Pectic substances from sugar beet pulp:
structural features, enzymatic modification, and gel formation

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VOORWOORD

Als laatste onderdeel van dit proefschrift rest mij nog dit voorwoord te schrijven. Een ietwat hachelijke zaak, omdat ik verwacht dat dit in enkele uren geschreven voorwoord door meer mensen en met meer aandacht gelezen zal worden dan het werk waar ik meer dan vier jaar de tijd voor had. In die periode heb ik pakweg 10.000 uur op de vakgroep doorgebracht, wat geleid heeft tot de negen hoofdstukken van dit proefschrift. Gelukkig heb ik in die tijd de steun gehad van veel mensen, waarvan ik de volgenden met name wil bedanken.

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Als laatste wil ik mijn ouders bedanken voor de kansen en de steun die ze me gegeven hebben. Jullie interesse in levensmiddelen is de mijne geworden.

En dan nu op naar de rest van het proefschrift!!

Lex

ABSTRACT

Pectic substances are present in high proportions in sugar beet pulp. This by-product is therefore a potential raw material for the pectin industry. However, sugar beet pectin has poor physico-chemical properties compared with pectins from other sources. In order to improve these properties, pectins obtained from beet pulp by autoclaving and acid extraction were chemically characterized and subjected to enzymatic modification and oxidative cross-linking.

The autoclave extracts contained two populations of feruloylated rhamnogalacturonans with highly branched arabinose side-chains having apparent molecular weights of 1300 and 120 kDa, respectively. Furthermore, the presence of homogalacturonans (21 kDa) and of feruloylated arabinans (10-15 kDa) was shown. The acid extracted beet pectin consisted mainly of galacturonic acid with galactose as predominant neutral sugar. The homogalacturonans and rhamnogalacturonans were still linked in this extract.

Cross-linking of the extracts obtained by autoclaving or acid treatment with hydrogen peroxide/peroxidase increased the viscosity and eventually led to the formation of a gel. Treatment of the autoclave extracts with ammonium persulfate decreased the viscosity. Cross-linking of the acid extracted pectin with ammonium persulfate slowly increased the viscosity. The ratio of the different ferulic acid dehydrodimers formed was similar for both oxidants. The total amount of dehydrodimers was lower for the ammonium persulfate cross-linked material. Cross-linking of purified rhamnogalacturonans with hydrogen peroxide/peroxidase led to an increase in viscosity or in gel formation at concentrations as low as 0.75 %. Oxidative cross-linking of the arabinans also led to gel formation, at a concentration of 4.0 %.

Treatment of acid extracted beet pectin with rhamnogalacturonan acetyl esterase plus rhamnogalacturonase showed that the rhamnogalacturonan backbone has little effect on the intrinsic viscosity of this pectin. Removal of the arabinans from purified rhamnogalacturonans showed that the arabinan side-chains contribute little to the intrinsic viscosity of the rhamnogalacturonans. Addition of pectin acetyl esterase plus pectin methyl esterase to a mixture of acid extracted beet pectin and Ca^{2+} increased the release of acetyl groups and of methyl esters as compared with addition of PE or PAE alone, and gave rise to a stiffer gel.

Stellingen

1. Het gebruik van xylanases om de invloed van ferulazuur op de stevigheid van de celwand van suikerbiet te bepalen ligt niet voor de hand, omdat suikerbiet een type I celwand bezit.
   K. W. Waldron, A. Ng, M.L. Parker, and A.J. Parr et al., *J. Sci. Food Agric.*, 74 (1997) 221-228.

2. De aanname dat de viscositeit en de brekingsindex van een populatie na scheiding middels size-exclusion chromatography op dezelfde plaats 'pieken', gaat voorbij aan het feit dat geëxtraheerde pectines per definitie heterogeen zijn en dat dus de viscositeitsrespons eerder 'piekt' dan de brekingsindex.
   M.L. Fishman, D.T. Gillespie, S.M. Sondey, and Y.S. El-Atawy, *Carbohydr. Res.*, 215 (1991) 91-104.

3. Aangezien zure extractie leidt tot afsplitsing van arabinose, dat zich met name in de 'hairy' regions bevindt, geeft het bestuderen van de 'hairy' regions verkregen na een dergelijke extractie een vertekend beeld.
   F. Guillon and J.-F. Thibault, *Carbohydr. Res.*, 190 (1989) 85-96.

4. Het gebruik van met zuur geëxtraheerde pectinestandaarden voor de molecuulgewichtsbepaling van rhamnogalacturonanen middels size-exclusion chromatography met conventionele calibratie of middels viscositeitsmeting leidt tot een onderschatting van het molecuulgewicht van rhamnogalacturonanen.
   Dit proefschrift, hoofdstuk 3.

5. De *in vitro* cross-linking van suikerbietenpectines resulteert in de vorming van meer typen ferulazuur-dimeren dan tot nu toe werd aangenomen.
   Dit proefschrift, hoofdstuk 4.

6. Het geven van cadeau's tijdens kerst, i.p.v. met sinterklaas, leidt tot een verminderde taal- en handvaardigheid, die immers gestimuleerd worden door het maken van gedichten en surprises.

7. Het gebruik van moderne opnametechnieken, waarmee muziek noot voor noot kan worden opgenomen en tot in de perfectie kan worden gecorrigeerd, leidt bij het publiek tot een verminderde belangstelling voor amateurmuziek.

8. Het gebruik van langeafstandswapens maakt het makkelijker om oorlog te voeren, omdat de soldaten meer het idee zullen hebben met een computerspel van doen te hebben, dan dat ze bezig zijn medemensen uit te roeien.

9. De adviezen van de 'zonverwachting' zetten mensen ertoe aan de fiets te laten staan en de auto te pakken om de zon te mijden.

10. De grootschaligheid van popconcerten heeft als consequentie dat zowel de muzikanten als het publiek gehoorbescherming zouden moeten gebruiken.
Slagwerkkrant, 49 (1992) 22-23.

11. Als televisiekijken het menselijk gedrag werkelijk beinvloedt, dan heeft het huidige beleid om gewelddadige films al vroeg op de avond te laten zien, en erotische films te beperken tot de late uurtjes, een negatief effect op het bevolkingsaantal.
Volkskrant, 1 november 1997.

12. Een triangel wordt ten onrechte beschouwd als een eenvoudig te bespelen instrument, zijn snelle klankopbouw en helderheid vergen echter een zeer goed gevoel voor timing van de bespeler.

13. 3D computerschietspelletjes versterken zowel de groepsbinding als de competitiviteit van de mensen die er aan meedoen, en vormen daarom een goed alternatief voor 'survival' weekends die met dit doel worden georganiseerd.

14. De schade aan monumenten veroorzaakt door 'wildplassers', zou door beheerders van monumenten verhaald moeten worden op de regering, wegens het ontbreken van openbare toiletten.

Stellingen behorende bij het proefschrift 'Pectic substances from sugar beet pulp: structural features, enzymatic modification, and gel formation' door A. Oosterveld. Wageningen, 16 december 1997.

CONTENTS

CHAPTER 1

General introduction

Origin and alternative utilization of beet pulp

Sugar beet pulp is a by-product of the industrial sugar extraction of sugar beets (*Beta vulgaris*). In the first stage of the sugar extraction process the sugar beets are washed and cut into thin strips, so-called cossettes. The sugar is then extracted from the cossettes by diffusion with hot water (70°C). As a result of the high temperature the cell walls of the beet tissue become permeable and the cell content, including the sugar, is released. This raw juice is then further refined to sugar. The remaining sugar beet pulp mainly consists of cell wall material. Its composition is shown in Fig. 1 [1, 2]. Beet pulp consists for approximately 77 % of polysaccharides. Pectin, including the (pectin associated) arabinans, are predominant and make up 40 % of the pulp, which makes it a potential raw material for the pectin industry. Cellulose accounts for another 23 % of the pulp. Besides polysaccharides, a relatively high amount of ash can be present. Furthermore, the pulp contains relatively low amounts of protein, lignin, and fat.

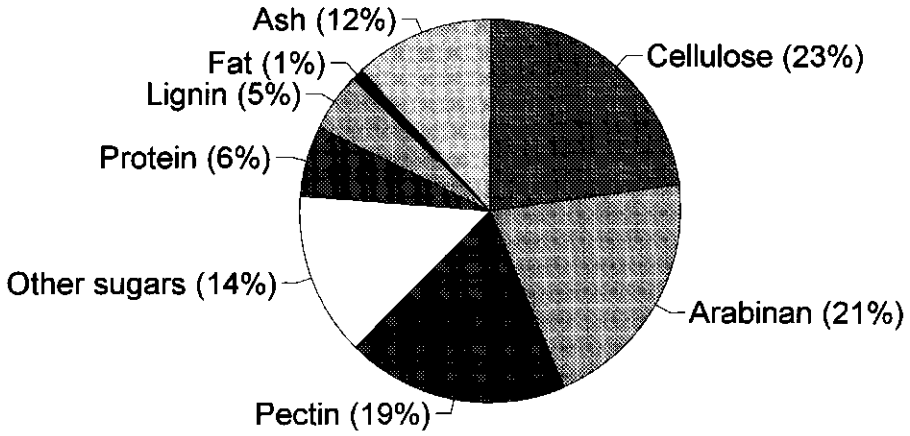

Fig. 1. Composition of sugar beet pulp.

The annual production of beet pulp in the Netherlands amounts to $1.3 * 10^6$ tons (22 % dry weight). Most of this pulp is used as animal feed, after fermentation at a dry matter content >22 %, or in dried form (89 % dry matter). The classical way of fermentation is ensilage. In this process *Bacilli* and *Lactobacilli* decrease the pH to below 4, by the production of lactic acid, thus increasing storage life. An alternative process is fermentation with specific fungi, which results in a protein enriched and better digestible product [3].

Since feed prices are decreasing, sugar industries have been looking for alternative utilizations of the beet pulp. Table 1 summarizes some of these potential applications [1, 3-9] for sugar beet pulp, as well as for poly-, oligo-, and monosaccharides obtained from beet pulp. There are small markets for sugar beet pulp as dietary fibre. The high content of polysaccharides make sugar beet pulp very suitable for this application. Sugar beet fibre has a high water holding capacity, which allows for the use in bakery products such as fibre enriched breads, soft cookies and muffins [1].

Sugar beet pulp is a rich source of polysaccharides. Various extraction processes are possible to produce polysaccharides from sugar beet pulp. Mild acid treatment is the classical way to extract pectins [10], whereas treatment with alkali results in the release of the arabinans [5, 11]. Pectin is generally used as gelling and thickening agent in e.g. jam, yoghurt drinks, and ice-cream [12]. Three mechanisms to form a gel from pectin are known: gel formation with acid and sugar (traditionally used for jams and jellies) [13], gel formation with calcium (used for milk products such as yoghurt drinks) [14] and, specific for sugar beet pectin, oxidative cross-linking through the ferulic acid groups [15]. The latter method is not used yet in the food industry.

Arabinans can be used as a fat replacer e.g. in low fat spreads, ice-cream and chilled or frozen desserts, after removal of the side-chains with the enzyme arabinofuranosidase [5, 16].

Table 1
Alternative utilization of sugar beet pulp [1, 3-9].

By-product	Polysaccharide	Oligosaccharide	Monosaccharide
Feed ingredient	Water binding	Functional Ingredients	Ethanol
Fibre	Thickener		Vanillin
Fermentation	Emulsifier		Sugars
	Gelling agent		
	Foam stabilizing		
	Fat replacer		

A relatively new area of research focuses on the role of physiologically functional oligomers in food. It is claimed that the presence of these oligosaccharides stimulates the growth of indigenous bacteria in the colon, causing a more acidic environment, which inhibits the growth of potential harmful bacteria in the intestinal tract [17]. This is supposed to have beneficial health effects, such as reduction of blood pressure, protection of the liver function and an anticancer effect [17]. Most of this research has been focused on fructo- and galacto-oligosaccharides. Not much is known about possible health effects of pectic oligosaccharides.

Another possibility is the use of sugar beet pulp as a source for monomers, which can be used as such, or as a raw material for the production of chemicals [3]. For example, sugars can be fermented for the production of ethanol [7]. Galacturonic acid and arabinose are possible precursors for the production of surfactants [18], rhamnose is a precursor for flavour and colour production through maillard reactions, whereas ferulic acid is a precursor for the production of vanillin [6].

The cell wall of plants

The cell walls of plants are responsible for their rigidity as well as for the intercellular adhesion [19]. In addition the cell wall is extensible to permit for growth of the cell. Two types of cell wall models have been defined in literature: (I) for the cell wall of all Dicotyledonae and some Monocotyledonae (type I wall); (II) for the cell wall of the Poaceae and related monocot families (type II wall). Sugar beet cells have the type I wall. Several models for the cell walls of dicot and non-graminaceous monocot plants (type I) have been proposed [20-22]. The model of McCann and Roberts (Fig. 2) describes the cell walls of onion parenchyma [20], but it is expected that the model is also applicable to parenchyma tissues from other plants with the type I cell walls. The primary cell wall (width ~75 nm) is flanked towards the inside of the cell by the plasma membrane and on the outside by the middle lamella (width ~50 nm). The cell wall is believed to consist of two independent networks: the cellulose-xyloglucan network and the pectic network. The cellulose-xyloglucan network contains a framework of cellulose micro fibrils. These micro fibrils are composed of linear chains of β-(1→4)-linked glucose residues. They are 5-15 nm wide and are spaced 20-40 nm apart. Although the cellulose chains themselves have a length of only ~1000 residues, the micro fibrils are much longer since each cellulose molecule starts and ends at a different point in the micro fibrils [21]. The micro fibrils are predominantly interlocked through xyloglucans (length is 30-400 nm), creating a space between micro fibrils. The basic repeating unit of the majority of xyloglucans consists of a backbone of four β-(1→4)-linked glucose residues of which in many species, such as apple, sycamore, and tamarind, three out

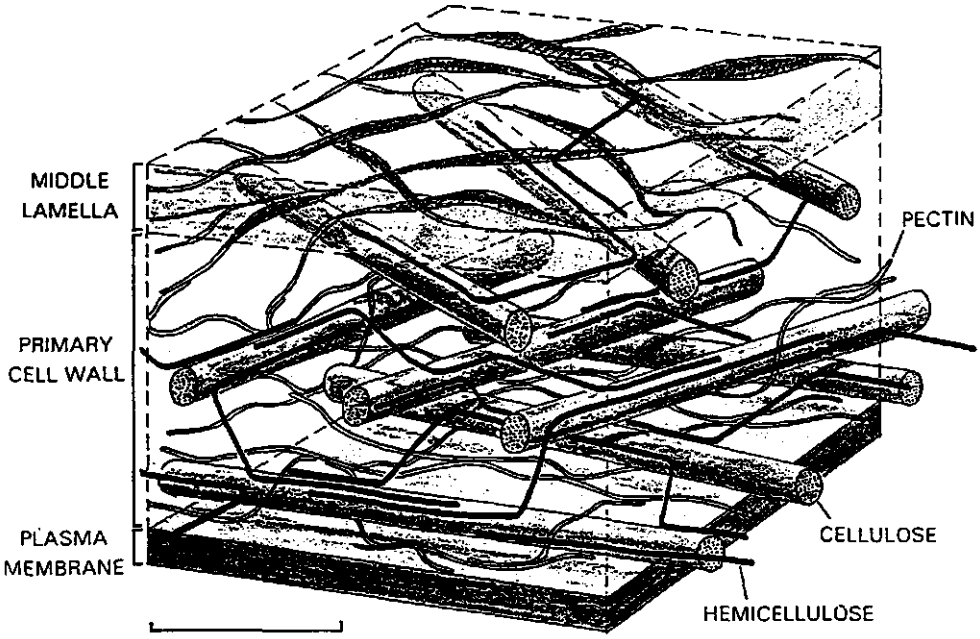

Fig. 2. A simplified and schematic representation of the onion cell wall. Reprinted from [20], by permission of the publisher Academic press Limited London.

of four residues are branched at the C-6 position with xylose. This in contrast to xyloglucans from potato and tomato, both belonging to the *Solanaceae*, for which the presence of two adjacent unbranched glucose residues is characteristic [23]. Additionally some of the xylose residues can be substituted with a β-galactose unit or an α-fucosyl-β-(1→2)-galactosyl unit [21] at C-2. The cellulose-xyloglucan framework (about 50 % of the cell wall mass) is embedded in a matrix of pectic polysaccharides (about 30 % of the cell wall mass) [21]. Pectins consist of homogalacturonan 'smooth' regions and of ramified 'hairy' regions, which contain most of the neutral sugars. The structure of pectins will be discussed in more detail in the next paragraph. Pectins are present in the cell wall as a highly concentrated gel [19]. Cell walls consist for approximately 60 % of water and for 40 % of polymers. The overall concentration of the pectins ranges from 8 to 14 % (w/w) [24]. The pectins influence various cell wall properties: the porosity, the surface charge, pH, ion balance, and are therefore of importance for the ion transport in the cell wall. The pectins also serve as recognition molecules that signal developmental responses to other organisms [25].

The structure and function of pectin

By definition pectins are the hetero polysaccharides from plant cell walls, which mainly consist of galacturonic acid with a certain amount of neutral sugars present as side-chains. All pectins are believed to contain essentially the same repeating structures, although the amount of each structure may vary [26]. The principal building units of pectins are the 'smooth' homogalacturonic regions and ramified 'hairy' regions in which most of the neutral sugars are located [27]. The composition of these building blocks will be discussed below. A structural representation of pectins is given in Fig. 3.

'Smooth' homogalacturonic regions. - The homogalacturonic regions predominantly consist of α-(1→4)-linked galacturonic acid residues. Some rhamnose may be present, leading to kinks in the homogalacturonan backbone [14]. For citrus and apple pectins an estimated minimum length of 72-100 galacturonic acid residues was determined [28]. Approximately 60-90% of the galacturonic acid residues carry a methyl ester.

Ramified 'hairy' regions. - Schols et al. proposed that the 'hairy' regions of pectins consist of three subunits which can be present in different ratios, as was shown for apple pectins [29]. Results found for pear, carrot, leek, onion, and potato are in agreement with this model [26]. Firstly, a rhamnogalacturonan subunit can be distinguished, consisting of a backbone of alternating (1→4)-linked galacturonic acid and (1→2)-linked rhamnose residues, partly substituted with single unit galactose residues (1→4)-linked to the rhamnose residues. Secondly, a rhamnogalacturonan subunit has been found, substituted with long arabinan side-chains. The arabinose residues were mainly terminally, (1→3)-linked and (1→3,5)-linked. Also the presence of a xylogalacturonan subunit has been proven to exist (apple) which consists of a galacturonan backbone, 3-substituted with single unit xylose residues. Apple contains relatively high amounts of this xylogalacturonan subunit, whereas the xylose content in 'hairy' regions from other sources is much lower [26].

Rhamnogalacturonan II. - Rhamnogalacturonan II is a minor component of plant cell walls. It was first isolated from suspension cultured sycamore cell walls by treatment with a fungal endo-polygalacturonase [30]. The presence of rhamnogalacturonan II has been reported in the cell walls of rice [31], onion [32], kiwi fruit [33], radish [34], and also in sugar beet pulp [35]. Rhamnogalacturonan II is a very complex polysaccharide containing the rare sugars 2-O-methyl-fucose, 2-O-methyl-xylose, apiose, aceric acid, 3-deoxy-manno-2-octulosonic acid (KDO), and 3-deoxy-lyxo-2-heptulosaric acid (DHA), besides the more common sugars rhamnose, arabinose, fucose, galactose and galacturonic acid [36]. Its backbone consists of galacturonic acid residues, some of which are esterified with methyl esters, and to which a variety of oligosaccharidic side-chains are attached [37, 38].

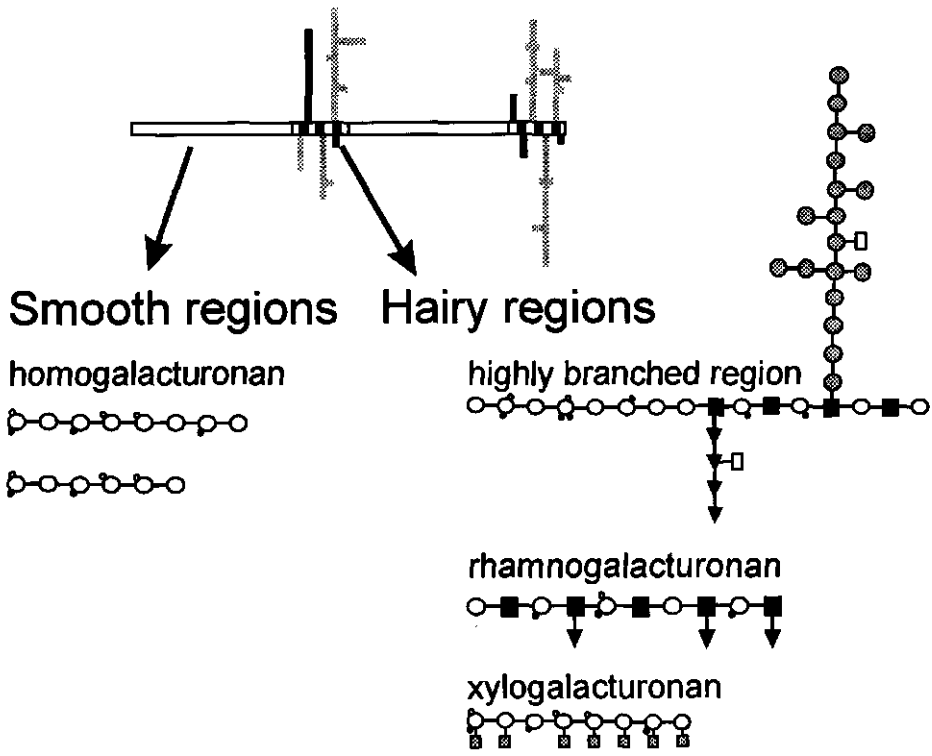

Fig. 3. Schematic structure of apple pectin. ▼: galactose; O: galacturonic acid; ■: rhamnose; ◍: arabinose; ▩: xylose; □: ferulic acid; •: acetyl group; °: methyl ester.

Structural features of sugar beet pectin. - Thibault et al. found a similar length for the homogalacturonan regions of sugar beet pulp as for apple [28]. The homogalacturonans are highly esterified with methyl esters. Also, 80-90 % of the acetyl groups are found on the homogalacturonic backbone in contrast to pectins from most

other sources and are located at the C-2 and/or C-3 position of the galacturonic acid residues [15].

Oligomers with a maximum length of 20 residues of alternating rhamnose and galacturonic acid for the rhamnogalacturonan backbone were isolated in a study using acid hydrolysis of sugar beet pectin. However, it is not known whether this is the *in vivo* length, because breakdown in the backbone might have occurred [39]. Sugar beet pectins contain relatively high amounts of neutral sugars compared with pectins from other sources. Arabinose is the predominant neutral sugar [40]. Galactose is predominantly present in the rhamnogalacturonan as single unit side-chains, as (1→4)-linked galactans of low DP or as (1→3,6)-linked galactans [26, 41].

Sugar beet is one of the few dicots which contain ferulic acid, besides e.g. spinach. It is attached to the O-2 position of (1→5)-linked arabinose residues in the arabinan side-chains and it can also be found to be attached to the O-6 position of galactose residues in (1→4)-linked galactans [41-45]. 50-55 % of the feruloyl groups in sugar beet pulp are linked to arabinose residues and approximately 45-50 % to galactose residues [44]. Ferulic acid dehydrodimers account for approximately 22 % of the total ferulates in sugar beet [40].

Applications for pectin. - Pectin is mainly used as gelling agent and thickening agent in e.g. jams, jellies, yogurt and ice-cream [12]. Also it can be applied as a texturizer, stabilizer or emulsifier in food. Most of the pectin used in food originates from citrus or from apple [10]. The paragraphs below will discuss the two gelling mechanisms, which are used in industrial applications: gel formation with acid and sugar, or with calcium ions.

Gel formation with acid and sugar. - Pectins are generally distinguished by their methoxyl content. High methoxyl (HM) pectins have a degree of esterification of $\geq$ 50 %, whereas low methoxyl (LM) pectins are defined to have a degree of esterification $\leq$ 50 %. HM pectins are known to form gels in the presence of sucrose (65 %) at a low pH (2.9-3.2). X-ray diffraction studies suggest that the HM molecules adapt a threefold helical conformation, which can associate with other molecules [13]. The structure formed is sustained by interchain and intermolecular hydrogen bonds and is additionally stabilized by hydrophobic interactions between methyl esters [46]. Sugar is required to decrease the water activity of the system. The presence of acetyl groups or neutral sugars as found in sugar beet pectins have a negative influence on the gel formation [47]. The gels formed with HM pectin are temperature and shear irreversible under the conditions encountered in food. This means that a HM pectin gel does not melt with heating and that a broken gel will not reform [12].

Gel formation with calcium. - LM pectin can be obtained from HM pectin by deesterification with acid or ammonia or by the use of the enzyme pectin methyl esterase. Depending on their origin (plant, microorganisms) a random or blockwise

distribution of the methyl esters can be obtained [48]. LM pectin is able to form a gel in the presence of calcium [14]. Gelation is due to the formation of intermolecular junction zones between homogalacturonic regions of different chains. The structure of such a junction is still controversial [49]. Besides the amount and distribution of methyl esters, also the occurrence and distribution of rhamnose and acetyl groups and the molecular weight are of importance [14]. The pH sensitivity of the calcium gels is low. A gel will be formed between pH 1.0 and pH 5.0. The gels are temperature and shear reversible: the gel will melt at a higher temperature but will set again after cooling. Also the gel will form again after breaking. Therefore in applications, LM pectins can be pumped, e.g. during filling of the product, without fear of damaging the gel [12].

Gel formation of sugar beet pectin. - Sugar beet pectin has been investigated as an alternative source of pectin, besides apple and citrus pectins. However, its structural characteristics make sugar beet pectin not very suitable as a gelling agent. The presence of high amounts of acetyl groups in the homogalacturonans is an important cause for the poor gelling characteristics [47]. The acetyl groups interfere with the optimal conformation and relative arrangement of the pectin molecules, in this way preventing the pectin from gelling. Also, the high amount of rhamnose in sugar beet pectin causes kinks in the homogalacturonan backbone, whereas the presence of arabinose side-chains may result in steric hinderence. Besides this, the molecular weight of sugar beet pectin is reported to be fairly low, between 15 and 48 kDa [4, 50, 51]. The large variation in molecular weights may be caused by the different methods used. An alternative method to obtain gels from sugar beet pectins will be discussed in the next paragraph.

Oxidative cross-linking of sugar beet pectins

It has been known for some time that ferulic acid groups bound to arabinoxylans from grasses are involved in the formation of cross-links in the presence of certain oxidants. For example treatment of wheat flour arabinoxylans with hydrogen peroxide and peroxidase resulted in cross-linking and in the formation of a gel [52]. Analogous to this Rombouts and Thibault [53] succeeded in coupling an acid extracted sugar beet pectin, which also contained ferulic acid.

Effect of oxidizing agents. - Several oxidising agents have been tested for their ability to cross-link feruloylated beet pectins. The results showed that various oxidants do not have the same effects on beet pectin. Treatment of beet pectin with e.g. potassium periodate, potassium permanganate, sodium chlorite, potassium ferricyanide, and hydrogen peroxide decreased the reduced viscosity up to 14 %. Only ammonium persulfate and hydrogen peroxide/peroxidase have been shown to be able to initiate oxidative cross-linking of beet pectins. As a result the molecular weight and viscosity

increased, and eventually a gel was formed [54]. Other experiments revealed that treatment of an acid extracted sugar beet pectin with ammonium persulfate leads to a continuous increase in viscosity, whereas the combination of hydrogen peroxide/peroxidase leads to an instantaneous increase in viscosity [54]. Treatment of an apple pectin with ammonium persulfate on the other hand decreased the reduced viscosity with 25 %, which indicates that degradation of the pectin molecule had occurred.

The cross-linking reaction. - Two possible reaction mechanisms have been proposed for the oxidative cross-linking of feruloylated pectins.

Hydrogen peroxide is known to create free radicals in the presence of peroxidase. Geissman and Neukom [52] suggested an intermolecular condensation of feruloyl residues for the treatment of feruloylated arabinoxylans with hydrogen peroxide/peroxidase. The reaction product was assumed to be the 5-5 dehydrodimer (Fig. 4A). This was confirmed by Vinkx et al. who stated that the gelation reaction of wheat pentosans is inhibited by ferulic and vanillic acid but not by fumaric acid [55]. From this they concluded that the aromatic ring is involved in cross-linking. Hoseney and Faubion on the other hand found that fumaric acid and not vanillic acid inhibited the cross-linking reaction and that therefore the propenoic double bond of ferulic acid was involved in the reaction, with the 8-8 dehydrodimer as reaction product (Fig. 4B) [56].

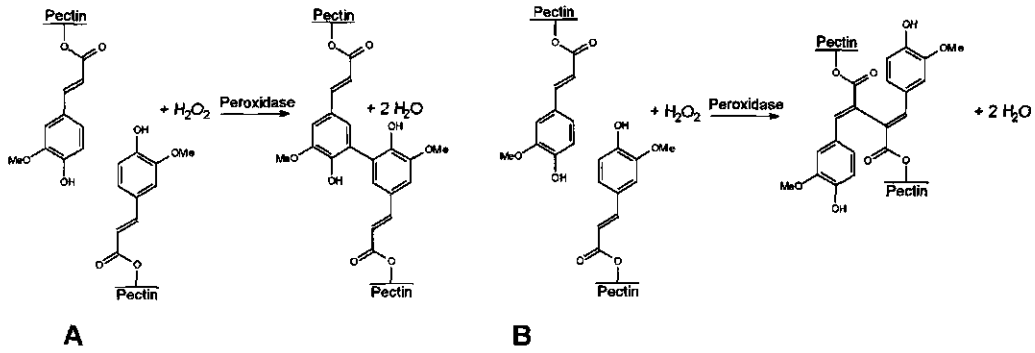

Fig. 4. Two hypothetical reaction schemes for the cross-linking of sugar beet pectins with hydrogen peroxide/peroxidase resulting in the formation of:
A) the 5-5 dehydrodimer, B) the 8-8 dehydrodimer.

Thibault et al. performed 1H-NMR studies on ferulic acid after treatment with ammonium persulfate [57]. It was shown that the propenoic double bonds of ferulic acid are involved in the cross-linking reaction. From this it was concluded that the 8-8 dehydrodimer was the reaction product. Recently Ralph et al. [58] described the presence of several types of ferulic acid dimers in grasses, e.g. the 5-5, the 8-5, the 8-O-4, and the 8-8 dehydrodimer. This indicates that *in vivo* cross-linking of ferulates leads to the formation of a variety of dimers. Cross-linking of feruloylated arabinoxylan in maize cell walls with hydrogen peroxide and peroxidase resulted in the formation of predominantly the 8-5 and the 8-O-4 dehydrodimers, whereas the 5-5 and the 8-8 dehydrodimers were formed in smaller quantities [59].

Influence of some structural characteristics on the cross-linking reaction. - Several structural characteristics influence the gel formation of sugar beet pectin by oxidative cross-linking. The hydrodynamic volume of the molecules determines largely the concentration at which gelling might occur. The hydrodynamic volume is determined by the molecular weight, the degree of branching and the stiffness of the molecule [60]. In addition, the amount of ferulic acid groups present and their localization in the molecule is of importance. Furthermore, Guillon and Thibault [61, 62] showed that the removal of arabinose by acid or by the enzyme arabinofuranosidase increased the gelling capacity of beet pectins.

Other factors influencing the cross-linking reaction. - Besides the structural characteristics several other factors determine the rate of cross-linking.

The concentration of the oxidants involved is an important factor in cross-linking. An increase in concentration of ammonium persulfate added to beet pectin decreased the induction time before gelling and increased the reaction rate, although this was followed by a rapid decrease in reduced viscosity at higher ammonium persulfate concentrations [54]. Hoseney and Faubion showed for wheat flour pentosans that with increasing hydrogen peroxide concentrations an increase in viscosity was seen up to an optimum concentration of ~5 mg hydrogen peroxide per mL [56]. The concentration of peroxidase has a large influence on the velocity of the free radical formation.

Increasing temperature decreased the induction period and increased the reaction rate after treatment of beet pectin with ammonium persulfate, although the maximum values of reduced viscosity decreased [54]. The temperature dependancy of the reaction with hydrogen peroxide and peroxidase is largely determined by the optimum temperature of the enzyme.

The pH plays an important role in the reaction of beet pectins with ammonium persulfate. Gelation occurs only in the range of pH = 3.8-5.7 [9].

The presence of certain additives can result in an inhibition of the cross-linking reaction. Reagents such as sodium acetate, disodium hydrogen phosphate, sodium dihydrogen phosphate and trisodium citrate completely inhibited the reaction of beet

pectin with ammonium persulfate. Addition of sodium chloride or sodium sulfate inhibited the reaction only slightly [54]. The cross-linking reaction of ferulates with hydrogen peroxide/peroxidase is known to be influenced by several reagents. Some of them have been reported to be involved in the cross-linking reaction, e.g. fumaric acid [56], vanillic acid and free ferulic acid [55]. Other inhibiting agents are cysteine and ascorbic acid, which are suggested to react with free hydrogen peroxide [55].

Effect of cross-linking on the physico-chemical properties. - The oxidative cross-linking of beet pectin increases the molecular weight and the intrinsic viscosity, and eventually leads to the formation of a gel [15]. The gels obtained are irreversible and cannot be dissolved in water. Solvent drying of the cross-linked gel yields a product with a very high water absorbing capacity (50-160 mL of water per gram of product, depending on the counter ion) [15].

Enzymatic modification of pectins

The structural characteristics of pectins determine their physico-chemical properties. Polysaccharide degrading enzymes are appropriate tools to modify the structure of pectins. The main reason for this is the specificity of most of these enzymes in comparison to non-specific chemical methods. The use of enzymes for this purpose is limited by the reaction conditions, by their availability in pure form, and by the relatively high price of enzymes. Many different enzymes are active towards beet pectin. The most important enzymes active on polymeric pectic polysaccharides are discussed below and summarized in Fig. 5.

Enzymes active towards the 'smooth' homogalacturonic regions. - The enzyme pectin methyl esterase (PE) can remove the methyl esters from the galacturonic acid residues in the homogalacturonans. Two types are known: plant PE, which is able to remove the methyl esters blockwise and fungal PE, which removes methyl esters block wise or randomly [63]. As a result of the demethylation the calcium sensitivity increases, in particular after blockwise removal of the methyl esters.

The acetyl groups of the homogalacturonans can be removed by the enzyme pectin acetyl esterase (PAE) [64]. Since it is known that the presence of acetyl groups inhibits gelation with acid/sugar or calcium [47], the removal of these groups probably improves the gelling performance of sugar beet pectin.

Endo-polygalacturonase (PG) is able to cleave the glycosidic bond of the α-(1→4)-polygalacturonan in a random fashion [65]. The activity of the enzyme decreases as the degree of methyl esterification increases [66]. Therefore, the enzyme is often used in

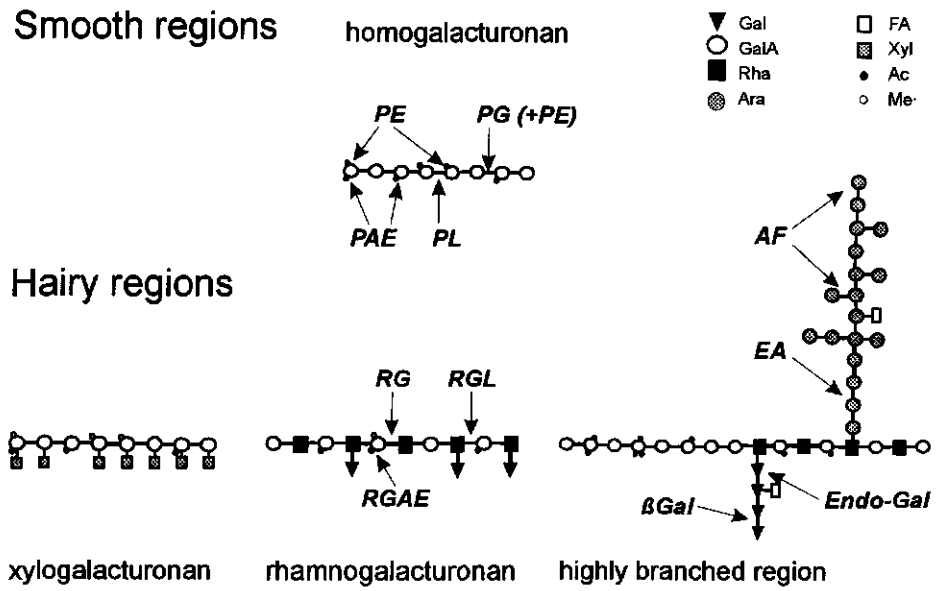

Fig. 5. Enzymes active on the 'smooth' and 'hairy' regions of pectins.

combination with PE. Since the viscosity of pectin is largely determined by the homogalacturonan backbone, treatment of a pectin solution with PG (+PE) rapidly decrease the viscosity.

Endo-pectin lyase (PL) is the only enzyme known to degrade highly methyl esterified apple or citrus pectins without the aid of other enzymes [66]. The enzymatic activity decreases as the degree of methyl esterification decreases. The products resulting from the action of the enzyme have a 4,5-unsaturated galacturonosyl residue at the non-reducing end of the cleaved substrate [66]. The effects on the physico-chemical properties are similar to the combination of PG+PE.

Enzymes active towards the ramified 'hairy' regions. - The rhamnogalacturonan subunit of the ramified 'hairy' regions can be degraded by the enzyme rhamnogalacturonan hydrolase (RGase). The enzyme cleaves the galactopyranosyluronic-rhamnopyranosyl linkages of the rhamnogalacturonan

backbone [67]. Another enzyme active on the rhamnogalacturonan subunit is rhamnogalacturonan lyase (RGL). This enzyme splits the rhamnopyranosyl-galactopyranosyluronic linkages of the rhamnogalacturonan backbone by ß-elimination [68]. Based on the assumption that pectins consist of alternating 'smooth' and 'hairy' regions, it is expected that cleavage of the backbone of the rhamnogalacturonan will result in a decrease of the viscosity.

The acetyl groups present in the rhamnogalacturonans can be removed specifically by the enzyme rhamnogalacturonan acetyl esterase (RGAE) [69]. Furthermore, addition of this enzyme was shown to be necessary for the degradation of modified hairy regions from apple with RGase [67].

Recently an enzyme has been described which is able to remove the feruloyl groups from oligomers obtained from beet pectin (FAE) [70]. However, no such enzyme has been found which is able to remove the feruloyl groups from pectin polymers.

The enzyme arabinofuranosidase B (AF) removes terminal arabinose residues from the arabinan side-chains of pectins [71]. Endo-arabinanase (EA) splits in an endo fashion in the arabinan side-chains [71]. A combination of these enzymes results in a synergistic effect, leading to a rapid degradation of the arabinans. Since the arabinan side-chains play an important role in the entanglement of pectins at high concentrations [50], it is expected that removal of these arabinans changes the rheological behaviour at such concentrations.

Relatively long (1→4)-linked galactan side-chains can be degraded by endo-galactanase [72]. β-galactosidase is able to remove terminal galactose residues from galactans or arabinogalactans [73]. Since galactose is only present in pectins as relatively short (1→4)-linked galactan side-chains and as single unit side-chains attached to the rhamnogalacturonan backbone of the ramified 'hairy' regions, it is expected that these enzymes will only have a small effect on the physico-chemical properties of pectins.

Aim and outline of this thesis

The aim of this thesis is twofold: (i) The extraction and (physico-)chemical characterization of pectic polysaccharides from sugar beet pulp in order to obtain knowledge about their structure. (ii) Specific modification of sugar beet pectins with appropriate enzymes or by oxidative cross-linking, in order to improve the physico-chemical properties, which may lead to a more efficient valorization of sugar beet pulp. Chapter 2 describes the extraction and characterization of pectic polysaccharides from sugar beet pulp. Effort was made to preserve the arabinan side-chains with or without the ester groups during the extraction. Two extraction methods are compared; a method

including mild and strong alkali treatments and a method including autoclaving and alkali treatment. Fractionation of the extracted pectins, by anion-exchange chromatography and size-exclusion chromatography, as well as the chemical and enzymatical characterization of several populations of pectic polysaccharides is described in Chapter 3. In Chapter 4 the analysis of the ferulic acid dehydrodimers formed during the oxidative cross-linking of an autoclave extract with hydrogen peroxide/peroxidase is described. The influence of the oxidative cross-linking with hydrogen peroxide/peroxidase or ammonium persulfate on the physico-chemical properties of the autoclave extract was compared with an acid extracted pectin in Chapter 5. The properties of the extracts after cross-linking were discussed in terms of viscosity, storage and loss modulus, and tan δ. Furthermore, the molecular weight distribution before and after cross-linking was determined. Also the populations present after cross-linking were characterized by sugar analysis. The pectic polysaccharides involved in cross-linking, the rhamnogalacturonans and arabinans, were isolated from the autoclave extract. The effect of oxidative cross-linking of these polysaccharides with hydrogen peroxide and peroxidase was investigated (Chapter 6) with respect to the physico-chemical properties. Enzymes are appropriate tools to specifically modify the structure of pectins. The effect of several combinations of enzymes on the molecular weight, intrinsic viscosity, and radius of gyration of a commercial acid extracted pectin and of several populations obtained from the autoclave extract is described in Chapter 7. The influence of acetyl groups on the gel formation of a commercial acid extracted pectin with calcium is determined by the use of the enzyme pectin acetyl esterase (Chapter 8). This chapter also describes the effect of some glycanases on gel formation with calcium.

