Wheat bran glucuronoarabinoxylans

biochemical and physical aspects

Promotor: dr. ir. A.G.J. Voragen
Hoogleraar in de Levensmiddelenchemie

Co-promotor: dr. G. Beldman
Universitair docent in de Levensmiddelenchemie

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M.E.F. Schooneveld-Bergmans

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van de Landbouwuniversiteit Wageningen,
dr. C.M. Karssen,
in het openbaar te verdedigen
op dinsdag 24 juni 1997
des namiddags te één uur dertig in de Aula

Schooneveld-Bergmans, M.E.F.

Wheat bran glucuronoarabinoxylans. Biochemical and physical aspects
Proefschrift Wageningen. - Met lit.opg. - Met samenvatting
in het Nederlands.
ISBN 90-5485-716-1

The research described in this thesis was financially supported by the
Dutch Innovation Oriented Programme Carbohydrates (IOP-k) and
Royal Gist-brocades (Delft, The Netherlands).

Stellingen

1. Homogeniteit, het percentage gesubstitueerde xylose-eenheden in de hoofdketen en de verdeling van de bindingstypen van arabinose en xylose-eenheden zijn bepalend voor de structurele karakterisering van arabinoxylanen.

   Dit proefschrift.

2. De veelheid aan xylanolytische enzymen garandeert nog niet dat elke gewenste modificatie van arabinoxylanen haalbaar is.

   Dit proefschrift.

3. De door Greenshields en Rees beschreven condities voor extractie van ferulazuurhoudend arabinoxylaan leiden niet tot het gewenste resultaat.

   Dit proefschrift.
   Greenshields R and Rees AL, 1993. UK Patent Application 9214392.

4. Het aantal ferulazuur-dimeren dat gevormd wordt bij *in vitro* koppeling van arabinoxylanen is groter dan tot nu toe werd aangenomen.

   Dit proefschrift.

5. Het feit dat journalisten conclusies trekken uit publicaties of interviews om een aandacht trekkende kop boven hun artikel te zetten leidt tot grote misvattingen.

   Van volkorenbrood word je verkouden. Trouw (mrt 1997).

6. Een verenigd Europa leidt tot meer taalgrenzen.

   Burger P, 1997. Onze taal 66: 103-106.

7. Derde rijstroken zijn een breedte-oplossing van het Nederlandse file-probleem.

8. De belangrijkheid van een persoon of organisatie wordt vaak bepaald door de mate waarin deze in staat is een proces te vertragen.

9. De vrije keuze bij het voeren van de familienaam leidt binnen de genealogie tot het ontstaan van `stambossen'.

10. Er dienen zo spoedig mogelijk stiltecoupés in de tweede klasse van treinen ingevoerd te worden.

11. Banken worden rijk van het tijdelijk laten verdwijnen van geld via Chipknip en Chipper.

12. Een onderzoeker op het gebied van enzymzuiveren heeft minder moeite met de aanduiding zuiveraar dan een onderzoeker van tarwezemel met zemelaar.

Stellingen behorende bij het proefschrift

Wheat bran glucuronoarabinoxylans
biochemical and physical aspects

Wageningen, 24 juni 1997

Margot Schooneveld-Bergmans

Abstract

Arabinoxylans are present in cereal cell walls and *in vitro* they have interesting physicochemical properties, such as viscosity and gelation. Although many studies on these properties were reported for wheat flour arabinoxylan, not much research has been directed towards exploitation of these polysaccharides as food gum. For that purpose glucuronoarabinoxylans of wheat bran, a cheap by-product of the cereal industry, were studied with regard to their extractability, their structural and physicochemical properties.

Approximately 50% of the glucuronoarabinoxylans of wheat bran cell wall material were recovered in high purity by barium hydroxide extraction at 70 to 95°C. Delignification or other treatments to open up the cell wall structure were not effective in increasing the yield. The extracted glucuronoarabinoxylans were very diverse in chemical structure and physicochemical properties. About 30% of them had a low degree of substitution, were easily degradable by xylanolytic enzymes and hardly influenced the viscosity of the solvent as a result of extensive aggregation. Over 50% of them had a high degree of substitution, were supposed to contain dimeric branches of arabinose and xylose, were scarcely degradable by xylanolytic enzymes, gave moderate viscosity to solutions and were very effective in stabilizing emulsions. The structure of these glucuronoarabinoxylans could only be speculated upon and it could not be enzymatically modified as a consequence of its complexity and the lack of appropriate enzymes. The remaining glucuronoarabinoxylans either had an intermediate or very high degree of substitution, of which the latter was presumed to be connected to lignin-fragments.

Gel-forming glucuronoarabinoxylans were recovered only in low yield by dilute alkali extraction and subsequent purification was necessary. These feruloylated glucuronoarabinoxylans gelled upon addition of oxidative agents, of which peroxide - peroxidase, glucose - glucoseoxidase - peroxidase and ammonium persulphate were investigated. In comparison with wheat flour arabinoxylans, those of wheat bran appeared to give less flexible networks at high concentration, which was ascribed to their high degree of substitution and high ferulic acid content. Of the dimers formed upon cross-linking, the generally known diferulic acid, being a 5-5 coupled dimer, was only present in relatively low amounts. Dimers, in which the 8-position of the ferulic acid residue is involved were preponderant. The distribution of the dimers was not affected by the type of cross-linking agent or the type of arabinoxylan. However, the presence of lignin fragments in the bran extract was presumed to cause a low ferulic acid recovery upon cross-linking.

Voorwoord

In de trein tussen Ede en Delft, al piekerend hoe deze pagina te beginnen, kwam ik op de vergelijking openbaar vervoer - promotie onderzoek. De vaart, ofwel de ups en downs van een onderzoek, is te vergelijken met intercity of boemeltrein. Met wat geluk kom je als reiziger of als AIO op de juiste plaats aan, maar meestal niet op de juiste tijd. De mensen waaraan ik mede te danken heb dat ik in deze promotie periode het spoor niet bijster ben geraakt, wil ik graag even noemen.

Fons Voragen, de mogelijkheid die je me gaf om nog snel een afstudeervak te doen voordat ik aan dit promotie onderzoek zou beginnen, heeft me geleerd onder druk te schrijven. Dit is me de afgelopen tijd goed van pas gekomen. Bedankt voor je steun en je vertrouwen dat ik het wel zou redden. En dat niet alleen in deze laatste tijd! Gerrit Beldman, hoewel enzymatische modificatie een grote rol had kunnen spelen in dit onderzoek, bleek dit bij tarwezemel toch niet zo eenvoudig te zijn. Bedankt voor de vrijheid die je me gaf en de kritische blik op dit koolhydraat- en fysisch-chemisch werk. Jozé Mutsaers, op enige afstand is onze samenwerking met dit project gestart en wordt nu van dichterbij voortgezet. Dank voor de vele malen dat jij met interesse de vergaderingen in Wageningen bijwoonde. Delft - Wageningen, het is een hele reis!

Màrgien Mutter en Katrien van Laere, door met één van jullie de kamer te delen, kreeg ik er twee vriendinnen bij. Het was fijn met jullie over werk en nóg belangrijkere zaken te kunnen praten. Harry Gruppen en Henk Schols, als gaand en komend arabinoxylaan-dan wel koolhydraatspecialist, wil ik jullie bedanken voor het nu en dan meedenken.

Yvette van Dijk, Anja Hopman en Mark Dignum, dank voor jullie substantiële bijdragen die duidelijk in dit proefschrift zijn terug te vinden. Pavla Lukešová, Judith de Vegt, Esther Conijn, Yvette van Dijk, Edith Korse en Marjon Matser hebben in het kader van hun studie aan dit onderzoek bijgedragen. Mijn labgenoten Marian Verbruggen, Toos Kroef en Miranda Huisman, bedankt voor de prettige sfeer op het lab en de theepauzes met of zonder lekkers. Ook alle andere collega's van Levensmiddelenchemie, bedankt voor de plezierige tijd en samenwerking.

Mijn ouders bedank ik voor de mogelijkheid en de steun die ze me hebben gegeven om voor Wageningen te kiezen in plaats van opvolging in hun *schitterende* vak. Jullie interesse en meeleven waren en zijn altijd geweldig. Paul, als ik dan toch dreigde te ontsporen, was er altijd jouw luisterend oor, je relativering en je steun om mij weer op de rails te zetten. Waar een congres in Frankrijk al niet toe kan leiden!

Contents

CHAPTER 1

General introduction

Wheat production, composition and uses

Wheat is the most important of all cereals grown. In the last few years its annual production was approximately 550 million tonnes, which amounts to more than 30% of the total cereal production. Two-thirds of the wheat is used for human consumption, approximately 20% for feed and the remainder for seed and other uses (Anonymous, 1991 and 1994). The major species grown is common wheat (*Triticum aestivum*). Durum wheat (*Triticum durum*) and club wheat (*Triticum compactum*) are of minor commercial value.

A wheat grain is composed of different tissues, of which the inner, the starchy endosperm, is of great importance for the food industry. It comprises approximately 84% of the grain and is covered with the aleurone layer, which accounts for 7% of the grains weight (Pomeranz, 1988). From a botanical point of view the aleurone layer is part of the endosperm (Bradbury *et al.*, 1956*b*). It is rich in protein, vitamins, minerals and enzymes, the latter play an important role in the mobilization of endosperm reserves during germination (Stone, 1985). The germ and the outer tissues of the grain comprise 2 and 7% of its weight, respectively (Pomeranz, 1988). The outer layers consist of nucellar tissue, seed coat, tube cells, hypodermis and epidermis, from the inside outward. The latter two tissues form a compact outer covering of the grain, which is also known as pericarp or beeswing. Between the hypodermis and tube cells certain regions of the grain also contain crushed and fragmentary thin-walled cells, intermediate and cross cells (Bradbury *et al.*, 1956*a*). A schematic representation of the location of the various tissues of a wheat grain is given in Figure 1. It is also shown that in technological point of view the aleurone layer is part of the bran. Industrial wheat bran usually accounts for 14 to 19% of the grains weight, because it comprises the outer coverings, the aleurone layer and remnants of the starchy endosperm (Pomeranz, 1988).

Most of the wheat used for human consumption is divided into endosperm, bran and germ for the production of flour-based foods, such as bakery and pasta products. This is achieved by milling, which is essentially a process of grinding and separating. The grains are broken open in a roller mill, resulting in wheat particles of different size and composition. By means of sifting the mixture of particles is graded by size. The largest particles, which consist of bran fragments and adhering endosperm, proceed to a second and further set of break rolls with a maximum of six breaks. The smaller particles, which consist of pure endosperm, composites of endosperm and bran, and small particles of bran, are purified by a combination of sifting and air classification. The purified material is then ground and sifted to different flour streams and small bran particles, the latter of which are also known as shorts (Bass, 1988). Usually, an extraction rate of 72% is obtained, resulting in straight run flours which are commonly used in food industry, for example for baking of white bread. Whole meal flour is composed of all flour, bran and germ streams

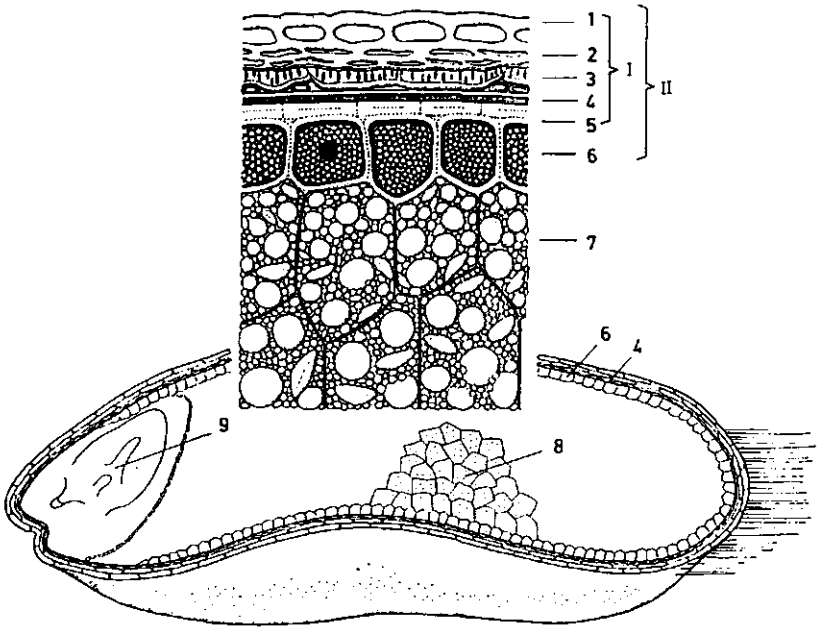

Figure 1: Longitudinal section of a wheat grain. I: bran, botanically; II: bran, technologically; 1: epidermis; 2: hypodermis; 3: tube cells; 4: seed coat; 5: nucellar tissue; 6: aleurone layer; 7: outer starchy endosperm cells; 8: inner starchy endosperm cells; 9: germ (adapted from Belitz and Grosch, 1987).

of the milling process (Hoseney, 1986). Interest in products prepared from whole meal has increased since a low intake of dietary fibre was assumed to be correlated with certain diseases in developed countries (Burkitt and Trowell, 1975). A lot of research has been directed to the use of bran in baked products ever since (Pomeranz *et al.*, 1976; Wootton and Shams-Ud-Din, 1986; Lai *et al.*, 1989; Gan *et al.*, 1992). Breakfast foods, such as flakes, porridge or bran-based products have gained interest also (Selvendran *et al.*, 1987). The remaining milling by-products, part of the shorts and coarse bran, are used as feed ingredients. The nutritive value of these products is poor as a result of their low degradability. However, differences in digestibility are observed for ruminants and monogastrics. A lower nutritive value of these products for monogastrics is generally ascribed to the absence of intestinal microorganisms producing enzymes, which are able to hydrolyse cell wall polysaccharides (Saunders *et al.*, 1972; Pettersson and Aman, 1988; Annison and Choct, 1991).

Composition of wheat bran

For milling of wheat to white flour various varieties of wheat, grown under different environmental and climatic conditions, are usually mixed to yield flours that meet the requirements for the specific product it is used for. Although variety, environmental and climatic conditions affect the precise composition of wheat and the resulting milling products (Lee and Stenvert, 1973; Davis *et al.*, 1981), the approximate composition of industrial wheat bran is rather constant.

Starch

The starch content of industrial wheat bran varies from 10 to 20% (Caprez *et al.*, 1986; Belitz and Grosch, 1987; Ralet *et al.*, 1990). However, contents of 30 to 50% are reported for brans obtained by laboratory milling (Brillouet and Mercier, 1981; Salomonsson *et al.*, 1984). The starch originates from residual endosperm attached to the bran. It is present in two types of granules, the small spherical and the large lenticular. A quarter of the starch is present as amylose, which is known as a linear polymer consisting of α-(1→4)-linked D-glucopyranosyl residues. The remaining starch, the amylopectin, also consists of α-(1→4)-linked D-glucopyranosyl residues, but it occurs as a highly ramified polymer as a result of branching through α-(1→6)-linkages (Lineback and Rasper, 1988).

Non-starch polysaccharides

Non-starch polysaccharides are the major constituents of wheat bran. The total content varies from 41 to 60% (Lee and Stenvert, 1973; Schwarz *et al.*, 1988), and is about 46% for industrial wheat bran (Ralet *et al.*, 1990). The main non-starch polysaccharides present are arabinoxylan, cellulose and (1→3)(1→4)-β-glucan. Minute amounts of glucomannan (Mares and Stone, 1973*a*; Gruppen *et al.*, 1989) and arabinogalactan (Fincher *et al.*, 1974), originating from aleurone and endosperm cells, are also present in bran. Small amounts of xyloglucan associated with arabinoxylans in the pericarp tissues are also reported (DuPont and Selvendran, 1987).

Arabinoxylan. Approximately 70% of the non-starch polysaccharides of wheat bran is composed of arabinoxylan (Selvendran *et al.*, 1980; Brillouet and Mercier, 1981; Carré and Brillouet, 1986; Ralet *et al.*, 1990). In general arabinoxylans of cereals consist of a backbone of β-(1→4)-linked D-xylopyranosyl residues, to which α-L-arabinofuranosyl residues are linked at the *O*-2, *O*-3 or both *O*-2 and *O*-3 position (Wilkie, 1979). The degree of arabinose substitution, which is usually expressed as the Ara/Xyl-ratio, is higher for wheat bran than for wheat flour arabinoxylan (D'Appolonia and MacArthur, 1976). However, the actual fine structure of arabinoxylans of different tissues of the bran are very

diverse. Arabinoxylans of the aleurone layer have a low degree of substitution with an Ara/Xyl-ratio of approximately 0.3 (Bacic and Stone, 1981; Schwarz *et al.*, 1988). These arabinoxylans have a relatively high amount of unsubstituted xylose residues. Only 20% of the xylose residues is branched at the *O*-3 position, whereas 5% is branched at both the *O*-2 and *O*-3 position. The branches are almost exclusively single α-L-arabinofuranosyl residues, although minute amounts of oligosaccharide branches containing 2-, 3- or 5-linked arabinose, or single uronic acid residues can be present also (Bacic and Stone, 1981). Ferulic acid is found in aleurone cell walls in relatively high concentrations (Fulcher *et al.*, 1972). This phenolic acid is esterified to the *O*-5 position of arabinose residues (Smith and Hartley, 1983; Kato *et al.*, 1983; Gubler *et al.*, 1985). Apart from ferulic acid, *p*-coumaric acid can also occur esterified to arabinose in cereal tissues (Mueller-Harvey *et al.*, 1986; Pussayanawin and Wetzel, 1987). A total phenolic acid content of 1.8%, consisting predominantly of ferulic acid, is determined for a cell wall fraction of the aleurone layer (Bacic and Stone, 1981).

Arabinoxylans of the pericarp or beeswing have a considerably higher degree of substitution, resulting in an Ara/Xyl-ratio of 1.0, and contain 6 to 9% of uronic acid (Adams, 1955; Schmorak *et al.*, 1957; Ring and Selvendran, 1980; DuPont and Selvendran, 1987; Brillouet and Joseleau, 1987; Schwarz *et al.*, 1988). The majority of the uronic acid, about 70%, is present as glucuronic acid, and the remainder as 4-*O*-methyl-glucuronic acid (Ring and Selvendran, 1980; Brillouet and Joseleau, 1987). These polysaccharides are therefore also named glucuronoarabinoxylans. Remarkable structural features of wheat pericarp glucuronoarabinoxylans are the presence of terminal xylose, about 20% of total xylose, non-terminal arabinose, about 40% of total arabinose, and a small amount of terminal galactose (Ring and Selvendran, 1980; Brillouet and Joseleau, 1987). Oligomeric branches terminated by arabinose, xylose or galactose have been suggested for arabinoxylans of grasses (Wilkie, 1979), but their precise composition and mode of linkage in the polysaccharides is not known conclusively. Esterified ferulic acid and *p*-coumaric acid are also present in the pericarp cell wall fraction, amounting to 0.5% and 0.01%, respectively (Ring and Selvendran, 1980).

Cellulose. Cellulose is a linear polymer characterized by long chains of β-(1→4)-linked D-glucopyranosyl residues, and occurs in semi-crystalline state. It comprises approximately 24% of the total non-starch polysaccharides of industrial wheat bran (Selvendran *et al.*, 1980; Carré and Brillouet, 1986) and is almost exclusively located in the pericarp (Ring and Selvendran, 1980; Schwarz *et al.*, 1988).

(1→3)(1→4)-β-glucan. The remaining 6% of the non-starch polysaccharides of the bran mainly consists of (1→3)(1→4)-β-glucan. This polysaccharide is composed of β-(1→3) and β-(1→4)-linked D-glucopyranosyl residues. It is predominantly present in the cell walls of

the aleurone layer, having a ratio of 3 to 7 of the 3- over 4-linked glucose residues (Bacic and Stone, 1981), which is similar to that determined for barley (1→3)(1→4)-β-glucan (Woodward *et al.*, 1983).

Protein

The content of protein in wheat bran ranges from 15 to 22% (Lee and Stenvert, 1973; Brillouet and Mercier, 1981; Carré and Brillouet, 1986; Caprez *et al.*, 1986; Schwarz *et al.*, 1988; Ralet *et al.*, 1990). A part of it originates from adhering endosperm, of which the protein can be divided into albumins, globulins, prolamins and glutelins, based on the solubility in various aqueous solutions (Belitz and Grosch, 1987). Wheat endosperm protein is relatively rich in glutamate, proline and leucine, and has a low content of lysine (Stevens *et al.*, 1963; Pomeranz, 1988; Gruppen *et al.*, 1989). However, most of the protein of wheat bran originates from the aleurone layer, which contains approximately 30% protein (Belitz and Grosch, 1987). The aleurone layer contains intracellular as well as cell wall protein. The intracellular protein has a different amino acid composition when compared to the main endosperm fractions. The relative glutamate and proline content is much lower, whereas especially a high arginine content is observed (Stevens *et al.*, 1963). The amino acid composition of a cell wall preparation of the aleurone layer resembles that of the intracellular proteins, with a little less arginine and glutamate and more cysteine in the wall preparation than in the intracellular protein (Bacic and Stone, 1981). The aleurone cell wall protein has a low hydroxyproline content, which is known to be the case for endospermic wheat cell wall proteins (Mares and Stone, 1973*b*). The remaining protein originates from the other tissues of the bran, in which they are present as cell wall proteins. The protein content of these tissues varies from 4 to 11% (Belitz and Grosch, 1987). The amino acid composition of beeswing cell wall material, differs from endosperm protein in a lower glutamate and proline content and a slightly higher aspartate, glycine, alanine and lysine content. Again, only minor amounts of hydroxyproline are detected (Ring and Selvendran, 1980; Pomeranz, 1988).

Lignin

The lignin content of wheat bran ranges from 4 to 8% (Lee and Stenvert, 1973; Carré and Brillouet, 1986; Schwarz *et al.*, 1988; Flint and Camire, 1992). Variations in the content are attributable to the origin of the bran and the method of analysis (Schwarz *et al.*, 1988; Flint and Camire, 1992). Lignin is a polymer composed of phenylpropanoid residues that are derived from *p*-coumaryl, coniferyl and sinapyl alcohols. These are referred to as *p*-hydroxyphenyl, guaiacyl and syringyl in lignins (Freudenberg and Neisch, 1968). Lignin of grasses contains approximately equal amounts of these three residues, to

which hydroxycinnamic acids, such as ferulic and *p*-coumaric acid, can be ether or ester-linked (Lewis and Yamamoto, 1990). In wheat bran lignin is not present in aleurone cell walls (Bacic and Stone, 1981), but is concentrated in the inner pericarp, seed coat and to a slightly lesser extent in the outer pericarp (Schwarz *et al.*, 1988). A lignin-isolate of wheat bran is reported to consist mainly of guaiacyl and syringyl residues, to which ferulic acid and minor amounts of *p*-coumaric acid are esterified (Schwarz *et al.*, 1989).

Minor constituents

Being a minor constituent of wheat bran, the crude fat content amounts to approximately 5% (Lee and Stenvert, 1973; Brillouet and Mercier, 1981; Caprez *et al.*, 1986). Most of it is concentrated in the aleurone layer, while the pericarp tissues contain less than 1% fat. However, some of the fat of bran can originate from contamination with germ, as this part of the grain has the highest concentration of fat (Morrison, 1978; Belitz and Grosch, 1987). In bran palmitic (C16:0), oleic (C18:1) and linoleic (C18:2) acid are the preponderant fatty acids (Morrison, 1978). Apart from triglycerides, wheat bran also contains minor amounts of phospholipids and glycolipids (Morrison, 1988).

The total free sugar content of wheat bran is approximately 6%, with the majority present in the aleurone layer. The main free sugars are sucrose and raffinose (Stevens, 1970; Brillouet and Mercier, 1981).

Minerals and vitamins are also concentrated in the aleurone layer of the wheat grain. In cereals vitamins B and E are prevailing (Belitz and Grosch, 1987). The main minerals are phosphorus, potassium, magnesium and calcium (Pomeranz, 1988). An indication for the total concentration of minerals is the ash content, which ranges from 4 to 6% for wheat bran (Lee and Stenvert, 1973; Brillouet and Mercier, 1981).

Cereal cell walls

The function of plant cell walls is to allow cell expansion during growth and to resist turgor pressures, with that determining the size and shape of the cell. Specialized cells, like tracheary elements or pericarp, need to withstand compressive forces better than parenchymatous tissues. Usually, these cells are therefore lignified (Bacic *et al.*, 1988). For that reason the composition of plant cell walls not only depends on family and species, but also on the type of tissue the cells originate from.

Unlignified and lignified cell walls

Parenchymatous tissues, such as endosperm and aleurone layer, are unlignified and predominantly consist of non-cellulosic polysaccharides, of which arabinoxylans and

(1→3)(1→4)-β-glucans are most common. These polysaccharides enclose the fibrillar structures of cellulose, which accounts for approximately 5% of the dry weight of these walls in wheat. Additionally, the cell walls contain small amounts of glucomannan, which is associated with cellulose fibrils (Mares and Stone, 1973*a*; Bacic and Stone, 1981; Gruppen *et al.*, 1989). Apart from polysaccharides, unlignified cell walls also contain minor amounts of structural protein, which is suggested to play a role in the cross-linking of cell wall polymers (Bacic *et al.*, 1988). The water content of these cell walls is relatively high, which gives the wall a flexible gel-like structure.

Lignified cell walls have a relatively low water content, because water is replaced by lignin (Bacic *et al.*, 1988). Lignification usually occurs during secondary wall formation after cells have ceased to grow. Thickening of cereal cell walls begins with deposition of cellulose and glucuronoarabinoxylans. Then lignin deposition starts at the cell corners and proceeds along the middle lamella and into the primary and secondary wall (Lewis and Yamamoto, 1990). Hydroxycinnamic acids esterified to glucuronoarabinoxylans are suggested to serve as recognition or anchoring sites for lignification (Gordon, 1975; Delmer and Stone, 1988; Lewis and Yamamoto, 1990). Finally, lignin encrusts the cellulose microfibrils, matrix polysaccharides and structural protein, by which means the cell wall stiffens and mechanical damage and biochemical degradation is prevented.

Cross-links between cell wall constituents

The majority of the constituents of cell walls are hydrophilic and would therefore be soluble in water. However, intact cell walls are insoluble in water, which implies that the constituents are interlinked or physically entrapped. Different types of links have been proposed and confirmed until now, for both unlignified and lignified cell walls.

Hydrogen bonds. Cell wall polymers can form many hydrogen bonds as a result of their numerous linked oxygen and hydroxyl groups and the presence of water in the wall. Individual hydrogen bonds are not very strong, but when acting in concert they give strong cohesion (Fry, 1989). The degree of hydrogen bonding depends on the conformation of the polymers. The linear ribbon conformation of cellulose gives rise to formation of many inter- and intramolecular hydrogen bonds, resulting in highly organized microfibrils (Preston, 1979). Apart from cellulose other cell wall polysaccharides, such as arabinoxylans, (1→3)(1→4)-β-glucans and glucomannans, can also bind to each other or to the cellulose surface by hydrogen bonds. The extent of hydrogen bond formation for these polysaccharides is limited as a result of the presence of substituents or irregularities in glycosidic linkages in the main chain (Fry, 1989).

Ionic bonds. The presence of oppositely charged groups gives rise to the possibility of ionic bonds. The most common ionic bond occurring in plant cell walls is the calcium ion

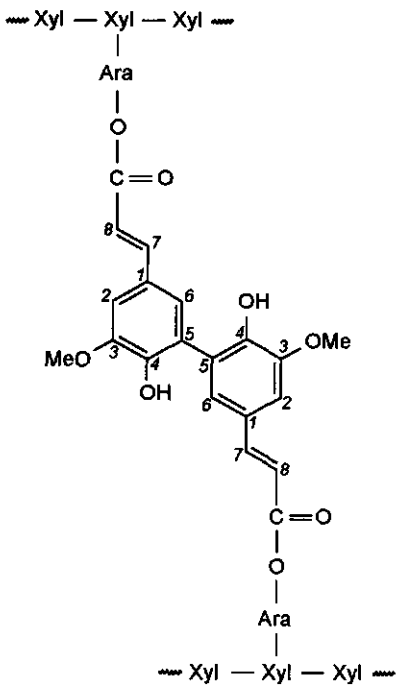

Figure 2: Dehydrodiferulic acid cross-link through 5-5 coupling of two ferulic acid residues of adjacent arabinoxylans.

bridging of negatively charged galacturonic acids in pectins. In cereal cell walls pectins are almost completely absent. Direct ionic bonds between negatively charged glucuronic acid and positively charged amino acids are also suggested, but have not been proved (Fry, 1989).

Covalent links. A variety of covalent links is suggested to occur in plant cell walls, involving linkage of polysaccharide-polysaccharide, protein-protein, polysaccharide-protein, polysaccharide-lignin and protein-lignin (Bacic *et al.*, 1988). Phenolic residues appear to be relevant in all of these linkages.

Polysaccharide-polysaccharide cross-linking is suggested to occur through dimerization of esterified ferulic acid residues of adjacent arabinoxylans as is shown in Figure 2. Dehydrodiferulic acid, a biphenyl formed by dehydrogenative coupling of the two ferulic acid residues at their 5-position, is detected in walls of wheat endosperm (Markwalder and Neukom, 1976), Italian ryegrass (Hartley and Jones, 1976) and rice endosperm (Shibuya, 1984). Evidence for covalent cross-linking of arabinoxylans through this type of

dehydrodiferulic acid is obtained by characterization of a diferuloyl arabinoxylohexamer in bamboo shoot cell walls (Ishii, 1991). Recently, other dehydrogenative coupling products, which have linkages at other positions, have been synthesized and identified in grass cell walls (Ralph *et al.*, 1994). Additionally, cyclobutane dimers, also known as truxillic or truxinic acid derivatives, arising from photodimerization of two ferulic or *p*-coumaric acid residues, are identified in grasses (Hartley *et al.*, 1988; Eraso and Hartley, 1990; Hartley and Morrison, 1991). Glycosidic and uronyl ester links are also suggested to occur between polysaccharides (Fry, 1989).

A protein-protein linkage identified in plant cell walls is isodityrosine. This is a diphenyl-ether formed by intramolecular coupling of two tyrosine residues in cell wall protein. Until now this type of linkage has not been proved to occur intermolecularly also (Fry, 1982).

Polysaccharide-protein linkages have been suggested frequently because of their coextraction and coelution. Recently, an arabinoxylan-protein complex was isolated from rye bran, but the nature of the linkage has not been established (Ebringerova *et al.*, 1994*b*). Linkages through esterified ferulic acid and tyrosine or cysteine are also proposed (Bacic *et al.*, 1988). These latter suggestions are based on oxidative coupling studies of isolated wheat endosperm arabinoxylans, in which protein appeared to play a role (Neukom and Markwalder, 1978; Hoseney and Faubion, 1981).

Polysaccharide-lignin linkages are suggested because of their close association in isolated lignin-carbohydrate complexes (Bacic *et al.*, 1988; Azuma, 1989). Some of these linkages are schematically represented in Figure 3. Polysaccharides can be glycosidically linked to hydroxyl groups of lignin, but this has not been proved until now (Bacic *et al.*, 1988). Linkage of lignin to polysaccharide through a direct ether-linkage is demonstrated in wood (Koshijima *et al.*, 1984; Watanabe *et al.*, 1989). Occurrence of direct ester-links is based on alkali instability of lignin-carbohydrate cross-links. The proposed presence of this type of linkage in jute (Das *et al.*, 1981), was actually proved in wood (Imamura *et al.*, 1994). Other polysaccharide-lignin linkages involve hydroxycinnamic acid ester-ether bridges. Ferulic acid is shown to be ether-linked to lignin in wheat straw (Scalbert *et al.*, 1985). The suggestion that ferulic acid serves as a bridging unit between lignin and carbohydrate is supported by the simultaneous ether and ester-linkage of this residue in wheat internodes (Lam *et al.*, 1992*b*). Dehydrodiferulic acid is shown to be similarly linked, which implies that a diester-ether bridge between lignin and carbohydrate can exist also (Lam *et al.*, 1992*c*).

Finally, protein-lignin linkages are suggested, but there is hardly any information on the type of linkage (Bacic *et al.*, 1988).

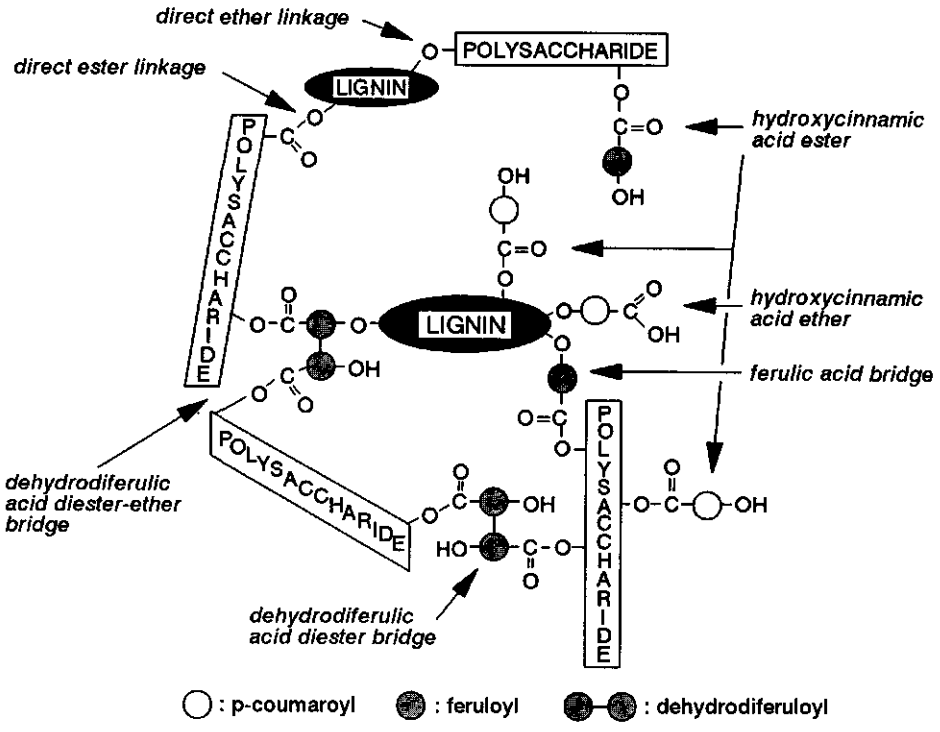

Figure 3: Schematic representation of suggested cross-linkages between polysaccharides and lignin in cereal cell walls (adapted from Lam *et al.*, 1992*a*; Iiyama *et al.*, 1994).

Physicochemical properties of arabinoxylans

Physicochemical properties of arabinoxylans, in particular those of wheat flour, have been of interest for a long time, because it is assumed that they play an important role in the distribution of water during dough preparation and baking of bread (Bushuk, 1966). This is attributed to their high water-binding capacity, for which values of 4 to 10 times their original weight are reported (Jelaca and Hlynka, 1971; Kim and D'Appolonia, 1977). The main physicochemical properties of arabinoxylans related to this water-binding capacity are viscosity and formation of gels by oxidative cross-linking.

Viscosity

The viscosity of polysaccharides is primarily dependent on intrinsic properties, such as molecular weight, chemical composition and conformation. Molecular weights reported for wheat arabinoxylans vary widely, which is mainly ascribed to the different techniques

used. Laser light-scattering analysis gave molecular weights of 250,000 to 650,000 Dalton for arabinoxylans of wheat flour (Gruppen *et al.*, 1992*b*). The conformation of crystalline un- or highly branched xylans is a three fold left-handed helix, having three xylose residues per turn, which is stabilized by an intramolecular hydrogen bond and interaction with surrounding water molecules (Nieduszynski and Marchessault, 1972; Atkins, 1992). This results in extended twisted ribbon-like strands. Un- or lowly branched xylans are only partly soluble upon heating (Blake *et al.*, 1970). Storage of solutions of these xylans gives formation of aggregates, which is a time and temperature dependent phenomenon (Blake and Richards, 1971). Branching of xylans by arabinose affects a higher solubility and more extended conformation (Dea *et al.*, 1973; Andrewartha *et al.*, 1979). The arabinose residues tend to stiffen the chain and decrease the possibility of aggregation (Andrewartha *et al.*, 1979). However, the branches do not completely limit aggregation, as participation of arabinose residues in lattice formation and stabilization of aggregates has been observed (Dea *et al.*, 1973).

