testing has shown that the okra HBSS pectin has a high complement-fixing activity.

To gain information about the relationship between the molecular weight of okra HBSS pectin structures and the corresponding anti-complementary activity, okra HBSS was digested with rhamnogalacturonan hydrolase and the different molecular weight fragments were tested. The molecular weight of okra RG I plays an important role in the complement-fixing activity, as higher molecular weight fragments (>50 kDa) showed higher activities. Also the effect of okra HBSS pectin on Swiss 3T3 fibroblast morphology, macrophage morphology, proliferation and cell cycle progression was investigated. The results showed that okra HBSS pectin can promote cell apoptosis and inhibit fibroblast proliferation through a block at the G1 phase of the cell cycle. Enhanced bioactivities may be due to substitution of rhamnosyl moieties within the RG I backbone with acetyl groups and α-galactosyl residues.

KEY WORDS: Okra, Pectin, Rhamnogalacturonan I, Complement-fixing activity, Cell behaviour, Biomaterial surface

Introduction

Pectin is an important cell wall component of plants and probably the most complex macromolecule in the nature. The pectin backbone has been found to be composed of two main structural elements, homogalacturonans or smooth regions and rhamnogalacturonans (RGs) or ramified regions. RG I contains a backbone of alternating rhamnosyl and galacturonosyl residues (Schols et al., 1990) in which galacturonosyl residues may be *O*-acetylated at position O-2 and/or position O-3 (Ishii, 1997; Perrone et al., 2002). Moreover, rhamnosyl residues may be branched at position O-4 with β-linked galactan (Gur'janov et al., 2007), α-(1, 5)-linked arabinan or branched arabinogalactan I (Vincken et al., 2003; Ridley et al., 2001) depending on source and method of extraction (McNeil et al., 1982; Lau et al., 1987; Ishii et al., 1989). Studies have shown that pectin may exhibit biological activity such as anti-complementary-, anti-inflammatory-, anti–ulcer-, IL-6 enhancing- and mutagenic activity and may inhibit cancer metastasis (Wagner and Kraus, 2000; Chen et al., 2006; Yamada, 2000).

Pectin has the ability to activate the complement system which plays an important role in the host defense mechanism (Michaelsen et al., 2000; Yamada et al., 1985). The ramified regions of pectin contain high complement-activating activity (Yamada et al., 1985) which were related to highly branched neutral sugar side chains with high levels of β-(1,6)- and β-(1,3,6)-linked Gal and low levels of β-(1,4) – linked Gal (Samuelsen et al., 2007). Moreover, Yamada and Kiyohara (1999) reported that the minimum requirement for the activity was the presence of β-(1,6) – linked galactose side chain attached to the RG backbone. In addition, arabinogalactan type II and/or arabinan structural elements from cabbage pectin were found to be of key importance for complement-fixing activity while removal of arabinosyl residues from arabinogalactan type II was found to decrease this activity (Westereng et al., 2006).

Recently, it was shown that pectin can serve as a biomaterial for surface modification of medical devices and implants. The biocompatibility of medical devices and implants are generally established by investigating the interactions of coating material with cells and adsorbed proteins at the implant/tissue interface (Roach et al., 2005). The potential of pectin for coating medical devices and implants has been studied by Morra et

al., 2004; Kokkonen et al., 2006; Morra, 2005; Nagel et al, 2008; Bussy et al., 2008, the relationship between the structure of pectin and direct/indirect biological effect has however hardly been investigated.

Okra pods are cultivated in many areas of the world and are used as a vegetable and health promoting ingredient in Asia. Okra pods are also used as a traditional medicine for treatment of gastric irritation, for treatment of dental diseases (Lengsfeld et al., 2004), lowering the cholesterol level and cancer prevention (Kahlon et al., 2007). Water extracted okra pectin showed anti-complementary and hypoglycaemic activities which were related to the presence of polysaccharides consisting of repeating Rha-1,4-GalA dimers linked by α-(1,2) linkages in okra (Tomoda et al. 1989).

Okra pods contain thick slimy, acidic polysaccharides consisting of galactose, rhamnose and galacturonic acid (Whistler and Conrad, 1954). The Okra Hot Buffer Soluble Solids (HBSS) pectin fraction was found to be composed of RG I structures including an uncommon substitution of the rhamnosyl residue with acetyl groups and terminally α-linked galactosyl residues present in rather short side chains. The Okra Chelating agent Soluble Solid (CHSS) pectin fraction was found to consist mainly of homogalacturonan segments (70%) in addition to much smaller levels of RG I (30%) having slightly longer β-(1,4)-linked galactan side chains (2 – 3 residues on average) when compared to CHSS RG I. In addition, some arabinan and arabinogalactan type II neutral sugar side chains might be present within the CHSS RG I (Sengkhamparn et al., 2009a, b).

In this chapter, we present the effect of the chemical fine structure and molecular weight of okra RG I on the complement-fixing activity. We also tested okra polysaccharide fractions for their effectiveness for surface modification of implant material in order to understand the effect of okra pectin structure on cell behaviour.

Results and discussion

The sugar composition of okra pectin fractions obtained by hot buffer (HBSS) and chelating agent (CHSS) extraction is shown in Table 1. Okra HBSS pectin consists of acetylated rhamnogalacturonan (RG) I containing rhamnosyl residues with acetyl groups and α-linked galactosyl residues attached. The neutral side chains in HBSS RG I are short,

containing 1 to 2 galactose units attached at O-4 of the rhamnosyl residues. The HBSS RG I is linked to minor amounts of homogalacturonan (HG) and is having an average degree of methyl esterification of 24%. Since RG I is assumed not to have any methyl esterification, a DM value of 72 could be calculated for the HG segments in HBSS. Since the acetyl groups were mainly linked to the rhamnosyl residues in the RG I backbone, the Degree of Acetylation (DA) for HBSS was, therefore, defined as moles acetyl per 100 moles of rhammnosyl residues instead of per 100 moles of galacturonosyl residues used more commonly. It was found that about 80% of all rhamnosyl moieties were acetylated. The okra CHSS pectins are slightly more complex than okra HBSS pectins. They are composed mainly of HG linked to minor amounts of RG I carrying acetyl substitution to both galacturonosyl and rhamnosyl residues. The CHSS RG I also carry arabinan and arabinogalactan type II side chains in addition to short galactose side chains (Sengkhamparn et al., 2009a and 2009b). Recently, Coenen et al. (2007) reported for apple pectin that HG may be linked to the RG I backbone directly or through a xylogalacturonan segment or may be present as a side chain linked through an O-3 or O-4 linkage to rhamnose. However, no xylogalacturonan was found in okra HBSS and CHSS pectin. Therefore, the HG segments within okra pectin may also directly be connected to RG I in the backbone of the pectin molecule as well as be linked as a side chains of RG I**.**

Table 1 Sugar composition (mol%) of HBSS and CHSS pectin isolated from okra alcohol insoluble solids (Sengkhamparn et al., 2009a)

	Rha	Ara	Gal	Glc	GalA	GlcA	DM[a] (%)	DA (%)	Total sugar[a]
HBSS	26	0	34	1	35	3	24	80 [b]	90
CHSS	14	3	17	1	63	2	48	18[c]	86

[a] gram qualities per 100 g of fraction moles acetyl or methanol per 100 moles of galacturonic acid

[b] moles acetyl or methanol per 100 moles of rhamnose

[c] moles acetyl or methanol per 100 moles of galacturonic acid

The complement-fixing activity

Effect of structural features

The hairy region of the pectin molecules seems to play a major role in its complement- fixing activity (Samuelsen et al., 2007; Samuelsen et al., 1996; Yamada and Kiyohara, 1999). Tomada et al. (1989) reported that water extracted okra pectins showed a high complement-fixing activity. Since okra HBSS pectin was found to have a rather unique structure differing from RG I structures of other plant origin (Sengkhamparn et al., 2009b), the complement-fixing activity of okra HBSS and CHSS pectins were measured in order to gain information of a possible effect of HBSS on the complement system. Additionally, the effect of methyl esters and acetyl groups on the activity was determined by measuring the activity of saponified pectins as well.

Figure 1 shows complement-fixing activity of HBSS, saponified HBSS (sHBSS), CHSS and saponified CHSS (sCHSS). The percentage of haemolysis inhibition which is the read out of the complement-fixing activity was slightly lower for all okra pectin samples when compared to the level found for PM II which was a positive control obtained from *Plantago major* L (Samuelsen et al, 1996). Comparing HBSS and saponified HBSS shows that the complement-fixing activity of both samples was similar, indicating that methyl esters and acetyl groups have no effect on the complement-fixing activity of HBSS. This is in contrast with the results found for CHSS and saponified CHSS where the saponified sample showed a slightly lower activity.

In contrast to the literature, stating that arabinosyl structures within pectic hairy regions such as (1,2,5)- and (1,3,5)– linked α-arabinosyl residues from *Acanthus ebracteatus* (Hokputsa et al., 2004) and α-(1,2,5)-arabinan structures isolated from *Zizyphus jujuva* (Yamada et al., 1985) are important for complement-fixing activity, okra HBSS pectin without any arabinose present showed a higher activity than okra CHSS pectin having slightly higher arabinose levels. Moreover, by using ICH_{50} values which is the concentration showing 50 % inhibition of haemolysis in the test system used, it was found that okra HBSS pectins (ICH_{50} about 1.00 mg/mL) express rather similar activities compare to white cabbage pectins and hot water extracted red Kale pectins, even though these pectins contained higher levels of arabinosyl residues (11.7 and 16.9 w/w% of total

carbohydrate content) (Samuelsen et al., 2007). Our results indicate that the level of arabinose plays no major role in okra pectin's complement-fixing activity.