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CHAPTER 2

Arabinose and ferulic acid rich pectic polysaccharides extracted from sugar beet pulp

Alexander Oosterveld, Gerrit Beldman, Henk A. Schols and Alphons G.J. Voragen

Abstract

Arabinose and ferulic acid rich polysaccharides were extracted from sugar beet pulp using two extraction methods: a sequential extraction with H_2O (2 times), NaOH/EDTA (2 times) and 4 M NaOH (2 times; Method A) and a sequential extraction in which the NaOH/EDTA extraction was replaced by an autoclave extraction (2 times; Method B). Both extraction method A and B yielded approximately 45 % of the sugar beet pulp polysaccharides. For both method A and B, three extracts with increasing neutral sugar content were obtained: two NaOH/EDTA extracts and a 4 M NaOH extract for method A, and two autoclave extracts and a 4 M NaOH extract for method B. The sugar linkage composition indicated the presence of arabinose rich rhamnogalacturonan ('hairy' regions) and homogalacturonan ('smooth' regions) in all extracts. The sugar compositions of the NaOH/EDTA extracts were very similar to the autoclave extracts. A remarkable difference was the higher amount of ester groups in the autoclave extracts: besides methyl esters and acetyl groups, they also contained relatively high amounts of feruloyl groups. Size-exclusion chromatography using RI and UV detection showed that all extracts were heterogeneous with respect to molecular weight distribution. Ferulic acid was particularly located in the high molecular weight populations of the autoclave extracts.

Introduction

Sugar beet pulp mainly consists of polysaccharides (approximately 67 % of the dry matter), of which pectin (~19 %), pectin associated arabinan (~21 %) and cellulose (~24 %) are prevailing. It is therefore a potential source of pectin and arabinan [1]. However, sugar beet pectin has a relatively low viscosity and a poor gelling capacity compared with citrus and apple pectins, which narrows its area of application. These poor physico-chemical properties have been attributed to the high amount of acetyl groups and the relatively low molecular weight [2]. The presence of ferulic acid, which is ester-linked to the arabinan and (arabino)galactan side-chains [3-7], makes it possible to cross-link sugar beet pectins [8].

Tradditionally, commercial pectins are being extracted under acidic conditions [1, 9]. This generally results in degradation of the arabinan side-chains [10] and therefore in a loss of feruloyl groups. Arabinans or arabinan rich pectic substances are generally extracted using hot alkali [11-12], releasing the feruloyl groups by saponification. Arabinans from sugar beet pulp can be used as a gelling product and fat replacer, after enzymatical linearization [11]. Also extraction procedures for pectic substances using hot water [8, 13], EDTA [1, 9], oxalate [1, 9] or chlorite/acetic acid [13] have been reported in literature. These extraction procedures often consist of several steps, each of which have a low yield. Autoclave treatment of beet pulp has been described as a means to improve its properties as dietary fiber, although no information was given about the feruloyl content of the water soluble fractions, which were obtained after autoclaving [14].

The aim of this study was to develop an extraction procedure for beet pulp pectic polysaccharides with a maximal yield and a minimal degradation of the arabinan side-chains accompanied by a maximal preservation of the feruloyl groups. Since both acid and strong alkali extraction result in a loss of feruloyl groups, we used a sequential extraction procedure introducing an autoclave treatment to obtain ferulic acid and arabinan rich pectins. This was compared with a sequential extraction procedure including mild alkali treatment.

Experimental

Materials.- Wet beet pulp (harvest 1991, 8.9 % dry weight) was obtained from CSM Suiker bv (Breda, the Netherlands).

Alkali extraction of polysaccharides.- Sugar beet pulp was extracted according to the procedure, presented in Fig. 1a. The pulp (1 kg) was ground in a Waring blender with 2 L distilled water (3 min, maximal speed). The mixture was successively extracted

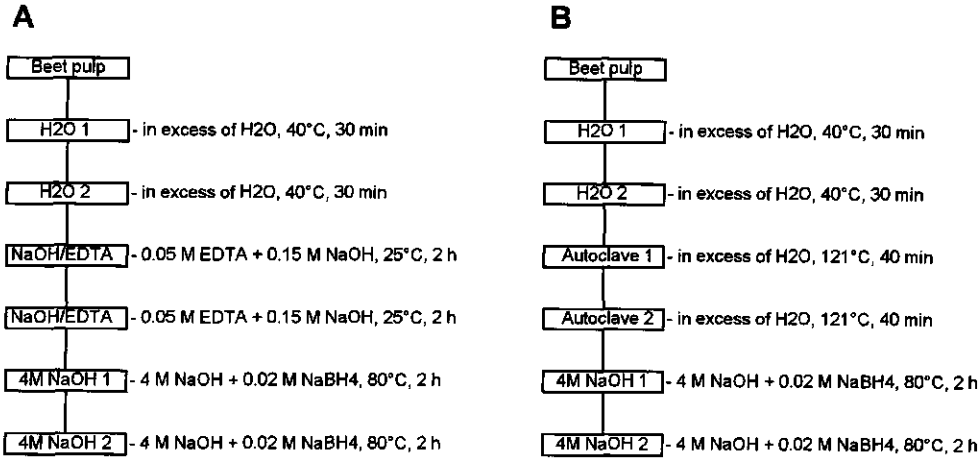

Fig. 1. Scheme for the extraction of polysaccharides using an alkali (A) and an autoclave (B) extraction method.

twice with distilled water, twice with 0.15 M NaOH in the presence of 0.05 M EDTA, and twice with 4 M NaOH in the presence of 0.02 M $NaBH_4$, in all cases using a total volume of 5 L. In between extractions the residues were filtered over a glassfilter (type 3, Ø 17 cm). Where applicable, the extracts were neutralized to pH 5.0. All extracts were dialyzed and freeze dried.

Autoclave extraction of polysaccharides.- The second extraction procedure (method B), was similar to method A, except for the third and fourth step (Fig. 1B) in which the sugar beet pulp was autoclaved twice for 40 min at 121°C (pH 5.2).

Analytical methods.- The uronic acid content was determined by the automated m-hydroxy biphenyl assay [15]. Neutral sugar compositions of the extracts were determined on a Carlo Erba 4200 system GLC, using a J & W DB 225 column, after hydrolysis with 2 M trifluoroacetic acid (1 h, 121°C) and conversion of the monomers to alditol acetates [16]. After pretreatment with 72 % H_2SO_4, hydrolysis with 1 M H_2SO_4 was used for the determination of neutral sugars in the sugar beet pulp. Inositol was used as internal standard. The degrees of methylation and acetylation were determined on a Spectra Physics 8800 system HPLC, using an Aminex HPX 87H column [17]. Feruloyl groups were determined spectrophotometrically (Beckman DU-62 Spectrophotometer) at 375 nm in freshly prepared pectin solutions adjusted to pH 10

with a 0.1 M NaOH solution. A molar extinction coefficient of 31,600 was used [8]. High-performance size-exclusion chromatography (HPSEC) was performed using Biorad Bio-Gel TSK 60XL, 40XL and 30XL columns in series in combination with a TSK XL guard column on a Spectra Physics 8700 XR system HPLC. Elution was carried out with 0.4 M sodium acetate buffer (pH 3.0) at a flow rate of 0.8 mL/min at 30°C. The eluate was monitored using refractive index (RI) detection (Shodex RI-71), and to monitor the presence of ferulic acid, UV detection (Kratos, Spectroflow 773) at a wavelength of 335 nm [6] was used. Molecular weights were determined using pectin standards obtained by mechanolysis. The molecular weights of these standards were calculated from their viscosities [18].

Methylation analysis.- Prior to methylation, carboxyl reduction was effected by the method of Taylor and Conrad [19] and was repeated three times. Polysaccharides were methylated according to the Hakomori [20] method, as modified by Sanford and Conrad [21], followed by hydrolysis with 2 M trifluoroacetic acid (1 h, 121°C), and conversion of the monomers into partially methylated alditol acetates [22]. The partially methylated alditol acetates were identified by GLC-MS using a Hewlett Packard mass selective detector 5970-B coupled to a HP 5890 GLC equipped with a Chrompack CPsil 19CB column and the partially methylated alditol acetates were quantified on a Carlo Erba HRGC 5160 system GLC using a J & W DB 1701 column.

Results

Alkali extraction of polysaccharides.- Beet pulp was extracted twice with distilled water to remove some residual water soluble and suspendable material. Both extracts had a low polysaccharide content and the second H_2O wash gave a very low yield (Table 1). Extraction A NaOH/EDTA 1 yielded 9.7 % of the total solids of beet pulp and the material consisted for 79.7 % of polysaccharides. Based on the composition of the original beet pulp, this fraction represented 12 % of the pulp polysaccharides (Table 1). The second extraction with mild alkali yielded another 5.4 % of the total solids of beet pulp, containing 6 % of the pulp polysaccharides. The highest yield was found in the first strong alkali extract, which contained 23 % of the pulp polysaccharides. The second 4 M NaOH extraction resulted in a very low yield, which indicated that nearly all the alkali extractable material had been removed.

The polysaccharides in extract A H_2O 1 mainly consisted of galacturonic acid, arabinose and galactose. The polysaccharides in extract A H_2O 2 contained less galacturonic acid and more glucose. The polysaccharides in extract A NaOH/EDTA 1 were mainly composed of galacturonic acid and arabinose. Galactose and rhamnose

Table 1

Yield and sugar composition of polysaccharides obtained with alkali (A) and autoclave extraction (B).

Extract	Rha[a]	Ara[a]	Xyl[a]	Man[a]	Gal[a]	Glc[a]	UA[a]	Yield[b]	Sugar content[c]
Sugar beet pulp	2.0	32.4	2.3	1.6	7.6	34.6	19.5	100.0	66.7
A H_2O 1	1.7	37.1	2.1	2.1	16.6	7.9	32.4	5.2	21.3
A H_2O 2	1.1	41.3	1.2	2.9	18.8	14.0	20.8	1.2	27.1
A NaOH/EDTA 1	3.6	37.7	0.0	0.0	5.0	0.1	53.6	9.7	79.7
A NaOH/EDTA 2	5.3	59.5	0.0	0.0	7.4	0.0	27.8	5.4	68.8
A 4 M NaOH 1	4.7	53.7	5.5	2.7	10.9	5.7	16.8	23.3	61.4
A 4 M NaOH 2	6.4	60.1	4.8	0.7	7.4	2.4	18.2	3.0	50.9
B H_2O 1	3.1	36.7	2.1	0.0	7.9	25.2	25.0	6.8	21.5
B H_2O 2	0.7	24.3	1.6	2.6	10.9	37.6	22.2	1.3	25.5
B Autoclave 1	2.6	29.8	0.4	0.4	3.9	1.1	61.9	12.0	66.8
B Autoclave 2	3.6	60.8	0.0	0.4	6.6	0.9	27.7	5.8	88.3
B 4 M NaOH 1	6.2	57.3	6.2	2.4	11.0	5.1	11.7	19.0	76.0
B 4 M NaOH 2	6.4	57.7	8.5	1.6	9.5	4.1	12.3	1.8	48.9

[a] Expressed as mole percentage. [b] Expressed as percentage dry weight of beet pulp. [c] Expressed as weight percentage of the extract.

were only present in minor quantities. The second NaOH/EDTA extraction yielded material containing relatively more arabinose and less galacturonic acid compared with the first extraction.

The sugar compositions of the A 4 M NaOH 1 and A 4 M NaOH 2 extracts were very similar. They mainly consisted of neutral sugars and 17-18 % of galacturonic acid. Compared with the NaOH/EDTA extracts, xylose, mannose and glucose were found in higher quantities, besides the pectic neutral sugars arabinose, rhamnose and galactose. The degrees of acetylation (DA) of the H_2O extracts were relatively high (Table 2). The degree of methylation (DM) was found to be 48 for A H_2O 1 and 31 for A H_2O 2. Only traces of acetyl and methyl were found in the alkali extracts. Despite the alkaline conditions ferulic acid was still present in the first and to a lesser extent in the second NaOH/EDTA extract, indicating the greater resistance of feruloyl esters to alkali conditions as compared with acetyl groups and methyl esters.

Autoclave extraction of polysaccharides.- Compared with the extraction method A, similar yields and sugar contents were found for the H_2O extractions of method B. The first autoclave extraction yielded 12.0 % of the dry weight of beet pulp, containing 12 % of the pulp polysaccharides. A second autoclave treatment yielded another 5.8 %, representing 8 % of the beet pulp polysaccharides. Again the highest yield was found for the 4 M NaOH extraction (19.0 %) and represented 22 % of the pulp

Table 2

Amount of acetyl, methyl and feruloylgroups present in extracts obtained with alkali (A) and autoclave extraction (B). Between brackets the degree of acetylation (DA), methylation (DM) and feruloylation (DF) is given.

Extract	Ac[a]	(DA)[b]	Me[a]	(DM)[c]	FerA[a]	(DF)[d]
A H_2O 1	1.1	(48)	0.6	(48)	0.00	(0.0)
A H_2O 2	0.9	(42)	0.4	(31)	0.00	(0.0)
ANaOH/EDTA 1	0.0	(0)	0.0	(0)	0.18	(0.4)
A NaOH/EDTA 2	0.0	(0)	0.0	(0)	0.09	(0.1)
A 4 M NaOH 1	0.0	(0)	0.0	(0)	0.03	(0.0)
A 4 M NaOH 2	0.1	(0)	0.0	(0)	0.03	(0.0)
B H_2O 1	1.0	(51)	0.4	(40)	0.00	(0.0)
B H_2O 2	1.0	(38)	0.5	(39)	0.00	(0.0)
B Autoclave 1	7.0	(45)	6.0	(73)	0.39	(1.5)
B Autoclave 2	5.4	(52)	3.2	(60)	1.05	(1.3)
B 4 M NaOH 1	0.0	(0)	0.0	(0)	0.04	(0.1)
B 4 M NaOH 2	0.1	(3)	0.0	(0)	0.06	(0.1)

[a] Expressed as weight percentage of the extract. [b] Moles of acetyl groups/100 moles anhydrogalacturonic acid residues. [c] Moles of methyl groups/100 moles anhydrogalacturonic acid residues. [d] Moles of feruloyl groups/100 moles arabinose + galactose residues.

polysaccharides. Similar to extraction method A, the second 4 M NaOH extraction resulted in a very low yield.

Although the H_2O extractions of method A and B were performed in the same way, both extract B H_2O 1 and B H_2O 2 contained more glucose and somewhat less galactose compared with A H_2O 1 and A H_2O 2. The sugar composition of extract B Autoclave 1 shows that this mainly consisted of galacturonic acid (61.9 %) and arabinose (29.8 %). Galactose and rhamnose were present in small quantities. Extract B Autoclave 2 contained relatively more arabinose (60.8 %) and galactose (6.6 %) and less galacturonic acid (27.7 %). The sugar compositions of the material released during the 4 M NaOH extractions of both methods were similar. Only the relative amounts of galacturonic acid were slightly lower in the extracts of method B.

The degree of acetylation of the extract B Autoclave 1 was found to be 45 (Table 2), and was even higher for B Autoclave 2 (52). Due to the alkali conditions only traces of acetyl groups and methyl esters were found in extract B 4 M NaOH 1 & 2. The degree of methylation of extract B Autoclave 1 (73) was higher than that of extract B Autoclave 2 (60). Based on the dry weight, extract B Autoclave 1 consisted of 0.39 % ferulic acid. This amount was much higher for B Autoclave 2, although the degree of feruloylation, expressed as mole ferulic acid per mole of galactose plus arabinose, did not change significantly. Traces of ferulic acid were found in extract B 4 M NaOH 1.

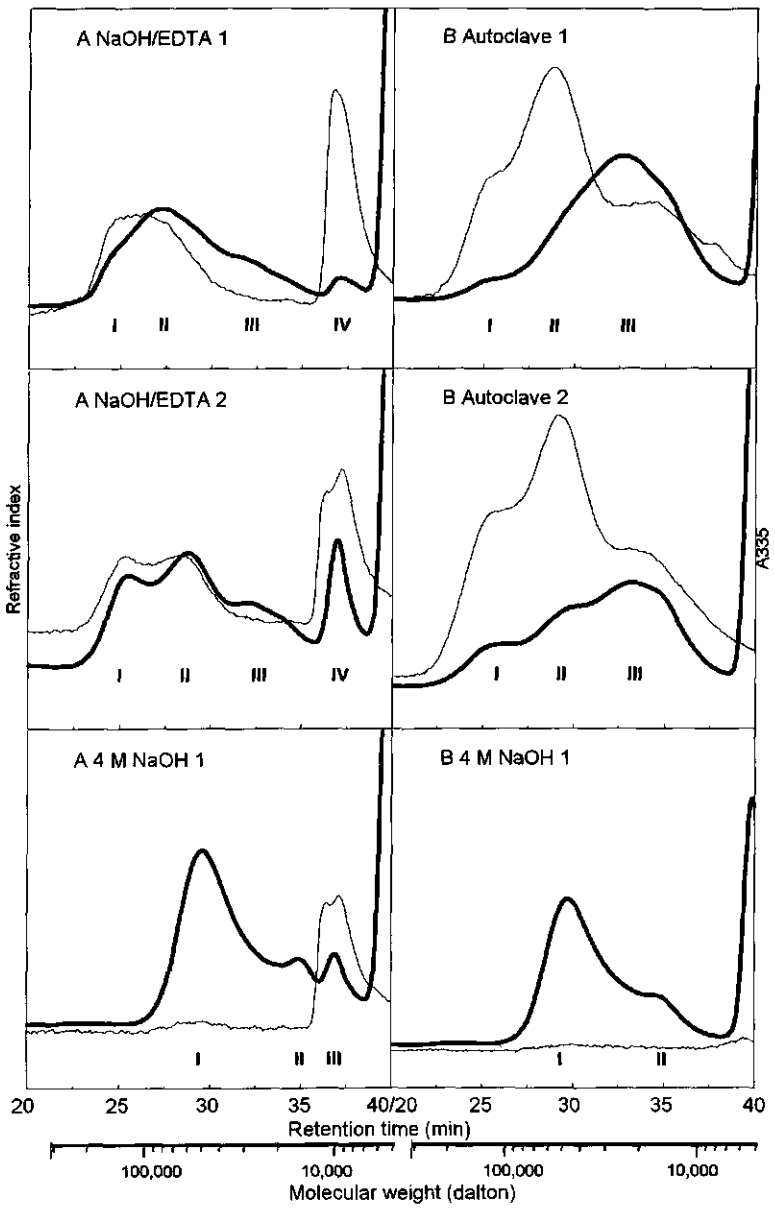

Fig. 2. High-performance size-exclusion chromatography of polysaccharides obtained with alkali (A) and autoclave extraction (B), Thick line : RI detection, thin line: UV detection.

Size-exclusion chromatography.- The molecular weight distribution as determined by HPSEC of the most important extracts is shown in Fig. 2. The concentration of the polymers in the eluate was determined using RI-detection and the presence of ferulic acid was monitored by UV-detection at 335 nm. Based on RI-detection three overlapping high molecular weight populations could be distinguished for extract A NaOH/EDTA 1 with molecular weights of approximately 100 (I), 65 (II) and 24 kDa (III), based on calibration with pectin standards. Population I and II contained most of the ferulic acid. Also some low molecular weight material (populaton IV), probably EDTA, was observed by UV-detection at a retention time of approximately 37 min. Extract A NaOH/EDTA 2 consisted of three populations with molecular weights of 93 (I), 46 (II) and 24 kDa (III) respectively. Again ferulic acid was mainly found in population I and II. Extract A 4 M NaOH 1 consisted of two populations with molecular weights of 36 (I) and 15 kDa (II). No UV-response was detected, except for peak III at 37 min (EDTA). Using RI detection two populations could be distinguished in B Autoclave 1; a small population (I) with a molecular weight of 96 kDa and the main population (III) with a molecular weight of 20 kDa. Using UV detection an additional population (II) with a molecular weight of 44 kDa showed up. B Autoclave 2 showed three populations on both RI and UV detection with molecular weights of 96 (I), 44 (II) and 20 kDa (III). The UV elution pattern of the second autoclave extract was very similar to the corresponding pattern of the first autoclave extract, however, the concentration of ferulic acid was higher. Extract B 4 M NaOH 1 gave similar results compared with extract A 4 M NaOH 1 mentioned earlier.

Sugar linkage composition.- Table 3 shows the results of the methylation analysis of the most prominent extracts. Reduction of galacturonic acid to galactose was carried out to include the linkage composition of this acid sugar. Not all galacturonic acid residues were recovered after reduction and methylation. This also influences the molar ratios of the other sugars. This might be related to the ß-eliminative degradation of galacturonan during the methylation treatment [23]. The amount of rhamnose was also found to be rather low in the non-reduced samples, which has been attributed to the relatively high resistance of the galacturonosyl-(1→2)-rhamnose linkage towards acid [23]. Little variation in sugar linkage composition was found between the extracts of the alkali extraction method (A) and those of the autoclave extraction method (B). Arabinose residues were mainly terminally linked, (1→5)-linked or (1→3,5)-linked. Also small amounts of (1→2,5)-linked and (1→2,3,5)-linked arabinose were present. Rhamnose was terminally linked, (1→2)-linked or (1→2,4)-linked. Galactose was found to be mainly present as terminally or (1→4)-linked residues. Galacturonic acid was in general (1→4)-linked, but small amounts of (1→3,4)- and terminally linked galacturonic acid residues were also present. In the 4 M NaOH extracts xylose, glucose and mannose, present as (1→4)-linked residues, were detected in higher concentrations compared

Table 3

Sugar linkage composition[a] of the extracts obtained by method A and method B.

Linkage	Method A			Method B		
	NaOH/EDTA 1	NaOH EDTA 2	4 M NaOH 1	Autoclave 1	Autoclave 2	4 M NaOH 1
Rhamnose						
T-Rha *p* [b]	0.4	0.5	0.5	0.5	0.3	0.2
(1,2)-Rha *p* [b]	0.4	0.8	1.1	0.4	1.0	1.0
(1,2,4)-Rha *p*	0.4	1.0	1.0	0.4	1.2	0.9
	1.2	2.3	2.6	1.3	2.5	2.1
Arabinose						
T-Ara *f*	17.6	27.3	25.3	17.4	125.7	25.4
(1,3)-Ara *f*	0.6	0.8	0.8	0.5	0.6	0.8
(1,5)-Ara *f*	15.4	21.7	23.1	14.2	22.5	21.7
(1,2,5)-Ara *f*	1.2	2.0	1.5	0.9	1.5	1.5
(1,3,5)-Ara *f*	13.4	19.0	16.9	11.8	18.8	16.7
(1,2,3,5)-Ara *f*	3.1	4.8	3.4	2.2	3.7	3.4
	51.2	75.6	71.0	47.0	72.8	69.5
Xylose						
T-Xyl *p*	0.4	0.4	1.2	0.8	0.4	1.4
(1,4)-Xyl *p*	0.4	0.3	5.3	0.3	0.3	5.9
(1,2,4)-Xyl *p*	0.1	0.1	0.2	0.4	0.1	0.1
(1,3,4)-Xyl *p*	0.1	0.1	0.2	0.4	0.1	0.1
	1.0	0.9	6.9	1.9	7.3	7.5
Galactose						
T-Gal *p*	1.7	2.7	2.7	1.8	2.2	3.1
(1,4)-Gal *p*	2.8	2.6	2.6	0.9	3.3	2.9
(1,6)-Gal *p*	1.4	1.4	1.5	0.4	1.0	1.4
(1,3,6)-Gal *p*	0.8	0.5	0.3	0.3	0.5	0.5
(1,4,6)-Gal *p*	0.3	0.5	0.8	0.3	0.3	1.4
	7.0	7.7	7.9	3.7	7.3	9.2
Glucose						
(1,4)-Glu *p*	0.4	0.1	2.8	0.9	0.6	2.9
(1,4,6)-Glu *p*	0.0	0.6	0.4	0.1	0.1	0.1
	0.4	0.7	3.2	1.0	0.7	3.0
Mannose						
(1,4)-Man *p*	0.5	0.2	2.2	0.8	0.6	2.3
	0.5	0.2	2.2	0.8	0.6	2.3
Galact. acid						
T-Gal *p* A	1.9	1.0	0.6	2.3	1.3	0.6
(1,4)-Gal *p* A	33.1	9.3	4.2	39.4	12.8	4.3
(1,3,4)-Gal *p* A	3.6	2.2	1.3	2.5	1.3	1.4
	38.6	12.5	6.1	44.2	15.4	6.3

[a] Linkage types in mol %. [b] T, terminal; 1,2-linked Rha, etc.

with the NaOH/EDTA and autoclave extractions.

Discussion

In this study an alkali (A) and a combined autoclave and alkali (B) extraction of sugar beet pulp were used to extract arabinose rich and ferulic acid rich pectic polysaccharides. The extraction methods were carried out repetitively; it was experienced that the results were reproducible. Both extraction methods included a repeated H_2O extraction to remove residual water soluble and suspendable material. The low total sugar content of the first H_2O extraction confirmed that most of the water soluble polysaccharides had been removed during the sugar extraction process. The low amount of material released by the second H_2O extraction indicates that one H_2O extraction is sufficient to remove residual water soluble and suspendable material. No explanation could be found for the difference in glucose content between method A and B.

Several similarities were found between the autoclave and NaOH/EDTA extracts. The yield of the first NaOH/EDTA extraction was comparable to the yield of the extract B Autoclave 1 and was also comparable to data reported in the literature obtained with mild alkali extraction [8]. The sugar compositions of the first autoclave and NaOH/EDTA extract were very similar, only the galacturonic acid content was somewhat lower in the latter. Rombouts and Thibault [8] found a lower arabinose content in their alkali extract (OHP), which is probably caused by the acid extraction which preceded the alkali extraction, causing hydrolysis of the arabinosyl linkages. Both the first autoclave and the first alkali extract contained high amounts of (1→4)-linked galacturonic acid, which indicates that these extracts mainly consisted of 'smooth' regions of pectin. The presence of (1→2,4)- and (1→2)-linked rhamnose, (1→3,5)-, (1→5)-, and terminally linked arabinose and terminally linked and branched galactoses in all extracts is indicative for rhamnogalacturonan type I [24]. Compared with acid extracted sugar beet pectins [1, 9] high amounts of arabinans were present in A NaOH/EDTA 1 and B Autoclave 1, which may affect the functional properties [25]. The arabinosyl linkage compositions of the extracted pectins were similar to those found in acid and alkali extracted pectins [3] and in arabinans from other sources [24]. The average molecular weight of the alkali extract was higher than the molecular weight of the autoclave extract and was also somewhat higher compared with literature values for beet pulp pectins [1, 8, 26], although calibration of the column using pectin standards may have led to an underestimation of the molecular weight of polysaccharides with high neutral sugar content. During the autoclave extraction apparently some breakdown occurs (pH 5.2), probably caused by ß-elimination. An obvious difference between extract B autoclave

1 and A NaOH/EDTA 1 is the amount of methyl esters and acetyl and feruloyl groups. The amounts of methyl esters and acetyl groups in the autoclave extract were comparable to various other beet pectins reported in the literature [8, 26, 27]. Neither methyl esters nor acetyl groups were present in the first NaOH/EDTA extract, due to saponification. Ferulic acid has been found in the 'hairy' regions of sugar beet pectin and is known to be linked both to arabinose and galactose [3-7]. The amount of ferulic acid found in the first autoclave extract is comparable to values found in EDTA, ammonium oxalate [4] and acid extracts [8, 9]. HPSEC was used to determine if the extracts were homogenious according to molecular size. In B Autoclave 1 three populations could be distinguished. Population I and II contained a relatively large amount of ferulic acid. This suggests that these populations predominantly contain 'hairy' regions. The third population contains only small amounts of ferulic acid and a high concentration of polysaccharides, which indicates the presence of 'smooth' regions. A lower amount of ferulic acid groups was found in the first NaOH/EDTA extract. Again, ferulic acid was predominantly present in the first two populations of the size-exclusion chromatogram.

A substantial yield of about 5 % was obtained for the second autoclave and NaOH/EDTA extraction, showing that not all the extractable material had been released. The sugar compositions of these extracts were similar. Compared with the first extractions a significantly higher amount of neutral sugars was found, which indicates the presence of more 'hairy' regions. Also a significantly higher amount of ferulic acid was present as compared with extract B Autoclave 1. These facts are in agreement with the observation that in both extracts the higher molecular weight populations, which contained the ferulic acid groups, predominated.

For both extraction method A and B residual arabinan rich polysaccharides were extracted using 4 M NaOH at 80°C during 2 h. In agreement with the literature [28, 29], high yields were obtained with 4 M NaOH (23.3 % and 19.0 % respectively for extraction method A and B). A single 4 M NaOH extraction proved to be sufficient to remove all the alkali soluble material. Only small differences were found in the composition of the resulting extracts A and B 4 M NaOH 1. Kobayashi et al. [28] found similar sugar compositions in their 1 M and 6 M NaOH extracts. The small amount of (1→4)-linked galacturonic acid in A & B 4 M NaOH 1 indicates the absence of a large amount of 'smooth' regions. The sugar linkages of arabinose, galactose, rhamnose and galacturonic acid show that arabinans are probably present as side-chains of a RG-I type polysaccharide [24]. Xylose was mainly present as (1→4)-linked xylan. The equimolar ratio of terminally linked xylose and (1→3,4)-linked galacturonic acid in the 4 M NaOH extracts indicate the presence of xylogalacturonan. A similar structure was found as part of the backbone of 'hairy' regions isolated from apples [30].

It can be concluded that both extraction method A and B resulted in three arabinose

rich pectin fractions. Increasing amounts of neutral sugars were observed for A EDTA/NaOH 1, A EDTA/NaOH 2 and A 4 M NaOH 1 respectively. The neutral sugar (linkage-) compositions of these extracts were very similar and indicated the presence of a rhamnogalacturonan with highly branched arabinans and some galactose attached to it. This in contrast to acid extracted pectins, which contain galactose as main neutral sugar since most of the arabinose has been removed by the acid conditions. For the extracts B Autoclave 1, B Autoclave 2 and B 4 M NaOH 1 also increasing amounts of neutral sugars were found. The composition of the extracts obtained in the subsequent extraction steps of method A and B were very similar, the only difference being the high amounts of methyl esters and acetyl and feruloyl groups present in B Autoclave 1 and 2. Especially the high amount of feruloyl groups is of interest, since it is possible to cross-link polysaccharides at the feruloyl groups, thus increasing the molecular weight.

Future research will include further elucidation of the structure of separate populations of the three most prominent extracts of both method A and B. Another topic of interest involves the oxidative cross-linking of the extracts containing high amounts of arabinose and ferulic acid, followed by physico-chemical characterization of the material formed.

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CHAPTER 3

Characterization of arabinose and ferulic acid rich pectic polysaccharides and hemicelluloses from sugar beet pulp

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Abstract

Pectic polysaccharides were extracted from sugar beet pulp to yield fractions representing homogalacturonans, rhamnogalacturonans and relatively small amounts of glucomannans and xyloglucans. The homogalacturonans had an apparent molecular weight of 21 kDa and contained relatively high amounts of methyl esters and relatively low amounts of acetyl groups as compared with the ramified 'hairy' regions. Three populations which originated from the ramified 'hairy' regions of pectin were distinguished. Two of these were rhamnogalacturonans with high apparent molecular weights of 1300 kDa and 120 kDa, respectively. These populations had a high arabinose and ferulic acid content. Despite the high neutral sugar content, these rhamnogalacturonans strongly bound to a DEAE column. The third population which originated from the ramified 'hairy' regions was a neutral population which did not interact with the DEAE column and had a low apparent molecular weight and a high arabinose and ferulic acid content. The arabinan side-chains of the rhamnogalacturonans were heavily branched in all populations. Enzymatic degradation of the xyloglucans showed some similarities with apple xyloglucans with respect to the substitution with fucose and galactose.

Introduction

The high content of pectic polysaccharides makes sugar beet pulp a potential source of pectins [1]. However, some structural characteristics (high acetyl content, low molecular weight) of sugar beet pectin limit its applicability for the traditional applications of pectin (gel formation with acid/sugar or calcium ions) [2].

Like all pectins, sugar beet pectins are considered to consist of 'smooth' homogalacturonan regions and ramified 'hairy' regions in which most of the neutral sugars are located [3]. The homogalacturonan regions of sugar beet pectin consist of a backbone of (1→4)-linked galacturonic acid, with an estimated minimum length of 72-100 galacturonic acid residues [4]. Both methyl esters and acetyl groups are present in the homogalacturonan. Of the acetyl groups 80-90 % is found on the homogalacturonic backbone and is located at the C-2 and/or C-3 position of the galacturonic acid residues [5].

The ramified 'hairy' regions of pectins extracted from apples are believed to consist of three subunits, as was shown by Schols et al. [6]. It is suggested that the ramified 'hairy' regions of pectins obtained from other sources are built from similar subunits, although principal differences in the relative amounts of the various subunits may exist [7]. Firstly, a rhamnogalacturonan subunit can be distinguished, consisting of a backbone of alternating (1→4)-linked galacturonic acid and (1→2)-linked rhamnose residues, partly substituted with single unit galactose residues (1→4)-linked to the rhamnose residues. Secondly, a rhamnogalacturonan subunit has been found, substituted with arabinan side-chains. And thirdly, the presence of a xylogalacturonan subunit has been proven, which consists of a galacturonan backbone, with single unit xylose residues linked at the C-3 position of galacturonic acid residues. A study using acid hydrolysis of sugar beet pectin showed a maximum length of 20 residues of alternating rhamnose and galacturonic acid for the rhamnogalacturonan backbone, although it is stated that breakdown in the backbone might have occurred [8]. Galactose is predominantly present in the ramified 'hairy' regions as (1→4)-linked galactans of low DP and as (1→3,6)-linked galactans in low proportions [9]. Ferulic acid is attached to the O-2 position of (1→5)-linked arabinose residues in the arabinan side-chains as well as to the O-6 position of galactose residues in (1→4)-linked galactans [9-13]. Approximately 50-55 % of the feruloyl groups in sugar beet pulp is linked to arabinose residues and approximately 45-50 % to galactose residues [12]. Ferulic acid dehydrodimers account for approximately 9 % of the total ferulates in an extract obtained from sugar beet pulp by autoclaving [14].

In a previous study, the extraction of pectic polysaccharides from sugar beet pulp has been described [15]. For this purpose a sequential extraction including autoclaving and an extraction with 4 M NaOH was used. Extracts obtained by autoclaving contained

pectic polysaccharides with highly branched arabinan side-chains [15]. The average length of the side-chains appeared to be much higher for the autoclaved extract (61-118) [15] than for acid extracted material (18) [9]. In the current study we describe the further structural elucidation of the most prominent pectic and hemicellulosic extracts. The information obtained will be used in future studies concerning the structure-function relationship of the pectic polysaccharides.

Experimental

Materials.- Wet beet pulp (8.9 % dry weight) was obtained from CSM Suiker bv (Breda, the Netherlands). Pectic and hemicellulosic polysaccharides were extracted from sugar beet pulp by two subsequent autoclave treatments (121°C, 40 min) and a strong alkali treatment (4 M NaOH + 0.02 M $NaBH_4$, 80°C, 2 h) as described previously [15]. The extracts were named Autoclave 1 and 2, and 4 M NaOH 1, respectively.

Analytical methods. - The uronic acid content was determined by the automated m-hydroxy biphenyl assay [16]. The neutral sugar composition was determined after hydrolysis with 2 M TFA (1 h, 121°C) as described previously [15]. Methylation analysis was performed as described previously [15], without reduction of galacturonic acid residues to galactose. Feruloyl groups were determined spectrophotometrically at 375 nm in freshly prepared pectin solutions adjusted to pH 10 with a 0.1 M NaOH solution. A molar extinction coefficient of 31,600 was used [5]. The degrees of methylation and acetylation were determined as described previously [17]

Chromatography. - High-performance size-exclusion chromatography (HPSEC) was performed on three Bio-Gel TSK columns in series (60XL-40XL-30XL) as described [9], using a combined RI detector and viscometer (Viscotek, model 250), a Right Angle Laser Light-Scattering detector (RALLS, Viscotek, LD 600) and a UV detector (Kratos, Spectroflow 773). Molecular weights were calculated using the light-scattering and universal calibration modules of the Trisec software (Viscotek). Also a calibration was performed comparing the elution times with those of pectin and dextran standards. The pectin standards were obtained by mechanical degradation through ball milling and the molecular weights were calculated from their viscosities [18]. Dextran standards were obtained from Pharmacia.

High-performance anion-exchange chromatography (HPAEC) was performed on a Dionex Bio-LC system with a (4 x 250 mm) PA100 column. Samples were applied onto the column using a Spectra Physics SP 8880 autosampler. The gradients were obtained by mixing 0.1 M NaOH and 1 M NaOAc in 0.1 M NaOH as shown in the corresponding figures. Rhamnogalacturonan [19] and xyloglucan [20] oligomers prepared from apple cell wall polysaccharides were used as standards. For detection of the mono- and

oligomers a pulsed electrochemical detector (PED) was used in the pulsed amperometric detection (PAD) mode.

Preparative size-exclusion chromatography was performed on two columns (50 x 10 cm) of Sephacryl S 500 (Pharmacia) in series, using a Biopilot system (Pharmacia). The sample (1.0 g) was eluted with 0.05 M NaOAc pH 5.0 at a flow rate of 37 mL/min.

Preparative anion-exchange chromatography was performed on a column (54 x 2.6 cm) of DEAE Sepharose Fast Flow (Pharmacia) using a Hiload System (Pharmacia). Samples (0.4-0.5 g) were applied onto the column at a flow rate of 2.6 mL/min (0.005 M NaOAc, pH 5.0). After 60 min, the flow rate was increased to 13.2 mL/min and a gradient was applied as indicated in the corresponding figures.

The fractions obtained by preparative size-exclusion chromatography (120 mL) and preparative anion-exchange chromatography (20 mL) were assayed for total neutral sugar [21] and uronic acid [16] content, using arabinose and galacturonic acid as standards. A correction was made for the response of uronic acids in the neutral sugar test. Calibration of the column was performed using pectins with degrees of methylation ranging from 30 to 70 (Obi Pektin), and soluble starch (Merck). The presence of ferulic acid in the fractions was monitored spectrophotometrically at 335 nm [12], using a Beckman DU-62 Spectrophotometer. Pooled fractions were dialyzed and freeze-dried.

Enzymatic degradation. - All the pools obtained by DEAE anion-exchange chromatography were treated with the following (combinations of) enzymes: arabinofuranosidase B (AF) [22], arabinofuranosidase B (AF) plus endo-arabinanase (EA) [22], endo-polygalacturonase (PG) [23] combined with pectin methyl esterase (PE) [24], and rhamnogalacturonase (RG) [25] in combination with rhamnogalacturonan acetyl esterase (RGAE) [26]. Measurement of activity towards a broad range of polysaccharides showed that these enzymes had no measurable activity on pectic substrates other than their main activity.

The DEAE pool 4 M NaOH 1a was treated with endo-glucanase I (EG I) [27] to degrade the excess of glucans/mannans/glucomannans. Following this treatment the pools were dialyzed, using microconcentrators, and treated with endo-glucanase V (EG V) [27]. The samples were analyzed by HPAEC to examine the presence of xyloglucan fragments.

The DEAE pools (5 mg/mL) were dissolved in 0.04 M sodium acetate buffer pH 5.0. All enzymes were added to obtain a final concentration of 1 μg of protein/mL, sufficient to obtain the maximum degradation possible. Incubations were carried out at 30°C for 20 h. The reactions were stopped by heating at 100°C for 5 min. The digests were analyzed by HPSEC and HPAEC.