The viscosity of arabinoxylans from wheat is also shown to be dependent on the wheat variety (Ciacco and D'Appolonia, 1982*a*; Izydorczyk *et al.*, 1991) and the origin of the tissue (Ciacco and D'Appolonia, 1982*b*; Brillouet *et al.*, 1982). Intrinsic viscosities reported for wheat arabinoxylans range from 2 to 8.5 dl/g, of which the lower values are generally observed for arabinoxylans derived from pericarp tissues (Ciacco and D'Appolonia, 1982*b*; Brillouet *et al.*, 1982) and for flour arabinoxylans of relatively low molecular weight (Izydorczyk and Biliaderis, 1992*a,b*).

Oxidative gelation

Already for a long time, the presence of ferulic acid attached to arabinoxylans has been known to be essential in the increase of viscosity upon addition of an oxidizing agent to water-soluble extracts of wheat flour (Fausch *et al.*, 1963). Initially, protein was assumed to be essential in the cross-linking as well (Fausch *et al.*, 1963; Geissmann and Neukom, 1973), but gelation of a protein-free flour extract refuted that (Morita *et al.*, 1974). However, the role for protein in the cross-linking is still a matter of debate. Cross-links of ferulic acid with tyrosine (Neukom and Markwalder, 1978) and cysteine (Hoseney and Faubion, 1981) have been suggested. A cross-linking product of ferulic acid and cysteine has been tentatively characterized (Jackson and Hoseney, 1986), but contradicting results have been obtained also (Vinkx *et al.*, 1991).

Upon oxidative treatment, the extent of viscosity increase or gelation is dependent on the arabinoxylan concentration (Moore *et al.*, 1990; Izydorczyk *et al.*, 1990), the molecular weight or intrinsic viscosity of the native arabinoxylan (Ciacco and D'Appolonia, 1982*a,b*; Izydorczyk *et al.*, 1991; Izydorczyk and Biliaderis, 1992*a*), the ratio of arabinose

to xylose (Izydorczyk and Biliaderis, 1992*a*), the type (Hoseney and Faubion, 1981; Izydorczyk *et al.*, 1990) and concentration of oxidant (Moore *et al.*, 1990; Izydorczyk *et al.*, 1990). Oxidants yielding free radicals, such as hydrogen peroxide in combination with peroxidase or ammonium persulphate, are able to induce gelation, whereas other oxidants, such as potassium bromate and potassium iodate, are not (Neukom and Markwalder, 1978; Hoseney and Faubion, 1981). The cross-linking product of the reaction is identified as dehydrodiferulic acid, which is coupled through the 5-position of each of the ferulic acid residues (Geissmann and Neukom, 1971). This dimer also occurs naturally in wheat endosperm walls (Markwalder and Neukom, 1976). Linkage through a carbon-carbon bond between the propenoic substituents of the phenyls, being at the 8-position, has also been suggested (Hoseney and Faubion, 1981).

Outline of the thesis

As a result of the assumed role of arabinoxylans in the preparation of dough and baking performance of white wheat flour, their chemical and physical characteristics have been studied extensively. Their functional properties, such as viscosity and ability to gel, seem to be attractive for application as a food additive. Because wheat flour contains only small quantities of arabinoxylans and these polysaccharides are ubiquitous in cereal cell walls, other sources are expected to be more advantageous for exploitation. For example by-products of the cereal industry, such as spent grains from the production of beer from barley and wheat bran from the production of white flour from wheat. These by-products are currently mainly used as ingredients of animal feed and can be considered as cheap sources for extraction of arabinoxylans. The product of choice for this research is wheat bran, which is one of the major by-products of the cereal industry.

The investigations were directed towards the development of extraction methods for wheat bran glucuronoarabinoxylans, whether or not feruloylated, and towards the relation between structural and physical characteristics of these polysaccharides. With regard to the latter, examination of adjustment of physical properties by means of enzymatic modification was also of interest. Firstly, extraction of glucuronoarabinoxylans was focused on a high yield and high purity. This was expected to result in non-feruloylated polysaccharides, because large amounts of arabinoxylans appear to be only extractable by the use of alkali at concentrations of 0.2 M or higher, which results usually in a loss of ester-linked ferulic acid. Prior to extraction, intracellular constituents, such as lipids, protein and starch, were removed from the bran (Chapter 2). Once considerable quantities of glucuronoarabinoxylans were selectively extracted, the composition and fine structure of the main populations was investigated by fractionation studies and methylation analysis.

Knowledge on the distribution of substituents was gained by using enzymes with known modes of action in degradation studies. For identification of degradation products well-characterized oligosaccharides of previous studies on wheat flour arabinoxylan were available (Chapter 3). Subsequently, functional properties, such as viscosity, interaction with other polysaccharides and emulsion stabilization, were investigated. Also the effect of minor variations in structure, which can be realized by enzymatic or chemical means, on these physical properties were studied. This was aimed at the exploration of relations between structural features and physical properties (Chapter 4). Secondly, extraction of glucuronoarabinoxylans was focused on obtaining feruloylated polysaccharides, which are able to form gels upon addition of oxidative reagents. For that purpose an extraction method was developed (Chapter 5). Finally, the feruloylated wheat bran arabinoxylans were compared with those of wheat flour, with regard to their gel-forming ability. Differences in intrinsic properties of the arabinoxylans, such as composition, structure and molecular weight, could then be related to the cross-linking behaviour (Chapter 6).

CHAPTER 2

Optimization of the selective extraction of glucurono-arabinoxylans from wheat bran: use of barium and calcium hydroxide solutions at elevated temperatures

This chapter has been published in *Journal of Cereal Science* **23** (1996): 235-245 by the authors M.E.F. Bergmans, G.Beldman, H. Gruppen and A.G.J. Voragen.

Abstract

Two approaches were investigated in attempts to obtain a high extraction yield of glucuronoarabinoxylans from water-unextractable cell wall material of wheat bran using saturated barium hydroxide containing 0.26 M sodium borohydride. Firstly, pretreatments, such as autoclave treatment, alkaline peroxide and chlorite delignification, of the cell wall material prior to extraction appeared to have no effect on the extract yield. Moreover, modifications to the composition and molecular weight distribution of the glucuronoarabinoxylans occurred when such treatments were used. Secondly, the effect of an increasing extraction temperature and concentration of alkali was investigated. Increasing the extraction temperature improved the yield of glucuronoarabinoxylans from 29% at 20°C to 50% at 95°C. Increasing the barium hydroxide concentration with the temperature resulted in no further improvements in yield up to 70°C. Above this temperature the yield decreased. Substitution of barium hydroxide by calcium hydroxide resulted in lower yields and a lower selectivity; the lower solubility of calcium hydroxide may have been responsible for this. Supplementary experiments to investigate the mechanism of the selectivity of the bivalent hydroxide extraction with addition of sodium borohydride indicated a possible role for borate, derived from borohydride.

Introduction

Wheat bran is produced world-wide in enormous quantities as a by-product of the milling of white wheat flour for human consumption. Because it comprises the outer tissues of the wheat kernel, including the aleurone layer and some adhering endosperm, wheat bran consists mainly of cell wall polysaccharides, such as glucuronoarabinoxylans, cellulose and (1→3)(1→4)-β-glucans, protein and lignin. Although the digestibility of these cell wall components by monogastrics is not very high (Saunders *et al.*, 1972), wheat bran has been used mainly as an ingredient for animal feed until now. Minor amounts are used as a source of dietary fibre for human consumption.

Presently, alternative applications of wheat bran and other agricultural by-products are of interest because their economic value as feed ingredients is decreasing. Research is now focused on the major component of wheat bran, the glucuronoarabinoxylans. Arabinoxylans, which are also known as pentosans, can be used as viscosity enhancer or thickener because they are able to hold large quantities of water. Water-holding capacities of 4 to 10 times their weight in water are reported, varying with the solubility and the wheat cultivar from which they are derived (Jeleca and Hlynka, 1971; Kim and D'Appolonia, 1977). Other recently reported functional properties of arabinoxylans are their ability to stabilize protein foams (Izydorczyk and Biliaderis, 1992*a*) and their applicability as a source for the production of oligosaccharides with possible physiological functions (Yamada *et al.*, 1994), or with anti-oxidative activity when substituted with phenolic acids (Ohta *et al.*, 1994).

For the development of new applications for wheat bran, and more specifically wheat bran glucuronoarabinoxylans, knowledge of their composition and structure is important. These glucuronoarabinoxylans are known to consist of a backbone of β-(1→4)-linked xylopyranosyl

units to which arabinofuranose is linked at the *O*-3 and *O*-2 positions and uronic acid at the *O*-2 position (Adams, 1955; Ring and Selvendran, 1980). Additionally, phenolic acids, such as ferulic and *p*-coumaric acid, are esterified to some of the arabinose residues (Smith and Hartley, 1983; Pussayanawin and Wetzel, 1987). The fairly linear structure of arabinoxylans results in a rod-like conformation, as a consequence of which these polysaccharides form viscous solutions upon hydration (Andrewartha *et al.*, 1979).

To investigate these related chemical and physical properties of glucuronoarabinoxylans from wheat bran, selective extraction would be favourable. Recently, barium hydroxide has been introduced as a highly selective extractant for arabinoxylans from cell wall material of wheat flour (Gruppen *et al.*, 1991). This extractant also proved to be selective for the extraction of glucuronoarabinoxylans from cell wall material prepared from barley (Viëtor *et al.*, 1992), sorghum (Verbruggen *et al.*, 1995) and rye (Vinkx *et al.*, 1995). The yields of the extracts from these latter cereals, however, appeared to be less than for wheat flour.

In this study the selective extraction of glucuronoarabinoxylans from cell wall material of wheat bran was investigated and optimized. For that purpose the effect of three different pretreatments of the cell wall material on the subsequent extraction was studied, as well as the effect of increased temperature and concentration of barium hydroxide during extraction. Additionally, the effect of a different bivalent hydroxide was examined. All extracts were compared on the basis of yield, composition and molecular weight distribution.

Experimental

Materials

Industrial wheat bran was obtained from Presco International (Weert, The Netherlands). The bran was milled using a Fritsch pulverisette mill (Marius Instruments, Utrecht, The Netherlands) equipped with a 0.5 mm sieve.

Isolation of water-unextractable cell wall material

The isolation of water-unextractable cell wall material (WUS) from milled wheat bran was performed by a slightly modified procedure as described for sorghum (Verbruggen *et al.*, 1993). Milled wheat bran (300 g) was defatted by Soxhlet extraction with 3.5 l of *n*-hexane for 6 h. Subsequently, the protein was extracted from the air-dried residue with 1.5 l of 1.5% (w/v) sodium dodecylsulphate, containing 0.05% (v/v) 2-mercaptoethanol, for 1 h with continuous stirring. After centrifugation (11,000 *g*; 20 min), the pellet was resuspended and the extraction was repeated twice. The residue was washed twice with 1.5 l of distilled water and afterwards suspended in 2 l of 0.01 M maleic acid, 0.01 M sodium chloride, 0.001 M calcium chloride, 0.05% (w/v) sodium azide, pH 6.5. After heating the suspension for 1.5 h at 85°C,

it was cooled to 30°C and 2 mg of porcine pancreatic α-amylase (Art. 16312, Merck, Darmstadt, Germany) was added. The suspension was incubated at 30°C in a shaking incubator for 20 h. The residue, obtained by centrifugation (11,000 *g*; 20 min), was washed with hot distilled water of 65°C and centrifuged again. The α-amylase incubation was repeated twice. The final residue was resuspended in distilled water and freeze-dried (WUS).

Pretreatments of WUS

Autoclave treatment. WUS was suspended in distilled water containing 0.01% sodium azide at a solid to liquid ratio of 1:100 (w/v) and autoclaved for 1 h at 121°C and 0.1 MPa overpressure. After centrifugation (17,700 *g*; 30 min), the residue was washed twice with distilled water and freeze-dried (AR). Supernatant and washings were combined and freeze-dried.
Alkaline peroxide delignification. WUS was suspended in 3% (w/v) hydrogen peroxide, which was adjusted to pH 11.5 with 5 M sodium hydroxide, at a solid to liquid ratio of 1:50 (w/v). The suspension was stirred for 4 h at room temperature, while the pH was kept constant by the addition of 2 M hydrochloric acid. The insoluble residue was recovered by filtration, washed extensively with distilled water and freeze-dried (PR). The filtrate and washings were treated as described above.
Chlorite delignification. 2 g of WUS was suspended in 100 ml of distilled water. The pH of the suspension was adjusted to 4.75 with glacial acetic acid and it was heated to 70°C. Over a period of 2 h, two portions sodium chlorite of 0.4 g each, and glacial acetic acid of 0.1 ml each, were added to the suspension, which was stirred continuously and maintained at 70°C. The insoluble residue (CR) and the solubles were recovered as described above.

Saturated barium or calcium hydroxide extraction

WUS and pretreated residues were extracted with saturated barium or calcium hydroxide solution with a solid to liquid ratio of 1:200 (w/v), based on the method described by Gruppen *et al.* (1991). The barium hydroxide solution was saturated at room temperature (BE.RT) or extraction temperature (BE.ET); the calcium hydroxide solution at extraction temperature (CE.ET). Saturation was achieved by stirring an excess of the hydroxide for 8 h at the desired temperature. After precipitation of the undissolved salts at that temperature the solution was taken off. Prior to extraction, sodium borohydride was added to this solution to give a concentration of 0.26 M. The suspensions were stirred for 16 h at temperatures ranging from 20 to 95°C. Extracted material was obtained by centrifugation (17,700 *g*; 30 min), and the pH was adjusted to pH 5 with glacial acetic acid. This procedure was repeated once with the residue. The two extracts were combined and dialysed extensively against 0.2 M sodium acetate buffer, pH 5 at 4°C, then against running tap water and finally against distilled water. After reduction of the volume by vacuum evaporation, the extracts were freeze-dried.

Analytical methods

Neutral sugar composition. Neutral sugars in wheat bran were analysed as total non-starch polysaccharides as described by Englyst and Cummings (1984). For WUS and extracts, neutral sugars were determined without prior starch removal. Hydrolysis of the polysaccharides with 1 M sulphuric acid for 3 h at 100°C was preceded by a treatment with 72% (w/w) sulphuric acid for 1 h at 30°C. Inositol was used as internal standard. Monosaccharides were analysed as their alditol acetates on a 15 m × 0.53 mm i.d. DB225 column in a Carlo Erba 4200 gas chromatograph. The temperature programme was set at: 180°C for 1 min, 180 to 210°C at 2.5°C/min and 210°C for 7 min. The FID-detector was set at 260°C and helium was used as carrier gas. Non-starch glucose in WUS was calculated as anhydro-glucose determined as glucitol acetate minus anhydro-glucose derived from starch. Cellulosic glucose in WUS was calculated as the difference between the anhydro-glucose contents determined with or without pretreatment with 72% (w/w) sulphuric acid.

Uronic acid was determined as anhydro-uronic acid by an automated *m*-hydroxydiphenyl assay using an auto-analyser (Thibault, 1979). To minimize the difference in response between glucuronic and galacturonic acid, 0.0125 M sodium tetraborate was added to the sulphuric acid. The results were corrected for interference by neutral sugars in the samples.

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

(1→3)(1→4)-β-glucan was determined enzymatically with a test kit (Biocon, Victoria, Australia), the liberated glucose was determined with a glucose test kit (Boehringer, Mannheim, Germany).

Protein content of wheat bran, WUS and pretreated WUS was determined by a semi-automated micro-Kjeldahl method. A conversion factor of 6.25 was used instead of the commonly used factor 5.7 for cereals, because the glutamate/glutamine content of the aleurone layer, which is a protein-rich tissue of wheat bran, is relatively low (Bacic and Stone, 1981). Protein contents of alkaline extracts were determined by a Coomassie Blue G-250 assay (Sedmak and Grossberg, 1977) using bovine serum albumin (Art. A-4503, Sigma, St. Louis, MO, USA) as standard.

Lignin was determined gravimetrically as Klason-lignin, which is the residue after hydrolysis with 72% (w/w) sulphuric acid for 1 h at 30°C followed by 1 M sulphuric acid for 3 h at 100°C, corrected for ash. Ash was determined by incineration at 700°C for 7 h.

Degree of acetylation of wheat bran, WUS and pretreated residues was determined by HPLC after saponification by 0.4 M sodium hydroxide (Voragen *et al.*, 1986).

Total esterified phenolic acids were determined by HPLC of the individual phenolic acids after saponification with 0.5 M potassium hydroxide for 16 h at room temperature (Gruppen *et al.*, 1989). The total content was calculated as *p*-coumaric plus ferulic acid.

High-performance anion-exchange chromatography (HPAEC). Soluble fractions of WUS pretreatments were analysed for presence of arabinose, xylose and arabinoxylooligomers by HPAEC as described by Gruppen *et al.* (1993*a*). Elution involved linear gradients of sodium acetate in 0.1M sodium hydroxide of 0-0.1 M over 10 min and 0.1-0.4 M over 35 min. *High-performance size-exclusion chromatography (HPSEC).* The molecular weight distributions of the extracts were determined by HPSEC, performed on a SP 8810 HPLC (Spectra Physics) equipped with three Bio-Gel TSK columns (each 300 × 7.5 mm; Bio-Rad Labs, Richmond, CA, USA) in series: 60XL (exclusion limit dextrans M_w $5 \cdot 10^7$); 40XL (exclusion limit dextrans M_w $1 \cdot 10^6$); 30XL (exclusion limit dextrans M_w $2 \cdot 10^5$) in combination with a TSK XL guard column (40 × 6 mm) and eluted at 30°C with 0.4 M sodium acetate buffer, pH 3, at a flow rate of 0.8 ml/min. The column effluent was monitored using a refractive index detector (Shodex SE-61, Showa Denko, Tokyo, Japan). For some samples the weight average molecular weight was determined using a Dawn-F/HT multi-angle laser light-scattering detector (Wyatt Technology, Santa Barbara, CA, USA) in combination with a refractive index detector (Jumel *et al.*, 1992).

Results and Discussion

Composition of WUS and pretreated residues

The composition of wheat bran, as well as the yields and compositions of WUS and its pretreated residues, namely autoclaved, peroxide and chlorite delignified WUS, are given in Table 1. WUS was isolated from wheat bran with a yield of approximately 58%. From the data given in Table 1 it can be calculated that WUS contained 86% of the non-starch neutral sugars, 7% of the starch, 18% of the protein and 84% of the lignin originally present in the bran. The predominant non-starch neutral sugars, arabinose, xylose and glucose in the WUS, represented 93%, 78% and 75%, respectively, of each of the sugars originally present in the bran. The minor components, acetic and phenolic acids, were recovered almost completely in WUS, whereas approximately 70% of the uronic acids were recovered. Two-thirds of the polysaccharides in WUS was accounted for by arabinose and xylose (Table 2). The remainder was mainly glucose, 65% and 14% of which was accounted for by cellulose and (1→3)(1→4)-β-glucan, respectively. Although the extent of milling and method of isolation of WUS or cell wall material influence its final composition, our results agreed well with findings of other workers (Selvendran *et al.*, 1980; Carré and Brillouet, 1986). Only minor differences were found in protein and starch contents of the isolated cell wall material. The higher lignin content of our material was probably due primarily to the method of determination, in which we did not correct for tannins and residual protein.

Table 1: Yield and composition of wheat bran, WUS and residues of WUS from wheat bran after autoclave, peroxide and chlorite treatment.

	Bran	WUS	AR	PR	CR
Yield[a]	-	100	87.5	65.4	75.6
Composition[b]					
neutral sugars[c]	66.8 (11.8)	75.7 (1.5)	78.7 (nd)	79.1 (nd)	74.9 (nd)
protein	15.9	5.0	4.5	3.4	3.1
lignin	9.8	14.3	9.8	8.7	6.1
uronic acid	2.7	3.2	4.0	3.4	3.5
acetic acid	0.6	1.2	1.1	tr	1.2
phenolic acid	0.4	0.8	0.8	0.1	tr

[a] expressed as weight percentage (dm) of WUS.
[b] based on 100 g of each fraction (dm).
[c] the figures in parenthesis represent the starch content.
nd = not determined; tr = trace amount.

The autoclave treatment resulted in only a minor loss of material, especially lignin-like material and protein, causing a slight increase of the neutral sugar content of AR (Table 1). Over 90% of the neutral sugars were recovered, with the lowest recovery being 80% for glucose. Residual starch and some mixed linkage β-glucans were presumably removed from WUS. The recovery of arabinose was slightly less than that of xylose, which is evident from the lower Ara/Xyl-ratio than was found in WUS (Table 2). This observation may be attributed to the higher sensitivity of furanoses towards degradation under weakly acidic conditions at elevated temperatures than that of pyranoses (Fry, 1988). The acidic conditions could have been provided by release of esterified acetic acid during the treatment as 20% of the amount originally present in WUS was not recovered in the residue. In the solubilized material monomeric arabinose and some small arabinose and xylose containing oligomers were detected by HPAEC-analysis (results not shown).

Peroxide delignification of WUS resulted in a 35% loss of material. From Table 1 the recovery of neutral sugars, protein and lignin-like substances in the residue (PR) were calculated and amounted to 68%, 45% and 40%, respectively, of each of the components originally present in WUS. Esterified acetic and phenolic acids were lost almost completely as a consequence of the alkaline conditions. From the molar sugar compositions a relative loss of arabinose and an enrichment of glucose could be observed (Table 2). These observations, together with the high overall loss of material, agreed with the results of a study on the delignification of rice straw with a similar peroxide concentration (Patel and Bhatt, 1992). As a result of the relative loss of arabinose, the Ara/Xyl-ratio of the residue was lower than that found in WUS. It can be argued that this was caused by selective extraction of a highly

substituted arabinoxylan. It is known that arabinoxylans of high Ara/Xyl-ratio are more easily extracted by dilute alkali than less branched arabinoxylans (DuPont and Selvendran, 1987). These dilute alkaline conditions were provided by the peroxide treatment. A polymeric fraction in the solubilized material was actually detected by HPSEC-analysis (results not shown).

The chlorite delignification resulted in a 25% loss of material (Table 1), which corresponds well with results of a similar treatment on wheat bran from which starch had been removed (Brillouet and Mercier, 1981). The solubilized material consisted of monomeric arabinose and xylose and some small oligomers on the basis of HPAEC-analysis. No polymeric material was detected by HPSEC-analysis (results not shown). The loss of lignin during chlorite delignification appeared to be higher than for peroxide treatment, which was calculated to be 70% and 60%, respectively (Table 1). The content of neutral sugars, nevertheless, was not increased compared with WUS. Solubilization of carbohydrates by chlorite treatment was reported previously (Ford, 1986). The recovery of the predominant neutral sugars in CR was 60% for glucose and over 80% for arabinose and xylose. This resulted in a slight enrichment in arabinoxylan, which had an Ara/Xyl-ratio equal to that of WUS (Table 2). This implies a similar sensitivity of arabinose and xylose to the chlorite treatment. The phenolic acids were lost completely, while 75% of the acetic acid was recovered (calculated from Table 1). This agreed with observations on pangola grass (Ford, 1986). From these results it can be concluded that chlorite delignification is more effective in removing lignin, and gives a lower loss of arabinose and xylose than peroxide delignification.

Barium hydroxide extracts of WUS and pretreated residues

Extraction of glucuronoarabinoxylans from the differently treated cell wall materials was performed by barium hydroxide solutions saturated at 20°C. The yields and molar sugar compositions of the extracts are given in Table 2. The extracts were designated BE, BE_A, BE_P and BE_C; the capital in subscript refers to the autoclave, peroxide and chlorite pretreatments, respectively. As observed for WUS from wheat flour (Gruppen *et al.*, 1991), the barium hydroxide extraction was also highly selective for glucuronoarabinoxylans from wheat bran. This is evident from comparison of the molar sugar compositions of the cell wall materials and their corresponding extracts (Table 2). The extracts had total sugar contents ranging from 84 to 92%, over 95% of which was glucuronoarabinoxylan, calculated as the sum of arabinose, xylose and uronic acid present. The different pretreatments of WUS carried out in attempts to improve the extraction yield did not result in pronounced increases compared with that for WUS, however. The highest increase in extraction yield was observed for chlorite delignification. Ring and Selvendran (1980), who investigated the influence of chlorite treatment prior to potassium hydroxide extraction on beeswing wheat bran, made similar observations. Their yields were higher than found here, probably because of the

Table 2: Yield and composition of WUS and residues of WUS from wheat bran after autoclave, peroxide and chlorite treatment and of their barium hydroxide extracts.

	yield[a]	total sugar content[b]	molar composition[c]						
			Ara	Xyl	Man	Gal	Glc	UA	Ara/Xyl
untreated									
WUS	100	78.9	27.7	40.7	0.2	1.1	27.0	3.3	0.68
BE	19.4	87.0	41.1	53.2	0	0.9	0.8	3.9	0.77
autoclave treated									
AR	87.5	82.7	27.3	44.6	0	1.2	22.9	3.9	0.61
BE_A	21.6(24.7)	86.8	42.0	53.2	0	0.9	0.4	3.5	0.79
peroxide treated									
PR	65.4	82.5	23.8	40.3	0.2	1.0	31.1	3.6	0.59
BE_P	16.5(25.2)	92.4	45.8	48.3	0	1.3	0.9	3.7	0.95
chlorite treated									
CR	75.6	78.4	29.3	43.1	0.1	0.8	24.3	2.5	0.68
BE_C	23.5(31.1)	84.2	41.8	50.0	0	1.4	1.9	5.0	0.84

[a] expressed as weight percentage (dm) of WUS. The figures in parentheses represent the yield based on AR, PR or CR.
[b] neutral sugars + uronic acids expressed as weight percentage (dm) of each fraction.
[c] expressed as percentage (mole per 100 mole).

higher concentration of alkali used, which was 1 M instead of 0.23 M for barium hydroxide saturated at room temperature (Linke, 1958), and because of coextraction of glucans, as is known to occur with potassium and sodium hydroxide. They also observed a slightly higher arabinose recovery in the extract after chlorite treatment compared with the xylose recovery. This was shown also in the present study by the higher Ara/Xyl-ratio of BE_C. The yields of glucuronoarabinoxylans extracted were very similar when based on WUS. These were 29.2%, 29.5%, 27.8% and 30.2% for WUS, AR, PR and CR, respectively. Only when calculated on the basis of the pretreated residues were significant differences found. In that case, the yields were 30.1%, 42.5% and 36.4% for AR, PR and CR, respectively.

The molecular weight distributions of these extracts were very similar, except for the extract of the chlorite treated WUS. The weight average molecular weights and the polydispersity indices of BE, BE_A and BE_P were approximately 400,000 Da and 1.8, respectively. The relatively higher polydispersity index of BE_C, which was 2.6 compared to 1.8 for the other extracts, proved a wider distribution. The weight average molecular weight of the chlorite extract of 570,000 also differed from those of the other extracts.

In conclusion, the pretreatments of WUS did not result in higher extraction yields. Furthermore, changes in composition and molecular weight distributions of the extracts occurred as a consequence of the pretreatments.

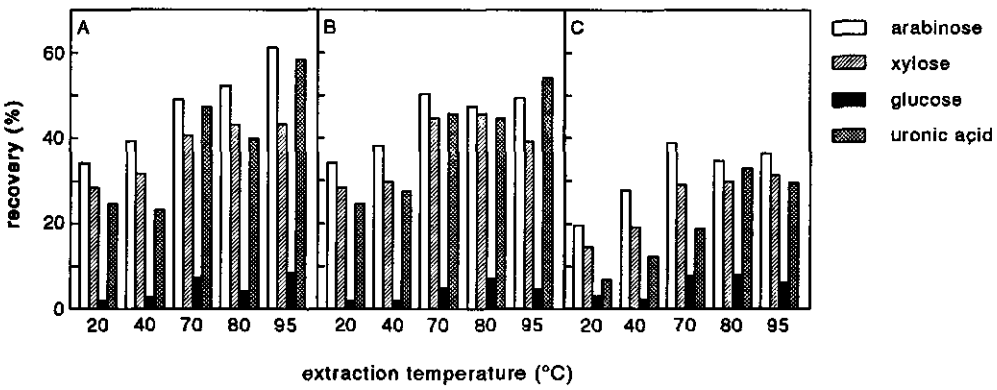

Figure 1: Individual recoveries (% w/w) of sugars in extracts obtained from WUS at different extraction temperatures with barium hydroxide saturated at room temperature (A), barium hydroxide saturated at extraction temperature (B) and calcium hydroxide saturated at extraction temperature (C).

Barium and calcium hydroxide extracts of WUS obtained at elevated temperatures

Since pretreatments of WUS were not very effective in increasing the yields, optimization of the saturated barium hydroxide extraction was investigated by increasing the temperature of extraction (BE.RT) and by increasing the concentration of barium hydroxide with temperature (BE.ET). The effect of another bivalent hydroxide, such as calcium hydroxide, was investigated also (CE.ET). The yields and compositions of the extracts at the temperature indicated are shown in Table 3. Most of these extractions were performed in duplicate; the maximum deviation in yield determined was 1.5%. In Figure 1 recoveries of the sugars, arabinose, xylose, glucose and uronic acid, are presented.

The BE.RT-series of extractions showed that with the same barium hydroxide concentration an increasing temperature resulted in an increase in yield (Table 3) and increased recoveries of the predominant sugars (Figure 1A). The Ara/Xyl-ratio of the extracts increased with temperature, with only minor changes between 40 and 80°C. The sugar content of the extracts, however, decreased slightly between 20 and 70°C. The selectivity of the extraction also decreased slightly as higher amounts of glucose were obtained in the extracts at higher temperatures. Gruppen *et al.* (1991) showed that the glucose originated not from residual

Table 3: Yield and composition of alkaline extracts obtained from WUS of wheat bran at temperatures ranging from 20 to 95°C by barium hydroxide saturated at room temperature (BE.RT), by barium hydroxide saturated at extraction temperature (BE.ET) and calcium hydroxide saturated at extraction temperature (CE.ET).

	yield[a]	protein content[b]	total sugar content[c]	molar composition[d]						
				Ara	Xyl	Man	Gal	Glc	UA	Ara/Xyl
BE.RT										
20	19.4	2.9	87.0	41.1	53.2	0	0.9	0.8	3.9	0.77
40	23.1	5.6	82.4	42.6	50.6	0	0.9	2.8	3.0	0.84
70	31.1	1.4	83.7	39.6	48.3	0.2	1.7	5.8	4.5	0.82
80	31.4	2.1	83.7	41.4	50.2	0	1.4	3.2	3.7	0.82
95	35.5	2.3	83.8	43.0	44.8	0	1.4	5.9	4.8	0.96
BE.ET										
20	19.4	2.9	87.0	41.1	53.2	0	0.9	0.8	3.9	0.77
40	20.8	3.9	88.4	43.1	49.4	0.1	1.4	2.3	3.7	0.87
70	32.1	2.2	82.8	39.4	51.5	0	1.2	3.7	4.2	0.77
80	33.9	2.0	78.9	37.0	52.3	0	1.2	5.4	4.1	0.71
95	31.9	1.9	79.3	41.0	47.7	0	2.3	3.8	5.3	0.86
CE.ET										
20	11.6	5.9	82.4	42.8	46.9	0.4	1.5	6.7	1.8	0.91
40	16.4	3.1	76.8	45.8	46.6	0	1.6	3.7	2.4	0.98
70	24.5	2.9	79.2	41.8	46.0	0.1	1.6	8.2	2.4	0.91
80	24.5	2.8	78.7	37.8	47.9	0	1.5	8.6	4.2	0.79
95	24.5	1.5	79.8	38.9	49.4	0	1.5	6.5	3.8	0.79

[a] expressed as weight percentage (dm) of WUS.
[b] expressed as weight percentage (dm) of each fraction.
[c] neutral sugars + uronic acids expressed as weight percentage (dm) of each fraction.
[d] expressed as percentage (mole per 100 mole).

starch but mainly from (1→3)(1→4)-β-glucan. Our WUS contained 3.3% (1→3)(1→4)-β-glucan and 1.5% residual starch. For the BE-extracts obtained at 20°C, the glucose may be derived mainly from (1→3)(1→4)-β-glucan. For the extracts obtained at higher temperatures glucose may originate elsewhere as well. In the WUS 21% of the non-starch glucose was not accounted for by cellulose or (1→3)(1→4)-β-glucan. It is not known whether this is attributable to shortcomings of the methods used or to the presence of other polysaccharides.

The BE.ET-series of extracts, which were obtained by increasing the temperature of extraction and the concentration of barium hydroxide from 0.23 M at 20°C to over 6 M at 95°C (Linke, 1958), showed an optimum in yield at 80°C (Table 3). This optimum of 33.9% was lower than the maximum observed in the BE.RT-series at 95°C. The individual recoveries of the sugars increased until 70°C, above which temperature no further increase occurred (Figure 1B). The sugar content of the extracts again showed a decrease with increasing

temperature (Table 3). This decrease was now more pronounced than in the BE.RT-series, presumably as a result of the increase of barium hydroxide concentration at elevated temperature. The protein content also decreased. This may have been caused by degradation as the cleavage of peptide bonds by barium hydroxide at elevated temperatures has been reported (Lamport, 1967). Because of the lower sugar and protein contents, a greater proportion of material is unaccounted for. Coextraction of lignin probably occurred because the colour of the extract became darker with increasing temperature. It is also reported that lignin can be extracted from plant material at higher temperatures with high concentrations of alkali (Vilpponen *et al.*, 1993). The Ara/Xyl-ratio of this series of extracts showed no relation with temperature and concentration of alkali. The increased yields at 70 and 80°C may have been caused partly by the high solubility of barium hydroxide. The rise in solubility of barium hydroxide with temperature is almost linear until 40°C, but increases exponentially above this temperature (Linke, 1958). Extraction at 95°C resulted in a decreased yield, probably because degradation of polymers occurred. This can be inferred from the small shift to low molecular weight as observed by HPSEC-analysis (results not shown). The total recovery of extract and residue after extraction at 95°C was also less than for the others.

The CE.ET-series showed lower overall yields of extracts than both BE-series (Table 3). This is due most probably to the lower solubility of calcium hydroxide, being 0.023 M compared with 0.23 M for barium hydroxide at 20°C. It is also noteworthy that the solubility of calcium hydroxide decreases as a function of temperature, from 0.023 M at 20°C to 0.014 M at 70°C (Linke, 1958). The maximum yield of 24.5% was obtained at 70°C (Table 3). This yield and the recovery of predominant sugars (Figure 1C) showed no increase above 70°C. This corresponds with the BE.ET-series, in which the alkali concentration increased.

In Figure 2 the course of glucuronoarabinoxylan recoveries as a function of temperature is shown. For the BE.RT-series a maximum glucuronoarabinoxylan yield of 50% was obtained at 95°C. An optimum in glucuronoarabinoxylan yield of 46% was found at 70°C for BE.ET, as a result of a decreasing yield and sugar content of the extracts above 70-80°C. The CE.ET-series of extractions gave a maximum glucuronoarabinoxylan yield of 32% at 95°C. The increase between 70 and 95°C was not very high, however. In order to make a comparison with common extraction conditions a yield of 53% of glucuronoarabinoxylan was obtained from wheat bran WUS by extraction with 1 M potassium hydroxide containing 0.26 M sodium borohydride at 20°C. The sugar content of this extract was only 76%, however, and the extract contained 20% glucose on a molar basis (no further results shown).

The molecular weight distributions of the extracts were quite similar. Their weight average molecular weights ranged from 400,000 to 540,000 and the polydispersity indices ranged from 1.5 to 1.8. No relation could be found between these molecular weights and the extraction variables.

