STRUCTURAL CHARACTERISTICS OF WHEAT FLOUR ARABINOXYLANS

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Structural characteristics of wheat flour arabinoxylans

Structuurkenmerken van arabinoxylanen uit tarwebloem

Proefschrift

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ABSTRACT

A procedure has been developed for the isolation of highly purified water-unextractable cell wall material (WUS) from wheat flour. From this material arabinoxylans were extracted via sequential extraction with $Ba(OH)_2$, KOH and NaOH solutions. The first extract comprised arabinoxylans only, which represented 80% of all arabinoxylans present in the WUS. The arabinoxylans in this extract were fractionated using anion exchange chromatography and graded-ethanol precipitation. Neutral sugar and glycosidic linkage analysis revealed great similarities between these alkali-extractable arabinoxylans and similarly fractionated water-extractable arabinoxylans, isolated from the same wheat flour. Enzymic analysis using different endo-(1→4)-ß-D-xylanases from *Aspergillus awamori* showed, however, that the water-extractable arabinoxylans were degraded both faster and to a larger extent than the alkali-extractable arabinoxylans.

Based on the structure and abundance of enzyme-resistant polymeric material and different oligosaccharides, the latter being identified with ^{1}H-NMR analysis, present in the enzyme digests, a structural model is proposed for the arabinoxylans. This model shows that the arabinoxylan contains highly branched regions, interlinked with less branched regions which include subregions of unsubstituted xylose up to 7 contiguous residues. Variation in arabinose/xylose ratio between different arabinoxylan is due to variation in the relative proportion as well as the composition of the less branched regions. With increasing arabinose/xylose ratio of the arabinoxylans a decreasing extent of enzymic degradation observed. The enzymes used had different effects on the degradation of WUS and extracted arabinoxylans as well as on the baking performance of wheat flour.

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TABLE III. Molar non-starch sugar composition of flour and flour fractions (mean value ± S.D.)[a]

		Fractions (mean ± S.D.)						
	FLOUR[b]	CWE[b]	GLUTEN[c]	STARCH[b]	WUS1[c]	WUS2[c]	HWE1[b]	HWE2[b]
Rhamnose	0	0.2 ± 0.3	0	0	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.2
Arabinose	32.3	37.7 ± 0.8	26.8 ± 2.8	31.3 ±10.2	30.4 ± 0.3	32.6 ± 0.2	27.0 ± 1.3	30.1 ± 1.2
Xylose	48.5	40.4 ± 0.1	15.4 ± 2.9	57.4 ±18.7	52.3 ± 0.4	57.7 ± 0.6	45.8 ± 2.3	48.2 ± 2.4
Mannose	1.6	0.2 ± 0.2	7.4 ± 1.2	0	1.9 ± 0.1	1.2 ± 0.1	2.6 ± 1.1	1.8 ± 0.2
Galactose	5.4	17.2 ± 0.4	27.0 ± 4.7	0	0.7 ± 0.1	1.1 ± 0.3	0.8 ± 0.2	1.9 ± 0.7
Glucose	12.2	4.2 ± 0.9	23.4 ±11.1	11.3 ± 3.7	14.7 ± 0.6	7.3 ± 0.4	23.7 ± 4.2	17.9 ± 4.0

[a] Average of four isolations.
[b] Sugars analysed as total non-starch polysaccharides according to Englyst and Cummings[12].
[c] Sugars released on 72%-1M H_2SO_4 hydrolysis, the glucose content is corrected for starch.

STELLINGEN

1. De verdeling van arabinose substituenten in tarwebloem arabinoxylanen is niet *random*.
   Dit proefschrift.

2. Verschillen in water-extraheerbaarheid tussen arabinoxylanen zijn niet primair terug te voeren op verschillen in arabinose/xylose verhouding.
   Dit proefschrift.

3. De door Girhammer en Nair gemaakte vergelijkingen tussen de structuurkenmerken van water-extraheerbare NSP uit tarwe en rogge zijn onzinnig.
   Girhammer, U. and Nair, B.M. *Food Hydrocolloids* **6** (1992) 285-299.

4. De naamgeving van polysachariden en de daarop inwerkende enzymen dient gebaseerd te zijn op de chemische structuur van polysachariden.

5. Een enzymzuivering leidt niet per definitie tot een zuiver enzym.

6. Met behulp van de geïsoleerde ABC genen van Arabidopsis, Anthirrhinum en Petunia is de fylogenetische verwantschap van lemma, lodicule en palea met petalen en sepalen te bepalen.
   Coen, E.S. and Meyrowitz, E.M. *Nature* **353** (1991) 31-37.
   Angenent, G.C. et al *The Plant Cell* **4** (1992) 983-993.

7. Met *in-vivo* experimenten dient nagegaan te worden in welke mate de door P. Reaven et al. *in-vitro* waargenomen gevoeligheid voor oxidatie van meervoudig onverzadigde vetzuurresiduen in LDL deeltjes in het menselijk lichaam van belang is voor het ontstaan van atherosclerose.
   P. Reaven et al *Am. J. Clin. Nutr.* **54** (1991) 701-706.

8. De naam 'functional foods' kan beter vervangen worden door 'bio-active foods'.

9. Ter verbetering van de eerlijkheid in de handel verdient het aanbeveling om eiwitstandaardisatie van melk middels wetgeving op Europees nivo te regelen.

10. Het gebruik om in lijsten van namen bij vrouwen de aanduidingen 'mevr' of 'mw' te vermelden en bij mannen de aanduiding 'dhr' of 'heer' achterwege te laten is discriminerend.

11. Voorstanders van het recht op vergoeding van seksuele diensten op sociaal/medische gronden vatten de term gemeenschapsgeld wel erg letterlijk op.

Stellingen behorende bij het proefschrift 'Structural features of wheat flour arabinoxylans' door Harry Gruppen. Wageningen, 4 december 1992.

CONTENTS

LIST OF ABBREVIATIONS

Ara*f*	α-L-Arabinofuranose
Ara/Xyl	Arabinose/xylose
AXH	(1→4)-ß-D-arabinoxylan arabinofuranohydrolase from *Aspergillus awamori* CMI 142717
BE1	First $Ba(OH)_2$ extract
BE2	Second $Ba(OH)_2$ extract
BE1-B	DEAE-bound fraction of BE1
BE1-B-RU	DEAE-unbound fraction after rechromatography of BE1-B
BE1-B-RB	DEAE-bound fraction after rechromatography of BE1-B
BE1-U	DEAE-unbound fraction of BE1
CWE	Cold water extractables
DEAE	Diethylaminoethyl
DMSO	Dimethylsulphoxide
DP	Degree of polymerization
endoI	Endo-(1→4)-ß-D-xylanase I from *Aspergillus awamori* CMI 142717
endoIII	Endo-(1→4)-ß-D-xylanase III from *Aspergillus awamori* CMI 142717
GC-MS	Gas chromatography-mass spectroscopy
^{1}H-NMR	Proton nuclear magnetic resonance
HOHAHA	Homonuclear Hartmann-Hahn spectroscopy
HPAEC	High performance anion exchange chromatography
HPSEC	High performance size exclusion chromatography
HWE	Hot water extractables
1M	1M KOH extract
4M	4M NaOH extract
MMNO	4-Methylmorpholino-N-oxide
NSP	Non-starch polysaccharides
RES	Unextractable residue after sequential extraction with $Ba(OH)_2$, 1M KOH, and 4M NaOH
ROESY	Rotating frame nuclear Overhauser enhancement spectroscopy
ROE	Rotating Overhauser effect
RS	Unextractable residue after $Ba(OH)_2$ extraction
TFA	Trifluoroacetic acid
WUS	Water-unextractable cell wall material
Xyl*p*	ß-D-Xylopyranose
●	Xyl*p*
◇	α-Ara*f*
●●	β-Xyl*p*-(1→4)-Xyl*p*
◇●	α-Ara*f*-(1→2)-β-Xyl*p*
◇●	α-Ara*f*-(1→3)-β-Xyl*p*

CHAPTER 1

General introduction

Origin and production

Wheat is among the oldest grown of all crops. It is generally believed that wheat evolved from wild grasses native to the arid lands in the Middle East. Humans probably first gathered wheat from the wild about 12,000 - 17,000 years ago and it is generally accepted that wheat was first grown as a crop 5000 years later. Wheat is placed in the family *Graminae* and the genus *Triticum*, comprising 18 species of which *aestivum* (common wheat), *durum* (durum wheat) and *compactum* (club wheat) are of commercial importance.

Nowadays, in terms of production, wheat ranks first among all cultivated plants. The estimated[1] world production of wheat in 1991 is 548 million tonnes, of which *Triticum aestivum* is the predominant species. The major use of wheat is for human consumption. It has been estimated that during 1984-1985 65% of the wheat production was for food, 21% for feed, 8% for seed and 6% for industrial uses. The major food outlet is the production

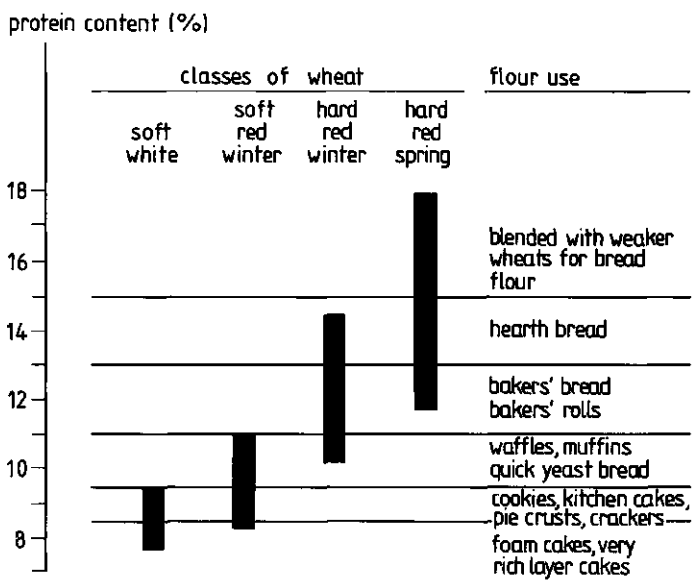

FIGURE 1. Protein content and flour uses of major classes of wheat (from Bushuk[2]).

of yeast-leavened products, biscuits and noodles (*Triticum aestivum*) and pasta products (*Triticum durum* and *Triticum aestivum*).

Grain of common wheat has been classified into different classes according to the technological properties of kernel hardness, bran colour, and protein content. The different uses of each of these classes are given in Fig. 1. The terms *hard* and *soft* are used to described the kernel texture. A hard wheat kernel requires greater force in order to disintegrate than a soft wheat kernel. The hardness of a kernel is genetically controlled and is not directly correlated to the protein content. For the production of yeast-leavened bread wheat flour must have a relatively high protein content of the right quality. Also, the amount of bran fragments in the flour is important. Flours with higher contents of bran material will yield a lower baking quality than flours with lower bran materials obtained from the same grain.

Wheat kernel and wheat flour

The wheat kernel is composed of various morphologically different tissues (Fig. 2). Botanically it is a one seeded fruit. However, from both commercial and technological viewpoints the kernel can be divided into the endosperm, bran, and germ. The separation into the three different grain parts is achieved by milling using a gradual reduction system with the objective of separating as much bran material from the endosperm as possible. This system is comprised of a sequence of breaking, grinding and separation operations. The wheat kernel is broken open through break rolls which results into endosperm particles (break middlings), large particles of bran fragments with adhering endosperm material, a small amount of fine flour (break flour) and the germ. The endosperm in the large particles is further scraped away from the bran in a second and further set of break rolls (commercially up to 6 break steps) resulting into different break flours, break middlings and a final (coarse) bran fraction. The different break middlings, being a mixture of pure endosperm, endosperm with bran particles attached, and small particles of bran, are purified by removing the bran fragments with sieves. These purified middlings (semolina or farina) are then gradually grinded and sieved (reduction process) into fine bran particles (shorts) and different flour streams. The early flour streams consists predominantly of endosperm whereas the latter ones contain more fine bran particles and are darker in colour. If all flour streams are combined, the result is known as a straight-run flour. Usually, by the choice of the milling procedure, a 70-72% net extraction rate (100 kg kernel gives 70-72 kg flour and 28-30 kg shorts+bran+germ) is obtained for straight run flours which are used for baking of white bread. Additional, low grade flour is obtained from the bran particles by bran finishing. When the streams are combined in different proportions different split-run flours (patent flours) are obtained, which have different applications. Both straight-run flour and patent flours are addressed to as white flour. Wholemeal flour is composed of all flour, bran and germ streams.

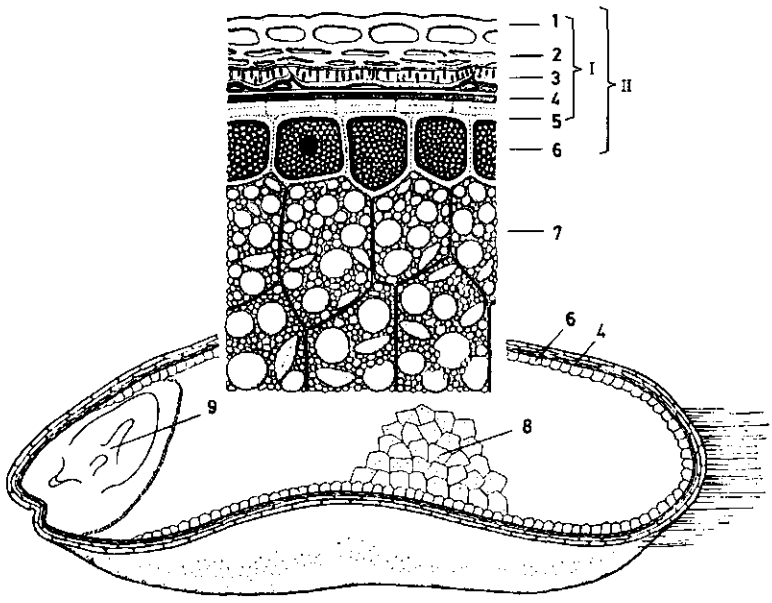

FIGURE 2. Longitudinal section of a wheat grain. I. Bran (botanically), II Bran (commercially), 1 epidermis (epicarp), 2 hypodermis, 3 tube cells, 4 seed coat (testa), 5 nucellar tissue, 6 aleurone layer, 7 outer starch endosperm cells, 8 inner starchy endosperm cells, 9 germ (adapted from Belitz and Grosch[3]).

Chemical composition of wheat flour.

As white wheat flour consists mainly of endosperm material, its composition reflects that of endosperm. Starch is the predominant component of wheat endosperm, making up 80% of the endosperm material. It is present as two populations of starch granules which differ in size. For the large granules diameters have been reported to vary from 14-40 μm[2,4,5]. This starch comprises of *c.* 25% amylose[6], which is considered as linear chains of (1→4)-linked α-D-linked glucopyranosyl residues having molecular weights of 10^5-10^6, although a limited number of branching points are believed to be present[7]. The amylopectin (75%) consists of chains of (1→4)-α-D-linked glucopyranosyl residues of which at every 20-25 glucopyranose residue a chain of (1→4)-α-D-linked glucopyranosyl residues is linked to via an (1→6)-α-D-glycosidic linkage and has a molecular weight as high as 10^7-10^8. Part of the kernel is present in a crystalline structure. Upon heating in the presence of abundant water starch granules swell and loose their crystallinity (58-64 °C).

Although in abundance second to starch, proteins (12%) are the most important components of wheat flour with respect to bread characteristics. The proteins of wheat endosperm can be separated sequentially into water-extractable proteins (albumins), 0.5M NaCl extractable proteins (globulins), 70% ethanol extractable proteins (gliadins) and

0.05M acetic acid extractable proteins (glutenins) and a residual fraction[8]. Albumins and globulins account for 20% of the total protein, gliadins and glutenins each for 35% with the remainder being residue protein although variation can occur due to overlap of the fractions[9]. The two most important protein fractions are the glutenins and gliadins which together represent the gluten proteins. The amino acid compositions of the gliadin and glutenin fraction reveal relatively high amounts of glutamic acid (mostly in amide form), proline and leucine and low amounts of lysine. The high glutamine content is important with respect to the functionality of the proteins. The albumins and globulins have less high glutamic acid and proline contents and more lysine and other amino acids resulting in a higher charge density compared to the gliadins and glutenins. Gliadins occur as globular, monomeric proteins with molecular weights of *c.* 40,000. Glutenins consist of subunits (Mw. 30,000 - 140,000) and disulphide linked aggregates thereof (Mw. $>10^8$). It is generally believed that the glutenins confer softness and elasticity whereas the gliadins are responsible for extensibility and firmness. Together, these two protein components are predominantly responsible for the formation of a viscoelastic dough after water addition and kneading. The unique rheological properties of wheat dough are responsible for the gas-holding capacity during leavening and produce a porous structure upon baking.

Wheat flour also contains 2.5 - 3.0% lipids[10]. The lipids can be classified into starch lipids (0.8-0.9%) and non-starch lipids (1.6-2.2%). The latter are mainly composed of triglycerides and galactolipids whereas the starch lipids are predominantly phospholipids. It has been proposed that the non-starch lipids play a role in gluten but there is ambiguity about the presence of molecular lipid-protein interactions[11,12].

Whereas the former three wheat flour components are predominantly reported to be intracellular c. 2-3% of the flour is composed of non-starch polysaccharides (NSP) mostly originating from cell wall material[13-15]. This cell wall material predominantly consists of NSP although the presence of some cell wall proteins can not be excluded. The NSP comprises different polysaccharides which are built up of pentose and hexose sugars. They are usually divided into a water-extractable and a water-unextractable part, which comprise 25 and 75%, respectively, of all NSP present in wheat flour[13].

Water-extractable NSP

The water-extractable NSP, also referred to as pentosans, can be separated from the bulk of the wheat flour by aqueous extraction and specific precipitation[16]. They can be divided into two fractions: arabinoxylans and arabinogalactan-proteins.

1. Arabinoxylans These heteroglycans of high molecular weight consist of a linear backbone of (1→4)-ß-D-xylopyranosyl units, to which single α-L-arabinofuranosyl substituents are attached through *O*-2, *O*-3, or *O*-2,3 linkages. Whereas the prevailing *O*-3 substitution has been widely accepted the relative proportion of *O*-2 substituted xylose residues has been reported either negligible[17] or as high as 30%[18]. The presence of substantial amounts of *O*-2,3 substituted xylose residues has recently been reported for

water-extractable wheat flour arabinoxylans by both methylation analysis of polymeric material[19,20] as well as ^{1}H-NMR analysis of enzymic derived fragments thereof[21,22]. Next to these single unit substituents a variety of di- and trimeric side chains have been identified as minor components of cereal grain arabinoxylans[17,19,20]. In addition, phenolic acids like ferulic and coumaric acid have been found esterified to arabinoxylans[23,24] via the arabinofuranosyl substituents[25].

Arabinoxylan preparations represent a population of arabinoxylan molecules which vary in structural features. Molecular weights have been reported ranging between 22,000-5,000,000[17,18,26-32], the latter probably being overestimated due to the technique used[33]. Arabinoxylans have been fractionated with either graded ethanol/ammoniumsulphate precipitation[19,20,32,34] or DEAE-borate chromatography[35-38] resulting into fractions with both different Ara/Xyl ratios and relative proportions of unsubstituted, single and double substituted xylose residues[19,20]. These structural features have a great effect on their functional properties. It is presumed that in unsubstituted form (1→4)-ß-D-xylans form threefold, left-handed helices which exist as a fully extended "twisted ribbon" in the solid state. Arabinosyl substitution "stiffens" the (1→4)-ß-D-xylan backbone into a more extended conformation[39], similar to the conversion of a shorter threefold helix to a more extended twofold helix on acetylation[40]. Higher degrees of substitution favour water solubility[30]. The distribution of substituents probably influences association between arabinoxylan molecules or with other polysaccharides. Arabinoxylans form highly viscous solutions upon hydration[28,38,41] which has been ascribed to the highly asymmetrical structure[33]. Due to the presence of ferulic acid arabinoxylans solutions can form gels upon oxidation[42,43]. During this process either diferulic acid bridges[23,29] or covalent linkages between ferulic acid and cysteine molecules[24], present in proteins, can be formed. Although the latter mechanism is disputable[14] other results[13] have indicated the presence of protein-arabinoxylan interaction via ferulic acid. Due to the attachment of ferulic acid to the high molecular weight arabinoxylans a three dimensional network is formed upon oxidation resulting in a gel-like structure[29].