Results

In the previous paper we described the extraction of pectic polysaccharides from sugar beet pulp by autoclaving and treatment with 4 M NaOH [15]. This method yielded pectic material containing highly branched arabinan side-chains. Furthermore, the extracts obtained by autoclave treatment also contained relatively high amounts of feruloyl groups, acetyl groups, and methyl esters. The presence of multiple polysaccharide populations in all extracts was shown. For this reason the most prominent extracts named Autoclave 1, Autoclave 2, and 4 M NaOH 1 were fractionated using anion-exchange chromatography.

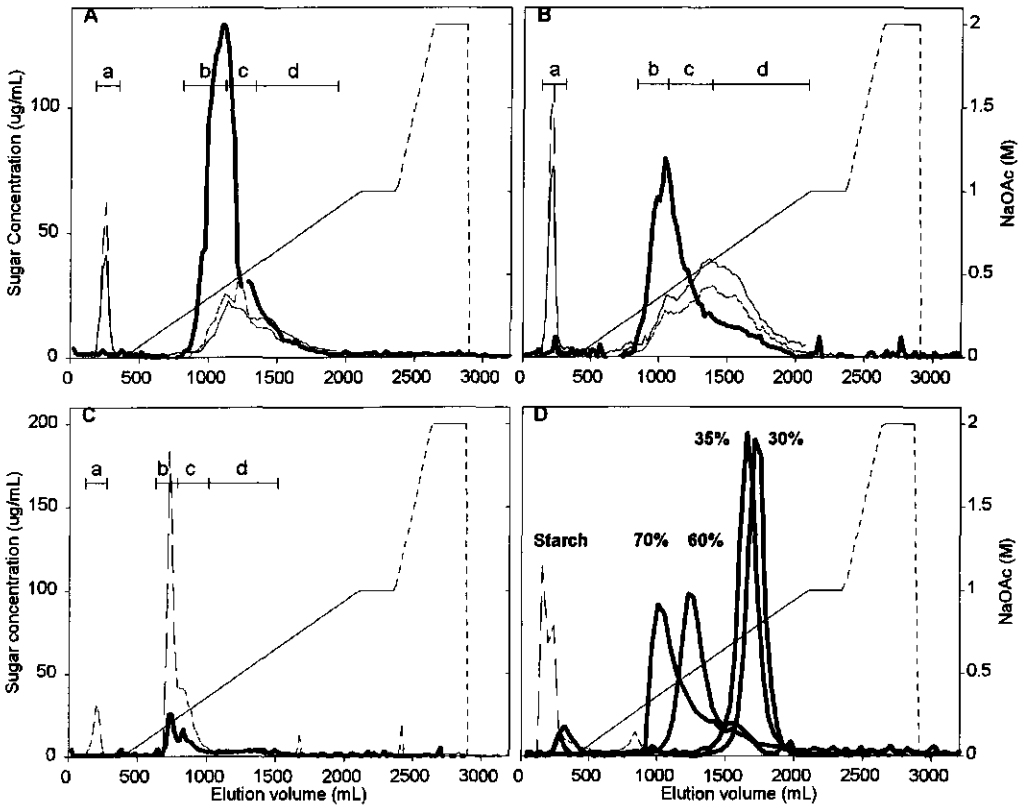

Fig. 1. Preparative anion-exchange chromatography on DEAE-Sepharose of the extracts obtained by autoclaving and extraction with 4 M NaOH. A) Autoclave 1, B) Autoclave 2, C) 4 M NaOH 1, D) Calibration of DEAE with pectins of various degree of methylation. Thin lines: ferulic acid, thick lines: uronic acid, dashed lines: neutral sugars.

Table 1

Characteristics of the populations present in the extracts obtained by autoclaving and extraction with 4 M NaOH upon anion-exchange chromatography.

Sample	Population	Elution volume (mL)	[NaOAc] (M)	Ferulic acid	AUA:ns
Autoclave 1	1	150-250	0.0	+++	-
	2	800-1250	0.4	+	+++
	3	1250-2000	0.6	+++	++
Autoclave 2	1	150-250	0.0	+++	-
	2	800-1250	0.4	+	+++
	3	1250-2200	0.6	+++	+
4 M NaOH 1	1	150-250	0.0	-	-
	2	700-800	0.2	-	+
	3	1000-1500	0.3	-	++

Anion-exchange chromatography. - Fig. 1 shows the results of fractionation of the extracts by anion-exchange chromatography on DEAE-Sepharose (Fig. 1A-C). Several commercial apple pectins, with degrees of methylation ranging from 30 to 70, and starch were used as standards (Fig. 1D). The populations which appeared to be present in the extracts and some of their characteristics are summarized in Table 1. From all extracts, one neutral population and two charged populations, eluting at different NaOAc concentrations, could be separated. The content of ferulic acid as well as the galacturonic acid to neutral sugar ratio varied for each fraction (Table I) and was an indication for their origin (homogalacturonan or rhamnogalacturonan). This was studied further by sugar composition and sugar linkage analysis.

Sugar composition. - The fractions from the DEAE column were pooled as indicated in Fig. 1. Since the DEAE elution pattern indicated that the two populations of Autoclave 1 & 2 which were bound to the column were not completely separated, these populations were collected into three pools: two pools containing relatively pure material from the populations and an intermediate pool containing a mixture of these populations. The sugar compositions of these pools and the original extracts are given in Table 2.

Pool a (the neutral population) of extract Autoclave 1 predominantly consisted of arabinans, based on its composition. Relatively high amounts of glucose and mannose were also found in this pool in a ratio of approximately 1 : 1, possibly indicative for the presence of glucomannans. Pool b mainly consisted of galacturonic acid, indicating that it represents homogalacturonan. Pool d mainly consisted of arabinose and galactose, besides smaller amounts of rhamnose and galacturonic acid, which is indicative for rhamnogalacturonan. Pool c was a mixture of the homogalacturonan and

Table 2

Sugar compositions (mol %) of the DEAE pools of the extracts obtained by autoclaving and extraction with 4 M NaOH.

	Rha	Ara	Xyl	Man	Gal	Glc	UA	DA	DM	Relative weight (%)	Ferulic Acid (%w/w)	Relative FA content (%w/w)
DEAE												
Autoclave 1	2.6	29.8	0.4	0.4	3.9	1.1	61.9	45	73	-	0.4	-
Pool a	2.6	76.9	0.7	7.2	1.7	7.1	4.0	-	-	6.7	-	19.6
b	2.2	12.1	0.5	2.4	3.6	2.0	77.3	-	-	62.6	-	11.5
c	4.3	27.2	1.0	1.2	7.2	1.8	57.5	-	-	16.5	-	31.8
d	4.4	43.9	1.9	2.6	9.3	1.9	35.9	-	-	14.3	-	37.1
Autoclave 2	3.6	60.8	0.0	0.4	6.6	0.9	27.7	52	60	-	1.1	-
Pool a	2.9	84.1	0.5	4.0	1.8	4.3	2.7	-	-	10.3	-	15.5
b	3.1	22.4	0.4	2.5	4.9	2.0	64.8	-	-	28.1	-	9.0
c	8.4	41.3	0.3	1.7	8.3	1.3	38.8	-	-	39.8	-	34.5
d	7.1	56.1	0.2	0.9	11.5	0.8	23.3	-	-	21.9	-	41.0
4 M NaOH 1	6.2	57.3	6.2	2.4	11.0	5.1	11.7	0	0	-	0.0	-
Pool a	2.1	33.6	10.0	16.1	4.7	30.0	3.5	-	-	13.6	-	-
b	5.8	69.1	2.7	0.5	11.1	2.2	8.8	-	-	55.0	-	-
c	9.3	57.8	1.9	0.4	13.8	1.8	15.2	-	-	23.3	-	-
d	6.6	24.6	1.6	0.1	7.5	1.4	58.4	-	-	8.1	-	-
SEC												
Autoclave 2												
Pool I	6.8	58.8	0.8	0.0	13.2	0.6	19.9	51	29	19.6	-	26.7
II	5.3	51.5	0.5	0.7	9.9	0.4	31.8	56	33	33.1	-	44.3
III	1.5	36.9	0.4	1.9	3.2	1.3	54.9	26	56	47.3	-	28.9

rhamnogalacturonan population.

Pool a (the neutral population) of extract Autoclave 2 also predominantly contained arabinans. Again glucose and mannose were found in a ratio of approximately 1 : 1, although the total amount of these sugars was lower. Pool b also contained 22 mol% of arabinose which was probably caused by contamination with the rhamnogalacturonan population. Pool c was again a mixture of rhamnogalacturonan and homogalacturonan. Arabinose was the main sugar in pool d, besides galacturonic acid, galactose and rhamnose.

Extract 4 M NaOH 1 was divided into four pools. Pool a (neutral population) consisted for 88 mol% of neutral sugars, predominantly arabinose, glucose, xylose and mannose. The high content of xylose, glucose and mannose might be an indication for the presence of xyloglucans and glucomannans. Pool b contained rhamnogalacturonans based on its composition. Pool c had a composition comparable to that of pool b. As expected from the elution pattern of this saponified pectin fraction, pool d contained relatively more galacturonic acid than pool b.

Preparative size-exclusion chromatography. - To obtain additional information about the molecular weight distribution of the populations present in the extracts obtained after autoclaving the extract Autoclave 2 was fractionated by preparative size-exclusion

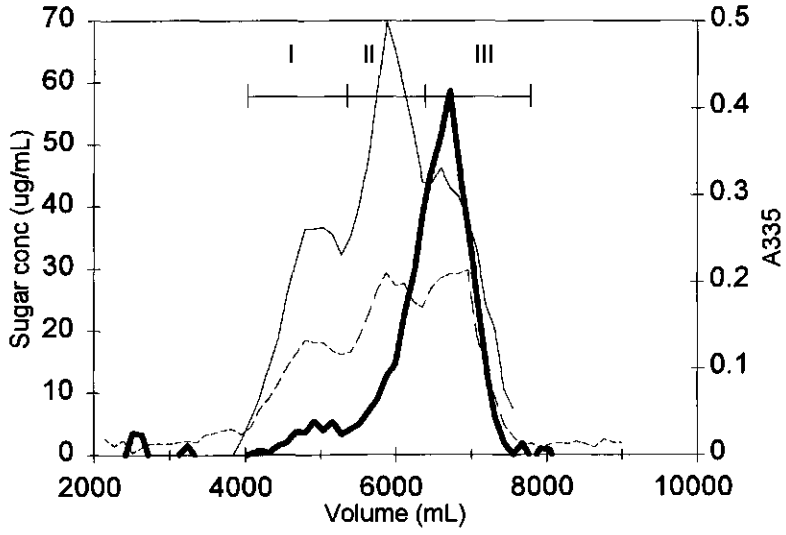

Fig. 2. Preparative size-exclusion chromatography (S 500) of Autoclave 2
Thin lines: ferulic acid, thick lines: uronic acid, dashed lines: neutral sugars.

chromatography (S 500, Fig. 2). Three populations could be distinguished, which were pooled as indicated. Pool I and II both mainly consisted of rhamnogalacturonan (Table 2), with a high ferulic acid content. Furthermore, the degree of acetylation (DA) in these populations was high, whereas the degree of methylation (DM) was relatively low. Pool III contained more homogalacturonans than the other pools. Also some mannose and glucose was present in this pool, again indicative for glucomannans. As compared with pool I and II the DM was high, whereas the DA was much lower. The DM of pool III was comparable to that of the parental extract. Pool I and II, which were present in much lower amounts, had a DM much lower than that of the parental extract.

Sugar linkage composition. - The neutral sugar linkage compositions of a selection of the pools obtained by anion-exchange chromatography were determined, and shown in Table 3. As compared with the sugar composition the amounts of rhamnose were rather low and the amounts of arabinose were somewhat high, as was seen in several other studies [28-30]. In all pools arabinose was predominantly present as terminal, (1→3)-linked and (1→3,5)-linked residues. Also some (1→2,3,5)-linked arabinose was found. In most of the pools rhamnose was determined in approximately equal amounts of (1→2)-linked and (1→2,4)-linked residues. However, in the unbound pool a of Autoclave 2 rhamnose was present primarily as (1→2,4)-linked residues. In most pools xylose was identified as terminally and (1→4)-linked, except for 4 M NaOH 1 pool a, where it was present as terminally and (1→2)-linked residues. This result is in contrast with previous results, in which mainly (1→4)-linked xylose residues were found in 4 M NaOH 1 [15]. Terminally and (1→4)-linked galactose residues were present in most pools in almost equal quantities. Also (1→6)-linked and, to a lesser extent (1→4,6)-linked galactose was found. Glucose was found to be mainly (1→4)-linked, although (1→4,6)-linked glucose was also found in some pools. Only (1→4)-linked mannose was found.

Molecular weight distribution and enzymic degradation. - The molecular weight distributions of the pools obtained by anion-exchange chromatography and SEC are shown in Fig. 3 and will be discussed below. Most of the pools consisted of different sub-populations. To determine the nature of these sub-populations as analysed by HPSEC, all pools were treated with EA+AF, with PG+PE, or with RG+RGAE.

Pool a from both extract Autoclave 1 and 2 eluted very similar (Fig. 3A & B, RI and UV detection). Both pools showed two populations based on molecular weight, one which eluted at 35 min with a high absorption at 335 nm (ferulic acid) and one at 36 min, which was present as a shoulder on the previous population. The latter was present in higher amounts in Autoclave 1 than in Autoclave 2. Treatment of these pools with EA+AF resulted in a shift of the population at 35 min towards lower molecular weights (data not shown), indicating that this pool contains the arabinan. At a retention time of 36 min still material remained, which could not be degraded by RG+RGAE and PG+PE. From this observation as well as from the sugar and methylation analyses (Tables 2 and

3) it was concluded that this population was a glucomannan or a mixture of glucan and mannan.

Table 3

Neutral sugar linkage composition of polysaccharides of the DEAE pools of the extracts obtained by autoclaving and extraction with 4 M NaOH.

Linkage	Glycosidic linkage composition[a]				
	Autoclave 1	Autoclave 2	Autoclave 2	4 M NaOH 1	4 M NaOH 1
Pool	b	a	d	a	b
Rhamnose					
T-Rha *p* [b]	0.9	0.1	1.3	0.0	0.3
(1,2)-Rha *p* [b]	2.8	0.0	1.8	0.4	0.8
(1,2,4)-Rha *p*	2.1	0.9	1.8	0.0	1.5
	5.7	1.0	4.9	0.4	2.6
Arabinose					
T-Ara *f*	27.2	31.5	30.9	19.4	30.4
(1,3)-Ara *f*	0.7	0.7	1.0	0.0	1.0
(1,5)-Ara *f*	21.4	31.8	27.3	13.3	25.8
(1,2,5)-Ara *f*	1.2	0.0	1.6	0.0	1.7
(1,3,5)-Ara *f*	17.5	24.2	16.8	10.2	23.0
(1,2,3,5)-Ara *f*	5.3	3.7	3.7	1.4	4.1
	73.3	92.2	80.6	44.2	86
Xylose					
T-Xyl *p*	1.9	0.2	0.9	6.7	0.5
(1,2)-Xyl *p*	0.0	0.0	0.0	6.1	0.0
(1,4)-Xyl *p*	2.5	0.6	0.0	0.0	2.6
(1,2,4)-Xyl *p*	0.7	0.0	0.3	0.0	0.1
(1,3,4)-Xyl *p*	0.6	0.0	0.2	0.0	0.1
	5.7	0.8	1.3	12.9	3.3
Galactose					
T-Gal *p*	4.1	0.2	4.8	2.2	2.5
(1,4)-Gal *p*	2.7	0.3	4.1	1.8	2.9
(1,6)-Gal *p*	1.8	0.0	2.3	0.1	1.7
(1,4,6)-Gal *p*	5.4	0.1	0.0	0.0	0.4
	13.9	0.6	11.1	4.0	7.5
Glucose					
(1,4)-Glu *p*	0.5	2.5	0.5	21	0.0
(1,4,6)-Glu *p*	0.8	0.0	1.2	10.1	0.5
	1.3	2.5	1.7	31.1	0.5
mannose					
(1,4)-Man *p*	0.0	2.8	0.3	7.3	0.1
	0.0	2.8	0.3	7.3	0.1

[a] Linkage types in mol %. [b] T, terminal; 1,2-linked Rha, etc.

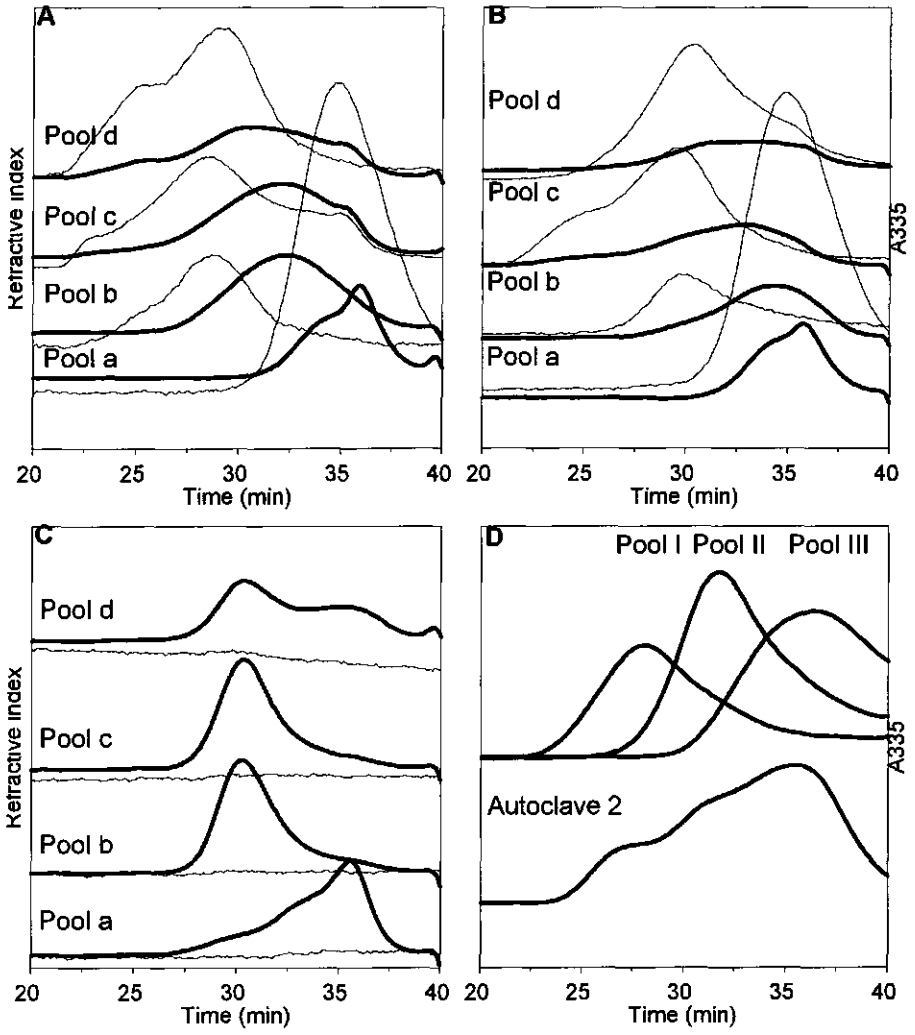

Fig. 3. High-performance size-exclusion chromatography of the DEAE pools of Autoclave 1 (A), Autoclave 2 (B) and 4 M NaOH (C) and of the S 500 pools of Autoclave 2 (D). Thin lines: UV detection, thick lines: RI detection.

Pool b of both Autoclave 1 and 2 showed a refractive index response at 34 min (Fig. 3A & B), which disappeared after treatment with PG+PE (not shown). Also a UV response at 28-29 min was observed, which shifted to a lower molecular weight after treatment with RG+RGAE (not shown). A decrease in RI and UV response was found after treatment with EA+AF as a result of the degradation of the arabinose side-chains to oligomers.

For both pools c and d from Autoclave 1 and 2 peaks were observed at 28-30 min by both RI and UV detection. An additional RI response was seen at 33 min, as well as an additional UV response at approximately 24 min (Fig. 3A and B). The populations at 24 and 28-30 min were degradable with RG+RGAE, resulting in a shift of both RI and UV to 34 min, as is shown in Fig. 4A for Autoclave 2 pool d. A decrease in intensity was found for both the RI and UV response at 24 and 30 min after treatment with EA+AF. As a result the amount of arabinose oligomers as detected by HPAEC increased (data

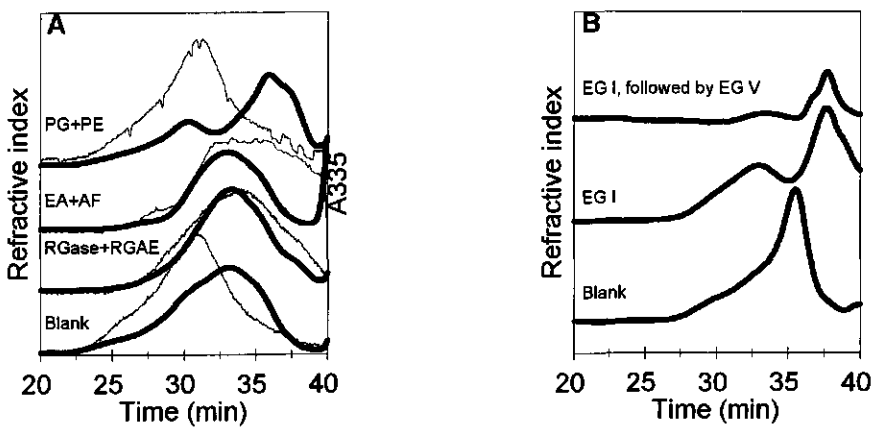

Fig. 4. High-performance size-exclusion chromatography of pool d from Autoclave 2 after treatment with EA+AF, RG+RGAE and PG+PE (A) and of pool a from 4 M NaOH 1 after treatment with EG I and EG V (B). Thin lines: UV detection, thick lines: RI detection.

not shown). The population which eluted at 33 min could be degraded with PG+PE and shifted to 37 min. This revealed the presence of two high molecular weight populations

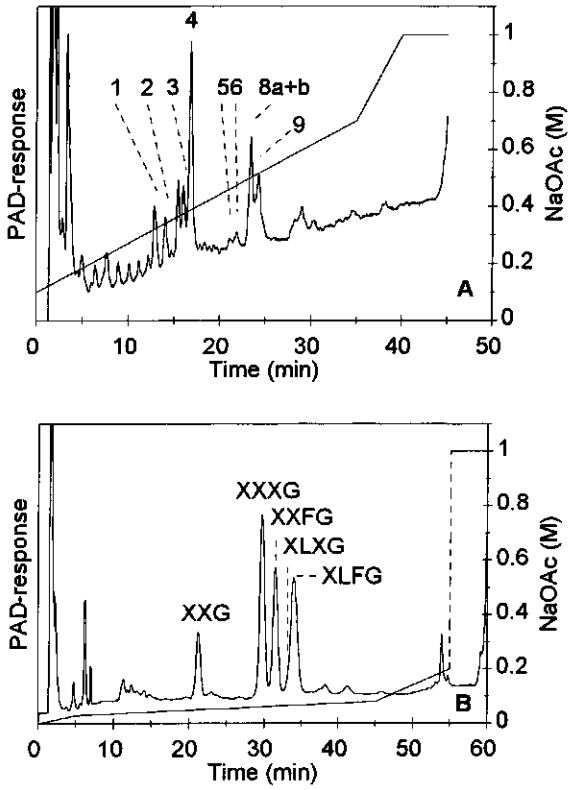

Fig. 5. High-performance anion-exchange chromatography of the enzyme digests of pool d from Autoclave 2, treated with RG+RGAE, (A) and of pool a from 4 M NaOH 1 after treatment with EG V (B).

1: ■-O-■-O; 2: ■-O-■-O; 3: ■-O-■-O; 4: ■-O-■-O; 5: ■-O-■-O-■-O

6: ■-O-■-O-■-O; 8a: ■-O-■-O-■-O; 8b: ■-O-■-O-■-O; 9: ■-O-■-O-■-O

where 2 represents: α-Rha*p*-(1→4)-α-Gal*p*A-(1→2)-α-Rha*p*-(1→4)-α-Gal*p*A.
β-Gal*p*-(1→4)

based on RI detection, a minor population at 24 min and a population at 30 min. The HPAEC results of the treatment of pool d with RG+RGAE are shown in Fig. 5a. The products were identified based on their retention times, which were compared with standard rhamnogalacturonan oligomers from apple 'hairy' regions [19]. It shows that after treatment of pool d with RG+RGAE several oligomers of various degrees of polymerization were formed with a backbone of alternating rhamnose and galacturonic acid residues. Most of these oligomers are branched with galactose residues linked to a rhamnose residue at the C-4 position.

From Fig. 3C it can be seen that HPSEC of the neutral population (pool a) of extract 4 M NaOH 1 gave a broad peak from 27 to 37 min indicating the presence of a wide range of molecular weights. From its sugar composition it was expected that arabinans, xyloglucans and glucomannans were present (Table 2). To prove this, the material was subsequently treated with EG I, EG V and EA+AF. Incubation with EG I, which is known to have a high activity towards glucans and a much lower activity towards xyloglucan [31], resulted in degradation of the material which eluted at 35.5 min towards lower molecular weight (38 min). Furthermore, a population could be distinguished at 33 min. After pretreatment with EG I, a subsequent treatment with EG V with high activity towards xyloglucan [31] was performed, resulting in degradation of the population at 33 min to lower molecular weight (38 min; Fig. 4b). Analysis of the reaction products by HPAEC showed the presence of typical xyloglucan oligomers (Fig. 5b), which also explains the presence of (1→2)-linked xylose residues in this population. As concluded from the elution behaviour, the oligomers found were: XXG, XXXG, XXFG and XLFG, named according to the nomenclature of Fry et al. [32], where G, X, L and F represent different substituted β-glucosyl residues of xyloglucan: G, β-Glcp; X, α-Xylp-(1→6)-β-Glcp; L, β-Galp-(1→2)-α-Xylp-(1→6)-β-Glcp; F, α-Fucp-(1→2)-β-Galp-(1→2)-α-Xylp-(1→6)-β-Glcp. A small amount of the XLXG oligomer seemed to be present as a shoulder of the XLFG oligomer. After treatment of pool a of 4 M NaOH 1 with EG I and V a population at 33 min remained, which could be degraded with EA+AF.

Pool b from extract 4 M NaOH 1 (Fig. 3C), eluting at 30 min, gave a symmetrical peak which is indicative for homogeneity, both on charge and on molecular weight.

Pool c showed a similar peak, but also a second population at 37 min showed up in minor amounts. The amount of the latter population was much higher in pool d. The population at 30 min could be degraded both with EA+AF and RG+RGAE (data not shown), indicating the presence of rhamnogalacturonans.

Fig 3D shows the HPSEC patterns of the pools obtained after separation of Autoclave 2 on Sephacryl S 500. The apparent molecular weights were calculated from the whole Autoclave 2 extract after separation by HPSEC, using light-scattering detection. The apparent molecular weight of pool I was 1300 kDa. The apparent molecular weight of pool II was 120 kDa, while the third pool had a low apparent

molecular weight (21 kDa). Since it is known that, in comparison to other techniques, often higher molecular weights are found for pectic polysaccharides when light-scattering techniques are used, also two other methods to determine the molecular weights by SEC were applied. It was found that the results obtained by laser light-scattering were similar to those obtained with universal calibration (1600 kDa, 120 kDa and 12 kDa, respectively) and to those obtained by comparison with dextran standards (pool I and II) and pectin standards (pool III) (1400 kDa, 110 kDa and 20 kDa, respectively).

Discussion

Three types of polysaccharides were present in both extracts obtained by autoclave treatment of beet pulp; homogalacturonans, rhamnogalacturonans and small amounts of glucose and mannose containing polysaccharides. In none of the incubations with enzymes active on the homogalacturonans (PG + PE) changes in the A_{335} pattern (which is indicative for ferulic acid) were observed, while enzymes active on the rhamnogalacturonans (RG+RGAE, EA+AF) never changed the elution patterns of the homogalacturonans upon HPSEC. Therefore, it was concluded that homogalacturonans and rhamnogalacturonans were present as separate populations in the extracts. The glucomannans and the xyloglucans in the neutral populations could specifically be degraded by endo-glucanases, indicating that they were present as separate populations as well.

The four building blocks of pectin from sugar beet pulp (high and low molecular weight rhamnogalacturonan, arabinan and homogalacturonan) which were found, are believed to be a result of degradation during the extraction. Although in this study rhamnogalacturonans and homogalacturonans were only found in the extracts as separate populations, it is believed that both polysaccharides are integral parts of the pectin molecule [33]. Also, compared with acid extracted commercial pectins the apparent molecular weight of the homogalacturonan was relatively low, indicating degradation of the pectins, as confirmed by the low intrinsic viscosity [34]. The molecular weight of the material which was formed after treatment of the rhamnogalacturonans with RG+RGAE was similar to the neutral arabinan population, which may indicate that the neutral population originates from degradation of the rhamnogalacturonans during autoclaving. Additional experiments showed that autoclaving of sugar beet pulp at higher temperatures results in larger amounts of low molecular weight material which coelutes with this population upon HPSEC (data not shown).

The apparent molecular weight of the homogalacturonans in Autoclave 2 (21 kDa)

implies a degree of polymerization of 120, which is comparable to the value found by Thibault et al. [4]. However, in both studies the backbone had been subjected to degradative conditions. Therefore, higher degrees of polymerization are expected for the homogalacturonans. The assumption that degradation occurred was supported for the extracts obtained by autoclaving by the lower molecular weight of the homogalacturonans of Autoclave 2 than those of Autoclave 1 as was observed by HPSEC (Fig. 3A and B). The degree of methylation (DM) for the homogalacturonans in the extract obtained by autoclaving was 56 for this population, which is similar to that of the whole Autoclave 2 extract. Comparable values were reported for CDTA and buffer soluble pectins from beet pulp [35], whereas that of the rhamnogalacturonans was higher. The degree of acetylation (DA) of the homogalacturonans was much lower than in the whole extract Autoclave 2.

The arabinans were found in the neutral population, upon anion-exchange chromatography (Autoclave 1 & 2, pool a). Since the neutral populations of Autoclave 1 and 2 represented only a small part of the total amount of arabinose present, it is concluded that most of the arabinans were present in charged polymers. Furthermore, the presence of small amounts of (1→2,4)-linked rhamnose and galacturonic acid in the neutral population indicates that it also originates from rhamnogalacturonans. Based on methylation analysis and its average molecular weight (10-15 kDa) it was concluded that this population consisted of highly branched arabinans, containing around 100 arabinose residues per molecule, assuming that the population was homogeneous.

The apparent molecular weights of the rhamnogalacturonans present in the extracts obtained by autoclaving were very high, 1300 kDa for the high molecular weight population and 120 kDa for the lower molecular weight population. This means that the 'hairy' regions from sugar beet pectins have higher apparent molecular weights than reported before for 'hairy' regions from beet [10, 15] or apple [36] and for acid extracted beet pectins [37-39]. Possibly this high apparent molecular weight is caused by aggregation phenomena, which are known to occur in pectic substances, and especially affect molecular weights determined by light-scattering. However, comparison with universal calibration and comparison with dextrans gave similar values, indicating that aggregation was not an important phenomenon in these samples. McNeil et al. suggested a length of the backbone of RG-I of up to 300 rhamnose and 300 galacturonic acid residues for suspension cultured sycamore cell-walls [40]. It can be calculated that this backbone has a molecular weight of approximately 100 kDa. Considering that the rhamnogalacturonans present in Autoclave 2 consist for less than 10 mol% of rhamnose and galacturonic acid, whereas it contains 85 mol% of neutral sugars [34], and assuming that the backbone of these rhamnogalacturonans have a comparable length as sycamore rhamnogalacturonans, a molecular weight can be calculated of approximately 1000 kDa. This value is in agreement with the results found

with the various methods described above. Another explanation for the high molecular weight found for this population is the possibility of the presence of ferulate cross-links. These could connect several rhamnogalacturonan molecules, which would result in a high molecular weight complex. A probable cause for the low molecular weights found in other studies is the use of (rather linear) pectin standards in some of these studies [15, 36] to calculate the molecular weight of the highly branched rhamnogalacturonans. Furthermore, in many studies [10, 37-39] the pectin has been subjected to acid conditions, thus removing arabinose and decreasing the molecular weight.

The rhamnogalacturonans (pool I & II, Fig. 2) had higher arabinose contents than reported before for the rhamnogalacturonans present in acid extracted beet pectins [10, 11]. From the results of the methylation analysis it could be calculated that the arabinose side-chains had an average DP of 45 residues or more, assuming that all (1→2,4)-linked rhamnose residues carry an arabinose side-chain of equal length. The oligomers released during incubation with RG+RGAE indicated that a part of the (1→2,4)-linked rhamnose residues are substituted with galactose. This made us to conclude that the average DP of the arabinose side-chains must be higher than 45. No additional evidence was found for the presence of xylogalacturonan subunits in the backbone of the ramified 'hairy' regions, as has been described for apple pectins [6], besides a small amount of (1→3,4)-linked galacturonic acid which was found previously in both autoclave extracts and in the alkali extract [15].

The DM of the rhamnogalacturonans was considerably lower than that of the homogalacturonans, explaining a stronger binding to the anion-exchange column. The DA on the other hand was higher than that of the homogalacturonans. These results were comparable to those for modified hairy regions from apples [6].

Since A_{335} is quite selective for ferulic acid and comparable chromatograms were obtained when detection was done by neutral sugar determination or A_{335} (on SEC as well as DEAE), it can be concluded that ferulic acid is evenly distributed amongst the neutral sugar containing populations. Degradation of the rhamnogalacturonans with EA+AF resulted in a sharp decrease of the UV response at 335 nm, therefore it was concluded that most of the ferulic acid was attached through the arabinan side-chains as opposed to the results found by Guillon et al. for acid and alkali soluble pectins from sugar beet pulp [10, 11], who indicated that only 30 % of the ferulic acid was removed by EA+AF. However, this might be caused by the degradation of arabinan side-chains as a result of the acid treatment during their extraction.

The sugar composition and the methylation analysis indicated that in the neutral populations of the extracts obtained after autoclave treatment (pools a) glucose and mannose containing polysaccharides were present. Treatment of this pool with EA+AF showed the degradation of the arabinans. Subsequently, a homogeneous population remained, probably a glucomannan.

The formation of specific xyloglucan oligomers after degradation of the neutral population of the 4 M NaOH extract with endo-glucanase V forms proof for the presence of xyloglucans. The oligomers found were XXG, XXXG, XXFG, XLFG and probably a small amount of XLXG. Therefore, three out of four glucose residues carry a side-chain, as was also seen for xyloglucans from many species such as apple, sycamore, and tamarind [20, 41]. This in contrast to xyloglucans from potato and tomato, both belonging to the *Solanaceae*, for which the presence of two adjacent unbranched glucose residues is characteristic [42]. In comparison with apple [43], xylose is only present in low amounts in sugar beet pulp (~2 mol%) [15], and therefore it can be concluded that xyloglucan plays a limited role in making up the architecture of sugar beet cell walls.

In conclusion it can be said that rhamnogalacturonans from sugar beet pulp obtained by autoclaving and strong alkali treatment have a much higher apparent molecular weight than reported before, which is partly caused by their high arabinose content. The DM of the rhamnogalacturonans is much lower than of the homogalacturonans, whereas the DA is much higher. When compared with extraction with acid these ferulic acid containing rhamnogalacturonans are well preserved during autoclaving. Therefore, these polysaccharides can be very suitable for gel formation through oxidative cross-linking. Future research will therefore be focused on such experiments with extracts obtained by autoclaving as well as with acid extracted pectins.

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CHAPTER 4

Formation of ferulic acid dehydrodimers through oxidative cross-linking of sugar beet pectin

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Abstract

Pectins isolated from sugar beet pulp by autoclaving contained significant amounts of ferulates, 8.8 % of which were ferulate dehydrodimers. The 8-8 and 8-O-4 dehydrodimers were predominant. Oxidative cross-linking with hydrogen peroxide/peroxidase lowered the amount of ferulic acid by 78 %, while an increase in ferulate dehydrodimers by a factor of 4.9 was observed. The highest increase was seen for the 8-5 and 8-O-4 dehydrodimers. The concentration of total ferulates decreased by 36 % after cross-linking, indicating that a part of the ferulates were converted to unidentified oxidation products. It was concluded that ferulic acid in beet pulp pectin is coupled into a variety of dehydrodimers by treatments that mediate oxidative cross-linking reactions.

Introduction

Pectins isolated from sugar beet, in contrast to those isolated from apple and citrus, contain significant amounts of ferulic acid [1]. Ferulic acid in beet pectin is attached to the O-2 position of (1→5)-linked arabinose residues in the arabinan side-chains and can also be found to be attached to the O-6 position of galactose residues in (1→4)-linked galactans [2-6]. Numerous studies with ferulate model compounds and with feruloylated arabinoxylans and cell walls from grasses have demonstrated that ferulate monomers are readily coupled into dehydrodimers by oxidases and one-electron oxidants [7-9]. Treatment of sugar beet pectin with a one-electron oxidant (ammonium persulphate) or hydrogen peroxide/peroxidase increased the viscosity and gelling of beet pectin, an effect apparently due to oxidative coupling of ferulate monomers into dehydrodimers [10-11]. However, no definite proof was given which ferulic acid dehydrodimers were formed during this process. We now report on the identification of ferulic acid dehydrodimers formed by hydrogen peroxide/peroxidase treatment of pectin obtained from sugar beet pulp.

Results and discussion

Pectin extracts from autoclaved beet pulp [10] contained 16.26 mg/g of total ferulates, 8.8 % of which were ferulate dehydrodimers (Table 1). The 5-5, the 8-5, the 8-8 and the 8-O-4 coupled dehydrodimers were detected in a ratio of 1:1.3:1.7:2.3, respectively (see Fig. 1). The 4-O-5 dehydrodimer was not detected. The predominance of the 8-O-4 coupled dehydrodimer is in contrast with the results obtained with grasses in which the 8-5 dehydrodimer is the most abundant type [8, 9]. The 8-5 dehydrodimer was only present in relatively small amounts in beet pectin.

Hydrogen peroxide/peroxidase treatment of the pectin extract reduced the concentration of ferulic acid monomers from 14.82 to 3.30 mg/g, indicating that about 78 % of the monomers were involved in reactions caused by the oxidant. Hydrogen peroxide/peroxidase treatment increased the concentration of 8-5 and 8-O-4 coupled dehydrodimers to 3.38 and 2.34 mg/g respectively, whereas the 5-5 type increased only to 1.02 mg/g and a small decrease was seen for the 8-8 type. The concentration of total ferulates (monomers plus dehydrodimers) decreased by 36 % following hydrogen peroxide/peroxidase treatment indicating that a portion of the ferulates were converted to products which were not detectable by GC (e.g. tetramers or oligomers).