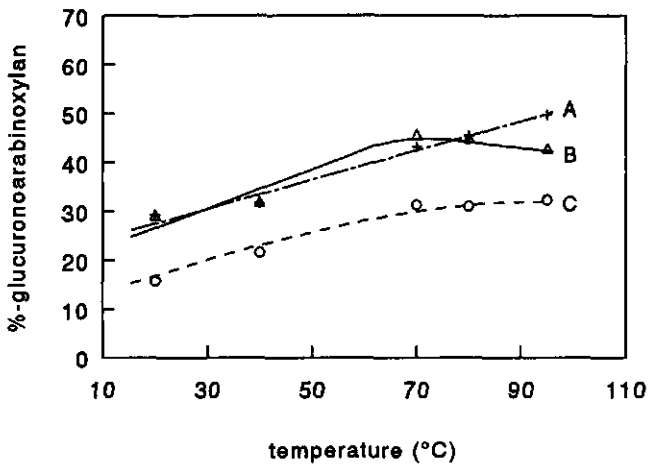

Figure 2: Yields of glucuronoarabinoxylans (% w/w) from WUS of wheat bran by extraction at different temperatures with barium hydroxide saturated at room temperature (A), by barium hydroxide saturated at extraction temperature (B) and by calcium hydroxide saturated at extraction temperature (C).

Selectivity of barium and calcium hydroxide

Although the selective extraction of glucuronoarabinoxylans with saturated barium hydroxide solutions containing 0.26 M sodium borohydride has already been used on different cell wall materials, the mechanism of its selectivity is still not clear.

The presence and concentration of hydroxyl ions is important in determining the yield. In general, a higher concentration of hydroxide results in a higher yield, when used at room temperature. Hydroxyl ions are believed to cause swelling of cellulose, disruption of hydrogen bonds between cellulose and hemicelluloses and hydrolysis of esters linked to hemicelluloses (Fry, 1988). The latter action may involve disruption of linkages between arabinoxylan and lignin as esterified phenolic acids are known to serve as cross-linking units (Lam *et al.*, 1992*b*).

Bivalent cations, such as Ba^{2+}, are considered to be responsible for the unextractability of (1→3)(1→4)-β-glucans (Gruppen *et al.*, 1991). The solubility of barium hydroxide of 0.23 M at 20°C gives a Ba^{2+} concentration of 0.1 M (Sillen and Martell, 1964), which appears to be sufficient for keeping the mixed linkage glucans unextractable. The concentration of Ca^{2+} of 0.01 M in a saturated calcium hydroxide solution seems not to be sufficient to maintain an equal selectivity. In an additional experiment extraction of wheat bran WUS at 20°C with

Table 4: Molar sugar composition of extracts obtained from wheat bran WUS after a single 16 h extraction at 70°C with saturated calcium hydroxide containing different ratios of borohydride and borate based on a total boron concentration of 0.26 M.

	molar composition[a]					
	Ara	Xyl	Man	Gal	Glc	UA
$CE_{260:0}$[b]	46.5	43.9	0	1.7	2.9	5.0
$CE_{180:80}$	40.7	46.4	0	1.5	7.9	3.5
$CE_{100:160}$	41.0	48.1	0	1.8	5.8	3.3
$CE_{20:240}$	38.8	51.8	0	1.2	5.1	3.1

[a] expressed as percentage (mole per 100 mole).
[b] the digits in subscript refer to the concentrations of boron (in mM) derived from borohydride and borate, respectively.

saturated barium hydroxide diluted to the concentration of saturated calcium hydroxide at 20°C, ruled out an effect of the cation, because the molar sugar compositions of the diluted barium hydroxide extract and the calcium hydroxide extract (CE.ET-20) were very similar (results not shown).

The presence of 0.26 M sodium borohydride was previously shown to be jointly responsible for a high selectivity of the extractant. A similar extraction on wheat flour WUS with a much lower concentration of 0.02 M sodium borohydride, which was calculated to be sufficient for prevention of alkaline peeling, resulted in more glucan being extracted (Gruppen *et al.*, 1991). The involvement of borate, arising from decomposition of sodium borohydride, was discussed by the same authors. A barium hydroxide extraction of wheat bran WUS with addition of 0.02 M sodium borohydride plus 0.026 M sodium borate showed no increase in selectivity compared with extraction with only 0.02 M sodium borohydride. In a new experiment with wheat bran WUS, using calcium hydroxide as extractant with different ratios of sodium borohydride and sodium borate, all based on a concentration of 0.26 M boron, an improved selectivity was shown with an increasing amount of borate after a single 16 h extraction at 70°C. The molar compositions of the extracts obtained are shown in Table 4, together with the composition of a similar extract using only 0.26 M sodium borohydride. This latter extract resulted in a higher selectivity, which was obvious from the low percentage of glucose. An explanation for this may be the influence of the slow evolution of borate from borohydride during extraction. The evolution of borate from borohydride is dependent on pH and temperature (Sillen and Martell, 1964). The lower the pH and the higher the temperature, the more rapidly will the borohydride decompose. The pH during extraction with barium or calcium hydroxide solutions at higher concentrations and higher temperatures is difficult

to predict because the dissociation of the hydroxide is temperature dependent. Additionally, the presence of sodium borohydride and the production of borate will have some influence on the pH of the solution. Both lowered the pH when added to a saturated barium hydroxide solution. It is impossible, therefore, to calculate the amounts of borate produced during extraction and to prove a possible relationship between the selectivity and the ratio of borohydride and borate. It is clear, nevertheless, that the concentration of hydroxyl ion, bivalent cation and borohydride, temperature and pH are inextricably linked, and that all take part in determining the yield and selectivity.

Conclusions

Pretreatments of WUS from wheat bran to remove lignin or loosen the structure of the cell wall were not very effective in increasing the yield in the subsequent saturated barium hydroxide extraction at room temperature when based on the original WUS. The treatments resulted in major losses of material or had no great impact on the extraction yield. Additionally, the extracts showed some detrimental modifications in composition and molecular weight distribution. Therefore, different conditions during extraction with barium hydroxide were investigated. By increasing the temperature of extraction, it was possible to improve the yield of glucuronoarabinoxylans extracted from 29% at 20°C to 50% at 95°C. By increasing the temperature of extraction, as well as the concentration of alkali, no additional improvement was observed. On the contrary, a decrease in the yields occurred above 70°C. The use of saturated calcium hydroxide solutions with increasing temperatures resulted in lower yields and selectivity, which could be explained by the lower solubility of calcium hydroxide than that of barium hydroxide. An indication was obtained of a possible role of borate, originating from decomposition of borohydride, in the selectivity of the extraction.

Acknowledgement

We thank Dr. J.H.G.M. Mutsaers (Royal Gist-brocades, Delft, The Netherlands) for fruitful discussions, Ms. P. Lukešová (Institute of Chemical Technology, Prague, Czech Republic) for her contribution to the experimental work, and Dr. K. Brunt for performing laser light-scattering analyses. This research was supported financially by the Dutch National Innovation Oriented Programme Carbohydrates (IOP-k) and Royal Gist-brocades (Delft, The Netherlands).

CHAPTER 3

Structural features of glucuronoarabinoxylans extracted from wheat bran by barium hydroxide

This chapter has been submitted for publication to *Journal of Cereal Science* by the authors M.E.F. Schooneveld-Bergmans, G. Beldman, A.G.J. Voragen.

Abstract

A glucuronoarabinoxylan extract obtained from water-unextractable cell wall material of industrial wheat bran was fractionated by means of graded ethanol precipitation, anion-exchange and reversed phase chromatography. Methylation analysis and enzymatic degradability were used to elucidate the structure. Approximately one-third of the extracted glucuronoarabinoxylan was very lowly substituted (Ara/Xyl~0.2). Substitution occurred predominantly by arabinose at the *O*-3 position of xylose residues. Enzymatic degradation showed a random distribution of the substituents, which are probably interrupted by stretches of 6 or more contiguously unsubstituted xylose residues. More than half of the extracted glucuronoarabinoxylan was highly substituted (Ara/Xyl≥1). The complexity of the structure was shown by poor enzymatic degradability and the presence of considerable amounts of branched arabinose and terminal xylose. Substitution of xylose occurred not only through *O*-3 mono-, *O*-2 and *O*-3 disubstitution by terminal arabinose and *O*-2 mono-substitution by (4-*O*-methyl)glucuronic acid, but also through dimeric arabinose, xylose and possibly galactose containing branches and through 2,3-linked arabinose. The remainder of the extracted glucuronoarabinoxylan (15%) was either intermediately substituted (Ara/Xyl~0.5) or very highly substituted (Ara/Xyl~1.2) and associated with protein and lignin.

Introduction

Wheat bran consists primarily of structural polymers, such as cellulose, glucuronoarabinoxylan and lignin, which give strength to the outer layers of the wheat kernel and protect it against external influences. In the 1980s wheat bran has received considerable interest as a source of dietary fibre for human consumption (Selvendran *et al.*, 1980; Ring and Selvendran, 1980; Brillouet and Mercier, 1981; Brillouet *et al.*, 1982; DuPont and Selvendran, 1987; Brillouet and Joseleau, 1987; Stevens and Selvendran, 1988; Schwarz *et al.*, 1988 and 1989). An important physiological effect of wheat bran is its faecal bulking capacity, which is ascribed to its low degradability in the digestive tract and its high water-holding capacity (Stevens and Selvendran, 1988). Arabinoxylans appeared to play a role in this. *In vitro* studies showed that human faecal bacteria preferentially degraded the aleurone layer (Stevens and Selvendran, 1988), which primarily consists of arabinoxylans and (1→3)(1→4)-β-glucans (Bacic and Stone, 1981). These arabinoxylans appeared to be of low degree of substitution (Ara/Xyl~0.3). The lignified outer layers, also known as beeswing bran, appeared to be resistant to degradation (Stevens and Selvendran, 1988). Besides lignin and cellulose these outer cell layers contain glucuronoarabinoxylans as a major constituent. Their degree of substitution appeared to be relatively high, because alkaline extracts of cell wall material from beeswing bran contained arabinose and xylose in a ratio of approximately 1.1 (Ring and Selvendran, 1980; DuPont and Selevendran, 1987; Brillouet and Joseleau, 1987).

Different fractionation techniques, such as graded ethanol precipitation (Brillouet *et al.*, 1982; DuPont and Selvendran, 1987) and anion-exchange chromatography (Selvendran *et al.*, 1980; DuPont and Selvendran, 1987) were used to get more insight in the diversity of wheat bran glucuronoarabinoxylans. Part of the glucuronoarabinoxylans of wheat bran

contained considerable amounts of terminal xylose and non-terminal arabinose (Selvendran *et al.*, 1980; Ring and Selvendran, 1980; Brillouet *et al.*, 1982; Brillouet and Joseleau, 1987) in addition to the known structural units of wheat endosperm arabinoxylans, namely un-, 2- or 3-mono- and disubstituted xylose and terminal arabinose.

Our renewed interest in wheat bran arabinoxylans is related to their possible application as food additive. Arabinoxylans are known to hold large amounts of water and give viscosity to solutions. As prices of animal feed, the major outlet of wheat bran, are decreasing, new uses are of interest. In this paper we report on the structural characteristics of wheat bran glucuronoarabinoxylans, which were extracted from cell wall material by a selective method. Fractionation, methylation analysis and enzymatic degradation studies were used to investigate the homogeneity and structure. It is envisaged that knowledge of structural characteristics will help us to understand the physical properties of the glucuronoarabinoxylans.

Experimental

Materials

Wheat bran water-unextractable cell wall material (WUS) was obtained from industrial wheat bran as described by Bergmans *et al.* (1996). Endo-(1→4)-β-D-xylanase I (EXI) (Kormelink *et al.*, 1993*a*) and (1→4)-β-D-arabinoxylan arabinofuranohydrolase (AXH) (Kormelink *et al.*, 1991) were purified from *Aspergillus awamori* CMI 142717. α-L-Arabinofuranosidase B (AfB) was purified from *Aspergillus niger* as described by Rombouts *et al.* (1988). Glucuronoxylan xylanohydrolase (GX) was a kind gift from Dr. D.J. Nevins (UCD, Davis, USA) and was purified from *Bacillus subtilis* (Nishitani and Nevins, 1991).

Extraction procedure

The selective extraction of glucuronoarabinoxylans from wheat bran WUS was performed as described before (Bergmans *et al.*, 1996). The ratio of solid material to extractant was changed from 1:200 to 1:100 (w/v) in order to facilitate large scale extraction of glucuronoarabinoxylans. Extraction was performed at 70°C by barium hydroxide solution saturated at room temperature, to which 0.26 M sodium borohydride was added. After dialysis, the resulting extract was concentrated by vacuum evaporation to a concentration of 7.2 mg/ml and stored at 4°C with the addition of 0.01% sodium azide. The extract corresponds with BE.RT-70 of the previous chapter and was designated here as BE.

Reversed Phase chromatography

Repetitive fractionation of 1 ml of centrifuged BE (25,000 *g*; 20 min; 5.9 mg/ml) was performed on a semi-preparative RP-column (25 × 1 cm) of Spherisorb 5 ODS-2 in combination

with a RP guard column (7.5 × 1 cm), using a SpectraSYSTEM P4000 gradient pump equipped with a membrane degasser (Thermo Separation Products, Fremont, CA, USA). The column was operated at room temperature at a flow rate of 4 ml/min. The starting conditions of the elution were 5% methanol in water for 5 min, then linear gradients were applied of 5-30% methanol in water during 17 min and 30-80% methanol in water during 18 min. This final condition was kept for the following 5 min. The column effluent was monitored by a SpectraSYSTEM UV1000 detector (Thermo Separation Products, Fremont, CA, USA) at a wavelength of 280 nm to detect lignin-like material. Corresponding fractions (2 ml) of various runs were collected in the same tubes each run and were finally analysed by an automated method for neutral sugar content (Tollier and Robin, 1979) using arabinose as standard. The fractions giving high neutral sugar content and low UV-absorption were pooled, designated BE.RP and stored after concentration as described above.

Ion-exchange chromatography

After centrifugation (25,000 *g*; 20 min) 50 ml of supernatant of BE (5.9 mg/ml) was applied on a column (55 × 2.6 cm) of DEAE-Sepharose Fast Flow, equilibrated with 0.005 M sodium acetate buffer, pH 5. After loading with sample, the column was washed with the same buffer (375 ml) and then eluted isocratically and with linear gradients of sodium acetate buffers of pH 5 in the following order: 0.005-1 M (1500 ml), 1 M (250 ml), 1-2 M (250 ml), 2 M (250 ml), 0.005 M (300 ml) and finally with 0.5M sodium hydroxide (600 ml) at a flow rate of 12 ml/min. Fractions of 20 ml were collected and analysed by automated methods for neutral sugar (Tollier and Robin, 1979) and uronic acid (Thibault, 1979) contents using arabinose and glucuronic acid as standards. The fractions were pooled, dialysed, concentrated by vacuum evaporation and designated BE.DU (unbound fraction), BE.DB (bound fraction) and BE.DBA (bound fraction eluted by alkali). They were stored as described above.

Graded ethanol precipitation

BE was diluted to a concentration of 2.5 mg/ml in distilled water and fractionated by incremental increases of 10% in ethanol concentration ranging from 10 to 80% (v/v). After each increase in ethanol concentration the mixture was stored at 4°C for 16 h. The precipitate was collected by centrifugation (25,000 *g*; 20 min) and dissolved in distilled water. The fractions were designated as BE.10, BE.20, etc. The last two digits refer to the ethanol concentration at which the precipitate was collected. The final supernatant was concentrated by vacuum evaporation, dialysed and designated BE.80S. All fractions were stored at 4°C with the addition of 0.01% sodium azide.

Enzyme incubations

Solutions of BE and fractions derived thereof were incubated with several combinations of xylanolytic enzymes (EXI, GX, AXH and AfB) with a substrate concentration of 1 mg/ml in 0.05 M sodium acetate buffer of pH 5. The total incubation volume was 1 ml and each enzyme was added in an amount of 0.5 µg protein per ml incubation mixture. The mixtures were incubated for 24 h at 40°C, mixed head-over-tail continuously and inactivated at 100°C for 10 min. The degree of degradation was determined by estimation of the release of reducing end-groups according to Somogyi (1952) using xylose as a standard. The change in molecular weight distribution and the production of arabinose and oligosaccharides was studied by HPSEC and HPAEC, respectively, as described in analytical methods.

Analytical methods

Neutral sugar composition, total uronic acid and *protein content* of extract and fractions were determined as described previously (Bergmans *et al.*, 1996).

Uronic acid composition was determined by high-performance anion-exchange and gas chromatography. For identification of galacturonic and glucuronic acid samples were heated for 16 h at 80°C with 0.5 ml anhydrous 2 M hydrochloric acid in absolute methanol. After cooling to room temperature the liquid was evaporated by a stream of air. The remaining carbohydrates were hydrolysed further with 2 M trifluoroacetic acid for 1 h at 121°C. The liquid was again evaporated after cooling. A standard mixture of galacturonic and glucuronic acid was treated likewise. Standard and samples were analysed by HPAEC as described by Verbruggen *et al.* (1995). Elution involved simultaneous linear gradients of sodium acetate and sodium hydroxide over 25 min of 0-0.25 M and 0-0.025 M, respectively. 4-*O*-methylglucuronic acid in BE was quantified by gas chromatography after reduction of the carboxyl groups according to Taylor and Conrad (1972), hydrolysis and derivatization to alditol acetates as described previously (Bergmans *et al.*, 1996).

Methylation analysis. BE and some fractions were methylated according to the Hakomori method, as modified by Sandford and Conrad (1966). Carboxyl groups in BE were reduced prior to methylation as described above. After methylation the samples were dialysed against distilled water, dried by evaporation and hydrolysed with 2 M trifluoroacetic acid as described above. The sugars were reduced using sodium borodeuteride and converted to alditol acetates. The samples were dried in a stream of air, dissolved in ethyl acetate and analysed by gas chromatography as described by Verbruggen *et al.* (1995). The derivatives were quantified according to their effective carbon response (ECR) factors (Sweet *et al.*, 1975). Identification of the compounds was confirmed by gas chromatography mass spectrometry. The 3,4- and 2,3-*O*-methylated xylitol acetates coeluted, as well as the 2- and 3-*O*-methylated xylitol derivatives. Their relative amounts were calculated from the relative

abundance of the ions at m/z 117 and m/z 118, and m/z 118 and m/z 130, respectively.

High-performance anion-exchange chromatography (HPAEC). Identification of oligosaccharides and quantification of arabinose produced during enzyme incubations was performed by HPAEC using a Dionex BioLC GPM-II gradient module (Sunnyvale, CA, USA) equipped with a CarboPac PA-1 column (250 × 4 mm) in combination with a CarboPac PA guard column (25 × 3 mm) at a flow rate of 1 ml/min. Elution involved linear gradients of sodium acetate in 0.1 M sodium hydroxide of 0-0.1 M over 5 min and 0.1-0.4 M over 35 min. The effluent was monitored using a Dionex PED detector in the pulsed amperometric detection (PAD) mode. A reference silver/silver chloride electrode was used with a working gold electrode using the following pulse potentials and durations: E_1 0.1 V and 0.5 s, E_2 0.6 V and 0.1 s, E_3 -0.6 V and 0.1 s. Arabinoxylooligosaccharides derived from EXI incubation of alkali-extractable wheat flour arabinoxylan purified and characterized at our laboratory (Gruppen *et al.*, 1992*c*) were used as standards for the identification of some oligosaccharides.

High-performance size-exclusion chromatography (HPSEC). Molecular weight distributions of extract, fractions and enzyme digests were determined by HPSEC using three Bio-Gel TSK columns in series as described elsewhere (Bergmans *et al.*, 1996). The weight average molecular weight and intrinsic viscosity of some samples were determined using a triple detection system composed of a dual refractometer/viscometer detector model 250 in combination with a right-angle laser light-scattering detector (Viscotek, Houston, TX, USA). The system was calibrated with dextran and pullulan standards.

Results and Discussion

Extraction and fractionation

Extraction. A relatively high yield of very pure glucuronoarabinoxylans was obtained by barium hydroxide extraction of wheat bran WUS (Bergmans *et al.*, 1996). Enlarged scale extraction of the same WUS yielded 29% dry matter (Table 1), containing 40% of the glucuronoarabinoxylans originally present in WUS, which corresponded well with the results of the small scale extraction (Bergmans *et al.*, 1996). However, the Ara/Xyl-ratios of the extracts differed, being 0.82 and 0.71 for small and large scale extract, respectively. The lower Ara/Xyl-ratio of the latter may have been caused by a different production rate of borate from borohydride as a result of the higher WUS content during extraction compared with the small scale extraction. Actually, a decrease in the Ara/Xyl-ratio was shown when relatively more boron was present as borate instead of borohydride (Bergmans *et al.*, 1996). The uronic acid in the extract appeared to be exclusively glucuronic acid, no galacturonic acid could be detected. Approximately half of the glucuronic acid was present as its 4-*O*-methyl-derivative. This corresponds fairly well with a 40% contribution of 4-*O*-methylglucu-

Table 1: Yield and composition of a barium hydroxide extract of wheat bran WUS and fractions derived thereof by centrifugation, reversed phase chromatography (RP), anion-exchange chromatography (DEAE) and graded ethanol precipitation (EtOH).

	yield[a]	protein content[b]	total sugar content[c]	molar composition[d]						
				Ara	Xyl	Man	Gal	Glc	UA	Ara/Xyl
extraction										
BE	28.9[e]	4.9	82.8	37.0	52.2	0.1	1.1	7.0	2.6	0.71
centrifugation										
BE.CP	17.3	nd	85.7	17.5	78.4	0	0.4	2.4	1.3	0.22
BE.CS	82.6	5.4	80.4	38.3	49.4	0.1	1.4	7.6	3.2	0.78
RP										
BE.RP	45.8	0.3	84.0	40.9	44.7	0.3	1.2	9.9	3.1	0.92
DEAE										
BE.DU	17.1	0.2	95.6	31.6	42.5	0.8	1.2	23.6	0.4	0.74
BE.DB	36.7	0.2	98.2	45.7	44.5	1.2	2.3	1.9	4.5	1.03
BE.DBA	6.2	0.9	96.2	18.0	70.8	2.8	2.8	3.0	2.5	0.25
EtOH										
BE.10	22.0	5.0	92.1	16.1	80.9	0	0.1	1.7	1.2	0.20
BE.20	8.0	4.7	95.0	17.4	68.8	0	0.1	12.3	1.4	0.25
BE.30	4.7	2.1	78.9	19.6	55.9	0	0.5	22.9	1.1	0.35
BE.40	3.0	2.3	78.8	21.5	47.1	0.1	0.8	28.5	1.9	0.46
BE.50	2.4	2.7	78.0	25.6	45.4	0.5	1.3	25.5	1.7	0.56
BE.60	20.8	3.2	96.5	44.5	44.2	0.4	1.7	5.1	4.1	1.01
BE.70	23.5	4.7	88.5	49.1	42.8	0.4	1.5	2.3	3.9	1.15
BE.80	3.2	1.9	71.4	47.3	40.1	0.8	2.5	5.4	3.9	1.18
BE.80S	11.0	17.1	49.2	42.2	33.2	1.8	5.1	10.0	7.7	1.27

[a] expressed as weight percentage (dm) of BE.
[b] expressed as weight percentage (dm) of each fraction.
[c] neutral sugars + uronic acids expressed as weight percentage (dm) of each fraction.
[d] expressed as percentage (mole per 100 mole).
[e] expressed as weight percentage (dm) of WUS.

ronic acid to the total glucuronic acid content as determined by Brillouet and co-workers (1982) in a sodium hydroxide extract of delignified industrial wheat bran.

Centrifugation. Prior to chromatographic fractionation, part of BE was centrifuged, as it showed some precipitation upon prolonged storage at low temperature. The yields and compositions of pellet and supernatant, BE.CP and BE.CS, respectively, are shown in Table 1. BE.CP represented 17% of the extract based on dry matter, containing 20% of the glucuronoarabinoxylans originally present. Brillouet and Mercier (1981) recovered 30% of their sodium hydroxide extract of delignified wheat bran cell wall material as a precipitate upon

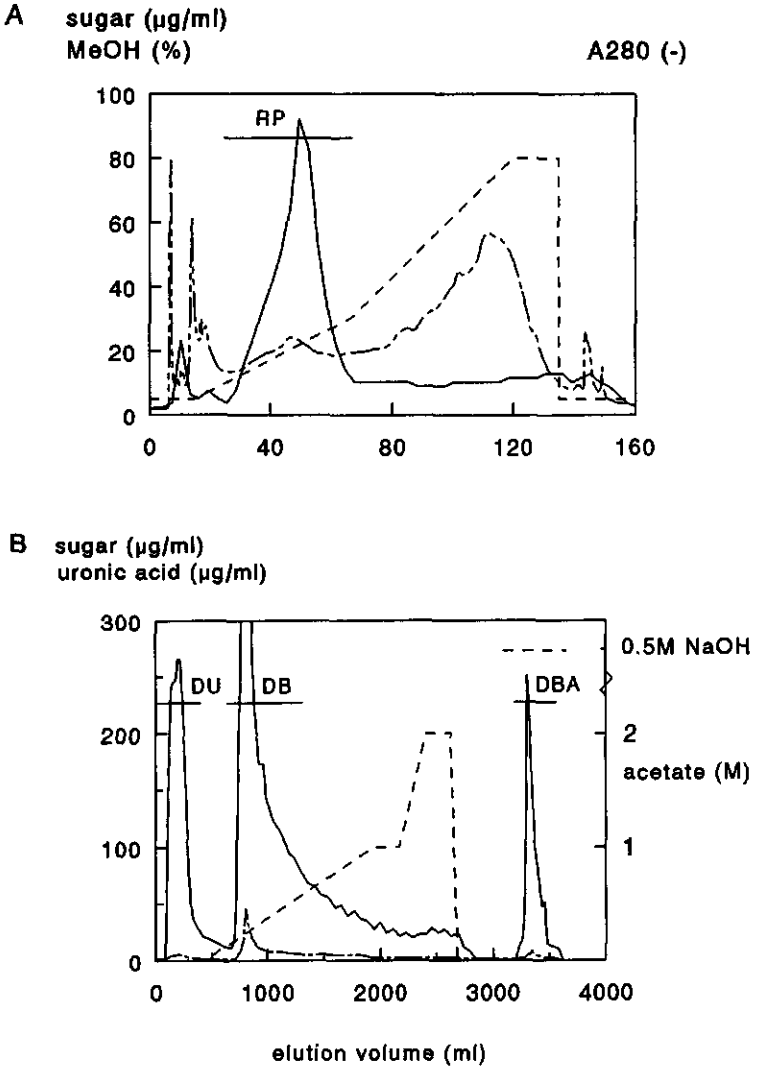

Figure 1: Chromatographic fractionation of a barium hydroxide extract from wheat bran WUS. A: Elution pattern on reversed phase chromatography: ——— neutral sugar content; — — — — A_{280}; – – – methanol concentration. B: Elution pattern on anion-exchange chromatography: ——— neutral sugar content; — – — uronic acid content; – – – sodium acetate or sodium hydroxide concentration.

acidification and centrifugation. Their precipitate and ours were of low degree of substitution (Ara/Xyl~0.2). It is known that lowly branched arabinoxylans easily aggregate, which was also shown to be time and temperature dependent (Blake and Richards, 1971).

RP-chromatography. A distinct population of glucuronoarabinoxylans almost devoid of lignin-like material (A_{280}) was obtained by reversed phase chromatography of the supernatant of centrifugation, as is shown in Figure 1A. This population yielded 46% dry matter (Table 1) and contained 45% of the glucuronoarabinoxylans originally present in BE. The fraction had a fairly high Ara/Xyl-ratio and was somewhat enriched in glucose. Its protein content was considerably lower than that of the supernatant from which it was obtained. The overall recovery of dry matter of this fractionation was calculated to be 90%, including the material removed by centrifugation and the other fractions collected. Although a good recovery and good separation between lignin-like material (A_{280}) and polysaccharides was obtained, this method appeared to be not appropriate for purification of large amounts of arabinoxylans.
DEAE-chromatography. The supernatant of centrifugation was also fractionated by anion-exchange chromatography, as is shown in Figure 1B. The yields and compositions of the three main populations are given in Table 1. The unbound fraction (BE.DU), being intermediately substituted, represented 15% of the glucuronoarabinoxylans of the extract, containing only 2% of the uronic acid. Moreover, 65% of the glucose of BE was recovered in this fraction, probably arising from coextracted (1→3)(1→4)-β-glucans. Relatively high glucose contents of DEAE-unbound fractions were also reported for barium and potassium hydroxide extracts of sorghum WUS (Verbruggen *et al.*, 1995). The major fraction of DEAE-chromatography was the bound fraction (BE.DB), which was eluted as soon as the acetate gradient was applied. It comprised 45% of the glucuronoarabinoxylans of the extract, containing approximately 70% of the uronic acid. The compositions of BE.DU and BE.DB corresponded fairly well with those of the two main fractions obtained by DEAE-Sephadex chromatography of an alkaline extract of delignified wheat bran (Selvendran *et al.*, 1980). A final fraction (BE.DBA) was eluted with 0.5 M sodium hydroxide, and comprised only 7% of the glucuronoarabinoxylans originally present in the extract. Although this fraction had a very low degree of substitution, similar to that of BE.CP, it was not removed by centrifugation. Its slightly higher uronic acid content may have caused a higher solubility. A blockwise distribution of these uronic acid residues may have caused the strong binding of this population to the column. But other factors, such as aggregation or presence of bound protein, as suggested by Gruppen and co-workers (Gruppen *et al.*, 1992*b*) for the binding of neutral wheat endosperm arabinoxylans to DEAE-Sepharose, may also be involved. The total recovery of this fractionation was 83% based on dry matter, including the residue of centrifugation and the other fractions collected. The three main populations had a very high sugar content and comprised 88% of the sugars applied to the column. The non-recovered sugars, of which the Ara/Xyl ratio was calculated to be 1.1, may have been linked to protein or phenolic complexes. The latter are known to give very low recoveries on anion-exchange chromatography (Selvendran and O'Neill, 1987).

EtOH-precipitation. The fractionation of the original extract by graded ethanol precipitation showed the presence of two main glucuronoarabinoxylan populations as can be seen from the yields and compositions of the fractions in Table 1. A lowly substituted fraction was recovered at 10 and 20% ethanol, which comprised 36% of the glucuronoarabinoxylans of the extract, containing only 18% of the uronic acid. A second major fraction was obtained at ethanol concentrations of 60 and 70%. These fractions represented 50% of the glucuronoarabinoxylans of BE, and contain 60% of the uronic acid. The intermediate fractions (BE.30, BE.40 and BE.50) represented only 7% of the glucuronoarabinoxylans of BE. These fractions, together with BE.20, were enriched in glucose. Glucose enrichment of fractions precipitating between 10 and 50% ethanol, as well as an increasing Ara/Xyl-ratio, uronic acid and galactose content with increasing ethanol concentration was also observed by Brillouet and co-workers (1982). The final two fractions (BE.80 and BE.80S) showed a substantially lower total sugar content. The dark brown color of these fractions was regarded as an indication for the presence of lignin-like material. However, the 80% ethanol-soluble fraction appeared to have a relatively high protein content also.

By combination of results of the different fractionation techniques presented in Table 1 it can be concluded that approximately 35% of barium hydroxide-extractable wheat bran glucuronoarabinoxylans consist of a very lowly substituted population, a fifth of which is of slightly higher uronic acid content (BE.DBA) than the remainder (BE.CP, BE.10 and BE.20). A major population, representing half of the barium hydroxide-extractable glucuronoarabinoxylans, shows an Ara/Xyl-ratio of approximately 1.1, the majority of which also has a relatively high uronic acid content (BE.DB). Only 7% of the glucuronoarabinoxylans had an intermediate degree of substitution (Ara/Xyl=0.4-0.6), whereas the remainder had a high degree of substitution (Ara/Xyl=1.2) and appeared to be associated with lignin and protein.

Glycosidic linkage composition

Table 2 shows the glycosidic linkage composition of the original extract and various fractions, as determined by methylation analysis. In the original extract over 50% of the xylose residues was unsubstituted. Approximately 20% of the xylose units was monosubstituted, predominantly at the *O*-3 position. Another 16% of the xylose residues was disubstituted at *O*-2 and *O*-3 position, whereas the remaining residues were terminal. The presence of relatively large amounts of terminal xylose was reported before for wheat bran (Selvendran *et al.*, 1980; Brillouet *et al.*, 1982; DuPont and Selvendran, 1987; Brillouet and Joseleau, 1987; Shiiba *et al.*, 1993), rye bran (Ebringerova *et al.*, 1990) and maize hulls (Saulnier *et al.*, 1993). In wheat endosperm usually less than 1% of the xylose residues is terminal (Gruppen *et al.*, 1992*a*). In addition to the terminal xylose, wheat bran arabinoxylans also differ from those of the endosperm by the presence of significant amounts of non-

Table 2. Glycosidic linkage composition of a barium hydroxide extract from wheat bran WUS and some fractions derived thereof, expressed as percentage (mole per 100 mole) of all partially methylated alditol acetates present.

alditol acetate of[a]	BE	BE.DU	BE.DB	BE.10	BE.20	BE.60	BE.70	BE.80S
2,3,5-Me_3-Ara	24.6	29.4	28.9	14.1	15.8	28.5	33.4	28.6
3,5-Me_2-Ara	1.6	1.1	2.3	0.2	0.2	2.5	2.6	2.0
2,3-Me_2-Ara	1.3	0.9	2.4	0.3	0.5	1.0	1.0	10.6
2,5-Me_2-Ara	4.7	2.1	7.9	0.7	0.7	6.8	8.0	6.5
5-Me-Ara	3.4	0.9	6.6	0.6	0.2	6.9	7.3	3.9
2,3,4-Me_3-Xyl	6.6	3.1	10.7	0.9	0.8	10.3	11.4	6.8
2,3-Me_2-Xyl	30.1	24.4	14.6	67.7	55.2	13.0	8.5	9.8
2-Me-Xyl	10.3	8.2	10.0	11.0	10.1	9.6	9.3	10.8
3-Me-Xyl	0.9	0.7	1.7	0.4	0.3	1.6	1.8	0.5
Xyl	9.0	9.3	12.1	1.8	2.4	13.3	14.2	13.5
2,3,4,6-Me_4-Glc	(1.5)[b]	0	0	0	0	0	0	0
2,3,6-Me_3-Glc	4.9	16.0	1.2	1.2	10.2	4.3	1.4	3.2
2,4,6-Me_3-Glc	1.0	3.1	0.2	0	2.0	0.9	0.3	0.7
Glc	0.9	0.4	0.8	0.6	0.2	0.6	0.3	2.1
Gal	0	0	0	0	0	0.1	0.1	0.2
Man	0.7	0.4	0.6	0.5	0.4	0.6	0.5	0.8

[a] 2,3,5-Me_3-Ara = 2,3,5-tri-O-methyl-1,4-di-O-acetyl-arabinose, etc.
[b] determined in carboxyl reduced BE, originally present as glucuronic acid or its 4-O-methyl-derivative.

terminal arabinose residues. It can be calculated that 30% of the arabinose residues is non-terminal, the majority of which is 3- and 2,3-linked. These non-terminal arabinose units were also reported in pericarp tissues of grasses (Selvendran *et al.*, 1980; Brillouet *et al.*, 1982; DuPont and Selvendran, 1987; Brillouet and Joseleau, 1987; Shiiba *et al.*, 1993; Ebringerova *et al.*, 1990; Saulnier *et al.*, 1993). Recently, the presence of α-L-Ara-(1→2)-α-L-Ara as a branch in sorghum endosperm glucuronoarabinoxylans was proved by NMR-analysis of purified enzymatic degradation products (Verbruggen, 1996). Carboxyl-reduction prior to methylation, increased the amount of terminal glucose by 1.5 mole-%, which is shown in Table 2 in parenthesis. This originated from glucuronic acid or its 4-O-methyl-derivative. In the original extract a uronic acid content of 2.6 mole-% was determined by a colorimetric assay (Table 1). A discrepancy between these two methods was observed before by Verbruggen *et al.* (1995). The majority of the glucose in the extract appeared to be mainly derived from coextracted (1→3)(1→4)-β-glucans, because both 3- and 4-linked glucose residues were identified. The ratio of 4- to 3-linked glucose was 5:1. An almost similar ratio was

observed before in an alkali extract of wheat bran (Selvendran *et al.*, 1980), but it is quite different from that of barley (1→3)(1→4)-β-glucans (Woodward *et al.*, 1983), in which it is 7:3. For maize a ratio of 3:1 and 4:1 was determined (Carpita, 1983), which increased when extraction was performed at a higher alkali concentration. In the latter publication, coextraction of xyloglucans or cellulose was suggested. The contents of galactose and mannose as determined by methylation analysis (Table 2) were different from those in Table 1. Moreover, both appeared only to be present as unmethylated alditol acetate, which was also true for a minor amount of glucose residues. This is probably the result of undermethylation.