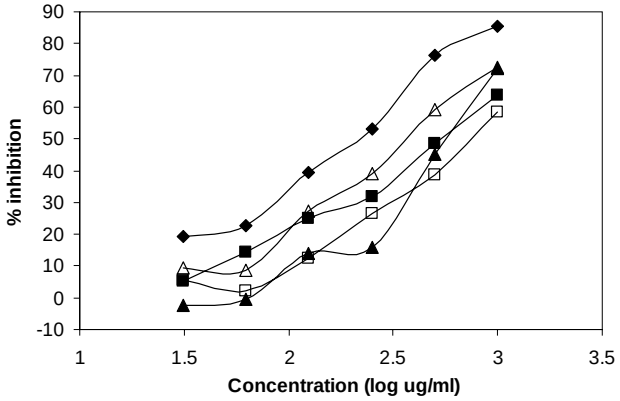

Figure 1 Haemolysis inhibition of okra pectins: HBSS (■), saponified HBSS (□), CHSS (▲), and saponified CHSS (Δ), PM II (♦) is positive control derived from *Plantago major* L (Samuelsen et al, 1996)

Another point which may have effect on the bioactivity of pectins is the amount of neutral sugar side chains other than arabinans. Samuelsen et al. (2007) mentioned that the bioactivity of pectins from *Brassica oleracea* (white cabbage, Kale and red Kale) was related to the amount of β-(1,6)- and β-(1,3,6)- linked galactose and the low amount of α-(1,4)-linked galacturonic acid. Therefore, galactan structures present may explain the high activity of the okra HBSS pectins although almost all galactosyl linkages were terminal or 1-4 linked. In addition, the α–galactosyl residues present may determine the complement-fixing activity as well. Unfortunately, it was not possible to remove the terminal α – galactose by α-galactosidases from various origin (Sengkhamparn et al., 2009b) preventing us to confirm the influence of this specific unit on the complement-fixing activity. The complement-fixing activity was expressed in percentage of PM II (positive control) activity at a concentration of 500 µg/mL in order to avoid day to day variations in the assay. For okra HBSS and CHSS pectin values of 63 and 59% were calculated, and these values are about similar to the values found for white cabbage pectin, 52 % (Samuelsen et al., 2007)

and red cabbage pectin, 60 % (Samuelsen et al., 2007) extracted by hot water (100 °C). Saponified HBSS pectin (50 %) also showed a similar activity as red cabbage pectin. All okra pectin expressed lower activities than the 90% inhibition mentioned for pectin from *Glinus oppositifolius* (L.) Aug. DC. a Malian medical plant (Inngjerdingen et al., 2005) and from *Acanthus ebracteatus*, a Thai medical plant (Hokputsa et al., 2004).

This different behaviour is probably due to the fact that the complement-fixing activity is affected by many factors such as size, three dimensional structure of the polymer, pectin source (Kweon et al., 2003; Suzuki et al., 2003) and is also influenced by differences in the conditions of the bioactivity assay itself.

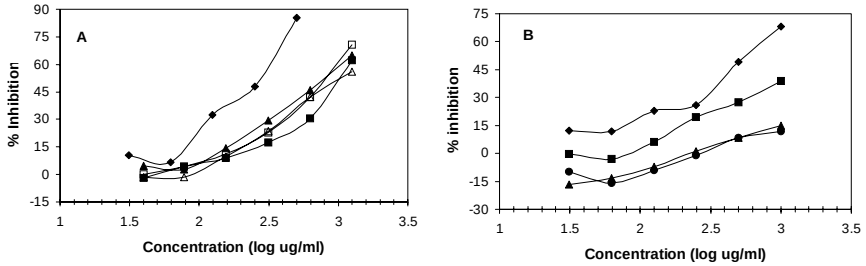

Figure 2 Haemolysis inhibition of A: HBSS (■), HBSS RGH digest (□), saponified HBSS (▲), and saponified RGH HBSS (Δ) and B: saponified CHSS (■),saponified RGH CHSS digest (▲)saponified CHSS PG digest (●), PM II (♦) is positive control derived from *Plantago major* L (Samuelsen et al, 1996)

To gain more information about the effect of the structure of okra pectins on its complement-fixing activity, the okra pectins were incubated with polygalacturonase (PG) and rhamnogalacturonan hydrolase (RGH). PG cleaves the glycosidic linkages in HG sequences by hydrolysis (Benen & Visser, 2003) while the RGH is able to cleave the GalA-Rha linkages in RG sequences having the requirement that the GalA residue do not carry acetyl substitution (Searle-van Leeuwen et al., 1996). Sengkhamparn et al. (2009b) showed that RGH can also split glycosidic linkages within the RG backbone of okra HBSS still having an acetyl group on the rhamnose moiety. PG was shown not to be active towards okra HBSS. Therefore, the okra HBSS pectins were incubated with RGH only. The

complement-fixing activity of okra HBSS and sHBSS after partial hydrolysis by RGH (incubation for 16 hrs.) is shown in Figure 2A. The digests of okra HBSS as well as sHBSS still retained a high activity which was only slightly lower than the activity of the original HBSS.

In contrast to the okra HBSS pectins, okra CHSS pectins are composed of mainly HG (70 %) next to RG I (30%) and both RGH and PG can act toward the okra CHSS pectins (Sengkhamparn et al., 2009b). Therefore, okra CHSS pectin was incubated with PG and RGH. The complement-fixing activity of the CHSS PG and CHSS RGH digests showed negligible activity (Fig. 2B). This might be explained by the structure and size of the remaining fragments after enzyme treatment. PG from *A. aculeatus* cleaves the α-1,4-D-galacturonosyl linkages of the HG-backbone by hydrolysis (Vincken et al., 2003) forming galacturonic acid oligomers. These were removed from the digest by dialysis. Therefore, the remaining polymers in the PG CHSS digest were supposed to be mainly RG I fragments. RG I obtained from many sources have been found to have complement-fixing activity. The PG treatment of CHSS showed a negligible degradation of the pectin. In general, pectin has been reported to mainly activate the complement system through the classical pathway (as examined through our test system) being activated through the complement protein 1 subunit q (C1q) (Michaelsen et al., 2000, Gaboriaud et al., 2003; Matsushita et al., 2004). It has been speculated that the activity might depend on carbohydrate backbone carrying the oligosaccharides with specific spatial organization and the unsuitable distances leading to inefficient binding (Westereng et al., 2006). Therefore, CHSS RG I fragment obtained from PG treated might provide insufficient affinity for a putative binding to the globular head of C1q.

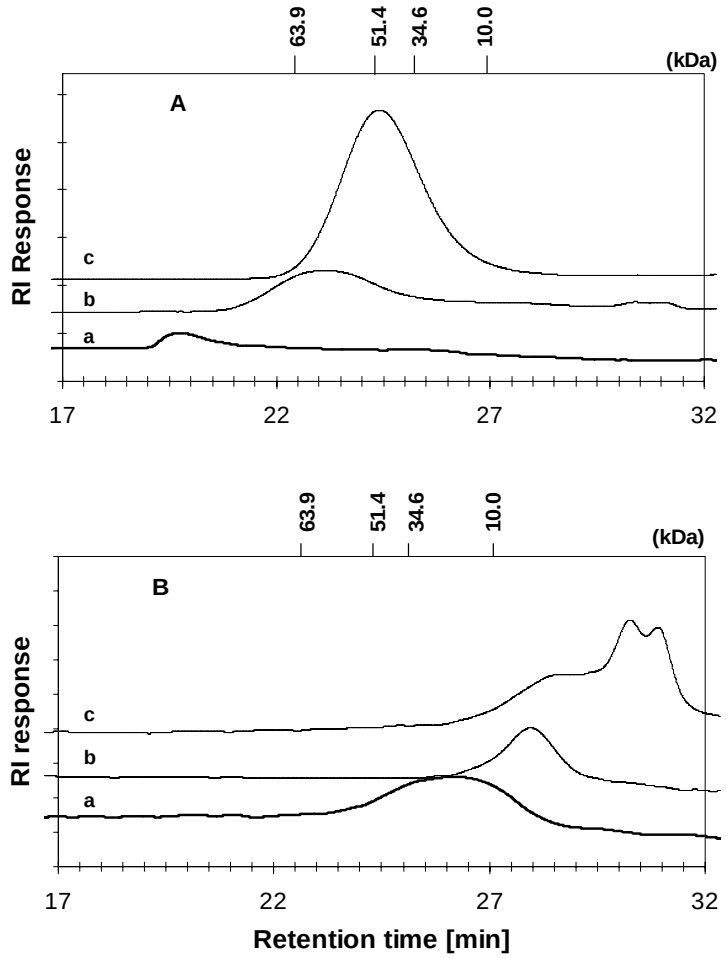

Figure 3 Molecular weight distributions of A) fractions obtained from RGH treated HBSS after separation on Sephacryl S500: pool 1 (S500-1, a), pool 2 (S500-2, b) and pool 3 (S500-3, c) and B) fractions obtained from RGH treated HBSS after separation of pool S500-4 on Sephacryl S300: pool 1 (S300-1, a), pool 2 (S300-2, b) and pool 3 (S300-3, c) (The molecular weight indication are based on pectin standards)

Effect of molecular weight

It has been reported in literature that the molecular weight of an arabinogalactan structure is quite important for its ability to activate the complement system: Mw should be higher than 5 kDa (Sakurai et al. 1999). In order to investigate the effect of the molecular weight (Mw) of RG I on the complement-fixing activity of our fractions, okra HBSS was incubated with RGH and then fractionated by size exclusion chromatography using Sephacryl S500 and Sephacryl S300 columns. The Mw distribution of each SEC pool is shown in Figure 3. All fragments were found to be in the range of 6 – 100 kDa based on a calibration curve obtained with defined pectins. The sugar composition of each pool confirmed the presence of similar RG I structures but having different degrees of polymerization (data not shown). No galactosyl residues were found within the RG I structure present in the last fraction S300-3.

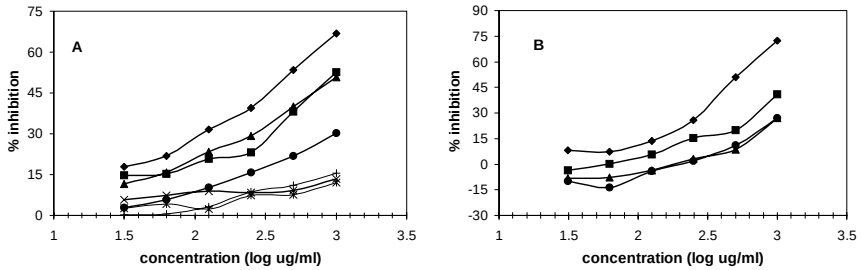

Figure 4 Haemolysis inhibition of A: HBSS (■),S500-1 (▲), S500-2 (●), S500-3 (×), S300-1(+) and S300-2 (*) and B**:** sHBSS (■), sS500-1 (▲) and sS500-2 (●), PM II (♦) is positive control derived from *Plantago major* L (Samuelsen et al, 1996)

The complement-fixing activity of the HBSS RGH digests obtained by Mw fractionation (Fig. 4A) showed that the S500-1 fraction (Mw > 100 kDa) clearly expressed the highest activity ($P<0.05$) and almost equals the activity of the original HBSS ($P<0.05$). The S500-2 fraction showed a significantly lower activity than S500-1 but significantly higher than the activity of S500-3 fraction ($P<0.05$). Consequently, the order of complement-fixing activity of the fractions is HB~S500-1> S500-2 > S500-3. Comparing

S500-1 with S500-2 shows that a reduction in the molecular weight with about 50% resulted in a decrease of complement-fixing activity of 40 %. Both the S300 fractions showed a decreased activity (-70%) when compared to the original okra HBSS pectin. From these results it can be concluded that the activity of okra HBSS pectins decreases with decreasing molecular weight. This trend can also be observed for the saponified okra HBSS pectins. The activity of saponifed S500-1 and sS500-2 fraction expressed both similar, very low activity (Fig. 4B). Therefore, the minimal size of okra RG I that possess significant complement-fixing activity according to these experiments is about 50 kDa.