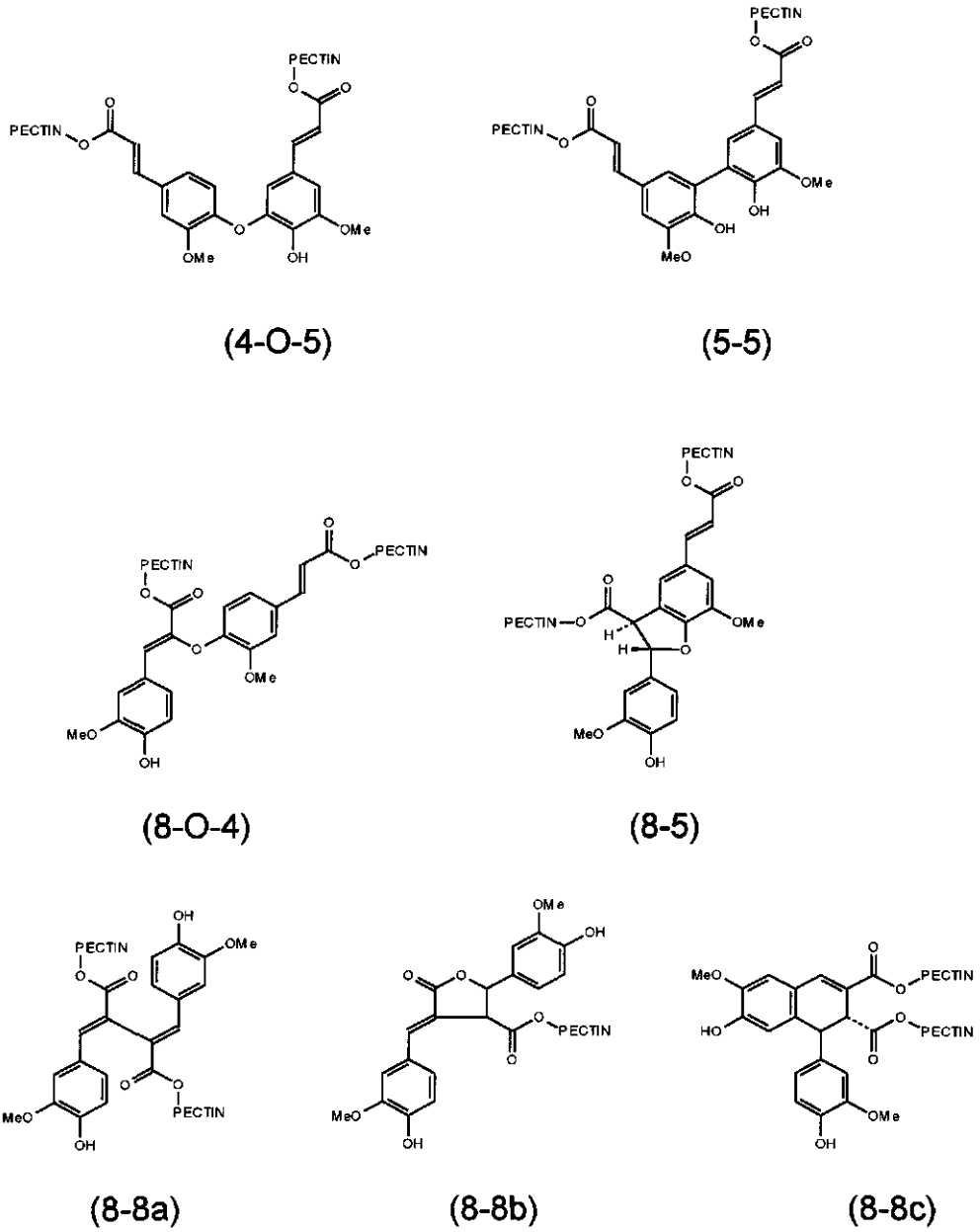

Fig. 1. Structural representation of ferulic acid dehydrodimers.

Table 1
Mono and diferulic acid composition of sugar beet pectin before and after oxidative cross-linking (mg/g of sample).

		Blank	Cross-linked
Monomers	ferulic acid	14.82	3.30
Dimers	8-8	0.38	0.29
	8-5	0.30	3.38
	8-O-4	0.53	2.34
	5-5	0.23	1.02
Total dimers		1.43	7.03
Total mono- + dimers		16.26	10.33

Therefore it can be concluded that ferulate monomers in pectin from sugar beet pulp are readily coupled into a variety of dehydrodimers by treatments that mediate oxidative cross-linking reactions. Cross-linking of the arabinan and galactan side-chains by ferulate dehydrodimers is responsible for increasing the viscosity and gelling of the hydrogen peroxide/peroxidase treated pectins from sugar beet pulp.

Experimental

Wet beet pulp (harvest 1991, 8.9 % dry weight) was obtained from CSM Suiker bv (Breda, the Netherlands). Arabinan and ferulic acid rich pectic polysaccharides were extracted from sugar beet pulp as described previously [10]. The extract used represented 5.8 % of the dry weight of the pulp and consisted for 88.3 % of polysaccharides. The extract contained 60.8 mol% arabinose, 27.7 mol% galacturonic acid, 6.6 mol% galactose and 3.6 mol% rhamnose [10]. Oxidative cross-linking of the extract was performed by adding 40 µL of horseradish peroxidase (0.5 mg/mL, Sigma) and 40 µL of hydrogen peroxide (0.5 M, Merck) to 4 mL of a 1.0 % (w/v) solution of beet pectin in 0.1 M phosphate buffer (pH = 6.0, 25°C). Ferulic acid and its dehydrodimers were analyzed by GLC after saponification and silylation as described by Ralph et al. [8].

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CHAPTER 5

Oxidative cross-linking of pectic polysaccharides from sugar beet pulp

Alexander Oosterveld, Gerrit Beldman, and Alphons G.J. Voragen

Abstract

The oxidative coupling of three pectin extracts from sugar beet pulp with hydrogen peroxide/peroxidase or ammonium persulfate were compared. Two extracts were obtained by sequential autoclave treatment and contained homo- and rhamnogalacturonan regions as separate populations. The extract obtained from the first treatment (Autoclave 1) contained more homogalacturonans than the second extract (Autoclave 2). A third pectin fraction was obtained by acid extraction (HP). It mainly contained homogalacturonans with part of the rhamnogalacturonan regions still linked to it. The arabinose content in the acid extract was relatively low.

Oxidative cross-linking of these extracts with hydrogen peroxide/peroxidase resulted in an increase in viscosity at low concentrations and in the formation of a gel at higher concentrations. Gels were formed using concentrations of 1.5 % for Autoclave 2 and HP and of 3 % for Autoclave 1. It was shown that in the autoclave extracts only rhamnogalacturonans and possibly the arabinans participated in the cross-linking reaction. Cross-linking with ammonium persulfate resulted in a viscosity decrease of the autoclave extracts, although ferulic acid dehydrodimers were formed. No high molecular weight material was formed. Treatment of the acid extracted pectin with ammonium persulfate gave a slow increase in viscosity and the formation of a high molecular weight population was observed.

Although there was a difference in reactivity with ammonium persulfate and hydrogen peroxide/peroxidase for the acid extracted pectin and the Autoclave 2 extract, the ratio of the various ferulic acid dehydrodimers formed was similar for both oxidants. The total amount of dehydrodimers was lower for the ammonium persulfate cross-linked material.

Introduction

The wide availability and low price of sugar beet pulp make this by-product of beet sugar production an interesting source of pectin. The presence of ferulic acid in sugar beet pectin allows gel formation through cross-linking of the ferulic acid groups [1], as an alternative for gel formation in an acid-sugar system, or in the case of low methoxyl pectin by calcium complexation.

Ferulic acid is present in the 'hairy' regions of sugar beet pectin and is attached by an ester linkage to the O-2 position of (1→5)-linked arabinose residues in the backbone of the arabinan side-chain and also to the O-6 positions of galactose residues in (1→4)-linked galactans [2-6]. Approximately 50-55 % of the feruloyl groups are linked to arabinose residues and approximately 45-50 % are linked to galactose residues [5]. From this, and from the sugar composition [5], it can be calculated that 1 out of 56 arabinose residues and 1 out of 16 galactose residues in sugar beet pulp carry a feruloyl group. During the traditionally used acid extraction process for pectin the arabinan side-chains are degraded and therefore the ferulic acid content decreases [7], while the galactan side-chains are better preserved.

Of several oxidising agents, only ammonium persulfate and hydrogen peroxide/peroxidase have been shown to be able to initiate oxidative cross-linking of beet pectins. This reaction increased the molecular weight and viscosity of beet pectins, and eventually a gel was formed [8]. Previous experiments revealed that treatment of an acid extracted sugar beet pectin with ammonium persulfate results in a gradual increase in viscosity, whereas the combination of hydrogen peroxide/peroxidase leads to an instantaneous increase in viscosity [8].

Recently we described the sequential extraction by autoclaving [9] and the characterization [10] of pectins from sugar beet pulp. This yielded two extracts named Autoclave 1 and 2. In Autoclave 1 galacturonic acid was the main sugar (61.9 mol%) although it also contained a relatively high amount of arabinose (29.8 mol%), whereas in Autoclave 2 arabinose was the predominant sugar (60.8 mol%) besides a relatively low amount of galacturonic acid (27.7 mol%) [9]. It was shown that four populations were present in the autoclave extracts. Two high molecular weight populations with apparent molecular weights of 1300 and 120 kDa, respectively, were found to consist of feruloylated rhamnogalacturonans with highly branched arabinose side-chains. Furthermore, the presence of a low molecular weight homogalacturonan (21 kDa) as well as a highly feruloylated low molecular weight arabinan (10-15 kDa) was shown.

The current study deals with the ability of oxidative cross-linking to increase viscosity and to form gels of the autoclave extracts, using hydrogen peroxide/ peroxidase or ammonium persulfate. The reactivity of these autoclave extracts is compared with that of acid extracted beet pectin as described by Thibault and

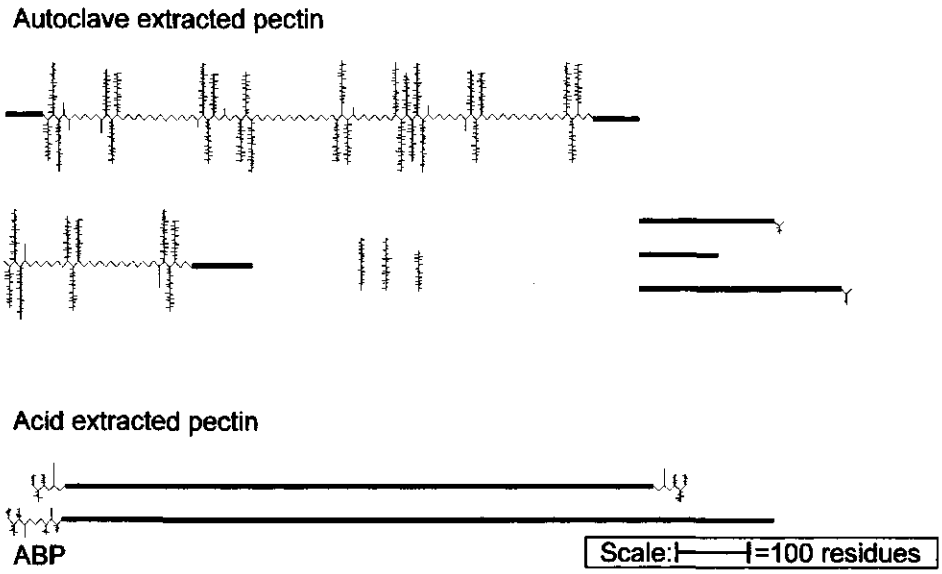

Fig. 1. Schematic structure of sugar beet pectins obtained by autoclave (Autoclave 2) and acid extraction (HP). The thin lines represent the rhamnogalacturonan regions (RG) and the thick lines represent the homogalacturonan regions (HG).

Rombouts [8]. This pectin was found to consist mainly of galacturonic acid (73.1 mol%) with galactose as predominant neutral sugar (14.1 mol%). The homogalacturonans and rhamnogalacturonans are still linked in this extract. Furthermore, most of the ferulic acid is linked to galactose. A schematic structural representation of the autoclave and acid extracted pectins, based on the model of Schols et al. [11], is given in Fig. 1.

Experimental

Materials.- Wet beet pulp (8.9 % dry weight) was obtained from CSM Suiker bv (Breda, the Netherlands). Autoclave extraction of sugar beet pulp was performed as described previously [9]. Two extracts were used: Autoclave 1 and 2. Autoclave 1 represented 12 % of the dry weight of the pulp and contained 66.8 % polysaccharides (61.9 mol% galacturonic acid, 29.8 mol% arabinose, 3.9 mol% galactose and 2.6 mol% rhamnose). It contained 0.4 % (w/w) ferulic acid and its degree of feruloylation (DF; Moles of feruloyl groups/100 moles arabinose + galactose residues) was 1.5. Autoclave 2 represented 5.8 % of the dry weight of the pulp and consisted for 88.3 % of polysaccharides (60.8 mol% arabinose, 27.7 mol% galacturonic acid, 6.6 mol% galactose and 3.6 mol% rhamnose) [10]. 1.1 % of the dry weight was ferulic acid and the DF was 1.3. The acid extracted pectin (HP) was a gift from Dr. J.-F. Thibault from INRA in Nantes (France) [8]. It had a DF of 4.8 and contained 0.8 % ferulic acid and 83.0 % polysaccharides (73.1 mol% galacturonic acid, 14.1 mol% galactose, 6.0 mol% rhamnose and 2.9 mol% arabinose).

Analytical methods. - Ferulic acid and its dimers were analyzed after saponification and silylation as described by Ralph et al. [12].

Chromatography. - High-performance size-exclusion chromatography (HPSEC) was performed on three Bio-Gel TSK columns in series (60XL-40XL-30XL) in combination with a combined RI detector and viscometer (Viscotek, model 250), a Right Angle Laser Light-Scattering detector (RALLS, Viscotek, LD 600) and a UV detector (Kratos, Spectroflow 773) as described before [10]. Preparative size-exclusion chromatography was performed on a column (75 x 2.6 cm) of Sephacryl S 500 (Pharmacia) using a Hiload system (Pharmacia). Samples (0.3 g) were eluted with 0.05 M NaOAc pH 5.0 at a flow rate of 2.5 mL/min. Fractions (2.5 mL) were assayed for total neutral sugar [13] and uronic acid [14] content using arabinose and galacturonic acid as standards, respectively. The presence of ferulic acid was monitored spectrophotometrically at 335 nm [5].

Oxidative cross-linking. - Oxidative cross-linking of the extracts with hydrogen peroxide/peroxidase was performed by adding 10 µL of horseradish peroxidase (0.5 mg/mL, Sigma) and 10 µL of hydrogen peroxide (0.5 M) to 1 mL of 0.1 M phosphate buffer (pH = 6.0, 25°C) containing various concentrations of beet pectin. Cross-linking with persulfate was performed by adding 10 µL of an ammonium persulfate solution (1 M) to 1 mL of a solution of pectin with various concentrations (25°C) in distilled water.

Rheological measurements. - The relative viscosity of polysaccharide solutions (0.25 - 4.0 % w/v) in 0.1 M phosphate buffer pH 6.0 before and after oxidative cross-linking was measured using Ubbelohde viscometers.

The formation of a gel network of the purified pools after cross-linking was

investigated by small amplitude shear strain oscillatory testing. A Bohlin VOR rheometer in oscillatory mode was used to monitor the gel structure development as follows. Polysaccharide solutions were cross-linked by the addition of the oxidant in the geometry and were measured immediately. A thin layer of soy oil was added to cover the bob and prevent evaporative losses throughout the measurements. All measurements were carried out at 25°C at a frequency of 1 Hz using a torsion bar of 20 g cm. It was ensured that the strain remained within the linear region of the material.

Results and discussion

Molecular size distribution. - The molecular size distribution and ferulic acid distribution of the extracts, as determined using HPSEC with simultaneous RI and UV detection, are presented in Fig. 2. Three populations could be distinguished in both autoclave extracts. In previous research [10] it was found that the two high molecular

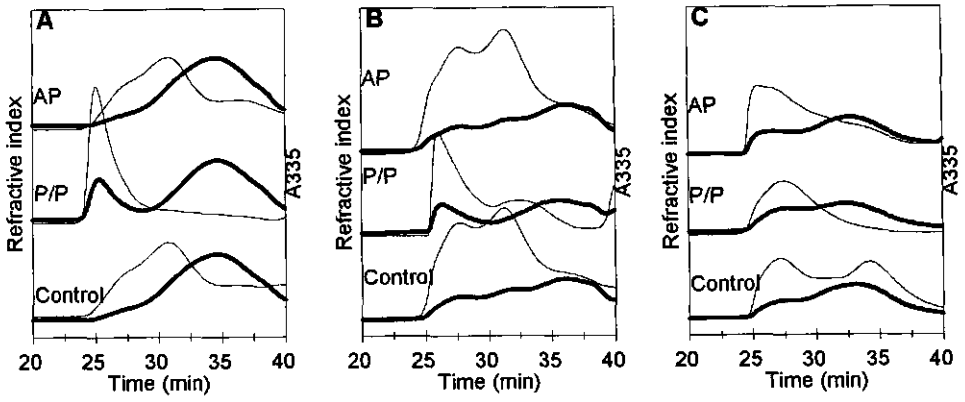

Fig. 2. High-performance size-exclusion chromatography elution patterns of Autoclave 1 (A), Autoclave 2 (B), and HP (C) before and after oxidative cross-linking with hydrogen peroxide/peroxidase (P/P) or ammonium persulfate (AP) for 6 h. Thin line: UV (335 nm), Thick line: RI.

Table 1

Apparent molecular weights (M_w), intrinsic viscosities ($[\eta]_w$), and radii of gyration (Rg_w) of the autoclave extracts (0.5 % w/v) before and after oxidative cross-linking (6 h) with hydrogen peroxide/peroxidase (P/P) or ammonium persulfate (AP).

Sample	M_w (kDa)	$[\eta]_w$ (dL/g)	Rg_w (nm)
Auto 1	77	0.54	9.9
Auto 1 P/P	1730	0.58	25.0
Auto 1 AP	56	0.58	9.4
Auto 2	295	0.43	13.7
Auto 2 P/P	12250	0.54	54.6
Auto 2 AP	245	0.53	14.1
HP	247	0.96	18.6
HP P/P	2692	1.32	45.9
HP AP	752	1.4	28.4

weight populations (26 and 31 min) contain the rhamnogalacturonans, while the low molecular weight population (35 min) contains both homogalacturonans and neutral arabinans. In contrast to the autoclave extracts, the HP extract contained only two populations; a high molecular weight population with a relatively high ferulic acid content and a low molecular weight population with a lower ferulic acid content.

Physico-chemical properties of the extracts before and after cross-linking were determined using HPSEC with detection of refractive index, light-scattering and viscosity (Table 1). The apparent molecular weight of Autoclave 1 was much lower than that of Autoclave 2. This was caused by the presence of a larger amount of the low molecular weight homogalacturonan population in Autoclave 1 (Fig. 2). The intrinsic viscosities of both autoclave extracts were very low, whereas pectins obtained by other extraction methods as described in the literature had higher intrinsic viscosities, ranging from 1.1 to 2.3 dL/g [15, 16]. The current data were confirmed by a series of viscosity measurements using Ubbelohde viscometers (data not shown). The average radius of gyration of Autoclave 2 was higher than that of Autoclave 1. This indicates that the rhamnogalacturonans, which are present in higher quantities in Autoclave 2 have a higher radius of gyration than the homogalacturonans. The apparent molecular weight of the HP extract was 247 kDa. The intrinsic viscosity of HP (0.96 dL/g) was higher than the intrinsic viscosity of the autoclave extracts and was comparable to that of an acid extracted beet pectin as described in the literature [15]. Furthermore, the radius of gyration of HP was much higher than that of Autoclave 2, although the apparent molecular weights were similar. An explanation for this is the high degree of branching in the rhamnogalacturonans, which results in a more compact molecule.

Oxidative coupling of the extracts. - After cross-linking the extracts (0.5 %) with hydrogen peroxide/peroxidase, a high molecular weight population was formed (Fig. 2). This population accounted for approximately 25 % of the total RI response for Autoclave 1 and for approximately 45 % for both Autoclave 2 and HP. A shift of the UV signal towards higher molecular weights was seen for both autoclave extracts. However, a part of the UV signal did not shift during cross-linking. The total UV response decreased after treatment with hydrogen peroxide/peroxidase, which was in agreement with the amount of ferulates recovered (see below). The apparent molecular weight increased 22 fold for Autoclave 1 and 44 fold for Autoclave 2 (Table 1). Only a small shift towards higher molecular size was found for the HP extract. The total UV response decreased significantly. The apparent molecular weight still increased 11 fold. Remarkably, only a small increase in intrinsic viscosities of the autoclave extracts was observed after cross-linking at this concentration. In contrast to the other samples the intrinsic viscosity of HP increased considerably (38 %). The larger molecular size as indicated by the relatively high radius of gyration in comparison with the other extracts probably causes this effect.

Treatment of the autoclave extracts with ammonium persulfate did not result in a shift towards higher molecular weights within the timespan (20 h) of the experiment (Fig. 2A & B). The apparent molecular weights of the extracts slightly decreased (Table 1), whereas only small changes in the intrinsic viscosities were observed. In the HP extract cross-linking did occur and a high molecular weight population was formed within 2 h (Fig. 2C). The apparent molecular weight increased to 752 kDa. Although this increase was lower than after cross-linking with hydrogen peroxide/peroxidase, the intrinsic viscosity increased to a similar value.

Effect of cross-linking on viscosity in time. - In order to obtain information about the speed of cross-linking, the reactions with hydrogen peroxide/peroxidase and ammonium persulfate were followed in time. Treatment of a 0.75 % solution of the extracts with hydrogen peroxide/peroxidase for 2 h indeed resulted in a fast increase in reduced viscosity of all extracts (Fig. 3). Within approximately 10 min the reaction was complete and the reduced viscosity remained stable for the duration of the experiment. The smallest increase in reduced viscosity was found for Autoclave 1 and HP (both to 1.3 times as high as the initial value, respectively), whereas the reduced viscosity of Autoclave 2 increased to 2 times the initial value.

Addition of ammonium persulfate resulted in a rapid decrease in reduced viscosity in Autoclave 1 and 2. Even after 20 h no increase was seen. This is somewhat unexpected since after oxidative cross-linking of the extract at a comparable concentration (0.5 %) a small increase in *intrinsic* viscosity was seen. However, the intrinsic viscosity was determined based on the polymeric material, whereas the reduced viscosity also includes the low molecular weight material. Therefore, a possible

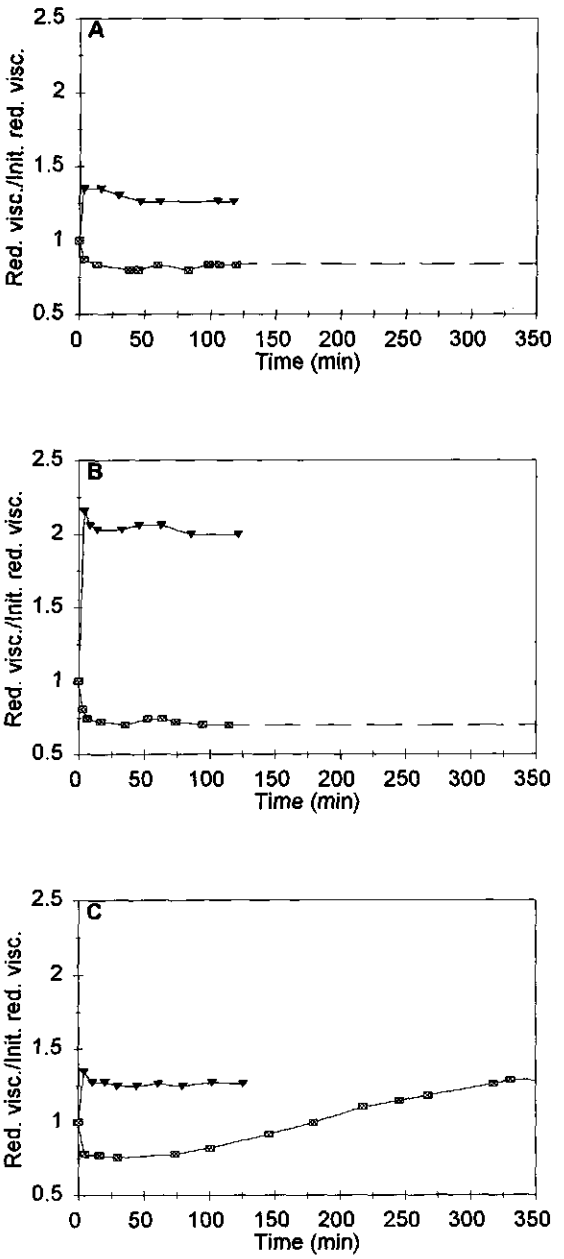

Fig. 3. Changes with time of the ratio of reduced viscosity to initial reduced viscosity for sugar beet pectins (0.75 % w/v, 25°C) after adding hydrogen peroxide/peroxidase (▼) or with ammonium persulfate (■).
A: Autoclave 1, B: Autoclave 2, C: HP.

Table 2

Ferulate composition of the extracts HP and Autoclave 2 (1.0 % w/v) before and after cross-linking with hydrogen peroxide/peroxidase or ammonium persulfate for 6 h (mg/g).

Sample (treatment)	monomer	8-8	8-5	8-O-4	5-5	total diferulates	total ferulates
HP (Control)	7.58	0.24	0.14	0.50	0.12	1.00	8.59
HP (Peroxide)	0.27	0.41	1.90	0.59	0.00	2.90	3.18
HP (Persulfate)	1.92	0.50	2.76	1.21	0.41	4.88	6.79
Auto 2 (Control)*	14.82	0.38	0.30	0.53	0.23	1.43	16.26
Auto 2 (Peroxide)*	3.30	0.29	3.38	2.34	1.02	7.03	10.33
Auto 2 (Persulfate)	12.75	0.46	0.88	0.85	0.27	2.46	15.21

* Data from [18].

explanation might be that both cross-linking and degradation of the polysaccharide occur at the same time. In the HP extract the reduced viscosity initially decreased as well, but this was followed by a slow increase to a maximum of 1.3 times the initial value after 5.5 h. After 40 h, however, the reduced viscosity had decreased again to 0.7 times the initial value (not shown). We showed that cross-links (dehydrodimers) were formed after 6 h. Since also a decrease of the reduced viscosity was observed, this implies that also some degradation of the polysaccharide must have occurred. These findings are in agreement with data found by Thibault and Rombouts [8], who found an increase in reduced viscosity for an extract similar to the HP extract, followed by a slow decrease. Also they found a decrease in reduced viscosity of 25 % for (ferulate free) apple pectin upon this treatment, which indicates that persulfate indeed degrades the pectin molecule [8]. Furthermore, the high arabinose content of the autoclave extracts may be a reason for the poor cross-linking properties, since Guillon and Thibault [7, 17] also found that cross-linking of sugar beet pectins with a high arabinose content using ammonium persulfate did not result in an increased viscosity, unless the arabinose side-chains had been removed.

Ferulic acid dehydrodimer composition. - Table 2 shows the ferulate composition of Autoclave 2 and HP. The structures of the ferulic acid dehydrodimers are given in Fig. 1 of Chapter 4. In Autoclave 2 [18] and HP, 9 and 12 % of the ferulates were present as dehydrodimers, respectively. This is considerably lower than the value reported for cell wall material from sugar beet (22 %) [19]. This might be explained by assuming that material with a relatively low amount of ferulate cross-links is easier released during autoclaving than material with a high amount of cross-links. The content of ferulate dimers in relation to the total amount of ferulates is also higher in other species e.g. (suspension cultured) corn, cocksfoot, and switchgrass, which range from 15 to 71 % [12, 20]. 8-O-4 dehydrodimers were predominantly present in both extracts besides the 8-8, the 8-5 and the 5-5 dehydrodimers. 4-O-5 dehydrodimers were not

detected. The predominance of the 8-O-4 coupled dehydrodimer over other types was in contrast with results found for most grasses in which the 5-5 dehydrodimer is prevailing [12].

Cross-linking of Autoclave 2 and HP using hydrogen peroxide/peroxidase resulted in a large decrease of the ferulic acid content and in an increase of the amount of ferulic acid dimers present (Table 2). A loss of ~60 % of total ferulates was found for the HP extract, indicating that other oxidative reactions might have occurred or that some ferulates had been lost during analysis. In the Auto 2 extract 36 % of the ferulates could not be recovered either. The largest increase was found for the 8-5 and 8-O-4 dimers for both extracts.

Ammonium persulfate cross-linking led to a larger decrease of ferulic acid in HP than in Autoclave 2. This was accompanied by a larger increase in dehydrodimers in the HP extract. The loss of total ferulates was smaller than occurred with the reactions with hydrogen peroxide/peroxidase, namely 21 % for HP and 6 % for Autoclave 2. Using ammonium persulfate the largest increase was also seen for the 8-O-4 and 8-5 dehydrodimers, whereas the 8-8 and 5-5 dehydrodimers increased only to a limited extent. It can be concluded that the 8-8 and 5-5 dimers have limited importance in the cross-linking reaction, either with hydrogen peroxide or with ammonium persulfate, as opposed to what has been suggested in previous studies on sugar beet pectins [21] and arabinoxylans [22].

Thibault et al. [21] showed that the reaction of ferulates with persulfate is a radical reaction and that the aromatic nuclei were not modified, but that double bonds in the propenoic moiety were involved in the reaction. Grabber et al. [20] showed that oxidative cross-linking of ferulates in maize with hydrogen peroxide/peroxidase indeed is a radical reaction, but that several active sites are present on the ferulic acid radicals and that several reaction products are formed. These active sites are assumed to be on C-5, C-8, and O-4 [20]. The reaction products we found were in agreement with these observations. The abundance of the 8-O-4 and 8-5 dimers is probably caused by the spatial arrangement of the ferulic acid groups on the pectin.

Since the reactions with hydrogen peroxide/peroxidase and ammonium persulfate resulted in the formation of the same types of dehydrodimers in similar ratios for both HP and Autoclave 2, it is assumed that the reaction mechanism was similar, although a relatively high amount of ferulates was not analyzed after cross-linking. In the literature several inhibition studies of the hydrogen peroxide/peroxidase system are presented. Vinkx et al. stated that the gelation reaction of wheat pentosans is inhibited by ferulic acid and vanillic acid but not by fumaric acid [23]. From this they concluded that the aromatic ring is involved in cross-linking. Hoseney and Faubion on the other hand found that fumaric acid and not vanillic acid inhibited the cross-linking reaction [24]. This may be due to the experimental setup, since it is very well possible that the

inhibiting agents just remove radicals or react with itself instead of reacting with ferulic acid attached to a polymer. From our results it was shown that both the propenoic moiety and the aromatic ring are involved in the cross-linking.

An increase of the reduced and intrinsic viscosity was observed together with the formation of dehydrodimers the samples, which were analyzed for their dehydrodimer composition, except for Autoclave 2 after treatment with ammonium persulfate, which showed a slight decrease in reduced viscosity. No explanation was found for the fact that no increase in viscosity occurred in this sample. Possibly, it is related to the location of the ferulic acid, which is mainly linked to galactose in the HP extract and to arabinose in the extract obtained by autoclaving. Furthermore, it might be caused by a difference in the initial release of free radicals, since the reaction with hydrogen peroxide/peroxidase occurs much more rapidly than the reaction with ammonium persulfate. Also, the high amount of arabinose may play a role, as was also suggested by Guillon and Thibault [7, 17].

Since the treatment of the autoclave extracts with hydrogen peroxide/peroxidase resulted in the largest increase in viscosity and in gel formation, further experiments were performed with hydrogen peroxide/peroxidase.

Preparative size-exclusion chromatography. - The Autoclave 2 extract cross-linked by hydrogen peroxide/peroxidase was applied onto a Sephacryl S 500 size-exclusion column (Fig. 4). Two populations could be distinguished: a high molecular weight population, which contained most of the ferulic acid and was collected in pool I, and a low molecular weight population which was collected in pool II. The sugar compositions of the pools are given in Table 3. Pool I mainly consisted of arabinose and galactose, besides low amounts of galacturonic acid and rhamnose. Pool II contained much galacturonic acid, but a considerable amount of arabinose was also present. The ferulic acid content of this pool was low.

These findings revealed that for the autoclave extracts only arabinan/galactan side-chains of the rhamnogalacturonans and possibly (free) arabinans participate in cross-linking and that very little homogalacturonan is linked to these rhamnogalacturonans. The composition of these rhamnogalacturonans is very similar to the "hairy fragments" from sugar beet pectin as isolated by Guillon and Thibault [3], except for the arabinose content, which was considerably lower than in the rhamnogalacturonans present in Autoclave 1 and 2.

For an extract similar to HP, Thibault and Rombouts [8] showed that the high molecular weight population formed after cross-linking with persulfate contained neutral sugars and galacturonic acid in a ratio of approximately 1 : 2. The high proportion of galacturonic acid suggests that the rhamnogalacturonans in their acid extracted pectin are still linked to the homogalacturonans.

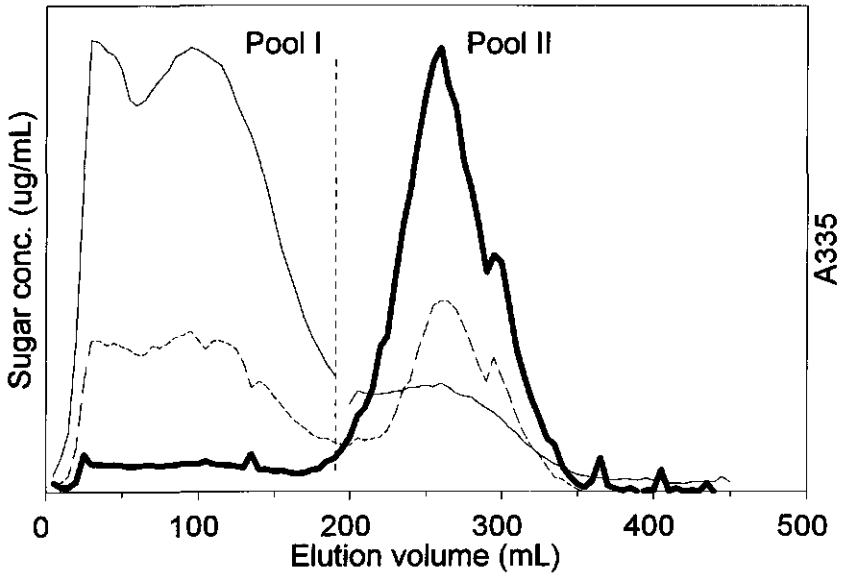

Fig. 4. Size-exclusion chromatography (S 500) of Autoclave 2 (0.5 % w/v) after cross-linking with hydrogen peroxide/peroxidase. Thick line: Galacturonic acid, thin line:UV (335 nm), dashed line: neutral sugars.

Table 3

Sugar composition of the pools obtained by preparative size-exclusion chromatography (S 500) of Autoclave 2 (0.5 % w/v) after oxidative cross-linking with hydrogen peroxide/ peroxidase.

	Pool I	Pool II
Rha[a]	2.7	1.0
Ara	76.2	26.9
Xyl	0.5	0.4
Man	0.7	1.9
Gal	10.8	3.7
Glc	2.4	1.7
UA	7.0	64.5
Relative weight[b]	36.7	63.3

[a]:Expressed as molar percentage. [b]:Expressed as weight percentage of the polysaccharides recovered.

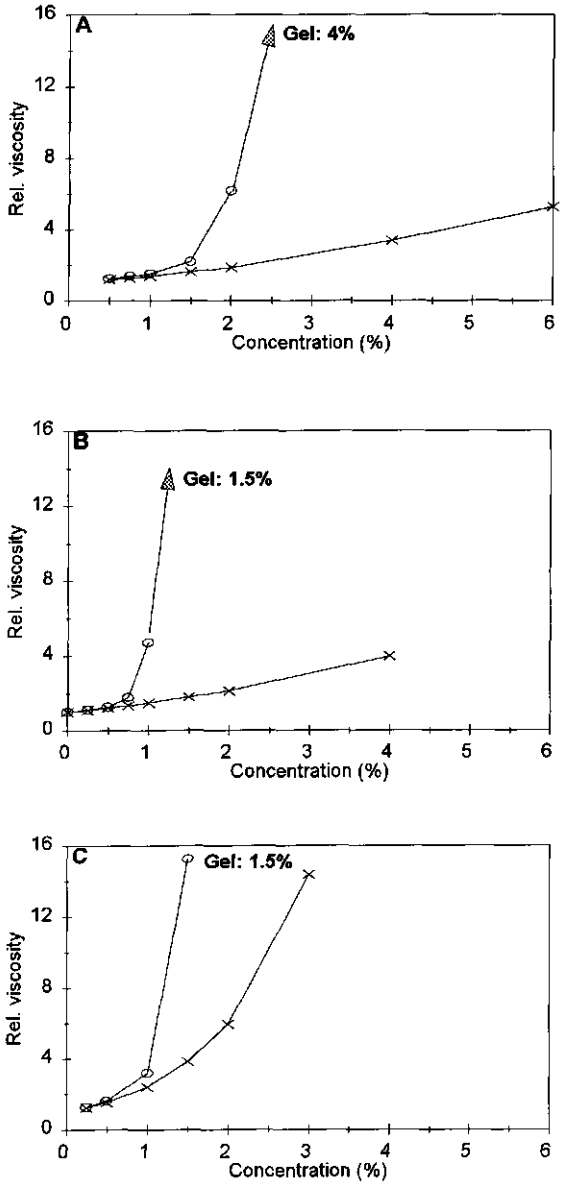

Fig. 5. Increase in relative viscosity with increasing concentration of sugar beet pectins before (x) and after cross-linking (○) with hydrogen peroxide/peroxidase (15 min, 25°C). A: Autoclave 1, B: Autoclave 2, C: HP.

Influence of the concentration of the extracts on the cross-linking reaction. - The influence of the concentration of the extracts on the relative viscosity of the extracts before and after cross-linking with hydrogen peroxide/peroxidase is presented in Fig. 5. At higher concentrations some overestimation of the relative viscosities may have occurred, due to non Newtonian behaviour in the Ubbelohde viscometers [25]. Of the controls the largest increase in relative viscosity with increasing concentration was found for HP, due to its higher intrinsic viscosity and therefore its lower coil overlap concentration. The coil overlap concentration was not determined from this experiment due to lack of measuring points, since insufficient material was available, but could be estimated from the intrinsic viscosity as determined by high-performance size-exclusion chromatography. For Autoclave 1 and 2 the estimated coil overlap concentrations were relatively high, (1.9 and 2.3 %, respectively), whereas the value for HP was lower (1 %).

Upon oxidative cross-linking with hydrogen peroxide/peroxidase a clear increase in the relative viscosity compared with the control was observed at a concentration of 1.5 % for Autoclave 1, at 0.75 % for Autoclave 2 and at 1 % for HP. This might be caused by a shift from intramolecular cross-linking to intermolecular cross-linking. At concentrations higher than the coil overlap concentration most of the cross-links formed will be intermolecular. Gels were formed at concentrations between 1.0 % and 1.5 % for Autoclave 2 and the HP extract. Autoclave 2 seems to form a gel at a somewhat lower concentration than HP. Autoclave 1 formed a gel at a concentration between 2 % and 4 %. Therefore, it can be concluded that even though HP has the highest intrinsic viscosity, Autoclave 2 showed the largest increase in relative viscosity and formed a gel at the lowest concentration, which is probably caused by the high ferulic acid content of Autoclave 2.

Gelation. - The formation of a gel network of the sugar beet pectins was investigated by small amplitude shear strain oscillatory testing. Fig. 6 shows the development of the storage modulus (G'), which is indicative for the amount of elastically effective cross-links formed and for the stiffness of the gel, at various concentrations of the extracts in time. Table 4 summarizes the rheological parameters of the networks formed after hydrogen peroxide/peroxidase treatment for 500 s. G' rapidly increased in time for all concentrations (Fig. 6). The G' values exceeded the loss modulus (G", loss modulus; indicative for the relaxation of cross-links in the gel) for all concentrations of the pectins (Table 4). The G' and also the initial speed of gel formation increased with increasing concentration. A comparison of all samples could only be made at a concentration of 3.0 %. The highest values for G' at this concentration were found for autoclave 2 and to a lesser extent for HP. A much lower value was found Autoclave 1.

Izydorczyk and Biliaderis [26] found a positive correlation between G' and the intrinsic viscosity of cross-linked arabinoxylans. However, comparing Autoclave 2 and

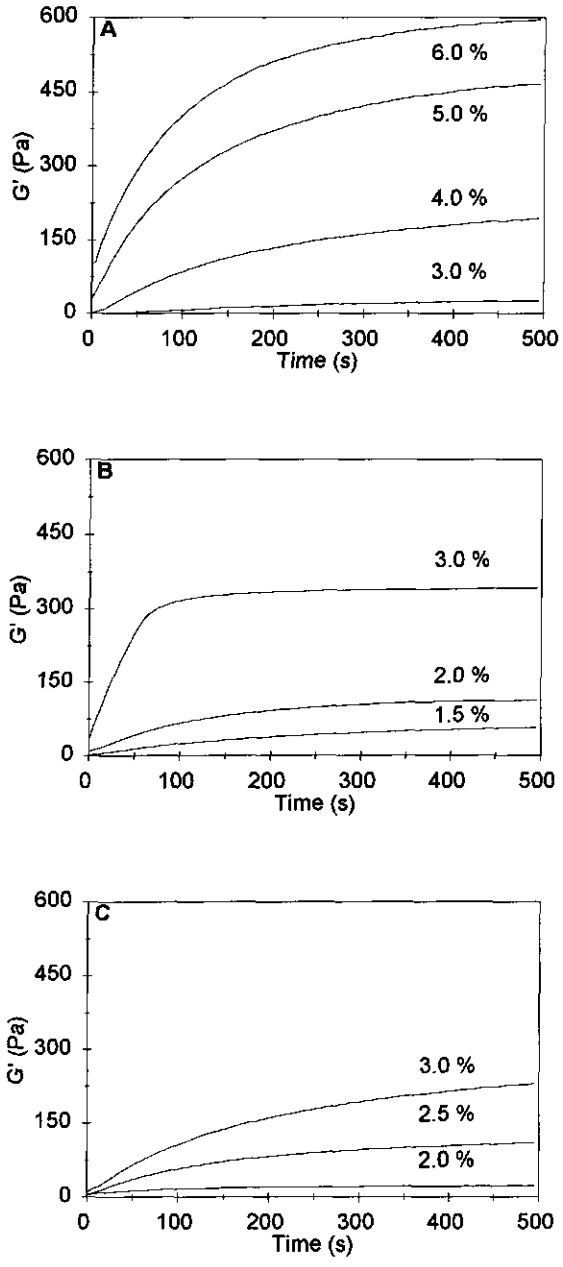

Fig. 6. Storage (G') moduli vs time for sugar beet pectins at various concentrations treated with hydrogen peroxide/peroxidase (25°C). A: Autoclave 1, B: Autoclave 2, C: HP.