2. *Arabinogalactan-proteins* Arabinogalactan-proteins are glycoproteins comprising of a galactose and arabinose containing polysaccharide moiety (92-94%) and a protein part (6-8%). The latter is for 15-20% composed of 4-hydroxyproline. The carbohydrate moiety consists of a (1→3) and (1→6) ß-D-linked galactose backbone to which single arabinose units are linked. The molar ratio arabinose to galactose is *c.* 0.8. Whereas some authors[44] have proposed that the basic galactose chain is branched, others have suggested a linear galactan structure[45,46]. The galactan chains are attached to a protein core via a galactose-hydroxyproline linkage[46,47]. Due to its high degree of branching the arabinogalactan-peptide complex is highly soluble in aqueous ethanol or ammoniumsulphate. In contrast to the arabinoxylans, arabinogalactan-proteins are not considered as cell wall components. They are thought to be associated with the plasma membrane[48]. Cell/cell recognition, lubrication and a function in morphogenesis has been proposed as their biological role[48,49]. No technological properties of arabinogalactans have been described.

Water-unextractable NSP.

The water-unextractable NSP can be isolated from the wheat flour via the preparation of water-unextractable cell wall material. The conditions must be mild and preferably not cause irreversible physical and chemical changes and not allow modification of the polysaccharides by endogenous or exogenous enzymes. Starch and protein must be removed as much as possible. Procedures have been worked out based on centrifuging flour/water or gluten-free flour/water suspensions in combination with enzymic breakdown of protein and/or starch to obtain purified water-unextractable cell wall material (WUS)[29,30,50-52]. Alternative methods have been based on removing protein and starch by wet-sieving in ethanol[53] or removal of starch and protein by extraction with specific organic solvents[54]. The water-unextractable cell wall material consists primarily of non-starch polysaccharides although the presence of small amounts of cell wall proteins, as reported for aleuron cell walls[55], may not be eliminated. No information on the presence of specific cell wall lipids have been reported.

The water-unextractable NSP comprises of arabinoxylans, (1→3,1→4)-ß-glucans, cellulose and glucomannans[33].

1. Arabinoxylans The arabinoxylans represent the major part (70%) of the water-unextractable NSP[32,56]. Their structure is basically the same as that for water-extractable arabinoxylans. It has been reported that the degree of branching is slightly lower than for water-extractable arabinoxylans[16-18,37,38,57-59], although similar degrees of branching have also been reported[32]. Also differences in molecular weight have been reported, being highest for the water-unextractable arabinoxylans. However, these differences do not seem to be sufficient to account for the differences in extractability and therefore other factors like chemical and physical interactions[33] are more likely.

2. (1→3,1→4)-ß-glucans The (1→3,1→4)-ß-glucans consists of linear chains of ß-D-glucopyranosyl residues joined by (1→3) and (1→4) glycosidic linkages. In contrast to (1→3,1→4)-ß-glucans from barley little information is known about the physical properties of (1→3,→4)-ß-glucans of wheat. Their presence has been established, both in whole grain as well as in endosperm material[60]. It has been reported that (1→3,1→4)-ß-glucans make up 0.3% of wheat flour[60] and 20% of the NSP present in WUS from wheat endosperm[56]. These (1→3,1→4)-ß-glucans are unextractable in water at 65 °C[61]. These two data are complete different to barley in which they represent 70-75% of the cell wall NSP[62,63] from which 25-30% is extractable at 65 °C[64,65].

3. Cellulose Cellulose consists of linear chains of several thousands of (1→4)-linked ß-D-glucopyranosyl residues. They make up only 4% of the endosperm NSP[32] and 0.3% of wheat endosperm[66]. It is usually determined as the residue after treatment with alkali. Electron microscopy of this residue has revealed the presence of fibrils typical for a cellulosic structure.

4. Glucomannans Glucomannans consists of varying proportions of (1→4)-linked ß-D-glucopyranosyl and ß-D-mannopyranosyl residues in linear chains. In wheat endosperm

their presence has been based on the determination of glucose and mannose in a borate containing alkaline extract upon acid hydrolysis[32]. However, other authors consider these sugars to be artefacts of alkaline extractions and not of endogenous nature[67].

Baking of bread

The essential ingredients for the production of bread are flour, water and limited amounts of yeast and salt. The latter are added to produce carbon dioxide and for stiffening the dough, respectively[68]. The first step in the production of bread is the formation of a dough. The added water binds to the damaged starch, protein (gluten), and cell wall polysaccharides. Bread quality is determined by a high bread volume and a fine crumb structure. This is achieved if during mixing many small gas bubbles are occluded and held in the dough during fermentation and baking. This is only achieved when the dough is properly developed. Dough development involves the formation of a gluten matrix obtained by a sequence of kneading and periods of rest, shaping and cutting. This gluten network is formed by interactions between the monomeric gliadins and a submatrix of disulphide linked glutenins. This results in a change in the conformation and alignment of the protein polymeric molecules. Due to production of carbon dioxide by the yeast during the periods of rest (proofs) the entrapped gas bubbles grow which results in an expansion of the dough. Finally, the expanded dough is transferred to the oven and baked, typically, at 240 °C for 20 min. During the initial state of baking the gas cell expands because of water vapour, thermal expansion of the gas, and enhanced production of carbon dioxide, due to increased yeast activity, resulting in a risening of the dough (oven-spring). In a later stage the starch granules gelatinize and the proteins denaturate, due to the higher temperatures in the dough. The structure of the dough with separate gas cells is transformed into a sponge-like structure, in which the gas cell are interconnected. This results in the final crumb structure of the bread.

Various wheat flours lack either quantity or quality of those proteins responsible for the formation of a gluten network. This can be partly solved by mixing these flours with those of wheat varieties being high in protein quality and quantity or by the addition of so-called vital gluten derived from the industrial separation of wheat flour into starch and gluten. Also other ingredients are added to the flour or dough in order to obtain optimal dough handling and bread quality. Fats and emulsifiers are added to improve the texture, loaf volume and shelf-life (softness)[69]. Oxidants like ascorbic acid may be added to increase the rate of dough development[70]. Exogenous amylases are added when insufficient endogenous α-amylase is present in the flour to produce low molecular weight sugars, necessary for the production of carbon dioxide by the yeast, the formation of a attractive brown colour due to Maillard reactions and the formation of a softer crumb structure. Protease addition is only used to modify proteins in flours from hard, high protein wheat, in order to optimize dough handling properties and loaf volumes. With the soft bread

wheat varieties produced in Europe this would only lead a to a more weaker gluten structure and is therefore not used for the production of breads. Lipoxygenase, added as soy flour is used for bleaching (carotenoids) and oxidation of polyunsaturated lipids of the flour resulting in improved dough handling and loaf volume[70]. Whereas the addition of the above enzymes has been common use for many years, only recently the addition of non-starch polysaccharidases (hemicellulases or pentosanases) has been practised. They hydrolyse the non-starch polysaccharides thereby modifying their effects on dough handling and baking.

Effects of non-starch polysaccharides on dough handling and baking performance

Despite being a minor component, NSP have been long considered to be functionally important in the production of yeast-leavened breads. They have a high water-binding capacity[28,71,72] and it has been suggested[73] that 23% of the water in a dough is associated with the NSP. Various studies have been performed to identify the functional role of either water-extractable as well as water-unextractable NSP. In order to study these effects, reconstitution experiments[74] of wheat flour prior to dough development have been performed. These experiments involved changing of the level of NSP enriched preparations in the final dough and also included modification of isolated NSP fractions before reconstitution. Although individual studies showed conclusive effects on loaf volume, crumb structure[75], dough handling[76] and their action as anti-staling agents[72,77], comparison of the results of all reconstitution experiments showed conflicting results[17,78]. These conflicting results are probably the effect of differences in the purity of the NSP fractions and modifications, due to differences in isolation. Especially the preparation of water-unextractable NSP preparations has encountered great difficulties of obtaining these in a purified form. The isolation techniques sometimes caused irreversible changes of the NSP which makes a comparison to native NSP difficult. In addition, the use of different varieties prevents a proper comparison of the effects of NSP.

An alternative method for the analysis of the effects of NSP has been the *in situ* use of NSP (arabinoxylan) degrading enzymes. This technique was first used by Tracey[79] using a crude enzyme preparation. McCleary *et al*[78] adopted this procedure by making use of a purified xylanase from *Trichoderma viride*. Application of this enzyme to a dough resulted in a loss of dough strength as well as loaf height, but in an increase of loaf volume. These effects could be restored by the addition of guar flour at a level similar to the level of NSP in flour. Another effect of the xylanase treatment was the up to 20 fold increase of the volume of the individual air cells.

In conclusion, it may be stated that the results of the reconstitution and enzyme-addition experiments have demonstrated the importance of cell wall fragments or NSP in breadmaking but as yet have not led to a better understanding of the process. The reason for this is both the lack of appropriate methods to isolate highly purified cell wall material

for reconstitution trials as well as the lack of detailed knowledge of the water-unextractable non-starch polysaccharides. Both are prerequisites for gathering information about the effects of NSP as well as the effect of enzymically modified NSP on dough handling and baking performance.

Aim and outline of this thesis

The aim of this thesis is (i) the development of a technique for the large-scale isolation of highly purified WUS (ii) the elucidation of the structural characteristics of the arabinoxylans present in wheat flour with emphasis on the water-unextractable arabinoxylans, and (iii) determination of the enzymic degradability of WUS and isolated arabinoxylans. First a method is developed for the mild isolation of highly purified water-unextractable cell wall material (Chapter 2). To enable a full comparison with previous methods also a complete mass balance with respect to the NSP constituent sugars is given. Next, the upscaling of the isolation method is described in order to yield large quantities (100 g) necessary for baking trials. (Chapter 3). As for a complete characterization solubilization is a prerequisite different primary extractants for wheat flour WUS are compared (Chapter 4). In Chapters 5 and 6 the sequential extraction, fractionation, and characterization of different alkaline extracts are given. The major alkaline fraction is compared to the water-extractable arabinoxylans. In order to enable a structure analysis of the arabinoxylans present in the WUS, oligosaccharides are generated with a purified endo-(1→4)-ß-D-xylanase from *Aspergillus awamori* and the primary structure of these oligosaccharides are elucidated (Chapter 7). In Chapter 8 a model for the major part of the arabinoxylans is proposed based on the abundance of different identified oligosaccharides present after enzymic degradation with different enzymes, the pattern of action of these enzymes, and the glycosidic linkage analysis of the arabinoxylans. The effect of the different xylanases on the breakdown of both extracted arabinoxylans and WUS is given in Chapter 9, together with their effects on water-holding capacity and baking characteristics.

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CHAPTER 2

Mild isolation of water-unextractable cell wall material from wheat flour. Composition of fractions obtained with emphasis on non-starch polysaccharides

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Summary

A method is described for the mild isolation from wheat flour of water-unextractable cell wall material (WUS), containing low amounts of starch and intracellular protein. The isolation procedure was based on the formation of a dough structure from which WUS was released by washing, followed by a wet-sieving procedure. A mass balance of the non-starch polysaccharides (NSP) over the various fractions obtained was made. WUS accounted for *c.* 50% of the total wheat flour NSP. Cold water-extractable cell wall material made up *c.* 30% of the total NSP. The remaining 20% was found in the starch and gluten fractions and as hot water-extractable cell wall material. WUS was obtained in two fractions, WUS1 and WUS2, which differed in size. WUS1 was recovered on a 32 μm sieve, whereas WUS2 passed through it. Composition studies revealed great similarities between WUS1 and WUS2. The WUS1 fraction consisted of approximately 93% NSP, 2% starch and 2% protein, whereas the WUS2 fraction contained more starch and protein. The protein present in the WUS fractions contained significantly more glycine than the protein in the other fractions. Besides NSP, protein and starch, the WUS fractions contained small amounts of ferulic acid and lipids. Only traces of uronic acids were detected.

Introduction

Non-starch polysaccharides (NSP) are believed to have an influence on the baking performance of wheat flours. Due to their high water-binding capacity they are considered to have an effect on loaf volume and dough development. Various studies on this effect, performed by adding isolated wheat endosperm cell wall fractions to (reconstituted) wheat flours, gave conflicting results as discussed by McCleary *et al*[1]. They studied the properties of NSP by adding purified xylanases to wheat flours. Addition of xylanases leads to a larger, mis-shapen loaf of bread. The baking properties of xylanase-treated doughs could be restored by adding guar flour.

For detailed studies on the structure, enzymic breakdown and physicochemical properties of NSP large amounts of intact and well defined NSP are necessary. Much attention has been given to water-extractable NSP. Even though the water-unextractable cell wall material (WUS) represents the larger part of the NSP present in flour, little attention has been given to this fraction. The presence of large amounts of intracellular protein and starch hinders the isolation of (relatively) pure WUS. Various isolation procedures for WUS from wheat flour have been developed. Recent isolations of WUS were based on centrifuging flour/water suspensions[2-4] and enzymic breakdown of protein and/or starch to obtain purified WUS. In two cases[2,3] the distribution of flour NSP among the fractions obtained was given. These isolations resulted in WUS preparations with a high protein (up to 34%) and glucose content (up to 28%).

An alternative method[5] is based on suspending wheat flour in 70% ethanol in water, followed by exhaustive sieving and ultrasonication to remove protein bodies and starch. The remaining starch is digested with salivary *alpha*-amylase. The resulting cell wall material consisted mainly of arabinose and xylose and contained about 10% protein. In the description of this isolation procedure[5] a quantitative distribution of the non-starch polysaccharides was not given.

Selvendran and Du Pont[6] reported that the use of ethanol in the isolation of WUS from cereals leads to co-precipitation of intracellular proteins and starch. In order to avoid this problem aqueous sodium deoxycholate, phenol/acetic acid/water and aqueous dimethylsulphoxide were used in the isolation of WUS from rye flour and wheat bran. They reported that the use of these organic solvents also resulted in solubilisation of some non-starch polysaccharides, which possibly originated from cell walls. Water-unextractable wheat proteins can be separated from water-unextractable cell wall material and starch by making use of gluten formation[7,8]. Gluten was obtained by the dough-kneading method and the starch and cell wall material were removed by handwashing. The starch and cell wall material was separated sequentially by centrifuging and wet-sieving. These isolations resulted in WUS preparations with varying amounts of protein (3-15%)[7,8] and starch (7-13%)[7]. No quantitative composition of the WUS or distribution of NSP composite sugars was given.

The main objectives of our study were: 1) To develop a method for the mild isolation

of WUS with minimum amounts of adherent intracellular material, 2) To determine the distribution of the NSP among the various fractions, including analysis of the NSP composition, 3) To analyse the non-carbohydrate components of WUS.

Experimental

Materials

White flour was prepared from wheat grain of *Triticum aestivum* cv. Arminda (1986 harvest), a soft milling wheat variety, using a Bühler MLU 202 laboratory mill. Six flour fractions were combined to obtain a 71% net extraction rate of straight-run flour (10.8% protein).

Isolation of WUS

Figure 1 shows a schematic diagram of the fractionation procedure for the isolation of WUS from wheat flour. To wheat flour (300 g) distilled water (168 ml) was added and the flour was kneaded in a Brabender farinograph. After 3.5 min distilled water was added to the dough at a flow rate of 13 ml/min for 2 min After this process, while still kneading, the dough was kept for 1 min to allow water to be absorbed. This addition/absorbing procedure was repeated 8-10 times until a starch/WUS slurry containing gluten strands was formed. Two batches of starch/WUS slurries were combined and subsequently the gluten was washed out (4 °C) and the water was combined with the starch/NSP slurry. The residual gluten was freeze-dried.

The starch/WUS slurry was filtered over a 32 μm sieve and washed with distilled water. The residue on the sieve was washed with distilled water and kept at 4 °C (as fraction WUS1). The filtrate (20 l) was centrifuged for 20 min at 6,100 *g*. After centrifugation two distinct layers were formed: a lower layer containing starch and an upper light brown layer. The starch layer was resuspended in distilled water and centrifuged again. The upper brown layer was combined with the former one and kept at 4 °C (as fraction WUS2). The resulting supernatants were combined and an aliquot was freeze-dried (CWE). The starch was resuspended in a small volume of water and freeze-dried (STARCH).

To remove residual starch the WUS1 and WUS2 fractions were each suspended in buffer solution (0.8 l; pH = 6.5) containing 10mM maleic acid - sodium maleate, 10mM sodium chloride, 1mM calcium chloride and 0.05% sodium azide. The suspension was heated at 63 °C for 1 h. After cooling to 30 °C, porcine *alpha*-amylase was added (Merck art. 16312, 10 units per g of residue) and the mixture was incubated for 30 h at 30 °C. The reaction mixture was then centrifuged for 20 min at 25,000 *g*. The *alpha*-amylase incubation was carried out twice. The residue after centrifugation was extracted for 1 h at

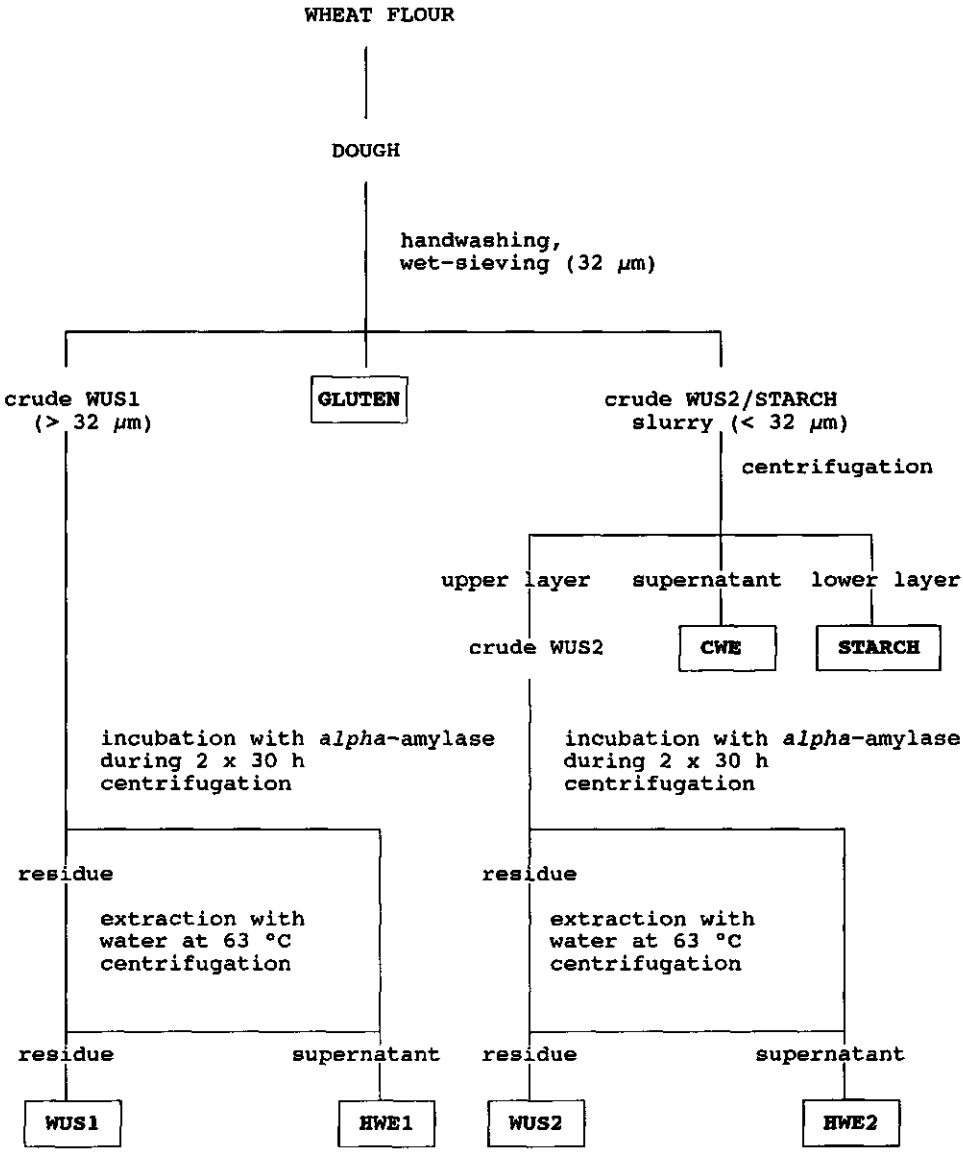

FIGURE 1. Fractionation procedure for the isolation of WUS from wheat flour.