Effect of presence of acetyl groups

To gain insight in the effect of acetyl groups on the activity, the activity of saponified fractions at a concentration of 1 mg/mL (maximum concentration) were compared to the activity of the original fractions (Table 2). Only the results for the sS500-1, sS500-2 fraction are reported since the other saponified fractions showed negligible activity. The original parental HBSS and S500-1 were significantly more active than saponified HBSS and sS500-1($P<0.05$). These results may suggest that the presence of acetyl groups has an effect on the activity since the removal of acetyl groups results in a decrease of the activity of particularly the RG I fragments in S500-1. However, S500-2 was significantly less active than sS500-2.

The substitution of okra HBSS RG-I by α-linked galactosyl moieties might be quite essential for the anti complementary activity. So far such effects on the activity have been mentioned only in literature for arabinose substitution. These results points once again that the bioactivity of pectins is very difficult to predict and strongly depend on structural features and size and 3D-stucture of the pectin (Yamada and Kiyohara, 2007; Kweon et al., 2003; Suzuki et al., 2003).

Table 2 Molecular weight distribution and level of haemolysis inhibition (at 1 mg/mL concentration) of the HBSS pools obtained from S 500

Sample	Mw (kDa)	Percentage of haemolysis inhibition	
		HBSS[a]	sHBSS[a]
Native		57	45
S500-1	> 100	58	35
S500-2	56	27	34

[a] percentage of haemolysis of PM II was 71.16; Results are presented as mean of duplicate determinations.

Influence on cell behaviour of okra HBSS

Lately, the modification of the surface of medical devices or implants by biomaterial has become a very important research topic. Pectin is a new candidate biomaterial for coating applications and the use of pectin with different structural features has been studied (Chen et al., 2006; Morra et al., 2004; Kokkonen et al., 2006; Verhoef et al, 2009). Moreover, RG I segments appear to be much more interesting for the surface modification due to the oriented immobilization of the RG backbone having side chains of controllable length and nature, affecting interfacial hydration and possibly showing bioactive properties (Morra et al., 2004). Therefore, the effect of okra HBSS pectins (almost pure RG I structure) on the behaviour of Swiss 3T3 fibroblasts and J774.2 macrophages was studied and compared with results obtained of RG I structures obtained from other sources as reported in the literature.

Table 3 Behaviour of Swiss 3T3 fibroblasts in the presence of okra HBSS – coated Petri dishes, 48 hours post –seeding

	TCPS	**Okra HBSS**
Morphology	spread	Round and aggregated
Proliferation index (N/N_0) (n=6)	6.3±0.3	0.7±0.06
Cell cycle % cells in G1 phase	61.05	86.3
Cell cycle % cells in S phase	23.75	3.6

The Swiss 3T3 fibroblasts were seeded on tissue culture polystyrene (TCPS) and okra HBSS coated polystyrene (okra HBSS-coated PS). The cell morphology of both samples after 48 hr seeding was observed. The cells in okra HBSS-coated PS Petri-dishes displayed rounded and aggregated morphology compared to spread morphology of cells in TCPS coated Petri-dishes. The proliferation index (Table 3) shows that cell growth was dramatically decreased on okra-HBSS coated PS, compared to control TCPS. The round cell morphology often indicates differentiation or apoptosis of adherent cells (Boudreau and Jones, 1999). Compared to other RG I structures (MHR – modified hairy regions), the Swiss 3T3 fibroblast morphology in contact with okra HBSS-coated PS was similar to the morphology found on apple MHR-α/A (recovered after liquefaction of apples using the enzyme Rapidase C600) as present on coated PS surfaces (Nagel et al., 2008), but differ from the cell morphology observed on apple MHR B (recovered after treating apples with the enzyme Rapidase Liq^+)-coated PS (Nagel et al., 2008; Kokkonen et al., 2007; Verhoef et al., 2009).

A cell cycle analysis was performed. In general, in the cell cycle 2 main phases (M- phase and S- phase) and 2 gap phases can be recognized: the G_1 phase which occurs before the S phase and the G_2 phase which occurs before the M phase (Morgan, 2007). Early in the cell cycle (the S phase), the replication of DNA and duplication of chromosomes occur, in the M phase typically nuclear division (karyokinesis) and cytoplasmic division (cytokinesis) take place (Morgan, 2007). In our study the cell cycle progression analysis (Table 3) showed that the level of cells in the G_1 phase were increased

but the level of cells in S phase were decreased on okra HBSS-coated PS, when compared to TCPS. It can be concluded that the cells were blocked in the G_1 phase and consequently the percentage of cells synthesizing DNA in S phase was decreased. It seems therefore that okra-HBSS acts in the same way as MHR-α/A which was demonstrated to inhibit fibroblast proliferation through a block at G1 phase of the cell cycle, and induce apoptosis (Nagel et al., 2008).

The interactions of cells at biomaterial surfaces may depend on many factors such as the protein adsorption capability, the wet ability of surface coating, type of cells (Vogler, 1999; Andrade and Hlady, 1987; Kokkonen et al., 2007). Kokkonen et al.(2007) suggested that the length of arabinan-rich side chain has an effect on the protein adsorption potential on the pectin-coated biomaterial surface by steric hindrance in which the adsorption of protein is necessary for cell attachment and growth on the biomaterial surface (Kokkonen et al., 2007; Cassinelli et al., 2002). Indeed, it was shown that the capability of fibronectin, a major serum adhesive protein responsible for cell behaviour, to adsorb onto surface and make its specific recognizing cell sites accessible, was completely different on MHR- α and MHR-B coatings. This situation could explain the opposite behaviour of fibroblasts when cultured on these two MHR coatings (Nagel et al., 2008). Okra HBSS contains very short galactosyl side chains consisting of sequences of approximately 1-3 galactosyl residues, attached to the rhamnosyl residues within the rhamnogalacturonan backbone. A possible explanation for the fewer cells spreading by okra HBSS-coated PS might be the different location of acetyl groups.

Macrophages play an important role in the host response to biomaterials and this interaction generally affects device potency and osseous integration of implants. Therefore, to evaluate the possible interaction of the pectin coating with macrophage growth and functions *in vitro*, the behaviour of J 774.2 macrophages towards okra HBSS coated-PS was studied by observing the macrophage morphology as shown in Figure 4. It was previously observed that the spreading of J774.2 macrophages is an indicator of cell activation (Bussy et al., 2008). It can be seen that macrophages adopt a round and aggregated cell morphology in contact with okra HBSS-coated PS, which probably means that they are not activated in accordance with the observation found for MHRα coating.

The round and aggregated morphology observed for okra HBSS-coated PS in the presence of lipopolysaccharides (LPS) (Fig. 4D) clearly differs from the morphology observed for TCPS in the presence of LPS (Fig. 4B) and also for okra HBSS-coated PS without LPS (Fig. 4C). These observations suggest that okra HBSS may bind LPS which might result in a decreased binding of LPS to its cell receptor as shown by Chen et al., (2006) and in this way should prevent macrophage activation. Further studies are necessary to verify this hypothesis.

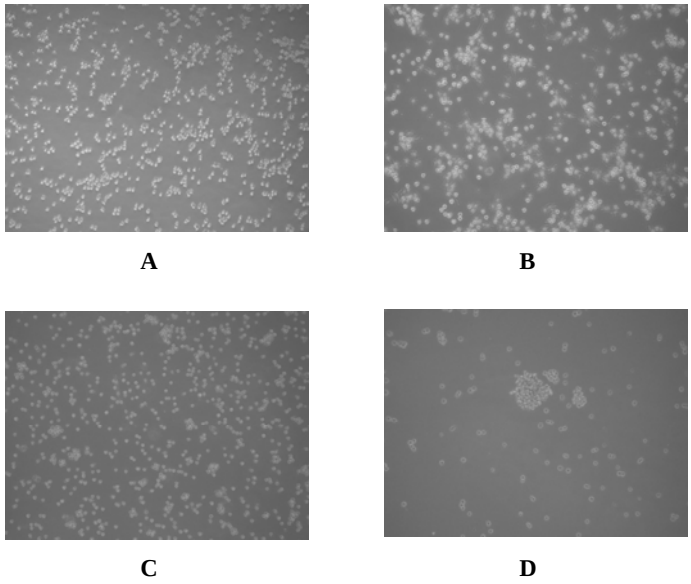

Figure 4 Inverted phase contrast micrographs showing morphology of J 744.2 macrophage 24 hours post seeding on TCPS (A), TCPS +LPS (B) , Okra HBSS coated PS (C) and Okra HBSS coated PS+LPS (D)

Conclusions

In this chapter, we present preliminary results of various assays to assess the bio-activity properties of okra pectins. Complement-fixing activity measured for okra pectins indicated that okra pectins in general and okra HBSS pectin in particular show high activity even though it lacks arabinose substitution which is commonly reported as being quite

beneficial for such activity. In contrast, HBSS is rich in galactose, present as short side chains and is having a rather unique alpha-galactosyl residue present in the side-chains. Furthermore, the activity is linked to the length of the rhamnogalacturonan (RG) I backbone but it seems to be independent from the acetyl groups and methyl esters. These results indicated that the factor for expressing complement-fixing activity of pectin is somewhat complicated to predict and strongly depend on structural features and size and 3D-structure of the pectin.

The use of okra pectins as a biomaterial for coating medical devices and implants is further enhanced by its ability to inhibit the colonization of the surface by adherent cells in promoting cell cycle arrest in G1 phase and apoptosis. Okra HBSS pectins also show an inability to activate macrophages and a potential beneficial effect in preventing macrophage activation by binding of LPS. These properties are promising for biomaterial anti-adhesive applications.