The lowly substituted fractions (BE.10 and BE.20) consisted of 81% unsubstituted xylose, 16% monosubstituted and only 3% disubstituted xylose residues. Arabinose was present almost exclusively as terminal residue. These results are in close agreement with the glycosidic linkage composition of wheat aleurone arabinoxylans (Bacic and Stone, 1981). From this, in combination with the absence of a similar fraction in beeswing wheat bran (Brillouet and Joseleau, 1987), it can be inferred that the lowly substituted fractions originate from the aleurone layer. The highly substituted glucuronoarabinoxylans generally consist of approximately 26% unsubstituted, 23% monosubstituted, 28% disubstituted and 23% terminal xylose, whereas approximately 40% of total arabinose is non-terminal. This corresponds well to the composition of the highly substituted extract of industrial wheat bran (Brillouet *et al.*, 1982), whereas in beeswing wheat bran somewhat less unsubstituted and more mono- and disubstituted xylose was present (Brillouet and Joseleau, 1987). The ratio of 4- over 3-linked glucose in all fractions was similar to that of BE. The ethanol-soluble fraction had a high degree of substitution, but differed from the other highly substituted fractions considering the non-terminal arabinose residues. The majority of these residues is 5- instead of 3-linked. The ratio of branched over terminal residues in this fraction was 1.2 for the arabinoxylan. So some undermethylation may have occurred, which may have been caused by the high protein and lignin content of this fraction.

Based on these methylation analyses it can be stated that the lowly substituted arabinoxylans are of rather simple structure with single unit branches and probably originate from the aleurone layer. For the highly branched fractions side chains of more than one unit are expected, which may be terminated by arabinose or xylose. The majority of these glucuronoarabinoxylans originate from the outermost cell layers of the bran. The assumed lignin-linked fraction appears to contain an arabinan like structure, which is unlike the other fractions.

Molecular weight distribution

Prior to HPSEC-analysis, all fractions were checked for their solubility in the eluent. Even of the lowly substituted fractions over 60% of the carbohydrates were recovered in solution after centrifugation. However, the higher the degree of substitution, the more carbohydrate

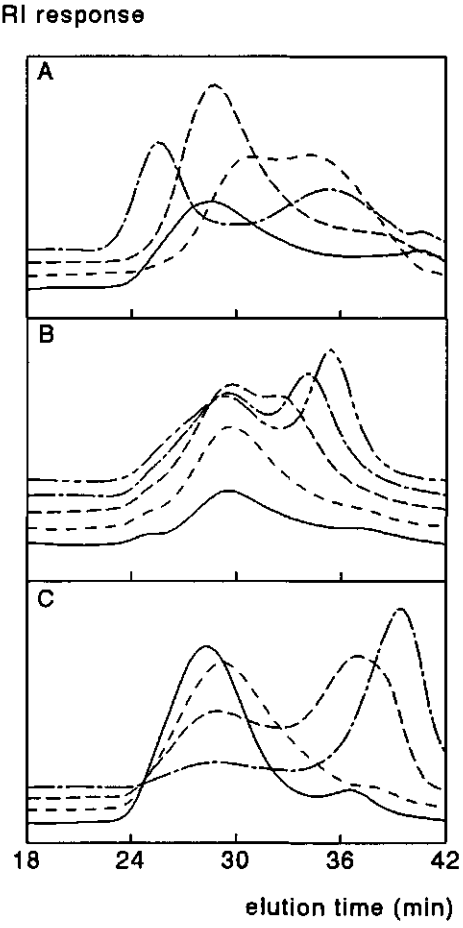

Figure 2: HPSEC-elution patterns of a barium hydroxide extract from wheat bran WUS and fractions derived thereof. A: ——— BE; – – – BE.DU; — — — BE.DB; — – — BE.DBA. B: ——— BE.10; – – – BE.20; — — — BE.30; — – — BE.40; — – – — BE.50. C: ——— BE.60; – – – BE.70; — — — BE.80; — – — BE.80S.

appeared to be soluble. Figure 2A shows that BE.DU consisted of two different populations. The first eluting population disappeared upon endoxylanase incubation, whereas the other population appeared to be degradable by (1→3)(1→4)-β-glucanase (results not shown). This justified the assumption of the presence of coextracted (1→3)(1→4)-β-glucan as suggested before. The DEAE- bound fraction (BE.DB) elutes at almost the same retention time as the original extract, but seems to be more homogeneous. The alkali-eluted fraction (BE.DBA) also consists of two populations, both of which are probably arabinoxylans based on the low

Table 3: Weight average molecular weight as determined by light-scattering ($M_{w;LS}$) and universal calibration ($M_{w;UC}$), polydispersity index (M_w/M_n) and intrinsic viscosity ([η]) of a barium hydroxide extract from wheat bran WUS and fractions derived thereof by graded ethanol precipitation.

	$M_{w;LS}$[a]	$M_{w;UC}$[b]	M_w/M_n[c]	[η][d]
BE	622,000	92,000	1.3	1.4
BE.10	613,000	137,000	1.3	2.0
BE.20	530,000	127,000	1.3	2.2
BE.30	411,000	105,000	1.8	2.2
BE.40	323,000	112,000	2.4	1.8
BE.50	298,000	118,000	2.8	1.6
BE.60	432,000	397,000	2.6	1.8
BE.70	296,000	267,000	2.9	1.5
BE.80	199,000	122,000	9.3	0.9
BE.80S	103,000	26,000	12.4	0.4

[a] $M_{w;LS}$: weight average molecular weight as calculated by light scattering data, expressed in Da.
[b] $M_{w;UC}$: weight average molecular weight as calculated by viscometry data, based on universal calibration, expressed in Da.
[c] M_n: number average molecular weight.
[d] expressed in dl/g.

glucose content of the fraction. In Figure 2B the existence of two populations for BE.30, BE.40 and BE.50 is shown. As these fractions had relatively high glucose contents, resemblance to BE.DU with respect to the order of elution of arabinoxylan and (1→3)(1→4)-β-glucan is suggested. Although in fraction BE.20 a fair amount of glucose was detected this was not visible in Figure 2B as a separate population. Probably the hydrodynamic volumes of the arabinoxylan and (1→3)(1→4)-β-glucan were very similar. In Figure 2C BE.60 and BE.70 showed higher hydrodynamic volumes than all other ethanol precipitated fractions. The fractions BE.80 and BE.80S revealed a low molecular weight population, which may be degradation products arising from the extraction procedure.

In Table 3 the weight average molecular weights and intrinsic viscosities of several fractions are given. The molecular weights were determined in two different ways; by means of light-scattering and by means of viscometry, both in combination with refractive index detection. Molecular weight determination by light-scattering analysis is independent of the shape of the molecule. With viscometry the shape of the molecule is taken into account as the product of viscosity and concentration. Universal calibration of the system with a set of polymers, which are not necessarily of a similar shape, can then be used (Grubisic *et al.*, 1967). From Table 3 it is clear that for all fractions the molecular weights are higher when determined by light-scattering in comparison with the universal calibration technique. The

difference appeared to be less obvious for highly substituted fractions (BE.60 and BE.70), which may indicate that the high molecular weights of lowly branched fractions as determined by light-scattering are the result of aggregation. Another indication for the occurrence of aggregates is the lower weight average molecular weight of all ethanol precipitated fractions compared to that of their parental extract, when based on light-scattering analysis. This was also observed for ethanol precipitated wheat flour arabinoxylan fractions and their parental extract (Gruppen *et al.*, 1992*b*). However, the decrease in molecular weight with increasing ethanol concentration observed here is opposite to the results with wheat endosperm arabinoxylan. Probably less aggregation occurred in lowly substituted endosperm fractions compared to the corresponding bran fractions. Furthermore, the bran fractions were contaminated with glucans, whereas the endosperm fractions were not (Gruppen *et al.*, 1992*b*). The molecular weights of the highly substituted wheat bran fractions as presented here may also serve as an indirect proof of the existence of terminal xylose in branches. If the terminal xylose residues in these fractions would only occur as non-reducing termini of the backbone, it can be calculated that the molecular weight would have been approximately 1,200 Da. As the degree of substitution of this fraction is very high, such extensive aggregation is not very likely to occur. The intrinsic viscosities presented in Table 3 correspond fairly well with reported values for arabinoxylans from wheat bran (Brillouet *et al.*, 1982) and maize bran (Chanliaud *et al.*, 1995).

From these results it can be concluded that wheat bran glucuronoarabinoxylans are disperse in molecular weight distribution. Additionally, it was clearly shown that molecular weight determinations of easily aggregating arabinoxylans by light-scattering and by universal calibration vary widely.

Enzymatic degradation

Endo-enzymes. The degradability of different fractions by EXI is shown to range from 2 to 26% (Table 4), the lowest of which was observed for BE.70 and the highest was found for BE.10. Substitution of the backbone is known to restrict the activity of endoxylanases in general. However, the activity of this endoxylanase is known to be only moderately restricted by arabinose substitution (Kormelink *et al.*, 1993*c*). Mono- or disubstitution of a xylose residue does not affect the hydrolytic activity at the non-reducing side. In order to hydrolyse a glycosidic linkage in the xylan backbone at the reducing side of a branched xylose, EXI requires one unsubstituted xylose residue in case of monosubstitution and two unsubstituted xylose residues in case of disubstitution (Kormelink *et al.*, 1993*c*). For sorghum glucuronoarabinoxylans it was shown that EXI also requires two unsubstituted xylose residues at the reducing side of a glucuronic acid substituted xylose residue, but none at the non-reducing side (Verbruggen, 1996). It can therefore be concluded that the highly substituted BE.70 did

Table 4: Degradation and release of arabinose from a barium hydroxide extract of wheat bran WUS and some fractions derived thereof, after 24 h incubation with EXI, GX, AXH, AfB and combinations thereof, expressed as percentage of reducing sugars produced from total sugars present and as percentage arabinose released of total arabinose present, respectively.

	BE	BE.DU	BE.10	BE.70	BE.80S
degradation					
EXI	13.8	6.6	25.6	1.6	4.9
GX	1.1	4.9	0.2	0.1	2.1
AXH	3.2	4.8	3.9	1.9	5.2
AfB	0.9	1.7	0.4	0.4	5.5
EXI+AXH	15.1	14.2	31.5	4.8	7.9
EXI+AfB	13.4	7.4	28.4	3.1	11.1
EXI+GX	12.7	10.4	26.2	2.4	6.7
EXI+GX+AXH	17.6	18.4	29.2	5.1	9.7
EXI+GX+AfB	17.4	11.9	29.7	3.6	11.7
arabinose release					
EXI	0	0	0	0	0
GX	0	0	0	0	0
AXH	8.4	16.0	24.9	5.0	9.3
AfB	2.9	7.6	5.2	2.1	17.1
EXI+AXH	6.4	10.9	23.4	4.1	8.8
EXI+AfB	5.0	6.1	17.4	2.3	15.4
EXI+GX	0	0	0	0	0
EXI+GX+AXH	7.3	13.6	22.3	4.1	7.2
EXI+GX+AfB	5.2	6.0	18.3	2.4	15.4

not contain many regions of two or more contiguously unsubstituted xylose residues. Brillouet and Joseleau (1987) also observed a lack of degradation of their highly substituted wheat bran arabinoxylan by an endoxylanase from *Polyporus tulipiferae*.

GX gave generally less degradation than EXI, ranging from 0 to 5% (Table 4). As the presence of glucuronic acid is known to be a prerequisite for this enzyme to hydrolyse the xylan backbone (Nishitani and Nevins, 1991), it is very peculiar that BE.DU, which contains almost no uronic acid, shows the highest degradability. The presence of a glucanase in this GX-preparation appeared to be a plausible reason for this, because the oligomers produced were identified as (1→3)(1→4)-β-glucooligosaccharides (results not shown). The very low degradability of BE.70 by GX, may be explained by the low content of unsubstituted xylose residues or by a specific pattern of substitution. GX is known to require at least one unsubstituted xylose at the reducing end of a glucuronic acid substituted xylose residue (Nishitani and Nevins, 1991). Furthermore, it was indicated by Verbruggen (1996) that GX did not

hydrolyse the xylan backbone when the glucuronic acid was methylated at the *O*-4 position. As half of the glucuronic acid in the original extract was determined to be the 4-*O*-methyl-derivative, this may have been a limiting factor for the action of GX also.

Exo-enzymes. AXH is a specific enzyme for release of arabinose monosubstituted at the *O*-3 position of xylose in polymeric glucuronoarabinoxylans (Kormelink *et al.*, 1991; Verbruggen, 1996). In Table 4 the enzyme is shown to release the largest amounts of arabinose from lowly substituted and neutral arabinoxylans (BE.10 and BE.DU). From methylation analysis (Table 2) it can be calculated that approximately 36% of the arabinose monosubstituted at the *O*-3 position of xylose was released from BE.10. For wheat endosperm arabinoxylans it was over 80% (Kormelink *et al.*, 1993*b*), and for sorghum glucuronoarabinoxylans it was 50% (Verbruggen, 1996). The latter value was explained by restriction of the enzyme by the high substitution of the polysaccharide. For BE.10 the low arabinose release may be caused by restriction through aggregation, which was already suggested before. For BE.DU approximately 65% of the arabinose monosubstituted at the *O*-3 position of xylose was hydrolysed. A specific substitution pattern or steric hindrance may have had some effect on the activity.

AfB released lower amounts of arabinose than AXH did, except for BE.80S. AfB preferentially hydrolyses α-(1→5)-linked arabinose residues in arabinans (Rombouts *et al.*, 1988), but is also able to hydrolyse the glycosidic linkage of an arabinose substituted at the *O*-3 position of a non-reducing monosubstituted terminal xylose residue (Kormelink *et al.*, 1993*b*). From the results of methylation analysis BE.80S appeared to contain relatively more 5-linked arabinose than all other fractions. So the high arabinose release from BE.80S probably arose from the preferential activity of AfB on these residues.

Combinations of enzymes. From Table 4 it can be calculated that incubation with combinations of enzymes showed no major improvements of the degradability of the substrates. Combination of EXI and AXH showed a minor synergistic effect for BE.DU, BE.10 and BE.70. Removal of arabinose by AXH most probably created new sites of hydrolysis for EXI. The slightly synergistic effect of EXI and AfB on BE.10 was probably affected by the creation of new sites of hydrolysis for AfB by EXI as a result of the production of arabinoxylooligosaccharides with arabinose monosubstituted at the *O*-3 position of a non-reducing terminal xylose residue. In case of BE.70 and BE.80S, AfB may have created new sites of attack for EXI by hydrolysis of arabinose from short branches, decreasing steric hindrance for EXI. Supplementation of EXI with GX and arabinose releasing enzymes had no additional effects.

From the percentages of arabinose released by combination of enzymes as presented in Table 4, it is shown that AXH always released less arabinose when EXI was also present. This may have been caused by a lower affinity of AXH towards oligomeric substrates. This assumption was proved by a time course study with polymeric wheat endosperm arabinoxylan and with the same substrate which was pretreated with EXI (results not shown).

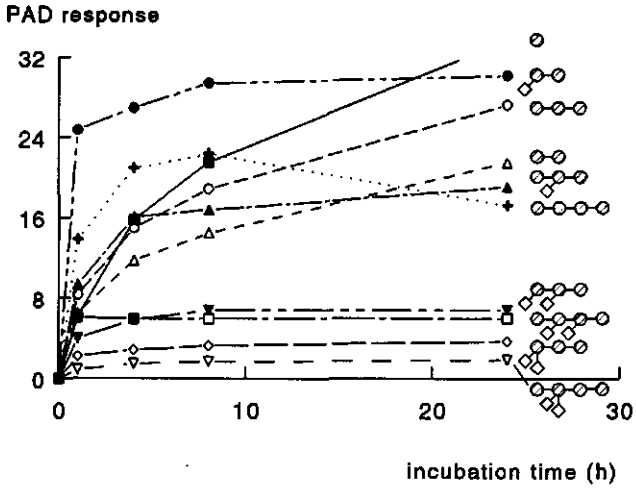

Figure 3: Production of different arabinoxylooligosaccharides in time by incubation of BE.10 with endoxylanase I from *Aspergillus awamori*. ○: Xyl; ◇: Ara.

In contrast, AfB showed the adverse effect, which can be explained by the same argument as pointed out above for the synergy observed for BE.10.

Production of oligosaccharides. In Figure 3 the production of several oligosaccharides in time by incubation of BE.10 with EXI is shown, as analysed by HPAEC. Large amounts of xylotetraose and xylotriose were produced and in the initial stages linear xylopentamer and -hexamer were observed. This is indicative for the presence of regions of at least 6 or 7 contiguously unsubstituted xylose residues. From Figure 3 it is clear that in BE.10 oligosaccharides with isolated *O*-3 monosubstituted xylose residues were more abundant than the ones with two consecutively monosubstituted xylose residues. As already expected from the results of methylation analysis, oligomers with disubstituted xylose residues were not present in large numbers. Degradation products with a combination of mono- and disubstituted or two disubstituted xylose residues, such as those purified from wheat flour endosperm arabinoxylan degraded with EXI (Gruppen *et al.*, 1992*c*), could not be identified in BE.10 incubation mixtures. For the highly substituted fraction (BE.70) the release of only minute amounts of xylose, xylobiose and -triose were observed, which corroborated the presence of only few regions of 2 or more contiguously unsubstituted xylose residues, as stated before. Hardly any other degradation products were detected. HPSEC-analysis of the EXI incubation mixture showed that the molecular weight distribution slightly broadened, implying that EXI was able to hydrolyse a few glycosidic linkages, but without the production

of oligosaccharides as was clear from HPAEC-analysis (results not shown). Very limited degradability of highly branched glucuronoarabinoxylans has been observed for wheat bran (Brillouet and Joseleau, 1987; Shiiba *et al.*, 1993) and sorghum (Verbruggen, 1996).

Structural features

By combination of results and comparison with literature data, the current structural knowledge of the main wheat bran glucuronoarabinoxylans is summarized and extended here.

Lowly substituted arabinoxylans. From methylation analysis (Table 2) it can be calculated that in the lowly substituted xylans (BE.10 and BE.20) 1 out of 7 or 8 xylose residues of the backbone is monosubstituted at the *O*-3 position by arabinose. If a regular distribution of arabinose residues is assumed, this would result in the release of a xylohexamer as the largest linear oligosaccharide from incubation with EXI. This was indeed observed for BE.10 by HPAEC-analysis. However, from Figure 3 it is clear that degradation with EXI also resulted in the release of oligosaccharides with 2 contiguously *O*-3 monosubstituted xylose residues or with an unsubstituted xylose at the non-reducing side of an *O*-3 monosubstituted xylose residue. This indicates that the substitution is not regularly distributed, which results in regions of more than 6 contiguously unsubstituted xylose residues. Methylation analysis and enzymatic degradation also showed the presence of disubstituted xylose, which was calculated to occur for 1 out of 30 to 45 xylose residues of the backbone. This type of substitution most probabaly occurs only in isolation, based on the oligomers produced (Figure 3). For lowly substituted regions of wheat flour arabinoxylan a model has been proposed, in which up to at least 7 contiguously unsubstituted xylose residues are present as well as paired disubstitution (Gruppen *et al.*, 1993*a*). Apart from this latter observation the lowly substituted wheat bran arabinoxylan also deviates from this model by the presence of uronic acid. From the *O*-2 monosubstituted xylose residues, which are generally known to carry uronic acid substituents (Wilkie, 1979), it can be calculated that 1 out of 200 xylose residues in the backbone is substituted with such residue. The recovery of an alkali-eluted fraction from anion-exchange chromatography suggests their possible blockwise substitution.

Highly substituted glucuronoarabinoxylans. The poor enzymatic degradability of the highly substituted wheat bran glucuronoarabinoxylan (BE.DB, BE.60 and BE.70) is very similar to that observed for sorghum glucuronoarabinoxylan (Verbruggen, 1996). It can be inferred from the uronic acid content and linkage composition that their structures differ. The model introduced by Verbruggen (1996) for sorghum glucuronoarabinoxylan is therefore not applicable to wheat bran. Because the actual appearance of a few structural units, such as 2-, 3- and 2,3-linked arabinose in wheat bran fractions, has not been elucidated conclusively until now, it is not possible to propose an acceptable model. Results obtained in this study and literature data were therefore used to speculate on the structure.

A first approach for this purpose is to look at the distribution of terminal arabinose residues. Until now disubstitution of xylose residues has been only proved to occur by terminal arabinose units. If the amount of terminal arabinose required for this disubstitution was subtracted from the total amount present, only 2 to 5 mole-% is left for the O-3 monosubstituted xylose. As these numbers are not in accordance with the actual presence of O-3 monosubstituted xylose (9-10 mole-%), this implies that at least half of the O-3 monosubstituted xylose is not substituted by terminal arabinose, or that not all disubstituted xylose residues are exclusively branched by terminal arabinose. Other branching possibilities occuring at the O-3 position of xylose are: β-Xyl-(1→2)-α-Ara (Wilkie, 1979; Kusakabe *et al.*, 1983); β-Xyl-(1→3)-α-Ara (Wilkie, 1979); β-Gal-(1→5)-α-Ara (Wilkie, 1979) and α-Ara-(1→2)-α-Ara (Verbruggen, 1996). Although branches of higher degree of polymerization were also reported (Wilkie, 1979) these are not taken into consideration here, because they all involved the presence of 2-linked arabinose, which is not very abundant in these wheat bran glucuronoarabinoxylans.

A second structural aspect is the distribution of the different O-3 linked dimeric branches mentioned above. In case the terminal xylose residues are distributed between the two xylose-containing dimeric branches, only 0.5 to 1 mole-% of terminal xylose is left. However, the 2-linked arabinose can also be terminated by arabinose (Verbruggen, 1996), which would result in approximately 3 mole-% of terminal xylose left. The actual amount of terminal xylose, not accounted for by these dimeric branches, will be between these two extremes. If we then consider the presence of the 5-linked arabinose and compare it with the amount of galactose in Table 1, the values are in quite close agreement. As all dimeric branches are reported to occur exclusively in O-3 monosubstitution (Wilkie, 1979), it can be calculated that the actual presence of O-3 monosubstituted xylose is not completely sufficient. This may imply that these dimeric branches also occur at disubstituted xylose residues. However, in both approaches the 2,3-branched arabinose was not taken into consideration, because it is not known whether this arabinose residue is present as a substituent attached to the xylan backbone, which then accommodates two substituents, or as a backbone unit, which carries one substituent. Yet this residue may play a crucial role in fitting in all substituents. Nevertheless, it is clear from this study that there is a need for enzymes, which are able to degrade highly substituted glucuronoarabinoxylans. Only by means of characterization of oligomers containing the unusual structural units revealed by methylation analysis the fine structure of this type of polysaccharides can be further elucidated.

Acknowledgement

This research was supported financially by the Dutch Innovation Oriented Programme Carbohydrates (IOP-k) and by Royal Gist-brocades (Delft, The Netherlands). The authors thank Dr. J.H.G.M. Mutsaers (Royal Gist-brocades, Delft, The Netherlands) for her helpful discussions.

CHAPTER 4

Physicochemical characteristics of wheat bran glucuronoarabinoxylans

This chapter has been submitted for publication to *Journal of Cereal Science* by the authors M.E.F. Schooneveld-Bergmans, Y.M. van Dijk, G. Beldman, A.G.J. Voragen.

Abstract

Graded ethanol precipitation of a wheat bran extract resulted in four fractions of glucuronoarabinoxylans, of which physicochemical properties were investigated in relation to their structure and molecular weight. Two fractions consisting of lowly substituted arabinoxylans appeared to be very sensitive to aggregation and gave hardly any viscosity to solutions. Moreover, the viscosity of these arabinoxylans was not affected by addition of salt, nor by changes in temperature. Interaction with other polysaccharides, such as xanthan and κ-carrageenan, based on observations with structurally similar lowly substituted galactomannans, was not observed. Additionally, these arabinoxylans gave no stabilization to emulsions. The other two fractions consisted of highly substituted glucuronoarabinoxylans, which had intrinsic viscosities of approximately 2.5 dl/g in 0.1 M potassium chloride. The viscosities were affected by temperature and addition of salt, the latter of which is in agreement with their polyelectrolyte nature. These polysaccharides appeared to be semi-flexible and not very sensitive to aggregation, although some aggregation was observed upon partial removal of arabinose substituents. Again, no interaction with xanthan and κ-carrageenan was observed. However, they were very effective in stabilizing emulsions.

Introduction

Functional properties of arabinoxylans have been a subject of research for a long time. Although arabinoxylans are only minor constituents of cereal grains, their presence is known to be of great importance to the properties of food and feed products they are part of.

The production of bread from flour exemplifies the significance of arabinoxylans in food industry. Arabinoxylans appear to influence dough characteristics and baking performance. This is generally believed to be caused by the high water-binding capacity of arabinoxylans (Jeleca and Hlynka, 1971), their high viscosity in solution (Andrewartha *et al.*, 1979) and their ability to form gels by oxidative cross-linking (Geissmann and Neukom, 1973). Only recently more insight has been gained into the underlying aspects by research on the structural characteristics and heterogeneity of these polysaccharides. Arabinoxylans of wheat and rye flour appear to be very heterogeneous in their degree of substitution, their distribution of substituents and their molecular weights (Gruppen, 1992; Vinkx, 1995). All of these aspects influence the physicochemical properties of arabinoxylans (Andrewartha *et al.*, 1979). For that reason research has been directed towards the effects of addition of xylanolytic enzymes on the baking performance of wheat flour (Gruppen *et al.*, 1993*b*; Rouau, 1993; Rouau *et al.*, 1994).

Another example of the functionality of arabinoxylans is from the feed industry. In feed for monogastrics the properties of arabinoxylans are believed to result in anti-nutritive effects. The high viscosity of arabinoxylans reduces the rate of digestion of cereal-based feeds, and for that reason absorption of nutrients from the gastrointestinal tract. It was suggested to be caused by the unability of the digestive system of monogastrics to degrade arabinoxylans (Antoniou *et al.*, 1981). Subsequent research on the effect of addition of xylanolytic enzymes

to these feeds justified this suggestion (Pettersson and Aman, 1988; Choct *et al.*, 1995).

In both examples viscosity is involved, which is also of interest in this study on wheat bran glucuronoarabinoxylans. Wheat bran is a by-product of the milling of wheat to white flour and it contains considerable amounts of arabinoxylans. Thorough investigation of the physicochemical properties of these arabinoxylans may open up possibilities for alternative uses of this waste material. In previous studies we showed that approximately 40% of the glucuronoarabinoxylans from wheat bran could be extracted by barium hydroxide at 70°C (Bergmans *et al.*, 1996) and that this extract consisted of two main populations; a lowly and a highly substituted glucuronoarabinoxylan (Schooneveld-Bergmans *et al.*, submitted *a*). Here, some functional properties of four fractions obtained from this extract are reported. Viscosity, possible interaction with other polysaccharides and emulsion stabilizing effects are examined in relation to chemical composition, structure and molecular weight.

Experimental

Materials

Wheat bran glucuronoarabinoxylans were extracted from cell wall material (WUS) of industrial wheat bran as described previously (Schooneveld-Bergmans *et al.*, submitted *a*). (1→4)-β-D-arabinoxylan arabinofuranohydrolase (AXH) was purified from *Aspergillus awamori* CMI 142717 (Kormelink *et al.*, 1991). Guar and locust bean galactomannan, Diaguar GH and Diagum LBG, respectively, were provided by Diamalt (München, Germany). Xanthan (Practical grade, Art. G-1253) and κ-carrageenan (Commercial grade, Type I, potassium form, Art. C-1013) were purchased from Sigma (St. Louis, MO, USA). Gum arabic was obtained from Meyhall (Switzerland) and paraffin oil (DAB 7, Art. 7160) from Merck (Darmstadt, Germany).

Fractionation

The glucuronoarabinoxylan extract (1000 ml of 2.5 mg/ml in distilled water) was fractionated by ethanol precipitation in three steps. After each increase in ethanol concentration the mixture was kept at 4°C for 16 h. The precipitates obtained at 10, 50 and 70% ethanol by centrifugation (25,000 *g*; 20 min) and subsequent dissolution in distilled water, were designated as BE.A, BE.B and BE.C, respectively. The final supernatant was concentrated by evaporation, dialysed and designated BE.D. The fractionation was performed in duplicate and all fractions were stored at 4°C with the addition of 0.01% sodium azide.

Removal of arabinose by AXH

BE.C was incubated with AXH at a concentration of 2 mg/ml in 0.05 M sodium acetate buffer of pH 5. The AXH concentration was 0.2 µg/ml. The mixture was incubated for 36 h at 40°C

with continuous mixing and AXH was inactivated at 100°C for 15 min. After centrifugation (25,000 *g*; 20 min) the supernatant was dialysed, concentrated and designated BE.C_{AXH}. The fraction was stored as described above.

Removal of arabinose by dilute acid

BE.C (5 mg/ml) was treated with 0.02 M trifluoroacetic acid for 4 h at 60°C. After cooling the solution was neutralized, dialysed and concentrated by evaporation. It was stored as described above and designated BE.C_{TFA}.

Analytical methods

Neutral sugar composition, uronic acid and *protein content* of extract and fractions were determined as described previously (Bergmans *et al.*, 1996).

Methylation analysis was performed according to the modified Hakomori method as was described before (Schooneveld-Bergmans *et al.*, submitted *a*).

High-performance size-exclusion chromatography (HPSEC). Molecular weight distributions of extract and fractions were determined by HPSEC using three Bio-Gel TSK columns in series as described by Bergmans *et al.* (1996). The weight average molecular weight and intrinsic viscosity were determined as described by Schooneveld-Bergmans (submitted *a*).

Rheological methods

Preparation of solutions and mixtures. Galactomannan solutions were prepared by dispersion of a mix of dry gum and potassium chloride in distilled water at 20°C, to give 0.15% or 0.7% (w/v) polymer solutions in 0.1 M potassium chloride. The dispersions were heated to 80°C for 30 min with continuous stirring to achieve total solubilization. Xanthan solutions were prepared in a similar way in distilled water of 80°C, which was maintained at this temperature for 30 min. κ-carrageenan and gum arabic solutions were prepared similarly, however without heating. Homogeneous solutions of glucuronoarabinoxylans were obtained by heating to 60°C for 30 min and slow cooling to 25°C. Mixtures of xanthan or κ-carrageenan with galactomannan or glucuronoarabinoxylan were prepared by mixing hot solutions of equal polymer concentration in different proportions. The blends were then kept at 80°C for 30 min with continuous stirring and cooled to room temperature.

Viscometry. Viscosities of polysaccharide solutions (0.04-0.70 g/dl) were measured with Ubbelohde capillary viscometers (Schott, Mainz, Germany) of varying capillary diameter (0.53-0.95 mm), submerged in a thermostatically controlled waterbath at 25°C or as stated. Flow times were measured with distilled water or 0.1 M potassium chloride solution as solvents. Intrinsic viscosities were obtained by extrapolation of the reduced and inherent viscosities to infinite dilution, using the Huggins and Kraemer equations (Huggins, 1942;

Kraemer, 1938). The influence of temperature and salt concentration on viscosity was examined at 0.25% (w/v) polysaccharide concentration.

Compression tests. Hot blends of κ-carrageenan with galactomannan or glucuronoarabinoxylan in a ratio of 2:1 and total concentration of 0.7% (w/v), were poured in moulds (h × Ø is 10 × 20 mm) to give gels upon cooling. Of each mixture six samples were tested by uniaxial compression at 25°C, using an Overlaod Dynamics equipped with a 50 N load cell. Fracture stress and relative deformation were calculated as averages of six measurements.

Emulsion stabilization. Resistance to coalescence of emulsions prepared with the addition of polysaccharides in the aqueous phase was determined according to a slightly modified procedure as described by Chanliaud (1995). Polysaccharide solutions in 0.1 M potassium chloride (4 ml, 0.04-0.8% w/v) were emulsified with 2 ml paraffin oil using an Ultraturrax T25 (20,000 rpm; 30 s). The emulsions were subsequently centrifuged (1,000 *g*; 100 min) and the oil separated was weighed. The percentage of oil recovered from the initial amount added is a measure for emulsion stabilization. All emulsions were prepared in duplicate.

Results and Discussion

Fractionation

Yield, composition and structural characteristics of barium hydroxide-extractable glucuronoarabinoxylans from wheat bran WUS have been discussed previously (Schooneveld-Bergmans *et al.*, submitted *a*). In that study it was shown that by means of incremental increases of 10% in ethanol concentration, fractionation in two main arabinoxylan populations was achieved. A lowly substituted population precipitated at 10-20% and a highly substituted fraction at 60-70% ethanol. Furthermore, coextracted (1→3)(1→4)-β-glucans precipitated at 20-50% ethanol. Phenolic material remained soluble at high ethanol concentrations. From these results it was deduced that four fractions of varying composition can be obtained in considerable amount by enlarged scale ethanol precipitation in three steps, namely at 10%, 50% and 70% ethanol. Yields and compositions of the fractions obtained in that way are presented in Table 1. The precipitate collected at 10% ethanol (BE.A), yielded 15% of dry matter. The fraction had an Ara/Xyl-ratio of 0.20 and contained only small amounts of glucose and uronic acid. The composition is very similar to that of the corresponding fraction (BE.10) described in our previous paper (Schooneveld-Bergmans *et al.*, submitted *a*). At 50% ethanol a relatively lowly substituted arabinoxylan was recovered, which contained most of the coextracted (1→3)(1→4)-β-glucans (BE.B). The presence of small amounts of intermediately substituted arabinoxylans (Ara/Xyl= 0.4-0.6) as was inferred from previous work (Schooneveld-Bergmans *et al.*, submitted *a*) explains the slightly increased Ara/Xyl-ratio of 0.28. The final two fractions were obtained at 70% ethanol. The

Table 1: Yield and composition of a barium hydroxide extract of wheat bran WUS and fractions derived thereof by graded ethanol precipitation.

	yield[a]	protein content[b]	total sugar content[c]	molar composition[d]						
				Ara	Xyl	Man	Gal	Glc	UA	Ara/Xyl
BE	-	4.9	82.8	37.0	52.2	0.1	1.1	7.0	2.6	0.71
BE.A	14.7	5.4	85.1	16.1	82.0	0	0.1	1.2	0.6	0.20
BE.B	17.4	2.8	84.7	18.6	66.1	0	0.3	14.5	0.5	0.28
BE.C	33.7	4.0	86.8	42.9	45.6	0.2	1.1	7.0	3.2	0.94
BE.D	32.6	9.8	69.5	49.5	41.6	0.2	1.7	2.7	4.3	1.19
$BE.C_{AXH}$	96.4[e]	2.4	88.9	42.0	46.5	0.2	1.0	7.1	3.2	0.90
$BE.C_{TFA}$	92.3[e]	1.7	89.2	41.0	47.4	0.2	1.0	7.2	3.2	0.86

[a] expressed as weight percentage (dm) of BE.
[b] expressed as weight percentage (dm) of each fraction.
[c] neutral sugars + uronic acids expressed as weight percentage (dm) of each fraction.
[d] expressed as percentage (mole per 100 mole).
[e] expressed as weight percentage (dm) of BE.C.

precipitate (BE.C) yielded 34% of the extracts dry matter and had an Ara/Xyl-ratio of 0.94. The fraction also contained some glucose and uronic acid. The supernatant of this last precipitation (BE.D) had a lower total sugar content and consisted of an arabinoxylan with an Ara/Xyl-ratio of 1.19. Its protein content was higher than for the other fractions, which corresponded with previous results (Schooneveld-Bergmans *et al.*, submitted *a*). However, the yields of the fractions as presented in Table 1 were not in complete agreement with the expectations from these previous results. The present fractionation gave precipitation at higher ethanol concentration. This may have been caused by enlarged scale and by fractionation in three steps instead of eight as used before.