63 °C with distilled water (1 l) and centrifuged (20 min, 25.000 *g*).

Extraction and centrifugation were repeated three times. The remaining residues were resuspended in a small volume of water and freeze-dried (WUS1, WUS2). The corresponding supernatants were combined and freeze-dried (HWE1,HWE2); no further purification of the HWE fractions was attempted. Two isolations (twice 2x300 g) were performed.

Analytical methods

Protein content/amino acid composition - The protein content (Nx5.7) was determined by a semi-automated micro-Kjeldahl method. Amino acid compositions were determined with a Biotronik LC 6000 E automatic amino acid analyzer. Samples were hydrolysed in 6M HCl for 21 h at 110 °C under nitrogen.

Lipid content - Samples (20 mg) were hydrolysed according to Anness[9] using margaric acid as internal standard. Subsequently lipids were extracted into chloroform and dried under nitrogen at 40 °C. Fatty acid methyl esters were prepared and analysed according to IUPAC[10] using screw cap tubes instead of distillation flasks.

Moisture contents were determined using the Karl-Fischer method.

Starch content was determined enzymically using the test kit supplied by Boehringer [Mannheim].

Phenolic acid content - WUS (20 mg) was treated with 0.5M KOH (5 ml; flushed with N_2) in a screw cap tube under N_2 at room temperature for 24 h. *p*-Hydroxybenzoïc acid was added as internal standard. 6M HCl (0.75 ml) was added to the mixture and the phenolic components were recovered from the acidified solution by extraction with ethylacetate (2x4 ml). The combined ethylacetate extracts were dried under N_2 at 40 °C. The residues were dissolved in methanol (1 ml) and transferred to 2 ml vials from which samples (20 μl) were injected for analyses by HPLC. A Spectra Physics SP8000 HPLC was used with a reversed phase Lichrosorb 10 RP18 column (Merck; 250 x 4.6 mm). The column was operated at room temperature and at a flow rate of 1.5 ml/min . The eluent was a gradient mixture of 4% (v/v) AcOH in MeOH (A) and 4% (v/v) AcOH in H_2O (B). The starting conditions were 10% A/ 90% B which were kept constant for 9 min. Then the gradient changed linearly to 50% A/ 50% B in 15 min and was kept in this ratio for the following 6 min. The eluent was monitored using a KRATOS spectroflow 773 UV detector set at 280 nm.

Uronic acid content - Uronic acids in WUS fractions were released by both 0.5M and 3M methanolic HCl and analysed as their trimethylsilyl derivatives according to Gerwig *et al*[11]. with minor modifications. The N-acetylation step was omitted. After derivatisation the mixture was evaporated to dryness and extracted with hexane. Samples (1 μl) were analysed by on-column injections on a fused silica capillary column (25 m x 0.34 mm; wall-coated with CPsil 5CB, Chrompack; Middelburg, The Netherlands) in a Carlo Erba Fractovap 4160 GC equipped with a F.I.D.detector set at 270 °C. The temperature was programmed from 130 - 175 °C at a rate of 1 °C/min.

TABLE I. Yield of flour fractions and NSP distribution over fractions[a]

		Fractions							
	FLOUR	CWE	GLUTEN	STARCH	WUS1	WUS2	HWE1[b]	HWE2[b]	TOTAL
Yield[c]	100	6.04	8.46	72.33	0.76	0.48	1.64	4.73	94.43
NSP[d]									
Rhamnose	0	0	0	0	0	0	0	0	0
Arabinose	0.74	0.25	0.01	0.04	0.21	0.13	0.05	0.04	0.72
Xylose	1.11	0.26	0.01	0.08	0.36	0.22	0.08	0.06	1.08
Mannose	0.05	0	0	0	0.02	0.01	0.01	0	0.03
Galactose	0.15	0.14	0.02	0	0.01	0	0	0	0.17
Glucose	0.34	0.03	0.01	0.02	0.12	0.03	0.04	0.04	0.28
Total	2.40	0.67	0.04	0.14	0.70	0.40	0.18	0.15	2.29

[a] Average of two isolations.
[b] The figures are corrected for the presence of buffer salts.
[c] Weight percentage (d.s.).
[d] NSP-sugars present in fractions based on 100 g flour (d.s.); sugars released on 72%-1M H_2SO_4.

Neutral sugar content Neutral sugars in flour, CWE, STARCH, HWE1 and HWE2 fractions were analysed as total non-starch polysaccharides[12] using inositol as internal standard and pretreatment with 72% (w/w) H_2SO_4 for 1 h at 30 °C prior to hydrolysis with 1M H_2SO_4 for 3 h at 100 °C. Neutral sugars in the WUS fractions were released directly by 72% (w/w) H_2SO_4 and/or 1M H_2SO_4 for 3 h at 100 °C, and analysed as their alditol acetates, whereas the GLUTEN fraction was first extracted with 4% (v/v) DMSO/ 16% (v/v) H_2O/ 80% (v/v) EtOH prior to hydrolysis with 72%-1M H_2SO_4. Non-starch glucose content in the WUS and GLUTEN fractions was determined from the difference between glucose content found with alditol acetates and with the Boehringer testkit. Cellulosic glucose was calculated as the difference between the contents of glucose obtained with and without the prehydrolysis step. Samples were checked for the presence of endogenous inositol. Alditol acetates were separated on a 3 m x 2 mm i.d. glass column (packed with Chrom WAW 80-100 mesh coated with 3% OV275) in a Carlo Erba Fractovap 2300 GC operated at 200 °C and equipped with a F.I.D. detector set at 270 °C.

Results and discussion

Yields of fractions and NSP distribution over the fractions

Table I shows the yield of the fractions and the distribution of NSP among the fractions (dry matter). Two isolations were performed. The variation in yield and NSP-

sugar content was 5-10% for the various fractions obtained. The overall yield amounted to 95%. The crude WUS1 and WUS2 fractions represent *c.* 1.2% of the total flour weight. The extracted starch made up *c.* 72% of the total flour. Digested and partially digested starch was still present in the two HWE fractions. The residual gluten after extensive washing accounted for *c.* 8% of the flour. The cold water-extractable material in the flour accounted for *c.* 6% of the flour.

The sum of the total NSP analysed in all fractions was lower than the total amount of NSP found in the flour; 2.29 and 2.40% respectively. The glucose content especially differed. This may be due to insufficient starch dispersion (and breakdown) in the flour during the Englyst procedure[12]. The WUS1 and WUS2 fractions together contained *c.* 48% of the total NSP-sugars analysed. The "squeegee or WUS" fraction obtained by Markwalder[2] accounted for 70% of the recovered NSP-sugars whereas Abdel-Gawad[3] found 73% of the total NSP present in his WUS fraction. These authors[2,3] found a total NSP content of 3.56 and 3.10%, respectively. The difference in the total amount of NSP-sugars is partly due to the high glucose content in their WUS fractions. Both authors ascribed this glucose totally to ß-glucans. No consideration was given to the presence of residual starch in their WUS fractions.

We performed a second series of two isolations in order to see whether the low yield of WUS material could be increased relative to the results reported previously[2,3]. With respect to future large-scale isolation of WUS1 we specifically tried to increase the yield of this fraction by shortening the washing and sieving procedure. An increase in the yield of WUS2 was attempted by increasing the centrifugal force (25,000 *g*) during the separation of the starch/WUS2/CWE slurry. The results (not shown) revealed that less washing leads to a higher yield of WUS1 (0.84 g) but this increase is caused by a higher starch content in this fraction since the mass balance revealed an equal amount of NSP in the WUS1 fraction (0.70 g). Due to the higher centrifugal force the crude weight and NSP content of the WUS2 fraction increased to 0.76 g and 0.64 g, respectively. This was

TABLE II. Composition of flour fractions[a]

	CWE	GLUTEN	STARCH	WUS1	WUS2	HWE1[b]	HWE2[b]
NSP	11.1	0.5	0.2	93.1	82.3	11.3	3.0
starch[c]	26.2	5.7	96.9	2.5	4.1	69.6	92.2
protein	32.5	87.1	0.3	2.5	5.3	8.9	4.3
lipids	-	-	-	0.7	2.5	-	-
ferulic acid	-	-	-	0.33	0.17	-	-
total	69.8	93.4	97.3	99.0	94.4	89.8	99.4

[a] Weight percentage (d.s.); average of two isolations.
[b] The figures are based on dry weight basis, corrected for the presence of buffer salts.
[c] Including degraded starch and/or free glucose.

accompanied by a decrease of NSP content in the STARCH fraction indicating a better separation of the slurry. The total amount of NSP found in all fractions was 2.37 g. With this isolation procedure the two WUS fractions account for 57% of the total NSP. These results are in better accordance with the work of both Markwalder[2] and Abdel-Gawad[3], especially considering the fact that both authors did not take into account the NSP present in their STARCH fractions and the possible presence of residual starch in their WUS fractions.

Table I shows that the CWE fraction (cold water-extractable material) contains *c.* 29% of the total NSP. This is in accordance with Meuser and Suckow[13] who reported that *c.* 25% of the total NSP in wheat flour is water extractable. With methods used in this study fructosans will accumulate in the CWE fraction. Since fructosans in wheat flour can be removed with boiling 80% ethanol[14], most of the fructosans are likely to have been removed during the Englyst procedure[12]. Furthermore, non-removed fructosans would have been converted with low efficiency to the alditolacetates of glucose and mannose. Since no mannose was detected in this fraction, significant interference of fructosans with the NSP distribution is not likely.

Composition of the fractions

Table II presents the NSP, starch and protein content of all fractions. The content of ferulic acid and lipids is also given for the WUS fractions. A typical lipid analysis of all fractions originating from one isolation is discussed later. The ferulic acid content was not determined in the other fractions. Since the results of the molar non-starch sugar, amino acid, and lipid composition showed minor variations the results of both series of two isolations were used in calculating the standard deviations and mean values (Table III, IV and V). In total over 89% of each fraction is accounted for by the components analysed with the exception of the CWE fraction. This fraction contains water-extractable components present in the flour other than protein, neutral sugars and lipids. This may explain the low recovery of materials found in the CWE fraction.

Carbohydrate composition. Table III shows the molar non-starch neutral sugar composition of each fraction. For the CWE, WUS and HWE fractions the results show small variations between the four replicates isolated. Larger variation was found in the STARCH and GLUTEN fractions. The amount of NSP was very low for these fractions (Table II) and therefore the analysis of the NSP composite sugars is less accurate. The use of inositol as internal standard was acceptable, since only traces of endogenous inositol (originating from phytate) could be detected in the WUS fractions. No endogenous inositol was detected in the other fractions using the Englyst procedure[12].

The WUS1 and WUS2 fractions differed mainly in their content of glucose. The remaining starch content was 2 to 4%. The arabinose/xylose (Ara/Xyl) ratio for WUS1 and WUS2 was 0.58 and 0.56, respectively. Previous studies on WUS from wheat

TABLE I. Yield and neutral sugar composition of wheat flour WUS and various extracts

		Extracts										
				Na_2CO_3		$NH_2OH.HCl$			MMNO		$Ba(OH)_2$	
	WUS	DMSO	Urea (8M)	4°C	20°C	pH5.0	pH7.2	NaOH (1M)	E1[a]	E2[a]	BE1[b]	BE2[b]
Molar composition (%)												
Arabinose	30.7	19.6	28.1	34.9	36.2	35.3	32.9	33.1	32.8	3.4	34.3	32.8
Xylose	54.7	35.1	47.0	63.4	62.0	61.5	62.8	59.0	59.1	7.8	65.4	48.7
Mannose	2.1	1.3	6.2	0.2	0.2	0.2	0.1	0.5	0.6	19.2	0.1	0.9
Galactose	0.6	2.5	1.0	0.2	0.2	0.2	0.2	0.3	0.3	0.9	0.1	0.7
Glucose	11.9	41.5	17.8	1.3	1.3	2.8	3.9	7.1	7.2	68.4	0.3	17.0
Total sugar content[c]	90.5	47.9	45.8	80.9	88.2	85.7	92.9	87.2	87.0	93.2	91.6	92.6
Yield[d] (%)	100	11	8	11	13	42	78	83	85	7	68	11

[a] E1: soluble fraction after dialysis; E2 insoluble fraction after dialysis.
[b] BE1: fraction solubilised directly after extraction; BE2: fraction solubilised after neutralising the $Ba(OH)_2$ residue.
[c] Expressed as weight percentage (as is basis) of each extract.
[d] The yield is expressed as weight percentage (as is basis) of WUS.

TABLE IV. Amino acid composition (mole %) of flour and flour fractions (mean ± S.D.)[a]

Amino	flour	Fractions (mean ± S.D.)					
		CWE	GLUTEN	WUS1	WUS2	HWE1	HWE2
Asx	4.3	6.5±1.1	3.1±0.0	7.7±0.2	7.9±0.1	5.4±0.8	6.4±0.4
Thr	3.4	3.6±0.3	2.6±0.0	5.5±0.1	5.2±0.3	4.7±0.3	4.8±0.3
Ser	5.5	5.6±0.2	6.0±0.0	5.4±0.2	6.8±0.5	5.4±0.7	5.9±0.7
Glx	35.8	29.6±1.1	38.2±0.2	10.7±0.7	9.7±0.0	30.3±1.4	22.3±1.4
Gly	5.8	6.0±0.2	5.6±0.1	17.1±1.5	13.1±0.8	7.9±0.7	9.1±0.5
Ala	4.2	6.8±1.1	3.5±0.1	8.4±0.3	8.8±0.2	5.3±0.3	7.2±0.3
Val	4.4	5.5±0.2	4.1±0.0	5.9±0.2	6.9±0.3	5.2±0.2	5.8±0.3
Met	0.7	0.8±0.3	0.4±0.0	1.1±0.1	1.2±0.1	0.8±0.1	0.8±0.3
Ile	3.3	3.5±0.1	3.4±0.0	2.9±0.2	3.7±0.3	3.3±0.0	3.6±0.1
Leu	6.8	7.1±0.1	6.7±0.0	8.1±0.1	10.1±0.1	7.2±0.1	7.5±0.1
Tyr	2.7	2.7±0.1	2.8±0.1	2.5±0.1	2.5±0.1	1.2±0.4	2.2±0.1
Phe	3.9	3.7±0.0	4.1±0.0	3.3±0.1	4.0±0.1	3.9±0.0	3.6±0.1
Lys	1.8	2.5±0.1	1.5±0.0	4.7±0.3	4.9±0.7	2.5±0.3	4.6±0.5
His	1.7	1.8±0.0	1.7±0.0	3.1±0.2	1.9±0.1	2.3±0.1	2.1±0.1
Arg	2.7	3.4±0.0	2.4±0.0	5.6±0.2	6.9±0.2	3.3±0.1	5.6±0.3
Pro	13.0	11.0±0.2	13.9±0.1	6.7±0.2	5.8±0.4	11.3±0.5	8.5±0.5
Hyp	0.0	0.0±0.0	0.0±0.0	1.2±0.1	0.4±0.2	0.0±0.0	0.0±0.0

[a] Average of four isolations.

TABLE V. Fatty acid molar composition (%) of lipids from flour and flour fractions

	FLOUR	Fractions						
		CWE	GLUTEN	STARCH	WUS1[a]	WUS2[a]	HWE1	HWE2
Myristic acid (C14:0)	0	0	0	0	0.8	1.0	0	0
Palmitic acid (C16:0)	27.2	22.2	22.9	36.3	43.4	68.8	36.3	40.3
Palmitoleic acid (C16:1)	0	0.6	0.2	0	1.0	1.3	1.4	0.6
Stearic acid (C18:0)	1.0	1.3	1.8	0.9	3.9	3.1	2.4	1.8
Oleic acid (C18:1)	7.7	11.2	10.3	6.6	16.1	13.5	10.9	11.0
Linoleic acid[b] (18:2)	64.0	64.6	64.8	56.2	34.6	12.3	49.0	46.4
Lipid content[c]	1.07	1.22	4.75	0.64	0.70	2.43	0.52	1.50

[a] Average of four isolations.
[b] Linolenic acid (C18:3) could not be determined quantitatively.
[c] Weight percentage (d.s.).

TABLE VI. Neutral sugar composition of WUS fractions[a,b]

	WUS1		WUS2	
Rhamnose	0.1		0.1	
Arabinose	27.5		26.2	
Xylose	47.3		46.7	
Mannose	2.0		1.2	
Galactose	0.7		0.9	
Glucose	15.5	(8.6)	7.2	(3.5)
Total	93.1		82.3	

[a] Weight percentage (d.s.); average of two isolations.
[b] Sugars released on 72%-1M H_2SO_4 hydrolysis; the figures in parentheses represent the glucose determined with 1M H_2SO_4.

flour[2-4] revealed an Ara/Xyl ratio varying from 0.61 to 0.68. Mares and Stone[5] found no significant differences in Ara/Xyl ratio of endosperm cell walls of three different varieties. Wheat bran arabinoxylans are reported to be more branched[6]. Contamination of the flour by bran particles will influence the Ara/Xyl ratio of the WUS. Therefore the lower Ara/Xyl ratio in our WUS could be due to different milling characteristics of Arminda flour, resulting in less bran contamination of the flour.

In addition to arabinose, xylose and glucose the WUS fractions contain some mannose. This could be derived from a ß-glucomannan which has been found in wheat endosperm cell walls[5]. A lower amount of mannose was present in the WUS fractions compared to data published previously[2-5]. Table VI shows that approximately half of the non-starch glucose could not be hydrolysed when the pretreatment with 72% H_2SO_4 was omitted. This indicates that half of the non-starch glucose is of a cellulosic nature. Although 1M H_2SO_4 hydrolysis might release some cellulosic glucose[15], cellulose is generally quantified as the difference in glucose content obtained with and without the prehydrolysis step. The presence of cellulose in endosperm cells was ascertained using the chlor-zinc-iodide staining method[16]. The other half of the non-starch glucose is most likely present as (1→3,1→4)-ß-glucans, which have been found in wheat endosperm cell walls previously[17].

The WUS1 fraction contained more cellulosic glucose as well as hemicellulosic glucose compared to WUS2 fraction, whereas the ratio of cellulosic/hemicellulosic glucose is roughly equal for both fractions. Microscopic studies (phloroglycine HCL; data not shown) revealed that the WUS1 fraction contains more non-endosperm particles than the WUS2 fraction. Visually, the degree of contamination was low. Previous studies on wheat bran[6,18] showed cellulose to be the predominant glucose polymer. Based on experiments with similarly extracted (water; 70 °C) wheat bran we have strong indications for the presence of substantial amounts of hemicellulosic glucose next to cellulose. Next to this, isolated aleurone cells which make up half the amount of wheat bran are found to contain significant amounts of (1→3,1→4)-ß-glucans[19]. Therefore the higher glucose content of WUS1 is likely to be due to bran contamination.

The CWE fraction contained a large amount of galactose in addition to arabinose and xylose. This corresponds with previous investigations on water-extractable NSP[12],[20]. These investigations showed that galactose was present as a constituent of an arabinogalactan-peptide complex in wheat flour.