Table 4

Storage (G') and loss (G") moduli, and tan δ for gels from sugar beet pectin after oxidative cross-linking with hydrogen peroxide/peroxidase after 500s.

Sample	Conc. (%)	G' (Pa)	G" (Pa)	tan δ (-)
Autoclave 1	3.0	27	1.5	0.055
	4.0	195	8.4	0.043
	5.0	466	11.9	0.026
	6.0	596	15.5	0.026
Autoclave 2	1.5	58	0.8	0.014
	2.0	114	0.4	0.003
	3.0	341	2.2	0.006
HP	2.0	23	0.6	0.025
	2.5	110	0.2	0.001
	3.0	230	0.3	0.001

HP, we found a higher G' value for Autoclave 2, whereas the HP extract had a much higher intrinsic viscosity. All arabinoxylans described by Izydorcyk and Biliaderis [26] had similar degrees of branching and similar ferulic acid contents, so that the amount of cross-links per molecule and the intrinsic viscosity were only dependent on the molecular weight. Autoclave 2, however, had a higher degree of branching than HP, leading to a lower intrinsic viscosity. The ferulic acid content, and therefore the amount of cross-links possible per molecule, was also higher in Autoclave 2 than in HP.

Whereas the storage modulus (G') can be related to the amount of cross-links formed and for the stiffness of the gel, tan δ (= G"/G') is also a good indicator for the nature of the gel. A low value for tan δ (< 0.1) is indicative for an elastic system, whereas high values (> 1) imply a more liquid-like character of the network [27]. The very low values of tan δ for HP and Autoclave 2 shows the formation of elastic networks, especially at higher concentrations, whereas the nature of the Autoclave 1 network is more liquid-like (see Table 4). The values found for tan δ were relatively low, and even lower when relatively high concentrations were used. The low values of tan δ, indicative for the absence of clear relaxation of cross-links, may be caused by the covalent nature of the cross-links, which do not easily allow relaxation.

Concluding remarks

Despite large structural differences, it was shown that pectins obtained by acid extraction and autoclaving could form a gel after oxidative cross-linking using polysaccharide concentrations as low as 1.5 %. A rhamnogalacturonan gel with only 7 % of galacturonic acid in the cross-linked population was formed with the Autoclave 2 extract, whereas it was shown that a gel with more than 60 % of galacturonic acid in the cross-linked fraction was formed with an extract similar to HP [8]. The properties of the gels as indicated by tan δ were in the similar range for both autoclave 2 and HP.

It was found that hydrogen peroxide/peroxidase is a better cross-linking agent than ammonium persulfate for the pectins described, since addition of the latter led to degradation of the pectin to some extent. Nevertheless, the reduced viscosity increased for HP after addition of persulfate, followed by a gradual decrease upon standing. The reduced viscosity of the autoclave extracts did not increase after addition of persulfate.

Although there was a difference in reactivity with ammonium persulfate and hydrogen peroxide/peroxidase for HP and the autoclave extracts, the ratio of the ferulic acid dehydrodimers formed was similar for both oxidants. Only the total amount of dehydrodimers was different.

The low intrinsic viscosity and good solubility of the extracts obtained by autoclaving will facilitate the handling (e.g. pumping) of the extracts in industrial applications. This makes them an interesting substrate for *in situ* gel formation. Future research will focus on the purification and oxidative cross-linking of the rhamnogalacturonans and arabinans from the Autoclave 2 extract.

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CHAPTER 6

Oxidative cross-linking of purified arabinans and rhamnogalacturonans from sugar beet pulp

Alexander Oosterveld, Irene E. Pol, Gerrit Beldman, and Alphons G.J. Voragen

Abstract

Rhamnogalacturonans and arabinans were purified from an autoclave extract of sugar beet pulp by size-exclusion and anion-exchange chromatography. Treatment with polygalacturonase and pectin methyl esterase was used to improve chromatographic separation of the rhamnogalacturonans from the homogalacturonans. Four populations were obtained: two rhamnogalacturonan populations with a high arabinose and ferulic acid content and with apparent molecular weights of 930 and 130 kDa, respectively, a feruloylated arabinan population and a (digested) homogalacturonan population with apparent molecular weights of 18 and 6 kDa, respectively.

Cross-linking of the high molecular weight rhamnogalacturonans with hydrogen peroxide and peroxidase gave an increase in viscosity and led to gel formation at concentrations < 1.0 %. The second rhamnogalacturonan population formed gels at concentrations as low as 0.75 %. Cross-linking of the arabinan resulted in gel formation at a concentration of 4.0 %. The properties of the gels are discussed in terms of the storage modulus (G') and tan δ.

Introduction

Sugar beet pectins contain high amounts of ferulic acid, in contrast to pectins obtained from other sources, e.g. citrus and apples [1]. Treatment of sugar beet pectin with ammonium persulfate or hydrogen peroxide/peroxidase improves the viscosity and gelling properties of beet pectin; an effect apparently due to oxidative cross-linking of the ferulate monomers into dehydrodimers [2-4]. Ferulic acid in beet pectin is located in the rhamnogalacturonans and is attached to the O-2 position of (1→5)-linked arabinose residues and to the O-6 positions of galactose residues in (1→4)-linked galactans [5-9]. The traditional extraction method for pectin, using acid treatment, degrades the arabinan side-chains of the rhamnogalacturonans [10], and therefore leads to a loss of ferulic acid.

Recently the extraction of pectic polysaccharides from beet pulp by autoclaving was described. In the extract high molecular weight rhamnogalacturonans to which ferulic acid is still attached were present. Besides rhamnogalacturonans, a separate population containing homogalacturonans and arabinans was found in this extract [11, 12]. Oxidative cross-linking of the autoclave extracts with hydrogen peroxide/peroxidase gives an increase in viscosity and eventually leads to the formation of a gel at a concentration as low as 1.5 % [4]. It was shown that only rhamnogalacturonans and possibly low molecular weight arabinans were involved in cross-linking.

The current study deals with the isolation and characterization of the rhamnogalacturonan and arabinan populations present in the autoclave extract, in order to study the effect of oxidative cross-linking with hydrogen peroxide and peroxidase of these individual populations, as determined by their viscosity increase and by the characteristics of the gels formed.

Experimental

Materials.- Wet beet pulp (8.9 % dry weight) was obtained from CSM Suiker bv (Breda, the Netherlands). Autoclave extraction of sugar beet pulp was performed as described previously [11]. Two subsequent autoclave treatments were performed. The extract obtained after the second autoclave treatment (Autoclave 2) was used in this study.

Analytical methods. - The uronic acid content of the extract was determined by the automated m-hydroxy biphenyl assay [13]. The neutral sugar composition was determined after hydrolysis with 2 M trifluoroacetic acid (1 h, 121°C), conversion to alditol acetates and analysis by gas chromatography as described previously [11]. Feruloyl groups were determined spectrophotometrically at 375 nm in freshly prepared

pectin solutions adjusted to pH 10 with a 0.1 M NaOH solution. A molar extinction coefficient of 31,600 was used [1]. The protein contents were determined according to the procedure of Sedmak and Grossberg [14]. BSA was used as a standard.

Enzyme treatment. - The extract Autoclave 2 was treated with a combination of purified endo-polygalacturonase (PG) [15] and pectin methyl esterase (PE) [16] to depolymerize homogalacturonan and facilitate its separation from rhamnogalacturonans by size-exclusion chromatography. The extracts (5 mg/mL) were dissolved in 0.05 M sodium acetate buffer pH 5.0. The enzymes were added to obtain a final concentration of 1 μg protein/mL. Incubations were carried out at 30°C for 20 h. The reactions were stopped by heating at 100°C for 5 min.

Chromatography. - High-performance size-exclusion chromatography (HPSEC) was performed on three Bio-Gel TSK columns in series (60XL-40XL-30XL) as described [12] using a combined RI detector and viscometer (Viscotek, model 250), a Right Angle Laser Light-Scattering detector (RALLS, Viscotek, LD 600) and a UV detector (Kratos, Spectroflow 773). Apparent molecular weights and intrinsic viscosities were calculated using the light-scattering module of the Trisec software (Viscotek).

Preparative size-exclusion chromatography was performed on two columns (50 x 10 cm) of Sephacryl S 500 (Pharmacia) in series using a Biopilot system (Pharmacia). Sample (3 g) was eluted with 0.05 M NaOAc pH 5.0 at a flow rate of 37 mL/min. The separation was repeated 4 times and corresponding populations were pooled after analysis of the fractions (135 mL) for total neutral sugar [17] and uronic acid [13] contents using arabinose and galacturonic acid as standards, respectively. A correction was made for the response of uronic acids in the neutral sugar test. The presence of ferulic acid was monitored spectrophotometrically at 335 nm [8]. Pooled fractions were dialyzed and freeze-dried.

Preparative anion-exchange chromatography was performed on a column (15 x 10 cm) of Source Q (Pharmacia) using a Biopilot system (Pharmacia). The sample (2.5 g) was applied onto the column at a flow rate of 15 mL/min (0.005 M NaOAc, pH 5.0). After 105 min the flow rate was increased to 80 mL/min and a linear gradient to 2 M NaOAc (pH 5.0) in 90 min was used to elute the polysaccharides. The elution with 2 M NaOAc was continued for another 15 minutes. The columns were washed with 0.5 M NaOH, followed by regeneration with 2 M NaOAc (pH 5.0) and equilibration with 0.005 M NaOAc (pH 5.0). The separation was repeated 4 times and corresponding fractions, identified by analysis of the fractions for total neutral sugar [17] and uronic acid [13], were pooled. Pooled fractions were dialyzed and freeze-dried.

Oxidative cross-linking. - Oxidative cross-linking of the purified polysaccharides with hydrogen peroxide/peroxidase was performed by adding 10 μL of horseradish peroxidase (0.5 mg/mL, Sigma) and 10 μL of hydrogen peroxide (0.5 M) to 1 mL of a solution of 0.25-4.0 % of beet pectin in 0.1 M phosphate buffer (pH = 6.0, 25°C).

Rheological measurements. - Relative viscosities of polysaccharide solutions (0.25 - 4.0 % w/v) in 0.1 M phosphate buffer pH 6.0 were determined using Ubbelohde viscometers before and after oxidative cross-linking.

The formation of a gel network of the purified pools after cross-linking was investigated by small amplitude shear strain oscillatory testing. A VOR rheometer (Bohlin) in oscillatory mode was used to monitor the gel structure development as follows. Polysaccharide solutions (various concentrations) were cross-linked. Immediately after addition of the oxidant, the samples were placed in the geometry. A thin layer of soy oil was added to cover the bob and prevent evaporative losses throughout the measurements. All measurements were carried out at 25°C at a frequency of 1 Hz using a torsion bar of 20 g cm. Effort was made to ensure that the strain remained within the linear region of the material.

Results and discussion

To study the cross-linking process of the rhamnogalacturonans and arabinans, individual populations of Autoclave 2 were purified by preparative size-exclusion chromatography and by preparative anion-exchange chromatography as described below.

Enzyme treatment. - In order to facilitate the isolation of the rhamnogalacturonans, the Autoclave 2 extract was treated with a combination of the enzymes endo-

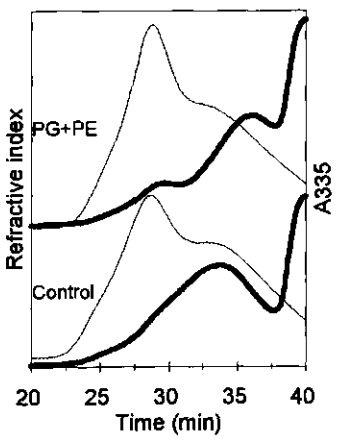

Fig. 1. High-performance size-exclusion chromatography of Autoclave 2 before and after treatment with PG+PE. Thick line: refractive index; thin line A_{335}.

polygalacturonase (PG) and pectin methyl esterase (PE). The elution patterns of the extract obtained by high-performance size-exclusion chromatography, before and after this enzyme treatment, are shown in Fig. 1. In the control three polysaccharide populations could be distinguished (23-27 min, 27-32 min, and 32-38 min), using UV and RI detection. The peak at 40 min was caused by the buffer. It has been shown that the two high molecular weight populations (23-27 min and 27-32 min) consisted of rhamnogalacturonans and that the low molecular weight population (32-38 min) mainly consisted of homogalacturonan, but also contained arabinans [12]. Treatment with PG and PE indeed resulted in a shift of the peak fraction of the low molecular weight population (from 33 to 37 min on RI), which was previously identified as a homogalacturonan [12]. The UV signal at 335 nm, which indicates the presence of ferulic acid which is attached to rhamnogalacturonans and arabinans [5-9], did not change significantly. It can be concluded that these polysaccharides were not degraded by PG+PE.

Preparative size-exclusion chromatography. - After treatment with PG+PE the extract Autoclave 2 was applied on a preparative S 500 size-exclusion column (Fig. 2). As expected from HPSEC three populations could be distinguished, which were pooled as indicated. The sugar content was relatively low for pool I (~60 %) in comparison with

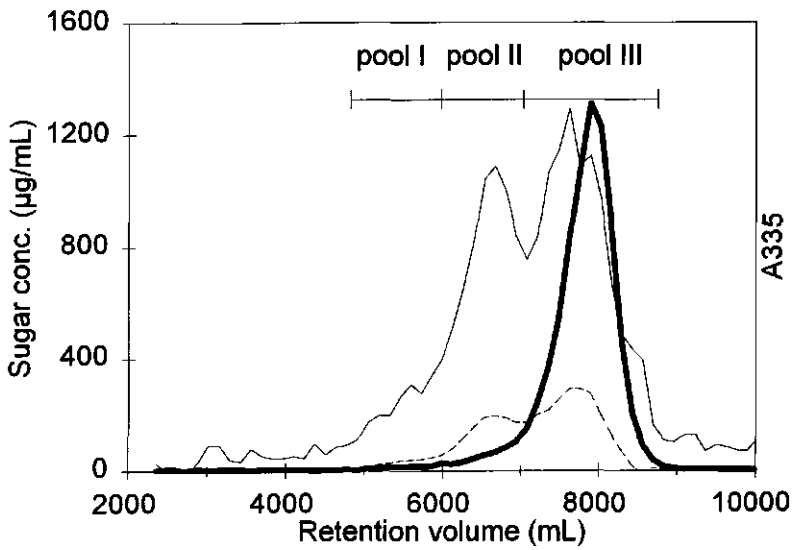

Fig. 2. Preparative size-exclusion chromatography (S 500) of Autoclave 2 after treatment with PG+PE. Thick line: uronic acid; dashed line: neutral sugars; thin line: A_{335}.

Table 1

Composition of Autoclave 2 and of the pools obtained by S 500 size-exclusion chromatography and by anion-exchange chromatography (Source Q).

	Autoclave 2[e]	S 500			Source Q	
		Pool I	Pool II	Pool III	Pool IIIa	Pool IIIb
Rha[a]	3.6	5.5	4.3	1.2	0	1.4
Ara[a]	68.9	68.9	61.1	27.6	82.6	8.8
Xyl[a]	0.0	0.7	0.3	0.4	4.7	0.6
Man[a]	0.4	0.5	0.6	2.8	3.7	1.8
Gal[a]	6.6	12.5	9.7	3.7	1.4	3.3
Glc[a]	0.9	0.9	0.5	1.4	4.7	0.6
Ua[a]	27.7	10.9	23.4	62.9	2.8	83.5
DA[b]	52	nd	nd	nd	nd	nd
DM[b]	60	nd	nd	nd	nd	nd
DF[c]	1.3	1.0	1.0	0.7	0.8	1.1
Sugar content[d]	88.3	60.1	71.5	73.1	95.3	57.9
Protein	0.4	0.4	0.2	0.1	0.2	0.1

[a] Expressed as mol%. [b] Expressed as moles acetyl/methyl per 100 moles of galacturonic acid. [c] Expressed as moles ferulic acid per 100 moles of galactose + arabinose. [d] weight percentage [e] From [11]. nd Not determined.

the other pools (~70 %), probably because not all buffer salts were removed during dialysis. The sugar compositions of the pools were comparable to previous results obtained for the extract before treatment with PG+PE [12] and are shown in Table 1. All the degrees of feruloylation (DF; moles of feruloyl groups/100 moles arabinose + galactose residues) were somewhat lower than found in the original extract. No explanation could be found for this observation. Pool I (eluting at around 5500 mL) mainly consisted of arabinose. Also some galactose, galacturonic acid and rhamnose were present, in a ratio of 2.4 : 2 : 1, respectively. The DF and the arabinose content of this pool were relatively high, and therefore also the absolute ferulic acid content. Pool II (eluting at 6600 mL) had a sugar composition comparable to pool I, although the galacturonic acid content was higher in this pool. This was probably due to some contamination with a separate homogalacturonan population of the same molecular size. From the sugar composition and the SEC elution pattern, it was concluded that both pool I and II consisted of rhamnogalacturonans with similar compositions, but with different apparent molecular weights. The composition of these rhamnogalacturonans is comparable to the high molecular weight fraction which was formed after cross-linking of the original extract Autoclave 2 with hydrogen peroxide/peroxidase [4]. Furthermore, the sugar composition of the rhamnogalacturonans was comparable to that of RG-I from sycamore cells [18] except for the arabinose content, which was higher. Pool III predominantly consisted of galacturonic acid. Nevertheless, this pool still contained a

substantial amount of arabinose (27.6 mol%). Previous studies suggested that this might be due to the presence of low molecular weight arabinans [12]. The DF of pool III was comparable to those of pool I and II, while the absolute ferulic acid content was relatively low.

The ferulic acid groups were spread relatively evenly over the populations, since the DF of the various populations did not differ much.

Preparative anion-exchange chromatography. - The arabinans, which elute together with the homogalacturonans were separated from the homogalacturonans by anion-exchange chromatography (Fig. 3). Two populations were found, which were pooled in pool IIIa and pool IIIb. Pool IIIa contained 70 % of the neutral sugars present in pool III (Table 1) and represented 15 % of the polysaccharides. Arabinose was the predominant neutral sugar in pool IIIa. Furthermore, relatively high amounts of xylose, mannose and glucose were present in this pool, whereas the uronic acid and galactose contents were very low. Pool IIIb accounted for 85 % of the polysaccharides of pool III and mainly consisted of galacturonic acid and low amounts of arabinose, galactose, and rhamnose.

High-performance size-exclusion chromatography. - The molecular size distributions of the purified pools from Autoclave 2 as determined by HPSEC are shown in Fig. 4. The peak eluting at >37.5 min was caused by the buffer, and was not included

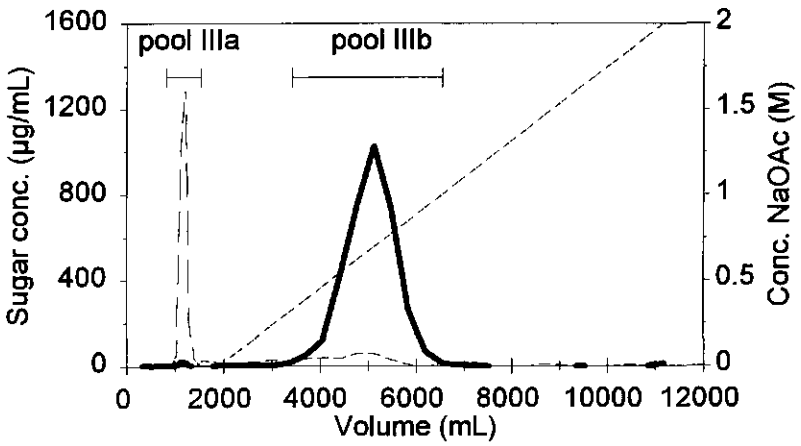

Fig. 3. Preparative anion-exchange chromatography (Source Q) of pool III obtained by S 500 size-exclusion chromatography. Thick line: uronic acid; dashed line: neutral sugars; dotted line: concentration NaOAc.

in the calculation of the apparent molecular weights. The ratio UV : RI was similar for both pool I (27 min), and II (30 min). After separation on Source Q anion-exchange chromatography almost all A_{335} response which was originaly present in pool III was found in the arabinan pool IIIa.

Table 2 shows some physico-chemical characteristics of the pools e.g. the apparent *molecular weight and intrinsic viscosity. The apparent molecular weight of pool* I was somewhat lower than we found for the pool I of the Autoclave 2 extract before treatment with PG+PE as determined by analytical scale separations of the whole extract [12]. This might be a result of the enzyme treatment or the pooling procedure of the population. An apparent molecular weight of 930 kDa for pool I implies an average

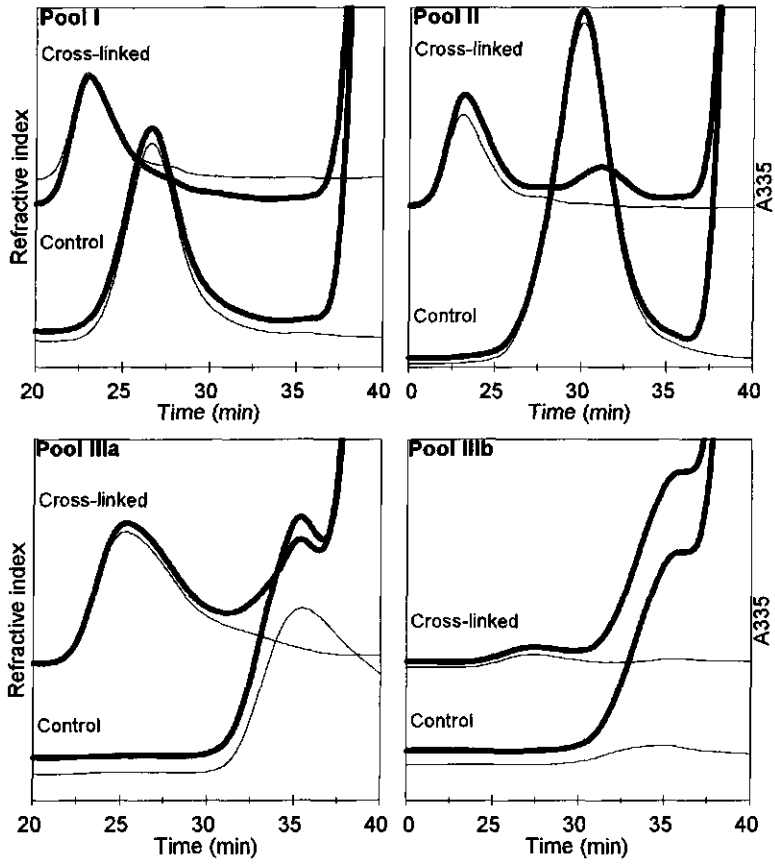

Fig. 4. High-performance size-exclusion chromatography of the pools purified from Autoclave 2 (0.5 % w/v) before and after oxidative cross-linking with hydrogen peroxide/peroxidase. Thick line: refractive index; thin line: A_{335}.

Table 2

Apparent molecular weight (M_w) and intrinsic viscosity ($[\eta]_w$) of the S 500 and Source Q pools (0.5 % w/v) before and after cross-linking with hydrogen peroxide/peroxidase.

Sample	M_w (kDa)		$[\eta]_w$ (dL/g)	
	Control	Cross-linked	Control	Cross-linked
pool I	930	7300	0.66	0.99
pool II	130	8800	0.33	0.56
pool IIIa	18	1900	0.19	0.24
pool IIIb	6	17	0.06	0.08

degree of polymerization (DP) of ~6000. Since the pool contains 80 mol% of arabinose and galactose, and 1 of every 100 arabinose and galactose residues carry a ferulic acid group, it can be calculated that on average every rhamnogalacturonan molecule carries 48 ferulic acid residues. The apparent molecular weight of pool II was comparable to that of a similar pool present in the extract before treatment with PG+PE [12]. It can be calculated that its average DP was approximately 900, whereas every molecule carries on average 6 feruloyl groups. The intrinsic viscosity was low for pool I and II, especially in relation to their high apparent molecular weights. This is probably caused by the high degree of branching of these polysaccharides. Pool IIIa had an apparent molecular weight of 18 kDa, which shows that it consists of approximately 120 arabinose residues. The apparent molecular weight and intrinsic viscosity of pool IIIb were much lower than of the homogalacturonan population present in the original extract [12], due to the degradation of the homogalacturonans with PG+PE. For this pool an average DP of 40 can be calculated.

High-performance size-exclusion chromatography after cross-linking. - After cross-linking the pools at a concentration of 0.5 % with hydrogen peroxide/peroxidase, a shift towards higher molecular weight for part of the material was seen as determined by HPSEC (Fig. 4). The peak at 27 min in pool I shifted entirely to the void. A large part of the material in pool II shifted towards 23 min. Approximately 25 % of this material did not shift to a higher molecular weight. This part did not contain ferulic acid and consisted probably of homogalacturonans. Most of the arabinan material, which was collected in pool IIIa, shifted towards higher molecular weight (25 min). The homogalacturonan population, which was collected in pool IIIb, contained a minute amount of material, which was able to cross-link, indicating the presence of some low molecular weight rhamnogalacturonans. It could be shown that for all pools the ferulic acid containing material shifted quantitatively towards higher molecular weight. So, it can be concluded that almost all ferulic acid containing material participates in the

cross-linking reaction. However, previously it was shown for the whole extract that not all ferulic acid participates in the cross-linking reaction [3, 4].

The physico-chemical properties of the pools after cross-linking at a concentration of 0.5 % with hydrogen peroxide/peroxidase are also presented in Table 2. The largest relative increase in apparent molecular weight was found for pool IIIa, which showed more than a 100 fold increase. The apparent molecular weight of pool II after cross-linking was higher than that of pool I, which confirms the results of the gelation experiments, in which it was found that pool II formed a gel at the lowest concentration (see further; Fig. 5). The largest relative increase in intrinsic viscosity was seen for pool I and II and a much smaller relative increase in intrinsic viscosity was seen for pool IIIa and pool IIIb. These results show that the relative increase in intrinsic viscosity is higher for larger molecules.

Effect of concentration of the pools on viscosity after cross-linking. - The pools I, II and IIIa were cross-linked at various concentrations to determine the effect on relative viscosity and the concentration at which gel formation occurs (Fig. 5). Pool IIIb was omitted because of its low ferulic acid content. Hydrogen peroxide and peroxidase were used as cross-linking agents, since previous results showed that this combination was more effective than ammonium persulfate [4]. Non cross-linked samples were used as controls. For these controls the largest increase in relative viscosity with increasing concentration was seen for pool I and the smallest increase for pool IIIa. This effect can be related to the coil overlap concentration, which can be estimated from the intrinsic viscosity. Pool I had the lowest estimated coil overlap concentration (1.5 %), whereas those of pool II and pool IIIa were much higher (3.0 and 5.3 %).

An increase in relative viscosity after oxidative cross-linking was seen in all pools. Pool II formed a gel at the lowest concentration (0.75 %), although its apparent molecular weight was much lower than from pool I, which formed a gel at 1.0 %. This may be partly due to the lower polysaccharide content of pool I in comparison with pool II. Surprisingly, considering its low apparent molecular weight and the fact that on average only one feruloyl group is present per arabinan molecule, pool IIIa was able to form a gel at a concentration of 4.0 %. These are the first results that show that rhamnogalacturonans and arabinans are able to form gels by cross-linking reactions. Gel formation of non cross-linked arabinans, based on physical entanglements, was reported previously by McCleary et al. [19, 20]. The arabinans in that study were linearized with arabinofuranosidase and had an intrinsic viscosity of 0.28 dL/g, which is comparable to the value we found (0.20 dL/g). Concentrations from 10 to 20 % w/v were needed to form gels from these linear arabinans, whereas we obtained a gel at a concentration between 1.5 % and 4 %. Furthermore, a setting time of >15 h found for

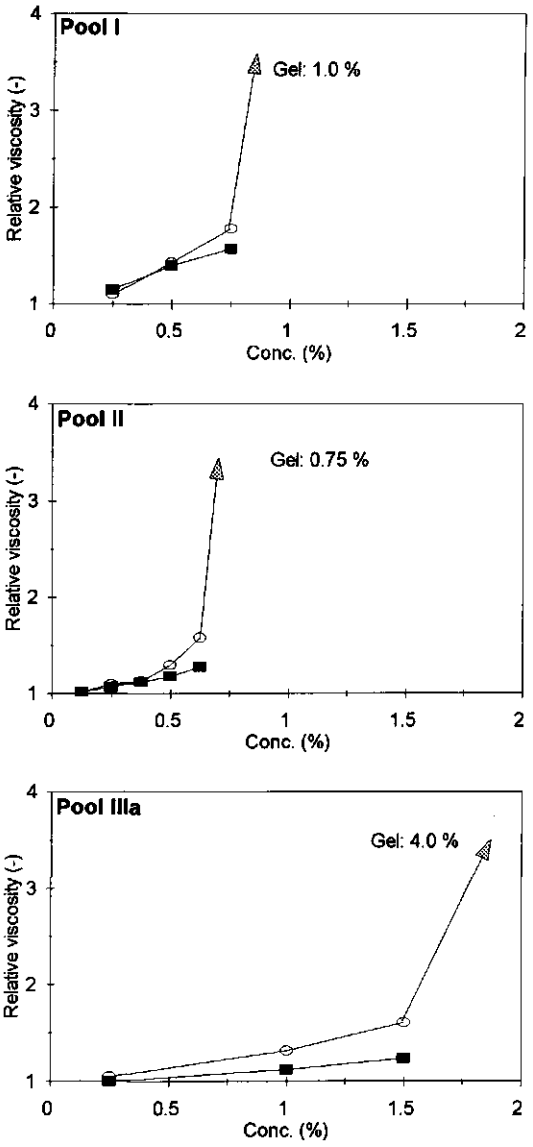

Fig. 5. Relative viscosity versus concentration of the pools purified from Autoclave 2 before (■) and after (O) oxidative cross-linking with hydrogen peroxide/peroxidase.

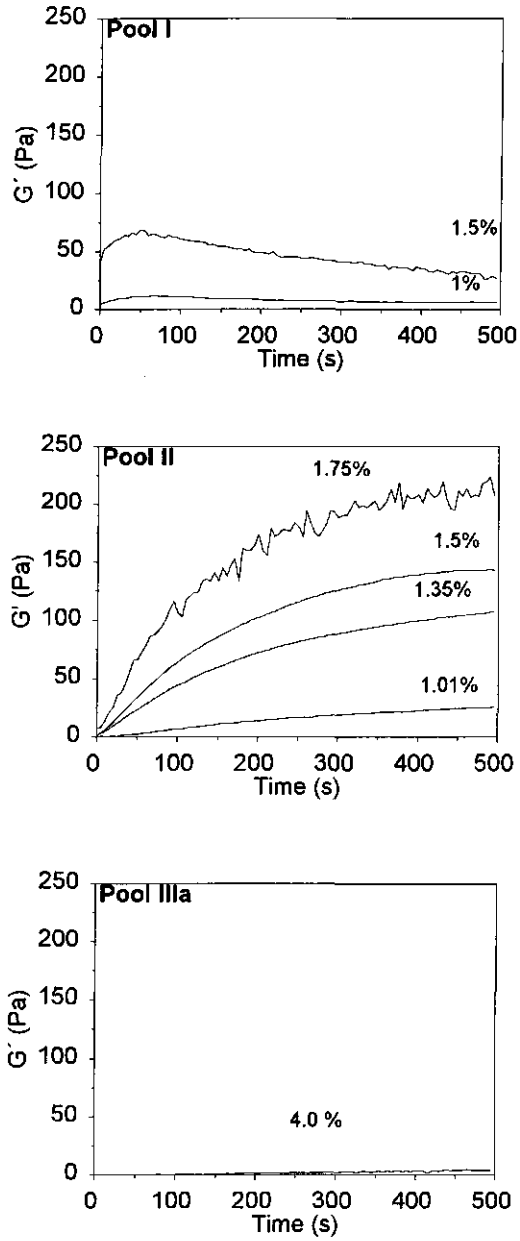

Fig. 6. Storage modulus (G') versus time of gels made by oxidative cross-linking with hydrogen peroxide/peroxidase of the pools purified from Autoclave 2 .

Table 3

Storage modulus (G'), loss modulus (G") and tan δ of the S 500 and Source Q pools after oxidative cross-linking with hydrogen peroxide/peroxidase (500 s).

Sample	Conc. (%)	G' (Pa)	G" (Pa)	tan δ (-)
Pool I	1.00	11	0.1	0.009
	1.50	68	1.2	0.018
Pool II	1.00	26	0.5	0.019
	1.35	107	1.0	0.009
	1.50	143	2.0	0.014
	1.75	208	17.5	0.084
Pool IIIa	4.00	4	0.3	0.075

the linear arabinans is considerably longer than the time needed to cross-link the arabinans in our system (<500 s). The behaviour of the linear arabinan gels was typical for disordered polysaccharide chains interacting solely by physical entanglement, whereas the cross-linked extracts mainly interact via covalent links [20].

Gelation. - The formation of the gel network of the purified pools after cross-linking was investigated by small amplitude shear strain oscillatory testing. Fig. 6 shows the development of the storage modulus (G'), which is indicative for the amount of elastically effective cross-links formed and for the stiffness of the gel, at various concentrations in time. Table 3 summarizes the rheological parameters of the networks formed after hydrogen peroxide/peroxidase treatment for 500 s. A rapid increase in G' was seen for pool I and II at all the concentrations investigated. However, after a large initial increase in G' for pool I during the first 10-20 s, a subsequent decrease was observed, even at low amplitudes. Possibly the settings of the apparatus were not optimal. Due to insufficient amounts of sample we could not fully optimize this measurement. The increase in G' for pool II was slower. The G' of pool IIIa increased very slowly to a relatively low value even at high concentration (4%), indicating that the amount of cross-links in the gel was relatively low.

Tan δ (= G"/G'; G" is indicative for relaxation of cross-links) is also a good parameter to describe the nature of the gel. A low value for tan δ (< 0.1) shows the presence of an elastic system, whereas high values (> 1) imply a more liquid-like character of the network [21]. The very small values of tan δ for Pool I and II show that elastic networks were formed, whereas the nature of the network of pool IIIa is more viscous than of most other pools (see Table 3). Only pool II gave a relatively high value for tan δ at a concentration of 1.75 %. No explanation could be found for this observation. The low values of tan δ may be caused by the covalent nature of the cross-links.

Outlook for applications of cross-linked polysaccharides. - Rhamnogalacturonans and arabinans have two advantages over acid extracted pectins from sugar beet pulp, namely the good solubility of the samples before cross-linking and their low viscosity prior to cross-linking, which makes them easier to handle in industrial applications, e.g. for *in situ* gel formation. Oxidative cross-linking of these polysaccharides can be an addition to the known applications for polysaccharides from sugar beet pulp, and may therefore play a role in the valorization of the pulp.

Concluding remarks

It was shown that both rhamnogalacturonan populations and the arabinan population, obtained from a sugar beet pulp extract by autoclave extraction, play a role in the oxidative cross-linking reaction. Cross-linking of the rhamnogalacturonan population with an apparent molecular weight of 930 kDa resulted in an increase of the viscosity at low concentrations and in gel formation at a concentration of 1.0 %. Although the second rhamnogalacturonan population isolated had a lower apparent molecular weight (130 kDa), it formed gels at concentrations as low as 0.75 %. It can be concluded that this population is the most suitable population to use as a gelling agent. Since it was also present in higher amounts than pool I and IIIa in the original autoclave extract this population probably is the most important for cross-linking of the whole extract. The gels obtained from both types of rhamnogalacturonans were relatively stiff networks in terms of G'. The gels were highly elastic as determined by tan δ, which can be explained by the covalent nature of the bonds formed. These results show that gels can be formed after oxidative cross-linking of rhamnogalacturonans, a feature which has not been described before. The arabinan fraction formed a gel as well after oxidative cross-linking with hydrogen peroxide/peroxidase at a considerably lower concentration (4 % w/v) than the gelling concentration for linear arabinan (10-20 % w/v) [19, 20].

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CHAPTER 7

Enzymatic modification of pectic polysaccharides obtained from sugar beet pulp

Alexander Oosterveld, Gerrit Beldman, and Alphons G.J. Voragen

Abstract

Two types of rhamnogalacturonans and an arabinan fraction, purified from an autoclave extract of sugar beet pulp, as well as an acid extracted beet pectin (ABP) were treated with enzymes in order to modify their physico-chemical properties. The (combinations of) enzymes used were arabinofuranosidase B (AF), endo-arabinanase plus arabinofuranosidase B (EA+AF), rhamnogalacturonase plus rhamnogalacturonan acetyl esterase (RGase+RGAE), and polygalacturonase plus pectin methyl esterase (PG+PE). During the enzyme treatments the intrinsic viscosity ($[\eta]_w$), apparent molecular weight (M_w), and radius of gyration (Rg_w) of the polysaccharides were monitored.

In most cases modification with these enzymes resulted in a decrease of the $[\eta]_w$. However, RGase+RGAE changed the $[\eta]_w$ only slightly, while the enzymes decreased the M_w of ABP significantly. From this it was concluded that the rhamnogalacturonan part contributes little to the $[\eta]_w$ of acid extracted beet pectin. It is further suggested that the rhamnogalacturonans in this pectin are located at the extremities of the molecules. For the rhamnogalacturonans purified from the extract obtained by autoclaving, treatment with AF had no effect on the $[\eta]_w$, although a significant decrease was found for the M_w. More extensive degradation of the arabinan side-chains in the rhamnogalacturonans with EA+AF decreased the $[\eta]_w$, but more effect was seen on the M_w. It was concluded that the arabinan side-chains of rhamnogalacturonans contribute little to the $[\eta]_w$.

Introduction

The structural characteristics of pectic polysaccharides from sugar beet have been the subject of many studies [1-10]. Also several studies have been attended towards the physico-chemical properties of these polysaccharides [11-15]. However, relatively little is known about the relationship between their structure and physico-chemical properties.

Due to their specificity, enzymes are appropriate tools to modify the structure of (sugar beet) pectins. In this way information can be obtained about the relation between chemical structure and physical characteristics. The knowledge obtained can be used to produce tailor made polysaccharides with specific (physico-chemical) properties.

All pectins are assumed to consist of essentially the same building blocks, while the amount of each building block may vary [16]. The principal building units of pectins are the 'smooth' homogalacturonic regions and ramified 'hairy' regions in which most of the neutral sugars are located [17].

The homogalacturonic regions of beet pectin consist of at least 72-100 galacturonic acid residues [6], which are partly esterified with methyl and acetyl groups.

The 'hairy' regions of apple pectins consist of three subunits, as was shown by Schols et al. [18]. Firstly, a rhamnogalacturonan subunit is present, in which part of the rhamnose residues are substituted with single unit galactose residues. Secondly, a rhamnogalacturonan subunit has been found, substituted with long arabinan and/or galactan side-chains. In sugar beet pectin ferulic acid is linked to these side-chains [2-4, 7, 8]. A xylogalacturonan subunit has been shown for apple pectin [18], but the presence of this subunit has not been proven yet in sugar beet pectin [10]. Both methyl esters and acetyl groups are present in the 'hairy' regions from beet pectin [10, 19].

Several studies have been carried out, addressing the enzymatical modification of beet pectins. Some of these studies focused on the enzymatic removal of acetyl groups from acid extracted sugar beet pectins. As a result the gelling properties of these pectins improved [11, 12, 19, 20]. McCleary et al. showed that arabinans extracted from sugar beet pulp could be used e.g. as fat replacer after debranching with the enzyme arabinofuranosidase [21, 22]. An improvement of the gel formation of pectins from sugar beet pulp through oxidative cross-linking with ammonium persulfate after pretreatment with the same enzyme was found by Guillon and Thibault [23, 24].

The current study deals with the enzymatic modification of three types of pectic polysaccharides from sugar beet pulp in order to gain insight in their structure function relationship and to improve their physico-chemical properties. The polysaccharides used were rhamnogalacturonans and arabinans obtained from beet pulp by autoclaving [9, 10], and a commercially available acid extracted pectin, mainly consisting of homogalacturonans.