Modification

The highly substituted BE.C fraction was subjected to two different debranching treatments, in order to study the effect of removal of arabinose branches on physical properties. AXH, which is known to be highly specific for the release of arabinose linked at the *O*-3 position of monosubstituted xylose in polymeric arabinoxylan (Kormelink *et al.*, 1993*b*) released only 5% of total arabinose from a highly substituted wheat bran glucuronoarabinoxylan with an Ara/Xyl-ratio of 1.15 (Schooneveld-Bergmans *et al.*, submitted *a*). Fraction BE.C was incubated with this enzyme, because no other arabinose releasing enzymes with higher activity towards such highly substituted arabinoxylans are presently known. In Table 1 the compo-

sition of this AXH-treated BE.C is shown. Approximately 4% of total arabinose present in this fraction was removed, which resulted in a slight decrease of the Ara/Xyl-ratio.

The other debranching treatment used was dilute acid hydrolysis. Although this method has been used before for selective removal of arabinose from a xylan backbone, conditions needed to be optimized as results from literature are very contradictory with respect to resistance of the backbone to simultaneous hydrolysis (Adams, 1955; Schmorak *et al.*, 1957; Brillouet *et al.*, 1982; Brillouet and Joseleau, 1987). Incubation of BE.C in 0.05 M trifluoroacetic acid at 100°C showed a quick release of arabinose, which coincided with a release of xylose and a decrease of the hydrodynamic volume as observed by HPSEC-analysis (results not shown). The latter observation was attributed to degradation of the backbone, giving a decrease of the molecular weight. When 0.01 M trifluoroacetic acid was used slightly less degradation occurred and less arabinose was released, namely a maximum of 8% of total arabinose in 4 hours instead of 12% as was observed for 0.05 M trifluoroacetic acid incubation at 100°C. Brillouet and co-workers (1982) showed a release of approximately 30% of total arabinose in 4 hours by a 0.01 M trifluoroacetic acid treatment of alkali-extractable arabinoxylans of industrial wheat bran. The fact that only 23% of the original extract was recovered as polymeric material after 6 hours of hydrolysis, indicated backbone degradation. Also, the presence of lowly substituted populations (Ara/Xyl = 0.1-0.6) in their extract, may have caused the relatively higher release of arabinose and more extensive backbone degradation when compared to our results. Eventually, a 0.02 M trifluoroacetic acid treatment at 60°C resulted in only a minute shift to lower molecular weight and removed 8% of total arabinose after incubation for 4 hours. Prolonged treatment gave no additional removal of arabinose, but resulted in a progressive decrease of the molecular weight (results not shown). The modified material produced by this treatment had an Ara/Xyl-ratio of 0.90 (Table 1). From the yield recovered from the parental fraction, it is suspected that some material was lost as arabinoxylooligosaccharides during dialysis. Production of small amounts of oligosaccharides was observed also by Brillouet and co-workers (1982).

From these results it can be concluded that the highly substituted glucuronoarabinoxylans of wheat bran can not easily be debranched neither by enzymatic nor by chemical means.

Glycosidic linkage composition

In Table 2 the glycosidic linkage composition of the various fractions is given. As can be calculated from this table, approximately 80% of the xylose residues of fractions BE.A and BE.B were unsubstituted. The remainder was mainly monosubstituted at the O-3 position of the xylose residue, being 83% and 72% of total substituted xylose residues for BE.A and BE.B, respectively. The arabinose residues occurred predominantly as single unit branches, because in both fractions 90% of total arabinose were terminal residues. In BE.B glucose

Table 2: Glycosidic linkage composition of barium hydroxide-extractable glucuronoarabinoxylan fractions of wheat bran WUS, expressed as percentage (mole per 100 mole) of all partially methylated alditol acetates present.

alditol acetate of[a]	BE.A	BE.B	BE.C	BE.C_{AXH}	BE.C_{TFA}	BE.D
2,3,5-Me_3-Ara	13.6	17.2	30.9	29.4	28.2	31.2
3,5-Me_2-Ara	0.3	0.3	1.9	1.9	2.1	2.9
2,3-Me_2-Ara	0.5	0.2	0.9	0.9	0.8	3.0
2,5-Me_2-Ara	0.7	0.7	6.2	6.3	6.3	8.0
5-Me-Ara	0.1	0.1	5.2	5.5	5.8	6.7
2,3,4-Me_3-Xyl	0.9	0.9	8.2	8.6	9.3	11.0
2,3-Me_2-Xyl	67.5	52.5	15.0	17.6	18.4	7.9
2-Me-Xyl	11.3	10.5	8.4	6.5	7.8	9.9
3-Me-Xyl	0.4	0.4	3.2	2.8	4.1	1.8
Xyl	1.9	3.7	13.0	13.4	10.9	13.6
2,3,6-Me_3-Glc	1.4	10.4	5.1	5.0	4.4	2.2
2,4,6-Me_3-Glc	0.2	2.2	1.0	1.1	0.9	0.5
Glc	0.4	0.3	0.3	0.4	0.3	0.6
Man	0.7	0.6	0.6	0.6	0.6	0.7

[a] 2,3,5-Me_3-Ara = 2,3,5-tri-O-methyl-1,4-di-O-acetyl-arabinose, etc.

residues were predominantly 4- and 3-linked in a ratio of 5:1 as was observed before (Selvendran *et al.*, 1980; Schooneveld-Bergmans *et al.*, submitted *a*). BE.C consisted of 31% unsubstituted, 24% mono- and 27% disubstituted xylose residues. In this fraction 70% of the arabinose residues were terminal, the remainder was predominantly 3- and 2,3-linked, which was observed before for wheat bran arabinoxylans (Selvendran *et al.*, 1980; Brillouet *et al.*, 1982; DuPont and Selvendran, 1987; Brillouet and Joseleau, 1987; Schooneveld-Bergmans *et al.*, submitted *a*). After AXH treatment 36% unsubstituted, 19% mono- and 27% disubstituted xylose residues were observed, showing the specificity of AXH to remove solely monosubstituted arabinose residues from a xylan backbone. However, the specificity for the *O*-3 position is not as obvious as expected, because the relative amount of *O*-2 monosubstituted xylose did not increase, when based on total xylose present. After trifluoroacetic acid treatment 36% unsubstituted, 23% mono- and only 22% disubstituted xylose residues were observed. So trifluoroacetic acid treatment gave a reduction of the disubstituted xylose residues, which was not observed after enzymatic treatment. However, the acid treated fraction did not show an increase of unsubstituted xylose residues compared with BE.C_{AXH}. This may indicate that one of the arabinose residues of a disubstituted xylose is resistant to dilute acid hydrolysis or that some of the arabinose residues *O*-3 linked to xylose

and susceptible to AXH were not removed by trifluoroacetic acid treatment. As the relative amount of O-2 monosubstituted xylose residues increased after dilute acid hydrolysis, the former suggestion appeared to be true. Unfortunately, this could not be confirmed by observations mentioned in literature, because these reports did not give separate values for O-2 or O-3 substituted xylose residues (Brillouet *et al.*, 1982; Brillouet and Joseleau, 1987). From Table 2 it can be calculated also that the relative proportion of substituted arabinose residues increased after trifluoroacetic acid hydrolysis, this corresponded with previous reports (Brillouet *et al.*, 1982; Brillouet and Joseleau, 1987). The relative amount of terminal xylose was almost similar before and after dilute acid treatment, although some degradation of the backbone occurred as a slight decrease of the hydrodynamic volume was observed on HPSEC-analysis (results not shown). Degradation may have been obscured by a decrease of terminal xylose residues which are thought to be linked to arabinose branches (Wilkie, 1979). The susceptibility of arabinose to dilute acid hydrolysis may have resulted in a loss of a dimeric branch composed of arabinose and terminal xylose during subsequent dialysis, along with other degradation products. Fraction BE.D consisted of 18% unsubstituted, 26% mono- and 31% disubstituted xylose residues. Of the arabinose residues 60% was terminal, whereas the remainder was mostly 3- and 2,3-linked. However, compared to the other fractions, BE.D appears to contain relatively more 5-linked arabinose.

Molecular weight

The molecular weights of the fractions are presented in Table 3. Because two different methods of molecular weight calculation were used, one based on light-scattering and the other one on universal calibration, two values are given. A considerable deviation between these two molecular weights was shown for the lowly substituted fractions (BE.A and BE.B), which was also observed previously (Schooneveld-Bergmans *et al.*, submitted *a*). The molecular weight based on light-scattering was even much higher in the present study. This may have been caused by more extensive aggregation, as a result of a higher concentration of the stock solution compared with the solution previously analysed. Aggregates were also indicated by a small peak near the void volume in the HPSEC-elution patterns of BE.A and BE.B, which was not observed before. Also the time of storage prior to HPSEC-analysis may have caused a difference, because aggregation is time and temperature dependent (Blake and Richards, 1971). If the molecular weights and gyration radii of the similar fractions BE.A ($M_{w;LS}$ = 2,800 kDa; R_g = 59 nm) and BE.10 ($M_{w;LS}$ = 613 kDa; R_g = 34 nm) from previous work (Schooneveld-Bergmans *et al.*, submitted *a*) are compared, it is possible to speculate on the mechanism of aggregation. The approximate 5 times increase in molecular weight resulted in less than doubling of the radius of gyration. This indicates that aggregation most probably occurred by a combination of parallel and random aggregation, which was sugges-

Table 3: Weight average molecular weight as determined by light-scattering ($M_{w;LS}$) and universal calibration ($M_{w;UC}$), polydispersity index (M_w/M_n), radius of gyration (R_g) and intrinsic viscosity ([η]) of fractions of barium hydroxide-extractable glucuronoarabinoxylans of wheat bran WUS.

	$M_{w;LS}$[a]	$M_{w;UC}$[b]	M_w/M_n[c]	R_g[d]	[η][e]
BE.A	2,796,000	159,000	1.2	59.4	2.4
BE.B	1,181,000	184,000	1.4	42.0	2.1
BE.C	371,000	293,000	2.8	25.8	1.6
BE.D	307,000	257,000	13.3	20.4	1.3
$BE.C_{AXH}$	775,000	326,000	1.3	33.8	1.5
$BE.C_{TFA}$	289,000	185,000	1.5	21.6	1.1

[a] $M_{w;LS}$: weight average molecular weight as calculated by light-scattering data, expressed in Da.
[b] $M_{w;UC}$: weight average molecular weight as calculated by viscometry data, based on universal calibration, expressed in Da.
[c] M_n: number average molecular weight.
[d] expressed in nm.
[e] expressed in dl/g.

ted before for arabinoxylans (Blake and Richards, 1971). Recently, parallel aggregation was observed for lowly substituted arabinoxylan from rye bran in dimethyl sulphoxide, because an enormous increase in molecular weight was hardly reflected in the radius of gyration (Ebringerova *et al.*, 1994). Parallel aggregation was also observed for (1→3)(1→4)-β-glucans isolated from beer (Grimm *et al.*, 1995). In that study the effect of concentration of the stock solution on the extent of aggregation, as was suggested above, was also demonstrated.

From Table 3 it is also clear that the deviation in the differently determined molecular weights is less obvious for more highly branched fractions (BE.C and BE.D). The effect of modification of BE.C on its molecular weight was dependent on the method used. The AXH-modified fraction showed an increased molecular weight and radius of gyration. Apparently, removal of only 4% of arabinose already resulted in partial aggregation. Andrewartha *et al.* (1979) suggested that arabinoxylans become more flexible upon removal of arabinose, causing a decrease of asymmetry of their rod-like shape and thereby a decrease in intrinsic viscosity. This was observed here for both modified fractions. However, for the dilute acid modified BE.C a decrease in molecular weight was observed, which was most probably attributable to some backbone-degradation. This affects the intrinsic viscosity markedly.

Viscosity

Temperature dependence. In Figure 1 the reduced viscosities of the glucuronoarabinoxylan

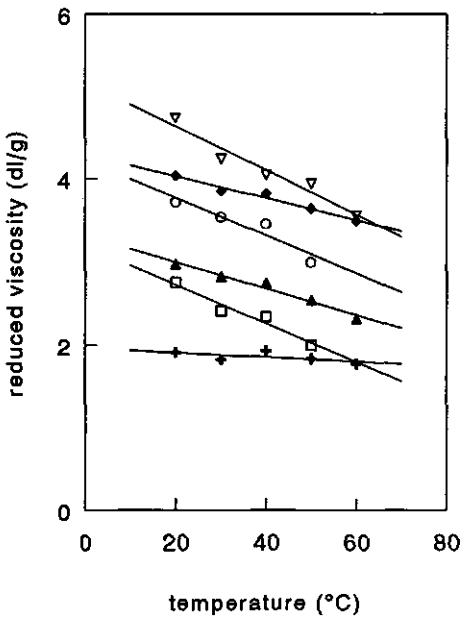

Figure 1: Reduced viscosities of wheat bran glucuronoarabinoxylan fractions with varying temperature at a polysaccharide concentration of 0.25% (w/v). + BE.A; ▲ BE.B; ◆ BE.C; ○ BE.C_{AXH}; □ BE.C_{TFA}; ▽ BE.D.

fractions at varying temperatures is shown. As is known for most polysaccharide solutions, viscosity decreases with increasing temperature, which usually shows a similar effect as a reduction in concentration (Glicksman, 1982). For BE.A hardly any change of viscosity with temperature was observed. When this temperature effect on viscosity is translated to a concentration effect and the speculations on the aggregation mechanism mentioned above are taken into consideration, the absence of a temperature effect is most probably the result of the extent of aggregation and the shape of the aggregate. For fraction BE.B, which is also lowly substituted, some effect of temperature on viscosity was found. This may be attributed to the presence of coextracted (1→3)(1→4)-β-glucans, which may have influenced the aggregation of the xylans as well. When the modified fractions were compared with their parental fraction a stronger temperature dependence was observed after modification. For BE.C_{AXH} this was shown by a slightly steeper slope, but for BE.C_{TFA} an overall reduction of the viscosity was observed also. Degradation, as was noticed before, may have caused this. The effect of temperature on viscosity can also be described by an Arrhenius-type of equation:

$$(\eta_0)_T = (\eta_0)_{Tref}\, e^{(\Delta H/R)(1/T-1/Tref)} \quad (1)$$

T_{ref} is a reference temperature (K), R is the gas constant (8.3 J/mol.K) and ΔH is the activation energy of viscous flow (kJ/mol) (Launay *et al.*, 1986). By plotting $\log(\eta_0)$ versus 1/T the activation energy can be determined from the slope $\Delta H/R$ (figure not shown). For the arabinoxylan solutions ΔH varied between 16 and 20 kJ/mol and for water it was 16 kJ/mol. When these values were compared with the slopes in Figure 1, it was observed that the higher ΔH, the steeper the slope. For the lowly substituted BE.A a ΔH of 16 kJ/mol, which is similar to that of the solvent, indicates that the arabinoxylan has no effect on the viscous flow. No additional energy is required for the flow of water when BE.A is added, and therefore no temperature effect is observed as is obvious from Figure 1. For locust bean gum a ΔH of 21.5 kJ/mol was determined under similar conditions as used here (Launay *et al.*, 1986). So, locust bean gum requires a higher energy input to initiate viscous flow, and accordingly shows a stronger temperature dependence than the arabinoxylan fractions.

Polyelectrolyte behaviour. Polyelectrolytes are known to give increasing reduced viscosities at decreasing concentration, because coil expansion can occur as a result of the presence of charged groups (Launay *et al.*, 1986). Reduced viscosities of the fractions at different concentrations were determined in distilled water, in order to investigate possible polyelectrolyte behaviour. Such behaviour was observed for BE.C, its modified fractions and BE.D, when the reduced viscosities were plotted versus the polysaccharide concentration (figure not shown). The reduced viscosities of the fractions were also determined at one polysaccharide concentration in solutions with increasing concentrations of salt. In Figure 2A this is shown for potassium chloride. It is clear from this figure that the viscosities of the polyelectrolytes were influenced by addition of salt, but that almost no further effect was observed at a concentration of 0.1 M. Essentially no effect of potassium chloride on the viscosity of the neutral fractions (BE.A and BE.B) was observed. The level of electrostatic repulsive forces for a certain polymer is reported to depend on factors such as number, location and degree of dissociation of ionizable groups, ionic strength of the solution and nature of the counterions. The latter two factors can be studied, using the equation:

$$[\eta] = [\eta]_\infty + S \cdot I^{-\frac{1}{2}} \quad (2)$$

$[\eta]_\infty$ is the intrinsic viscosity at infinite ionic strength (I) and S is a constant (Launay *et al.*, 1986). As viscosities were only determined at a single low polysaccharide concentration, the intrinsic viscosities were estimated by single point extrapolation of the combined relation of Huggins and Kraemer (Morris, 1984):

$$[\eta] = 1/C \cdot \{2(\eta_{sp} - \ln \eta_{rel})\}^{\frac{1}{2}} \quad (3)$$

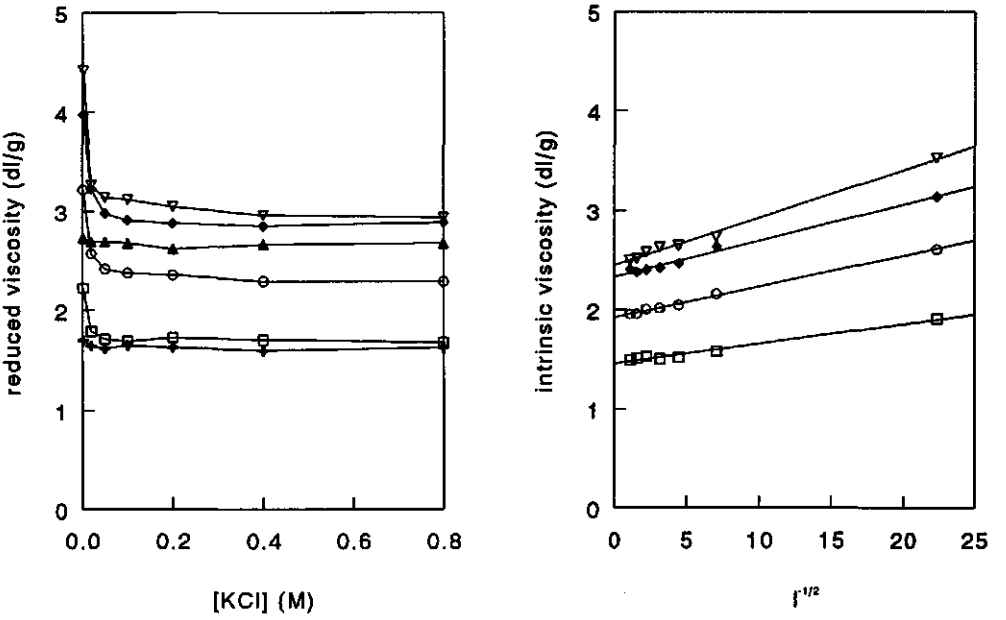

Figure 2:Viscosities of wheat bran glucuronoarabinoxylan fractions with varying concentration of potassium chloride at a polysaccharide concentration of 0.25% (w/v). A: reduced viscosity as a function of potassium chloride concentration. B: intrinsic viscosity as a function of $I^{-½}$. + BE.A; ▲ BE.B; ◆ BE.C; ○ BE.C_{AXH}; □ BE.C_{TFA}; ▽ BE.D.

In Figure 2B these intrinsic viscosities are plotted against $I^{-½}$. The slope (S) can be used to calculate an empirical stiffness parameter (B) with the equation:

$$S = B \cdot ([\eta]_{0.1})^{v} \qquad (4)$$

$[\eta]_{0.1}$ is the intrinsic viscosity in 0.1 M potassium chloride and v~1.3 (Smidsrød and Haug, 1971). The values of $[\eta]_{\infty}$ varied between 1.5 and 2.4 dl/g, as can be observed from Figure 2B. For maize bran heteroxylans these were 1.4 to 1.8 dl/g (Chanliaud, 1995). The parameter B varied between 0.012 and 0.015, which corresponds with values of semi-flexible chains, such as high-methoxyl pectin (B=0.02) (Michel *et al.*, 1984). They are lower than found for maize bran heteroxylans, for which B was determined to be 0.024 (Chanliaud, 1995), but not as low as was reported for xanthan (B=0.005) (Rinaudo and Milas, 1978).

Intrinsic viscosities. The intrinsic viscosities of the polyelectrolytes were also determined in 0.1 M potassium chloride by the Huggins and Kraemer equations. These were 2.4; 2.2; 1.6 and 2.5 dl/g for BE.C, BE.C_{AXH}, BE.C_{TFA} and BE.D, respectively. When these values were

compared with the ones determined by single point extrapolation in 0.1 M potassium chloride, a deviation of 1 to 8% was observed. The single point extrapolation therefore appeared to be justified at the polymer concentration used. However, comparison of intrinsic viscosities determined by the Huggins and Kraemer equations and the ones determined by viscometry during HPSEC-analysis (Table 3), showed a stronger deviation than was observed for single point extrapolation. This may be due to different conditions of measurement, because HPSEC-analysis was performed in 0.4 M sodium acetate buffer of pH 3 at 30°C, whereas Ubbelohde measurements were done in 0.1 M potassium chloride at 25°C. However, the trends for BE.C and its modified fractions were comparable for both methods. This was not true for BE.D when compared to BE.C. Probably the lower total polysaccharide content of BE.D, which was taken into account in the Ubbelohde measurements but not in HPSEC-analysis, may have caused this. The intrinsic viscosities of wheat bran glucuronoarabinoxylans were higher than those of maize bran heteroxylans (Chanliaud, 1995). This may be attributed to the slightly higher molecular weights and higher stiffness (B-parameter) of the former. Moreover, the intrinsic viscosity of BE.C was in close agreement with that of a similar fraction of industrial wheat bran isolated by Brillouet *et al.* (1982). Intrinsic viscosities of wheat flour arabinoxylans ranged from 2 to 8.5 dl/g (Izydorczyk and Biliaderis, 1992*a,b*). For galactomannans, which are assumed to be structurally quite comparable to arabinoxylans, intrinsic viscosities of approximately 12 dl/g in 0.1 M sodium chloride are reported (Goycoolea *et al.*, 1995*a*). The intrinsic viscosities of the gums used in this study were 11.5 and 14.0 dl/g for guar (Gal/Man = 0.57) and locust bean gum (Gal/Man = 0.28). Reported molecular weights as determined by light-scattering ranged from 300 to 1,700 kDa for these galactomannans (Dea and Morrison, 1975). The high values may have resulted from aggregration. Although the diequatorial β-(1→4)-linkages in galactomannans and xylans may imply similar flow properties, this is apparently not true as demonstrated by the diverse intrinsic viscosities. This is probably caused primarily by differences in extent of aggregration resulting from dissimilarities in fine-structure and conformation. Galactomannans usually adopt random coil conformation in solution, but intermolecular association between unsubstituted regions has been proved recently (Goycoolea *et al.*, 1995*a*). Regions of association are expected to be terminated by the protruding galactose substituents. In arabinoxylans it is suggested that the arabinose substituents can be incorporated in the lattice of the backbone conformation and that the substituted regions can also participate in the association of neighbouring arabinoxylan chains (Dea *et al.*, 1973). This may probably be attributed to differences in linkage position of the substituents, being galactose at *O*-6 of the mannose residues in galactomannans and arabinose at *O*-3 and *O*-2 of xylose residues in arabinoxylans. Furthermore, substitution at the 5-position of the backbone residues is different, being hydroxymethyl for mannose residues and hydrogen for xylose residues (Atkins, 1992).

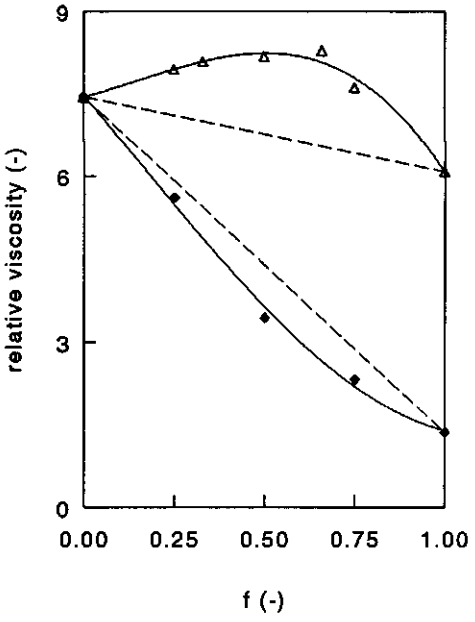

Figure 3: Relative viscosities of mixtures of xanthan and guar galactomannan (△) or BE.C (◆) with different proportions of galactomannan or BE.C (f) at total polysaccharide concentration of 0.15% (w/v). —— observed viscosity; - - - expected viscosity.

Interaction with other polysaccharides

Xanthan. Xanthan is known to give synergistic interaction with locust bean gum, resulting in gelation at sufficiently high polymer concentration (>0.5% w/v), whereas both polysaccharides do not gel separately. Guar galactomannan, which is more highly branched, is not able to form gels with xanthan, although synergy in viscosity is observed at low polymer concentrations (~0.15% w/v) (Dea and Morrison, 1975). The existence of such synergistic effect on viscosity was investigated for some glucuronoarabinoxylan fractions of wheat bran. Guar gum was used as control. In Figure 3 observed and expected relative viscosities of xanthan blends, containing different proportions of guar galactomannan or BE.C, are shown. No synergy was observed for xanthan-arabinoxylan blends. BE.B and the modified BE.C fractions showed similar behaviour as BE.C (results not shown). The viscosity decrease upon addition of arabinoxylan may suggest that a certain interaction with xanthan takes place, but that it leads to contraction of the hydrodynamic volume. This was also observed for mixtures of κ-carrageenan and locust bean gum at low concentration (Goycoolea *et al.*, 1995*b*).

Mixtures of xanthan and locust bean gum or glucuronoarabinoxylan, in equal proportions

and at a total concentration of 0.7% (w/v) were also prepared to examine possibilities of gelation. For the arabinoxylans no gelation was observed, whereas a strong gel was obtained for xanthan-locust bean gum (results not shown). The mechanism of gelation is supposed to be based on intermolecular binding between xanthan and galactomannan, in which a low degree of substitution of galactomannan plays an important role (Dea and Morrison, 1975). In subsequent papers other factors, such as galactose distribution (McCleary, 1979), xanthan side chains (Tako *et al.*, 1984) and denaturation of the xanthan helical structure (Cairns *et al.*, 1987) were supposed to play a role in gelation also. Because of the complexity of the mechanism, the unability of glucuronoarabinoxylans of different Ara/Xyl-ratio to form gels with xanthan is difficult to explain. Structural and conformational differences between galactomannans and arabinoxylans as already mentioned before may have caused this.

κ-carrageenan. κ-carrageenan is known to form gels in the presence of potassium ions after a heating-cooling cycle. The gels become less brittle and more elastic when galactomannan, particularly locust bean gum, are added (Dea and Morrison, 1975). Interaction of κ-carrageenan and glucuronoarabinoxylans was investigated. Gel strength and relative deformation at fracture of these gels, as well as for pure κ-carrageenan and its blends with galactomannan are shown in Table 4. It is obvious that locust bean gum had a strong synergistic effect, because gel strength and relative deformation increased considerably. The arabinoxylan mixtures had an effect which was less than observed for guar gum and quite similar

Table 4: Stress and relative deformation at fracture of gels composed of κ-carrageenan and mixtures of κ-carrageenan and galactomannan or arabinoxylan in a ratio of 2:1 and of total concentration of 0.7% (w/v).

gel[a]	fracture stress (kPa)	relative deformation (%)
CAR	27.7	40
CAR⅔	14.1	37
CAR - GG	20.1	38
CAR - LBG	57.4	49
CAR - BE.A	14.5	34
CAR - BE.B	15.1	37
CAR - BE.C	14.6	37
CAR - BE.C_{AXH}	15.1	36
CAR - BE.C_{TFA}	14.3	36
CAR - BE.D	15.3	38

[a] CAR = κ-carrageenan; CAR⅔ = κ-carrageenan at two-thirds of the original concentration; GG = guar gum; LBG = locust bean gum.

to κ-carrageenan by itself at two-thirds of its original concentration. Therefore it can be concluded that if any interaction between κ-carrageenan and arabinoxylan occurred, it certainly did not have the same effect as locust bean gum, nor did it disturb the gelation of κ-carrageenan. Again the exact mechanism of gelation and the synergistic effect of locust bean gum is not completely clear. Apart from a coupled network with specific heterotypic junction zones, in which potassium ions appear to play a role (Tako and Nakamura, 1986), it was also suggested that the network structure consists of a galactomannan solution in a continuous κ-carrageenan network (Cairns *et al.*, 1987). Also phase separation in enriched regions of κ-carrageenan and locust bean gum was supposed to result in a firm gel (Miles *et al.*, 1984). The suggestion of a galactomannan solution in a continuous κ-carrageenan network may explain why arabinoxylans did not disturb gelation. The absence of synergy may have been caused by the same reasons as mentioned before.

Emulsion stabilization

In Figure 4 the resistance to coalescence of emulsions of paraffin oil and glucuronoarabinoxylan or gum arabic solutions of varying concentrations is shown. Because hydrocolloids stabilize emulsions primarily by increasing the viscosity of the continuous phase (Glicksman, 1982), the results for arabinoxylans are clear. BE.A hardly gave viscosity to solutions and showed only a low oil recovery at high concentrations. BE.C had a relatively high viscosity and appeared to stabilize the emulsion already at 0.05 g/dl. Gum arabic, which is often used for stabilization, was needed in higher concentration to achieve a similar effect. This polymer stabilizes emulsions by a different mechanism. It absorbs to the oil-water interface giving the globules a charged surface, which then results in stabilization by electrostatic repulsive forces (Glicksman, 1982). As the highly substituted arabinoxylans BE.C and BE.D also contain uronic acid substituents, electrostatic repulsive forces may play a role in the stabilizing effect also. The modified fractions of BE.C (not shown in figure) appeared to be slightly less efficient than their parental fraction, which was to be expected from the lower intrinsic viscosities when compared with BE.C. A similar high efficiency of stabilization of paraffin oil emulsions as shown for BE.C, was observed for maize bran heteroxylans (Chanliaud, 1995).

Conclusions

Separation of barium hydroxide-extractable glucuronoarabinoxylans from wheat bran in four fractions by ethanol precipitation made investigation of some functional properties possible. The lowly substituted arabinoxylans gave almost no viscosity to solutions. These fractions were neutral, thereby showing no influence of addition of salt on viscosity. Temperature did not affect viscosity either. Functional similarity of these xylans with lowly substituted locust

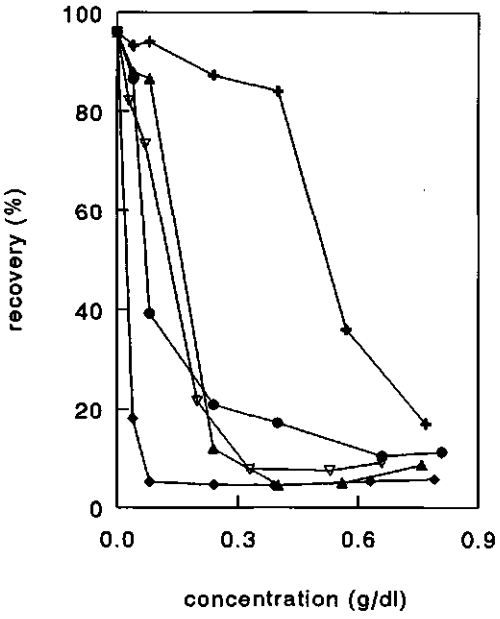

Figure 4: Recovery of paraffin oil from emulsions prepared with the addition of wheat bran glucuronoarabinoxylan or gum arabic to the aqueous phase in varying concentrations. + BE.A; ▲ BE.B; ◆ BE.C; ▽ BE.D; ● gum arabic.

bean gum galactomannan was not evident from interaction with other polysaccharides, such as xanthan and κ-carrageenan. Moreover, the xylans appeared to be sensitive to extensive aggregation. In short, their functional properties are not attractive for thickening of solutions. The highly substituted fractions gave moderate viscosity to solutions. Their viscosity was sensitive to small additions of salt, revealing their polyelectrolyte nature, and to changes in temperature. Aggregation was not very dominant. Only after removal of a small amount of arabinose, some aggregation occurred. The highly substituted fractions did not show any interaction with xanthan and κ-carrageenan either, but they may find application in food as viscosity enhancers and, more importantly, as emulsion stabilizers.

Acknowledgement

The authors thank Dr. J.H.G.M. Mutsaers (Royal Gist-brocades, Delft, The Netherlands) for stimulating comments, and Ms. K. Grolle of the Division of Food Physics of Wageningen Agricultural University for her assistence with the compression tests. This research was supported financially by the Dutch Innovation Oriented Programme Carbohydrates (IOP-k) and by Royal Gist-brocades.

CHAPTER 5

Extraction and partial characterization of feruloylated glucuronoarabinoxylans from wheat bran

This chapter has been submitted for publication to *Carbohydrate Polymers* by the authors M.E.F. Schooneveld-Bergmans, A.M.C.P. Hopman, G. Beldman, A.G.J. Voragen.

Abstract

Feruloylated glucuronoarabinoxylans were extracted from wheat bran by means of cold water, steam and dilute alkali, yielding approximately 1, 20 and 3%, respectively, of the glucuronoarabinoxylans originally present in bran. The extracts had ferulic acid contents ranging from 0.1 to 0.6% (w/w). Both the cold water and steam extract had a low total sugar content and a relatively low content of high molecular weight material. Moreover, the ferulic acid content of the water extract was low (0.1%) and the amount of degradation products in the steam extract was relatively large. Both extracts gave no increase in viscosity upon addition of oxidative reagents, such as hydrogen peroxide plus peroxidase or ammonium persulphate. The alkaline extracts obtained with saturated calcium hydroxide or 0.05 M barium hydroxide had high contents of sugars, the majority of which was accounted for by glucuronoarabinoxylan (> 90%). The extracts had moderate ferulic acid contents (0.3%) and consisted predominantly of high molecular weight material. Addition of oxidative reagents resulted in an increase of viscosity. The extent of this increase in viscosity was shown to be influenced by the oxidative reagent used, the purity of the extract, its molecular weight distribution and the degree of substitution of the glucuronoarabinoxylan.

Introduction

Already several decades it has been known that ferulic acid plays a significant role in the increase of viscosity of water-soluble extracts from wheat flour by the addition of hydrogen peroxide and peroxidase (Fausch *et al.*, 1963; Geissmann and Neukom, 1971 and 1973; Neukom and Markwalder, 1978). Because the water-binding capacity of wheat flours is of importance in dough preparation and baking performance (Meuser and Suckow, 1986), the components involved and the mechanism of reaction have been of interest ever since.

The increase in viscosity of water-extractable arabinoxylans, to which ferulic acid is esterified, has been shown to be the result of oxidative coupling of two ferulic acid residues after addition of hydrogen peroxide and peroxidase (Geissmann and Neukom, 1971 and 1973). Involvement of protein in this reaction was suggested also by these authors, but it appeared not to be essential (Morita *et al.*, 1974). However, the amino acids tyrosine (Neukom and Markwalder, 1978) and cysteine (Hoseney and Faubion, 1981; Jackson and Hoseney, 1986) were presumed to participate in the cross-linking. For rye flour water-soluble arabinoxylans it was shown that the cysteine residues of the coextracted protein seemed not to be involved in the gelation (Vinkx *et al.*, 1991). Whether protein has any role in cross-linking or not is still not clear. Nevertheless, factors that have been shown to influence the extent of cross-linking are the wheat variety the extract originates from (Ciacco and D'Appolonia, 1982*a*; Izydorczyk *et al.*, 1991), the ash content of the flour (Neukom and Markwalder, 1978; Ciacco and D'Appolonia, 1982*b*), the intrinsic viscosity of the extract (Ciacco and D'Appolonia, 1982*a,b*; Izydorczyk and Biliaderis, 1992*a,b*) and the structure of the arabinoxylans, more precisely the degree of substitution (Izydorczyk and Biliaderis, 1992*a*).