The GLUTEN fraction contained, next to 6% starch, 0.5% non-starch polysaccharides, representing 2% of the total NSP present in flour (Table I and II). Application of the Englyst procedure[12] to gluten led to insufficient dispersion of the starch and therefore the GLUTEN fractions were first extracted with 4% DMSO/16% H_2O/80% EtOH prior to analysis. When GLUTEN was analysed directly galactose was predominantly present as non-starch neutral sugar (Table VII). The presence of galactose has been previously ascribed to the presence of arabinogalactans[3,21] and glycolipids (digalactosyldiglyceride)[22] in gluten. We extracted gluten separately with hexane and chloroform/methanol 1/1 (v/v)

TABLE VII. Sugar composition[a] of gluten, defatted gluten and extracted lipids[b]

	GLUTEN[c]	Hexane residue	MeOH/CHCL3 residue	Hexane extract	MeOH/CHCl3 extract
Molar composition (%)					
rhamnose	0	0	0	0	0
arabinose	4.7 (14.1)	4.3	4.4	0	0
xylose	1.8 (5.7)	1.6	1.8	0	0
mannose	1.3 (4.4)	1.3	1.2	0	0
galactose	19.7 (62.2)	19.2	5.4	100	87.5
glucose	72.4 (13.6)	73.7	86.7	0	12.5
Neutral sugar content[d]	4.1 (1.4)	3.9	4.1	0.02	0.15

[a] Sugars released on 72%-1M H_2SO_4 hydrolysis, including starch.
[b] Extraction in a soxhlet for 3 h.
[c] The figures in parentheses represent the molar sugar composition corrected for the presence of starch.
[d] Weight percentage, extracts are calculated as weight percentage of original gluten this cell wall material.

and analysed both residue and extract for neutral sugar composition (including starch). The results are given in Table VII. The hexane extract contained galactose as the only neutral sugar. Extraction with chloroform/methanol resulted in substantially lower amounts of galactose in the residue. Due to clotting of the gluten during chloroform/methanol extraction no quantitative extraction with this extractant could be performed. Nevertheless, these results confirm the presence of galactolipids in the GLUTEN fraction. These galactolipids will be extracted during the Englyst procedure[12] (or with 4% DMSO/16% H_2O/80% EtOH) and therefore not contribute to the NSP content of flour and gluten. Although, due to the low NSP content, the sugar analysis is not very accurate the NSP composition (Table III) suggests the presence of an arabinogalactan and arabinoxylan in

gluten which are not removed during handwashing. The large variation in glucose content suggests that the relatively high glucose content is likely due to errors in the correction for starch content rather than representing a relatively high amount of (hemi)cellulosic glucose. The HWE fractions have the same molar neutral sugar composition as the WUS fractions except for a somewhat higher glucose content (Table III). The glucose may be present as a constituent of hot water-extractable (1-3,1-4)-ß-glucans. The Ara/Xyl ratio is *c.* 0.60. The ratio tends to be higher than the Ara/Xyl ratio in the WUS1 and WUS2 fractions (0.58 and 0.56, respectively).

Protein content/amino acid composition The protein content of the various fractions is given in Table II and the amino acid composition is given in Table IV. Because of the low protein content in the STARCH fraction no amino acid analysis was attempted for this fraction. The WUS2 fraction contained more protein compared to the WUS1 fraction: 5.3 and 2.5%, respectively. This higher protein content suggests contamination with intracellular material. Since the amino acid composition of WUS2 does not show a higher glutamic acid and proline content relative to WUS1, this contamination is not likely to originate from gluten. The additional amount of protein in the WUS2 fraction is probably inherent to the isolation procedure (e.g. centrifugation).

The protein content of the WUS fractions is substantially lower compared to those of previous isolations. Using centrifugation, Markwalder[2] found a protein content of 34% in his cell wall material and Abdel-Gawad[3] isolated WUS containing approximately 17% protein. Mares and Stone[5] used 70% ethanol in water instead of water as extraction medium and calculated the protein content to be 10%, however co-precipitation of intracellular protein took place. A second isolation[17] without the use of *alpha*-amylase did result in cell wall material with a low protein content, 4.7%. The sugar composition of the WUS however differed from the previous isolation. When water is used for the isolation of WUS from wheat flour the development of a gluten structure is presumably necessary for preventing contamination with intracellular protein.

The WUS fractions differ markedly from the other fractions with respect to their low glutamic acid (and proline) content and their high glycine content. The high amount of glutamic acid found in the other fractions is in agreement with the literature[13,23]. The amino acid composition, except for the glycine content, was similar for both WUS fractions. The presence of high amounts of glycine in the WUS fractions is possibly specific for this cell wall material. Cell wall material with quite similar amino acid composition is found in (beeswing) wheat bran[6,24] and wheat endosperm cell walls[25]. The amount of glycine found in our fractions is however significantly higher. Since the formation of a dough structure enables interaction of polysaccharides and glutenins[26], glutenin-polysaccharide complexes may be present in the WUS fractions. The amino acid composition of glutenin subunits of Arminda flour[27] reveals a roughly equal amount of glycine compared to the WUS1 fraction. However, the amount of glutamic acid in the WUS1 fraction is significantly lower relative to glutenin[27], indicating the possible presence

of a structural cell wall protein. Bacic and Stone[19] found relatively high amounts of glycine present in aleurone cell walls although earlier studies[28] revealed that the major part of the protein is of intracellular origin. The relatively high glycine content in the WUS fractions may lead to the conclusion that in wheat endosperm a glycine-rich protein is a structural cell wall component. This is consistent with the work of Condit and Meagher[29] who stated that glycine rich proteins are structural cell wall components and will be found in a wide variety of plants. Low amounts of hydroxyproline are present in both WUS1 and WUS2. This suggests the absence of substantial amounts of extensin or loosely associated arabinogalactan-proteins[30].

Minor components. Ferulic acid (4-hydroxy-3-methoxycinnamic acid) and p-coumaric acid (4-hydroxycinnamic acid) have been found in wheat cell walls[2,25] and in particular wheat bran cell walls[31]. As listed in Table II, ferulic acid is found in both WUS fractions. For WUS1 the content is 0.33% and for WUS2 0.17%. p-Coumaric acid is only found in WUS1 (0.007%, data not shown). According to Pussayanawin and Wetzel[31] the higher ferulic acid content of WUS1 compared to WUS2 would be due to the presence of bran particles. The amount of ferulic acid in the flour was 0.006% (data not shown). This means that approximately half of the total amount of ferulic acid present in flour is found in the two WUS fractions.

The total lipid content and fatty acid composition are given in Table V. According to Anness[9] the lipid content refers to compounds containing fatty acids within their structure. Linoleic acid usually makes up more than half of the fatty acid content of wheat and flour milling products[32]. As showed in Table V, this is found for the several flour fractions obtained with the exception of the WUS fractions. Because of the sensitivity of linoleic acid to oxidation several extraction conditions were compared. The highest recovery of linoleic acid was achieved at the selected hydrolysis conditions, which is in agreement with the findings of Anness[9]. Therefore the different fatty acid composition seems to be characteristic for the isolated cell wall material. The total fat content of WUS1 and WUS2 is 0.7 and 2.5% respectively. The molar fatty acid composition between these fractions differ markedly in palmitic and linoleic acid content. On a weight basis, WUS2 contains more palmitic acid and oleic acid compared to WUS1. Markwalder[2] found in his WUS fraction 7.7% lipids present. Our results indicate that isolating WUS by a wet-sieving procedure (e.g. WUS1) leads to lower lipid content of the WUS.

Traces of galacturonic acid and glucuronic acid seemed to be present in both WUS fractions even though uronic acids could not be determined accurately due to the presence of high amounts of neutral sugars. No influence of the hydrolysis conditions on the uronic acid content could be detected. Mares and Stone[5] used an enzymic assay to determine uronic acid content. Although no uronic acids could be determined in the cell wall material they found 1.5% uronic acids in KOH-extractable and unextractable cell wall material.

Conclusions

It can be concluded that for the mild isolation of highly purified WUS the formation of a gluten structure is necessary to prevent contamination with intracellular proteins. Starch can be largely removed by extensive washing. Since the mass balance reveals which part of the total flour NSP is accounted for as WUS, we now have developed a mild method to obtain well defined, highly purified water-unextractable cell wall material. Both the gluten formation and the sieving procedure can be scaled up to yield large quantities of WUS1.

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CHAPTER 3

Large-scale isolation of water-unextractable cell wall material from wheat flour

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Summary

Water-unextractable cell wall material (WUS) from wheat flour was isolated on a large scale using a dough-kneading method. The method involved the formation of a dough which is transformed into a slurry from which the WUS, gluten and starch were separated on a stack of sieves under extensive washing. The WUS was reclaimed on the 32 and 50 μm sieves and residual starch was removed by *alpha*-amylase digestion. With the method described 100 g quantities of WUS could be isolated. The WUS comprised of 95% non-starch polyssaccharides, 2.4% protein, 0.6% lipids, 0.2% residual starch and 0.3% ferulic acid.

Introduction

Non-starch polysaccharides (NSP) are believed to have an influence on the baking performance of wheat flours, due to their high water-binding capacity. Much attention has been given to the isolation and characterization of water-extractable NSP. The chemical structure as well as the physico-chemical and baking properties of the water-unextractable NSP have been less well characterized. In order to study the relation between chemical structure and physico-chemical/baking properties, large amounts of water-unextractable cell wall material (WUS) are necessary.

Various procedures have been reported for the isolation of water-unextractable cell wall material. Most isolations involve centrifugation of flour/water suspensions[1-4] or dough washings[5], resulting in a sludge layer which is further purified. In order to get a distinct sludge layer, high centrifugation speeds must be applied which means that only small volumes can be centrifuged at a time. Alternative isolation methods were based on wet-sieving and ultrasonication in aqueous ethanol[6] or removal of starch and intracellular protein by organic solvents[7]. Most of the isolation methods yield cell wall material which contains large amounts of intracellular protein and/or starch. Usually quantities up to 5 g of cell wall material are obtained. The centrifugation of large amounts of material as a first step of the isolation procedure hinders rapid upscaling of the procedure. Therefore, when larger quantities of WUS are required these methods become very labour-intensive. Recently we reported the isolation of highly purified WUS based on dough washing followed by wet-sieving[8]. This method circumvents the labour-intensive centrifugation as a first step of the isolation procedure.

In this study we describe the upscaling of this previous reported method to yield 100 g quantities of WUS.

Experimental

Flour

Wheat flour was prepared from grain of *Triticum aestivum* cv. Arminda (1986 harvest), a soft milling wheat variety, using a Bühler MLU 202 laboratory mill. Six flour fractions were combined to obtain a 71% net extraction rate of straight-run flour. On a dry weight basis the flour contained 10.8% protein (Nx5.7).

Isolation of WUS

Figure 1 shows a schematic diagram of the WUS isolation procedure. A dough was prepared by mixing 5 kg of flour and 1.5 l distilled water in a Hobart type D 300 mixer. After 30 and 50 s, respectively, 0.76 and 0.38 l distilled water were added. The dough was kneaded for 7 min. Next, distilled water was added to the dough at a flow rate of 100

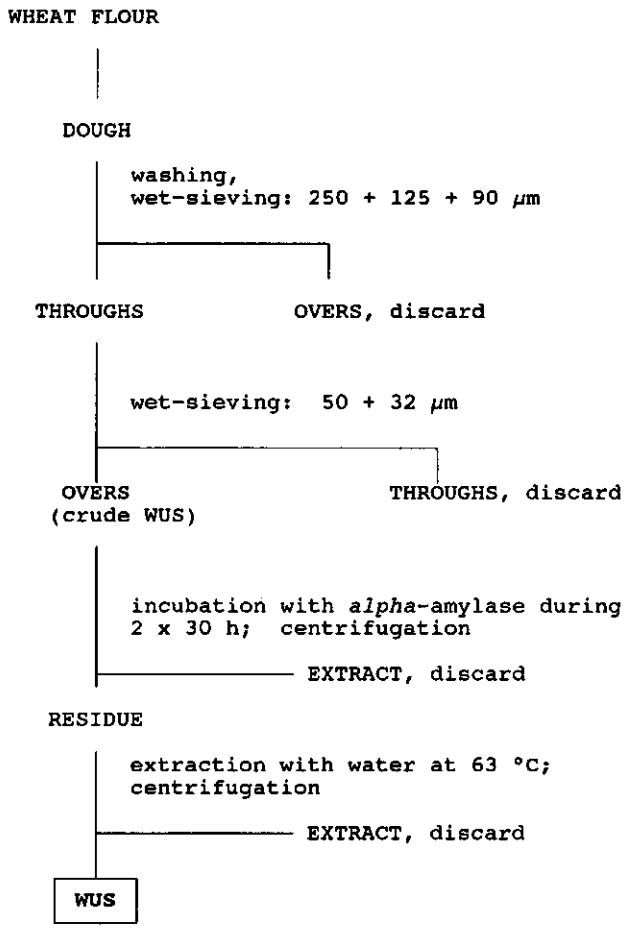

FIGURE 1. Schematic diagram for the large-scale isolation of WUS from wheat flour.

ml/min for 1.5 min. After this addition, the dough was kneaded for 1 min to allow the water to be absorbed. This addition/absorbing process was repeated three times. Then the addition and absorbing times were changed to 1 and 0.75 min, respectively. After 50 min this procedure was stopped. Next, the gluten/starch/WUS slurry was diluted with 10 l of distilled water and further separated as described by Weegels *et al*[9]. Following this procedure the gluten/starch/WUS slurry was pumped onto a Vortair Vibrating sieve (HEUB 90-S4, BBC KEG;4) onto which a stack of five sieves of 250, 125, 90, 50 and 32 μm was mounted. Fresh tap water was sprinkled over the sieves for washing.

Five runs of 5 kg flour were processed sequentially and the fractions on the 32 and 50 μm sieves were combined (crude WUS slurry). Residual starch in this fraction was removed as described previously[8]. For this purpose, the crude WUS slurry was diluted to 10 l, buffered to pH 6.5 and heated to 63 °C for 1 h. After cooling to 30 °C the mixture was incubated for 30 h with 33 mg *alpha*-amylase (Merck no. 16312) and centrifuged.

The *alpha*-amylase activity amounted to 303 U/mg. One unit was expressed as the amount of enzyme liberating one μmol reducing endgroups per minute from soluble potato starch (Merck no. 1252) at pH 6.5 (sodium maleate buffer) and 30 °C under saturated substrate conditions. Maltose (Merck no. 5912) was used as a reference. The resulting residue was resuspended in buffer, incubated with *alpha*-amylase and centrifuged. The residue was four times extracted with water (2 l; 63 °C) and freeze-dried (WUS). The *alpha*-amylase showed no activity towards arabinoxylan and (1→3,1→4)-ß-glucan.

Analysis

The WUS fraction was analyzed for starch content, protein content (Nx5.7), lipid content, ferulic acid content, neutral sugar composition and amino acid composition as described previously[8]. (1→3,1→4)-ß-Glucan content was determined enzymically using the test kit supplied by Biocon Ltd. The liberated glucose was determine enzymically using the glucose test kit from Boehringer (cat. no. 716251).

Results and Discussion

Table I shows the yield and composition of the large-scale isolated WUS together with the previous published data on small-scale isolated WUS. The two fractions differed mainly in residual starch content. On large-scale isolation a lower starch content, 0.2%, was found. This was attributed to the extensive washing which could be performed using the pilot plant sieving set. Previous studies[8] have revealed that extensive washing results in a lower residual starch content.

The NSP composition of both fractions is very similar. When isolated on a large scale, the WUS contains *c.* 95% NSP. This NSP consists mainly of xylose, arabinose and glucose which are present as arabinoxylans and ß-glucans[6]. The figures in parentheses represent the amount of glucose found on 1M H_2SO_4 hydrolysis after correction for the residual starch content, indicating it to be of non-cellulosic origin. This is in correspondence with the (1→3,1→4)-ß-glucan content (7.7%, no further data shown) we found for the large-scale isolated WUS with the Biocon testkit. Therefore, approximately half of the glucose is present as (1→3,1→4)-ß-glucans, the other half probably being a mixture of glucomannans and cellulose[6].

Both large- and small-scale isolated WUS fractions had similar protein contents of 2.4 and 2.5%, respectively (Table I). The amino acid data for both fractions (Table II) likewise showed similar compositions except for somewhat lower glycine and glutamic acid contents of the large-scale isolated WUS. The porcine *alpha*-amylase was tested for proteolytic activities under the conditions used to remove starch. Although it showed some breakdown of casein, no solubilization of wheat gluten could be measured with E_{280}. Analysis of protein according to Sedmak and Grossberg[10] determined that less than 0.02%

of wheat gluten was solubilized. Therefore, the relative high glycine content is likely typical for wheat cell wall material[8].

The yield of large-scale isolated WUS is *c.* 0.42% of the flour. With small scale isolation using only a 32 μm sieve we previously found a yield of 0.76%[8]. The reason for this difference is that a substantial part of the cell wall material was retained on the larger sieves (250, 125 and 90 μm) used in the present study. These sieves not only retain gluten particles which are loosened during the dough washing but also retain larger WUS particles. We only used material retained on the 32 and 50 μm sieves (these were visually free of gluten particles) and thereby introduced a lower yield of WUS.

TABLE I. Composition of isolated WUS (% weight)

Material	Large-scale Isolation	Small scale Isolation[a]
NSP[b]	94.7	93.1
rhamnose	0.2	0.1
arabinose	28.2	27.5
xylose	49.6	47.3
mannose	2.3	2.0
galactose	0.7	0.7
glucose	13.7 (8.3)	15.5 (8.6)
Starch	0.2	2.5
Protein	2.4	2.5
Lipids	0.6	0.7
Ferulic acid	0.3	0.3
Total	98.1	99.0
Yield[c]	0.42	0.76

[a] Data from Gruppen *et al*[8].

[b] Sugars released on 72%-1M H_2SO_4 hydrolysis; the figures in parentheses represent the glucose content determined with 1M H_2SO_4 hydrolysis.

[c] Percentage WUS recovered from flour.

Highly purified WUS has been previously isolated by Kim and D'Appolonia[3] by making use of centrifugation and alkali treatment. They obtained a WUS yield of 0.2%, starting with 200 g of flour. The NSP composition of their WUS resembled ours. However the alkali treatment implicates that ester bonds within the WUS are broken during the isolation. Therefore substantial information about alkali-sensitive linkages (e.g. feruoyl and acetyl groups) in the WUS is lost. In the present study all alkali-sensitive linkages are still present in the WUS.

The above results show, that for Arminda flour we can isolate highly purified WUS on a large scale. With five runs of 5 kg flour we prepared 2-3 l crude WUS suspension within one day, yielding 100 g of purified WUS after *alpha*-amylase treatment. The main advantage of this method is that by making use of the pilot plant sieving set the labour-

TABLE II. Amino acid composition (mole %) of WUS

Amino acid	Large-scale isolation	Small scale isolation[a]
Asx	7.9	7.7
Thr	5.3	5.5
Ser	6.7	5.4
Glx	9.2	10.7
Gly	14.1	17.1
Ala	9.0	8.4
Val	6.3	5.9
Met	1.7	1.1
Ile	3.2	2.9
Leu	8.7	8.1
Tyr	1.9	2.5
Phe	3.3	3.3
Lys	4.8	4.7
His	2.6	3.1
Arg	5.5	5.6
Pro	8.1	6.7
Hyp	1.5	1.2

[a] Data from Gruppen *et al*[6].

intensive centrifugation of large amounts of flour/water slurry can be avoided. In addition, extensive washing, necessary for obtaining highly purified WUS, can be done. The resulting crude WUS suspension has a relatively small volume and can further be purified. This purification includes prolonged *alpha*-amylase treatment followed by removal of the starch digest by washing with water using centrifugation. Although time-consuming, the *alpha*-amylase incubation requires minimal manuel input. The subsequent removal of the starch digest is more labour-intensive but can, due to the relatively small volume of crude WUS suspension, easily be carried out on laboratory scale.

We also prepared WUS from a commercial soft wheat flour blend ('Zeeuwse bloem') and analyzed starch and protein content. With this flour we found similar low amounts of residual starch and protein; 0.15% and 2.3% respectively. In order to see whether WUS could be isolated from hard wheat varieties we also used Katepwa flour (Canadian Red Spring) and a Northern Spring wheat flour blend. In both cases we could isolate large amounts of crude WUS (before *alpha*-amylase digestion) indicating a good separation of gluten and non-starch polysaccharides.

This study indicated that highly purified water-unextractable cell wall material can be obtained in large quantities by making use of dough washing followed by wet-sieving and *alpha*-amylase treatment.

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CHAPTER 4

Barium hydroxide as a tool to extract pure arabinoxylans from water-unextractable cell wall material of wheat flour

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Summary

Water-unextractable cell wall material (WUS) prepared from wheat flour was extracted with various solvents. The extracts were compared in terms of yield, sugar composition, amino acid composition and molecular weight distribution.

With 1M NaOH, 1M hydroxylamine hydrochloride, saturated $Ba(OH)_2$ and 4-methylmorpholino-N-oxide (MMNO) most of the WUS was extracted, whereas 0.05M Na_2CO_3, 8M urea and dimethylsulphoxide (DMSO) only extracted a small part. Fractions enriched in glucan content could be extracted using MMNO and DMSO. Pure arabinoxylans were extracted in limited amounts with Na_2CO_3. $Ba(OH)_2$ extraction, however, yielded a pure arabinoxylan fraction which represented *c.* 80% of all the arabinoxylan present in the WUS and a second fraction containing arabinoxylans and glucans.