Experimental

Materials.- Wet beet pulp (8.9 % dry weight) was obtained from CSM Suiker bv (Breda, the Netherlands). Pectins were extracted by autoclave treatment as described previously [9]. The acid extracted pectin obtained from sugar beet pulp (ABP) was a gift from the Copenhagen Pectin Factory Ltd.

Analytical methods. - The uronic acid contents of the extracts were determined by the automated m-hydroxy biphenyl assay [25]. The neutral sugar composition was determined after hydrolysis with 2 M trifluoroacetic acid (1 h, 121°C) as described previously [9]. Feruloyl groups were determined spectrophotometrically at 375 nm in freshly prepared pectin solutions adjusted to pH 10 with a 0.1 M NaOH solution, using a molar extinction coefficient of 31,600 [1].

Chromatography. - High-performance size-exclusion chromatography (HPSEC) was performed on three Bio-Gel TSK columns in series (60XL-40XL-30XL) as described [10] using a combined RI detector and viscometer (Viscotek, model 250), a Right Angle Laser Light-Scattering detector (RALLS, Viscotek, LD 600) and a UV detector (Kratos, Spectroflow 773). Apparent molecular weights, intrinsic viscosities and radii of gyration were calculated using the light-scattering module of the Trisec software (Viscotek).

Preparative size-exclusion chromatography was performed as described previously [10].

Preparative anion-exchange chromatography was performed on a column (15 x 10 cm) of Source Q (Pharmacia) using a Biopilot system (Pharmacia). The sample (~0.4 g) was applied onto the column at a flow rate of 15 mL/min (0.005 M NaOAc, pH 5.0). After 1575 mL the flow rate was increased to 80 mL/min and a linear gradient to 2 M NaOAc (pH 5.0) was applied for 7200 mL to elute the polysaccharides. Subsequently, the elution was proceeded using elution with 1200 mL 2 M NaOAc. The columns were washed with 0.5 M NaOH, followed by regeneration with 2 M NaOAc (pH 5.0) and equilibration with 0.005 M NaOAc (pH 5.0). Fractions (90 or 356 mL) were assayed for total neutral sugar [26] and uronic acid [25] content using arabinose and galacturonic acid as standards. A correction was made for the response of uronic acids in the neutral sugar test. Pooled fractions were dialyzed and freeze-dried.

Enzymatic modification. - The pools obtained by DEAE anion-exchange chromatography and S 500 size-exclusion chromatography were treated with the following (combinations of) purified enzymes: arabinofuranosidase B (AF) [27], AF plus endo-arabinanase (EA) [27], endo-polygalacturonase (PG) [28] combined with pectin methyl esterase (PE) [29], and rhamnogalacturonase (RGase) [30] in combination with rhamnogalacturonan acetyl esterase (RGAE) [31].

All pools were dissolved in 0.04 M sodium acetate buffer containing 0.01% of NaN_3 (pH 5.0) to a final concentration of 5 mg/mL. Enzymes were added to a final

concentration of 1 μg of protein/mL. Incubations were carried out at 20°C for 42 h. The digests were analyzed at several stages of the incubation by HPSEC.

Results and discussion

Composition of the substrates. - Two rhamnogalacturonan fractions and an arabinan fraction were purified from an autoclave extract of sugar beet pulp by S 500 size-exclusion chromatography and characterized as described previously (Table 1) [10]. Additionally the degree of feruloylation (DF) of these substrates was determined, as well as the apparent molecular weight (M_w), intrinsic viscosity ($[\eta]_w$), and radius of gyration (Rg_w) (Table 2).

Three fractions are distinguished (see also Table 1). Pool I mainly contains rhamnogalacturonans with a very high M_w (1020 kDa, Table 2). The pool is highly acetylated and feruloylated. The degree of methylation is relatively low. The $[\eta]_w$ of this pool was relatively low for a molecule with such a high M_w, which can be explained by the high degree of branching.

In pool II rhamnogalacturonans with an apparent molecular weight of 225 kDa and with a relatively low intrinsic viscosity are predominantly present. Except for the galacturonic acid and arabinose content, its composition is similar to that of pool I. The DF is somewhat lower than that of pool I.

Table 1

Sugar compositions (mol %) of the pools of the extracts obtained by autoclaving and of the acid extract.

	ABP[d]	Autoclave 2				
		pool I[e]	pool II[e]	pool III[e]	pool IIIA	pool IIIB
Rha[a]	5.4	6.8	5.3	1.5	0.1	3.8
Ara[a]	7.1	58.8	51.5	36.9	91.0	19.3
Xyl[a]	1.2	0.8	0.5	0.4	0.1	1.0
Man[a]	2.0	0	0.7	1.9	2.2	3.3
Gal[a]	11.2	13.2	9.9	3.2	0.9	7.7
Glc[a]	0.4	0.6	0.4	1.3	2.5	2.9
UA[a]	72.9	19.9	31.8	54.9	3.2	62.0
DA[b]	15.3	51.1	56.3	25.8	nd	25.6
DM[b]	57.7	28.8	33.4	56.0	nd	60.5
DF[c]	1.2	1.7	1.4	0.9	1.0	0.8

[a] Expressed as mol%. [b] Expressed as moles acetyl or methyl per 100 moles of galacturonic acid. [c] Expressed as moles ferulic acid per 100 moles of galactose + arabinose. [d] Molar sugar composition from [19]. [e] Molar sugar composition from [10]. nd not determined.

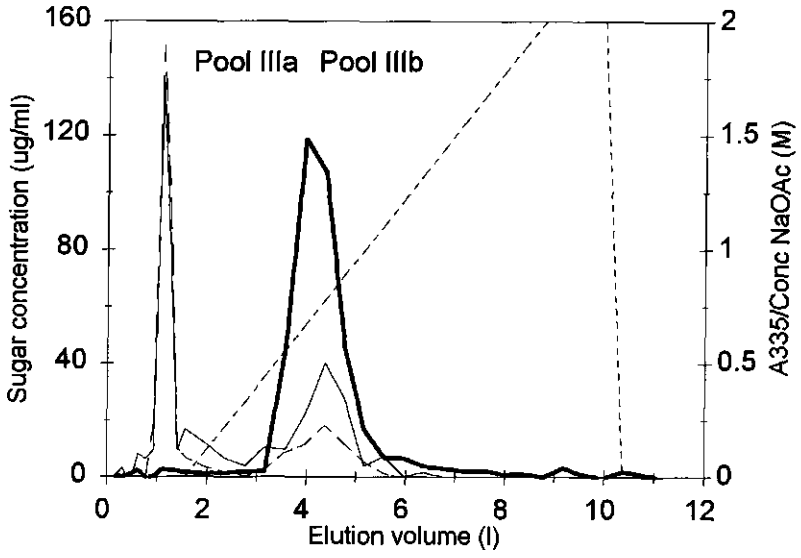

Fig. 1. Preparative anion-exchange chromatography (Source Q) of pool III obtained by S 500 size-exclusion chromatography. Thick line: uronic acid; dashed line: neutral sugars; thin line: A335; dotted line: concentration NaOAc.

The third pool (III) is a mixture of homogalacturonans and arabinans with low M_w as determined by light-scattering (~20 kDa). This fraction is highly methylated, while the DA and DF are low. The arabinans and homogalacturonans present in pool III were separated by Source Q anion-exchange chromatography (Fig.1). Pool IIIA consisted almost exclusively of arabinans, with a high ferulic acid content. The composition of this arabinan population was similar to the neutral fraction of the autoclave extract as obtained after DEAE anion-exchange chromatography [10]. However, the M_w of this fraction as calculated by light-scattering was somewhat higher than determined before using dextran standards [10]. Most of the galacturonic acid eluted in pool IIIB, together with 38 mol% of neutral sugars. This fraction had a high DM, whereas the DA was lower than in pool I and pool II.

The acid extracted sugar beet pectin (ABP) consisted for 72.9 mol% of galacturonic

acid and galactose was the predominant neutral sugar [19]. Substantial amounts of both methyl esters and acetyl groups were present. The DF was 1.2, but since this is calculated based on the amount of galactose plus arabinose, the absolute amount of ferulic acid was relatively low. The composition of ABP was comparable to other acid extracted sugar beet pectins described in the literature [32, 33].

Effect of enzymatic modification. - Pool I, II, and IIIA of the autoclave extract, as well as ABP, were subjected to the following combinations of enzymes: rhamnogalacturonase (RGase) in combination with rhamnogalacturonan acetyl esterase (RGAE), arabinofuranosidase B (AF), AF plus endo-arabinanase (EA), and endo-polygalacturonase (PG) combined with pectin methyl esterase (PE). During enzymatic treatment for 42 h, changes in the apparent molecular weight (M_w), intrinsic viscosity ($[\eta]_w$), and radius of gyration (Rg_w) were monitored in time, as shown in Figs. 2-5. The

Table 2

Apparent molecular weight (M_w, kDa), intrinsic viscosity ($[\eta]_w$, dL/g), and radius of gyration (Rg_w, nm) of ABP and pools obtained from Autoclave 2, before and after treatment with various enzyme combinations for 42 h.

		ABP	Autoclave 2		
			pool I	pool II	pool IIIA
Blank	M_w	271	1020	225	23
	$[\eta]_w$	4.54	0.93	0.66	0.20
	Rg_w	33.1	30.8	15.6	5.2
RGase+RGAE	M_w	112	157	60	18
	$[\eta]_w$	4.01	0.52	0.45	0.18
	Rg_w	22.7	12.7	9.2	4.8
AF	M_w	nd	712	131	9
	$[\eta]_w$	nd	0.99	0.67	0.18
	Rg_w	nd	28.4	14.2	3.8
EA+AF	M_w	207	99	126	4
	$[\eta]_w$	4.24	0.79	0.42	0.11
	Rg_w	29.2	14.0	12.2	2.3
PG+PE	M_w	5	735	156	nd
	$[\eta]_w$	0.12	0.83	0.46	nd
	Rg_w	2.5	26.4	12.3	nd

nd not determined.

values of these parameters after 42 h are shown in Table 2. The homogalacturonan pool IIIB was not subjected to enzymatic modification, because of its low M_w, which indicates that this pool was severely degraded during autoclave treatment. ABP was used as an example of a beet pectin with relatively intact homogalacturonan regions.

Effect of enzymatic modification on Autoclave 2 pool I. - The changes in M_w, $[\eta]_w$, and Rg_w during enzyme treatment of Autoclave 2 pool II are shown in Fig. 2a-c, while the HPSEC elution patterns before and after enzymatic treatment are shown in Fig. 2d.

Treatment of Autoclave 2 pool I with RGase+RGAE caused a rapid decrease of the M_w from 1020 kDa to 157 kDa. Moreover, the $[\eta]_w$ and the Rg_w decreased approximately by a factor 2 and an additional population with a high ferulic acid content appeared

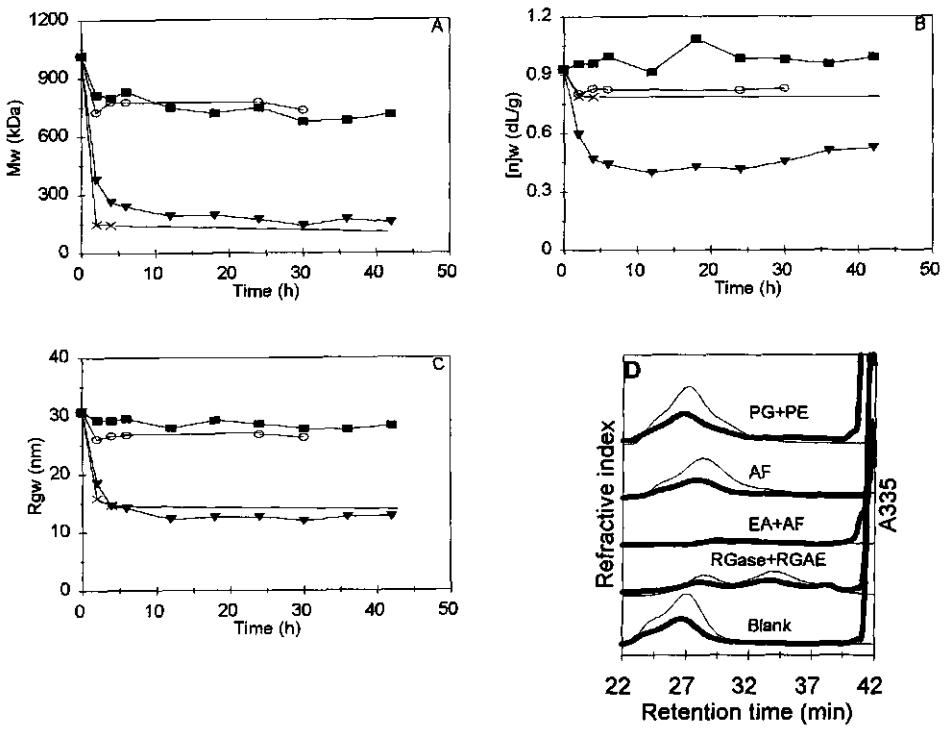

Fig. 2. Effect of enzymatic modification of Autoclave 2 pool I on M_w (A), $[\eta]_w$ (B), and Rg_w (C) in time, and on the HPSEC elution pattern (after 42 h; D). ■: AF; X: EA+AF; ▼: RGase+RGAE; O: PG+PE. Fig. 2D: Thick line: refractive index; thin line A_{335}.

upon HPSEC (34 min). The M_w of this new pool was approximately 50 kDa, whereas its $[\eta]_w$ was approximately 0.37 dL/g. Since RGase is known to degrade the more linear rhamnogalacturonan backbone only substituted with single unit galactose residues, the material which remains is assumed to consist of the rhamnogalacturonan subunit highly branched with arabinose.

The addition of AF to pool I reduced the M_w to 712 kDa. Interestingly, this resulted in a small increase of the $[\eta]_w$, whereas the Rg_w decreased slightly. As a consequence of the lower Rg_w, this pool eluated somewhat later upon HPSEC. Apparently a significant part of the arabinose can be removed from this pectin, without a loss (and in fact a small increase) of the $[\eta]_w$. AF is known to be active both on the terminal arabinofuranosyl residues in side-chains and on the non-reducing end of the arabinan backbone. Since the arabinan side-chains are present in much higher amounts than the non-reducing ends, AF degrades the side-chains in the arabinans faster than the backbone [34]. Because only part of the arabinose had been removed, considering the M_w after treatment with AF, it is assumed that mainly the side-chains of the arabinans had been removed and that these contribute little to the intrinsic viscosity of the rhamnogalacturonans. In the HPSEC chromatogram, the ratio A_{335} : RI increased after treatment with AF, which indicates that feruloylated arabinose is not removed, or with less preference than the non-feruloylated arabinose. Apparently, AF is hindered by the ferulic acid residues. Another possible explanation is the observation that the galactose residues in sugar beet pulp have a higher degree of feruloylation than the arabinose residues [7].

Hydrolysis of the arabinan side-chains with EA+AF showed a decrease in M_w to 99 kDa. The $[\eta]_w$ of this population remained relatively high, apparently due to the fact that the backbone of the rhamnogalacturonan was not degraded by EA+AF. Since 58.8 mol% of this pool consisted of arabinose, which was present as side-chain of the rhamnogalacturonan, and since mono- and oligomers were not included in the calculation of the M_w, a maximum decrease in M_w of approximately 60 % was expected. Therefore, the resulting low M_w after treatment with EA+AF is striking and might be ascribed to the degradation of the backbone of arabinans that participate in cross-links between two different rhamnogalacturonan molecules in the original material. Diferulic acid bridges are present in the autoclave extract [35], and are able to link multiple molecules together. ~10 % of the ferulates present in the original autoclave extract is present as dimer [35]. Since the M_w of pool I is ~1000 kDa, a degree of polymerization can be estimated of 6700. About 70 mol% of the sugars is made up by arabinose and galactose and the ferulic acid content was 1.7 molecule per 100 molecules of arabinose and galactose. From this it can be calculated that on average every molecule contains 4 diferulates, which shows that cross-linking might occur to some extent.

It can be concluded that extensive degradation of the arabinan side-chains with

EA+AF has little influence on the $[\eta]_w$ of a rhamnogalacturonan. These findings are in agreement with the results of Hwang and Kokini, who investigated the contribution of side-chains to the rheological properties of apple pectins with different neutral sugar contents [13], although the neutral sugar contents were relatively low in all the pectins investigated. They found that the degree of branching had little effect on the intrinsic viscosity, which is determined at low concentrations, but that the side-chains of pectins were significantly involved in entanglements of the pectin molecules in concentrated solutions.

PG+PE caused a small decrease in M_w, $[\eta]_w$, as well as in Rg_w. Also, a small shift in hydrodynamic volume was observed upon HPSEC. This limited decrease in M_w shows that this enzyme was only slightly active on this pool, showing that no homogalacturonan regions are present internally in this rhamnogalacturonan population. Addition of pectin acetyl esterase, which is known to increase the activity of PE [19] and therefore indirectly increases the activity of PG, besides PG+PE to the parental extract did not increase the degradation of this rhamnogalacturonan population (unpublished data), but only increased the degradation of the separately present homogalacturonan populations (pool III). These results indicate that pieces of homogalacturonan are possibly located at the extremities of the rhamnogalacturonan molecules or that some homogalacturonans are present as a separate population. Since PG+PE are active on the 'smooth' regions and not on the 'hairy' regions, the small decrease in M_w indicates that the 'hairy' regions of sugar beet pectin may have a very high M_w.

Effect of enzymatic modification on Autoclave 2 pool II. - Autoclave 2 pool II, a highly branched rhamnogalacturonan with a lower M_w than pool I, was incubated with the same enzyme combinations as pool I (Fig. 3). The results were rather similar to the results found for pool I. Hydrolysis with the enzymes RGase+RGAE caused a large decrease in M_w, $[\eta]_w$, and Rg_w. Degradation of the arabinan side-chains of pool II by AF decreased the M_w, while the $[\eta]_w$ and the Rg_w changed relatively little. Hydrolysis of the arabinan side-chains of pool II by EA+AF led to a large decrease in M_w. Although M_w decreased to a value similar to the value found after treatment with AF, the $[\eta]_w$ and Rg_w decreased to lower values. Hydrolysis of homogalacturonan regions in pool II by PG+PE led to a relatively large decrease in M_w. If possible homogalacturonans stretches are only located at the extremities of the molecule, as was suggested for pool I, this decrease seems rather large. Possibly some high M_w homogalacturonans eluted together with the rhamnogalacturonans in small amounts, as was also indicated by the higher galacturonic acid content in this pool as compared with pool I. The formation of an additional peak with a low A_{335} absorption at 37 min upon HPSEC after degradation with PG+PE is in agreement with this assumption. The $[\eta]_w$ and Rg_w also decreased to relatively low values, which shows that this homogalacturonan fraction has a relatively

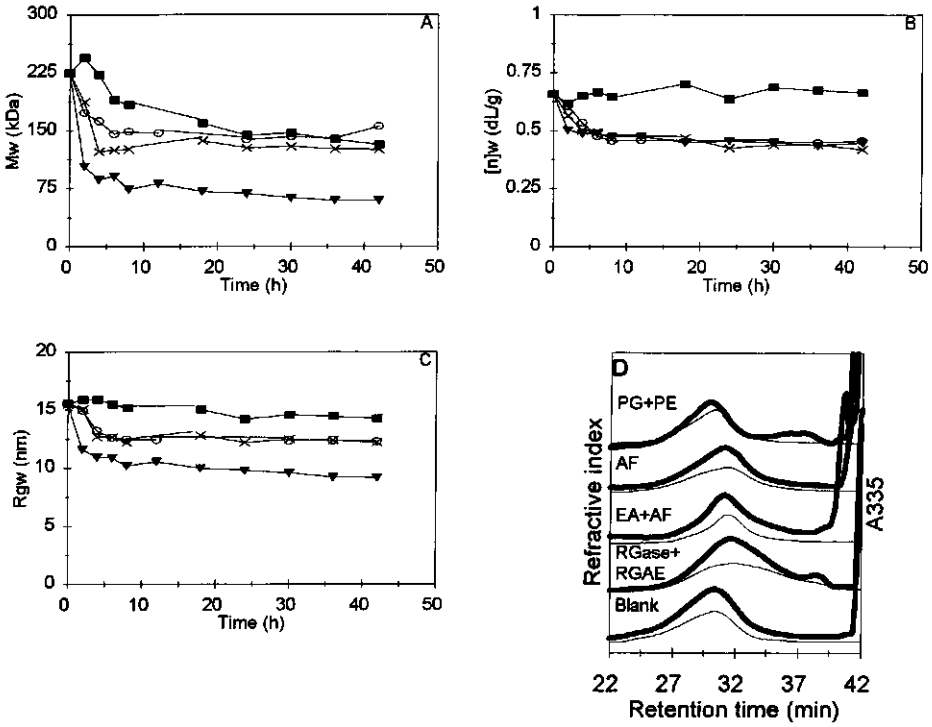

Fig. 3. Effect of enzymatic modification of Autoclave 2 pool II on M_w (A), $[\eta]_w$ (B), and Rg_w (C) in time, and on the HPSEC elution pattern (after 42 h; D). ■: AF; X: EA+AF;▼: RGase+RGAE; O: PG+PE. Fig. 3D: Thick line: refractive index; thin line A_{335}.

large influence on these parameters.

Effect of enzymatic modification on Autoclave 2 pool IIIA. - The arabinan fraction Autoclave pool IIIa was also treated with the enzyme combinations mentioned above (Fig. 4).

Modification by RGase+RGAE resulted only in a small change in M_w. This is in agreement with the assumption that some arabinans are still linked to a small piece of rhamnogalacturonan backbone, too small to bind to the anion-exchange column, as was previously shown by the methylation analysis of the arabinans [10]. The $[\eta]_w$ did not change, since this is determined by the longest backbone of the fragment, in this case probably the arabinan backbone. The HPSEC diagram hardly changed as compared with the blank.

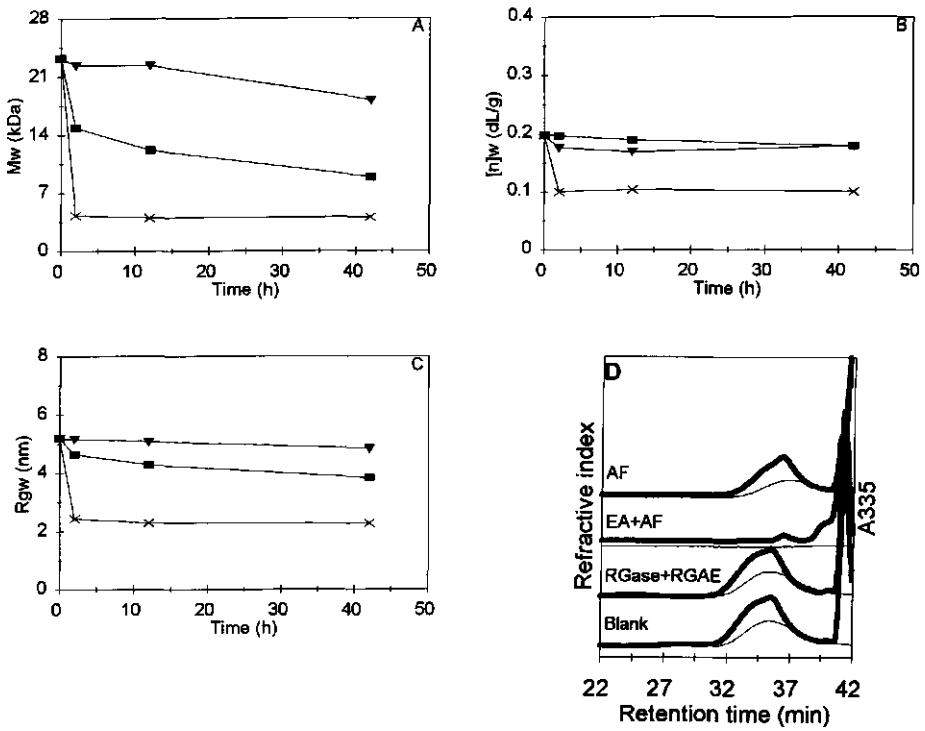

Fig. 4. Effect of enzymatic modification of Autoclave pool IIIa on M_w (A), $[\eta]_w$ (B), and Rg_w (C) in time, and on the HPSEC elution pattern (after 42 h; D). ■: AF; X: EA+AF;▼: RGase+RGAE. Fig. 4D: Thick line: refractive index; thin line A_{335}.

Hydrolysis of the arabinans with AF decreased the M_w from 23 kDa to 9 kDa. From these results a degree of polymerization of the arabinans can be calculated of 130-170 residues before and 60-70 after modification with AF. However, the $[\eta]_w$ did not change. This can be explained by the fact that AF predominantly removes the side-chains from the arabinans, having little effect on the length of the backbone, which determines the $[\eta]_w$ to a large extent. So, probably the backbone has a length of 60-70 residues. This would implicate that more than 45-65 % of the arabinose residues are present as single unit or oligomeric side group of the arabinan main-chain. Also, methylation analysis showed that approximately 35 % is present as terminal residues indicating that on

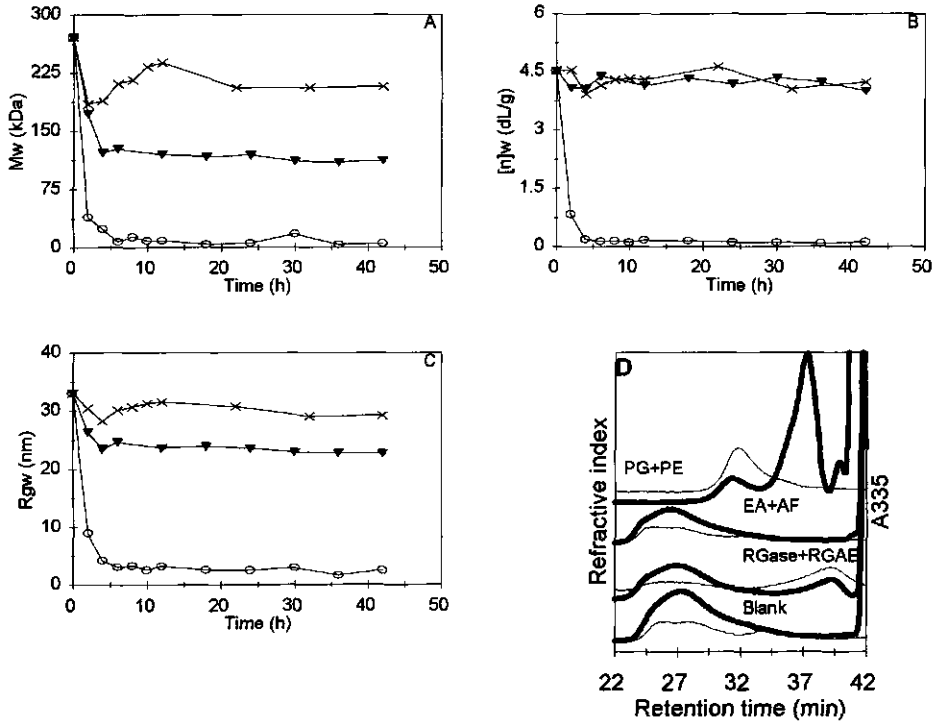

Fig. 5. Effect of enzymatic modification of ABP on M_w (A), $[\eta]_w$ (B), and Rg_w (C) in time, and on the HPSEC elution pattern (after 42 h; D). X: EA+AF; ▼: RGase+RGAE; O: PG+PE. Fig. 6D: Thick line: refractive index; thin line A_{335}.

average the side-chains of the arabinans are relatively short. Cooper et al. described the production of linear arabinans with AF [22]. They estimated a length of 50-80 residues after linearization, which is in good agreement with the value described in this paper.

Incubation of the arabinans with the enzymes EA+AF led to a decrease in both M_w and $[\eta]_w$ of the arabinan population. In the HPSEC elution pattern only a very small population remained. It is speculated that this population consists of glucomannans, which represent less than 5 mol% of the sugars initially present in the arabinan fraction. The M_w of this fraction was 4 kDa.

The effect of treatment of the arabinans with PG+PE was not determined, since the galacturonic acid content of the arabinans was very low.

Effect of enzymatic modification on an acid extracted beet pectin (ABP) - Besides

rhamnogalacturonans and arabinans obtained from an autoclave extract from beet pulp, also an acid extracted pectin from sugar beet pulp (ABP) was incubated with the enzyme combinations RGase+RGAE, PG+PE, and EA+AF (see Fig. 5). ABP consists primarily of homogalacturonans. As the arabinose content of this pectin is low, and the effect of arabinan degrading enzymes on the physico-chemical properties was expected to be neglegible, treatment with AF was omitted.

Treatment of ABP with RGase+RGAE decreased the Mw from 271 kDa to 112 kDa, due to hydrolysis of the rhamnogalacturonan backbone present in the 'hairy' regions of ABP. Surprisingly, the $[\eta]_w$ hardly changed. Most of the material eluted at the same retention time upon HPSEC. However, a low M_w population with a high A_{335} absorption appeared at 38 min in low quantities. It made us conclude that the 'hairy' regions in this pectin are located at the extremities of the molecules. It is also possible that a pectin population is degraded by this enzyme, which is present in small amounts and with a very high M_w. Berth et al. [36] described the presence of a pectin population, which was present in minute amounts. This population had such a high M_w that it significantly influenced the M_w of the whole pectin extract. Our results show that the rhamnogalacturonan backbone from ABP contributes little to the $[\eta]_w$. On the other hand treatment with RGase+RGAE causes a loss of the gelling properties with Ca^{2+}, as we showed in another study [19].

Modification of the arabinan side-chains by addition of EA+AF to ABP decreased the M_w to 207 kDa. This decrease was somewhat larger than was expected based on the low arabinose content (7 mol%). Both the Rg_w and the $[\eta]_w$ hardly changed. Upon HPSEC no clear changes were observed. Guillon and Thibault found a decrease from 46 kDa to 14 kDa in the viscosity average M_w of an acid extracted sugar beet pectin after treatment with EA+AF [23]. However, this seems an extremily large decrease, regarding the low amount of arabinose (13.2 mol%) present in their extract. They also found that AF resulted in a very small decrease in M_w, which is in agreement with our results.

Modification of the 'smooth' homogalacturonan regions of ABP with PG+PE decreased the average M_w rapidly to a value of 5 kDa. Furthermore, the $[\eta]_w$, and Rg_w also decreased rapidly. This is in agreement with the results found by Guillon and Thibault, who also found a rapid decrease in M_w and $[\eta]_w$ for an acid extracted beet pectin during treatment with PG+PE [23]. After treatment of ABP with PG+PE only some 'hairy' fragments remained. Based on our results the average M_w of these fractions is 50 kDa, which is much lower than those obtained by autoclaving (see above). On the other hand, it is somewhat higher than the results found by Guillon and Thibault, who found a viscosity average M_w of 12 kDa after treatment of an acid extracted beet pectin with PG+PE [23].

Concluding remarks

In the current study various types of pectic polysaccharides, extracted from beet pulp by autoclaving and acid extraction, were enzymatically modified. Changes in physical parameters resulting from the enzyme treatments were analyzed. From these data conclusions were made regarding the relation between the molecular structure of the pectic polysaccharides and their physical properties.

It was shown that the (free) arabinans present in an autoclave extract from sugar beet pulp have a degree of polymerization of 130-170, whereas the backbone contains 60-70 arabinose residues. Furthermore, some indications were found that the rhamnogalacturonans present in this extract are linked though the arabinose side-chains, maybe by diferulic acid cross-links, however, further study is needed to confirm this.

In most cases we found that enzymatic modification decreased the $[\eta]_w$ of the polysaccharides investigated. Obviously, this occurred when backbone degrading enzymes were used. However, the backbone degrading enzyme (RGase) decreased the M_w of ABP significantly, but not the $[\eta]_w$. It made us conclude that the backbone of the rhamnogalacturonan has relatively little influence on the $[\eta]_w$ and that the 'hairy' regions in this pectin are probably located at the extremities of the molecules. Removal of the side-chains of the arabinans present in the rhamnogalacturonans with AF had no effect on the $[\eta]_w$, although the M_w of the populations decreased significantly. When these arabinan side-chains were degraded with EA+AF, the $[\eta]_w$ decreased relatively little as compared with the M_w. This shows that the arabinan side-chains contribute little to the $[\eta]_w$ of acid extracted beet pectins.

Acknowledgements

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CHAPTER 8

Effect of enzymatic deacetylation on gelation of sugar beet pectin in the presence of calcium

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Abstract

This study deals with the effect of methyl esters, acetyl groups, and neutral sugar side-chains on the gelation properties of sugar beet pectin with Ca^{2+}. Sugar beet pectin was treated in the presence of Ca^{2+} with the enymes pectin methyl esterase (PE), pectin acetyl esterase (PAE), rhamnogalacturonan acetyl esterase (RGAE), arabinofuranosidase B (AF) and rhamnogalacturonase (RGase) in various combinations.

Addition of RGAE plus PE or PAE plus PE to the pectin-Ca^{2+} mixture significantly increased the release of acetyl groups and methyl esters, in comparison to the addition of only PE or PAE. This indicates that pectin methyl esterase activity is hindered by the presence of acetyl groups both in the 'smooth' and in the 'hairy' regions. Also the pectin acetyl esterase activity is hindered by the presence of methyl groups in the 'smooth' regions. Treatment with PAE plus PE led to a stiffer gel, as determined by the storage modulus (G'), than treatment with PE alone, while RGAE plus PE did not improve the gel forming properties. Addition of only PAE to the pectin-Ca^{2+} mixture did not result in gel formation. A lower stiffness of the gel was found when RGase combined with RGAE and PE were added to the pectin-Ca^{2+} mixture, in comparison to treatment with PE alone. Addition of arabinofuranosidase B plus PE to the pectin-Ca^{2+} mixture gave similar rheological effects as treatment with only PE.

A fraction representing the 'smooth' homogalacturonan regions, which was obtained after treatment of the beet pectin with rhamnogalacturonase and subsequent size-exclusion chromatography, was also able to form a gel with Ca^{2+} and PE. However, the gel formation was much slower, and the stiffness of the gel was lower than when the parental extract was used. Also with the modified pectin the treatment with PAE plus PE gave an increased stiffness of the gel in comparison to PE alone.

Introduction

Sugar beet pectins are characterized by a high acetyl content, a high arabinose content and by the presence of feruloyl groups attached to the galactose and arabinose side-chains [1-5]. The acetyl groups can be located on both the C-2 and C-3 position of the galacturonic acid residues [6]. It was shown that approximately 75 % of the acetyl groups in an acid extracted beet pectin are located in the 'smooth' homogalacturonan regions [7]. The high acetyl content, together with a high arabinose content and a relatively low molecular weight, limit the use of sugar beet pectin in the traditional applications of pectin: gel formation with acid/sugar or Ca^{2+} ions [8].

Several attempts have been made to improve gel formation of beet pectins with calcium by the removal of acetyl groups using acetyl esterases. Pippen et al. (1950) reported that deacetylation of an artificially acetylated citrus pectin with a citrus acetyl esterase was unsuccessful, and suggested that the presence of the methyl groups was the main reason [8]. Another study showed that treatment of sugar beet pectin with a partial purified pectin acetyl esterase, which also had pectin methyl esterase activity, led to an improved gel formation with Ca^{2+} [9]. Matthew et al. [10] reported an improvement of the gelling properties of sugar beet pectin with Ca^{2+} following treatment with an enzyme preparation derived from *Aspergillus niger*. This treatment resulted in deacetylation, demethylation and in a large reduction of the arabinose content. However, the use of partially purified and poorly defined enzymes or artificially acetylated substrates in these studies make it difficult to draw conclusions.

Recently, two types of acetyl esterases active on beet pectin were purified and characterized in our laboratory. The enzyme pectin acetyl esterase (PAE) specifically removes part of the acetyl groups from the 'smooth' homogalacturonan regions of (sugar beet) pectins [11]. The enzyme rhamnogalacturonan acetyl esterase (RGAE) specifically removes part of the acetyl groups present in the 'hairy' regions of pectin [12]. The current study deals with the effect of methyl esters, acetyl groups, and neutral sugar side-chains on the gelation properties of acid extracted sugar beet pectin. For this purpose sugar beet pectin was treated in the presence of Ca^{2+} with the enzymes pectin methyl esterase (PE), PAE, RGAE, arabinofuranosidase B (AF) [13] and rhamnogalacturonase (RGase) [14] in various combinations.

Experimental

Materials.- The acid extracted pectin obtained from sugar beet pulp (ABP) was a gift from Copenhagen Pectin Factory Ltd.

Enzymes. - A rhamnogalacturonase from *Aspergillus aculeatus* cloned in

Aspergillus oryzae (RGase) [15] was used for the large scale treatment of ABP, whereas a RGase purified from a technical preparation of *Aspergillus aculeatus* was used for rheological experiments [14]. Rhamnogalacturonan acetyl esterase was purified from *Aspergillus aculeatus* [12], while pectin methyl esterase (PE) [16], pectin acetyl esterase (PAE) [11], and arabinofuranosidase B (AF) [13] were isolated from *Aspergillus niger*.

Analytical methods. - The uronic acid content of the extract was determined by the automated m-hydroxy biphenyl assay [17]. The neutral sugar composition was determined after hydrolysis with 2 **M** trifluoroacetic acid (1 h, 121°C) as described previously [16]. Feruloyl groups were determined spectrophotometrically at 375 nm in freshly prepared pectin solutions adjusted to pH 10 with a 0.1 M NaOH solution. A molar extinction coefficient of 31,600 was used [7]. Methylation analysis was performed as described previously [18], without reduction of galacturonic acid residues to galactose.

Enzyme treatments. - ABP was treated with rhamnogalacturonase (RGase) [14] in order to degrade the 'hairy' regions. Pectin (0.5 % w/v) was dissolved in 0.04 **M** sodium acetate buffer pH 5.0. The enzyme was added to obtain a final concentration of 1 µg protein per mL. The sample was incubated for 20 h at 30°C. The reaction was then stopped by heating at 100°C for 5 min. The digest was analyzed by high-performance size-exclusion chromatography.

Gel formation in the presence of Ca^{2+} and enzymes. - The acid extracted beet pectin (ABP), the pools obtained after treatment with RGase and subsequent separation on Sephacryl S 300, were treated with the following (combinations of) enzymes to investigate their effect on the gel formation with Ca^{2+}: PE, PAE, PE plus PAE, PE plus RGAE, PE plus RGase plus RGAE, PE plus AF. The pectins (1 % w/v) were dissolved in 3 mL 0.1 M bis-tris buffer pH 6.0 and 120 µL of 1 M $CaCl_2$ solution in water was added. The enzyme combinations were added to obtain a final concentration of 1 µg of protein per mL and incubated at 25°C for 20 h.

The formation of the gel networks in the preparations was investigated by small amplitude shear strain oscillatory testing. A Bohlin VOR rheometer in oscillatory mode was used to monitor the gel structure development as follows. Immediately after addition of the enzyme(s), the samples were placed in the geometry. A thin layer of mineral oil was added to cover the bob and prevent evaporative losses throughout the measurements. All measurements were carried out at 25°C at a frequency of 1 Hz using a torsion bar of 20 g cm. Ensured was that the strain remained within the linear region of the material at all times.

Concentration of acetyl groups and methyl esters. - The enzyme treatments of ABP and the pools obtained after treatment with RGase were repeated as described above. The digests were analyzed for their acetyl and methyl content by HPLC as described

[19].

Chromatography. - High-performance size-exclusion chromatography (HPSEC) was performed on three Bio-Gel TSK columns in series (60XL-40XL-30XL) and analyzed with a combined RI detector and viscometer (Viscotek, model 250), a Right Angle Laser Light-Scattering detector (RALLS, Viscotek, LD 600) and a UV detector (Kratos, Spectroflow 773) as described before [20]. Preparative size-exclusion chromatography of the extract after degradation with RGase was performed on a column (75 x 2.6 cm) of Sephacryl S 300 (Pharmacia) using a Hiload system (Pharmacia). Samples (0.05 g) were eluted with 0.05 M NaOAc buffer pH 5.0 at a flow rate of 2.5 mL/min. The procedure was repeated 7 times. Fractions (2.5 mL) were assayed for total neutral sugar [21] and uronic acid [17] content using arabinose and galacturonic acid as standards. The presence of ferulic acid was monitored spectrophotometrically at 335 nm [5]. Corresponding fractions were pooled, dialyzed and freeze-dried.

Preparative anion-exchange chromatography was performed on a column (54 x 2.6 cm) of DEAE Sepharose Fast Flow (Pharmacia) using a Hiload System (Pharmacia) as described previously [20]. Fractions (20 mL) were assayed for total neutral sugar [21] and uronic acid [17] content. Pooled fractions were dialyzed and freeze-dried.