Material and methods

Material

The soft part of immature pods of okra (*Abelmoschus esculentus* (L.) Moench) was collected from okra grown in Thailand. The okra alcohol insoluble solids were prepared and were sequential extracted by aqueous hot buffer and chelating agent solutions according to Sengkhamparn et al. (2009a). Saponification of polysaccharide fractions were performed by adding 0.05 M NaOH at low temperature (Sengkhamparn et al., 2009b).

Analytical methods

Enzymatic degradation

Pectin solutions (final concentration of 3 mg/mL in 50 mM NaOAc buffer of pH 5) of Okra HBSS and saponified HBSS were incubated with 0.547 µg of rhamnogalacturonan hydrolase (RGH) from *Aspergillus aculeatus* per mg substrate (Sengkhamparn et al., 2009b). Solutions (final concentration of 4 mg/mL in 50 mM NaOAc buffer of pH 5) of Okra CHSS and saponified CHSS pectin were incubated with 0.547 µg

of RGH per mg substrate and 0.016 units of endo-polygalacturonase (PG) from *A. aculeatus* (Sengkhamparn et al., 2009b). The incubations were performed at 40 ^{o}C for 24 h and were stopped by heating at 100 ^{o}C for 5 min. The digests were then desalted and freeze dried.

Size exclusion chromatography (SEC)

HBSS solutions (6 mg/mL in water, 100 mL) were incubated with Rhamnogalacturonan hydrolase (RGH) for 24 h at 40 ^{o}C, the enzyme was then inactivated at 100 ^{o}C for 5 min. The digest was fractionated using SEC, Sephacryl S500 (S500-1, 2, 3) and Sephacryl S300 (S300-1, 2, 3) according to Sengkhamparn et al. (2009b). After pooling and desalting, each fraction was saponified.

Molecular weight distribution

The molecular weight distribution was established by High-Performance Size-Exclusion Chromatography (HPSEC) system (Thermo Separation Products, USA) according to Chen et al. (2004). The HPSEC was equipped with three Tosoh Biosep TSK-Gel G columns in series (4000PW_{XL}-3000PW_{XL}-2500PW_{XL}) in combination with a PW_{XL} – guard column (Tosoh, Japan). The sample was eluted with 0.8 mL/min of 0.2 M sodium nitrate Detection was performed by refractive index detector (Shodex SE-61, Showa Denko K.K., Japan).

Complement-fixing activity assay

To measure complement-fixing activity samples were dissolved in a Veronal Buffer in a concentration of 1 mg/mL and further diluted with VB:BSA buffer solution to solutions containing 0.25, 0.0625 0.0313 and 0.015 mg pectin/mL. The human serum used as a complement source was diluted with VB:BSA to a concentration giving about 50% haemolysis of sheep erythrocytes. 50 µL of diluted human serum was added to 50 µL of the sample solution in a 96- well microtiterplate. The sample solution was then incubated on a shaker at 37°C for 30 min. After adding antibody-sensitized sheep erythrocytes (50 µL) and

incubation at 37°C for 30 min, the microtiter plate was centrifuged at 1600 x g, for 5 min and 100 µL of the supernatants were transferred to a flat bottomed microtiter plate for measurement of the absorbance (*A*) at 405 nm. Results are presented as mean of duplicate determinations. The percent inhibition of lysis is calculated by $[(A_{control} - A_{sample}) / A_{control}]$ x 100 % in which the absorbance of the mixture of VB:BSA, diluted serum and sensitized sheep erythrocytes is $A_{control}$. The percent inhibition of haemolysis results were plotted versus concentrations (Michaelsen et al., 2000; Mollnes et al., 1995)

Fibroblast and macrophage culture assay

Surface modification of polystyrene (PS) Petri dishes was performed according to Morra et al. (2004). Swiss 3T3 albino mouse embryo fibroblasts (ATCC ref. CCL-92) and the murine macrophage cell line J774.2 (European collection of cell cultures) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % foetal bovine serum (FBS), 4 mM L-glutamine and penicillin/streptomycin (Gibco Invitrogen, Cergy-Pontoise France). Both cells lines were grown at 37°C under humidified atmosphere (10% CO_2) and were seeded at 10^4 cell/cm^2 on tissue culture polystyrene (TCPS) and Petri dishes coated with okra polysaccharide fractions for cell behaviour investigation.

Proliferation study

To assess Swiss 3T3 fibroblast proliferation, cells were dissociated in 0.25% trypsin + 1 mM EDTA solution. Trypsin reaction was stopped by adding half a volume of FBS, then cells were counted in a Malassez haemocytometer. The proliferation index was the ratio between total cell count (N) and cell number seeded (N_0) (Nagel at al, 2008).

Cell cycle analysis

Swiss 3T3 fibroblasts were dissociated in 0.25% trypsin + 1 mM EDTA solution,washed with 1 mL PBS containing 5 mM EDTA, then fixed for 45 min at 4°C in 1 mL of 75% ethanol in PBS with 5mM EDTA. Cells were washed and suspended in PBS,

5mM EDTA, containing 0.1% Triton X-100, mixed with 40 µg RNase A and 25 µg propidium iodide (Sigma Aldricht, Saint-Quentin Fallavier, France), and incubated for 15 min protected from light . The stained samples were analysed by Epics XL-MCL flow cytometer according to Nagel et al. (2008).

Statistical Analysis

The One-way analysis of variance (ANOVA) with Duncan and Least Significant Difference (LSD) were used for testing significant differences ($P<0.05$) between complement-fixing activity of samples as performed with SPSS for Windows (Version 13.0).

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Chapter 6

General discussion

Motivation and aim of the research

Okra pods, *Abelmoschus esculentus* (L.) Moench, found in many areas of Thailand, are often used as a vegetable, as a thickening agent, and as a traditional medicine for treatment of gastric irritation, dental diseases, lowering cholesterol level and preventing cancer (BeMiller et al., 1993; Lengsfeld et al., 2004; Kahlon et al., 2007). Water extracted okra polysaccharides showed anti-complementary and hypoglycaemic activity (Tomoda et al., 1989). The structure of okra polysaccharide has been studied with conventional methods and reported to be composed of a repeating unit of rhamnosyl and galacturonosyl residues with dimeric β-(1,4)-linked galactan side chains while the pectin was also found to be acetylated (Tomada et al., 1980).

The absence of detailed information on specific pectin structures and the lack of information concerning other polysaccharides present in okra cell walls encouraged us to start research towards the precise structure of okra polysaccharides especially since such knowledge would contribute to the understanding of its unusual physical behaviour and health promoting effects.

To better understand the physical behavior of the vegetable okra and the properties of water extracted okra polysaccharide preparations, we extracted all polysaccharides presented in okra pods cell walls and re-examined their chemical fine structure using specific enzymes as analytical tools and using up to date separation techniques combined with NMR and MS techniques with emphasis on pectic and hemi-cellulosic materials. The rheological properties of the okra pectin extracts were examined under various conditions in order to understand the unusual slimy behaviour of okra pectins. The complement fixing activity and effectiveness for surface modification of implant material of the pectin extracts and molecular weight fractions, with and without chemical and enzymatic modification, were studied to gain information on the relation between their structure and some biological activities.

Hemicellulosic polysaccharides in okra

The polysaccharides in okra cell wall material were extracted from okra pods alcohol insoluble solids by sequential extraction including hot buffer, chelating agent and both diluted and concentrated alkali. The extracted material, okra pulps, represented the major part of the okra pods. All fractions obtained were analyzed for their sugar and glycosidic linkage composition and these data indicated that the okra hemicelluloses mainly ended up in the concentrated alkali extracted fraction (chapter 2).

The presence xylose and terminally linked fucose, together with the presence of 1,4,6–linked glucosyl residues pointed to the presence of a xyloglucan. This was confirmed by incubating this fraction with a specific xyloglucanase, and analysis of the released oligomers by HPAEC and MS. The results showed that XXXG, XXFG and XLFG were the main building blocks; others were XLXG, XXLG and XLLG. This indicated that poly-XXXG type xyloglucan carrying both galactose and fucose substitutions were a part of this fraction. Mass spectrometry of xyloglucanase digests of okra cell wall material showed the presence of low levels of acetylated oligomers in the digest. However, since only a relative small part of the xyloglucan was solubilized in this way, the level of xyloglucan acetylation may still be underestimated.

Furthermore, glucuronic acid was found in the concentrated alkali extracted fraction indicating that acidic xylans were also part of this fraction. The presence of glucuronoxylans was demonstrated by treating the extract with a Glycosyl Hydrolase family 11 xylanase and analyzing the digests by mass spectrometry. The series of xylo-oligomers substituted with one 4-*O*-methylglucuronic acid identified showed that the acidic substituent is rather randomly distributed over the xylan backbone.

The structural features of the xylan and xyloglucan present in okra cell wall were similar to those found in other dicotyledonous plants (olives: Vierhuis et al., 2001; Asteridae: Hoffman et al., 2005; berries and currants: Hilz et al., 2006a).

Pectic polysaccharides in okra

Pectins were found to be the main polysaccharides in okra cell wall and were obtained in the first 3 extracts (hot buffer, chelating agent and diluted alkali). The extracted pectic polysaccharides were characterized by conventional and new analytical techniques including enzymatic fingerprinting by using specific enzymes as analytical tools, up to date separation techniques combined with NMR and MS techniques.

Pectin structural elements in okra pectin

Molecular weight determination of all pectic polysaccharide extracts from okra showed only one population to be present in the high molecular weight range, however the molecular weight appeared to be lower for pectic polysaccharides extracted under more severe extraction conditions (chapter 2). Since also only one molecular weight population was shown to be present by anion exchange chromatography, it is clear that the different pectic structural elements in both okra pectin fractions studied are interlinked.

The pectins present in the 3 extracts obtained showed different characteristics. Based on the proportions of rhamnose and galacturonic acid and on the fact that the backbone of rhamnogalacturonan (RG) I is composed of alternating rhamnosyl and galacturonosyl residues, the percentage of homogalacturonan (HG) within the pectin fraction can roughly be estimated. The hot buffer extract was found to contain 15% HG; the chelating extract 75% HG and the diluted alkali extract 57% HG. The small amounts of mono-galacturonic acid and galacturono-oligomers in saponified hot buffer extracted pectins are indicative for the relative low amounts of HG segment present. The high proportion of HG in the chelating agent extract compared to the hot buffer extract was confirmed by the difference in degradability of these extracts by endo-polygalacturonase (PG) from *Aspergillus aculeatus* and the pattern of galacturono-oligomers released (chapter 3).