The various extracts showed almost no differences in protein contents and amino acid compositions. Most extracts had similar molecular weight distribution patterns. Light scattering analyses showed the molecular weight of these fractions to be *c.* 850,000.

Application of $Ba(OH)_2$ extraction to wheat flour WUS from other wheat varieties, wheat bran, oat spelt xylan and starch-containing wheat flour WUS also resulted in selective extraction of arabinoxylans. The presence of $NaBH_4$ in the saturated $Ba(OH)_2$ solution appeared to be important for the selective extraction.

Introduction

The components of cell walls from wheat flour are only partly extractable in water. So far research has been predominantly focused on water-extractable components. Water-unextractable cell wall material (WUS) has been less well characterized because of the problems of obtaining them in a purified form[1,2].

In order to enable a full characterization the WUS has to be brought into solution. Sequential extraction is one of the more generally-used methods in cell wall fractionation. Usually, pectic material is first removed by a chelating agent (eg. EDTA) and/or dilute Na_2CO_3, followed by subsequent extraction of cell wall components with 1M KOH, 4M KOH, 4M KOH and boric acid[1].

Wheat flour WUS contains mostly arabinoxylans with cellulose, (1→3,1→4)-ß-glucans and glucomannans and proteins as other minor constituents[2,3]. The arabinoxylans can be largely extracted with 1M NaOH; however, (1→3,1→4)-ß-glucans are co-extracted. The presence of these glucans in the alkali extract may hinder further analysis of the arabinoxylans (e.g. molecular weight determination).

Separation of arabinoxylans and (1→3,1→4)-ß-glucans has been attempted by ethanol precipitation or DEAE-chromatography and, although some purified arabinoxylan fractions can be obtained, these fractionations usually did not succeed in a complete separation of (1→3,1→4)-ß-glucans and arabinoxylans[4,5]. Removal of (1→3,1→4)-ß-glucans may also be achieved by specific enzymolysis. However, this requires the availability of substantial amounts of highly purified ß-glucanases since any xylanase impurity will lead to degradation of arabinoxylans.

Another approach is the use of selective extractants. Mares and Stone[6] compared several extractants for wheat flour WUS in terms of protein and polysaccharide extractability. They found the highest protein and polysaccharide extraction yields with dilute alkali. Since their WUS-isolation procedure has led to the removal of the (1→3,1→4)-ß-glucans[7] these authors found no difference between extractants with respect to neutral sugar composition.

The present study was undertaken to investigate whether the major part of the arabinose and xylose present in the wheat flour WUS could be selectively extracted as a pure arabinoxylan fraction. Various extractants were compared in terms of yield, sugar composition, amino acid composition and molecular weight distribution of the extracted material.

Experimental

Materials

Wheat flour water-unextractable cell wall material (WUS) was obtained from Arminda flour as described previously[8]. WUS from Granta, Okapi, Taurus and Camp Remy wheat

flour (75% net extraction rate) was obtained on a small scale (designated as WUS1) as described previously[2]. Wheat bran WUS was obtained from commercial wheat bran (1 g) after ethanol washing (6 x 200 ml; 20 °C) and starch removal with *alpha*-amylase after gelatinization (70 °C) as described previously[2]. Oat spelt xylan and barley glucan [a hot water-extractable (1→3,1→4)-ß-glucan] were purchased from Sigma (St. Louis, Missouri, USA) and Biocon (Kilnagleary, Cork, Ireland), respectively.

Extraction procedures

Extraction with DMSO, 8M urea, 1M hydroxylamine hydrochloride ($NH_2OH.HCl$) in phosphate buffer and 1M NaOH (+ 260mM $NaBH_4$) were performed as follows: Wheat flour WUS samples (200 mg) were extracted with solvent (100 ml) for 16 h at 20 °C under continuous stirring. After centrifugation (50,000 *g*; 20 min) residues were re-extracted with solvent (50 ml; 1h) and distilled water (4x100 ml; 1h). After centrifugation (50,000 *g*; 20 min) the corresponding supernatants were combined and extensively dialysed (Visking V40 cellulose membrane; Viskase, Chicago, USA) against distilled water. The 1M NaOH extract was first neutralized with acetic acid before dialysis. Hydroxylamine hydrochloride solutions were made in 0.2M sodium phosphate buffer adjusted to pH 5.0 or 7.2.

Extraction with 0.05M Na_2CO_3 (+ 20mM $NaBH_4$) was carried out as follows: WUS (200 mg) was extracted with Na_2CO_3 (100 ml) for 16 h at 4 °C. After centrifugation (50,000 *g*; 20 min) the extract was neutralized with acetic acid and dialysed against distilled water; this extract is referred to as Na_2CO_3-4°C. The residue was resuspended in 0.05M Na_2CO_3 + 20mM $NaBH_4$ (200 ml) and extracted for 3 h at 20 °C. After centrifugation (50,000 *g*; 20 min) the residue was re-extracted with solvent (50 ml; 1h) and distilled water (4x100 ml; 1 h). After centrifugation (50,000 *g*; 20 min) the corresponding supernatants were combined, neutralized and dialysed against distilled water; this extract is referred to as Na_2CO_3-20°C.

MMNO extraction was performed according to Voragen *et al*[9]. Part of the extracted material precipitated during dialysis against distilled water (MMNO-E2), whereas the remainder stayed in solution (MMNO-E1).

Extraction with saturated $Ba(OH)_2$ solution (+ 260mM $NaBH_4$) was carried out with wheat flour WUS, wheat bran WUS, oat spelt xylan and barley glucan. The cell wall materials (200 mg) were extracted with $Ba(OH)_2$ (100 ml) for 16 h at 20 °C. After centrifugation (50,000 *g*; 20 min), the residue was re-extracted with solvent (50 ml; 1 h) and centrifuged (50,000 *g*; 20 min). The supernatants were combined and neutralized (acetic acid) and dialysed successively against sodium acetate buffer (0.2M; pH 5) and distilled water; this extract is referred to as BE1. The residue was first acidified to pH 5 (acetic acid) and subsequently extracted with distilled water (4x100 ml; 1h). After centrifugation (50,000 *g*; 20 min) the corresponding supernatants were combined and extensively dialysed against distilled water resulting in a second extract (BE2).

All extracts were kept at -18 °C and aliquots were thawed or freeze-dried according to needs. All residues (RS) were freeze-dried.

Analytical methods

Neutral sugar composition. Polysaccharides were hydrolysed by pretreatment with 72% (w/w) H_2SO_4 for 1 h at 30 °C, followed by hydrolysis with 1M H_2SO_4 for 3 h at 100 °C. Next, neutral sugars were converted to their alditol acetates[10] and analysed by gas chromatography as described previously[2]. Inositol was used as internal standard.

(1→3,1→4)-ß-glucans were degraded to glucose using the (1→3,1→4)-ß-glucan test kit supplied by Biocon. The liberated glucose was determined enzymically using the glucose test kit from Boehringer (cat. no. 716251).

Protein content / amino acid composition. The protein content in the extracts was determined according to Sedmak and Grossberg[11] (Coomassie Blue G-250 assay). For amino acid analysis, samples were ethylated with 2-vinylpyridine[12] followed by vapour-phase hydrolysis with 6M HCl containing 1% (v/v) redistilled phenol for 20 h at 110 °C under nitrogen. Amino acids released were derivatized using phenylisothiocyanate[13] and analysed on a SP 8000 HPLC (Spectra Physics) using the separation conditions described by Janssen *et al*[14].

Chromatography. High performance size exclusion chromatography (HPSEC) was performed on a SP 8700 HPLC (Spectra Physics) equipped with three Bio-Gel TSK columns (each 300x7.5 mm; Bio-Rad Labs) in series: 60XL (exclusion limit dextrans: $5 \cdot 10^7$ Da); 40XL (excl. limit dextrans: $1 \cdot 10^6$ Da); 30XL (excl. limit dextrans: $2 \cdot 10^5$ Da) in combination with a TSK XL guard column (40x6 mm) and eluted at 30 °C with 0.4M or 0.005M sodium acetate buffer (pH 3) at a flow rate of 0.8 ml/min. The eluate was monitored using a Shodex SE-61 refractive index detector.

Phenolic acids. The presence of phenolic acids was determined as described by Rombouts and Thibault[15].

Results

Extraction of wheat flour WUS

Yield and neutral sugar composition Figure 1 shows the percentage of each individual sugar extracted from WUS by the different solvents. The total yield and the molar neutral sugar composition of each extract is given in Table I. DMSO and 8M urea extracted only

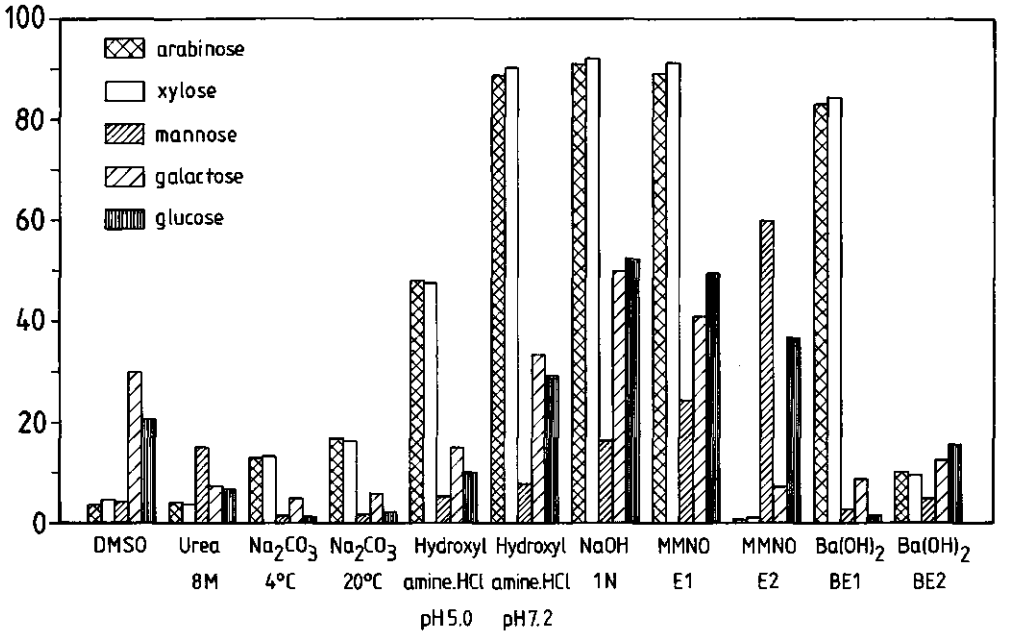

FIGURE 1. Extraction of WUS with different solvents. The different shaded bars relate to the amount (per cent w/w) of individual sugars extracted.

a small proportion of the water-unextractable cell wall material. Both extracts were enriched in glucose. The urea extract also contained a relatively high amount of mannose which represented *c.* 20% of the total mannose present in WUS.

Higher yields were found with successive 0.05M Na_2CO_3 extraction (4°C and 20°C) and with hydroxylamine hydrochloride extraction at different pHs. Na_2CO_3 solubilised a fraction containing only arabinose and xylose, both at 4°C (Na_2CO_3-4°C) and 20°C (Na_2CO_3-20°C). In total, 24% of the cell wall material could be solubilised with this extractant. Hydroxylamine hydrochloride extracted different amounts of cell wall material depending on the pH of extraction. At pH 7.2, 78% cell wall material was extracted whereas at pH 5.0 the yield was only 42%. Both on a weight, as well as on a molar basis, more glucose was extracted at pH 7.2.

The highest yields were found with NaOH, MMNO and $Ba(OH)_2$. NaOH extracted *c.* 90% of all the arabinose and xylose, and 50% of the glucose. MMNO treatment yielded two fractions after dialysis: a water-soluble fraction (MMNO-E1) which had the same composition as the 1M NaOH extract, and a water-insoluble fraction (MMNO-E2), which contained mainly glucose and mannose. $Ba(OH)_2$ extraction resulted in two fractions, BE1 and BE2. The BE1 fraction was released from the WUS directly, being extractable in $Ba(OH)_2$. The BE2 fraction was only released from the WUS-residue after lowering the pH and washing with water. The two fractions differed in composition: BE1 containing only arabinose and xylose (34 and 65%, respectively), and BE2 containing substantial

TABLE I. Yield and neutral sugar composition of wheat flour WUS and various extracts

		Extracts										
				Na_2CO_3		$NH_2OH.HCl$			MMNO		$Ba(OH)_2$	
	WUS	DMSO	Urea (8M)	4°C	20°C	pH5.0	pH7.2	NaOH (1M)	E1[a]	E2[a]	BE1[b]	BE2[b]
Molar composition (%)												
Arabinose	30.7	19.6	28.1	34.9	36.2	35.3	32.9	33.1	32.8	3.4	34.3	32.8
Xylose	54.7	35.1	47.0	63.4	62.0	61.5	62.8	59.0	59.1	7.8	65.4	48.7
Mannose	2.1	1.3	6.2	0.2	0.2	0.2	0.1	0.5	0.6	19.2	0.1	0.9
Galactose	0.6	2.5	1.0	0.2	0.2	0.2	0.2	0.3	0.3	0.9	0.1	0.7
Glucose	11.9	41.5	17.8	1.3	1.3	2.8	3.9	7.1	7.2	68.4	0.3	17.0
Total sugar content[c]	90.5	47.9	45.8	80.9	88.2	85.7	92.9	87.2	87.0	93.2	91.6	92.6
Yield[d] (%)	100	11	8	11	13	42	78	83	85	7	68	11

[a] E1: soluble fraction after dialysis; E2 insoluble fraction after dialysis.
[b] BE1: fraction solubilised directly after extraction; BE2: fraction solubilised after neutralising the $Ba(OH)_2$ residue.
[c] Expressed as weight percentage (as is basis) of each extract.
[d] The yield is expressed as weight percentage (as is basis) of WUS.

TABLE II. Protein content (g/100 g, as is basis) and amino acid composition (mole %) of wheat flour WUS and various extracts

		Extracts									
				Na_2CO_3		$NH_2OH.HCl$				$Ba(OH)_2$	
	WUS	DMSO	Urea (8M)	4°C	20°C	pH5.0	pH7.2	NaOH (1M)	MMNO E1[a]	BE1[b]	BE2[b]
Protein content	2.4[a]	2.3	2.9	1.0	1.2	1.1	1.1	1.7	1.1	1.1	1.8
Amino acids											
Asx	9.7	3.7	8.7	8.0	7.9	7.6	7.0	8.5	6.8	8.0	8.3
Glx	10.7	8.7	11.0	10.9	10.0	8.0	10.3	12.2	9.8	11.1	11.3
Ser	8.2	10.7	11.9	10.3	9.4	8.9	15.9	8.3	7.7	8.2	11.7
Gly	14.7	11.0	12.1	12.9	11.3	11.2	14.2	9.8	13.2	10.5	14.2
His	3.9	1.5	1.5	1.5	1.4	1.8	1.5	1.5	1.6	1.6	1.5
Arg	4.7	4.9	4.4	3.4	4.4	5.1	4.1	3.6	1.5	3.9	3.9
Thr	4.9	6.4	5.2	5.3	5.2	5.3	5.1	3.8	5.4	4.2	3.8
Ala	8.4	10.5	9.6	9.0	10.0	9.0	9.5	9.3	8.8	9.9	10.2
Pro	8.3	6.7	5.4	6.8	5.7	5.9	5.2	6.1	6.6	6.2	4.6
Tyr	4.0	4.2	2.4	3.1	3.7	4.8	4.0	3.7	3.2	5.9	3.1
Val	5.5	8.3	6.4	6.6	7.5	6.6	5.3	9.3	8.4	8.8	7.0
Met	1.1	0.9	1.2	1.3	1.1	0.8	0.9	1.0	1.3	1.3	0.8
Cys	3.1	3.1	2.7	2.8	2.7	6.6	3.6	0.8	0.5	1.1	0.6
Ile	2.6	3.5	3.3	2.9	3.2	2.7	2.2	4.3	4.8	3.7	3.8
Leu	6.9	10.6	7.9	7.8	8.7	8.0	5.5	10.6	9.9	9.1	8.6
Phe	1.9	3.7	3.1	3.3	3.5	3.2	2.4	4.7	4.4	3.8	3.7
Lys	4.5	2.4	3.0	4.0	4.3	4.4	3.2	2.5	6.2	2.8	2.8

[a] determined by the Kjeldahl method (Nx5.7).

amounts of glucose (17%) in addition to arabinose (33%) and xylose (49%).

Protein content and amino acid composition With the exception of the MMNO-E2 extract each fraction was analysed for protein content and amino acid composition (Table II). The protein content and amino acid composition of all extracts were quite similar with the exception of the hydroxylamine hydrochloride-pH 7.2 extract, which had a relatively high serine and a low leucine content. The amino acid composition of the extracts largely resembled that of the WUS, being rich in glycine, glutamic acid and alanine. However, some clear differences between the extracted materials and WUS were observed. Leucine was higher in most extracts and valine was relatively high in the NaOH, MMNO-E1 and BE1 extracts. Traces of hydroxyproline were found in all extracts.

Phenolic acids A qualitative determination of the presence of phenolic acids was

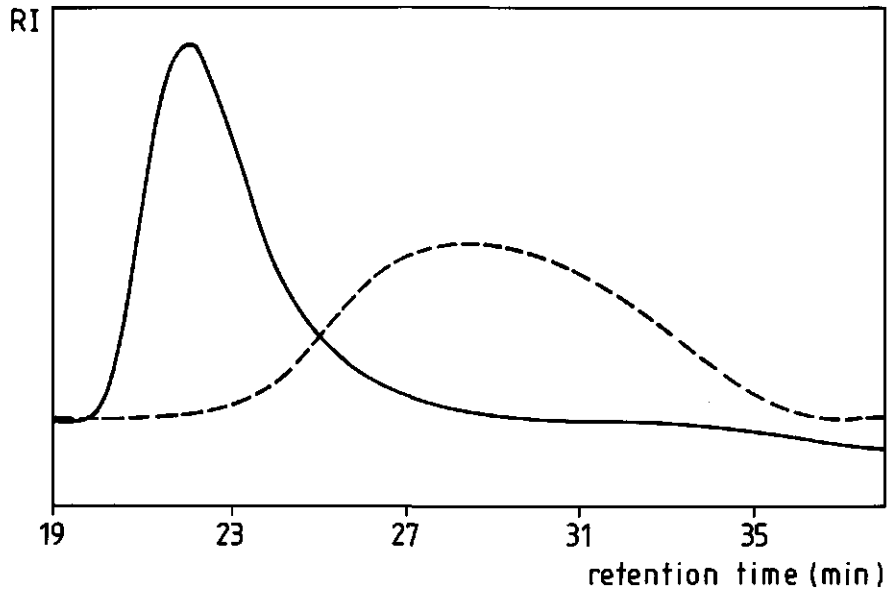

FIGURE 2. Elution pattern (HPSEC) of the BE1 (—) and MMNO-E1 (----) extracts using 0.4M sodium acetate buffer pH 3.

performed by measuring the bathochromic shift[16] in UV absorption of WUS extracts as the pH of the extract was raised from 4.8 to 10. Bathochromic shifts were detected in the DMSO, urea, Na_2CO_3-20°C, Na_2CO_3-4°C and the hydroxylamine hydrochloride-pH 5.0 extract. The double absorption peak at 300 and 325 nm shifted to a single peak at 375 nm upon increasing the pH. No bathochromic shift was showed by the other extracts. No further quantification or identification of phenolic acids in the extracts was attempted.

Molecular weight distribution The molecular weight distribution of the various extracts (except MMNO-E2 and BE2) was analysed using a tandem set of HPSEC columns. With the exception of the MMNO-E1 extract, all extracts had the same elution pattern. For a typical extract, BE1, the elution pattern, together with that of MMNO-E1, is given in Fig. 2. The bulk of the BE-1 material eluted in a sharp peak with a retention time of $\pm$ 22 minutes. The weight average molecular weight was calculated to be *c.* 850,000 using a DAWN-F multi angle laser light scattering detector[17] in combination with the HPSEC column set; the M_w/M_n ratio was 1.51. The MMNO-E1 extract had a longer relative retention time, indicating a markedly lower molecular weight.