In this study it was investigated whether feruloylated arabinoxylans can be extracted from wheat bran. Wheat bran is known to contain 0.4 to 0.7% (w/w) alkali-extractable ferulic acid (Smith and Hartley, 1983), which is predominantly located in the aleurone layer and to a lesser extent in the pericarp (Fulcher *et al.*, 1972; Pussayanawin and Wetzel, 1987). Isolation of feruloylated arabinoxylans from wheat bran, which can form gels upon oxidative cross-linking, can open up possibilities for new uses of this by-product. Different extraction methods were therefore investigated for their ability to give extracts that can be cross-linked upon addition of oxidative reagents. In order to induce cross-linking not only hydrogen peroxide and peroxidase, but also ammonium persulphate was used, because both were reported to form gels with wheat flour arabinoxylans (Izydorczyk *et al.*, 1990).

Experimental

Materials

Industrial wheat bran was obtained from Presco International (Weert, The Netherlands). Wheat bran was destarched using a heat-stable α-amylase (Termamyl 120L, Novo Industri A/S, Copenhagen, Denmark). A wheat bran suspension with a solid to liquid ratio of 1:9 (w/v), containing 0.05 ml Termamyl per 100 g of bran and 30 ppm calcium was incubated for 2 h at 95°C with continuous stirring after adjustment to pH 6. Finally, the suspension was autoclaved for 1 h at 121°C. The residual solids were collected by centrifugation (17,700 *g*; 30 min) and subsequent washing with distilled water. Water-unextractable cell wall material (WUS) from wheat bran was prepared as described previously (Bergmans *et al.*, 1996).

Cold water extraction

Water-extractable material was isolated from wheat bran according to a slightly modified method of Fincher and Stone (1974). The milled bran was boiled in 80% ethanol and filtered, which was repeated twice. The alcohol-insoluble residue was suspended in distilled water with a solid to liquid ratio of 1:5, and stirred continuously at 4°C for 64 h. After removal of the solids by centrifugation (17,700 *g*; 30 min) the liquid was adjusted to 0.01 M citric acid, 0.01 M sodium chloride, 0.001 M calcium chloride, pH 4.6 and 0.05% sodium azide. After incubation of this solution with amyloglucosidase (Boehringer, Mannheim, Germany) at 30°C for 40 h it was heated to 100°C for 15 min, cooled, centrifuged, dialysed and freeze-dried (WE).

Steam explosion

Destarched wheat bran was treated in a laboratory defibrator with saturated steam (Institute of Wood Chemistry, Hamburg, Germany). Steam treatments were performed at temperatures of 180 to 200°C for periods of 5 to 15 min. At the end of the treatment the defibrator

was operated for 15 s, the steam was released and the humid material was removed with water. The solids were collected by centrifugation (17,700 *g*; 30 min) and washed three times with distilled water. Solids and combined liquids were freeze-dried. Soluble fractions were designated $180SE^5$; $190SE^5$; $190SE^{10}$; $190SE^{15}$; $200SE^5$. The first three digits refer to the steam temperature (°C) and the superscript to the time of steaming (min).

Dilute alkali extraction

WUS was extracted with bivalent hydroxide solutions with a solid to liquid ratio of 1:100 (w/v). Calcium hydroxide was used as saturated solution as described previously (Bergmans *et al.*, 1996) and barium hydroxide at a concentration of 0.05 M. Addition of 0.26 M sodium borohydride as used before (Bergmans *et al.*, 1996) was omitted. Extraction lasted for 1 to 16 h and was performed at 20 or 70°C. The extracted material was recovered by centrifugation, neutralization and dialysis as described before (Bergmans *et al.*, 1996). The extracts were either freeze-dried or concentrated by vacuum evaporation. The concentrated solutions were stored at 4°C with the addition of 0.05% sodium azide. Large scale extracts were designated $0.02CE_4$ and $0.05BE_2$, for calcium and barium hydroxide, respectively. The first numbers refer to the concentration of alkali (M) and the subscript to the extraction time (h).

Purification of extracts

Solubilized extracts (2 ml of 2.0 mg/ml) were mixed with cold ethanol, methanol or acetone giving mixtures with 70 or 80% (v/v) of organic solvent. These mixtures were stirred for 1 h at 4°C and then left at the same temperature for 16 h. Precipitate and supernatant were recovered by centrifugation (25,000 *g*; 20 min). The precipitate was dissolved in 2 ml of distilled water, and the supernatant was dried in a stream of air and redissolved in 2 ml of distilled water. The original solution, redissolved precipitate and air-dried supernatant were analysed for total neutral sugar content by an automated colorimetric method using arabinose as standard (Tollier and Robin, 1979) and for UV-absorbance at 280 nm. Large scale purification was also performed at a concentration of 2 mg/ml.

Cross-linking of glucuronoarabinoxylans

Hydrogen peroxide - peroxidase. Samples were cross-linked in 0.1 M sodium phosphate buffer of pH 6, by addition of 0.05 mg horse-radish peroxidase (Type I, Art. P-8125, Sigma, St. Louis, CA, USA) and 2 µmoles of hydrogen peroxide per ml of solution. The samples were diluted to the appropiate concentration, which ranged from 5 to 15 mg/ml, with phosphate buffer containing 0.05% sodium azide. The reaction was performed at 25°C.

Ammonium persulphate. Cross-linking of samples by addition of ammonium persulphate at a concentration of 0.01 M was performed in distilled water containing 0.05% sodium azide.

The concentration of the samples and reaction temperature was similar as described above.
Viscometry. Viscosities of the solvent and of the polysaccharide solutions before and after addition of reagents were measured with an Ubbelohde capillary viscometer (Schott, Mainz, Germany) with a capillary diameter of 0.63 mm, submerged in a thermostatically controlled waterbath at 25°C. Measurements were taken 15 min or 24 h after addition of peroxide and ammonium persulphate, respectively.

Analytical methods

Neutral sugar composition and *uronic acid content* of the wheat bran materials, extracts and residues were determined as described previously (Bergmans *et al.*, 1996).
Ferulic acid content. Samples were saponified with 5 ml of 0.5 M potassium hydroxide for 16 h at room temperature in nitrogen atmosphere and shielded from light. *p*-Hydroxybenzoic acid was added as internal standard. The samples were acidified with 0.75 ml of 6 M hydrochloric acid and extracted twice with 4 ml ethyl acetate. The combined ethyl acetate extracts were dried under nitrogen. The residues were dissolved in 1 ml methanol and analysed by HPLC. A SpectraSYSTEM P4000 gradient pump equipped with a membrane degasser (Thermo Separation Products, Fremont, CA, USA) was used with a reversed phase Spherisorb 10 ODS column. The column was operated at a flow rate of 1 ml/min at room temperature. Elution was performed with a gradient of 0.01 M acetic acid of pH 5 and methanol. Starting conditions were 5% methanol in 0.01 M acetic acid for 5 min, then linear gradients were applied of 5-50% methanol in 0.01 M acetic acid during 20 min and 50-90% methanol in 0.01 M acetic acid during 10 min. This final condition was kept for the following 5 min. The column effluent was monitored by a SpectraSYSTEM UV1000 detector (Thermo Separation Products, Fremont, CA, USA) at a wavelength of 280 nm. A standard of *trans*-ferulic acid was used to quantify the amounts present in the samples. As isomerization of *trans* to *cis*-ferulic acid can occur during sample work-up, part of the standard solution was irradiated for 4 h at 360 nm in order to give partial conversion to the *cis*-isomer. By this means quantification of both isomers was performed.
High-performance size-exclusion chromatography (HPSEC). Molecular weight distributions of the extracts were determined by HPSEC using three Bio-Gel TSK columns in series as described elsewhere (Bergmans *et al.*, 1996).

Results and Discussion

Cold water extraction

Water-extractable material was isolated from wheat bran by cold water in attempt to obtain feruloylated glucuronoarabinoxylan. In Table 1 yield and composition of this extract is given.

Table 1: Yield and composition of extracts obtained from wheat bran (WB) by water extraction, from destarched wheat bran by steam treatment or from wheat bran WUS by dilute alkali extraction.

	yield[a]	ferulic acid content[b]	total sugar content[c]	molar composition[d]						
				Ara	Xyl	Man	Gal	Glc	UA	Ara/Xyl
WB as is										
WE	1.2	0.1	53.8	32.2	52.2	1.3	5.1	5.8	3.3	0.62
WB destarched										
$180SE^{10}$	20.6	0.6	46.1	35.5	37.7	0.5	2.5	19.9	3.9	0.94
$190SE^{5}$	19.5	0.6	54.4	35.8	38.1	0.6	2.4	19.5	3.6	0.94
$190SE^{10}$	30.7	0.5	44.2	26.2	51.2	0.7	2.2	15.6	4.1	0.51
$190SE^{15}$	31.1	0.5	42.1	31.0	47.1	0.5	2.4	14.6	4.4	0.66
$200SE^{5}$	20.6	0.6	51.8	34.4	38.7	0.5	2.4	20.5	3.5	0.89
WB WUS										
$0.02CE_4$	2.0	0.3	75.8	42.9	46.2	0.3	2.0	5.7	3.0	0.94
$0.05BE_2$	2.9	0.2	72.8	41.6	51.9	0.2	1.5	2.6	2.2	0.80

[a] expressed as weight percentage (dm) of original wheat bran.
[b] expressed as weight percentage (dm) of each fraction.
[c] neutral sugars + uronic acids expressed as weight percentage (dm) of each fraction.
[d] expressed as percentage (mole per 100 mole).

Only 1% of the original bran appeared to be extractable in cold water. The sugar and ferulic acid content of the extract were 54% and 0.1%, respectively. The extract contained 1.5% of the glucuronoarabinoxylans originally present in bran. From Table 1 it can be calculated that 1 out of 100 arabinose residues in the extract was esterified with ferulic acid, for whole bran it was 1 out of 20 arabinose residues. This showed that ferulic acid was predominantly bound to polymers, which were not water-extractable. These polymers are most probably held in the cell wall matrix by covalent linkages. Ferulic acid is known to be a bridging unit in lignified grass cell walls between polysaccharide and lignin by means of an ester and ether-linkage, respectively (Scalbert *et al.*, 1985; Lam *et al.*, 1990). However, esterified ferulic acid is also known to be predominantly present in the aleurone layer (Fulcher *et al.*, 1972; Pussayanawin and Wetzel, 1987), which contains hardly any lignin (Bacic and Stone, 1981; Akin, 1995). The insolubility of the feruloylated arabinoxylans of the aleurone layer is very likely caused by extensive hydrogen bonding, because these arabinoxylans have a low degree of substitution (Bacic and Stone, 1981; Schooneveld-Bergmans *et al.*, submitted *a*).

The Ara/Xyl-ratio of 0.62 for WE was low compared to previous alkaline extracts obtained from wheat bran (Bergmans *et al.*, 1996). As this ratio corresponds quite well with the ones

reported for water-extractable wheat flour arabinoxylan (Izydorczyk *et al.*, 1990; Cleemput *et al.*, 1993), WE or part of it may as well have originated from residual endosperm material in the bran. The amount of starch in the bran of 12% (w/w) (Bergmans *et al.*, 1996) is indicative for this assumption, however it was not studied any further. HPSEC-analysis of the extract showed the presence of populations of varying hydrodynamic volumes (Figure 2A).

From these results it can be inferred that for the isolation of large amounts of feruloylated glucuronoarabinoxylans from wheat bran techniques need to be used that disintegrate the dense cell wall structure without hydrolysis of ester-linked ferulic acid.

Steam explosion

From literature it is known that disintegration of lignified cell walls can be achieved by steam explosion treatment, resulting in solubilization of partially depolymerized hemicellulose without loss of esterified ferulic acid (Puls *et al.*, 1985; Puls and Poutanen, 1989). Preliminary experiments of mild steam treatments for 10 min at 180°C with wheat bran resulted in strong browning of the material. Very likely this was caused by decomposition of starch, because conversion of sugars to furfural-like compounds is known to occur during severe steaming of wood chips (Puls *et al.*, 1985). Moreover, formation of Maillard and tannin-like condensation products during heat treatment of cereals has also been reported (Theander and Westerlund, 1986; Castro *et al.*, 1994). Browning appeared to be reduced when destarched bran was used. However, as a result of the destarching treatment a loss of approximately 12% of glucuronoarabinoxylans of the original bran was observed (results not shown). This was most likely caused by the hot water incubation and autoclave treatment. Previously, a loss of approximately 10% of the glucuronoarabinoxylans originally present in wheat bran WUS was reported as a result of a 1 h autoclave treatment (Bergmans *et al.*, 1996).

By applying different steam treatments, which varied in temperature and time, it was attempted to solubilize polymeric feruloylated glucuronoarabinoxylans from destarched wheat bran. In Table 1 yields and compositions of the steam extracts are presented. The yields ranged from 20 to 31%, and the extracts contained 19 to 28% of the glucuronoarabinoxylans present in the parental bran. The purity of the extracts was not very high, because their total sugar contents were only approximately 50%, of which 15 to 20 mole-% was accounted for by coextracted glucose. The glucose may originate from starch, as destarched bran had a residual starch content of 4% (w/w), or it may originate from (1→3)(1→4)-β-glucans. Cellulose has been shown not to get solubilized by steam treatment (Puls *et al.*, 1985). The ferulic acid content of the steam extracts was considerably higher than that of the water-extractable fraction. It was calculated that 1 out of 10 to 20 arabinose residues in these extracts was esterified with ferulic acid, which corresponds well with the ratio in whole bran. Approximately 20% of the ferulic acid originally present in the bran was recovered in

the extracts. From Table 1 it can be observed that higher temperature and longer time of steaming resulted in a higher dry matter yield, but also in a lower total sugar content of the extract. The latter is likely caused by the aforementioned decomposition of sugars to furfural-like compounds and formation of condensation products. HPSEC-analysis of the steam extracts showed that only $180SE^{10}$, $190SE^{5}$ and $200SE^{5}$ contained a minor fraction eluting at relatively high hydrodynamic volume. In Figure 2B the HPSEC-elution pattern of $200SE^{5}$ is shown. For the other extracts the steaming conditions appeared to be too rigorous to yield polymeric material.

From these results it can be stated that steam explosion can be used for the extraction of feruloylated glucuronoarabinoxylans from destarched wheat bran. However, steaming conditions need to be mild in order to recover any polymeric material.

Dilute alkali extraction

As a third attempt to isolate feruloylated glucuronoarabinoxylans from wheat bran, dilute alkali extraction was used. Although it is generally known that esterified ferulic acid is hydrolysed in alkaline solutions, it is also reported that 0.1 M sodium hydroxide treatment for 1 h released only 10 to 20% of the alkali-labile ferulic acid of grass cell wall materials (Hartley and Morrison, 1991). The extractability of feruloylated glucuronoarabinoxylans from wheat bran WUS was therefore followed in time for three different alkali treatments. Bivalent hydroxides, such as barium and calcium hydroxide, were preferred for the extraction, because of their previously observed selectivity (Gruppen *et al.*, 1991; Bergmans *et al.*, 1996). The use of sodium borohydride, which further improved the selectivity of the extractants and prevented alkaline peeling, was now omitted. Literature data on possible reduction of the α,β-unsaturated carbonyl side chain of hydroxycinnamic acids by borohydride were contradictory. Carpita (1986) claimed that the unsaturated bond was reduced, whereas Ford (1989) recovered almost all phenolic acids without modification when 0.2 M borohydride solution was used as extractant for polysaccharides from pangola grass. Because the majority of the extractable glucuronoarabinoxylans from wheat bran WUS are highly substituted (Schooneveld-Bergmans *et al.*, submitted *a*) alkaline peeling was not expected to play a role during extraction. It was therefore decided not to use sodium borohydride. In Figure 1 yields of extracted glucuronoarabinoxylans and the ferulic acid content of the extracts are presented. Figure 1A shows that saturated calcium hydroxide, of which the concentration is approximately 0.02 M, extracted up to 10% of the glucuronoarabinoxylans of WUS in 16 h. After 6 h of extraction the ferulic acid content of the extract was 0.1%. Extraction at 70°C with the same extractant resulted in a maximum yield of more than 30% of the glucuronoarabinoxylans originally present in WUS. However, already after 1 h of extraction the ferulic acid content of the solubilized material was less than 0.1%. The effect of temperature on

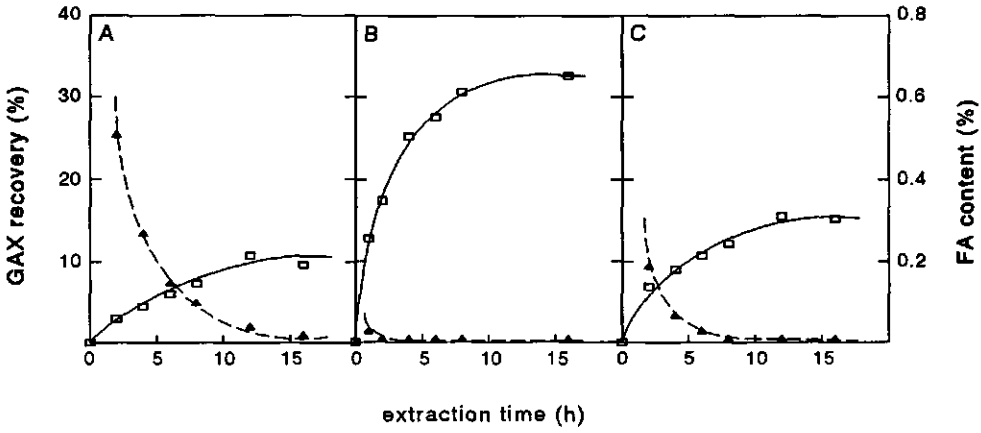

Figure 1: Recovery of glucuronoarabinoxylan (——) and ferulic acid content (- - -) of extracts obtained from wheat bran WUS by saturated calcium hydroxide at 20°C (A), saturated calcium hydroxide at 70°C (B) or 0.05M barium hydroxide at 20°C (C).

ferulic acid content of the extract was further investigated by performing extractions with saturated calcium hydroxide at temperature intervals of 10°C in the range of 20 to 70°C. Substantial losses of ferulic acid were observed starting at 40°C (results not shown). Extraction at slightly higher hydroxide concentration was performed with 0.05 M barium hydroxide at 20°C. In 16 h 15% of the glucuronoarabinoxylans of WUS were extracted. After 4 h of extraction the ferulic acid content of the extract was less than 0.1%. Greenshields and Rees (1993) described the use of 0.1 to 0.6 M sodium or potassium hydroxide typically for 0.5 to 5 hours at 60 to 85°C for extraction of feruloylated polysaccharides from testaceous plant material. Based on the results described above it is suspected that their range of conditions is not appropriate to prevent the loss of ferulic acid in all cases. However, minor differences in extraction procedure, such as solid to liquid ratio and particle size of the plant material, may have an effect on the results, but this was not further investigated.

From the extractions shown in Figure 1 two conditions were selected for enlarged scale extraction of feruloylated glucuronoarabinoxylans from wheat bran WUS. These conditions were saturated calcium hydroxide for 4 h ($0.02CE_4$) and 0.05M barium hydroxide for 2 h ($0.05BE_2$), both at 20°C. The yields and compositions of the extracts are given in Table 1. Only 2 and 3% of the glucuronoarabinoxylans present in bran were extracted by calcium and barium hydroxide, respectively. This corresponds with 4 and 7% of the glucuronoarabinoxylans of WUS as is shown in Figure 1. The selectivity of the extractions appeared to be quite high compared to the steam treatments, because the total sugar contents amounted

Table 2: Recovery of neutral sugar (NS) and absorption at 280 nm (A_{280}) of redissolved residues of water (WE), steam (200SE[5]) and alkali ($0.02CE_4$ and $0.05BE_2$) extracted glucuronoarabinoxylans from wheat bran, expressed as percentage of neutral sugars and absorption at 280 nm of the original extract.

	WE		200SE[5]		$0.02CE_4$		$0.05BE_2$	
	NS	A_{280}	NS	A_{280}	NS	A_{280}	NS	A_{280}
ethanol								
70%	69	36	37	23	65	30	55	47
80%	75	51	44	29	85	63	73	61
methanol								
70%	59	44	29	14	41	22	30	36
80%	64	34	32	19	71	37	51	47
acetone								
70%	79	54	45	27	81	35	67	54
80%	81	54	61	38	90	57	60	59

to more than 70% of the dry matter and coextraction of only 3 to 6 mole-% of glucose was observed. The extracts contained approximately 0.3% ferulic acid. As ferulic acid contents of the residues of extraction were also determined (results not shown), the total ferulic acid recovery was determined to be approximately 60%. HPSEC-analysis showed the presence of a relatively high content of material of high hydrodynamic volume (Figure 2C and D).

In conclusion, dilute alkali can be used for the extraction of polymeric feruloylated glucuronoarabinoxylans from wheat bran, as long as the concentration of alkali, time and temperature of extraction are properly balanced.

Purification of extracts

Preliminary studies on the viscosity behaviour of the extracts upon addition of hydrogen peroxide and peroxidase showed no increase of the viscosity even at high concentrations of glucuronoarabinoxylans. Use of 80% ethanol (v/v) is reported for purification of feruloylated arabinoxylans from wheat flour, resulting in a considerable improvement of the gelling capacity (Moore *et al.*, 1990). Studies on the homogeneity of the dilute alkaline extracts of wheat bran WUS ($0.02CE_4$ and $0.05BE_2$) by fractionation using graded ethanol precipitation, showed that the fractions soluble at high concentrations of ethanol had low sugar and ferulic acid contents (results not shown). Because of this, these highly ethanol-soluble fractions were assumed not to participate in the cross-linking, on the contrary they may even limit cross-linking as a result of the presence of lignin and protein, which may scavenge radicals needed for coupling of ferulic acid residues.

Purification of various extracts was performed by precipitation at 70 or 80% (v/v) ethanol, methanol or acetone. The efficiency was evaluated based on the recovery of neutral sugars and absorbance at 280 nm in the redissolved residue. This wavelength was chosen, as it is characteristic for condensed aromatics (Akin and Hartley, 1992; Akin, 1995), such as lignin, which was expected to interfere in the cross-linking of arabinoxylans. Because ferulic acid esters are known to give some absorbance at 280 nm also, and these esters needed to be recovered in the redissolved residue, the purification was assumed to be optimal when the recovery of neutral sugars was high with a simultaneous relatively low UV-absorbance. The results of these precipitations are presented in Table 2. Generally, when acetone was used the sugar recovery and UV-absorbance were highest. The lowest recovery of UV-absorbance was observed for methanol, but it also resulted in great losses of carbohydrates. Although ethanol gave intermediate results, the use of acetone was preferred because it reduced the absorbance relatively well without great losses of sugars. The latter criterion was therefore considered to be more important, as the extract yields were already very low. For the water and steam extracts purification with 80% acetone was chosen, whereas 70% acetone was used for the calcium and barium hydroxide extracts.

In Table 3 the yields and compositions of the purified extracts are shown. Comparison of the Ara/Xyl-ratios of the extracts before (Table 1) and after purification (Table 3), showed a decrease for all extracts. This is mainly caused by the higher solubility of highly substituted arabinoxylans in organic solvents compared with lowly substituted populations (Gruppen *et al.*, 1992*b*; Schooneveld-Bergmans *et al.*, submitted *a*). The very sharp decrease in Ara/Xyl-ratio for the steam extract is most probably also caused by the extraction method. It has been reported that mild acidic conditions can occur during steam treatment (Puls *et al.*, 1985), as a result of which the highly acid-sensitive furanosidic linkages can be hydrolysed. In the subsequent purification the hydrolysed arabinose was lost in the supernatant as a result of its solubility in 80% acetone. For the WE purification resulted in a loss of glucose, and 80% of the glucuronoarabinoxylans of the original extract were recovered. However, an increased sugar content was not obtained by purification. This latter observation was also noticed for the steam extract, which consisted of only 50% of the glucuronoarabinoxylans of the parental extract. The solubility of mono- and oligosaccharides, which were produced during steaming, in high concentrations of organic solvent most probably caused this low recovery. Both alkaline extracts showed an increased total sugar content as well as ferulic acid content. A recovery of 80 and 60% of the original glucuronoarabinoxylans was obtained for $0.02CE_4$ and $0.05BE_2$, respectively, whereas almost all ferulic acid was recovered.

It is therefore concluded that the best purification was obtained for the alkaline extracts, because an increase in total neutral sugar content and ferulic acid content coincided with a loss of coextracted glucose and non-sugar material.

Table 3: Yield and composition of acetone purified extracts obtained from wheat bran by water extraction (WE·P), from destarched wheat bran by steam treatment (200SE[5]·P), or from wheat bran WUS by dilute alkali extraction (0.02CE_4·P and 0.05BE_2·P).

	yield[a]	ferulic acid content[b]	total sugar content[c]	molar composition[d]						
				Ara	Xyl	Man	Gal	Glc	UA	Ara/Xyl
WE·P	79.8	0.1	44.6	33.2	55.6	0.7	5.6	1.4	3.4	0.60
200SE[5]·P	49.7	0.7	46.0	24.4	44.4	0.3	2.9	21.5	6.5	0.55
0.02CE_4·P	67.5	0.5	85.3	43.7	48.3	0.2	2.4	1.5	4.0	0.90
0.05BE_2·P	53.3	0.4	78.8	40.1	53.8	0.2	1.2	1.4	3.3	0.75

[a] expressed as weight percentage (dm) of original extract.
[b] expressed as weight percentage (dm) of each fraction.
[c] neutral sugars + uronic acids expressed as weight percentage (dm) of each fraction.
[d] expressed as percentage (mole per 100 mole).

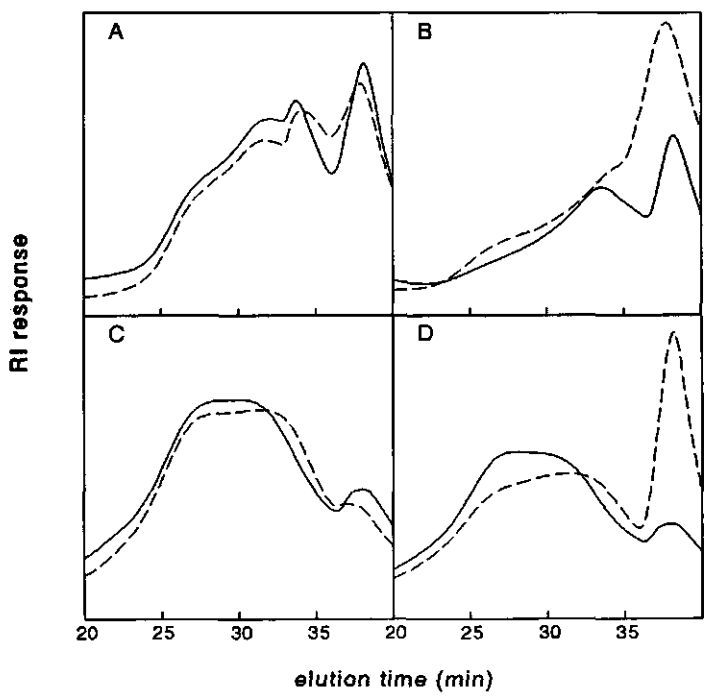

Figure 2: HPSEC-elution patterns of extracts of feruloylated glucuronoarabinoxylans from wheat bran before (- - -) and after purification (——) by acetone precipitation. A: WE; B: 200SE[5]; C: 0.02CE_4; D: 0.05BE_2.

Molecular weight distribution

The molecular weight distributions of the extracts before and after purification are shown in Figure 2. It is clear that purification resulted in a loss of material eluting at retention times of 35 minutes and further. This is most clear for $0.05BE_2$ (Figure 2D). From the purification of the steam extract (Figure 2B) it is also obvious that the relative amount of material of low hydrodynamic volume decreased. Both alkaline extracts contained relatively more material of high hydrodynamic volume than the water and steam extracts. After purification the molecular weight distributions of the former two extracts narrowed, which was not observed for the water and steam extracts.

Cross-linking of extracts

Crude extracts. The relative viscosity of all crude extracts was not affected in the concentration range of 5 to 15 mg/ml by addition of hydrogen peroxide and peroxidase. As was mentioned before this may be caused by the presence of phenolic compounds that scavenge radicals. However, inhibition of the enzyme by phenolic compounds may also have occurred. In case of ammonium persulphate as cross-linking agent, an increase in viscosity was observed for both alkaline extracts (results not shown). The production of sulphate ion radicals from persulphate therefore appears not to be limited by the aforementioned phenolics. When the viscosity increase of the alkaline extracts of corresponding concentration were compared, it appeared to be highest for the barium hydroxide extract for the whole range of concentrations tested. As the barium hydroxide extract had a slightly lower glucuronoarabinoxylan and ferulic acid content compared with the calcium hydroxide extract, the arabinoxylan and ferulic acid content appear not to be the only crucial factors in cross-linking. For wheat flour arabinoxylans intrinsic viscosity (Ciacco and D'Appolonia, 1982*a,b*; Izydorczyk and Biliaderis, 1992*a,b*) and Ara/Xyl-ratio (Izydorczyk and Biliaderis, 1992*a*) were also reported to have an effect on cross-linking. From Figure 2C and D it can be observed that the molecular weight distribution of the extracts differed only in the low molecular weight region, which is not expected to have an effect on their intrinsic viscosities. From Table 1 it is clear that the Ara/Xyl-ratio of the barium hydroxide extract was lower than that of calcium hydroxide. A high degree of substitution of arabinoxylans is reported to result in a limited flexibility of the polymer (Andrewartha *et al.*, 1979). Most likely this causes a decreased accessibility of the ferulic acid residues, which consequently may diminish the number of cross-links formed. So the slightly higher amount of ferulic acid in the calcium hydroxide extract did not participate as effectively in cross-linking as in the barium hydroxide extract.

Purified extracts. After purification, all extracts showed increased viscosities upon addition of hydrogen peroxide and peroxidase. In Figure 3 the relative viscosities of the extracts before and after addition of the reagents are presented. From this figure it is clear that for

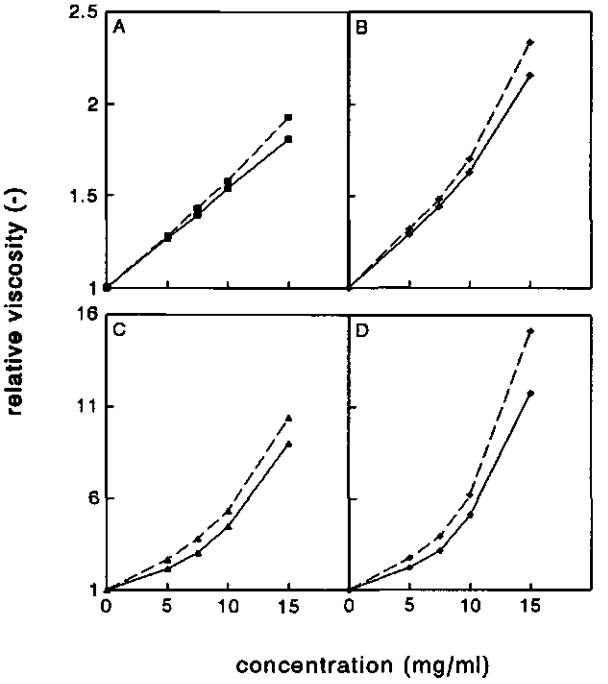

Figure 3: Viscosities of purified extracts of feruloylated glucuronoarabinoxylans from wheat bran at different concentrations before (——) and after (- - -) addition of hydrogen peroxide and peroxidase, expressed relative to the viscosity of the solvent. A: WE·P; B: 200SE[5]·P; C: 0.02CE_4·P; D: 0.05BE_2·P.

the water and steam extracts the low viscosities only increase slightly. This is most probably attributable primarily to the low purity of the extracts (Table 3) and the relative low content of high molecular weight material (Figure 2). Figure 3 also shows that the increase in viscosity is generally larger at higher extract concentration, with the exception of the calcium hydroxide extracted material.

By determination of the ferulic acid recovery after cross-linking it was investigated whether the amount of monomeric esterified ferulic acid residues consumed by the reaction was correlated with viscosity increase. No correlation was observed (results not shown), which may indicate that not all dimers formed contribute to the viscosity increase to a similar extent or that not all ferulic acid residues consumed result in formation of dimers. As viscosities were only measured at one point in time after addition of reagents, it is not known whether oxidative degradation of arabinoxylan, which can take place competitively with the

cross-linking reaction and which is caused by hydroxyl radicals produced from hydrogen peroxide (Ciacco and D'Appolonia, 1982*a*), influenced the viscosities of the extracts also.

Cross-linking of the purified extracts by addition of ammonium persulphate resulted in no increased viscosities for the water and steam extracts at all concentrations, whereas the dilute alkali extracts formed gels over the whole range of concentrations tested. The flow time of these gels could not be measured in a capillary viscometer and it was not attempted to characterize possible differences originating from the differences in concentration or composition of the extracts by any other means.

However, it can be stated that the cross-linking ability of feruloylated glucuronoarabinoxylans of wheat bran is dependent on their molecular weight distribution, Ara/Xyl-ratio, purity and type of cross-linking reagent used. Determination of types and amounts of ferulic acid dimers formed, as was recently reported by Ralph and co-workers (1994), most likely leads to a better understanding of the mechanism of coupling and is currently under investigation.

Conclusions

Extraction of feruloylated glucuronoarabinoxylans from wheat bran appeared to be most successful by dilute alkali extraction, in relation to their viscosity behaviour upon oxidative cross-linking. Extracts obtained by water and steam extraction had a lower glucuronoarabinoxylan content, contained relatively less high molecular weight material and showed hardly any increase in viscosity after addition of cross-linking reagents, when compared to the dilute alkali extracts. During alkali extraction the concentration of hydroxyl ions, the temperature and time of extraction appeared to be of major importance in the recovery of ferulic acid esterified to the glucuronoarabinoxylans. Purification of the extracts by acetone precipitation resulted in a higher increase of the viscosity upon cross-linking compared with the crude extracts. Substantial differences were found in the increase of viscosity when hydrogen peroxide and peroxidase was compared with ammonium persulphate as cross-linking reagent. Apart from the purity of the extract and the type of cross-linking reagent, the Ara/Xyl-ratio was also shown to have an effect on the increase of viscosity. A high Ara/Xyl-ratio resulted in a smaller viscosity increase.

Acknowledgement

The authors thank Dr. J. Puls of the Institute of Wood Chemistry (Hamburg, Germany) for giving the opportunity and assistance with the steam explosion treatments, and Dr. J.H.G.M. Mutsaers (Royal Gist-brocades, Delft, The Netherlands) for her stimulating comments. This research was carried out with the financial support of the Dutch Innovation Oriented Programme Carbohydrates (IOP-k) and Royal Gist-brocades (Delft, The Netherlands).

CHAPTER 6

Studies on the oxidative cross-linking of feruloylated arabinoxylans from wheat flour and wheat bran

This chapter will be submitted for publication to *Carbohydrate Polymers* by the authors M.E.F. Schooneveld-Bergmans, M.J.W. Dignum, J.H. Grabber, G. Beldman, A.G.J. Voragen.

Abstract

Feruloylated arabinoxylans isolated from wheat flour and wheat bran were compared in their cross-linking behaviour with respect to viscosity properties and cross-linking products formed when various oxidative agents were applied to dilute solutions. For each oxidative agent optimal conditions, resulting in relatively rapid viscosity increase without subsequent decline, were investigated. In case of hydrogen peroxide - peroxidase, similar conditions were found for both types of arabinoxylans. Yet, wheat bran arabinoxylans gave a larger viscosity increase upon cross-linking than those of wheat flour starting from an arabinoxylan concentration of 3.5 mg/ml. The distribution of coupling products was approximately 5.5:3:1:1 for 8-5, 8-O-4, 8-8 and 5-5, respectively, in both cases. When glucose - glucoseoxidase - peroxidase or ammonium persulphate were used, differences in concentration of reagent needed to induce cross-linking were observed. Additionally, the maximum viscosity increase varied for both extracts. However, the distributions of dimers formed by cross-linking were similar to that in case of peroxide - peroxidase. The presence of lignin-fragments in the bran extract was assumed to cause the lower observed ferulic acid recovery after cross-linking when compared to the flour extract. Cross-linking of mixtures of flour arabinoxylan and feruloylated pectin, using hydrogen peroxide - peroxidase, resulted in a synergistic effect on viscosity, which was at a maximum when mixed in a 1:1 ratio. Both polysaccharides were shown to participate in cross-linking.