HG segments in okra pectin

Assuming that no methyl esters were present in the RG I structural elements (Kravtochenko et al., 1992; Ishii, 1997; Perrone et al., 2002), (almost) all galacturonosyl residues present in the HG segments of hot buffer extracted okra pectin are methyl esterified. In the chelating agent extracted okra pectin the DM of the HG segment present can be calculated to be 64%. Depending from the type of distribution of these methyl ester groups these HG segments might be cross linked in the cell walls via calcium ions and be solubilized by extracting with chelating agents. Where acetyl substitution of homogalacturonan is well established for potato and sugar beet pectin (Albersheim et al., 1996), NMR analysis of the pectins did not reveal any acetylation of galacturonosyl residues within HG segments.

Rhamnogalacturonan I segments in Okra pectin

In addition to the typical 1:1 ratio of rhamnose:galacturonic acid, RG I is also characterized by the presence of acetyl groups substituted to the galacturonosyl residues (Schols and Voragen, 1994; Albersheim et al., 2006). Variation on the precise location and distribution may vary depending on plant source. O-3 acetylated galacturonosyl residues in the RG I backbone have been mentioned for pectins from carrot, cotton, tobacco and tomato (Komalavilas and Mort, 1989) and spinach (Perrone et al., 2002). Ishii (1995 and 1997) found acetyl groups to be mainly located at O-2 of galacturonic acid in the RG I from bamboo. Double substitution of a single galacturonosyl residue at O-2 and O-3 has been reported for RG I from suspension-cultured sycamore cells (Lerouge et al., 1993).

The degree of acetylation (DA) of the pectin in the hot buffer extract of okra pods was much higher (58%) than in the chelating agent extracted pectin (18%). Since rhamnogalacturonan hydrolase (RGH) is inhibited by the presence of acetyl groups, it was expected that the hot buffer extracted okra pectin would not be degradable by this enzyme. Surprisingly, RGH was quite active towards hot buffer extracted okra pectin and this activity even increased upon removal of the acetyl groups (chapter 3). It was hypothesized that the acetyl groups would be located at other sugar moieties in the RG I backbone which would be in accordance with NMR data obtained where we could not identify signals

belonging to O-acetylated galacturonosyl residues (Lerouge et al., 1993). These observations were pointing to the existence of a new type of RG I having new structural features.

Using mass spectrometry technique and NMR techniques, it was demonstrated that for the HBSS RG I, the major part of the acetyl groups was substituted to O-3 of the rhamnosyl moiety, where only a minor part was substituted to O-2 and/or O-3 of the galacturonosyl residues as has been commonly reported so far. The presence of acetyl groups attached to a rhamnosyl moiety in hot buffer extracted RG I has never been reported. The different sites for acetylation (okra typical RG I versus common RG I) could be confirmed by use of different enzymes. RG hydrolase showed to be able to degrade the rhamnosyl-acetylated RG I (chapter 3), while the enzyme was not able to degrade galacturonosyl-acetylated RG I (Schols et al., 1990). HG segments are dominating in the CHSS fraction. However, within the rhamnogalacturonans, the RG I part consisting of acetylated galacturonosyl residues was prevailing next to only minor amounts of RG I consisting of acetylated rhamnosyl residues. It is hypothesized that okra pectin extracted by diluted alkali also consisted of two types of RG I segments with minor amounts of the okra-specific RG I.

Neutral sugar side chains and other pectin structural elements in okra pectin

So far, it has been reported that the level of branching in RG I structures may vary significantly ranging from 20-80% of all rhamnosyl residues. The composition and length of RG I side chains at O-4 or the rhamnosyl residues depends on plant species and origin of plant tissue (McNeil et al., 1982; Lau et al., 1987; Ishii et al., 1989; Albersheim et al., 1996). These side chains are mainly reported to consist of galactosyl and /or arabinosyl moieties such as arabinan, galactan and arabinogalactan type I and II. RG I structures as present in pectin from apple and sugar beet are rich in arabinan side chains, while e.g. potato-, soy-, and flax pectins are known to be rich in RG I segments containing mainly galactan side chains (Voragen et al., 1995). The length of arabinan and galactan side chains range from one or two residues to around 26 residues (Voragen et al., 1995; Gur'janov et al., 2007).

The sugar linkage composition of okra HBSS pectin indicated the presence of highly branched RG I structures carrying short galactan side chains composed of, on the average, 1 or 2 galactosyl residues, although these side chains appeared a little bit longer in chelating agent extracted pectin. Arabinose was absent in the new type of okra RG I from HBSS pectin and is only a minor constituent in okra RG I from CHSS pectin containing some higher levels of commonly present RG I. Diluted alkali extracted okra pectin was less branched and contained arabinan and a mixture of type I and II arabinogalactan structures as a side chain.

Where galactosyl residues have been reported to be present within beta-linked galactans, NMR showed the presence of *two* types of galactosyl residues in RG I. Next to (1,4- and terminally) beta-linked galactoses, the signals for the other type did not fit with any data reported before for RG I structures. These signals could be ascribed to terminally linked alpha-galactosyl residues. These α-linked galactose were linked to O-4 of a β-1,4-linked galactosyl residue or O-4 of a rhamnosyl residue in about equal amounts (chapter 3). This t-α-galactosyl residue was also found to be present in chelating agent extracted pectin but only in minor amounts, confirming the relatively low level of okra HBSS-typical RG I in the CHSS fraction.

Although the majority of RG I structures described in literature are mentioned to contain relative long side chains (either rich in arabinose, galactose or both), short galactose-side are reported to be present in flax RG I (Naran et al., 2007) although these galactoses were in the L-configuration rather than in the D-configuration as found for okra RG I and all other RG I structures described in literature. A water extractable RG I from Arabidopsis seed mucilage was found to be a linear rhamnogalacturonan, devoid of any side chains (Deng et al., 2006).

Xylo-substitution as present in pectic xylogalacturonan structural elements (Schols et al., 1995; Coenen et al., 2007) was not found for okra pectins. RG II, another structural element of pectin having 4 well conserved side chains of many different sugar residues linked to a sequence of 8 galacturonosyl residues, and usually associated to HG (Albersheim et al., 1996; Hilz et al., 2006b), was only present in minor amounts (1-2 %).

A new type of RG I as structural element of pectin?

The most striking result of the elucidation of the structural features of the polysaccharides in the okra pods extracts is the presence of a new type of RG I characterized by the occurrence of acetylated rhamnosyl residues, the absence of methyl ester groups and carrying a terminal α-galactosyl residue either directly linked at O-4 or linked to O-4 of a β-1,4-galactosyl residue. This structural element of pectin is almost completely extracted in the first extraction step with hot buffer and may explain the typical physical behavior of okra pectins. This new RG I structure seems to coexist next to the other, more common RGI structure and some HG in one single population in okra HBSS. The same is true for okra CHSS pectin, although the ratio between the three pectic structural elements differ completely from the ratio established for okra HBSS pectin.

Based on the new structural insights gained in this thesis, we hypothesized that the overall structure of okra hot buffer and chelating agent extracted pectins consists of HG segments directly connected to RG I forming the backbone of the pectin molecule with side chains attached to RG I (Coenen et al., 2007). The proposed overall structure of okra pectin is shown in Figure 1. It should be stated that HG segments may be present in both the backbone of the pectin molecule as well as side chains linked to rhamnosyl residues present in the backbone (Coenen et al., 2007; Ralet and Thibault, 2009). Whether the new RG I structure as present in the okra hot buffer extracts should be considered as a new structural element fully depend on the frequency of occurrence in pectins from other material.

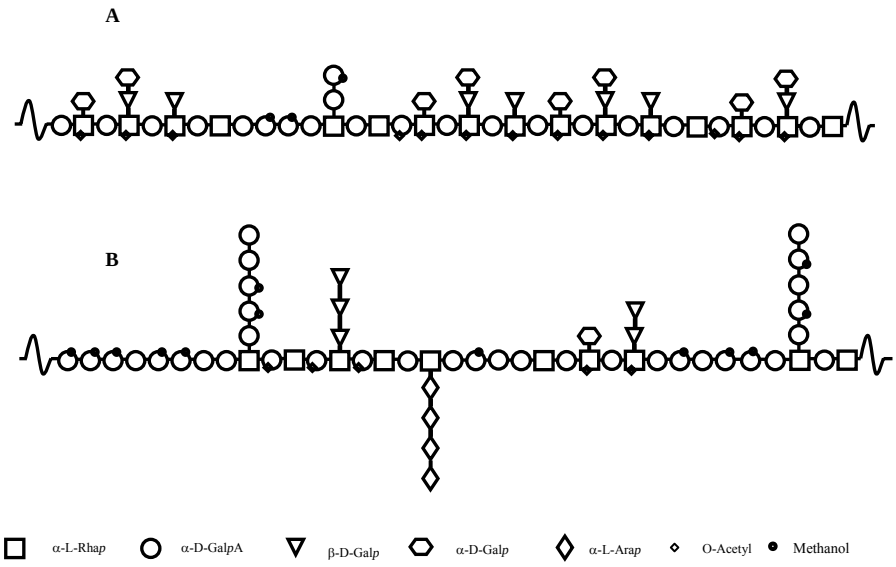

Figure 1: Proposed structure of hot buffer extracted okra pectin (A) and chelating agent extracted okra pectin (B). No evidence is found for okra pectin on the precise location of the HG segments within the pectin molecule.

Structure-physical properties relationship of okra pectins

Okra extracted pectins show a deviant viscous and rheological behaviour. It can be questioned whether this behaviour can be explained by the deviations in the chemical structure of okra pectins described in our study (chapter 4).

Ratio RG to HG structural elements determine the viscosity of solutions

In our studies on the physical properties of hot buffer and chelating agent extracted okra pectin we observed that the viscosities of solutions of hot buffer extracted pectins were much higher than the viscosity of solutions of chelating agent extracted pectin. The hot buffer extracted okra pectin also showed a much higher viscosity than pectins from other, commercially used sources like lime, orange and apple pectin (chapter 4).

Okra pectin exhibited a much larger shear thinning behaviour at low concentration (0.1%) when compared to pectins of pumpkin (Evageliou et al., 2005) and mango (Iagher et al., 2002). The flow behaviour index of okra hot buffer extracted pectin, derived from the Ostward- De Waele equation, was lower than the index established for apple pectin pointing to a more pseudo-plastic nature for okra pectins. Hwang and Kokini (1992) reported that a highly branched RG backbone with long side chains contributed to a higher viscosity and pseudo-plastic behaviour. However, a 0.5 % (w/v) solution of okra hot buffer extracted pectin showed a higher viscosity than highly branched soy RG I with long side chains and modified hairy regions from apple at the same concentration. The viscosity of hot buffer extracted pectin solution was higher than found for water soluble soybean polysaccharides at 10 % w/w concentration (64 mPa s at 129 s^{-1} and at 20 ^{o}C) (Furuta et al., 1999; Furuta and Maeda, 1999).