Barium hydroxide extraction of other cell wall materials

Since the results on wheat flour WUS showed that $Ba(OH)_2$ selectively extracted the major part of the arabinose and xylose, we applied $Ba(OH)_2$ to other cell wall materials.

TABLE III. Yield and neutral sugar composition of WUS and BE-1 extracts of wheat flour from different varieties

Variety	WUS						BE1						
	Total sugar content[a]	molar composition[b]					Yield	Total sugar content	molar composition				
		ara	xyl	man	gal	glc			ara	xyl	man	gal	glc
Granta	92.4	28.1	51.8	4.0	0.5	16.6	62.0	87.8	33.9	65.8	0	0	0.3
Okapi	88.2	29.5	53.3	2.5	1.2	13.7	65.1	90.2	36.7	62.9	0.1	0.1	0.3
Taurus	89.5	30.4	54.4	2.6	0.6	12.1	69.1	89.2	38.0	61.6	0	0	0.4
Camp Remy	90.2	27.5	48.8	3.1	0.9	19.7	59.1	88.4	37.1	62.5	0.1	0	0.3

[a] Expressed as weight percentage (as is basis) of each extract.

[b] Abbreviations: ara = arabinose; xyl = xylose; man = mannose; gal = galactose; glc = glucose.

[c] The yield is expressed as weight percentage (as is basis) of WUS.

TABLE IV. Yield and neutral sugar composition of cell wall material and extracts using $Ba(OH)_2$ containing 260mM $NaBH_4$

	Yield[a]	Total sugar content[b]	(1→3,1→4)-ß-glucan distribution[c]	Molar composition[d]					
				ara	xyl	man	gal	glc	
Wheat flour									
WUS		90.5		30.7	54.7	2.1	0.6	11.9	(7.1)
BE1	68.1	91.6	2.1	34.3	65.4	0.1	0.1	0.3	(0.2)
BE2	10.7	92.6	24.8	32.8	48.7	0.9	0.7	17.0	(12.7)
RS	21.2	82.8	73.1	17.7	24.9	7.4	1.2	48.9	(25.8)
Wheat bran									
WUS		66.5		25.7	47.0	0.4	1.2	25.6	(4.8)
BE1	23.3	84.1	2.6	41.5	57.1	0	0.8	0.5	(0.4)
BE2	8.5	79.4	47.4	27.1	47.9	0	1.9	23.0	(20.5)
RS	68.2	59.3	50.0	21.5	41.6	0.5	1.0	35.4	(3.7)
Oat spelt xylan (OS)									
OS		86.0		8.4	84.7	0	0.5	7.2	(0.5)
BE1	69.7	87.1	19.2	10.7	88.4	0	0.6	0.3	(0.1)
BE2	3.4	88.3	63.4	10.3	62.9	0	1.3	25.5	(9.7)
RS	26.9	84.9	17.4	5.2	81.3	0	0	13.5	(0.3)
Barley glucan (BG)									
BG		94.4		0.4	1.0	0.2	0	98.6	
BE1	5.6	92.6		0.2	0.5	0	0	99.3	
BE2	42.2	93.0		0.4	1.5	0.2	0	97.9	
RS	52.1	96.0		0.3	0.2	0.3	0	99.2	

[a] The yield is expressed as weight percentage (as is basis) of WUS, OS or BG.

[b] Expressed as weight percentage (as is basis) of each extract.

[c] Distribution of (1→3,1→4)-ß-glucan among fractions expressed as weight percentage (as is basis) of all (1→3,1→4)-ß-glucan present.

[d] Abbreviations: ara = arabinose; xyl = xylose; man = mannose; gal = galactose; glc = glucose. The figures in parentheses represent the molar percentage glucose present as (1→3,1→4)-ß-glucan.

Wheat flour WUS was isolated from four wheat varieties. Table III shows the molar sugar composition of WUS and BE1 extracts from these wheat varieties. The isolated WUS fractions contained less than 0.3% starch when determined using the enzyme kit supplied by Boehringer (including the pretreatment with HCl/DMSO). All varieties yielded a BE1 extract which contained only arabinose and xylose as constituent sugars. The results of $Ba(OH)_2$ extraction of oat spelt xylan, wheat bran WUS and barley glucan together with those of wheat flour WUS are shown in Table IV. Both oat spelt xylan and wheat bran WUS yielded a BE1 extract which contained predominantly arabinose and xylose. For wheat bran WUS, 23% of the material was found in the BE1 extract, versus 70% for oat spelt xylan. The corresponding BE2 extracts each contained *c.* 25% glucose and represented less than 10% of the starting material. For wheat flour WUS, wheat bran WUS and oat spelt xylan the (1→3,1→4)-ß-glucan content was determined for each of the fractions obtained. From the data, it can be seen that for the BE1 and BE2 extracts from wheat flour WUS and wheat bran WUS, most of the glucose is present as (1→3,1→4)-ß-glucans. The barley glucan material could also be fractionated into 3 fractions. The two extracts made up *c.* 48% of the material. Sugar analysis showed that impurities were mostly present in the BE2 and RS fraction. The residue after $Ba(OH)_2$ treatment could easily be dissolved by increasing the temperature to 60-70 °C.

To investigate the effect of $NaBH_4$ concentration on the extraction of (1→3,1→4)-ß-glucans, we also extracted the different cell wall material with $Ba(OH)_2$ containing 20mM $NaBH_4$ instead of 260mM $NaBH_4$. The results of the different extractions are given in Table V. For all cell wall materials, both the BE1 and BE2 extract contained more glucose when compared with those obtained after extraction with $Ba(OH)_2$ containing 260mM $NaBH_4$. For barley glucan, it was found that 43% of the material was then present in the BE1 extract, 39% in the BE2 extract and 18% was rendered unextractable (results not shown). No neutral sugar analyses on these barley glucan fractions were performed.

The gel permeation patterns of the wheat flour WUS, wheat bran WUS, oat spelt xylan and barley glucan extracts [$Ba(OH)_2$-260mM $NaBH_4$] are shown in Fig. 3. Two different buffer molarities were used: 0.005M and 0.4M. The elution pattern with 0.4M buffer shows that the BE1 extracts of both wheat flour and wheat bran WUS clearly eluted earlier than the BE2 extracts, implying a higher molecular weight. The behaviour of the extracts towards a change of buffer molarity varied with the type of raw material. Most extracts showed a marked shift in retention time when lowering the buffer strength. The extracts obtained with $Ba(OH)_2$-20mM $NaBH_4$ extraction showed similar gel permeation patterns to those obtained with $Ba(OH)_2$-260mM $NaBH_4$ (results not shown).

Discussion

Comparison of extractants

Only low amounts of WUS material were extracted using DMSO and 8M urea. The freeze-dried extracts contained *c.* 50% neutral sugars and were relatively rich in glucose.

TABLE V. Yield and neutral sugar composition of cell wall material and extracts using $Ba(OH)_2$ containing 20mM $NaBH_4$

	Yield[a]	Total sugar content[b]	(1→3,1→4)-ß-glucan distribution[c]	Molar composition[d]					
				ara	xyl	man	gal	glc	
Wheat flour									
WUS		90.5		30.7	54.7	2.1	0.6	11.9	(7.1)
BE1	68.8	92.3	12.6	34.8	63.8	0	0.1	1.4	(1.3)
BE2	9.5	91.6	34.7	26.0	38.9	1.2	1.2	34.0	(25.2)
RS	21.7	83.2	52.7	18.5	26.2	8.3	1.0	46.0	(20.4)
Wheat bran									
WUS		66.5		25.7	47.0	0.4	1.2	25.6	(4.8)
BE1	19.6	84.0	19.4	36.6	56.8	0.1	1.2	5.4	(3.8)
BE2	9.2	53.5	55.0	20.4	32.3	0.1	0.5	43.3	(37.8)
RS	71.2	68.2	25.6	22.1	47.2	0.5	0.9	29.4	(1.8)
Oat spelt xylan (OS)									
OS		86.0		8.4	84.7	0	0.5	7.2	(0.5)
BE1	65.9	86.1	82.0	11.0	86.8	0	1.1	1.2	(0.7)
BE2	3.7	68.8	7.1	8.1	50.6	0.4	2.1	38.4	(1.3)
RS	30.4	86.1	10.9	6.1	86.7	0	0	7.2	(0.1)

[a] The yield is expressed as weight percentage (as is basis) of WUS or OS.

[b] Expressed as weight percentage (as is basis) of each extract.

[c] Distribution of (1→3,1→4)-ß-glucan among fractions expressed as weight percentage (as is basis) of all (1→3,1→4)-ß-glucan present.

[d] Abbreviations: ara = arabinose; xyl = xylose; man = mannose; gal = galactose; glc = glucose. The figures in parentheses represent the molar percentage glucose present as (1→3,1→4)-ß-glucan.

Starch can only account for at most 5% of the glucose in these extracts since the WUS contained 0.2% starch[8]. Therefore most of the glucose must originate from other glucans[3], presumably (1→3,1→4)-ß-glucans which make up *c.* 8% of the WUS[8]. In the urea extract a relatively high amount of mannose was found next to glucose. Mannose-enriched fractions were previously found in chaotropic extracts of other cell walls[18,19].

Both Na_2CO_3 extracts contained mostly arabinose and xylose, presumably present as arabinoxylans[3]. The low amount of extracted glucose indicates that the glucans in WUS are firmly self-associated or associated with the other polysaccharides. However, on a weight basis, DMSO (and urea) dissolved more glucose than 0.05M Na_2CO_3 (Fig. 1). A possible explanation may be the involvement of specific hydrophobic or hydrogen bonds which may be broken by urea or DMSO but not by 0.05M Na_2CO_3.

Hydroxylamine hydrochloride gave different yields depending on the pH. At pH 7.2 more glucans as well as arabinoxylans were extracted than at pH 5.0 (Fig. 1). Similar data have been found for wheat endosperm cell walls[6]. In the pH 5.0 extract a bathochromic shift could be detected. This bathochromic shift is indicative of esters of cinnamic acid-type phenols[15,16]. The isolated wheat flour WUS contains 0.3% ferulic acid[8]. Ferulic acid esterified to arabinoxylans[3] was probably responsible for the bathochromic shift. In the pH 7.2 extract no bathochromic shift could be observed. This indicates that at pH 7.2 more ester linkages are broken than at pH 5.0, which might be responsible for extracting higher quantities of WUS material.

Both with MMNO and 1M NaOH large amounts of cell wall material with similar neutral sugar composition were extracted. Whereas 1M NaOH (KOH) extraction is commonly used[1], MMNO extraction is not wide spread. With regard to the neutral sugar composition, the main difference between both extractants is the precipitation of a mannose-rich polymer from the MMNO extract after dialysis (MMNO-E2). This was previously found for barley flour cell walls[9]. For wheat endosperm cell walls, a mannose-rich fraction was previously found in a 1M KOH residue and attributed to a glucomannan fraction[20].

The MMNO-E1 fraction differed from all other fractions showing a lower molecular weight on HPSEC. Although MMNO was reported not to degrade oat spelt xylan[9] it is believed to cause mid-chain cleavage of wheat bran arabinoxylans[21,22]. We also found extensive breakdown of water-extractable arabinoxylans from wheat flour when treated with MMNO (unpublished results). Therefore, the low molecular weight of the MMNO extract is likely due to breakdown of the polysaccharide during the extraction. No degradation of water-extractable arabinoxylans was observed when treated with the other extractants.

With $Ba(OH)_2$ *c.* 80% of the arabinoxylan could be extracted as a pure fraction which almost equals the amount of arabinoxylan extracted with 1M NaOH and MMNO.

Comparison of extracts in terms of amino acid composition showed only minor differences between the various extracts with the exception of the hydroxylamine hydrochloride-pH 7.2 extract (Table II). This corresponds to previous investigations on

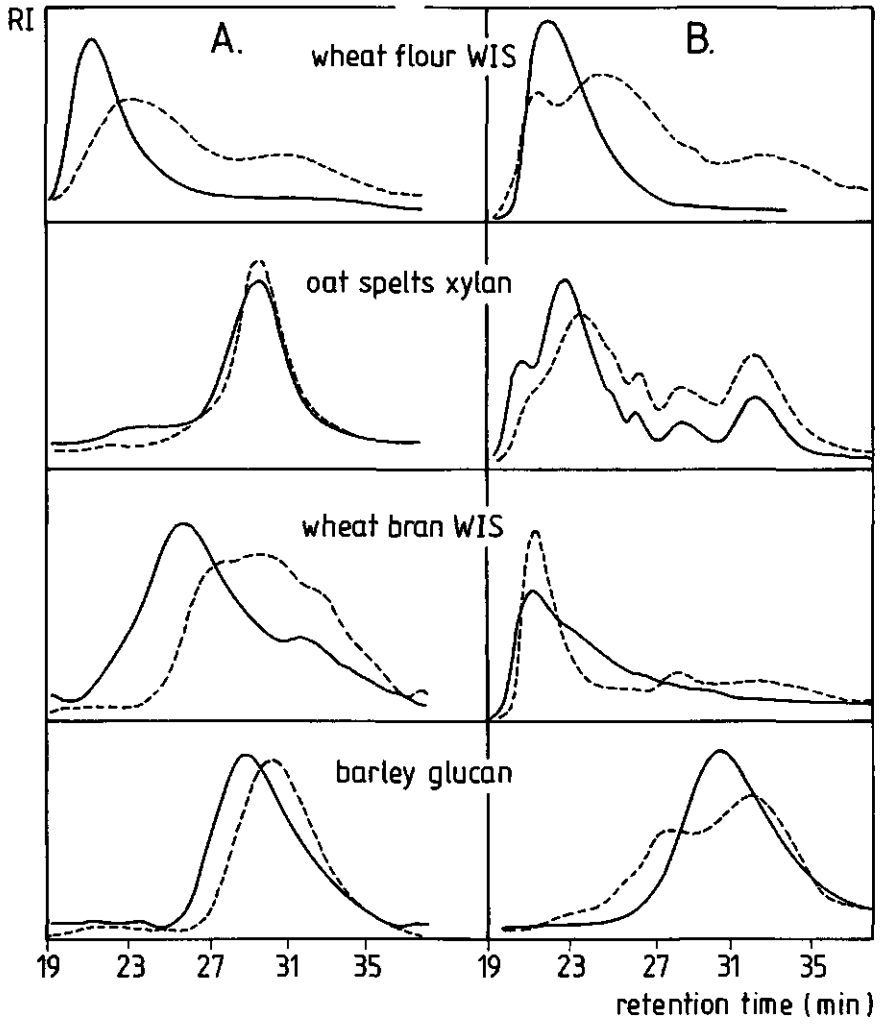

FIGURE 3. Elution pattern (HPSEC) of BE1 (—) and BE2 (----) extracts from different material using 0.4M (A) and 0.005M (B) sodium acetate buffer pH 3.

wheat endosperm cell wall extracts[6]. However, a precise comparison between both studies is hindered by the fact that the endosperm WUS[6] contained precipitated intracellular protein[3].

Use of barium hydroxide as a primary extractant

$Ba(OH)_2$ is known to be a complexing agent for saccharides[23]. In cell wall analysis $Ba(OH)_2$ has been used as a precipitating agent for mannans[1] and as a peptide splitting agent at elevated temperatures[24]. To our knowledge the use of $Ba(OH)_2$ as a primary extractant for arabinoxylans has not been published previously.

$Ba(OH)_2$ could be successfully applied to different cell wall materials. Pure arabinoxylans could be obtained in high yield from WUS from different wheat varieties (Table III). Application of $Ba(OH)_2$ to oat spelt xylan and wheat bran WUS gave similar results to wheat flour WUS (Table IV). In both cases it resulted in a BE1 fraction with almost no glucose and a BE2 extract containing *c.* 25% glucose.

Most of the extracted glucose originated from (1→3,1→4)-ß-glucans. Although the isolated wheat flour WUS and wheat bran WUS contained 0.2 and 1.0% residual starch, enzymic analysis (starch testkit-Boehringer Mannheim, including pretreatment with HCl/DMSO) revealed that the different extracts contained only minimal amounts of starch (results not given). This implies that the starch is kept unextractable in the presence of $Ba(OH)_2$ which is consistent with previous work[25].

To verify whether it was also possible to extract arabinoxylans from WUS containing large amounts of starch, $Ba(OH)_2$ was applied to crude wheat flour WUS containing *c.* 18% starch (designated as crude WUS1 c.q. WUS[2,8]). It showed that the obtained BE1 extract contained only arabinose and xylose (arabinose/xylose ratio = 0.55) when analysed by GLC. Therefore $Ba(OH)_2$ extraction in combination with large-scale isolation of crude WUS[8] is a suitable method to obtain large amounts of highly purified water-unextractable wheat flour arabinoxylans.

The mechanism of the selective extraction of arabinoxylans from cell wall material using $Ba(OH)_2$ is not clear. At room temperature the solubility of $Ba(OH)_2$ in water is *c.* 0.23M and from the dissociation constant[26] the OH^- concentration can be calculated to be *c.* 0.33M. When WUS was extracted with 0.33M NaOH a neutral sugar composition similar to the 1M NaOH extract was found, indicating that not the OH^- concentration but the presence of Ba^{2+} ions was responsible for the unextractability of these (1→3,1→4)-ß-glucans. It is supposed that the polysaccharides in the BE2 fraction were released from the cell wall due to the alkaline conditions but were kept unextractable by the Ba^{2+} ions. For acidic arabinoxylans the formation of an insoluble barium salt has been suggested[27] although the existence of $Ba(OH)_2$ soluble acidic arabinoxylans has also been reported[28]. The presence of acidic groups within the polysaccharide structure can be shown by HPSEC using different buffer strengths. In the 0.4M buffer polysaccharides are separated by differences in hydrodynamic volume. When applying the 0.005M buffer charged groups will not be shielded by buffer ions. As a result, ionic polysaccharides will exhibit increased exclusion because of swelling of the polysaccharides[29] or repulsion of the polysaccharides by column material[30].

Comparison of the HPSEC patterns (Fig. 3) of the $Ba(OH)_2$ extracts showed that the BE1 and BE2 patterns differed for each type of material. From Fig. 3b it can be seen that with the exception of the wheat flour and barley glucan BE1 extract, all extracts showed a marked acidic character. This implies that the complexation of $Ba(OH)_2$ with polysaccharides can not be explained by the presence of acidic groups within the polysaccharide only.

Another observation indicating the intricacy of the mechanism of $Ba(OH)_2$ complexation was the impossibility to precipitate polysaccharides by adding $Ba(OH)_2$ to either a neutralized and dialysed 1M NaOH extract or a crude alkaline 1M NaOH extract from wheat flour WUS. Moreover, the concentration of $NaBH_4$ used in the $Ba(OH)_2$ extraction appeared to be important for the interaction with (1→3,1→4)-ß-glucans. In cell wall extraction, $NaBH_4$ concentrations varying from 10mM up to 260mM are used[1,4,6,19] to prevent alkaline peeling[31]. Table V shows that when applying $Ba(OH)_2$ containing 20mM $NaBH_4$ instead of 260mM $NaBH_4$ higher glucose contents were found in both BE1 and BE2 extracts. This is confirmed by the data on barley glucan. At 20mM $NaBH_4$ concentration *c.* 82% of the barley glucan material was extracted versus 48% at 260mM $NaBH_4$. When barley glucan was treated with water (20 °C) *c.* 46% of the material could be extracted. This means that $Ba(OH)_2$ containing 260mM $NaBH_4$ does not extract additional (1→3,1→4)-ß-glucans in comparison to water. Therefore it can be concluded that using a low $NaBH_4$ concentration less (1→3,1→4)-ß-glucans are kept unextractable by $Ba(OH)_2$. It can be estimated[32] that during the extraction *c.* 0.5% of the $NaBH_4$ is converted to borate, which interacts with saccharides[1,33]. As a result different borate concentrations are present in the two extracts. Extraction of wheat bran WUS with $Ba(OH)_2$ containing 20mM $NaBH_4$ and 26mM sodium borate resulted in an extract with the same sugar composition as $Ba(OH)_2$-20mM extract. This means that the effect of $NaBH_4$ concentration does not seem to be related to the presence of borate in the extract.