Results and discussion

Composition of the acid extracted pectin. - The acid extracted sugar beet pectin (ABP) consisted for 72.9 mol% of galacturonic acid (Table 1). Galactose was the main neutral sugar, besides arabinose and rhamnose. This composition was in agreement with several other acid extracted sugar beet pectins reported in literature [22-25]. The high galacturonic acid content shows that it predominantly consists of 'smooth' homogalacturonan regions. The degree of methylation was 57.7 and the degree of acetylation was 15.3. These values are slightly lower than those found by Guillon and Thibault for acid extracted sugar beet pectin [26].

The apparent molecular weight of the sample as determined by light-scattering detection (271 kDa) was rather high in comparison to values in the literature as determined by viscometry and universal calibration (46 kDa and 141 kDa, respectively) [26, 27], but was in good agreement with light-scattering results obtained previously for a different acid extracted beet pectin (247 kDa) [28]. A possible explanation is the fact that light-scattering detection might reveal small amounts of high molecular weight populations (or aggregates), which contribute (much) more than proportional to the average molecular weight and are not easily detected by other methods, as has been shown by Berth et al. for citrus pectin [29]. The intrinsic viscosity (4.6 dL/g) was high

Table 1

Composition of ABP and fractions thereof obtained by DEAE anion-exchange chromatography, and by size-exclusion chromatography after treatment with the enzyme RGase.

		DEAE		SEC (S 300) RGase	
	ABP	pool A	pool B	pool I	pool II
Rha[a]	5.4	2.1	9.1	2.4	20.2
Ara	7.1	4.0	11.8	5.4	15.4
Xyl	1.2	0.5	1.0	0.7	0.2
Man	2.0	2.8	1.9	2.6	0.9
Gal	11.2	5.5	21.4	8.5	30.1
Glc	0.4	0.9	3.6	0.5	0.6
UA	72.9	84.3	51.1	79.9	32.7
DA[b]	15.3	18.2	20.1	17.1	26.7
DM[c]	57.7	71.4	48.8	54.3	25.8
DF[d]	1.2	0.8	1.9	1.4	2.1
Relative weight[e]	-	74.	26.	76.	24.
Mw[f]	271.	nd	nd	97.	14.
[n][g]	4.5	nd	nd	2.2	0.1

[a] Sugar composition (mol%); [b] Degree of acetylation (moles of acetyl groups/100 moles anhydrogalacturonic acid residues); [c] Degree of methylation (moles of methyl groups/100 moles anhydrogalacturonic acid residues); [d] Degree of feruloylation (moles of feruloyl groups/100 moles arabinose + galactose residues); [e] Expressed as weight percentage of the polysaccharides recovered; [f] Apparent molecular weight (kDa); [g] Intrinsic viscosity (dL/g).

as compared with literature values (1.1 - 2.5 dL/g) [26, 27, 30].

DEAE chromatography. - To determine the homogeneity of the acid extracted pectin with respect to charge, the sample was separated using DEAE anion-exchange chromatography (Fig. 1). Most of the material eluted in a major peak at 0.35 M NaOAc (pool A), and consisted for 84 mol% of galacturonic acid (Table 1). Its degree ofmethylation (DM) was 71.4 and the degree of acetylation (DA) was 18.2. The degree of feruloylation (DF) was relatively low (0.8). These findings show that this pool predominantly consists of homogalacturonans. Furthermore, a tailing population (pool B) eluted between 0.45-0.85 M NaOAc. This pool contained a relatively high amount of neutral sugars (40.9 mol%), indicating that it contained relatively more 'hairy' regions than pool A. Since pool B eluted later from the anion-exchange column than pool A, it was concluded that it had a higher charge or charge density than the 'smooth' regions. This was confirmed by the lower DM of this pool. Both pool A and B had a higher DA

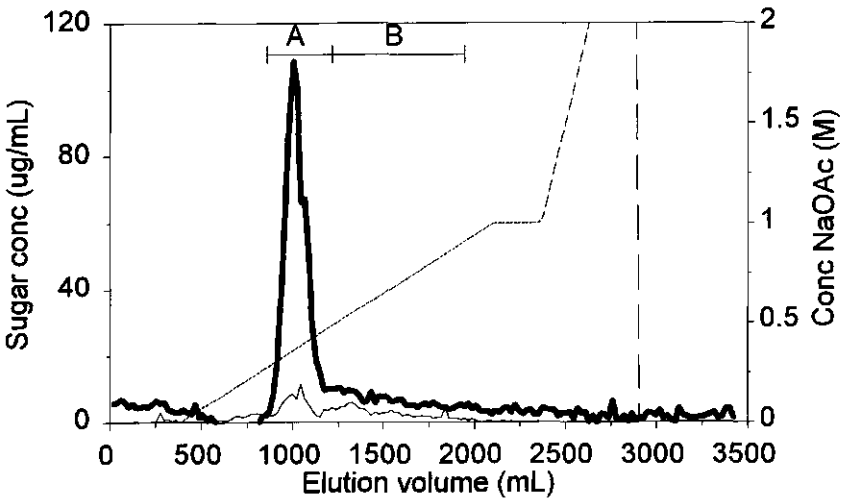

Fig. 1. DEAE anion-exchange chromatography of ABP. Thick line: uronic acid, thin line: neutral sugars, dotted line: NaOAc.

than the original extract. This is possibly caused by a loss of material with a low ester content on the anion-exchange column, as the total recovery of polysaccharides after anion-exchange chromatography was approximately 85 %. The DF in pool B was higher than in pool A.

Methylation analysis. - The nature of the glycosidic linkages of the neutral sugar residues in the acid extracted beet pectin and in the DEAE pool B was determined by methylation analysis (Table 2) in order to obtain information about the presence and degree of branching of the side-chains of the 'hairy' regions. Rhamnose was found in these samples in almost equal amounts of (1→2)- and (1→2,4)-linked residues, as was also observed for sugar beet pectins obtained by autoclaving [18, 20]. Terminally linked rhamnose was present in small amounts. Arabinose was mainly terminally and (1→5)-

Table 2

Neutral sugar linkage composition of ABP and ABP pool B (mol%).

		ABP	ABP pool B
Rhamnose	T-Rha *p* [a]	1.2	1.2
	1,2-Rha *p* [a]	10.0	11.0
	1,2,4-Rha *p*	8.0	10.5
		19.2	22.7
Arabinose	T-Ara *f*	18.2	13.8
	1,3-Ara *f*	1.6	0.0
	1,5-Ara *f*	9.6	10.0
	1,2,5-Ara *f*	1.7	1.9
	1,3,5-Ara *f*	4.1	4.5
	1,2,3,5-Ara *f*	2.7	0.4
		37.9	30.6
Xylose	T-Xyl *p*	1.2	0.0
	1,2-Xyl *p*	0.0	0.0
	1,4-Xyl *p*	0.6	1.4
	1,2,4-Xyl *p*	0.4	0.0
	1,3,4-Xyl *p*	0.3	0.0
		2.5	1.4
Galactose	T-Gal *p*	16.1	11.1
	1,4-Gal *p*	12.9	11.9
	1,6-Gal *p*	8.4	8.0
	1,3,6-Gal *p*	0.2	0.0
	1,4,6-Gal *p*	0.1	4.6
		37.7	35.5
Glucose	1,4-Glc *p*	0.7	3.4
	1,4,6-Glc *p*	1.8	4.6
		2.5	8.0
Mannose	1,4-Man *p*	0.0	1.7
		0.0	1.7

[a] T, terminal; 1,2-linked Rha, etc.

linked. (1→3,5)- and (1→2,3,5)-linked residues were found in relatively small amounts as compared with pectins obtained by autoclaving [18, 20]. This suggests that on average the arabinan side branches are relatively short and more linear, which is probably caused by the acid extraction conditions. High amounts of terminally linked galactose were found in both extracts, as well as (1→4)- and (1→6)-linked residues. The high amount of terminally linked galactose is in agreement with the presence of rhamnogalacturonans with single galactose residues attached to it, as described by Schols et al. [31] for apple modified 'hairy' regions (MHR). A difference in the neutral sugar linkage composition between ABP and ABP pool B was the relatively high amount of glucose in the latter, which had mainly (1→4)- and (1→4,6)-linkages. Possibly this glucose originates from xyloglucans. It can be speculated that the fact that it was found in a population which bound to the DEAE column might indicate that xyloglucans and rhamnogalacturonans are linked together. So, further investigation is needed to confirm this observation.

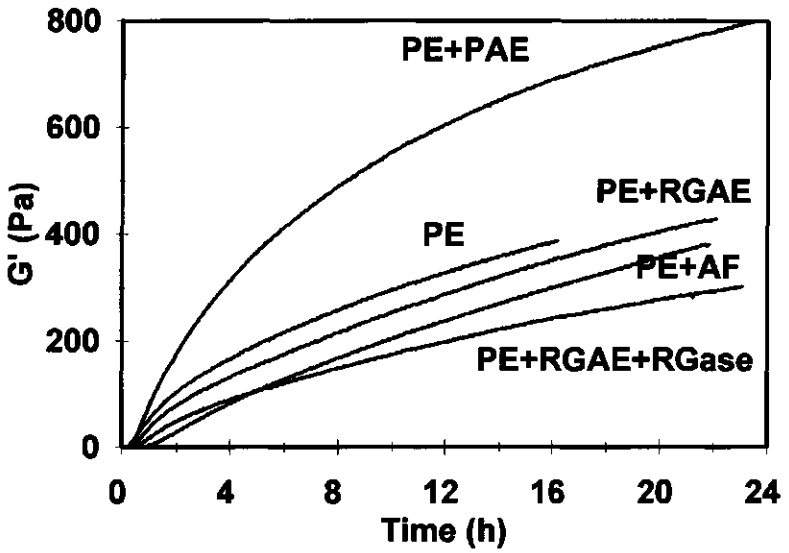

Fig. 2. Development in time of the storage modulus (G') of ABP+Ca^{2+} during treatment with several enzyme combinations.

Effect of enzymatic modification on gel formation of ABP with Ca^{2+}. - ABP was treated with the following (combinations of) enzymes to investigate their effect on the gel formation with Ca^{2+}: PE, PAE, PE+PAE, PE+RGAE, PE+RGase+RGAE, PE+AF. Ca^{2+} was necessary to obtain a gel with pectin upon treatment with PE. Previously it was shown that Ca^{2+} is also necessary for optimal activity and stability of PAE [11]. The formation of the gel networks was investigated by small amplitude shear strain oscillatory testing. Fig. 2 shows the development of the storage modulus (G') in time, which is indicative for the amount of elastically effective cross-links formed, as well as for the stiffness of the gel. Table 3 summarizes the rheological parameters of the networks formed after enzyme treatment for 16 h and Table 4 shows the percentage of acetyl groups and methyl esters released after 20 h.

Both the presence of Ca^{2+} and PE proved to be necessary to obtain a gel from the acid extracted sugar beet pectin. The storage modulus of the gel thus formed leveled of within 16 h. The value of G' at this point was 383 Pa. Approximately 15 % of the methyl groups were released in this period. This combination was used as a reference sample for the other combinations.

Treatment of the pectin-Ca^{2+} mixture with PAE alone did not lead to gel formation. Approximately 8.5 % of the acetyl groups initially present in ABP was released by this treatment. This acetyl release was much lower than found by Searle-van Leeuwen et al. [11], who measured an acetyl release of 30 % with PAE. The lower acetyl release in the current study was probably due to the use of another buffer system.

Table 3

Storage modulus (G'), loss modulus (G") and tan δ of gels formed with ABP or ABP pool I in combination with Ca^{2+} and various enzyme combinations (16 h).

		G' (Pa)	G" (Pa)	tan δ (-)
ABP	Ca^{2+}	-	-	-
	PE	-	-	-
	PAE+Ca^{2+}	-	-	-
	PE+Ca^{2+}	383	21.9	0.057
	PE+PAE+Ca^{2+}	690	32.6	0.047
	PE+RGAE+Ca^{2+}	350	18.8	0.054
	PE+RGase+RGAE+Ca^{2+}	240	14.3	0.060
	PE+AF+Ca^{2+}	306	16.6	0.054
ABP pool I	PE+Ca^{2+}	30	1.6	0.052
	PE+PAE+Ca^{2+}	56	1.9	0.034

-: no gel formed

Table 4

Percentage of acetyl groups and methyl esters released after 20 h by PE, PAE, and RGAE, alone and in combination with each other and RGase and AF, during treatment of ABP or ABP pool I in combination with Ca^{2+}.

		20 h	
		% Ac	% Me
ABP	PE	0.0	10.7
	PAE+Ca^{2+}	8.5	0.0
	PE+Ca^{2+}	0.0	14.8
	PE+PAE+Ca^{2+}	13.8	27.2
	PE+RGAE+Ca^{2+}	13.5	28.9
	PE+RGAE+RGase+Ca^{2+}	20.4	24.8
	PE+AF+Ca^{2+}	5.5	14.2
ABP pool I	PE+Ca^{2+}	0.0	11.0
	PE+PAE+Ca^{2+}	15.0	27.9

The addition of PE+RGAE to the pectin-Ca^{2+} mixture resulted in an increased hydrolysis of acetyl groups as well as methyl esters by approximately 14 % in comparison with PE alone. This indicates that the acetyl groups hindered the action of PE, as was already suggested by Pippen et al. [8]. Since RGAE is only active on the 'hairy' regions and the activity of PE increased when RGAE was added, it can be concluded that PE is able to remove methyl groups from these 'hairy' regions. The gel formation curve, however, was similar to the curve without RGAE. Another conclusion from this experiment is that the addition of RGAE did not result in a higher amount of Ca^{2+} cross-links between pectin molecules.

The addition of PE+RGase+RGAE to the pectin-Ca^{2+} mixture led to lower values for G', which was probably caused by partial degradation of the rhamnogalacturonan backbone of the 'hairy' regions by RGase, resulting in a lower apparent molecular weight. The total release of acetyl groups was higher than in the absence of RGase.

The combination of PE+PAE released more acetic acid and methanol from the pectin-Ca^{2+} mixture, originating from the 'smooth' homogalacturonan regions, than PE or PAE alone. The values for G' were twice as high as with PE alone. This can be explained by the fact that the formation of Ca^{2+} cross-links increased with an increasing amount of free carboxyl groups, due to the removal of the methyl groups. Here it is shown that the PE activity increased when it acts together with PAE and that the PAE activity increased when it is combined with PE. This observation suggests that the acetyl groups interfere with the action of PE and that methyl groups interfere with the action of PAE. Searle-van Leeuwen et al. showed that demethylation of an acid

extracted beet pectin with PE also increased the activity of PAE [11]. In literature, it is shown [8] that the acetyl groups interfere with the "egg-box" formation with Ca^{2+}, however, this could not be concluded directly from our experiments.

Treatment of the pectin-Ca^{2+} mixture with a combination of PE+AF resulted in similar values for the storage modulus as when AF was omitted. This is in agreement with the assumption made by Hwang et al. that side branches of pectin have a relatively small influence on gel formation with Ca^{2+} [32]. Furthermore, the arabinose content of pectins obtained by acid extraction is relatively low and will have relatively little influence on the physico-chemical properties [33].

Additionally, tan δ (= G''/G') is a good parameter to describe the nature of the gel. G'' is indicative for relaxation of cross-links. A low value for tan δ (< 0.1) shows the presence of an elastic system, whereas high values (> 1) imply a more liquid-like character of the network [34]. All the values found for tan δ were lower than 0.060, showing that for all combinations in which gel formation was observed an elastic system was formed. While relatively large differences were found for G', the differences for tan δ were much smaller. The lowest value for tan δ was found for PAE+PE, which shows that the gel formed had a somewhat more elastic nature in comparison to the other combinations. It can be concluded that the enzyme treatments did not change the elastic nature of the gels as much as they changed the stiffness of the gels.

Treatment with rhamnogalacturonase. - To investigate the role of the 'smooth' regions in gel formation with Ca^{2+}, the original beet pectin was treated with RGase in order to degrade the 'hairy' regions. Although it is known that the removal of acetyl groups by RGAE is necessary for RGase to work optimally on modified 'hairy' regions from apple [11], we found that ABP was degraded to the same extent without the use of RGAE based on the HPSEC results (data not shown). This indicates that sufficient acetyl free regions must have been present in beet pectin for RGase to act on. Based on their molecular size, two populations were present after the enzyme treatment, as shown by HPSEC (Fig. 3). A population, containing most of the material as indicated by refractive index readings, eluted at 26 min after the RGase treatment. This population had a low A_{335} absorption (indicating a low ferulic acid content). Since it is known that ferulic acid is only present in the 'hairy' regions [1-5], it was concluded that this population contained a low amount of 'hairy' regions and a high amount of 'smooth' regions. Upon RGase treatment a small population was formed, which eluted at 38 min. Based on the high A_{335} absorption, it was concluded that this population contained a relatively high proportion of 'hairy' regions.

The pools were separated using a S 300 preparative size-exclusion column (Fig. 4). Only approximately 65 % of the polysaccharides originally present in the extract were recovered. 15 % of the material was lost during dialysis after the enzyme treatment, which was caused by removal of the rhamnogalacturonan oligomers and by

some other oligosaccharides already present in the original pectin. Furthermore, some material was lost during the purification. The high galacturonic acid content of pool I (79.9 mol%) shows that it predominantly consists of homogalacturonans (Table 1). The amount of neutral sugars had decreased from 27.1 mol% to 20.1 mol% as compared with the untreated beet pectin. Pool I had a lower intrinsic viscosity than the original material, which is in contradiction with a previous study [33], in which it was shown that treatment of ABP with a RGase+RGAE decreases the molecular weight but not the intrinsic viscosity. Possibly the solubility of highly viscous pectins is decreased after degradation of the rhamnogalacturonans with RGase+RGAE and subsequent purification and freeze drying. Pool II contained a high proportion of neutral sugars, confirming that this pool mainly consists of 'hairy' regions. Rhamnose was present in a high proportion as compared with the neutral sugar composition of the original material. The DM of pool I was much higher than that of pool II, confirming that the 'smooth' regions have a lower charge or charge density than the 'hairy' regions.

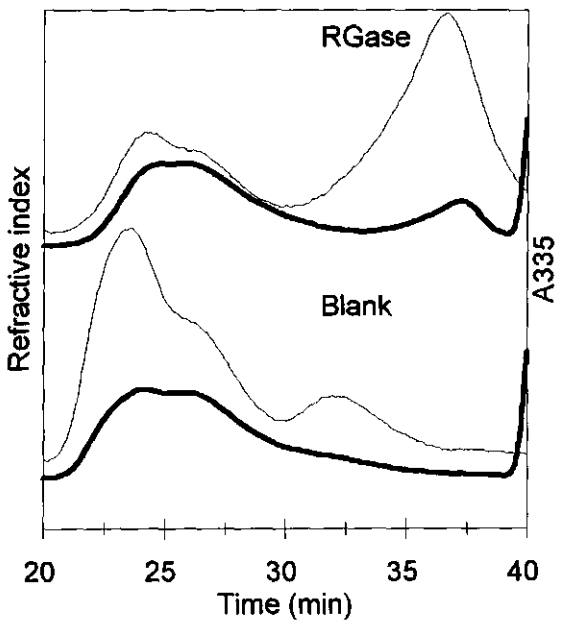

Fig. 3. HPSEC elution pattern of ABP before and after treatment with rhamnogalacturonase (RGase). Thick line: RI, thin line A_{335}.

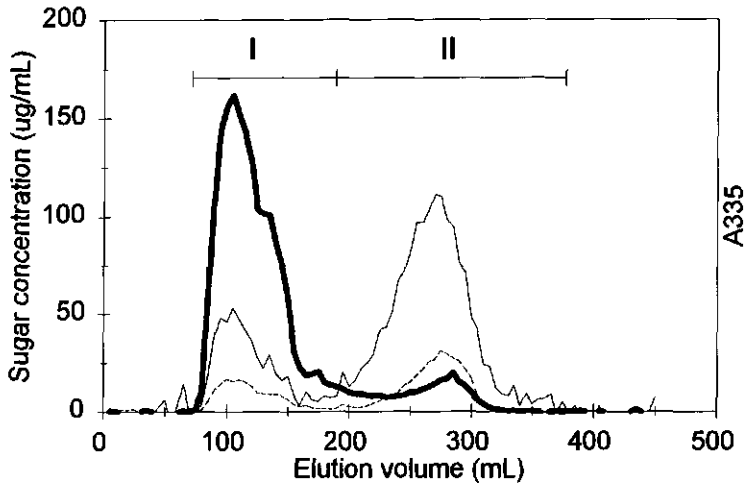

Fig. 4. S 300 SEC elution pattern of ABP after treatment with rhamnogalacturonase (RGase). Thick line: uronic acid; dashed line: neutral sugars; thin line: A_{335}.

The DA in the 'hairy' regions was higher than in the 'smooth' regions. This can be partly explained by the observation that the RGase activity is higher on pectin regions without acetyl groups [14, 35]. These acetyl free regions are then degraded to oligomers, which are (partially) lost during dialysis. Consequently, the remaining polymeric material will have a higher acetyl content. On the other hand, it was shown that 'hairy' regions purified from an autoclave extract from beet pulp had a comparable DA [20]. As was already seen in the HPSEC analysis, most of the ferulic acid was located in pool II.

Effect of enzymatic modification on gel formation of ABP pool I with Ca^{2+}. - To determine the effect of the 'hairy' regions on gel formation with Ca^{2+} and PE, a mixture of Ca^{2+} and pool I, representing the 'smooth' homogalacturonan regions of ABP was treated with PE or with PE+PAE. Fig. 5 shows the development of the storage modulus (G') in time. The values of G', G" and tan δ after 16 h are shown in Table 3. A gel was formed after addition of PE to the mixture of pool I and Ca^{2+}. The maximum value for G', indicative for the stiffness of the gel, was lower than that of the original pectin, which was probably caused by the lower apparent molecular weight of ABP pool I (Table 1). Furthermore, the gel was formed much slower. The amount of acetyl groups and methyl esters released was of the same level as that released from ABP.

Again, a higher maximum value for G' was found after addition of PE+PAE to the mixture in comparison with PE alone. Tan δ was somewhat lower for the mixture of pool I plus Ca^{2+} with PE+PAE than with PE alone, again showing that the addition of these enzymes gives a more elastic gel.

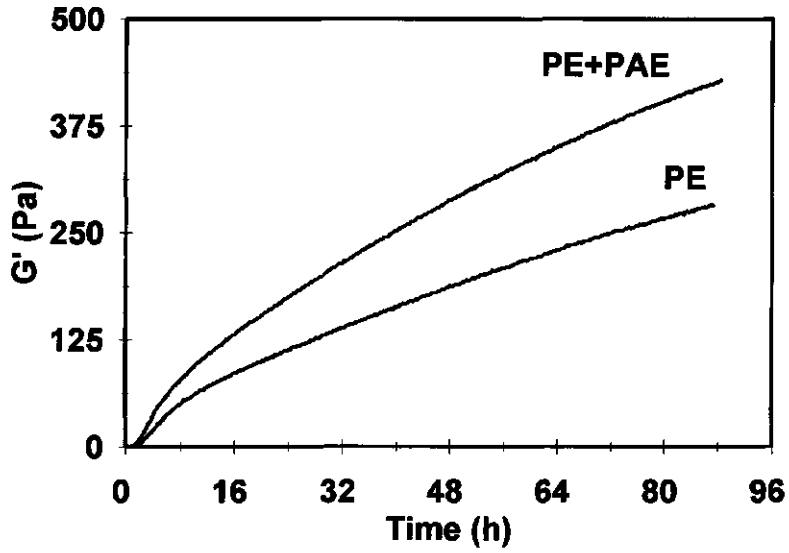

Fig. 5. Development in time of the storage modulus (G') of ABP pool I +Ca^{2+} during treatment with PE or PE+PAE.

Also for pool I addition of PAE led to a higher release of both acetyl groups and methyl esters during this experiment, confirming that the acetyl groups interfere with the action of PE (Table 4).

Concluding remarks

The acid extracted sugar beet pectin used in this study contained 'smooth' regions as well as a relatively low amount of 'hairy' regions. Relatively high amounts of ester groups (acetyl, methyl, feruloyl) were present. Both DEAE anion-exchange chromatography as well as degradation with the enzyme RGase, followed by preparative size-exclusion chromatography, showed that the 'smooth' regions had a higher DM and a lower DA than the 'hairy' regions, which were linked to them in the original extract. Fractionation and characterization of fractions obtained from ABP treated with RGase also suggested that the original ABP contained on average a low

number of 'hairy' regions per molecule.

The ABP formed a gel in the presence of Ca^{2+} only after partial demethylation by PE. Addition of only PAE to the pectin-Ca^{2+} mixture did not result in gel formation. Treatment of the pectin-Ca^{2+} mixture with PE+RGAE or PE+PAE increased the release of both acetyl groups and methyl esters in comparison with PE or PAE alone, indicating that PE is hindered by the presence of acetyl groups and that PAE is hindered by the presence of methyl esters. The increased release of methyl esters also explains the increased stiffness of the gel due to the addition of PE+PAE to the pectin-Ca^{2+} mixture. Since addition of RGAE, which is only active on the 'hairy' regions, also led to an increase of methyl groups released, it is assumed that PE is also active on methyl esters apparently present in the 'hairy' regions.

Treatment of the pectin-Ca^{2+} mixture with PE+RGAE did not increase the stiffness of the gel as compared with treatment with PE, although additional acetyl groups and methyl esters were released from the 'hairy' regions in comparison with PE alone. Also, addition of PE+AF to the pectin-Ca^{2+} mixture did not increase the stiffness of the gel in comparison with PE. From these results it can be concluded that the acetyl groups in the 'hairy' regions, as well as the arabinose side-chains do not interfere with the gel formation with Ca^{2+}. The combination PE+RGAE+RGase did result in a lower stiffness of the gel than PE alone. It was shown that the various enzyme treatments did not change the elastic nature of the gels as much as they changed the stiffness of the gels.

A fraction representing the 'smooth' homogalacturonan regions, which was obtained after treatment of the beet pectin with rhamnogalacturonase and subsequent size-exclusion chromatography, was also able to form a gel with Ca^{2+} and PE. However, the gel formed much slower, and the stiffness of the gel was lower in comparison with PE alone. Also with this modified pectin the treatment with PAE plus PE gave an increased stiffness of the gel in comparison to PE alone.

It was shown that removal of acetyl groups from the 'smooth' regions of sugar beet pectin by the enzyme pectin acetyl esterase increases the action of pectin methyl esterase and therefore improves the gelling capacity of this type of pectin with Ca^{2+}. Since the poor gelling properties of sugar beet pectins hampered its widespread application in industry, these observations may lead to a more general use of beet pectins in the pectin industry. However, the availability of relatively pure enzymes in large quantities is essential.

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CHAPTER 9

General discussion

Already for a long time, sugar beet pulp has been recognized as a potential source of pectins [1] because of its high pectin content, high availability, and low price. However, beet pulp pectin has never been used on a large scale because of its poor gelling characteristics. This has been attributed to the high acetyl content of sugar beet pectin, besides a high neutral sugar content and a fairly low molecular weight [2].

An important impulse to the research on pectins from sugar beet pulp was given by the work of Rombouts and Thibault in 1983 [3], who showed that feruloyl esters are present in sugar beet pectin obtained by acid extraction. They found that these pectins can be used for gel formation through oxidative cross-linking. Furthermore, the chemical characterization of beet pectins obtained by treatment with acid and mild alkali and the mechanism of cross-linking were subject of investigation by this group [4-14].

Research on pectins from sugar beet pulp also focused on the removal of the acetyl groups from these pectins, which were assumed to be responsible for their poor gelling characteristics [15, 16]. Indications were found that treatment of sugar beet pectin with acetyl esterase improves the gel formation with calcium. However, a serious drawback in these studies was the use of partially purified and poorly characterized enzymes, which makes it difficult to draw conclusions.

Another application for pectic polysaccharides from sugar beet pulp was described by Cooper et al. [17]. They showed that arabinans extracted by strong alkali from beet

pulp were able to gel after linearization with the enzyme arabinofuranosidase [17, 18].

Despite the developments of the last 20 years the production of pectins from sugar beet pulp has not been established on a large scale. The research described in this thesis deals with the development of alternative extraction methods aimed at preserving the feruloyl groups and/or the neutral sugar side-chains of beet pectin. The extracts thus obtained may be a better representation of pectins present in the cell wall of sugar beet than the traditionally used acid extracted pectins. The extracts were characterized chemically and physically after fractionation, in order to obtain knowledge about their structure and physical properties. A further aim was to improve the physico-chemical properties of sugar beet pectins by specific modification through oxidative cross-linking or with appropriate enzymes.

Validity of cell wall models for the cell wall of sugar beet

Several models for the cell walls of dicot and non-graminaceous monocot plants have been proposed [19-21]. In these models the cell wall is believed to consist of a framework of cellulose micro fibrils. The micro fibrils are interlinked through xyloglucans, thus creating a space between these fibrils. The cellulose-xyloglucan framework (about 50 % of the cell wall mass) is imbedded in a matrix of pectic polysaccharides (about 30 % of the cell wall mass) [20]. Pectin is present in the cell wall as a highly concentrated gel [22]. The pectin matrix determines various cell wall properties: the porosity, the surface charge, pH, and ion balance. The pectins also serve as recognition molecules that signal developmental responses to other organisms [23]. Most of these functions are attributed to the homogalacturonan region of the pectins. Limited information is available about the role of the ramified 'hairy' regions in the cell wall .

In sugar beet the amount of pectins, and in particular of the 'hairy' regions, is high compared with the plant material on which the cell wall models were based (onion, pea, and sycamore). It has been reported [24] that in sugar beet cell walls the sugar moieties of pectic polysaccharides (arabinose, rhamnose, galactose and galacturonic acid) account for more than 50 % (w/w) of the cell wall material, whereas the cellulose-xyloglucan matrix accounts for less than 30 %. It can be calculated from the sugar composition of beet pulp as presented in Chapter 2 [25] that approximately 70 % of the pectin in sugar beet pulp consists of branched rhamnogalacturonan, assuming a rhamnose to galacturonic acid ratio of 1. However, the rhamnogalacturonan chain accounts only for about 20 % of the pectic backbone. In sugar beet both values are probably somewhat lower, since sugar beet is subjected to an extraction with water during the sugar extraction process, which leads to the removal of a galacturonic acid

rich fraction from the beet pectin [26]. In the 'hairy' regions isolated from beet pulp only pectin related arabinans were found and no free arabinans/arabinogalactans (Chapter 3) [27], this being a major difference with the cell wall model as proposed for pea [21]. Furthermore, sugar beet pectin contains high amounts of acetic acid in both homo- and rhamnogalacturonans and ferulic acid mono- and dimers in the rhamnogalacturonans, as opposed to pectins in many other species.

Some observations described in literature may give an indication for the role of branched rhamnogalacturonans in sugar beet cell-walls. The arabinan side-chains of the rhamnogalacturonans influence the hydratation properties of pectin matrix and might play a role in the entanglement of the pectin in the cell wall [28]. Also, it is suggested that rhamnogalacturonans can be cross-linked to other pectins through diferulic acid bridges [29].

Another cross-linking mechanism of pectic polysaccharides in the cell wall is calcium binding, which is mainly determined by the amount of methyl esters present [22]. Apart from these methyl esters, the acetyl groups play a role as well in the regulation of the calcium binding properties of the pectin, which is important for both cell-cell adhesion and the porosity of the cell walls [30]. The elution behaviour of the rhamnogalacturonans on the anion-exchange column (Chapter 3) [27] might implicate that in these polysaccharides carboxyl groups are located in each others vicinity, which leads to a relatively high charge density. Therefore, calcium binding might be possible, although the presence of side-chains to the rhamnogalacturonans might interfere with this.

Although much has been published about the structure of pectins, some of which is also presented in this thesis, many questions about the implications for the cell wall architecture remain. Especially the role of rhamnogalacturonans, arabinans, and acetyl and feruloyl groups, which are all present in high amounts in sugar beet cell walls, is not fully clear. The role of these structural elements in the cell wall needs further study, in order to understand the complex structure of plant cell walls.

Structural features of sugar beet pectic and hemicellulosic polysaccharides

Cell wall polysaccharides. - Based on its sugar composition as determined in Chapter 2, it can be calculated that the polysaccharide part of sugar beet pulp consists for 60-62.5 % of pectic polysaccharides, for 30-32.5 % of cellulose, and for 5-7.5 % of xyloglucan [25]. In Chapter 3 indications were found that some glucomannan was also present in sugar beet pulp, probably less than 3 % [27]. Our research mainly focused on the pectic polysaccharides from sugar beet pulp. Also, some structural characteristics of xyloglucans from sugar beet pulp are described in Chapter 3.

The structure of beet pectin. - Based on the structural characteristics of extracted pectins an image can be obtained of pectins in their native state. These native pectins are assumed to consist of alternating 'smooth' homogalacturonan regions and highly branched 'hairy' regions [31]. This was also found to be the case for extracted beet pectins [32], which was confirmed in Chapter 7, since degradation of the homogalacturonan regions of an acid extracted beet pectin with a combination of pure PG+PE resulted in a shift of the 'hairy' regions to later retention times upon size-exclusion chromatography [33], indicating a lower hydrodynamic volume. A schematic representation of a native sugar beet pectin is given in Fig. 1A.

'Smooth' regions. - In Chapter 3 a degree of polymerization was found of 120 galacturonic acid residues for the 'smooth' regions of beet pectin obtained by autoclaving (Fig. 1B, pool IIIb) [27], which was comparable to the value of 72-100 found by Thibault et al. after acid hydrolysis [34]. It is very likely that in both studies the backbone has been subject to degradation (β-elimination in the autoclave extracted pectin; hydrolysis in the acid treatment). Therefore, higher degrees of polymerization are expected for the native homogalacturonans. Assuming that rhamnose and galacturonic acid are present in equal amounts in the rhamnogalacturonans, it can be calculated from the sugar composition that the rhamnogalacturonan backbone represents 4 % of beet pulp, whereas homogalacturonan represents 17 % [25]. The length of the homogalacturonan regions in Fig. 1A was based on these percentages. However, in this model it is assumed that all pectin in beet has a similar structure, although it is known that variations of the pectin structure occur, depending on the location in the cell wall and on the nature of the tissues in which the cell wall is present [22].

'Hairy' regions. - The autoclave extracts contained 'hairy' regions with apparent molecular weights as high as 120 kDa and 1300 kDa as determined by light-scattering (Fig. 1B, pool I and II). The value of 1300 kDa is high in comparison with literature values for pectic 'hairy' regions from beet pulp, as determined by viscosity measurements [11] or in comparison with values based on retention times of pectin standards as measured by size-exclusion chromatography [25]. Considering that the rhamnogalacturonans present in the extract obtained by autoclaving consist for less than 10 mol% of rhamnose and galacturonic acid, whereas it contains 85 mol% of neutral sugars [35], it can be calculated that the rhamnogalacturonan backbone has a molecular weight of approximately 100-150 kDa (= 650-1000 residues). This value is in agreement with the results found by McNeil et al., who suggested a length of the backbone of RG-I of up to 300 rhamnose and 300 galacturonic acid residues for suspension cultured sycamore cell-walls [36], representing a molecular weight of approximately 100 kDa. Another possible explanation for the high apparent molecular weight found for the rhamnogalacturonans from sugar beet pulp is the presence of

ferulate dimers as shown in Chapter 4 and 5 [35, 37], which covalently cross-link several rhamnogalacturonan molecules, as is shown in Fig. 1A and B. Some indications for this assumption were found after treatment of the rhamnogalacturonans with endo-arabinanase and arabinofuranosidase (Chapter 7) [33].

The DM in the 'hairy' regions of beet pectins obtained by acid extraction or autoclaving (~25) was considerably lower than that of the 'smooth' regions (~60), whereas the DA was much higher (~60 vs ~20 for the 'smooth' regions) [27]. These values show that the acetyl groups are not evenly distributed amongst the subunits present in pectins, which may be related to their function in the cell wall.

In apple 'hairy' regions three types of subunits were found [38]. The rhamnogalacturonan subunit with single unit galactose side-chains and the highly branched rhamnogalacturonan subunit were also present in the autoclave extracts from beet pulp (Chapter 3) [27]. 30-50 % of the galactose in the autoclave extracts was present as terminally linked residues, and was probably located in the rhamnogalacturonan subunit with single unit galactose side-chains. The remaining galactose residues were predominantly (1→4)- and (1→6)- linked, and are assumed to be present in the highly branched subunit. Sakamoto and Sakai [39] suggested that stretches of (1→4)-linked galactans may interlink the rhamnogalacturonan backbone and part of the arabinan side-chains, based on a degradation study of an alkali extract from beet pulp with an endogalactanase. The arabinan side-chains of the rhamnogalacturonans present in an autoclave extract from sugar beet pulp were found to be highly branched and a DP was calculated of more than 45 arabinose residues for these side-chains (Chapter 3) [27]. The highly branched subunit had an apparent molecular weight of approximately 50 kDa [33]. In the extracts obtained by autoclaving and by treatment with alkali some (1→3,4)-linked galacturonic acid was found (Chapter 2) [25], which may be an indication for the presence of the xylogalacturonan subunit. However no additional evidence for the presence of this subunit was found. The 'hairy' regions isolated from a cross-linked autoclave extract by size-exclusion chromatography consisted for more than 95 % of rhamnogalacturonans. However, these 'hairy' regions contained a minor amount of xylose (0.5 mol%; Chapter 5) [35]. Further study is needed to determine whether this xylose originates from xylogalacturonan.

The free arabinans (see Fig. 1B, pool IIIa) present in the autoclave and alkali extracts probably originated from the rhamnogalacturonans as was shown in Chapter 3, and had a degree of polymerization of 130-170. It was estimated that the backbone of these arabinans contained 60-70 arabinose residues (Chapter 7) [33]. The latter was in agreement with the observations of Cooper et al., who found similar values for arabinans which were linearized with the enzyme arabinofuranosidase [17].

Ferulic acid. - Thibault et al. [10-14] reported that ferulic acid in sugar beet pectin

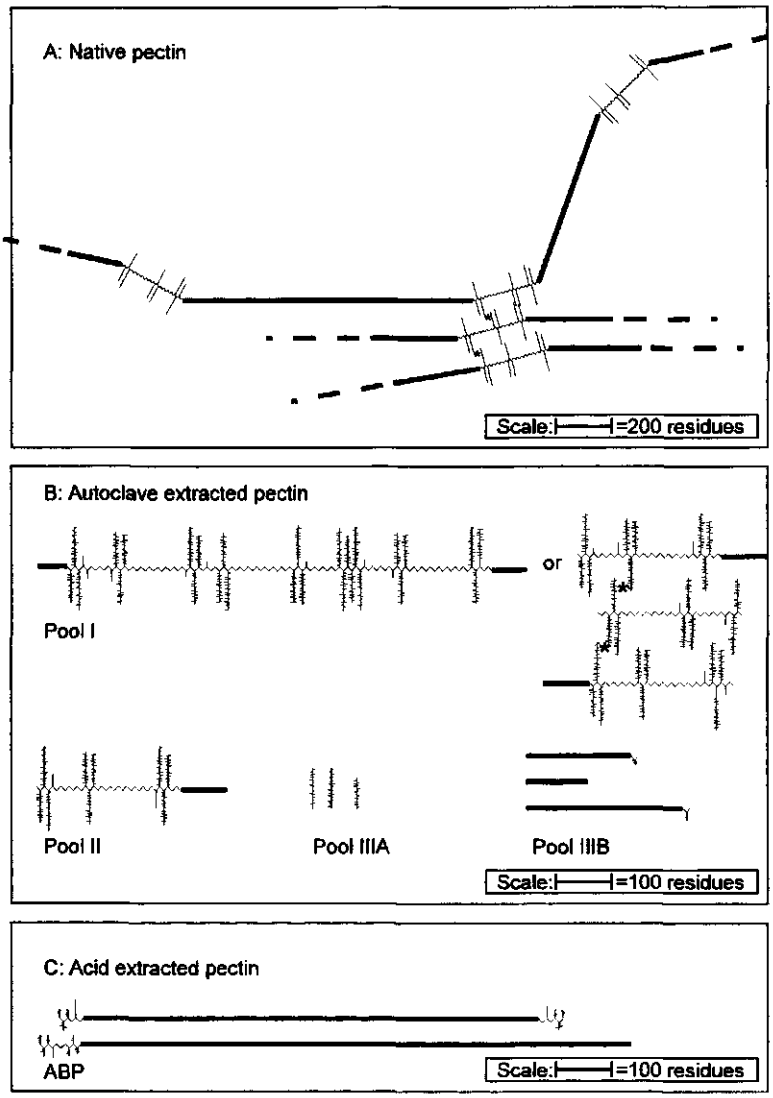

Fig. 1. Schematic representation of a native sugar beet pectin (A), of the pools purified from an autoclave extract from sugar beet pulp (B), and of an acid extracted beet pectin (C). Thick lines: 'smooth' homogalacturonan regions, thin, corrugated lines: rhamnogalacturonans, * ferulic acid cross-links.

is solely located in the 'hairy' regions. This was confirmed by our results, since the ferulic acid present in the extracts obtained by autoclaving and acid treatment coeluted with the neutral sugars both upon size-exclusion and anion-exchange chromatography [27, 40]. In Chapter 4 it was shown that ferulic acid dehydrodimers represented 9 % of all ferulates in an autoclave extract [37]. Waldron et al. reported that in sugar beet over 20 % of the ferulates are present as dimers [41]. From this it can be concluded that 'hairy' regions with a low amount of dimers are first released during autoclave extraction. The above is consistent with the suggestion by Waldron et. al that ferulic acid cross-linking plays a role in the cell-cell adhesion, and therefore also in the texture of sugar beet [41]. Since degradation of the rhamnogalacturonans (obtained from an autoclave extract from beet pulp) with endo-arabinanase and arabinofuranosidase B resulted in a more extensive decrease in apparent molecular weight than was expected (based on the apparent molecular weight and the sugar composition before enzyme treatment), it was suggested that the extracted rhamnogalacturonans are also linked by diferulic acid cross-links [33]. In Fig. 1B an example of two rhamnogalacturonans of the same molecular weight is given (pool I), one of which is composed of three rhamnogalacturonans cross-linked by diferulic acid bridges. Degradation of the arabinan side-chains in the cross-linked molecule will result in a lower molecular weight of the remaining material than degradation of the molecule without cross-links. However, further experiments are needed to establish the presence of these cross-links between rhamnogalacturonans. Four types of ferulic acid dehydrodimers were identified in the autoclave extract [37]. The 8-O-4 dehydrodimer was the predominant dimer, whereas the 8-5, the 8-8, and the 5-5 dehydrodimer were found in lower amounts. These results show that the oxidative cross-linking process through ferulic acid in cell walls follows several reaction routes [35, 37].