Introduction

Oxidative cross-linking of water-extractable arabinoxylans of wheat and to a lesser extent of rye flour as effected by hydrogen peroxide - peroxidase, has been investigated extensively over the past years (Fausch *et al.*, 1963; Geissmann and Neukom, 1971; Hoseney and Faubion, 1981; Ciacco and D'Appolonia, 1982*a*; Izydorczyk *et al.*, 1990; Moore *et al.*, 1990; Vinkx *et al.*, 1991; Girhammar and Nair, 1992). Ferulic acid, linked to arabinose branches of the xylan, was generally recognized to play a crucial role (Fausch *et al.*, 1963; Geissmann and Neukom, 1971). However, contradictions about the linkage position in the coupling product, being at the 5- or 8-position of ferulic acid (Hoseney and Faubion, 1981; Moore *et al.*, 1990; Vinkx *et al.*, 1991), and about possible participation of protein (Fausch *et al.*, 1963; Morita *et al.*, 1974), through cysteine (Hoseney and Faubion, 1981; Vinkx *et al.*, 1991) and tyrosine (Neukom and Markwalder, 1978) have been awaiting clarification ever since. Only lately direct analysis of ester-linked dehydrodiferulic acid isomers having various linkage positions has been developed (Ralph *et al.*, 1994). Consequently, it was shown that the 5-5 coupled dimer, which so far was the only type actually known to be produced during coupling (Geissmann and Neukom, 1971) and to exist naturally in grasses (Markwalder and Neukom, 1976; Ishii, 1991), was present in relatively low amount in grass cell walls. Dimers in which the 8-position was involved prevailed (Ralph *et al.*, 1994; Grabber *et al.*, 1995).

Since extraction of feruloylated glucuronoarabinoxylans from wheat bran has been accomplished (Schooneveld-Bergmans *et al.*, submitted *b*), comparison of their cross-linking behaviour with that of wheat flour arabinoxylans appears to be of interest. In that respect,

not only viscosity properties, but also cross-linking products formed when different types of oxidative agents were used, are of importance. In order to investigate possibilities of cross-linking arabinoxylan to other phenolic materials, studies were also performed on arabinoxylans with the addition of tyrosine or feruloylated pectin.

Experimental

Materials

Feruloylated arabinoxylan was isolated from commercial wheat flour by cold water extraction according to a procedure previously described for wheat bran (Schooneveld-Bergmans *et al.*, submitted *b*). The extract was concentrated by vacuum evaporation, stored at 4°C with addition of 0.01% sodium azide and designated WF.WE. Extraction and purification of feruloylated glucuronoarabinoxylan from cell wall material (WUS) of wheat bran by saturated calcium hydroxide solution at 20°C and 70% acetone precipitation was performed as described before (Schooneveld-Bergmans *et al.*, submitted *b*). The extract was designated WB.CE, which corresponds to $0.02CE_4$·P of the previous paper (Schooneveld-Bergmans *et al.*, submitted *b*). Feruloylated beet pectin (Lot no. X-6938/61-735-0, Copenhagen Pectin Factory, Lille Skensved, Denmark) was dissolved in distilled water and dialysed before use.

Cross-linking

Hydrogen peroxide - peroxidase. Samples (4 mg/ml) were incubated in 0.1 M sodium phosphate buffer of pH 6, to which 5 µg horse-radish peroxidase (Type I, Art. P-8125, Sigma, St. Louis, CA, USA) and 1.6 µmoles of hydrogen peroxide per ml of arabinoxylan solution were added. Quantities of 50 µg horse-radish peroxidase and 0.2 to 2 µmoles of hydrogen peroxide per ml of solution were also tested. Wheat flour arabinoxylan was also incubated with the addition of 1.6 or 3.2 µmoles of tyrosine per ml of arabinoxylan solution. Additionally, both arabinoxylan samples were incubated with beet pectin solution of equal concentration, when mixed in different ratios. The sample concentrations were 4 or 7 mg/ml. All incubations were performed at 25°C and were monitored by viscometry for periods up to 24 h.

Glucose - glucoseoxidase - peroxidase. Samples were incubated in a similar way as described above, with the addition of 20 µg glucose, 5 µg glucoseoxidase (Type II from *Aspergillus niger*, Art. G-6125, Sigma, St. Louis, CA, USA) and 5 µg horse-radish peroxidase per ml arabinoxylan solution. Incubations were also performed with 5 and 50 µg glucose and 50 µg of glucoseoxidase and peroxidase per ml solution.

Ammonium persulphate. Incubation of samples with ammonium persulphate at concentrations of 0.01 M or 0.03 M was performed in phosphate buffer of pH 6 and in distilled water. Concentration of samples, temperature and time-scale were similar as described above.

Viscometry. Viscosities of arabinoxylan solutions or its mixtures with tyrosine or beet pectin were measured before and after addition of reagents with Ubbelohde capillary viscometers (Schott, Mainz, Germany) with capillary diameters ranging from 0.53 to 0.95 mm, submerged in a thermostatically controlled waterbath at 25°C.

Analytical methods

Neutral sugar composition, uronic acid content and *protein content* of the extracts were determined as described previously (Bergmans *et al.*, 1996).

Ferulic acid and ferulate dehydrodimers. Blanks and dialysed cross-linked samples were treated with 2 M sodium hydroxide (4 ml) for 20 h at room temperature in nitrogen atmosphere. 2-Hydroxycinnamic acid was added as internal standard. The samples were acidified to pH 1 with 0.7 ml of 12 M hydrochloric acid and extracted twice with 3 ml diethyl ether. After drying, the extracts were silylated with pyridine (5 µl) and *N,O*-bis(trimethylsilyl)-trifluoroacetamide (30 µl) for 20 min at 60°C. Trimethylsilylated phenolic acid derivatives were separated on a 30 m × 0.25 mm i.d. DB1 column in a Perkin Elmer 8500 gas chromatograph. The temperature programme was set at: 210°C for 1 min, 210 to 230°C at 3°C/min, 230 to 310°C at 10°C/min and 310°C for 9.5 min. The injector and detector were set at 310°C and helium was used as carrier gas. Amounts of individual esterified phenolic acids were calculated using response factors as described by Ralph *et al.* (1994).

Size-exclusion chromatography. Solutions of beet pectin and wheat flour arabinoxylan in 0.1 M phosphate buffer of pH 6, each with a concentration of 7 mg/ml, were mixed in a ratio of 1:1. Mixtures (7 ml) were applied to a column (95 × 2.5 cm) of Sephacryl S500 (fractionation range for dextrans: M_w $4 \cdot 10^4$ to $2 \cdot 10^7$; Pharmacia, Uppsala, Sweden) as such, or after incubation with hydrogen peroxide and peroxidase. The column was eluted with 0.05 M sodium acetate buffer of pH 5 at a flow rate of 2.5 ml/min. Fractions of 2.5 ml were collected and assayed by automated methods for total neutral sugar (Tollier and Robin, 1979) and uronic acid content (Thibault, 1979), using arabinose and galacturonic acid as standards.

High-performance size-exclusion chromatography (HPSEC). Molecular weight distributions were determined by HPSEC using three Bio-Gel TSK columns in series as described elsewhere (Bergmans *et al.*, 1996). The weight average molecular weight and intrinsic viscosity were determined as described by Schooneveld-Bergmans *et al.* (submitted *a*).

Results and Discussion

Characterization of extracts

Yield, composition and some physicochemical characteristics of feruloylated arabinoxylan extracts of wheat flour and wheat bran are presented in Table 1. From this table it is clear

Table 1: Yield, composition, molecular weight and intrinsic viscosity of isolated feruloylated arabinoxylans from wheat flour (WF.WE) and wheat bran (WB.CE).

	WF.WE	WB.CE
Yield[a]	0.5	1.2
Composition[b]		
total sugar	94.2	85.8
protein	0.5	12.6
ferulic acid	0.2	0.5
Molar sugar composition[c]		
Ara	36.3	40.1
Xyl	44.0	48.9
Man	1.9	1.8
Gal	16.0	2.7
Glc	1.1	2.7
UA	0.6	3.8
Molecular weight		
$M_{w;LS}$[d]	243.5	191.1
M_w/M_n[e]	2.5	1.8
Viscosity		
$[\eta]$[f]	2.3	1.6

[a] expressed as weight percentage (dm) of flour or bran
[b] expressed as weight percentage (dm) of each extract.
[c] expressed as percentage (mole per 100 mole).
[d] $M_{w;LS}$ = weight average molecular weight as calculated from light-scattering data; expressed in kDa.
[e] M_n = number average molecular weight.
[f] $[\eta]$ = intrinsic viscosity (dl/g).

that the yield of the bran extract, based on dry matter, was over two times higher than that of flour. However, the total sugar content of the flour extract was slightly higher than that of bran. From the molar sugar composition it can be observed that the bran extract was of high purity, because it contained only minor amounts of mannose, galactose and glucose. In the flour extract the galactose content was relatively high. This was the result of coextraction of arabinogalactans as is known to occur during water extraction of wheat flour (Fincher and Stone, 1974). The yield calculated as the amount of arabinoxylan recovered from total dry matter is therefore more indicative for the efficiency of the extractions. The total feruloylated glucuronoarabinoxylan content of the bran extract, calculated as the sum of arabinose, xylose, uronic acid and ferulic acid, amounted to 79.0% (w/w). For wheat flour arabinoxylan correction of the arabinose content was necessary, because of coextracted arabinogalactan. This component had an Ara/Gal-ratio of 0.67 when obtained from wheat flour by the same procedure as was used here (Fincher and Stone, 1974). As the composition of our extract

was in good agreement with their crude extract, the content of feruloylated arabinoxylan can therefore be estimated by the sum of arabinose, xylose, uronic acid and ferulic acid minus 0.67 × galactose. This resulted in an arabinoxylan content of 61.4% (w/w). From this it followed that the arabinoxylan yields based on total dry matter were 0.3% and 0.9% for flour and bran, respectively. For both extracts the Ara/Xyl-ratio was 0.82. However, after correction for presence of arabinogalactan it was 0.53 for the flour extract, which was in good agreement with previous results (Izydorczyk *et al.*, 1990; Hoffmann *et al.*, 1991; Gruppen *et al.*, 1992*b*). The protein content of our extract was low compared with extracts obtained without hot 80% ethanol treatment (Ciacco and D'Appolonia, 1982*a*; Izydorczyk *et al.*, 1990; Girhammar and Nair, 1992; Cleemput *et al.*, 1993). The protein and ferulic acid content of the bran extract were higher than that of flour. The latter observation corresponded with the higher ferulic acid content of bran compared with flour (Pussayanawin and Wetzel, 1987). The weight average molecular weight of the flour extract was higher than that of bran. This was in agreement with their molecular weight distributions observed by HPSEC-analysis (results not shown) and those observed for the alkali-extractable arabinoxylans from flour and bran (Gruppen *et al.*, 1991). The higher polydispersity index of the present flour extract was very likely caused by the coextracted arabinogalactans, which were shown to be of low molecular weight (Fincher and Stone, 1974). As it was demonstrated that arabinogalactans have very low intrinsic viscosities (Izydorczyk *et al.*, 1991) and do not participate nor affect oxidative cross-linking of arabinoxylans (Izydorczyk *et al.*, 1990), it was decided not to purify the wheat flour extract further for studies on cross-linking behaviour. The intrinsic viscosity of our extract corresponded well to that determined by Izydorzyk *et al.* (1991), and was higher than for the bran extract, which was in agreement with the higher molecular weight.

Cross-linking with different oxidative agents

Hydrogen peroxide - peroxidase. The most extensively investigated cross-linking agent is hydrogen peroxide in combination with peroxidase. The amounts of enzyme and peroxide used varied widely. In early reports addition of 20 µg peroxidase and 10 µmoles hydrogen peroxide per ml arabinoxylan solution of approximately 3 mg/ml, and even larger quantities of reagents, have been reported (Geissmann and Neukom, 1973; Hoseney and Faubion, 1981; Ciacco and D'Appolonia, 1982*a,b*). In more recent reports approximately 0.5 µg peroxidase and 0.05 to 0.2 µmoles hydrogen peroxide per ml arabinoxylan solution of 3 to 20 mg/ml have been used (Izydorczyk *et al.*, 1990 and 1991; Vinkx *et al.*, 1991). These latter concentrations resulted in a slower viscosity increase, but gave no degradation upon longer incubations, as was reported for the high quantities of reagents (Ciacco and D'Appolonia, 1982*a*). By using different amounts of peroxide and peroxidase, optimal conditions were investigated for our flour and bran extracts (see experimental).

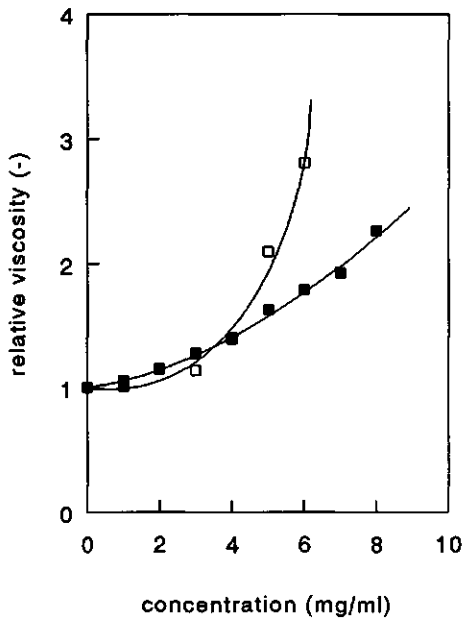

Figure 1: Viscosities of wheat flour (■) and wheat bran (□) extract at varying dry matter concentrations 4 min after addition of hydrogen peroxide - peroxidase, expressed relative to the viscosity before addition of reagents.

A maximum viscosity increase after 2 min was observed for both extracts, when 5 µg peroxidase and 1.6 µmole hydrogen peroxide per ml of arabinoxylan solution of 4 mg/ml were used. The viscosity increase was 40% for both extracts and it lasted for at least 10 h.

In Figure 1 the effect of extract concentration on the increase of viscosity, 4 min after addition of peroxidase and hydrogen peroxide is shown. It is clear from this figure that the relative viscosity increase for the bran extract was larger than for that of flour, starting from an extract concentration of 4 mg/ml. As the arabinoxylan contents of the extracts were different, being 61.4% and 79.0% for flour and bran, respectively, it can be calculated from the figure that the larger viscosity increase for bran compared with flour started from 3.5 mg/ml based on arabinoxylan concentration. The initially smaller viscosity increase for bran arabinoxylan is most probably caused by its lower molecular weight compared with that of flour. A similar explanation was given by Vinkx *et al.* (1991) for the larger viscosity increase of rye flour arabinoxylan upon cross-linking when compared with those of wheat flour. However, differences in composition and structure of arabinoxylans may also have influenced their viscosity behaviour. The lower Ara/Xyl-ratio of flour arabinoxylans is expected to give a

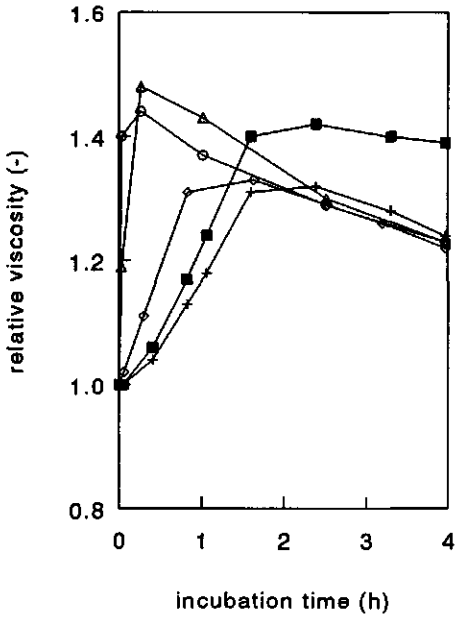

Figure 2: Viscosities of wheat flour extract as function of time after addition of varying amounts of glucose - glucoseoxidase - peroxidase, expressed relative to the extract viscosity before addition of reagents. ○ 50-50-50; △ 20-50-50; ◇ 5-50-50; + 20-5-50; ■ 20-5-5; in which the numbers give the amounts of glucose - glucoseoxidase - peroxidase, respectively, expressed as µg per ml of extract.

higher flexibility of the polymers (Andrewartha *et al.*, 1979), and may have resulted in a better accessibility of the available ferulic acid residues. At higher concentrations the stiffness of the highly substituted bran glucuronoarabinoxylans may have resulted in formation of a stronger, more closely packed, network. Additionally, the bran arabinoxylan had a higher ferulic acid content, which may also give a high cross-link density.

Glucose - glucoseoxidase - peroxidase. A similar oxidative system, which has not been reported before in gelation studies of feruloylated arabinoxylans, consists of glucose, glucoseoxidase and peroxidase. The former two reagents generate hydrogen peroxide, which is converted into radicals by peroxidase, giving rise to cross-linking of ferulic acid residues. In Figure 2 relative viscosities of the flour extract at a concentration of 4 mg/ml after addition of different amounts of reagents are shown. From this figure it is clear that the higher the amounts of reagents used, the quicker the viscosity increase as well as its subsequent decrease. When the amount of glucose was limited, being 5 µg per ml solution, the maximum

viscosity was smaller than when 20 or 50 µg glucose were added. A decreased maximum viscosity was also observed when the amount of glucoseoxidase was limited, being 5 µg per ml solution. The best conditions for viscosity increase and its subsequent maintenance were addition of 20 µg glucose and 5 µg of each of the enzymes per ml arabinoxylan solution. The viscosity increase then observed corresponded to that of the hydrogen peroxide - peroxidase cross-linking. These conditions gave almost no viscosity increase with bran glucurono-arabinoxylan. Only when 50 µg glucoseoxidase was used per ml solution, the increase was similar to that of flour (results not shown). This difference may have been attributable to presence of inhibitors of glucoseoxidase or radical scavengers in the bran extract. The effects of these compounds appeared to be surmountable when an excess of glucoseoxidase was used. No further research was directed towards the nature of the compounds, although it is suspected that lignin-like compounds may have acted as such in both cases.

Ammonium persulphate. Ammonium persulphate has been used before as cross-linking agent for pectin (Thibault and Rombouts, 1986) and wheat flour arabinoxylan (Izydorczyk *et al.*, 1990). In Figure 3 relative viscosities of flour and bran extract in different solutions are shown. Use of 0.01 M ammonium persulphate in a flour extract of 4 mg/ml in water or phosphate buffer gave no viscosity increase. Phosphate has been shown to inhibit the cross-linking of pectin by ammonium persulphate (Thibault and Rombouts, 1986). However, the absence of a viscosity increase in water was in contradiction with results of Izydorczyk *et al.* (1990). This was probably caused by the low concentrations of ammonium persulphate and arabinoxylan used in the present study. When 0.01% sodium azide was present in the solution slow development of a network was shown. This is most likely attributable to propagation of the radical reaction by the azide ion. It has been shown that the azide ion reacts very rapidly with hydroxyl radicals to produce an azide radical, which can oxidize phenols and phenolate ions rapidly (Alfassi and Schuler, 1985). At a concentration of 0.03 M ammonium persulphate in water containing 0.01% sodium azide the viscosity increase was quicker and followed by a decrease. A similar effect of persulphate concentration on cross-linking behaviour of pectins was shown by Thibault and Rombouts (1986), who did not add sodium azide to their pectin. Probably azide is not essential for a viscosity increase of the flour extract, as Izydorczyk *et al.* (1990) did not use it either. It is very likely that cross-linking of the present flour extract can also be induced without presence of sodium azide, but with higher concentration of ammonium persulphate, which was not investigated. The bran extract gave a slight viscosity increase in phosphate buffer (Figure 3) and resulted in gel formation in 16 h when water or water containing azide was used (results not shown). The higher arabinoxylan and ferulic acid content, the lower flexibility, or the presence of other phenolic compounds may have caused the stronger effect on viscosity when compared with the flour extract. The latter suggestion becomes more clear from analysis of cross-linking products in the next section.

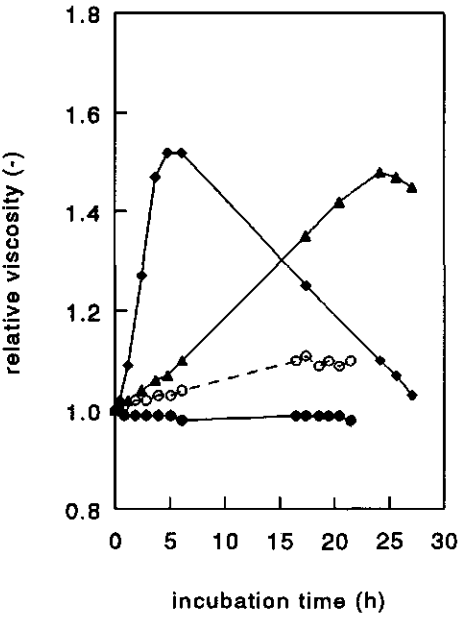

Figure 3: Viscosities of wheat flour or wheat bran extract as a function of time after addition of ammonium persulphate, expressed relative to the viscosity of the extract before addition of reagent. ● wheat flour extract in water or phosphate buffer containing 0.01 M ammonium persulphate; ▲ wheat flour extract in water containing 0.01% sodium azide and 0.01 M ammonium persulphate; ♦ wheat flour extract in water containing 0.01% sodium azide and 0.03 M ammonium persulphate; ○ wheat bran extract in phosphate buffer containing 0.01 M ammonium persulphate.

Cross-linking products

The dehydrodiferulic acids occurring most frequently as cross-linking products in plant material are presented in Figure 4. The notation of the various cross-linking products indicates the linkage position, based on the numbering of the carbon atoms in the ferulic acid monomer. It should be noted that other isomers of 8-5 and 8-8 coupled dimers may be present also (Ralph *et al.*, 1994).

In Figure 5 the recovery and distribution of ferulic acid residues amongst the various dimers and remaining monomer, calculated as percentage of the number of µmoles of ferulate per gram of untreated material, is presented for the untreated and differently cross-linked flour and bran extracts. It is clear from Figure 5A that the flour extract only contained monomeric ferulic acid. After cross-linking using hydrogen peroxide - peroxidase or glucose - glucoseoxidase - peroxidase approximately 80% of total ferulate was recovered, 85% of

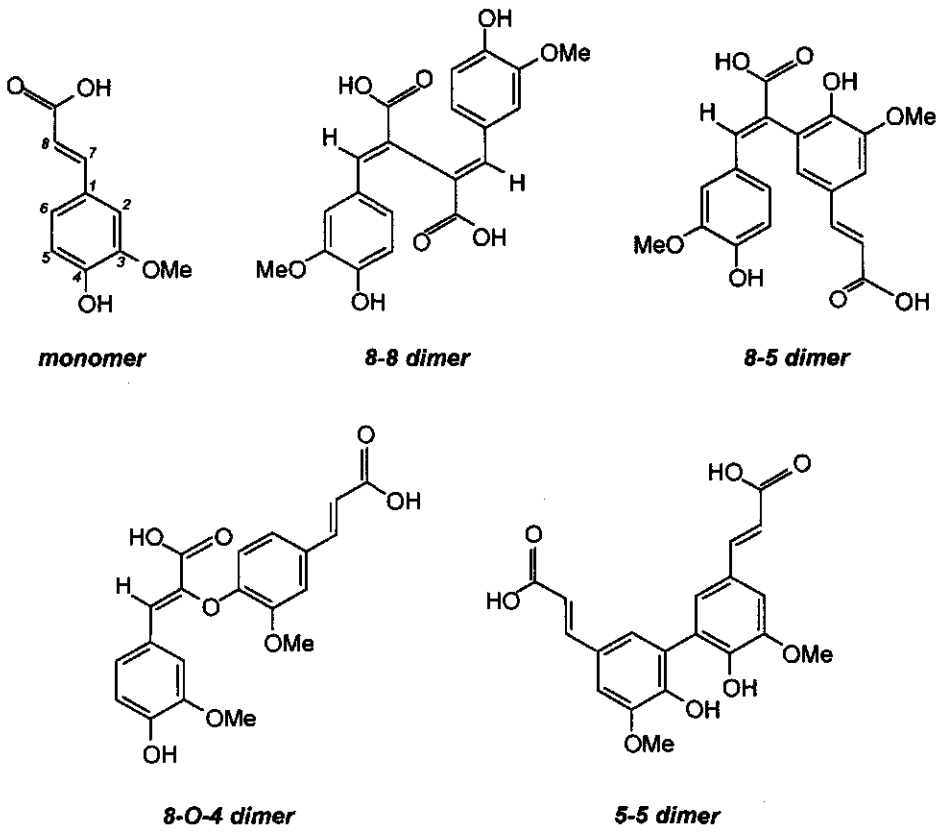

Figure 4: Ferulic acid monomer and the most frequent dehydrodiferulic acid residues in plant material, as designated by the type of coupling.

which was present as dimer. From the proportions of the differently linked dimers present in the cross-linked extracts it is obvious that the 5-5 coupled dimer, which has long been assumed to be the only coupling product, was not prevalent. Up to 10% of the dimers was coupled by such linkage or by an 8-8 linkage, whereas approximately 55% and 30% was linked by 8-5 and 8-O-4 linkage, respectively. This distribution of dimers was quite similar to results on arabinoxylans cross-linked *in vivo* in suspension cultured maize cell walls (Grabber *et al.*, 1995) and on isolated beet pectins cross-linked *in vitro* using hydrogen peroxide - peroxidase (Oosterveld *et al.*, in press). The distribution of dimers in the coupled extracts in the present study appeared almost not to be affected by the direct addition of hydrogen peroxide or its production from glucose by glucoseoxidase in the solution.

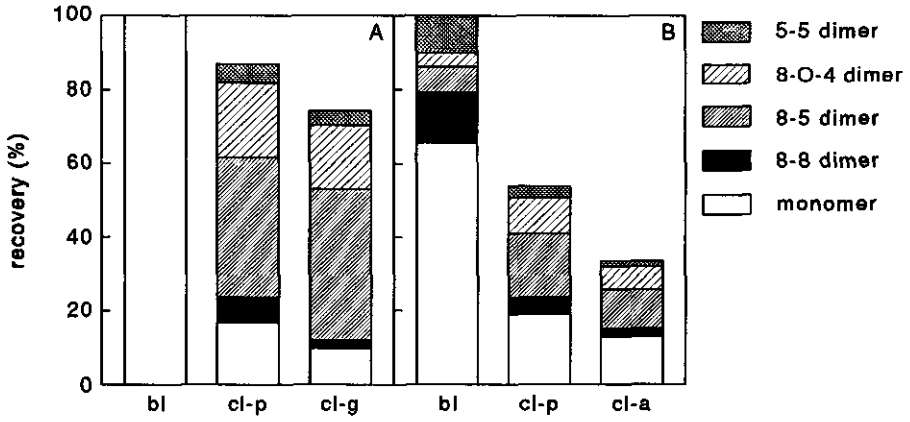

Figure 5: Ferulate distribution and recovery before and after incubation with oxidative agents, expressed as percentage of the µmoles of ferulate present per gram of untreated extract of wheat flour (A) and wheat bran (B). Blank: untreated; cl-p: cross-linked by hydrogen peroxide - peroxidase; cl-g: cross-linked by glucose - glucose-oxidase - peroxidase; cl-a: cross-linked by ammonium persulphate.

Figure 5B shows that the bran extract contained approximately 45% of ferulate as dimers. The distribution of the dimers was 40, 20, 10 and 30% for 8-8, 8-5, 8-O-4 and 5-5, respectively. In the whole bran this distribution was about 15, 25, 45 and 15%, and in the organic solvent soluble material of the purification treatment it was approximately 15, 35, 35 and 15% for 8-8, 8-5, 8-O-4 and 5-5, respectively (no further results shown). These varying distributions may indicate that 8-8 and 5-5 coupled dimers occur more frequently as cross-links between arabinoxylans, whereas 8-5 and 8-O-4 seem to be more frequent in lignaceous material. However, this needs further research, because possible differences in alkali sensitivity may have affected the recovery of the various dimers in the extract, as it was obtained by dilute alkali extraction, and this was not taken into account here. After cross-linking by hydrogen peroxide - peroxidase or ammonium persulphate the dimer content of the extract increased to 65% relative to the total amount of ferulate recovered as mono- or dimer. The dimer distribution was then similar to that mentioned above for the cross-linked

flour extract. However, the recovery of ferulate after cross-linking was low when compared to the flour extract. Particularly the extract cross-linked by ammonium persulphate gave a low recovery. This was even reduced when cross-linking was performed in the presence of sodium azide. The low recovery may have been caused by decomposition of the ferulate under these conditions, as oxidative degradation in side chains or the aromatic ring of phenolics can occur at high oxidant concentrations (Taylor and Battersby, 1967). Because of the observed stability of the viscosity using hydrogen peroxide - peroxidase for cross-linking the oxidant concentration was not expected to be too high, whereas the ferulate recovery was only 55%. This may have been caused by cross-linking of ferulate and its dimers to lignin-like fragments (Lam *et al.*, 1992*b,c*), which escape our analysis. This may have resulted also in phenolics of higher degree of polymerization, which may have been attached to lignin additionally by linkages that resist alkaline hydrolysis (Ralph *et al.*, 1992).

From these results it can be concluded that *in vitro* cross-linking of arabinoxylans results in production of dimers, of which the distribution is not affected by the type of cross-linking agent used or the original distribution of dimers in the sample. The 5-5 coupled dimer is only present in minority. It should be noted that the presence of other phenolic compounds very likely influences the recovery of ferulate as mono- or dimer.

Cross-linking with addition of other phenolic materials

Tyrosine. In order to get more insight in a role of protein, particularly of tyrosine, in the cross-linking of arabinoxylans, the flour extract was studied for its viscosity behaviour when tyrosine was added and hydrogen peroxide and peroxidase were used as oxidizing agents. Addition of tyrosine in equal molarity as hydrogen peroxide showed a quick viscosity increase with a maximum that was slightly less than in the extract without added tyrosine. After reaching the maximum a rapid decrease in viscosity was observed. When the concentration of tyrosine was doubled similar observations were made (results not shown), suggesting that concentration of tyrosine has no influence on the cross-linking. Analysis of cross-linking products formed showed that no ferulate was present as monomer or dimer in the cross-linked material. At a pH of 6, as used in this study, it has been shown that tyrosine can give di-, tri-, and even polymeric products of tyrosine residues after addition of peroxide and peroxidase (Fry, 1987). It may be hypothesized that tyrosine or oligomers produced from it by cross-linking, can be linked to ferulic acid residues of arabinoxylan, giving a viscosity increase. If the cross-linking products are composed of three or more phenolic compounds they can not be determined by the described procedure. On the other hand addition of tyrosine may also have resulted in high concentrations of radicals, which may have given decomposition of phenolics, as was already mentioned before. It is therefore still not clear whether protein participates in the cross-linking of arabinoxylans through this particular amino acid.

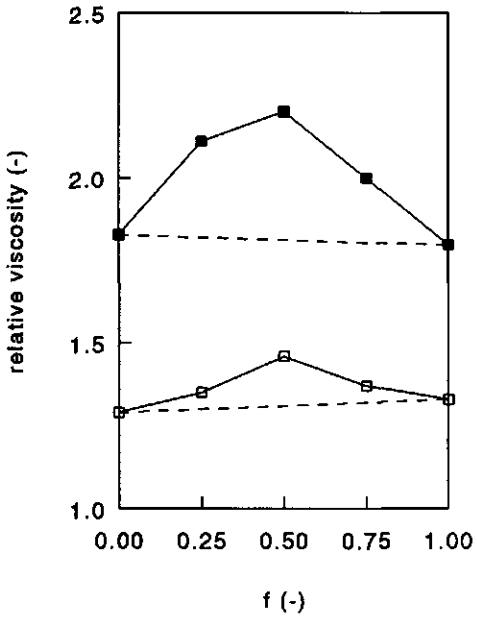

Figure 6: Viscosities of mixtures of feruloylated flour arabinoxylan and beet pectin with different proportions of arabinoxylan (f) at concentrations of 4 mg/ml (□) and 7 mg/ml (■), as determined 4 min after addition of hydrogen peroxide - peroxidase, expressed relative to the viscosity of the mixture before addition of reagent. —— observed viscosity; - - - expected viscosity.

Feruloylated pectin. Mixtures of flour arabinoxylan and beet pectin as well as bran extract and beet pectin were incubated with hydrogen peroxide - peroxidase. Solutions of equal concentrations were mixed in different ratios, all amounting to a similar total dry matter concentration. The viscosities were measured before, and 4 min after addition of reagents. For the flour extract a synergistic effect was observed, which reached a maximum when arabinoxylan and pectin were present in a 1:1 ratio. This is shown in Figure 6 for a total extract concentration of 4 and 7 mg/ml. This effect was less obvious in case of the bran extract, for which it was only investigated at a concentration of 4 mg/ml. In Figure 7 the elution pattern of a blank and cross-linked sample of flour arabinoxylan and beet pectin in a ratio of 1:1, with a concentration of 7 mg/ml, is shown. As beet pectin consisted predominantly of galacturonic acid, being 75% of the total sugar content of 70%, it can be observed from the neutral sugar and uronic acid distributions in Figure 7A that it eluted separately from the arabinoxylan and is of larger hydrodynamic volume than the flour arabinoxylan. The elution volumes of pectin

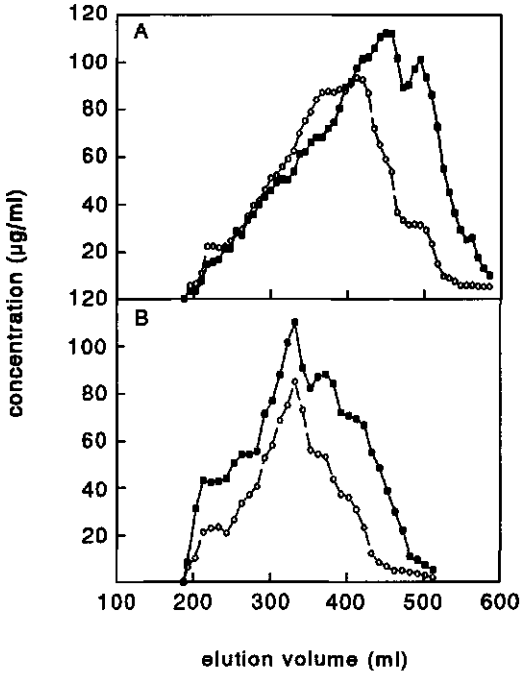

Figure 7: Gel permeation elution patterns of mixtures of equal amounts of feruloylated beet pectin and wheat flour arabinoxylan before (A) and after (B) incubation with hydrogen peroxide - peroxidase. ■ neutral sugar; ○ uronic acid.

and arabinoxylan were approximately 400 and 470 ml, respectively. Upon cross-linking an increase in hydrodynamic volume occurred, in addition to a redistribution of neutral sugar and uronic acid as is clear from Figure 7B. The cross-linked material eluted as one peak at an elution volume of approximately 330 ml. This, and the synergistic effect on viscosity, indicated that both polysaccharides participate in the cross-linking and even appear to cross-link to one another.

These results indicate that cross-linking of different feruloylated polysaccharides might give rise to interesting cross-linking behaviour, which needs further research to explore new uses.

Conclusions

Wheat flour and bran arabinoxylans were shown to behave differently upon cross-linking with various oxidative agents. When hydrogen peroxide and peroxidase were used the relative viscosity increase was larger for the bran extract starting from an arabinoxylan concentration of 3.5 mg/ml, whereas below this concentration the opposite was observed. The distribution of coupling products for both extracts was approximately 5.5:3:1:1 for 8-5, 8-O-4, 8-8 and 5-5 dimers, respectively. This showed that the 5-5 dimer was only of minor importance in *in vitro* cross-linking of isolated arabinoxylans. The distribution was similar when glucose - glucoseoxidase - peroxidase or ammonium persulphate were used as oxidative agents, but the ferulate recovery seemed to be affected by the presence of other phenolic material. The concentrations of reagents needed to induce cross-linking and the increase in viscosity were very different when glucose - glucoseoxidase - peroxidase or ammonium persulphate were used. Cross-linking of the flour extract in the presence of tyrosine resulted in viscosity increase without formation of detectable coupling products. Therefore, no definite answer on the role of tyrosine can be given at this time. In case of cross-linking of mixtures of feruloylated arabinoxylan and pectin with hydrogen peroxide - peroxidase it was shown that both polysaccharides participate in the cross-linking and that it results in a synergistic effect on viscosity.