Conclusions

Extracts from wheat flour WUS obtained with several solvents differed markedly in yield and sugar composition but were almost equal in amino acid composition. With MMNO, 1M NaOH, $Ba(OH)_2$ and 1M hydroxylamine hydrochloride-pH 7.2, most of the cell wall material was extracted, minor amounts were extracted with 0.05M Na_2CO_3, 8M urea, DMSO and 1M hydroxylamine hydrochloride-pH 5.0. Polysaccharide-bound phenolic acids were only present in these latter four extracts.

With $Ba(OH)_2$ it was possible to extract the major part of all arabinoxylan present in wheat flour WUS as a pure arabinoxylan fraction, whereas with most other solvents, glucans were co-extracted. Pure arabinoxylans could also be extracted from wheat flour WUS derived from other wheat varieties, wheat bran WUS, oat spelt xylan and wheat flour WUS containing substantial amounts of starch. A sufficiently high concentration of $NaBH_4$ used during the extraction with $Ba(OH)_2$ appeared to be important for the selective extraction of the arabinoxylans.

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CHAPTER 5

Water-unextractable cell wall material from wheat flour. I. Extraction of polymers with alkali

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Summary

Water-unextractable cell wall material (WUS) prepared from wheat flour was extracted sequentially with saturated $Ba(OH)_2$, 1M KOH and 4M NaOH. The resultant extracts, which together contained 96% of the total WUS in the flour were enriched in arabinoxylans, (1→3,1→4)-ß-glucans and glucomannans, respectively. The remaining 4% of the wheat flour WUS was mainly cellulose. Methylation analysis of the arabinoxylans showed that the xylopyranosyl residues are substituted at *O*-3 or *O*-2,3 with mainly terminal arabinofuranosyl residues. Only minor amounts of *O*-2 substituted xylose were found. The degree of substitution of xylopyranosyl residues was highest in the NaOH extract and lowest in the first $Ba(OH)_2$ extract, with that in the second $Ba(OH)_2$ extract and the KOH extract being intermediate. Only minor differences in protein content and amino acid composition were observed among the extracts. Alkali-extractable arabinoxylans had slightly higher apparent molecular weights than water-extractable arabinoxylans. The alkali-extractable (1→3,1→4)-ß-glucans and (gluco)mannans clearly had lower apparent molecular weights than the arabinoxylans.

Introduction

Wheat flour contains 2-3% (w/w) of cell wall material, which consists largely of non-starch polysaccharides although minor amounts of protein are also present[1,2]. This cell wall material partly determines the dough characteristics and baking properties of wheat flours, effects that have been ascribed to its high water absorbing capacity[3,4]. Only a proportion of the cell wall material is extractable in water[2,5]. So far, research has been focused predominantly on this water-extractable material. Less attention has been given to the water-unextractable cell wall material (WUS). Furthermore, that research which has been carried out on the WUS has been focused on physico-chemical properties[6-10], and less attention has been given to the mode of substitution[11] of arabinoxylans or molecular weight distribution of individual classes of polysaccharides. Both the molecular weight distribution and substitution of xylose residues by arabinose are believed to have important consequences for the physico-chemical properties of arabinoxylans[12,13].

The most commonly used extractants for cereal hemicellulosic polymers are concentrated NaOH or KOH, which leave cellulose as an unextracted residue[1,14-17]. In wheat flour WUS, arabinoxylans predominate, but there are also minor amounts of cellulose, (1→3,1→4)-ß-glucans and glucomannans[1]. Using NaOH or KOH, a mixture of arabinoxylans and (1→3,1→4)-ß-glucans is extracted which is difficult to fractionate[1]. Recently, we introduced the use of $Ba(OH)_2$ as a primary extractant for wheat flour WUS, which extracts *c.* 80% of the arabinoxylan present in the WUS as a pure arabinoxylan fraction[18].

In the present research we have extended our studies on the extraction of WUS from wheat flour using 1M KOH and 4M NaOH. We have also characterized the extracted polymers in terms of their neutral sugar compositions, protein contents, amino acid compositions, their molecular size distributions, and the mode of linkage of arabinose residues to the xylose units within the arabinoxylan components.

Experimental

Materials

The preparation and characterization of water-unextractable cell wall material (WUS) from wheat flour of *Triticum aestivum* cv. Arminda (1986 harvest) has been described previously[19].

Extraction of WUS

WUS (3 g) was extracted with saturated $Ba(OH)_2$ solution (500 ml) containing 260mM $NaBH_4$ for 16 h at room temperature with continuous stirring. After centrifugation (50,000 *g*; 20 min), the residue was re-extracted with the same extractant (250 ml; 1 h) and

centrifuged (50,000 *g*; 20 min). The supernatants were combined, acidified to pH 5 (acetic acid) and dialysed successively against sodium acetate buffer (0.2M; pH 5) and distilled water (BE1). The residue was acidified to pH 5 (acetic acid) and extracted with distilled water (4x300 ml; 1 h) and centrifuged (50,000 *g*; 20 min). The supernatants were combined and dialysed against distilled water (BE2). This step was introduced to remove polysaccharides released from the cell wall due to the alkaline conditions but which were kept unextractable by the Ba^{2+} ions[8]. The residue from the water extraction step was further extracted with 1M KOH containing 260mM $NaBH_4$ (200 ml) for 16 h at room temperature. After centrifugation (50,000 *g*; 20 min) the residue was re-extracted with the same extractant (100 ml; 1 h) and centrifuged (50,000 *g*; 20 min). The supernatants were combined, acidified (pH 5; acetic acid) and dialysed against distilled water (1M). The residue from the KOH extraction was finally extracted with 4M NaOH containing 260mM $NaBH_4$ (200 ml) for 16 h at room temperature. After centrifugation (50,000 *g*; 20 min) the residue was re-extracted successively with the same extractant (100 ml; 1 h) and distilled water (4x100 ml; 1 h). After centrifugation (50,000 *g*; 20 min) the remaining residue was freeze-dried (RES). The supernatants were combined, acidified (pH 5; acetic acid) and dialysed against distilled water (4M). All extracts were kept at -18 °C. Aliquots were thawed or freeze-dried as needed.

Water-extractable arabinoxylans (weAX)

These were isolated from the same Arminda wheat flour according to the method of Fincher and Stone[20] using amyloglucosidase (Boehringer, no. 102849) instead of salivary *alpha*-amylase for the removal of starch material. After dialysis, the arabinoxylan containing fraction was kept at -18 °C and aliquots were thawed or freeze-dried as needed.

Analytical methods

Neutral sugar composition. Neutral sugars in WUS, extracts, and residue were released by pretreatment with 72% (w/w) H_2SO_4 for 1 h at 30 °C (solubilization of semi-crystalline structures[21]), followed by hydrolysis with 1M H_2SO_4 for 3 h at 100 °C. The sugars present in the hydrolysates were converted to their alditol acetates[22] and analysed as described previously[2]. Sephacryl S1000 fractions were hydrolysed with 2M trifluoroacetic acid (TFA) for 1 h at 120 °C. After cooling to room temperature the TFA was removed by evaporation (airstream, 40 °C). The sugars released were reduced by adding 0.2 ml of a 1.5M ammonia solution containing 75 mg $NaBH_4$/ml and then converted to alditol acetates[22] and analysed by gas-liquid chromatography[2]. For both procedures inositol was used as internal standard.

Protein content / amino acid composition. The protein contents of the WUS and RES

fraction were determined by Kjeldahl analysis (Nx5.7). In order to use as little material as possible a Coomassie Blue G-250 assay[23] was used to estimate the protein contents of the extracts. Bovine serum albumin was used as standard. Amino acid compositions were determined as described previously[18].

(1→3,1→4)-β-glucans were degraded to glucose using the (1→3,1→4)-ß-glucan test kit supplied by Biocon (Kilnagleary, Cork, Ireland). The liberated glucose was determined enzymically using the glucose test kit from Boehringer (cat. no. 716251).

Methylation analysis. Polysaccharides were methylated according to the Hakomori method[24], as modified by Sandford and Conrad[25]. After methylation, the samples were dialysed against water, dried by evaporation (airstream, 40 °C), and hydrolysed with 2M TFA for 1 h at 120 °C. The TFA was removed by evaporation (airstream, room temperature). Next, sugars were reduced by adding 0.2 ml of a freshly prepared 1.5M ammonia solution containing 75 mg $NaBD_4$/ml, and converted to alditol acetates[22]. Samples (0.5 μl) were analysed by on-column injections on a fused silica capillary column (30 m x 0.32 mm; wall coated with DB 1701; 0.25 μm; J & W Scientific, Folsom, California, USA) in a Carlo-Erba Fractovap 4160 gas chromatograph equipped with a flame ionization detector (FID) set at 280 °C. The temperature program was 80→180°C at 20°C/min, 180→230°C at 2°C/min, 230°C for 3 min. Identification of the compounds was confirmed by gas chromatography mass spectrometry (GC-MS) using a Hewlett Packard mass selective detector 5970-B coupled to a HP 5890 GC equipped with a CPsil 19CB column (26 m x 0.22 mm, 0.18 μm; Chrompack Nederland B.V., Middelburg, The Netherlands) and using a PAW-HP 300 Chem Station (Hewlett Packard). Derivatives were quantified from the FID detector response according to their effective carbon response[26].

Gel permeation chromatography. Solutions of the extracts (2-3 mg) in 0.1M sodium acetate buffer, pH 5.0 (1-2 ml) were applied to a column (110 x 1.1 cm) of Sephacryl S1000 (Pharmacia; separation range for dextrans: mol. wt.: 5 x 10^5 - est.> 10^8) and eluted with 0.1M sodium acetate buffer, pH 5.0. Fractions (2.3 ml) were assayed by automated methods[27,28] for total neutral sugar and uronic acid content, using arabinose and glucuronic acid, respectively, as standards. For the uronic acid procedure 96% (w/w) H_2SO_4 containing 0.0125M sodium tetraborate was used in order to quantify glucuronic acid as well as galacturonic acid residues. In all experiments almost complete recoveries (> 95%) were obtained. Every second fraction was analysed for neutral sugar composition. The void and total volumes of the column were measured from the elution volumes of *Escherichia coli* (ATCC 11229) cells and glucose, respectively. Dextran T70 (mol. wt. 70,000), T150 (mol. wt. 150,000) and T2000 (mol. wt. 2,000,000) were used to calibrate the column.

TABLE I. Yields, protein contents and neutral sugar compositions of fractions

Fractions	Yield[a]	Total sugar content[b]	Protein content[c]	(1-3,1-4)-ß-glucan distribution[d]	Molar composition (%)[e]				
					ara	xyl	man	gal	glc
WUS	100	90.5	2.4	100	30.7	54.7	2.1	0.6	11.9 (7.1)
BE1	68.1	91.6	1.1	2.3	34.3	65.4	0.1	0.1	0.3 (0.2)
BE2	10.7	92.6	1.8	23.5	32.8	48.7	0.9	0.7	17.0 (12.7)
1M	9.6	80.3	2.0	61.6	17.5	26.7	2.9	0.9	52.0 (43.2)
4M	7.8	84.1	1.9	12.0	14.1	17.2	26.1	1.7	41.1 (19.7)
RES	3.8	89.7	1.0	0.6	9.0	8.7	3.7	0.5	78.2 (1.1)
weAX	-	90.1	1.3	-	34.9	63.1	0.1	0.1	1.8 (0.2)

[a] The yield is expressed as weight percentage (as is basis) of WUS.

[b] Expressed as weight percentage (as is basis) of each extract.

[c] Protein content, expressed as weight percentage (as is basis); for WUS and RES fractions determined by the Kjeldahl method, the others by the Coomassie Blue method.

[d] Distribution of (1→3,1→4)-ß-glucans among fractions expressed as weight percentage (as is basis) of all (1→3,1→4)-ß-glucans present.

[e] Abbreviations: ara = arabinose; xyl = xylose; man = mannose; gal = galactose; glc = glucose. The figures in parentheses represent the molar proportion of glucose present as (1→3,1→4)-ß-glucan.

Results and discussion

Extraction and composition of polymers

In total, 96% of the WUS could be extracted using sequential extraction with saturated $Ba(OH)_2$, 1M KOH and 4M NaOH in the presence of $NaBH_4$. The latter was used to prevent alkaline peeling[29]. Furthermore, a concentration of 260 mM is needed for the selective extraction[18] of arabinoxylans by $Ba(OH)_2$. Saturated $Ba(OH)_2$ extracted the bulk (68%) of the WUS as a first extract and an additional 11% by subsequent washing with water. A similar proportion (10%) was extracted by 1M KOH, with only a small proportion (4%) of the WUS being present in the 4M extract. More non-arabinoxylan polymers were extracted with successive steps in the extraction procedure used. This is illustrated in Fig. 1. The BE1 fraction comprised arabinose and xylose only, and this fraction represented *c.* 80% of all the arabinose and xylose present in the WUS. Its sugar composition resembled that of the water-extractable arabinoxylan (weAX), which was prepared with a yield of 0.3% of flour weight. Both the BE2 and 1M extract contained substantial amounts of glucose, which was present mainly in the form of (1→3,1→4)-ß-glucans. The latter fraction contained *c.* 62% of the (1→3,1→4)-ß-glucans present in the WUS. The 4M extract contained substantial proportions of mannose, representing 80% of all the mannose present in the WUS, in addition to glucose, arabinose, and xylose. In this fraction, *c.* 50% of the glucose was accounted for as (1→3,1→4)-ß-glucans. Glucose was

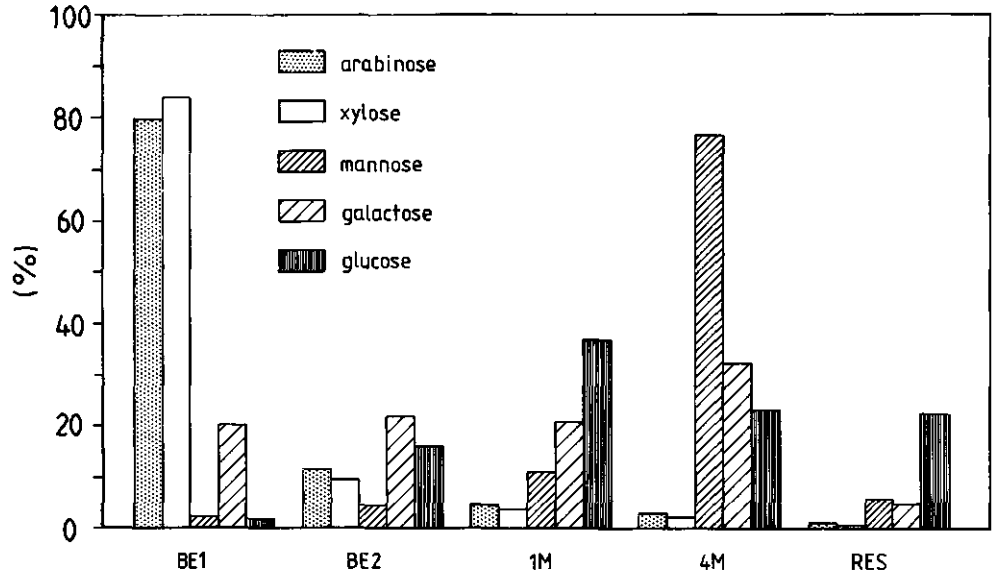

FIGURE 1. Distribution of individual sugars among the various fractions obtained after extraction. The different shaded bars relate to the amount of individual sugars extracted, expressed as percentage (w/w) of the amount present in WUS.

by far the most important sugar in the unextractable residue, but little of it was in the form of (1→3,1→4)-ß-glucan.

In cell wall extraction some authors have used either NaOH[17,30] or KOH[15,31], whereas others have used both[14]. KOH is reported to leave more mannose containing polysaccharides unextracted than NaOH[32]. When only NaOH was used as extractant less mannose was recovered in the 4M extract (results not shown). Therefore, it is essential to use KOH prior to NaOH in the sequential extraction procedure. Whereas 4-methylmorpholino-N-oxide (MMNO) has been reported to be a selective extractant for mannans[33], comparison of the above data with those for MMNO extraction of wheat flour WUS[18] shows that the sequential extraction used in this study is even more selective than MMNO extraction.

The extracts contained only low amounts of protein (1-2% w/w; Table I). The amino acid compositions of the 1M and 4M extracts (data not shown) resembled those of the WUS, BE1 and BE2 fractions[18], except for low contents of aspartic acid in the 1M and 4M extract (2.9 and 3.3 mole%, respectively) and a high content of leucine in the 4M extract (12.0 mole%). Minor differences in amino acid composition between extracts were also reported for beeswing wheat bran[15] and wheat endosperm[34]. Hydroxyproline, as a site of attachment of arabinose, is a major component of dicotyledonous cell walls[35] but was detected only at very low levels in the WUS, RES and BE1 fractions (1.2, 0.3 and 1.7%, respectively). It is not usually found in cereal cell wall preparations, although in rice

TABLE II. Glycosidic linkage compositions of fractions

Alditol acetate of	Amount (mole %)[a]						
	weAX	WUS	BE1	BE2	1M	4M	RES
2,3,5-Me_3-Ara [b]	32.0	30.1	35.6	28.7	17.6	11.9	7.5
3,5-Me_2-Ara	0.9	1.0	1.0	2.1	0.9	0.4	0.3
2,5-Me_2-Ara	0.3	0.2	0	0.8	0.5	0.3	0.2
2,3-Me_2-Ara	0.8	0.6	0.4	0.3	0.3	0.2	0.3
2,3,4-Me_3-Xyl	0.2	0	0.3	1.4	0.9	0.3	0.3
2,3-Me_2-Xyl	40.9	35.0	39.0	28.3	17.4	6.4	3.8
2[3]-Me-Xyl [c]	12.6(9.3)	11.5(8.2)	12.3(8.3)	8.3(4.2)	7.7(7.0)	4.3(6.1)	2.1(1.4)
Xyl	11.2	9.9	11.0	10.4	5.2	4.4	2.6
2,4,6-Me_3-Glc	0.3	1.3	0	3.1	9.7	3.5	0.6
2,3,6-Me_3-Glc	2.2	8.4	0	16.0	37.1	38.2	82.3
2,3,6-Me_3-Man	0	2.1	0	0.7	2.8	30.2	0
2,3-Me_2-Xyl / 2(3)-Me-Xyl+Xyl	1.7	1.6	1.7	1.5	1.4	0.7	0.8
Xyl / 2(3)-Me-Xyl	0.9	0.9	0.9	1.2	0.7	1.0	1.2

[a] Expressed as percentage (mol per 100 mol) of all partially methylated alditol acetates present.

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

[c] Sum of 2-Me-Xyl and 3-Me-Xyl as determined by GC-FID; the figures in parentheses represent the ratio 2-Me-Xyl/3-Me-Xyl as determined by GC-MS.

endosperm the protein material in a 4M KOH unextractable cell wall fraction[36] contained 30% hydroxyproline on a molar basis.

Glycosidic linkage composition

The results of methylation analysis of the extracted polysaccharides are given in Table II. Partially methylated alditol acetates present in trace amounts (< 0.1%) were not taken into account. The data show that xylose was present as unsubstituted, mono-, and di-arabinosylated pyranosyl residues. Most of the arabinose in each fraction was present as terminal furanosyl residues, as indicated by the presence of 2,3,5-Me_3-Ara. The small amounts of 2,5-,3,5- and 2,3-Me_2-Ara suggests that up to 10% of the arabinose in the fractions was present in longer sidechains. Arabinose containing oligomeric sidechains have also been found in arabinoxylan fractions from beeswing wheat bran[15,37] and in water-extractable wheat flour arabinoxylans[38]. The arabinose residues were linked to the xylan backbone through positions *O*-2 or *O*-3 of the individual xylose residues, as shown by the presence of 3-Me-Xyl and 2-Me-Xyl, respectively, or at both positions of the same

xylose residue as indicated by the presence of Xyl.

With the separation conditions used, GLC-FID analysis could not separate 2-Me-Xyl and 3-Me-Xyl. They were also not separated by GC-MS. The ion-spectrum of the top of the 2(3)-Me-Xyl peak showed predominantly ion m/e 118, indicative[39] of deuterated 2-Me-Xyl. On scanning over the whole peak, however, ions m/e 129 and 130, indicative[39] of deuterated 3-Me-Xyl, were detected in substantial amounts in the tail of the peak. Analysis of almost pure 3-Me-Xyl, obtained from a fraction having a high m/e (129+130) to m/e 118 ratio showed that 25% of the total ion count was present as m/e (129+130). For a pure 2-Me-Xyl fraction (no m/e 129+130 present) it was found that 20% of the total ion count was present as m/e 118. Taking into account the above factors of 20 and 25%, the ratio 3-Me-Xyl to 2-Me-Xyl was calculated by GC-MS from the relative abundance of m/e 118 and m/e (129+130), respectively. The ratio of 2-Me-Xyl to 3-Me-Xyl varied in the WUS subfractions between 8.3 (BE1) and 1.4 (RES). For the WUS and weAX fraction these values were 8.2 and 9.3, respectively.