From this it appears that not only the ratio of RG to HG, the degree and nature of branching but also other factors play a role like the sites of acetylation and the charge of the backbone and the exposure of the charge. For the time being the acetylation of rhamnosyl residues within the RG structural element seems to be unique for okra and very well may explain the unique features of okra pectins in solution. The role of alpha-linked galactosyl moieties as present in okra HBSS pectin in influencing physical properties is less clear and should be addressed in future research.

Effect of location and level of acetyl groups on physical properties

The viscosity of okra pectin extracted by hot buffer or by hot buffer with chelating agent was reduced upon saponification 5 and 2 times respectively, even though saponification results in an increased level of total negative charge and/or higher charge densities which, in general, will lead to an increased viscosity (Williams and Phillips, 2000). The observed loss in viscosity indicates that the removal of the acetyl groups located at O-3 of rhamnosyl residues of RG I has much more influence on the viscosity than the removal of methyl esters which introduce charge to the polymer. It was also observed that solutions of okra hot buffer extracted pectin at low concentration showed a crossover point at lower frequency values than corresponding solutions of chelating agent extracted pectin

indicating that the former can more easily form elastically active zones. The concentration at which this entanglement occurs with hot buffer extracted pectin is much lower than the concentration established for other pectins (0.6 %w/w, Kjoniksen et al., 2005), but is close to the value reported for xanthan gum (0.13 % w/w, Rodd et al., 2000). However, the Cox-Merz superposition indicated that the aggregation does not lead to the formation of a weak associative network, as observed in for example xanthan solutions (chapter 4). The presence of acetyl groups in HG has a negative effect on the gel formation as established for sugar beet pectins (Oosterveld et al., 2000). The tendency of hot buffer extracted okra pectin to easily form elastically active zones may be due to the presence and location of acetyl groups on O-3 of the rhamnosyl residues in the RG I backbone which might enhance formation of such elastically active zones via hydrophobic interaction.

The aggregation behaviour of pectins as monitored by dynamic light scattering of their solutions in buffer showed a lower tendency to aggregate for okra hot buffer extracted pectin compared to okra chelating agent extracted pectin. This indicted a partially screening effect by salts on the electrostatic repulsion between the molecules which decreases the possibility of hydrophobic association via acetylated rhamnosyl residues. On the other hand divalent cations may cause formation of cross-links between non-esterified galacturonosyl residues which are more abundant in chelating agent extracted pectin and thus enhance their aggregation. Since the light scattering experiments could not be performed at the same concentration as the rheological measurements due to limitation of multiple scattering at higher concentrations, results could not be compared. However, from the big difference in aggregation behaviour between saponified buffer extracted okra pectin and original buffer extracted okra pectin, it can be concluded that chain association driven by hydrophobic interactions is an important factor for okra pectins.

Increasing the amounts of acetyl groups by increasing the concentration of hot buffer extracted okra pectin in solutions was found to cause a shift of the crossover point to lower frequency values indicating that the polymer behaves more elastic. This behaviour can be ascribed to an increase in elastically active zones with increasing concentration. The hydrophobic association of acetylated rhamnosyl residues seems to be the main factor to

explain the viscoelastic behaviour of okra pectin when dissolved in water. This specific structure of okra pectin makes it different from all other pectins known at this moment.

Modeling Okra pectins in solutions

Proposed models for okra pectin interaction in aqueous solutions are shown in Figure 2. At a pH above pKa of the carboxylic group, the okra pectin can associate through hydrophobic interaction and hydrogen bonding. The numerous hydroxyl groups within the RG I regions of okra pectin can lead to hydrogen bonding (Ralet et al., 2002). The acetyl groups of rhamnosyl residues in okra RG I will lead to hydrophobic interaction. Moreover, calcium bridges might be present within HG segments as well.

Structure-Bioactivity relationship of okra pectins

Pectins are gaining more and more interest for their health promoting properties and these activities could be attributed to various pectic polysaccharides from various sources, particularly medical herbs (Yamada et al., 2009). Through their ability to activate the complement system they also belong to the group of immunomodulators (Yamada et al., 1985; Michaelsen et al., 2000; Diallo et al., 2001).

Okra pectin and its immunomodulating activity

The complement-fixing activity of okra pectin fractions in relation to its chemical fine structure has been one of the subjects of this thesis. Hot buffer extracted okra pectin was found to show a higher complement-fixing activity than the chelating agent extracted okra pectin. The calculated activity, which are expressed in percentage of PM II (positive control) activity, of hot buffer extracted okra pectin is about similar to the values found for hairy regions of pectins extracted from white and red cabbage (Westereng et al., 2006). For these two cabbage pectins, it has been reported that RG I structures with short β-(1,3,6) galactan chains or with galactosyl and arabinosyl residues attached were important for expressing this activity. This results are pointing out that arabinose plays no major role in

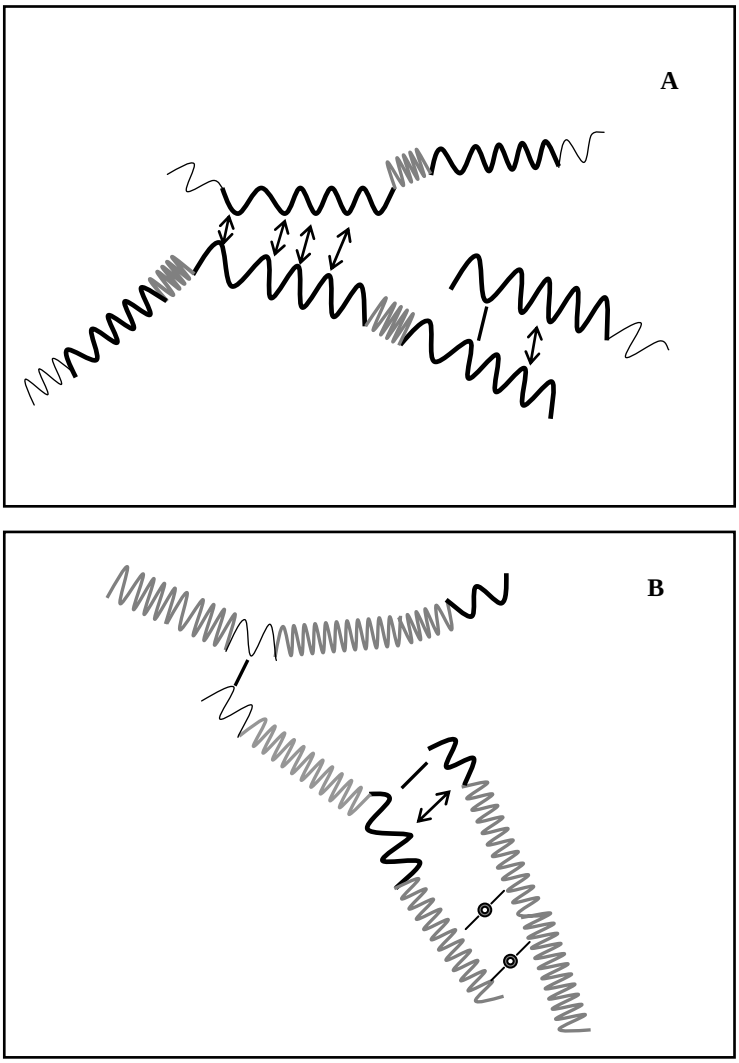

Figure 2: Proposed models for okra pectin extracted by hot buffer without chelating agent (A) and with chelating agent (B) interaction in water. (okra specific RG I , common RG I, HG segments, ↔ hydrophobic interaction between acetyl group in RG I region, – hydrogen bonding between hydroxyl group and —◎— calcium bridge between galacturonic acid in HG segment)

okra pectin's complement-fixing activity. The complement-fixing activity of okra HBSS pectin might therefore be attributed to the monomeric and dimeric galactosyl side chains.

The size of RG I molecules in hot buffer extracted okra pectin was also found to be of importance, larger RG I fragments obtained by degradation with RGH showed higher activity than smaller fragments. The minimal size of okra RG I able to activate the complement system is about 50 kDa which is higher than the value reported for an immunomodulating arabinogalactan (Sakurai et al., 1999).From the results reported here and from published data it appears that differently structured side chains of RG I from different sources may activate the complement system and that a minimum size for an active RG I structure is required. Also the 3-D-structure of the polysaccharide might be important (Kweon et al., 2003; Suzuki et al., 2003; Coenen, 2007; Yamada and Kiyohara, 2007;). However, the complement-fixing activity measured is also affected by many factors of the methodology like cell type, serum type, sheep blood and buffer solutions used and this hampers comparison and evaluation of published results.

Okra pectin and its biocompatibility

Pectin hairy regions have been found to be potential biomaterial for coating applications of medical devices due to their anti-inflammatory properties and the ability to control these properties by tailoring their structure (Morra et al., 2004; Chen et al., 2006; Kokkonen et al., 2007; Nagel et al., 2008).

Chapter 5 describes the potential of hot buffer extracted okra pectin as a coating material for medical devices established by observing cell behaviour and cell cycle analysis of Swiss 3T3 fibroblasts and J774.2 macrophage. Swiss 3T3 fibroblasts exposed to hot buffer extracted okra pectins showed round cell morphology indicating differentiation or apoptosis of adherent cells. Hot buffer extracted okra pectin was also found to resist attachment of cells more than pectic hairy regions from other plant sources. The proliferation index and cell cycle analysis of Swiss 3T3 fibroblasts also showed prevention of cell cycle progression indicating that okra pectin is able to prevent the colonization of adherent cells. The attachments of cells to biomaterial coated surfaces may depend on many factors such as the protein adsorption capability, the wet-ability of surface coating, type of

cells (Andrade and Hlady, 1987; Vogler, 1999; Kokkonen et al., 2007). Kokkonen et al.(2007) suggested that the length of arabinan-rich side chains has an effect on the protein adsorption potential on the pectin-coated biomaterial surface (Cassinelli et al., 2002; Kokkonen et al., 2007). Because the fewer cells spreading found for okra pectin compared to apple MHR B (Kokkonen et al., 2007; Bussy et al., 2008; Verhoef et al., 2009), the coating potential of hot buffer extracted okra pectin seems to be influenced by the position of the acetyl groups and α-galactosyl residues.