Acknowledgement

The authors thank Dr. J.H.G.M. Mutsaers (Royal Gist-brocades, Delft, The Netherlands) for stimulating comments and suggestions. This research was financially supported by the Dutch Innovation Oriented Programme Carbohydrates (IOP-k) and Royal Gist-brocades (Delft, The Netherlands).

CHAPTER 7

Concluding remarks and summary

Research motives

The research described in this thesis was initiated by two main reasons. Firstly, current knowledge of structural and physicochemical properties of arabinoxylans, those of wheat flour in particular, raised the question whether these polysaccharides could be of use as additive in food industry. Secondly, decreasing prices of cereals on the European market brought up the issue of giving a surplus value to by-products of the cereal industry. The by-product of choice for this investigation was wheat bran, which is produced in large amounts by the cereal industry and has a relatively high glucuronoarabinoxylan content.

Accordingly, the aims of the investigations were to develop extraction methods for wheat bran glucuronoarabinoxylans, whether or not feruloylated, reveal their structural features, relate these to their measured physical properties and examine potentials for enzymatic modification to adapt physical properties to certain needs.

Extractability of glucuronoarabinoxylans

In order to obtain glucuronoarabinoxylans from cereals in acceptable purity, different methods of cell wall purification prior to extraction have been described in literature. The one chosen here for purification of cell wall material from wheat bran (Chapter 2) was based on a method developed for sorghum grain, in which fat, protein and starch were removed satisfactorily (Verbruggen *et al.*, 1993). For wheat bran this method was also successful and it was comparable to a method published before, in which protein was removed by pronase digestion (Carré and Brillouet, 1986). Purification of cell wall material from rye bran and barley spent grains by our procedure gave similar results as compared with wheat bran (unpublished results). Since the glucuronoarabinoxylan and ferulic acid content was highest for cell wall material of wheat bran, this material was also of highest interest for this investigation.

The subsequent extraction of glucuronoarabinoxylan from cell wall material needed to meet two prerequisites. On the one hand a high yield of pure glucuronoarabinoxylan was required, as this is advantageous for investigation of structural and physical properties and for future exploitation. On the other hand presence of ferulic acid esterified to the arabinoxylans was required, because this is expected to give new openings for application by making their oxidative cross-linking possible, by which means viscosity can be manipulated from liquid to gel. However, these conditions are in direct conflict with each other, because ester-linked phenolic acids have been shown to play a role at the interlinking of cell wall polymers (Chapter 1), which is assumed to result in the unextractability of glucuronoarabinoxylans. The two prerequisites were therefore investigated separately.

Firstly, extraction of large amounts of glucuronoarabinoxylan of high purity was investigated. Alkali solutions are known to be very efficient in the extraction of arabinoxylans from cell wall material, because the hydroxyl ions are believed to cause disruption of hydrogen bonds between cellulose and arabinoxylan, and hydrolysis of esters linked to the arabinoxylans. Lately, the role of phenolic esters in the cross-linking of cell wall polymers, involving arabinoxylans and lignin, becomes more and more clear (Lam *et al.*, 1992*b,c*; Ralph *et al.*, 1994 and 1995; Grabber *et al.*, 1995). Here, saturated barium hydroxide solution with addition of sodium borohydride was used to extract glucuronoarabinoxylans from wheat bran (Chapter 2). This extractant had been demonstrated to yield the majority of the arabinoxylans from wheat flour cell wall material, almost without coextraction of (1→3)(1→4)-β-glucans (Gruppen *et al.*, 1991). The latter will occur when sodium or potassium hydroxide are used. When the barium hydroxide extraction was applied to other cereal products, such as barley (Viëtor *et al.*, 1992), rye (Vinkx *et al.*, 1995), sorghum, maize and rice (Verbruggen, 1996) the yield was considerably lower. This was also observed for wheat bran (Chapter 2). The lower yields appeared to be related to a higher Ara/Xyl-ratio of the arabinoxylans, presence of uronic acid linked to the arabinoxylans or presence of lignin in the cell wall material. Probably these factors indicate a relatively high frequency of cross-linking between cell wall polymers, as a result of which extraction of arabinoxylans is hindered.

Different pretreatments of the wheat bran cell wall material to remove lignin or open up the cell wall structure failed to increase the yield (Chapter 2). However, a very efficient way of increasing the yield, with only a minor decrease in selectivity, was found in performing extraction at elevated temperature with barium hydroxide as extractant. With regard to selectivity this method was superior to the use of a higher concentration of alkali, such as 1 M potassium hydroxide at room temperature, whereas equal glucuronoarabinoxylan yields were obtained (Chapter 2). At the same time this temperature effect on the yield of arabinoxylans extracted by alkali was demonstrated for maize bran (Chanliaud *et al.*, 1995). Replacement of barium with calcium hydroxide caused a lower yield and slightly lower selectivity, which was attributed to its lower solubility compared with that of barium hydroxide. Apart from the concentration of bivalent cations, sodium borohydride also played a role in the selectivity. A previous study had shown a higher glucan content when the concentration of sodium borohydride in the extractant was decreased (Gruppen *et al.*, 1991). It was indicated in the present study on wheat bran that borate, which is produced from borohydride during extraction, may participate in determining the selectivity (Chapter 2). However, the applied procedure and the composition of extractant, in which concentration of hydroxyl ion, bivalent cation and borohydride, temperature and pH are mutually linked, makes complete understanding of its mechanism almost inconceivable.

Secondly, the extraction of feruloylated glucuronoarabinoxylans from wheat bran was investigated. Gel-forming arabinoxylans are commonly obtained by water extraction from wheat flour. For wheat bran this method was ineffective (Chapter 5), which also indicates that these polysaccharides are covalently bound to one another or to other cell wall polymers. Steam extraction is known to preserve phenolic esters but usually gives some degradation of arabinoxylan. Although conditions were adjusted to recover polymeric material, no gel-forming fractions were obtained, even though ferulic acid was present and removal of degradation products arising from the steaming treatment was attempted (Chapter 5). From the different methods investigated, dilute alkali extraction proved to be the only one yielding feruloylated glucuronoarabinoxylans which were able to gel. The selectivity of the extraction was again assured by use of calcium and barium hydroxide, in this case at concentrations of 0.02 M and 0.05 M, respectively. Only low yields were gained, as an increase of yield was only possible at the expense of a decrease in ferulic acid content. A role of ferulic acid in keeping arabinoxylans unextractable was demonstrated by applying elevated extraction temperatures. A high temperature had a detrimental effect on ferulic acid recovery, whereas it positively affected the arabinoxylan yield (Chapter 5). Extraction with 0.02 M calcium hydroxide for 2 hours at 70°C resulted in a glucuronoarabinoxylan yield of 17% and ferulic acid content of the extract of 0.01%. At room temperature the results were 3% and 0.51% for yield and ferulic acid content, respectively. Based on these results the appropriateness of the extraction conditions for feruloylated polysaccharides from pericarp tissues of cereals, being 0.1 to 0.6 M sodium or potassium hydroxide for 0.5 to 5 at 60 to 85°C, as described by Greenshields and Rees (1993), are questioned.

In an attempt to increase the yield without additional loss of ferulic acid wheat bran cell wall material was incubated with cellulolytic enzyme preparations after an autoclave treatment to open up the cell wall structure. Only minor degradation of cellulose was observed, which did not result in increased solubilization of wheat bran glucuronoarabinoxylans during subsequent dilute alkali extraction (unpublished results).

Structural aspects of wheat bran glucuronoarabinoxylans

Studies on the structural features of the glucuronoarabinoxylans from wheat bran were performed by investigation of the homogeneity of a barium hydroxide extract by means of fractionation using anion-exchange chromatography and graded ethanol precipitation. Subsequently, methylation analysis and degradability by enzymes with known mode of action were used to reveal the structures (Chapter 3). The isolated glucuronoarabinoxylans consisted primarily of a lowly substituted population with an Ara/Xyl-ratio of 0.2,

and a highly substituted population with an Ara/Xyl-ratio of 1.0. The lowly substituted population comprised 30% of the total extract and branching occurred predominantly through monosubstitution at the *O*-3 position of xylose residues with terminal arabinose. Some disubstitution with arabinose and monosubstitution at the *O*-2 position, probably with glucuronic acid, was also observed. The substituents were randomly distributed, and interrupted by stretches of up to six or more contiguously unsubstituted xylose residues, as was observed from enzymatic degradation studies (Chapter 3). The highly branched population comprised over 50% of the total extract and was very different from the lowly substituted fraction and the well characterized alkali-extractable wheat flour arabinoxylans (Gruppen, 1992). This was clear from the very high Ara/Xyl-ratio, but more importantly from the presence of relatively large amounts of terminal xylose and non-terminal arabinose. The occurrence of these residues has been observed before in arabinoxylans of pericarp tissues of cereals. In Table 1 the distribution of differently linked arabinose and xylose residues and the Ara/Xyl-ratio of several cereal products are summarized.

From this table it is clear that the arabinoxylans from wheat bran are very similar to those of rye bran, as both consist of a lowly and highly substituted population. Wheat and rye originate from the same subfamily (*Triticeae*), just as barley, whereas maize, rice and sorghum have different origins and are grown in subtropical and tropical regions. The arabinoxylans of the latter three do not consist of such differently substituted populations but appear to be very homogeneous. Wheat flour arabinoxylans consist of a continuum of populations with Ara/Xyl-ratios ranging from 0.4 to 0.7. Very similar structural features have been observed for barley flour arabinoxylans, which only deviated from those of wheat flour by a relatively higher content of 2,4-linked xylose (Viëtor *et al.*, 1992). With regard to the arabinose residues, being mainly terminal, the lowly substituted arabinoxylans of wheat bran and rye bran are quite similar to those of wheat and barley flour. This is not true for the highly substituted populations of these two by-products. Moreover, when these populations are compared with other highly substituted arabinoxylans, such as those of maize bran, rice bran and sorghum flour, dissimilarities in distribution of differently linked arabinose and xylose residues are observed. So, even when the Ara/Xyl-ratio or the percentage of substituted xylose residues are similar, the actual structure of the arabinoxylans may be very different. Most likely these differences are manifested in various types of oligomeric branches linked to the xylan backbone. In the past, dimeric branches, such as β-Xyl-(1→2)-α-Ara, β-Xyl-(1→3)-α-Ara and β-Gal-(1→5)-α-Ara have been tentatively characterized (Wilkie, 1979). Recently, α-Ara-(1→2)-α-Ara as dimeric branch substituted at the *O*-3 position of a xylose residue of sorghum glucuronoarabinoxylan has been characterized by NMR-analysis of enzymatic degradation products (Verbruggen, 1996). More complete understanding of the structures of highly substituted

Table 1: Distribution of differently linked arabinose and xylose residues, expressed as percentage of each sugar individually, percentage of xylose residues substituted and Ara/Xyl-ratio of alkali-extractable glucuronoarabinoxylans of various cereal products (for wheat and rye bran the lowly and highly substituted populations are indicated by L and H, respectively).

linkage position	wheat flour[a]	wheat bran[b] L	wheat bran[b] H	rye bran L[c]	rye bran H[d]	maize bran[e]	rice bran[f]	sorghum flour[g]
Arabinose								
terminal	94-97	89	64	100	78	55	78	88
2	2-3	1	5	0	3	23	6	5
3	tr	4	15	0	11	16	10	0
5	0-2	2	2	0	8	6	6	7
2,3	0	4	14	0	0	0	0	0
Xylose								
terminal	1-2	1	25	tr	16	23	6	2
4	60-70	83	19	88	35	17	24	28
3,4	12-22	13	21	12	23	40	55	45
2,4	1-3	1	4		4			8
2,3,4	7-26	2	31	0	22	20	15	17
% Xyl sub[h]	29-40	16	75	12	59	78	75	72
Ara/Xyl	0.4-0.7	0.2	1.0	0.1	0.7	0.6	1.0	1.0

[a] Gruppen *et al.*, 1992
[b] Schooneveld-Bergmans *et al.*, submitted *a*
[c] Hromadkova *et al.*, 1987
[d] Ebringerova *et al.*, 1990
[e] Chanliaud *et al.*, 1995
[f] Shibuya and Iwasaki, 1985
[g] Verbruggen *et al.*, 1995
[h] percentage of substituted xylose residues; calculated from (2,4- + 3,4- + 2,3,4-Xyl)/(total Xyl - terminal Xyl) × 100%

arabinoxylans is hindered by their low enzymatic degradability (Verbruggen, 1996; Chapter 3). For wheat bran glucuronoarabinoxylan it was therefore only possible to speculate on the structure by distributing the differently linked residues based on the current knowledge of oligomeric branches (Chapter 3). However, not all substituents could be accommodated to the backbone, which was most probably caused by a lack of knowledge on the placement of the unusual 2,3-linked arabinose in this polysaccharide. Characterization of a degradation product containing such an arabinose residue is necessary to further reveal the structure of this polysaccharide. With that, availability of enzymes or combinations thereof, that can degrade the polysaccharides to oligomers is

inevitable. Arabinofuranosidases that are able to hydrolyse arabinose from doubly substituted xylose, or xylosidases that are able to hydrolyse terminal xylose from oligomeric branches will be of great value. During these studies such enzymes were not at our disposal. Yet, the substrate to search for them is available.

Physical aspects of wheat bran glucuronoarabinoxylans

The very diverse structural features of the main populations of glucuronoarabinoxylans extracted from wheat bran affected the physical properties largely. Arabinoxylans are known to give viscosity to solutions, which is generally assumed to be the result of their extended rod-like conformation. It is also known that lowly substituted arabinoxylans easily aggregate. This latter phenomenon was observed for the lowly substituted fraction of the wheat bran extract. Aggregation was indicated to occur partly in a parallel mode, because the increase in molecular weight upon prolonged storage exceeded the increase of the radius of the aggregate by far. As a result of extensive aggregation these arabinoxylans hardly gave any viscosity to solutions (Chapter 3 and 4). The structural resemblance between these lowly substituted arabinoxylans and the lowly substituted locust bean galactomannan was not reflected in equal intrinsic viscosities or in equal interaction with other polysaccharides. This latter aspect is known to occur for locust bean galactomannan in combination with κ-carrageenan or xanthan. Small differences in structure, such as location and distribution of substituents, conformation and extent of aggregation most probably caused this. An interesting possibility to reveal the importance of these aspects would be modification of an arabinoxylan resulting in a more similar distribution of the substituents as observed for locust bean galactomannan. The lowly substituted population was not considered in this respect, because redistribution of substituents is hardly possible. The highly substituted glucuronoarabinoxylan appeared to be structurally too complex for modification. Available arabinose releasing enzymes scarcely removed any arabinose from the backbone (Chapter 3 and 4). No improvement in the arabinose release was observed when these enzymes were used in combination with a xylosidase. The addition of xylosidase was aimed at the removal of terminal xylose from the suspected oligomeric branches, thereby creating new sites of hydrolysis for the arabinose releasing enzymes. However, the xylosidase was not active towards this substrate (unpublished results). A high release of arabinose by chemical means using dilute acid, was only possible with coinciding degradation of the backbone. So, at present, wheat bran glucuronoarabinoxylans seem to be not suitable for modification by these methods.

However, the observed moderate viscosity of these highly substituted glucuronoarabinoxylans can be of interest for application as such. Additionally, they effectively

stabilize emulsions (Chapter 4). Similar properties were observed for the highly substituted maize bran heteroxylans (Chanliaud, 1996). Both types of xylans were shown to be polyelectrolytes. Yet, the wheat bran glucuronoarabinoxylans had a higher intrinsic viscosity, which was approximately 2.5 dl/g compared with 1.8 dl/g for those of maize bran under similar conditions. A difference in molecular weight in favour of those of wheat bran was noticed, being approximately 350,000 Da and 230,000 Da for wheat bran and maize bran xylans, respectively. Also, the lower flexibility, which was expressed as the emperical stiffness parameter B, may have caused the higher intrinsic viscosity of wheat bran arabinoxylans when compared with those of maize bran (Chapter 4). This difference in flexibility may be attributable to the higher content of doubly substituted xylose residues in the wheat bran glucuronoarabinoxylans compared with those of maize bran (Table 1). When this wheat bran population is compared with alkali-extractable wheat flour arabinoxylan, which had an intrinsic viscosity of 7.5 dl/g and a weight average molecular weight of 450,000 Da (Gruppen, unpublished results), the relatively high intrinsic viscosity of the flour arabinoxylans is remarkable. Most probably this is caused by the relatively low degree of substitution of the flour arabinoxylans, which makes some physical interaction or entanglement of polysaccharide chains in solution possible. These results also indicate some resemblance of arabinoxylan to galactomannan, as occurence of entanglement in locust bean gum galactomannan has recently been proved (Goycoolea *et al.*, 1995a). Direct comparison of wheat bran arabinoxylans with those of other origin, such as rye, barley and oats (Girhammar and Nair, 1992; Ebringerova and Hromadkova, 1992) is very difficult, as different techniques and solvents are used for molecular weight and viscosity determinations. However, the main conclusion that can be drawn from these investigations is that the chemical structure can influence viscosity largely by affecting flexibility and possibility of interaction.

As adaptation of the viscosity properties of the glucuronoarabinoxylans by means of removal of arabinose residues was shown to be not possible at present, the ability of these polysaccharides to cross-link to one another appeared to be an interesting alternative. In Figure 1 the viscosity of oxidatively cross-linked arabinoxylans originating from rye flour (Vinkx *et al.*, 1991), wheat flour and wheat bran (Chapter 6), relative to the viscosity before addition of hydrogen peroxide and peroxidase, and based on arabinoxylan concentration are shown. It is obvious that the sudden viscosity increase of rye flour arabinoxylan at about 2 mg/ml is not equalled by both the wheat flour and bran arabinoxylan. Yet, the bran arabinoxylan also shows an exponential increase in viscosity, whereas that of flour is more gradual. The differences can be attributed to (i) molecular weight, which had been shown to be highest for rye arabinoxylan (Vinkx *et al.*, 1991; Girhammar and Nair, 1992), (ii) flexibilty, which is expected to be greatest for wheat flour

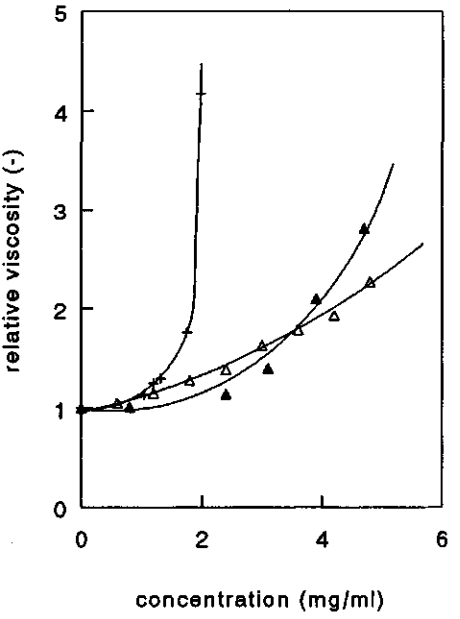

Figure 1: Viscosities of cross-linked arabinoxylan derived from rye flour (+), wheat flour (△) and wheat bran (▲), relative to the viscosity before addition of hydrogen peroxide and peroxidase, and based on arabinoxylan concentration.

arabinoxylan as inferred from the degree of substitution, and (iii) content of phenolics, which was highest for wheat bran glucuronoarabinoxylan. It was also shown in the present study that cross-linking can be induced by glucose - glucoseoxidase - peroxidase, which can be favourable for food application in view of legislation. The distribution of cross-linking products formed was not affected by the arabinoxylan origin, its original distribution of ferulate residues or the type of cross-linking agent used (Chapter 6). However, presence of other phenolic material was suspected to influence the ferulate recovery determined as mono- or dimer. The distribution of recovered dimers was approximately 5.5:3:1:1 for 8-5, 8-O-4, 8-8 and 5-5 coupled dimers, respectively. This confirmed previous suggestions and results that the 5-5 dimer, which has long been suspected to be the only coupling product, is present in only relatively low amount (Ralph *et al.*, 1994 and 1995; Grabber *et al.*, 1995). Finally, combination of feruloylated pectin and arabinoxylan was shown to open up possibilities for further exploration of the use of these types of polysaccharides, because synergy was demonstrated upon cross-linking of a mixture with equal proportions of both polymers (Chapter 6).

Perspectives for application

The current use of wheat bran, mainly as an ingredient of feed, may be expanded in the field of food, based on the properties of its glucuronoarabinoxylans as described in this thesis. The lowly substituted arabinoxylans probably have limited use, as their functional properties are not very attractive. However, they may find application as filling agent or fat replacer. In this respect they may be comparable to debranched araban from sugar beet pulp, which can be used as fat substitute (McCleary *et al.*, 1989; Cooper *et al.*, 1992). The highly substituted glucuronoarabinoxylans showed interesting characteristics, which may give rise to application as a moderate thickener or an effective emulsion stabilizer. Application as foam stabilizer may be of interest also, as previous reports demonstrated advantageous interfacial properties of arabinoxylans in protein foams (Izydorczyk *et al.*, 1991; Chanliaud, 1996). From the cross-linking point of view the feruloylated glucuronoarabinoxylans may be used as thickener or gelling agent. Apart from use in food, application in the pharmaceutical industry for drug delivery, or use as water absorbent in other fields of industry based on renewable resources, may be of interest also. Before that, the economic feasibility of the isolation of these polysaccharides needs to be studied further. The extractions in these investigations were aimed at a high purity in addition to a high yield, in order to reveal the physical properties of the glucuronoarabinoxylans as conclusively as possible. However, results of these studies indicate that coextraction of (1→3)(1→4)-β-glucans was not disadvantageous to the viscosity of the glucuronoarabinoxylans. With regard to the cross-linking of bran glucuronoarabinoxylans, it was indicated that coextraction of phenolics, such as lignin, has a detrimental effect on the viscosity increase upon addition of oxidative agents.

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Samenvatting

Arabinoxylanen komen voor in celwanden van onder meer grassen en granen. Na extractie bezitten deze koolhydraten aantrekkelijke functionele eigenschappen, zoals het geven van viscositeit of verdikking in waterige oplossing of de mogelijkheid tot het vormen van gelen. Voor arabinoxylaan afkomstig uit tarwe- en roggebloem zijn deze eigenschappen uitgebreid onderzocht, in verband met de rol die aan dit koolhydraat werd toegeschreven bij de waterbinding in deeg en de uiteindelijke bakeigenschappen van de bloem. Onderzoek naar mogelijke toepassing van arabinoxylanen in levensmiddelen als verdikkings- of geleermiddel is echter nauwelijks uitgevoerd. Behalve de genoemde functionele eigenschappen kunnen arabinoxylanen ook als voedingsvezel van fysiologische betekenis zijn. Ze worden niet verteerd in het spijsverteringskanaal waardoor ze geen energie leveren, maar wel verzadiging geven. Als geschikte grondstoffen voor isolatie van arabinoxylanen komen graanprodukten in aanmerking. Daarbij zijn bijprodukten van de graanverwerkende industrie, zoals tarwezemel, zeer aantrekkelijk, omdat ze een hoog gehalte aan celwandmateriaal en dus arabinoxylaan bevatten, maar ook omdat ze van lage economische waarde zijn en daardoor interessant voor opwaardering. Het hier beschreven onderzoek richtte zich op de extraheerbaarheid van arabinoxylanen uit tarwezemel. Aangezien arabinoxylanen uit deze grondstof ook glucuronzuur bevatten is een juistere benaming glucuronoarabinoxylanen. Na extractie werden de chemische structuur en daaraan gerelateerde fysische eigenschappen onderzocht. Behalve viscositeit werd ook het gelerend vermogen bestudeerd.

Voor onderzoek naar de extraheerbaarheid van arabinoxylaan uit tarwezemel werd eerst vet, eiwit en zetmeel verwijderd om het arabinoxylaan-bevattende celwandmateriaal zo zuiver mogelijk in handen te krijgen. In hoofdstuk 2 wordt de chemische samenstelling van tarwezemel en het daaruit geïsoleerde celwandmateriaal beschreven, evenals de verschillende strategieën die gevolgd zijn om een zo hoog mogelijke opbrengst aan zuiver glucuronoarabinoxylaan te verkrijgen. Door voorbehandelingen, zoals verwijdering van lignine uit het celwandmateriaal of het losser maken van de compacte structuur van de celwanden, werd gepoogd de opbrengst te verhogen bij de opvolgende extractie. Voor extractie werd gebruik gemaakt van verzadigde barium hydroxide oplossing, waarvan in eerder onderzoek aan arabinoxylanen uit tarwebloem was gebleken dat het zeer selectief was. Bovengenoemde voorbehandelingen bleken echter niet effectief te zijn voor verhoging van de opbrengst. Deze was in alle gevallen, ook als geen voorbehandeling werd toegepast, ongeveer 30% van de oorspronkelijke hoeveelheid glucuronoarabinoxylaan in het celwandmateriaal. Door extractie bij verhoogde temperatuur uit te voeren, bleek het wel mogelijk een duidelijk hogere opbrengst te verkrijgen.

Extractie bij 70°C resulteerde in een opbrengst van 45% aan glucuronoarabinoxylaan. Verdere verhoging van de temperatuur leidde wel tot hogere opbrengst, maar daarbij werd geringe afbraak van de koolhydraat-keten geconstateerd. De aanwezigheid van natrium boorhydride in het extractiemiddel om alkalische afbraak te voorkomen was bij deze hoge temperatuur niet voldoende. Naast verhoging van temperatuur gaf gelijktijdige verhoging van de concentratie barium hydroxide geen verdere verbetering. Indien calcium hydroxide werd gebruikt als extractiemiddel was de opbrengst en zuiverheid van het extract lager in vergelijking met barium hydroxide. Dit is grotendeels aan de lage oplosbaarheid van calcium hydroxide toe te schrijven. Door vergelijking van deze extractiemiddelen, de daarmee verkregen extracten en nader onderzoek naar de rol van natrium boorhydride, werd meer duidelijkheid verkregen over de specificiteit en complexiteit van dit extractiemiddel voor glucuronoarabinoxylaan.

Vervolgens werd glucuronoarabinoxylaan op grote schaal uit het celwandmateriaal geëxtraheerd met behulp van barium hydroxide bij 70°C voor onderzoek naar structurele en fysische eigenschappen. Hoofdstuk 3 beschrijft de structurele eigenschappen, die door middel van fractionering, methyleringsanalyse en enzymatische afbraakstudies zijn ontrafeld. Fractionering werd uitgevoerd met anionenwisselingschromatografie en stapsgewijze ethanol precipitatie, om de homogeniteit van het materiaal te bepalen. De glucuronoarabinoxylanen van tarwezemel bleken zeer heterogeen te zijn. Enerzijds werd een populatie geïsoleerd met zeer lage vertakkingsgraad, dat wil zeggen een lage verhouding arabinose tot xylose. Anderzijds bevatte het extract een populatie met zeer hoge vertakkingsgraad. Deze vertakkingen, voornamelijk bestaand uit arabinose-eenheden, komen voor op de *O*-3 positie of op zowel de *O*-2 als de *O*-3 positie van xylose-residuen. In beide tarwezemel populaties werden deze vertakkingsplaatsen aangetroffen. De proporties daarvan varieerden met de vertakkingsgraad. Ook werden xylose-residuen aangetroffen met een enkele vertakking op de *O*-2 positie. Dit werd voornamelijk toegeschreven aan glucuronzuur-eenheden, waarvan een gedeelte ook als 4-*O*-methylglucuronzuur voorkwam. De hoog vertakte populatie bevatte daarnaast relatief veel eindstandig xylose, en arabinose-eenheden die indicaties gaven voor aanwezigheid van zijketens bestaande uit meer dan één eenheid. In voorgaand onderzoek aan arabinoxylanen uit de buitenste cellagen van granen was dit ook opgemerkt. Door gebruik te maken van xylanolytische enzymen met bekend werkingsmechanisme werd gepoogd meer informatie te krijgen over de fijn-structuur van beide populaties. De laag vertakte populatie bleek goed afbreekbaar te zijn door deze enzymen. Door identificatie van de afbraakprodukten kon afgeleid worden, dat de vertakkingen op willekeurige wijze waren verdeeld. De hoog vertakte populatie werd nauwelijks door de enzymen afgebroken. Enerzijds gaf de hoge vertakkingsgraad aanleiding tot sterische hindering, anderzijds

bevatte het polymeer specifieke structuurelementen die blijkbaar geen aangrijpingspunt voor de huidig bekende enzymen vormen. Zodoende kon slechts gespeculeerd worden over de fijn-structuur van het hoog vertakte glucuronoarabinoxylaan.

In hoofdstuk 4 worden fracties, verkregen uit het barium hydroxide extract, vergeleken wat betreft hun fysische eigenschappen. De laag vertakte glucuronoarabinoxylanen gaven weinig viscositeit aan een waterige oplossing, bovendien bleek verhoging van temperatuur of ionsterkte hierop geen invloed te hebben. Dit laatste aspect geeft aan dat deze populatie geen of nauwelijks geladen groepen bevat, zoals het eerder genoemde glucuronzuur. De lage viscositeit werd toegeschreven aan het voorkomen van compacte aggregaten van arabinoxylanen, zoals werd aangetoond door middel van molecuulgewichtsbepalingen en gelpermeatiechromatografie. Op grond van de lage vertakkingsgraad en globale structurele overeenkomsten met galactomannanen werd de mogelijkheid van een synergistische interactie met andere koolhydraten, zoals voor xanthaan en κ-carrageenaan met galactomannaan is aangetoond, onderzocht. Synergistische interactie werd met genoemde koolhydraten in combinatie met laag vertakt arabinoxylaan niet aangetoond. Ook met hoog vertakt glucuronoarabinoxylaan bleek dit niet het geval te zijn. Hun viscositeit was wél hoger dan van de laag vertakte arabinoxylanen, en werd beïnvloed door verhoging van temperatuur of ionsterkte. In vergelijking met bekende verdikkingsmiddelen, zoals galactomannaan en pectine, is hun viscositeit niet meer dan gemiddeld. Een belangrijk voordeel van de glucuronoarabinoxylanen bleek echter hun stabiliserende werking op emulsies. Hierin overtreffen ze het als stabilisator vaak gebruikte arabische gom. Door middel van enzymatische of chemische verwijdering van arabinose-eenheden van het hoog vertakte glucuronoarabinoxylaan werd onderzocht of de fysische eigenschappen gericht konden worden aangepast. Door gebrek aan geschikte enzymen en door grote afbraakgevoeligheid van de polymeerketen bij milde chemische modificatie kon de structuur slechts in geringe mate worden gemodificeerd, waardoor geen vergaande conclusies getrokken konden worden over de mogelijkheid tot gerichte aanpassing van de functionele eigenschappen.

Voor verkrijging van tarwezemel glucuronoarabinoxylanen met gelvormende eigenschappen werd een andere extractiemethode ontwikkeld, omdat gelering alleen mogelijk is indien veresterd ferulazuur, een fenolzuur dat van nature gebonden aan arabinoxylanen voorkomt, aanwezig is. Bij de bovenbeschreven extractiemethode wordt als gevolg van de relatief hoge concentratie loog alle ferulazuur gehydrolyseerd. Hoofdstuk 5 beschrijft het gebruik van verdunde hydroxide oplossing gedurende korte tijd als een mogelijkheid ferulazuur bevattende glucuronoarabinoxylanen uit tarwezemel te extraheren. De opbrengst was niet verder te verhogen dan 4 tot 7% van de oorspronkelijke hoeveelheid glucuronoarabinoxylaan in het celwandmateriaal. Isolatie van water-oplos-

baar materiaal of behandeling van tarwezemel met stoom bij hoge druk en temperatuur gaven geen geleerbaar extract. Gelering werd bewerkstelligd door gebruik van oxidatieve reagentia, zoals de combinatie van waterstofperoxide en peroxidase of ammonium persulfaat. De koppelingsreaktie van ferulazuureenheden van naburige arabinoxylaanketens, waarbij radicalen een rol spelen, bleek beïnvloed te worden door aanwezigheid van ander fenolisch materiaal. In tarwezemel extracten bleek namelijk enig lignine aanwezig te zijn, dat koppeling van ferulazuur eenheden zou kunnen verhinderen door het wegvangen van radicalen. Op grond van eerdere observaties bij precipitatie in organische oplosmiddelen, waarbij lignine enigiszins van glucuronoarabinoxylaan gescheiden kon worden, werd zuivering door middel van precipitatie met aceton toegepast. De mate van koppeling tussen arabinoxylaan-ketens bleek daardoor aanzienlijk verbeterd.

In hoofdstuk 6 worden tenslotte tarwezemel arabinoxylanen vergeleken met die van tarwebloem wat betreft hun koppelingsgedrag en de invloed van chemische samenstelling, structuur, molecuulgewicht en type koppelingsreagens daarop. Hoewel het molecuulgewicht van de bloem arabinoxylanen hoger was, geven de resultaten aan dat tarwezemel arabinoxylanen een minder flexibel netwerk bij hoge concentratie vormen, indien waterstofperoxide - peroxidase als koppelingsreagens werd gebruikt. De hoge vertakkingsgraad van de tarwezemel arabinoxylanen werd verondersteld hierin van belang te zijn, maar ook het hoge ferulazuur gehalte en de aanwezigheid van andere fenolische verbindingen kunnen aanleiding hebben gegeven tot een steviger netwerk. Dit laatste aspect kon niet eenduidig aangetoond worden, als gevolg van de afgenomen hoeveelheid ferulazuur die na koppeling aangetoond kon worden ten opzichte van het ongekoppeld materiaal. Binding van ferulazuur aan lignine fragmenten zou daarbij een rol gespeeld kunnen hebben. Bij de analyse van de koppelingsprodukten bleek dat het type reagens of de oorsprong van het arabinoxylaan geen invloed had op de verdeling van de koppelingsprodukten. Het bekende diferulazuur, ontstaan door een 5-5 koppeling van twee ferulazuur eenheden, werd het minst aangetroffen. Andere typen, ontstaan door een 8-5 of een 8-O-4 koppeling, werden voornamelijk aangetroffen. Koppeling van ferulazuur-houdende arabinoxylanen bleek ook mogelijk door gebruik van glucose in combinatie met glucoseoxidase en peroxidase. Hierbij wordt met behulp van glucoseoxidase uit glucose waterstofperoxide geproduceerd. Voor toepassing in levensmiddelen kan een dergelijk reagens, wettelijk gezien, gunstig zijn.

Kortom, arabinoxylanen uit tarwezemel bezitten aantrekkelijke eigenschappen voor toepassing in levensmiddelen, als matig verdikkingsmiddel, geleermiddel, waterbinder of effectieve emulsiestabilisator, maar de economische haalbaarheid daarvan zal nog nader onderzocht moeten worden.

Curriculum vitae

Margot Elisabeth Françoise Bergmans werd geboren op 3 april 1968 te Blerick. Het Gymnasium-β diploma werd behaald aan het Collegium Marianum te Venlo in 1986. In dat jaar begon zij aan haar studie Levensmiddelentechnologie aan de Landbouwuniversiteit te Wageningen. De studie werd afgerond met een stage aan het Food Research Institute, Werribee, Melbourne, Australië, en met afstudeeropdrachten bij de vakgroep Levensmiddelentechnologie, zowel bij de sectie Proceskunde als Levensmiddelenchemie en -microbiologie, en bij de vakgroep Organische Chemie. In maart 1992 behaalde ze het ingenieursdiploma met lof. Van april 1992 tot april 1996 werkte ze als Assistent in Opleiding aan de Landbouwuniversiteit Wageningen bij de vakgroep Levensmiddelentechnologie, sectie Levensmiddelenchemie en -microbiologie, onder begeleiding van dr. G. Beldman en prof. dr. ir. A.G.J. Voragen. Het onderzoek uitgevoerd in deze periode staat beschreven in dit proefschrift. Gedurende de maand maart 1997 is ze als Post-doc bij de sectie Proceskunde, van laatstgenoemde vakgroep, werkzaam geweest. Sinds april 1997 werkt ze als Cereal Chemist bij Gist-brocades te Delft.