Xyloglucans. - For the first time the presence of xyloglucans in beet cell walls was demonstrated (Chapter 3) [27]. This was done by enzymatic degradation of the neutral population of an alkali extract from beet pulp with an endo-glucanase. The oligomers found in the digest were XXG, XXXG, XXFG, XLFG and probably a small amount of XLXG according to the nomenclature of Fry et al. [42]. So, three out of four glucose residues carried a side-chain, as was also seen for xyloglucans from many species such as apple, sycamore, and tamarind [43, 44]. Vincken et al. [45] named this class of xyloglucans the poly-XXXG type. This is in contrast with xyloglucans from potato and tomato, both belonging to the *Solanaceae*, for which the presence of two adjacent unbranched glucose residues is characteristic [46], and which belong to the poly-XXGG type.

Extraction of sugar beet pectic polysaccharides

Extraction methods. - On an industrial scale pectins are usually obtained from apple and citrus residues using hot acid treatment (pH = 1-2) [47]. Arabinans on the other hand can be extracted from sugar beet pulp with hot alkali [48]. The latter treatment results in β-eliminative degradation of the homogalacturonan backbone and in loss of all ester groups, caused by saponification. In the current research we predominantly investigated pectins obtained by autoclaving or acid treatment. Both treatments degrade the native pectin, and therefore, the structure of the resulting pectic polysaccharides is different from the native form, as described below.

Acid extracted beet pectins. - A large amount of sugar beet pulp (~19 % w/w) is solubilised by hot dilute acid (0.05 N HCl, 85°C) [32]. Guillon and Thibault [5] showed that subsequent treatment of such an acid extracted sugar beet pectin with diluted acid (TFA) results in further degradation of the pectin. Arabinose is the first sugar to be released, followed by galactose, ferulic acid, and rhamnose. Galacturonic acid is released at the lowest rate. If the same processes are assumed to take place in native pectin during acid extraction, acid treatment will primarily degrade the arabinan side-chains, and to a lower extent the rhamnogalacturonan backbone. The results described in Chapter 7 showed that the average molecular weight of the 'hairy' fragments which remained after treatment of an acid extracted pectin with PG+PE is 50 kDa, which is much lower than the values obtained by autoclaving [33]. The material which remained after treatment with RGase+RGAE and which was considered to be a homogalacturonan, had an apparent molecular weight of ~100 kDa as determined by light-scattering, implicating a DP of ~550, which is much higher than the value found by Thibault et. al (72-100) based on the viscosity average molecular weight [5]. However, 10 mol% of neutral sugars was still present in this 'homogalacturonan', indicating that not all rhamnogalacturonan was degraded by the enzymes. The intrinsic viscosity of the acid extracted pectin hardly changed after treatment with RGase+RGAE, although the molecular weight decreased significantly. From these results it is concluded that acid extracted pectins can be viewed as a very heterogeneous material containing homogalacturonan stretches, at their extremities attached to 'hairy' regions with remnants of degraded arabinan side-chains. A schematic representation of acid extracted beet pectin is shown in Fig. 1C.

Autoclave extraction. - For the current research we were interested in obtaining pectic polysaccharides in a more native state, preserving the arabinan side-chains and the ester groups, in order to be able to cross-link and enzymatically modify these polysaccharides and in this way manipulate the functional properties. Therefore, we used an extraction method based on autoclaving (Chapter 2) [25]. Non-solubilized

arabinans and rhamnogalaturonans were extracted by subsequent strong alkali treatment. The autoclave extraction yielded arabinose rich pectic polysaccharides with a high ester content, and with a yield comparable to that obtained with mild acid or alkali extractions (~15 %) [4]. Subsequent strong alkali treatment yielded another extract which contained 19 % of the original pulp and mainly consisted of rhamnogalacturonans with a high arabinose content and a very low ester content.

The populations present in the autoclave extract were characterized after purification by anion-exchange chromatography and preparative size-exclusion chromatography (Chapter 3) [27]. Four populations were characterized: two populations mainly consisting of high molecular weight rhamnogalacturonans (see Fig. 1B, pool I and II), a population containing homogalacturonans (Fig. 1B, pool IIIb), and a population consisting of arabinans (Fig. 1B, pool IIIa). Indications were found that during autoclaving the homogalacturonans were degraded to a DP of 120, resulting in a low intrinsic viscosity. The homogalacturonans were highly methylated and contained less acetyl groups than the 'hairy' regions. The rhamnogalacturonans derived from the autoclave extract had relatively high apparent molecular weights of 1300 and 120 kDa, a high degree of acetylation, and a low degree of methylation. Also some arabinans were released during autoclaving, which probably originated from the rhamnogalacturonans.

The crude autoclave extracts were very suitable for gel formation by oxidative cross-linking as shown in Chapter 5 [35], but are probably not very useful for the gel formation with acid/sugar or calcium ions as a result of the low molecular weight of the homogalacturonans present and the relatively high degree of esterification (in the case of a calcium gel).

These results show that extraction of beet pectins by autoclaving can be a valuable addition to the known extraction procedures (acid, alkali). The crude extracts can be used after oxidative cross-linking as a gelling agent and thickening agent (Chapter 5) [35], or possibly as a water absorbing agent [4].

Oxidative cross-linking

Reaction products of oxidative cross-linking. - In 1983 Rombouts and Thibault showed that it was possible to cross-link feruloylated sugar beet pectins with peroxidase plus hydrogen peroxide or with ammonium persulfate [3]. As a result a gel was formed, which had a high water holding capacity after freeze drying [4]. Thibault [8] suggested that the reaction with ammonium persufate led to the formation of the 8-8 dehydrodimer as only product. For feruloylated arabinoxylans Geissman and Neukom [49] proposed an intermolecular condensation of feruloyl residues after treatment with

hydrogen peroxide/peroxidase, with the 5-5 dehydrodimer as reaction product. However, these proposals were not confirmed by analysis of the reaction products. In this thesis we showed that four types of ferulic acid dehydrodimers (the 5-5, 8-O-4, 8-5, and the 8-8 dehydrodimer) were found in higher amounts than in the original extract after oxidative cross-linking of pectins obtained by autoclave extraction or acid treatment (Chapter 4 and 5) with hydrogen peroxide/peroxidase or ammonium persulfate [35, 37]. Since not all ferulates were recovered during the analysis, it is possible that other reaction products were formed as well. These results are in agreement with the work of Grabber et al., who showed that the same set of dehydrodimers were formed by peroxidases in cell walls isolated from maize cell suspensions, after addition of hydrogen peroxide [50]. From the above it can be concluded that the cross-linking reaction is less specific than assumed previously.

Hydrogen peroxide/peroxidase vs ammonium persulfate. - An important difference between the cross-linking reaction of feruloylated beet pectins with hydrogen peroxide/peroxidase and ammonium persulfate is that the former leads to an instantanious increase in viscosity, whereas the latter results in a slow viscosity increase [32]. Furthermore, it was shown that treatment with ammonium persulfate results in an initial decrease in the reduced viscosity, which suggests that some degradation of the pectic polysaccharides takes place. In Chapter 5 [35] of this thesis it is shown that a gel could be formed of the arabinose rich autoclave extracts only with hydrogen peroxide/peroxidase and not with ammonium persulfate. Similarly, Thibault et al. showed that the presence of high amounts of arabinose in beet pectins inhibited the reaction with ammonium persulfate, which could be improved by removal of the arabinose side-chains with enzymes or mild acid hydrolysis [5]. An explanation for this difference could not be found, although it was shown in Chapter 5 [35] that the same dehydrodimers were found in similar ratios after cross-linking with hydrogen peroxide/peroxidase or ammonium persulfate. Only the total amount of dehydrodimers was lower for the ammonium persulfate cross-linked material. The acid extracted beet pectin was able to form a gel in presence of either one of these reagents.

Cross-linking of rhamnogalacturonans and arabinans. - Feruloylated rhamnogalacturonans and arabinans were isolated from the 'hairy' regions present in an autoclave extract obtained from beet pulp (Chapter 6) [51]. The rhamnogalacturonans (see Fig. 1B, pool I and II) were characterized by a high apparent molecular weight (1300 kDa and 120 kDa, respectively) and a low intrinsic viscosity, whereas the arabinans (Fig. 1B, pool IIIa) had both a low apparent molecular weight and a low intrinsic viscosity. Despite their low viscosities, it was possible to form gels from these polysaccharides by oxidative cross-linking. Gel formation of the rhamnogalacturonans occurred at concentrations as low as 0.75 %, which is comparable to acid extracted beet pectins. The arabinans formed a gel at a

concentration of 4 % after cross-linking, which is still considerably lower than the concentration used by Cooper et al. to form gels from linear arabinans by physical entanglement (10-20 %) [17, 18].

The different physico-chemical properties of the rhamnogalacturonans and arabinans obtained by autoclaving as compared with acid extracted pectin will determine their possible applications as gelling agent. The first difference, the low viscosity of the extracted rhamnogalacturonans and arabinans, makes polysaccharide solutions easy to handle before gel formation/thickening [35, 51]. Then, it will be necessary to cross-link the polysaccharides in the final product, since the gels are irreversible. Another difference is the easy solubility in water of the autoclave extracted pectins [35], which is in contrast with the acid extracted pectins.

Gel formation by oxidative cross-linking or with calcium. - A difference between the gelling properties of the cross-linked beet pectins and gels formed from beet pectin with calcium, after partial demethylation and deacetylation, is the fact that the gels formed by oxidative (covalent) cross-linking are irreversible gels [4], whereas the calcium gels are reversible and will reform a gel after fracture [52]. As a consequence of the covalent bonds formed by oxidative cross-linking, the loss modulus (G"; indicative for relaxation of cross-links) is lower for the gels formed in this way in comparison with calcium gels (Chapter 5 and 8) [35, 40]. This gives the calcium gels a more viscous nature as compared with gels formed by oxidative cross-linking, which are highly elastic. Furthermore, the gels obtained by oxidative cross-linking remain intact after freeze drying and can afterwards be used as a water absorbing agent [4].

Application. - A major problem for food applications of gels obtained by oxidative cross-linking is the fact that hydrogen peroxide and ammonium persulfate are not allowed in (the production of) food. A possible solution for this problem is the use of glucose and glucose oxidase, which are able to form hydrogen peroxide *in situ*. The combination of glucose oxidase and peroxide should therefore also be able to cross-link ferulic acid containing polysaccharides in a similar way as hydrogen peroxide and peroxidase, as was previously shown for arabinoxylans [53].

An interesting option is the chemical feruloylation of polysaccharides [54]. This would make it possible to make gels by oxidative cross-linking from all kinds of polysaccharides, or increase their molecular weights. The degree of feruloylation could be varied in that case. However, to our knowledge no additional information is available on this area of research and further study is needed to gain more insight in the effect of cross-linking on the physico-chemical properties of the polysaccharides used.

Enzymatic modification

A thorough knowledge of the structure of polysaccharides and the relation to its physico-chemical properties opens the way to modify specific parts of the polysaccharide molecule. Enzymes are appropriate tools to elucidate the fine structure of polysaccharides and to specifically modify them and thus their functionality. Two types of enzymes can be used for this: esterases are able to remove acetyl goups, feruloyl groups and methyl esters, whereas glycanases are able to degrade the polysaccharide.

Modification with esterases. - Deacetylation of sugar beet pectins by acetyl esterases is in principal a very promising way of enzymatic modification [2, 15, 16]. However, the use of partially purified and characterized enzymes, or artificially acetylated substrates in previous studies made it difficult to draw conclusions. In Chapter 8 of this thesis the purified enzyme pectin acetyl esterase (PAE) was used to remove the acetyl groups from the homogalacturonan regions of a commercial sugar beet pectin. The action of PAE was studied in the presence of calcium and pectin methyl esterase (PE) [40]. The combination of the two enzymes resulted in a large increase of the amount of acetyl groups and methyl esters released as compared with PE and PAE alone. Therefore, it was concluded that on one hand the acetyl groups hindered the action of PE and that on the other hand the methyl groups hindered the activity of PAE. With respect to gel formation, an increase in the stiffness of the gels (as monitored by the storage modulus (G')) was observed after addition of PAE, besides PE, indicating that indeed this combination of enzymes is responsible for the improved gel formation of beet pectins with calcium.

Besides acetyl and methyl esterases, feruloyl esterases can theoretically be used for the modification of sugar beet pectin. However, at this point only feruloyl esterases are known which can remove ferulic acid groups from oligomers and not from polymers [55]. Furthermore, the extent of oxidative cross-linking can already be varied by changing the amounts of cross-linking reagents used [6], making feruloyl esterases not very interesting for enzymatic modification.

Modification with glycanases. - In Chapter 7 the enzymatic modification of rhamnogalacturonans and arabinans obtained from an autoclave extract from sugar beet pulp, and of an acid extracted beet pectin with several (combinations of) glycanases was described [33]. In most cases we found that enzymatic modification resulted in a loss of the intrinsic viscosity and apparent molecular weight. As was expected, this occurred almost always when backbone degrading enzymes were used. However, it was found that the backbone degrading enzyme rhamnogalacturonase only significantly decreased the apparent molecular weight of the acid extracted beet pectin, but not the intrinsic viscosity. These results showed that the rhamnogalacturonan

backbone contributes little to the intrinsic viscosity of acid extracted beet pectins.

Removal of the side-chains of the arabinans attached to the rhamnogalacturonans with arabinofuranosidase B had little effect on the intrinsic viscosity, although the apparent molecular weight of the polysaccharide decreased significantly. More extensive degradation of the arabinan side-chains with endo-arabinanase and arabinofuranosidase B resulted in a large decrease in apparent molecular weight and in a moderate decrease in the intrinsic viscosity. These results show that the presence of high amounts of arabinan side-chains had relatively little effect on the intrinsic viscosity of the rhamnogalacturonans, which is in agreement with the findings of Hwang and Kokini [56], who investigated the contribution of side-chains to the rheological properties of apple pectins with different neutral sugar contents. However, the arabinose contents of their apple pectins were low in comparison with the pectic polysaccharides used in this thesis work.

Several other studies have been described in the literature dealing with the enzymatic modification of pectic polysaccharides from sugar beet pulp with glycosidases. Cooper et al. modified arabinans extracted from sugar beet pulp by debranching with the enzyme arabinofuranosidase [17]. This resulted in improved gelling properties at high polysaccharide concentrations of these arabinans, which were suitable for use as fat replacers in various food products. An improvement of the gel formation of sugar beet pectins through oxidative cross-linking with ammonium persulfate was found by Guillon and Thibault, after treatment with the same enzyme [5, 7]. They suggested that the removal of a part of the arabinose residues would result in an improved accessibility of the feruloyl groups.

Outlook for enzymatic modification. - From our results it is concluded that pectin acetyl esterase is the most promising enzyme for the enzymatical modification of beet pectins on industrial scale, besides pectin methyl esterases, which can be used to alter the calcium sensitivity of pectins in general. It was shown that pectin acetyl esterase is able to significantly improve the gel forming properties of beet pectin, which may lead to a more general use of beet pectins in the pectin industry. Feruloyl esterases are not expected to be important as was already mentioned. In our experiments enzymatic modification with glycanases never led to significantly improved physico-chemical properties of the pectins used. Therefore, glycanases are assumed only to be used for specific purposes such as the production of debranched arabinans or the improvement of the oxidative cross-linking reaction of beet pectins by removal of the arabinan side-chains [5, 7].

An important prerequisite for introduction of enzymatic modification of beet pectins with pectin acetyl esterase on industrial scale, is the availibility of sufficient amounts of enzymes at reasonable prices. The use of cloned polysaccharides degrading enzymes as described by Kofod [57] is assumed to be an important step towards the

introduction of these enzymes on industrial scale.

Possible applications for sugar beet pectic polysaccharides

The viability of an industrial production process is largely determined by the costs of the raw materials and of the process, as well as of the yield and possible price of the products. Depending on the price of the beet pulp and of the end products, a choice can be made between the efficient extraction of one component or a sequential extraction of several polysaccharides from beet pulp, with useful applications for all fractions obtained. In Table 1 the most important applications for pectic polysaccharides from sugar beet pulp are summarized, which will be discussed below. Furthermore, an indication is given for the way of production, the estimated yield, and additional process steps.

Applications for the autoclave extract. - In our research we mainly used pectic polysaccharides obtained by autoclave extraction. These autoclave extracts contained three types of polysaccharides: rhamnogalacturonans, homogalacturonans and arabinans [25, 27]. The total yield was 15 % after two subsequent autoclave extractions. After isolation by SEC and DEAE chromatography, the yields of the rhamnogalacturonan and arabinan fractions were relatively low (5 and 2 %, respectively). However, two additional autoclave extraction steps yielded another 5 %, with a high rhamnogalacturonan and arabinan content (unpublished data). The rhamnogalacturonans and arabinans can be used as gelling agents after oxidative cross-linking (Chapter 6) [51]. Another 19 % was extracted with strong alkali. The high content of the arabinan side-chains present in this extract makes it suitable for the production of arabinans, which can be used as fat replacer in foods after enzymatic modification [17, 18]. It has been shown that galacturonic acid oligomers have physiological functions in plants [58]. Galacturonic acid from the homogalacturonans and arabinose from the arabinans can be used as a precursor for the production of surfactants after hydrolysis [59]. Rhamnose is a precursor for flavour and colour production through Maillard reactions, whereas ferulic acid is a precursor for the production of vanillin [60]. What remained after extraction was the residue, which mainly contained cellulose. Possible applications are in the paper industry [61]. Also, the cellulose can be hydrolysed to glucose and further fermentation to ethanol [62].

Applications for the acid extract. - Homogalacturonan rich pectins as obtained by acid extraction of sugar beet pulp [19] can be used as a gelling agent after oxidative cross-linking [4, 35]. In addition to that, they can be used to form gels with calcium after partial deacetylation and demethylation [40]. Sugar beet pectins can also be used as

Table 1

Possible applications, production methods, yields, and additional treatments for the fractions obtained from sugar beet pulp.

Fraction	Beet pulp	Polysaccharides			
Amount (%)		Homogalacturonans(18)	Rhamnogalacturonans(40)	Arabinans(32*)	Cellulose(30)
Production (% Yield)	Drying (90-100)	Acid (10-15) Autoclaving (7-10)	Alkali (15-20) Autoclaving (5-8)	Autoclaving (2-?) Alkali (2-?)	Residue (30)
Additional Treatment	-	Cross-linking Enzymes (PAE)	Cross-linking	Cross-linking Enzymes (AF)	-
Application	Fibre Feed ingredient Fermentation	Gelling agent Water holding Thickener Emulsifier Foam stabilizing	Gelling agent Water holding Thickener	Gelling agent Fat replacer Water holding Thickener	Paper

Fraction	Oligosaccharides	Monosaccharides			
Amount (%)	Galacturonic Acid	Rhamnose (2)	Arabinose (30)	Galacturonic acid (20)	Glucose (30)
Production (% Yield)	Acid (10-15) Autoclaving (7-10)	Enzymes Acid Fermentation	Enzymes Acid Fermentation	Enzymes Acid Fermentation	Enzymes Acid Fermentation
Add. treatment	Enzymes(PG+PE)/Acid	Maillard reactions	-	-	-
Application	Physiological functions in plants	Colour Flavour	Surfactants Ethanol	Surfactants Ethanol	Ethanol

* when considered as individual polysaccharides.

a foam stabilizer or emulsifier [63], although it is not known which structural feature is responsible for this effect. The acid extraction can also be combined with a subsequent alkali extraction [17, 18, 48]. The residue can be used in a similar way as described above.

Concluding remarks

In this thesis several new structural features are described of pectic polysaccharides from sugar beet pulp obtained by autoclaving, alkali treatment, and acid extraction. These structural data were used to explain the physico-chemical characteristics of these polysaccharides. The knowledge thus obtained was used to specifically modify the structure by oxidative cross-linking or enzymatic treatment. It was shown that some modifications improved the physico-chemical properties of pectins obtained from sugar beet significantly.

An important finding was the improvement of the gel formation of an acid extracted beet pectin in the presence of calcium by partial deacetylation and demethylation. This may lead to acceptance of beet pectins for a large field of applications.

The gel formation by oxidative cross-linking of the pectic polysaccharides will probably be limited to special applications. The knowledge that rhamnogalacturonans and arabinans can also form a gel by oxidative cross-linking, as was previously shown for acid extracted beet pectins, may be of interest, since they are present in high amounts in sugar beet. Possible applications for these cross-linked polysaccharides are gelling agent in e.g. low sugar jams or water absorbing agents e.g. in sanitary products.

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Summary

The aim of this thesis was (i) the extraction and (physico-)chemical characterization of pectic polysaccharides from sugar beet pulp in order to obtain knowledge about their structure, (ii) to use this knowledge to improve the physico-chemical properties of sugar beet pectins by specific modification with appropriate enzymes or by oxidative cross-linking, which may lead to new applications and a more efficient valorization of sugar beet pulp.

A brief overview of the possible ways for utilization of sugar beet pulp are presented in Chapter 1. The present knowledge of the structure of beet pectins and their role in the cell wall is explained. Also attention is paid to developments concerning the oxidative cross-linking and enzymatic modification of pectins from sugar beet pulp and their effect on the physico-chemical properties of these pectins.

In Chapter 2 an extraction procedure including two sequential autoclave treatments and two strong alkali treatments was introduced. This procedure yielded approximately 45 % of the sugar beet pulp polysaccharides. The sugar linkage composition indicated the presence of increasing proportions of arabinose rich rhamnogalacturonan in the subsequent extraction steps, as well as decreasing proportions of homogalacturonan. The autoclave extracts (Autoclave 1 and 2) contained relatively high amounts of feruloyl groups, besides methyl esters and acetyl groups. In the alkali extracts the ester groups were lost due to hydrolysis under these conditions. This procedure was compared with an extraction procedure including two sequential EDTA/mild alkali treatments and two strong alkali treatments. The sugar compositions of the mild alkali/EDTA extracts were very similar to the corresponding autoclave extracts, but the ester groups were removed in these extracts. Size-exclusion chromatography using RI and UV detection showed that all extracts were heterogeneous with respect to molecular weight distribution.

It was shown in Chapter 3 that the autoclave extracts contained four fractions of pectic polysaccharides. Two highly branched rhamnogalacturonan populations with apparent molecular weights of 1300 and 120 kDa, respectively, were present. These populations were rich in neutral sugars, of which arabinose was predominant, and contained most of the ferulic acid. The third population only consisted of neutral sugar residues and had a low apparent molecular weight and a high arabinose and ferulic

acid content. Also homogalacturonans were present in high proportions with apparent molecular weights of 21 kDa. The extract obtained with 4 M NaOH contained low amounts of xyloglucans, besides rhamnogalacturonans, arabinans and some homogalacturonans. Enzymatic degradation of the xyloglucans showed some similarities with apple xyloglucans with respect to the substitution with xylose, galactose, and fucose.

In Chapter 4 it was shown that the Autoclave 2 extract contained significant amounts of ferulates, 8.8 % of which were ferulate dehydrodimers. The 8-8 and 8-O-4 dehydrodimers were predominant. Oxidative cross-linking with hydrogen peroxide/peroxidase increased the amount of ferulate dehydrodimers by a factor of 4.9. The highest increase was seen for the 8-5 and 8-O-4 dehydrodimers. The concentration of total ferulates decreased by 36 % after cross-linking, indicating that a part of the ferulates were converted to unidentified oxidation products. It was concluded that ferulic acid residues in beet pulp pectin are coupled by oxidative cross-linking through several mechanisms, leading to a variety of dehydrodimers.

Oxidative cross-linking of the Autoclave 1 and 2 extracts, as well as an extract obtained by acid treatment, with hydrogen peroxide/peroxidase resulted in a large increase in viscosity and eventually in the formation of a gel as described in Chapter 5. It was shown that in the autoclave extracts only rhamnogalacturonans and possibly the arabinans participated in the cross-linking reaction. Cross-linking with ammonium persulfate decreased the viscosity of the autoclave extracts. Treatment of the acid extracted pectin with ammonium persulfate gave a slow increase in viscosity. Although there was a difference in reactivity with ammonium persulfate and hydrogen peroxide/peroxidase for the acid extracted pectin and the autoclave extracts, the ratio of the various ferulic acid dehydrodimers formed was similar for both oxidants. Only the total amount of dehydrodimers was lower for the ammonium persulfate cross-linked material.

Chapter 6 describes the purification of the two rhamnogalacturonan populations and the arabinan population from Autoclave 2. For this polygalacturonase and pectin methyl esterase were used in order to facilitate the separation of the rhamnogalacturonans from the homogalacturonans also present in this extract. Cross-linking of the high molecular weight rhamnogalacturonans with hydrogen peroxide and peroxidase led to an increase in viscosity or in gel formation at a concentration of 1.0 %. The second rhamnogalacturonan population formed gels at concentrations as low as 0.75 %, and since it is present in the initial extract in relatively high amounts, this population will largely determine the properties of the gels made from the whole extract. Cross-linking of the arabinan led to gel formation at a concentration of 4.0 %. The properties of the gels are discussed in terms of the storage modulus (G') and tan δ.

The rhamnogalacturonans and arabinan, obtained from the Autoclave 2 extract,

and a commercial acid extracted beet pectin were treated with several (combinations of) enzymes in order to study the effect of these enzymes on their physico-chemical properties (Chapter 7). During the enzyme treatments the intrinsic viscosity, the apparent molecular weight, and the radius of gyration of the polysaccharides were monitored. In most cases we found that enzymatic modification decreased the intrinsic viscosity. However, rhamnogalacturonase plus rhamnogalacturonan acetyl esterase decreased the apparent molecular weight of the acid extracted beet pectin significantly, but not the intrinsic viscosity. It made us conclude that the 'hairy' regions in this pectin are located at the extremities of the molecules. Furthermore, it can be concluded that the rhamnogalacturonan backbone has little effect on the intrinsic viscosity of acid extracted beet pectins. Partial removal of the arabinan side-chains of the arabinans present in the rhamnogalacturonans with arabinofuranosidase B had little effect on the intrinsic viscosity, although the apparent molecular weight of the populations decreased significantly. More extensive removal of the arabinans from the rhamnogalacturonans led to a relatively small decrease in intrinsic viscosity as compared with the decrease in molecular weight. These results show that the arabinan side-chains contribute little to the intrinsic viscosity of the rhamnogalacturonans.

In Chapter 8 it was shown that the addition of rhamnogalacturonan acetyl esterase (RGAE) plus PE, or pectin acetyl esterase (PAE) plus PE to a mixture of acid extracted beet pectin and Ca^{2+} increased the release of acetyl groups and of methyl esters as compared with addition of PE or PAE alone, indicating that pectin methyl esterase activity is hindered by the presence of acetyl groups and that pectin acetyl esterase activity is hindered by the presence of methyl groups. The treatment with PAE plus PE led to a stiffer gel as determined by the storage modulus (G') than after treatment with PE, while RGAE plus PE did not improve the gel forming properties. Addition of only PAE to the pectin Ca^{2+} mixture did not lead to gel formation. A lower stiffness of the gel was found when RGase plus RGAE plus PE were added to the pectin-Ca^{2+} mixture as compared with treatment with PE. Addition of arabinofuranosidase B plus PE to the pectin-Ca^{2+} mixture gave similar results as the treatment with PE alone. A fraction representing the 'smooth' regions, which was isolated after degradation with RGase, was also able to form a gel with Ca^{2+} and PE. However, the gel formation was much slower than with the parental extract, and the stiffness of the gel as determined by G' was lower. Treatment with PAE plus PE again resulted in an increased stiffness of the gel.

In Chapter 9 the results of this thesis are discussed with special emphasis on the feasibility of the applications of pectic polysaccharides from beet pulp as described in this thesis and in the literature.

Samenvatting

Een eerste doel van het in dit proefschrift beschreven onderzoek was de extractie en (fysisch-) chemische karakterisering van pectine gerelateerde polysacchariden verkregen uit suikerbietenpulp om zo kennis te verkrijgen over de structuur van deze polysacchariden. Een tweede doel was het gebuiken van de opgedane kennis om de fysisch-chemische eigenschappen van deze pectines te verbeteren via specifieke modificatie met enzymen of door oxidatieve koppeling. Dit kan dan leiden tot nieuwe toepassingsmogelijkheden en opwaardering van suikerbietenpulp.

Een overzicht van de toepassingsmogelijkheden van bietenpulp staat beschreven in hoofdstuk 1. Ook wordt daar de huidige kennis van de structuur van bietenpectines en van hun rol in de plantencelwand beschreven. Tevens wordt een overzicht gegeven van recente ontwikkelingen omtrent de oxidatieve koppeling en enzymatische modificaties van pectines uit suikerbietenpulp en het effect hiervan op de fysisch-chemische eigenschappen van deze pectines.

In hoofdstuk 2 wordt een nieuwe extractieprocedure gepresenteerd, die bestaat uit twee autoclaaf behandelingen en twee extracties met geconcentreerde loog. Met deze methode werd 45 % van de in bietenpulp aanwezige polysacchariden geëxtraheerd. In de vier extractiestappen werd in toenemende mate arabinaan-rijke rhamnogalacturonanen verkregen, terwijl de gehaltes aan homogalacturonaan afnamen. De twee autoclaaf extracten (Autoclave 1 en 2) bevatten relatief veel ferulazuur, methylesters en acetylgroepen. In de loogextracten verkregen met 4 M NaOH waren geen estergroepen meer aanwezig, als gevolg van verzeping gedurende de extractie. Deze procedure is vergeleken met een extractiemethode waarbij achtereenvolgens tweemaal werd behandeld met EDTA in een lage concentratie loog en daarna tweemaal met geconcentreerde loog. Extractie met EDTA/loog leverde pectines op met een vergelijkbare suikersamenstelling als in de autoclaafextracten, maar zonder estergroepen. Uit size-exclusion chromatografie met detectie van de brekingsindex en de UV absorptie, bleek dat de molecuulgrootteverdeling van alle extracten zeer heterogeen was.

De autoclaafextracten bevatten vier populaties van aan pectine gerelateerde polysacchariden (hoofdstuk 3). Twee populaties bestonden uit sterk vertakt rhamnogalacturonaan met een molecuulgewicht van respectievelijk 1300 kDa en 120

kDa. Deze populaties bevatten veel neutrale suikers, voornamelijk arabinose, en het grootste deel van de ferulazuren. De derde populatie bestond bijna uitsluitend uit neutrale suikers. Deze had een laag molecuulgewicht en een relatief hoog arabinose en ferulazuurgehalte. Daarnaast was een populatie aanwezig die voornamelijk uit homogalacturonaan bestond en een schijnbaar molecuulgewicht had van 21 kDa. Het extract verkregen met geconcentreerde loog bevatte kleine hoeveelheden xyloglucaan, en verder rhamnogalacturonaan, arabinaan en een kleine hoeveelheid homogalacturonaan. Via enzymatische afbraak werd aangetoond dat het xyloglucaan uit biet veel gelijkenis vertoont met xyloglucaan uit appel, wat betreft de substituie met xylose, galactose, en fucose.

In hoofdstuk 4 werd aangetoond dat het extract Autoclave 2 significante hoeveelheden ferulazuurachtige stoffen bevatte, waarvan 8,8 % uit dehydrodimeren van ferulazuur bestond. De 8-8 en de 8-O-4 dimeren werden in dit extract het meest gevonden. Oxidatieve koppeling met waterstofperoxide/peroxidase verhoogde de hoeveelheid dehydrodimeren met een factor 4,9. De grootste verhoging werd gevonden voor het 8-5 en het 8-O-4 dimeer. De totale concentratie aan ferulazuurachtige stoffen nam af met 36 %, wat erop kan wijzen dat een gedeelte van de ferulazuurachtige stoffen werden omgezet in niet-geïdentificeerde oxidatieproducten. Er werd geconcludeerd dat ferulazuur residuen in suikerbietenpulp middels verscheidene mechanismen gekoppeld worden tijdens oxidatieve koppeling, wat leidt tot de vorming van een aantal verschillende dehydrodimeren.

Oxidatieve koppeling met waterstofperoxide/peroxidase van de extracten Autoclave 1 en 2, en van een door zure behandeling verkregen extract, leidde tot een grote viscositeitsverhoging en bij hogere polysaccharideconcentraties tot gelvorming (Hoofdstuk 5). Er werd aangetoond dat in de autoclaafextracten alleen rhamnogalacturonanen en mogelijk vrije arabinanen participeerden aan de koppelings reactie. Koppeling met een andere oxiderende stof, ammonium persulfaat, verminderde de viscositeit van de autoclaaf extracten. Behandeling van het met zuur verkregen extract met ammoniumpersulfaat leidde wel tot een (langzame) viscositeitsverhoging. Er werden verschillen gevonden in de reactiviteit van het met zuur verkregen extract en van de door autoclaveren verkregen extracten met ammoniumpersulfaat en waterstofperoxide/peroxidase. De ratio van de gevormde dehydrodimeren was echter gelijk voor beide oxidanten. Alleen de totale hoeveelheid dehydrodimeren die gevormd werd was lager in het met ammoniumpersulfaat behandelde materiaal.

In hoofdstuk 6 wordt de opzuivering beschreven van de twee rhamnogalacturonaan populaties en van de arabinaan populatie uit het extract Autoclave 2. Om de scheiding tussen de rhamnogalacturonanen en homogalacturonanen te bevorderen, werden de laatsten enzymatisch afgebroken met polygalacturonase (PG) en met pectine methyl esterase (PE). Oxidatieve koppeling van de rhamnogalacturonaan populatie met het

hoogste molecuulgewicht met waterstofperoxide/peroxidase leidde tot een steiging van de viscositeit of tot gelvorming bij concentraties vanaf 1.0 %w/w. De tweede rhamnogalacturonaan populatie vormde al gelen bij een concentratie vanaf 0.75 %w/w. Aangezien deze populatie in relatief grote hoeveelheden aanwezig is in het oorspronkelijke extract, wordt aangenomen dat deze populatie grotendeels de eigenschappen bepaald van dit extract na oxidatieve koppeling. Oxidatieve koppeling van de arabinaanfractie resulteerde in gelvorming bij concentraties vanaf 4.0 %w/w. De eigenschappen van de gevormde gelen worden besproken aan de hand van de opslag modulus (G') en van tan δ.

De rhamnogalacturonaan en arabinaan populaties verkregen uit Autoclave 2, en een commercieel verkrijgbaar zuur geëxtraheerd bietenpectine zijn behandeld met verschillende enzymen of combinaties daarvan om het effect op de fysisch-chemische eigenschappen te bepalen (Hoofdstuk 7). Tijdens de enzymbehandelingen werden de intrinsieke viscositeit, het schijnbare molecuulgewicht en de gyratiestraal bepaald. Voor de pectines verkregen door autoclaveren of zure extractie werd in de meeste gevallen gevonden dat enzymatische modificatie de intrinsieke viscositeit verlaagde. Echter, behandeling met rhamnogalacturonase (RGase) plus rhamnogalacturonaan acetyl esterase (RGAE) verlaagde het schijnbare molecuulgewicht van zuur geëxtraheerd bietenpectine aanzienlijk, maar niet de intrinsieke viscositeit. Hieruit werd geconcludeerd dat de 'hairy' regions in dit pectine aan de buitenkant van het molecuul gelocaliseerd zijn. Verder kan geconcludeerd worden dat de rhamnogalacturonaan hoofdketen weinig invloed heeft op de intrinsieke viscositeit van dit extract. Gedeeltelijke afbraak van de zijketens van de arabinanen, die aanwezig zijn in de rhamnogalactronanen, met het enzym arabinofuranosidase B (AF) had weinig invloed op de intrinsieke viscositeit, terwijl het molecuulgewicht wel aanzienlijk afnam. Afbraak van de hele arabinaan zijketens van de rhamnogalacturonanen resulteerde in een relatieve kleine verlaging van de intrinsieke viscositeit vergeleken met de afname van het schijnbare molecuulgewicht. Deze resultaten geven aan dat de arabinaan zijketens van de rhamnogalacturonanen weinig invloed hebben op de intrinsieke viscositeit van een dergelijk pectinefragment.

In hoofdstuk 8 werd aangetoond dat door het toevoegen van RGAE plus PE of pectine acetyl esterase (PAE) plus PE aan een pectine/calcium mengsel, de hoeveelheid vrijgemaakte acetylgroepen en methylesters toeneemt in vergelijking met behandeling met alleen PE of PAE. Hieruit werd geconcludeerd dat de activiteit van PE gehinderd wordt door de aanwezigheid van acetylgroepen en dat de activiteit van PAE gehinderd wordt door methylesters. Modificatie met PE plus PAE resulteerde in een stijver gel gemeten als G', dan behandeling met alleen PE. Behandeling met RGAE plus PE verbeterde de gelvorming niet. Modificatie van het pectine/calcium mengsel met alleen PAE leidde niet tot gelvorming. Na behandeling van het pectine/calcium

mengsel met RGase plus RGAE plus PE, werd een lagere stijfheid van het gel gevonden in vergelijking met PE. Het toevoegen van AF plus PE aan het pectine/calcium mengsel gaf vergelijkbare geleigenschappen als wanneer alleen met PE behandeld werd. Een fractie die verrijkt was met homogalacturonanen, welke geïsoleerd werd na behandeling met RGase, kon ook een gel vormen in aanwezigheid van calcium en PE. Het gel werd echter langzamer gevormd, en de stijfheid van het gel, zoals bepaald door G', was minder. Behandeling van deze fractie met PAE plus PE in aanwezigheid van calcium resulteerde ook in een stijver gel dan na behandeling met alleen PE.

In hoofdstuk 9 worden de in dit onderzoek gevonden resultaten besproken met speciale nadruk op de haalbaarheid van applicaties voor pectines uit suikerbietenpulp.

Curriculum vitae

Lex Oosterveld werd op 1 november 1966 geboren te Lunteren. In 1985 behaalde hij het VWO* diploma aan het Christelijk Streeklyceum te Ede. In datzelfde jaar begon hij de studie Levensmiddelentechnologie aan de Landbouwuniversiteit te Wageningen. In juni 1991 studeerde hij af met afstudeervakken in de levensmiddelenchemie en levensmiddelenmicrobiologie en met een stage bij de afdeling Animal Science van de University of Manitoba te Winnipeg (Canada). Daarna werd hij assistent-in-opleiding bij de Sectie Levensmiddelenchemie en -Microbiologie van de Landbouwuniversiteit te Wageningen, waar het in dit proefschrift beschreven onderzoek werd uitgevoerd, in het kader van het IOP-koolhydraten, mede gefinancierd door CSM Suiker bv. Sinds augustus 1997 is hij werkzaam als Post-doc bij deze afdeling.