Although the behaviour of okra HBSS as biomaterial seems to be promising, the relationship between okra RG I structure and its biological behaviour is far from clear and therefore more engineering of the structure of pectins should be performed in order to understand and optimize the health aspects of these polysaccharides.

Pectin from other Thai plants

Today many Thai plants are used as vegetable in Thai cuisines or for medical treatment based on ancient medical traditions. Only few of these plants have been studied for their chemical composition and physicochemical and biological properties. Mucilage's obtained from Thai plants have been reported to be acidic polysaccharides, mainly pectin with a high proportion of homogalacturonan. Krueo Ma Noy (*Cissampelos pareira*), commonly used as a medical herb for treatment of asthma, dysentery, diuretic and traumatic (Mukerji and Bhandari, 1959), grows in many areas in Thailand. The water extracts from its leaves easily form gels which are consumed as a dessert in Northeastern Thailand (Singthong et al., 2004 and 2005). The aqueous extract from the leaves was reported to contain mainly low methoxyl homogalacturonan (Singthong et al., 2004). The jelly made from Malva Nut (*Scaphium scaphigerun*), known in Thailand as Pungtalay or Sumrong, is consumed as a food product (dessert) on the one hand but is also used for relief of cancer sores and cough, on the other hand. Linkage analysis of Malva nut mucilage, extracted by alkali, showed that it was a branched pectin-like polysaccharide with a backbone consisting 1,4-linked D-Gal*p*, 1,4-linked D-GalA*p* and some 1,2-linked L-Rham*p*. The side chains were found to consist mostly of arabinosyl residues which were located at position O-2 of galactosyl and O-3 and O-4 of rhamnosyl residues within the backbone (Somboonpanyakul

et al., 2006). The intrinsic viscosity of malva nut pectin (10 dL/g) was much higher than Kreuo Ma Noy pectin (2.3 dL/g) (Singthong et al., 2004; Somboonpanyakul et al., 2006).

The Ngueak Plaa Mo (*Acanthus ebracteatus* Vahl) plant is common in South-East Asia. In Thailand, the root and stem are used for treating skin diseases and for longevity, the leaves are used as a poultice on inflamed joints and the whole plant is used for therapies associated with inflammation (Panthong et al., 1986; Ong et al., 2001; Laupattarakasem et al., 2003). Its stems contain a pectin-type polysaccharide with a RG-I backbone and showed potent effects on the complement system (Hokputsa et al., 2004).

Future perspective in pectin research

It is clear that we are still missing detailed structural information of okra pectins to explain physico-chemical and biological properties of specific pectin structural elements. For our studies on okra pectin, the availability of an α-galactosidase active on the terminal galactosyl groups in okra pectin and a rhamnogalacturonan acetyl esterase specific for acetyl groups linked to rhamnosyl residues would have been very helpful. Okra pectin was found to have unique structural features and the variation in pectin structures seems to be endless making this complex molecule even more complex. More dedicated and advanced analytical techniques and novel, specific enzymes are necessary to make progress. Also the bio-activity assays used may need more standardization to enhance comparison of results.

One important lesson should be learned from this thesis: the variation in pectin structures is extremely large. It is quite difficult to predict the pectin structures of specific plants which have not been studied in detail. The same is true for making correlations between techno- and bio-functionalities: no pectin molecule is alike and predictions may not be easy to make. Therefore, the need for more structural knowledge of pectin seems everlasting.

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Summary

Samenvatting

Summary

In Thailand, many plants have been used as vegetables as well as for traditional medicine. Okra, *Abelmoschus esculentus* (L.) Moench, is an example of such a plant. Examples for the medical use are treatment of gastric irritation, treatment of dental diseases, lowering cholesterol level and preventing cancer. These biological activities are ascribed to polysaccharide structures of okra in particular pectin structures. However, the precise structure of okra pectins and also of other polysaccharides in okra pods have been lacking so far.

In order to obtain detailed information of the different polysaccharides present in okra, okra cell wall material was prepared from the pulp of okra pods and was then sequentially extracted with hot buffer, chelating agent, diluted alkali and concentrated alkali. The sugar (linkage) composition indicated that okra cell wall contained, next to cellulose, different populations of pectins and hemicelluloses.

The pectic polysaccharides were mainly obtained in the first three extracts having slightly different chemical structures. The okra pectin extracted by hot buffer was almost a pure rhamnogalacturonan (RG) I with a high degree of acetylation (DA), covalently linked to a minor amount of homogalacturonan (HG) having a high degree of methyl esterification (DM). The chelating agent extractable pectin and the diluted alkali extractable pectin predominantly contained HG with only minor amounts of RG I. Okra pectins extracted by hot buffer and with chelating agent had in common that both contained highly branched RG I with very short side chains containing not more than 3 galactosyl units attached to the rhamnosyl residues in RG I backbone. Chelating agent extracted okra pectins also carried arabinan and arabinogalactan type II as neutral side chains and these side chains were even more abundantly present in the diluted alkali extracted okra pectin.

The hemicellulosic polysaccharides ended up in concentrated alkali extract. From the sugar (linkage) composition and enzymatic degradation studies using pure and well defined enzymes, it was concluded that this fraction contained a XXXG–type xyloglucan and 4-methylglucuronoxylan. The cellulosic polysaccharides were retained in the residue.

The okra hot buffer extractable RG I having a high level of acetyl substitution appeared to be very well degradable by rhamnogalacturonan hydrolase which was known to

be hindered completely by acetylated substrates. In contrast, an acetylated galacturonic acid-specific rhamnogalacturonan acetyl esterase was unable to remove acetyl groups from the RG I molecule of hot buffer extracted okra pectin. For these reasons, the precise position of the acetyl groups present on enzymatically released oligomers were determined by Electron Spray Ionization Ion Trap Mass Spectrometry (ESI-IT-MS) and Nuclear Magnetic Resonance (NMR) spectroscopy. The acetyl groups were found to be predominantly located at position O-3 of the rhamnosyl moiety, while the methyl esters seemed to be present only on the HG part of the hot buffer extracted okra pectin. Another novelty of okra RG-I was the presence of terminal alpha-linked galactosyl substitution at position O-4 of the rhamnosyl residues within the RG I backbone. These specific features (acetylated rhamnosyl- and alpha-galactosyl-substitutions) were almost absent in the chelating agent extracted okra pectin where more commonly known substitutions were present, including acetylated galacturonosyl residues in the RG I backbone. The unique structure features of hot buffer extracted okra pectin led to the assumption that these features may contribute to the rather typical physical properties as well as to the biological properties found for okra pectin.

In order to understand the effect of the specific structural features of RG I on its physical properties, the rheological properties of hot buffer extracted okra pectin were determined and compared to those found for chelating agent extracted okra pectin and for pectins from other plant materials as reported in the literature. The solutions of hot buffer extracted okra pectin showed a high viscosity and predominant elastic behaviour which most probably is caused by strong hydrophobic associations through its acetylated rhamnosyl residues rather than by methyl esterified galacturonosyl residues as is commonly the case for pectins. The removal of acetyl groups and methyl esters decreased the association of the pectin molecules as observed by the light scattering experiment, meaning that not only viscosity and rheological properties but also association of pectin molecules were as result of both hydrophobic interactions and charge effects.

The effect of the position of acetyl groups on the bioactivity of okra pectin was also determined. The complement-fixing activity of okra pectins was found to be affected by many factors like e.g. the presence of acetyl groups, the size of RG segments and the

presence of terminal alpha galactosyl groups and even the three dimensional conformation of the molecules. The hot buffer extracted okra pectin was also examined for its potential to modify surfaces of medical devices and implants. The results showed that okra pectin can be used in coating medical device since it promotes cell apoptosis and shows no macrophage activation.

The knowledge described in this thesis provided us with novel information on the unique structures of okra pectins and may lead to a better understanding of the functional properties of okra polysaccharides in general and okra pectin in particular and to optimize the use of okra pectins within the food industry and in medical applications. However, despite our efforts, at the moment the dependency of the (bio) functionality of okra pectins on the precise chemical structure are not yet completely understood.

Samenvatting

In Thailand worden veel planten zowel gebruikt als groenten als ook als traditionele medicijnen. Okra, *Abelmoschus esculentus (L)* Moench, is een voorbeeld van zo'n plant. Voorbeelden van zijn medicinale toepassing zijn behandeling van maagaandoeningen, behandeling van tandpijn, verlaging van het cholesterol gehalte in het bloed en het voorkomen van kanker. Deze biologische activiteiten worden toegeschreven aan de polysacchariden die in okra aanwezig zijn met name aan de complexe pektine strukturen. De chemische fijn structuur van okra pektine en ook van de andere aanwezig polysacchariden zijn nog altijd onvoldoende bekend.

Om gedetailleerde informatie te verkrijgen over de verschillende typen polysacchariden aanwezig in okra werd okra celwand geisoleerd uit een pulp gemaakt van okra peulen. Hieruit werden door opeen volgende extracties met een warme buffer oplossing, met een oplossing van een chelaterend middel, met een verdunde loog oplossing en tenslotte met een geconcentreerde loog oplossing verschillende polysaccharide fracties verkregen. De suikersamenstelling en de geanalyseerde bindingstypen gaven aan dat de okra celwanden naast cellulose diverse populaties pektine en hemi-cellulose bevatten.

Pektine-achtige polysacchariden werden voornamelijk verkregen in de eerste drie extracten en deze vertoonden enkele verschillen in chemische structuur. Okra pektine ge-extraheerd met warme buffer oplossing was een bijna zuiver rhamnogalacturonaan (RG) I met een hoge acetyleringsgraad dat covalent gekoppeld was aan kleine hoeveelheden homogalacturonaan (HG) dat een hoog gehalte aan methylesters (DM) bevatte. De pektines ge-extraheerd met het chelaterend middel en met verdunde loog waren hoofdzakelijk homogalacturonanen (HG) met slechts kleine hoeveelheden RG I. Okra pektines ge-extraheerd met warme buffer en met het chelaterend middel hadden met elkaar gemeen dat ze beide sterk vertakt RG I bevatten met korte zijketens die uit niet meer dan 3 galactose eenheden bestonden, gekoppeld aan rhamnose bouwstenen in de RG I hoofdketen. Pektine ge-extraheerd met het chelaterend middel bevatte ook arabinaan en arabinogalactaan type II als zijketens. Deze zijketens waren nog meer aanwezig in het pektine ge-extraheerd met verdunde loog.

Polysacchariden van het hemi-cellulose type werden teruggevonden in het extract verkregen met geconcentreerde loog. Uit de analyse van de bindingstypen tussen de suikerbouwstenen en analyse van de afbraakprodukten verkregen met zuivere en goed gekarakteriseerde enzymen bleek dat deze fractie XXXG-type xyloglucan en 4-methylglucuronxylan bevatte. Het cellulose bleef achter in het residue van de extracties.

RG I met een hoog acetylgehalte, aanwezig in het warm-buffer extract van okra celwanden, bleek onverwacht zeer goed afbreekbaar met rhamnogalacturonaanhydrolase. Tot nu toe was steeds gevonden dat dit enzym niet in staat was geacetyleerd RG I af te breken. Rhamnogalacturonaan acetyl esterase, specifiek voor RG I, bleek daarentegen niet in staat acetylgroepen af te splitsen van het RG I ge-extraheerd met warme buffer. Om deze waarnemingen te kunnen verklaren werd met behulp van Electron Spray Ionization Ion Trap Mass Spectroscopy (ESI-IT-MS) en Nuclear Magnetic Resonance (NMR) Spectroscopy de preciese positie van de acetylgroepen in RG I oligomeren, verkregen door enzymatische afbraak, bepaald. De acetylgroepen bleken hoofdzakelijk voor te komen op de O-3 positie van rhamnosyl bouwstenen, terwijl de methyl esters hoofdzakelijk leken voor te komen in de HG segmenten. Een andere nieuwe vinding was de aanwezigheid van terminale galactosyl eenheden alpha-gebonden aan positie O-4 van de rhamnosyl bouwstenen in de hoofdketen van okra RG I in het warm-buffer extract. Deze kenmerkende karakteristieken (geacetyleerd rhamnosyl bouwstenen en alpha gebonden galactosyl eenheden) bleken nagenoeg afwezig in de pektine fractie ge-extraheerd met het chelaterend middel. Deze fractie bleek de algemeen bekende substitutuenten te bevatten waaronder geacetyleerde galacturonosyl bouwstenen id de hoofdketen van RG I. De unieke, structurele kenmerken van de pektine fractie in het warme buffer extract waren aanleiding voor de veronderstelling dat deze afwijkende structuren bij zouden kunnen dragen aan zowel de nogal typische fysisch-chemisch eigenschappen als ook aan de biologische eigenschappen vastgesteld voor okra pektine.

Om de effecten van de specifieke, structurele kenmerken van RG I op zijn fysische eigenschappen te kunnen begrijpen werden rheologische parameters van de met warme buffer ge-extraheerde okra pektine vergeleken met de parameters vastgesteld voor de met chelateringsmiddel ge-extraheerde okra pektine en met pektines van andere plantaardige

bronnen zoals vermeld in de literatuur. Oplossingen van met warme buffer ge-extraheerde okra pektine vertoonden een hoge viscositeit en een overheersend elastisch gedrag waarschijnlijk veroorzaakt door sterke hydrofobe associaties van de geacetyleerde rhamnosyl bouwstenen. Dit in tegenstelling tot de tot nu toe bekende pectines in oplossing waar methyl veresterde galacturonosyl bouwstenen de eigenschappen vooral bepalen. Het verwijderen van de acetyl groepen en methyl esters verlaagde de associatie van de pektine moleculen zoals waargenomen kon worden uit lichtverstrooiings metingen. Dit duidt erop dat niet alleen de visceuze en rheologische eigenschappen maar ook de associatie van de pektine moleculen het gevolg zijn van zowel hydrofobe interacties als van ladingseffecten.

Het effect van de positie van de acetylgroepen op de bioactiviteit van okra pektine werd ook bepaald. De anti-complement activiteit gemeten voor okra pektines bleek beinvloed te worden door vele factoren zoals bijvoorbeeld de aanwezigheid van acetylgroepen, de grootte van de RG I segmenten, de aanwezigheid van terminale alpha-galactosyl bouwstenen en zelfs van de 3-dimensionale structuur van de pektine moleculen. De met warme buffer ge-extraheerde pektine fractie werd ook onderzocht op zijn vermogen het oppervlak van medische apparaten en implantaten te veranderen. Er bleek dat okra pektine hiervoor gebruikt kan worden omdat hierdoor cell apoptosis bevorderd werd en er geen macrophage activering optrad.

De nieuwe kennis beschreven in dit proefschrift verschaft ons aanvullende, waardevolle informatie over de unieke structuur van pektine verkregen uit okra celwanden en helpt de functionele eigenschappen van okra polysschariden in het algemeen en van okra pektines in het bijzonder beter te begrijpen en kan bijdragen de toepassing van deze pektines in de voedselindustrie en in medische toepassingen te optimaliseren. Echter, ondanks onze inspanningen is vooralsnog het verband tussen de (bio) functionaliteit van okra pektines en de preciese chemische structuur nog onvoldoende duidelijk.

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"The need for more structural knowledge on pectin seems to be everlasting".

During my stay in Wageningen I have learned a lot about pectin, particular of structural, analytical and biological aspects. It was sometimes difficult for me and I think I still need to learn more. Also writing this thesis in English was hard for me, as is writing this acknowledgement since I do not want to miss anybody. My thesis would not be possible without lending a hand from many people.

"Success comes from chance and endeavour".

After graduation for my Master Degree, it seemed very difficult for me to enrol in a PhD program. Fortunately, I had a chance to meet Dr. Tanaboon Sajja-anatakool who made it possible for me to get started with a PhD study. A Pom: thank you for encouraging me and giving me an opportunity to work in the Netherlands. This was made possible by Prof. Fons Voragen who, during a visit to Kasetsart University in Bangkok, invited me to come to Wageningen University and do research in the Laboratory of Food Chemistry. Therefore, I would like to first express my gratitude to my promoter, Prof. Fons Voragen and my co-supervisor Dr. Tanaboon Sajja-anatakool. Fons, thank you for giving me this opportunity. Your encouragement kept me going through the work for the PhD degree. I received valuable help from you not only in writing the manuscript but also personal advice while living in Wageningen.

"Yet different cultures but not difficult to live".

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"Learning in a new environment with new colleagues gives a lot of fun and happiness".

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"The big support comes from my warm family".

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and I would never have come this far without the secure feeling of my family's love, support and guidance.

Nipaporn, Tai

Curriculum vitae

Nipaporn Sengkhamparn (Tai) was born on 1 November 1977 in Nongkhai Province, Thailand. In 1995, she started her bachelor degree in Food Technology at Khonkaen University and graduated in 4 years later with diploma project of 'Production of reduced-fat chicken frankfurters with konjac and carrageenan'. Afterwards she started her master degree in Food Technology at Chulalongkorn University, 2000 – 2003. Her diploma thesis was entitled 'Production of concentrated santol *Sandoricum indicum* Cav. Juice and santol juice supplemented with calcium and vitamin D'. Tai had continually studied her Ph D in Food Science at Kasertsart University since June 2003 under financial support from the Commission on Higher Education Ministry of Education, Thailand, under the Commission on Higher Education Staff Development project for the Joint Ph.D program in Food Science at Kasetsart University, Thailand. From November 2005, she went aboard to Laboratory of Food Chemistry, Wageningen University and Research Centre, the Netherlands and worked on the research in topic of 'Chemical, physical and biological features of Okra pectin'. The research on structural characterization of cell wall polysaccharides from okra including phyochemical properties of okra pectin within this thesis was carried out at Food Chemistry Group and Food Physic Group, Wageningen University and Research Centre, from November 2005 until September 2009. Some part of this thesis, biological properties of okra pectin was carried out at Norwegian Food Research Institute-Matforsk, Norway for 3 weeks.

List of publications

Full papers

Sengkhamparn, N., Verhoef, R., Schols, H. A., Sajjaanantakul, T. & Voragen, A.G.J. (2009a) Characterization of Cell Wall Polysaccharide from Okra (*Abelmoschus esculentus* (L.) Moench). *Carbohydr. Res*., 344, 1824-1832.

Sengkhamparn, N., Verhoef, R., Bakx, E.J., Schols, H.A., Sajjaanantakul, T. & Voragen, A.G.J. (2009b) Okra pectin contains an unusual substitution of its rhamnosyl residues with acetyl and alpha –linked galactosyl groups. *Carbohydr. Res*., 344, 1842-1851.

Sengkhamparn, N., Sagis, L.M.C., de Vries, R., Schols, H.A., Sajjaanantakul, T. & Voragen, A.G.J.Physicochemical properties of pectins from Okra (*Abelmoschus esculentus* (L.) Moench). *Food Hydrocolloid.*, 2010, 24, 35-41.

Book chapter

Sengkhamparn, N., Verhoef, R., Bakx, E.J., Schols, H.A., Sajjaanantakul, T. & Voragen, A.G.J. (2009). Structural elucidation of cell wall polysaccharides from Okra *(Abelmoschus esculentus* (L.) Moench). *Pectins and pectinases* (49-58).the Netherlands: Wageningen Academic Publishers.

Abstracts

Sengkhamparn, N., Schols, H.A., Sajjaanantakul, T. & Voragen, A.G.J. (2007) Structural elucidation of cell wall polysaccharides from Okra (*Abelmoschus esculentus* (L.) Moench). Abstacts of papers , XI th Cell Wall Meeting, Copenhagen, Denmark, 12-17 August, 2007; *Physiol. Plantarum*., 130.

Overview of completed training activities

Discipline specific activities

Courses

Glycosciences Summer School (Wageningen, The Netherlands, June 2006)
Food and Health (Cluj Nacopa, Romania, February 2007)
Polysaccharides as Food Colloids and Biomaterials: Fundamentals and Applications (Wageningen, The Netherlands, October 2007)
Fundamentals of Hydrocolloid Technology Course (Nottingham, UK, March 2007)

Meetings

XI Cell Wall Meeting (Copenhagen, Denmark, August 2007)
Pectins and Pectinases (Wageningen, The Netherlands, April 2008)

General Courses

Techniques for Writing and Presenting a Scientific Paper (July 2007)
Scientific Publishing (November 2007)
Food Chemistry Colloqia (2005-2008)
Food Chemistry Seminar (2005-2008)

Additional activities

Norwegian Food Research Institute-Matforsk, Bio-activity Testing (June 2007)
Food Chemistry PhD Excursion to Belgium, France and United Kingdom (2006)
Food Chemistry PhD Excursion to China (2008)