The water-extractable arabinoxylans exhibited a somewhat higher average Ara/Xyl ratio (0.55) than the alkali-extractable arabinoxylans (0.52) in the BE1 extract which represented the major part of the water-unextractable arabinoxylans. This is in accordance with data on wheat aleurone arabinoxylans[40]. For water- and alkali-extractable arabinoxylans from wheat endosperm, however, similar Ara/Xyl ratios were found[14]. The water-extractable arabinoxylans examined here had ratios of unsubstituted to substituted xylose and single substituted xylose to double substituted xylose similar to those of the BE1 extract. In a recent study[38] on water-extractable arabinoxylans from the soft wheat variety Kadet, the ratios of unsubstituted to substituted xylose and double substituted xylose to single substituted xylose were found to be 2.2 and 0.6, respectively. Therefore, these parameters do not seem to account directly for the difference in water-extractability between the water-extractable and water-unextractable arabinoxylans. Other factors, such as non-covalent and covalent (e.g. phenolic linkages) interactions[1] have been suggested as being responsible for the difference in extractability. For the alkaline extracts the more heavily substituted arabinoxylans were found, in this study, to be less extractable. Since increased arabinosylation increases the water-solubility of the xylan backbone[6], this difference in solubility does not seem to be related to the difference in alkali-extractability. As with differences in water extractability, other factors must be responsible for the difference in extractability in alkaline solutions. In this case, the effect of different ratios of unsubstituted to substituted xylose and double substituted xylose to single substituted xylose may be significant.

In both the water-extractable and the water-unextractable arabinoxylans the arabinose was linked predominantly at *O*-3 of the xylose residues. This is in agreement with the literature[1], although for water-extractable arabinoxylans from wheat flour up to 33% of *O*-2 linked arabinose has been reported[41]. In our study, such a high proportion (36%) of *O*-2 linked arabinose was found only in the RES fraction. The ratio of unsubstituted to substituted xylose (2,3-Me_2-Xyl/(2[3]-Me-Xyl+Xyl) decreased slightly from fraction BE1 through BE2 to fraction 1M, but fraction 4M had a considerably lower value. The ratio of double substituted to single substituted xylose residues varied from 0.7 to 1.2 but not in a

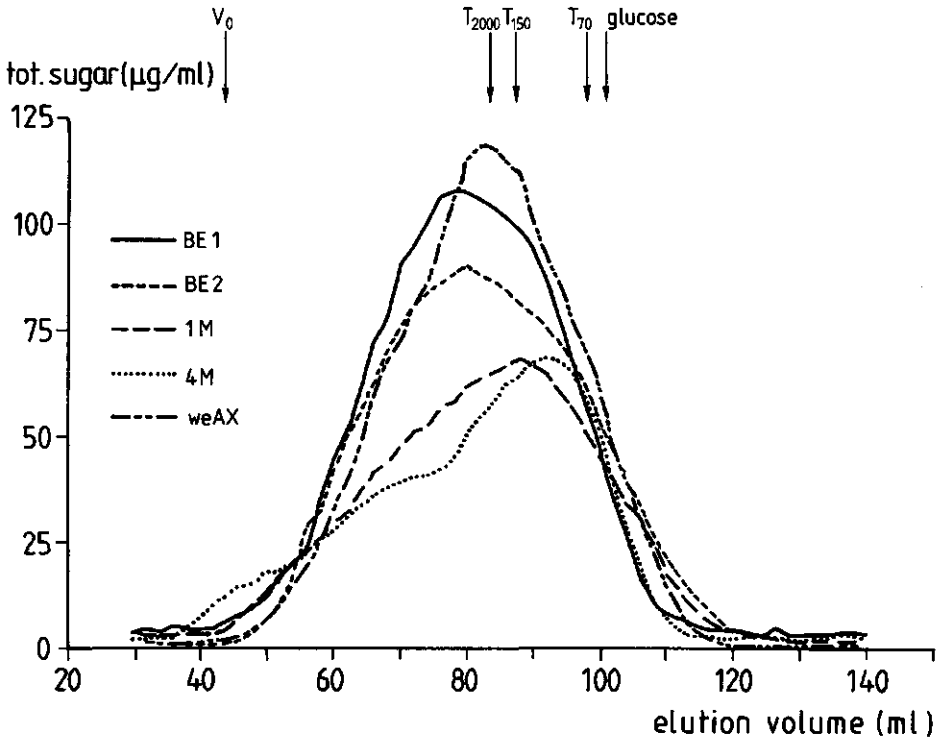

FIGURE 2. Gel permeation chromatography of alkaline extracts of WUS and water-extractable arabinoxylans on Sephacryl S1000. Elution profile for total neutral sugars determined by the orcinol method.

systematic fashion. The large amounts of 2,3,6-Me_3-Man in the 4M extract indicate the presence of glucomannans, which have been found to be present in various cereal cell walls[1]. The presence of 2,4,6- and 2,3,6-Me_3-Glc indicates the presence of (1→3,1→4)-ß-glucans and/or (1→4) linked glucans (cellulose, glucomannans). The (1→3,1→4)-ß-glucan content is given for each fraction in Table I. Assuming all other glucose to be present in ß-(1→4)-linked form, e.g. in cellulose or glucomannans, and subtracting this from the total amount of 2,3,6-Me_3-Glc (Table II), the ratio of (1→3)-linked-glucose to (1→4)-linked-glucose for the (1→3,1→4)-ß-glucans in the extracts was calculated to be 0.26-0.31. Higher ratios were found in (1→3,1→4)-ß-glucans from barley and oat[1] (0.43) and in wheat aleurone cell walls[40] (0.35-0.50). In the final residue almost all the glucose was present as (1→4)-linked glucose. Taken together with its unextractability in concentrated alkali and the low level of (1→3,1→4)-ß-glucan determined using the enzymic assay, the data indicate that these glucosyl residues are predominantly cellulosic.

Molecular size distribution

Figure 2 shows the gel permeation patterns of weAX and WUS extracts on Sephacryl S1000. Only neutral sugars, and no uronide-containing material, were detected in each

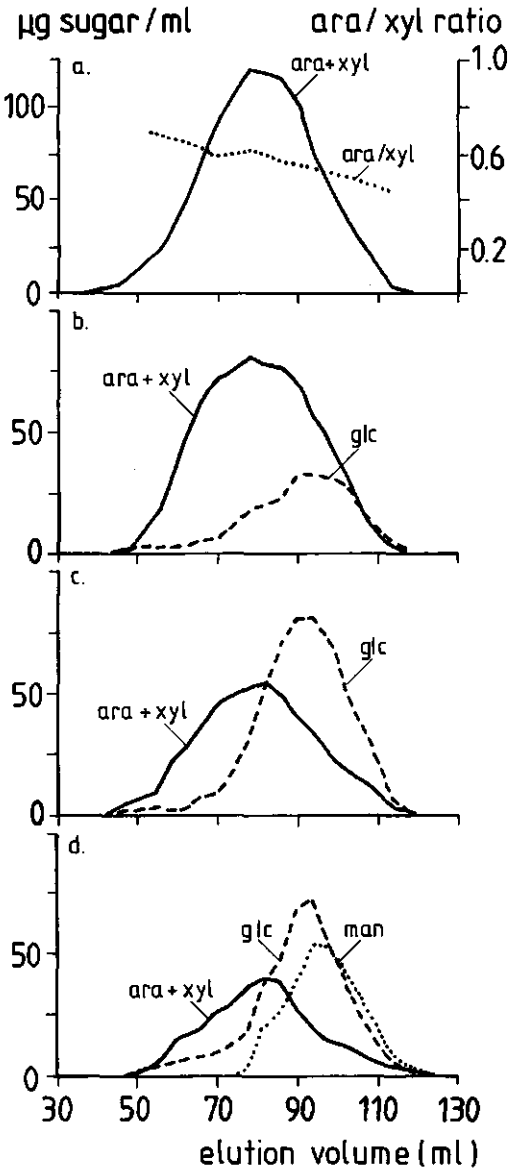

FIGURE 3. Gel permeation chromatography of alkaline extracts of WUS on Sephacryl S1000: (a) elution profile of BE1 showing arabinose + xylose values (ara+xyl) and arabinose/xylose ratios (ara/xyl), (b) elution profile of BE2 showing arabinose + xylose (ara+xyl) and glucose (glc) values, (c) elution profile of 1M showing arabinose + xylose (ara+xyl) and glucose (glc) values, (d) elution profile of 4M showing arabinose + xylose (ara+xyl), glucose (glc) and mannose (man) values.

fraction. The extracted polymers were clearly polydisperse, with apparent molecular weights in the fractionation range of the column: mol. wt 5.10^5 - $>10^8$ (Pharmacia,

dextran standards). The bulk of the polysaccharides in the 1M, 4M and weAX fractions were eluted at higher volumes than those in the BE1 and BE2 extracts.

For each WUS extract a neutral sugar profile was obtained by analysis of the monosaccharide composition of the column fractions. The amount of each constituent sugar versus the elution volume is shown in Fig. 3a-d. Since no, or only low amounts of 2,3-Me_2-Glc and 2,3,4-Me_3-Xyl, indicative of xyloglucans, were detected, all the arabinose and xylose was likely to have been present as arabinoxylans, hence the use in Fig. 3 of values for arabinose plus xylose rather than values for the two individual sugars. Comparison of Fig. 2 and 3 shows that (1→3,1→4)-ß-glucans and (gluco)mannans are the polysaccharides in the 1M and 4M extracts that were eluted at higher volumes as detected by the unspecific orcinol assay (Fig. 2), whereas arabinoxylans were present in all column fractions. These higher elution volumes of mannose and glucose relative to arabinose and xylose indicate that wheat glucomannans and (1→3,1→4)-ß-glucans have lower apparent molecular weights than arabinoxylans. Based on dextran standards, the (1→3,1→4)-ß-glucans and mannans have apparent mean molecular weights of *c.* 150,000 and 100,000, respectively. The lower apparent molecular weight of (1→3,1→4)-ß-glucans relative to arabinoxylans is in contrast with results obtained from the analysis of water-extractable polysaccharides from barley cell walls[42].

With increasing elution volume a decrease in arabinose/xylose (Ara/Xyl) ratio from 0.69 to 0.44 was observed for BE1 [Fig. 3(a)]. For the BE2, 1M and 4M extracts, the decrease in Ara/Xyl ratio with increasing elution volume was less pronounced or even absent (results not shown). Variation in Ara/Xyl ratio with molecular weight of wheat endosperm arabinoxylans has been reported[34], although not in a systematic fashion. Based on comparison with dextran standards, the polysaccharides in the BE1 and weAX fraction had apparent mean molecular weights of *c.* 16.10^6 and 1.10^6, respectively. A recent study[10] showed that both water- and alkali-extractable pentosans (arabinoxylans) were excluded from Sepharose CL-4B columns indicating apparent molecular weights exceeding 5.10^6. Similar results were found in studies[43,44] on water-extractable wheat flour arabinoxylans although lower apparent molecular weights have also been reported[20,37,45].

The alkali-extractable arabinoxylans examined here had higher apparent molecular weights than water-extractable arabinoxylans. Similar results were obtained for wheat endosperm[34] and wheat flour arabinoxylans[46], although in the latter study lower apparent molecular weights were observed. Because arabinoxylans are highly linearly branched, gel permeation chromatography often results in overestimation of molecular weights using dextran standards[1]. Since no calibration standards of similar shape are available, determination of the exact molecular weights using only gel permeation chromatography is not possible.

Conclusions

With a sequential extraction procedure using saturated $Ba(OH)_2$, 1M KOH, and 4M NaOH fractions enriched in arabinoxylans, (1→3,1→4)-ß-glucans and glucomannans, respectively, were obtained from wheat flour WUS. The arabinoxylans extracted at successive stages of

this procedure had progressively lower ratios of unsubstituted to substituted xylose. The arabinose was present mostly as single-unit sidechains, although longer arabinose containing sidechains also appeared to be present. Only minor differences in protein content and amino acid composition were observed amongst the fractions. Methylation analysis showed no difference between the water-extractable and the major water-unextractable arabinoxylan fraction. Gel permeation chromatography showed that alkali-extractable arabinoxylans had slightly higher apparent molecular weights than water-extractable arabinoxylans. Alkali-extractable (1→3,1→4)-ß-glucans and glucomannans had lower apparent molecular weights than arabinoxylans.

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CHAPTER 6

Water-unextractable cell wall material from wheat flour. II. Fractionation of alkali-extracted polymers and comparison with water-extractable arabinoxylans

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Summary

Alkaline extracts of water-unextractable cell wall material (WUS) from wheat flour were fractionated by DEAE anion exchange chromatography. Neutral sugar analysis showed that, for each extract, the arabinoxylans that did not bind to the ion exchanger had lower overall arabinose/xylose (Ara/Xyl) ratios than those that did bind. When the extract contained a mixture of polysaccharides, the fractions that were bound to the DEAE column contained more arabinoxylans than the unbound fractions. With increasing buffer strength, arabinoxylans with increasing Ara/Xyl ratios were eluted. The unbounded DEAE fraction (BE1-U) of the major alkaline extract was fractionated further by graded ethanol precipitation. Water-extractable arabinoxylans from the same wheat flour were fractionated similarly. This resulted in arabinoxylan fractions with Ara/Xyl ratios increasing from *c.* 0.4 to 1.1. Glycosidic linkage analysis showed that, as the Ara/Xyl ratio increased, so too did the ratio of substituted to unsubstituted xylose and the ratio of double substituted to single substituted xylose. The single substituted xylose was mainly *O*-3-substituted xylose, although, in fractions with high Ara/Xyl ratios, substantial proportions of *O*-2 substituted xylose were present. No difference in linkage composition between arabinoxylans from the BE1-U fraction, representing 65% of the arabinoxylans present in the WUS, and water-extractable arabinoxylans was observed. HPSEC/light scattering analysis showed that, with increasing Ara/Xyl ratio, the weight average molecular weights of BE1-U fractions increased from 260,000 to 640,000.

Introduction

In the preceeding paper[1] we showed that 96% of the polysaccharides in water-unextractable cell wall material (WUS) from wheat flour could be solubilized by sequential extraction with $Ba(OH)_2$, 1M KOH and 4M NaOH, leaving an unextracted cellulosic residue. Separation based on gel permeation chromatography revealed that the extracted arabinoxylans exhibited higher apparent molecular weights than the extracted (1→3,1→4)-ß-glucans and glucomannans.

Other techniques that have been used to fractionate various types of arabinoxylan preparations from wheat include DEAE-chromatography and graded ethanol or ammoniumsulphate precipitation[2-5]. In the work described here we have applied these techniques to the fractionation of alkali-extracted polysaccharides from wheat flour, and have characterized the fractionated polysaccharides in terms of their sugar compositions and by carrying out glycosidic linkage analysis. The data for the alkali-extractable arabinoxylans are compared with those for water-extractable arabinoxylans obtained from the same wheat flour.

Experimental

Materials

The isolation and characterization of alkali-extractable cell wall extracts (BE1, BE2, 1M and 4M) from water-unextractable cell wall material (WUS) of Arminda wheat flour has been described previously[1]. The isolation of water-extractable arabinoxylans (weAX) were isolated from Arminda wheat flour, according to the method of Fincher and Stone[6] using amyloglucosidase (Boehringer, no. 102849) instead of salivary *alpha*-amylase, and characterization has been described previously[1].

Ion-exchange chromatography

Frozen cell wall extracts were thawed and diluted (300-700 ml; 0.3 mg/ml) in 0.005 M sodium acetate buffer, pH 5.0 and applied to columns (50x5.5 cm) of DEAE-Sepharose CL-6B, equilibrated with the same buffer. After loading with sample the column was washed with buffer (400 ml) and then eluted successively with a linear gradient (3 l) of 0.005-1.0M sodium acetate buffer, pH 5.0, 1.0M sodium acetate buffer, pH 5.0 (500 ml) and 0.5M NaOH (500 ml) at 60 ml/h. Fractions (23 ml) were assayed by automated methods[7,8] for total neutral sugar and uronic acid contents using arabinose and glucuronic acid (GluA) as standards, respectively. In the latter procedure, 96% (w/w) H_2SO_4 containing 0.0125M Na tetraborate was used in order to quantify GluA residues also. For all extracts, recoveries exceeded 90%. DEAE-bound fractions were pooled, dialysed and designated as BE1-B, BE2-B, 1M-B and 4M-B, whereas the corresponding unbound fractions were named BE1-U, BE2-U, 1M-U and 4M-U. All DEAE-bound fractions were rechromatographed, after dialysis, under exactly the same conditions as above. For the

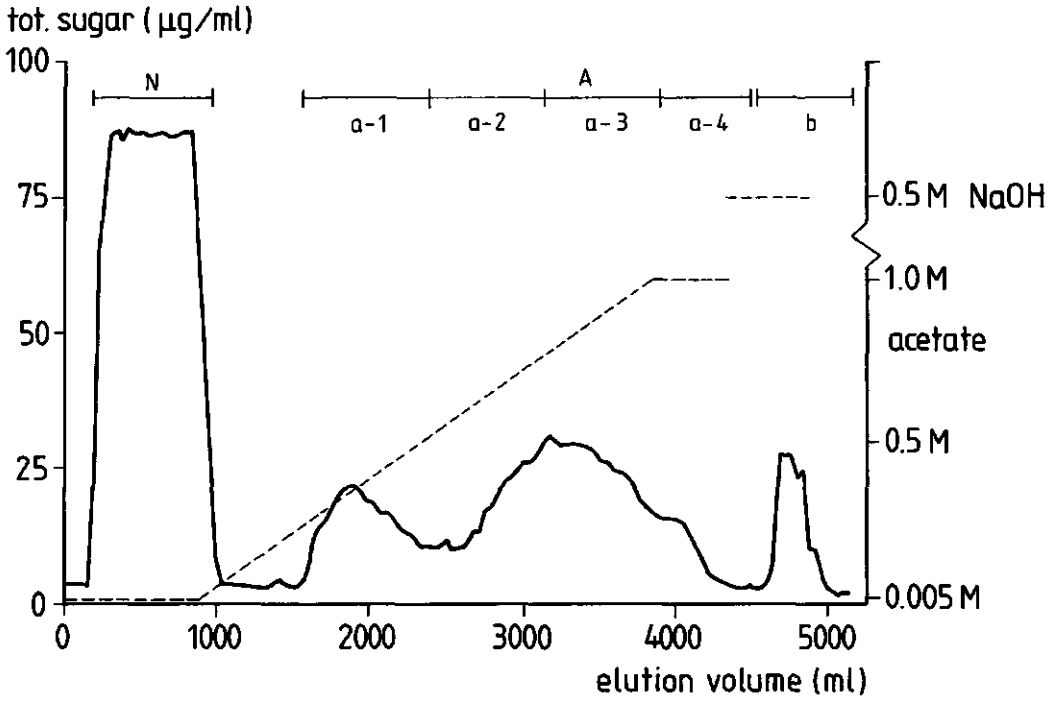

FIGURE 1. Fractionation of BE2 extract on DEAE-Sepharose CL-6B; Aa-n: pooled fractions eluted with Na acetate buffer, pH 5, the digit corresponds with order of elution. A-b: fraction eluted with 0.5M NaOH. The total sugar content is expressed as arabinose equivalents, as determined by the orcinol method.

BE1-B fraction this resulted in a BE1-B-RB (bound) and BE1-B-RU (unbound) fraction, the other extracts showing no further fractionation. Fractions were dialysed extensively against distilled water and kept at -18 °C. Aliquots were thawed or freeze-dried when required. For neutral sugar analysis, fractions were pooled as indicated in Fig. 1.

Graded ethanol precipitation

The BE1-U fraction and the water-extractable arabinoxylans (weAX), both as 0.25% solutions in water, were further fractionated by incremental increases in ethanol concentration (increments of 10%). After each increase in ethanol concentration the mixture was stored at 4° C for 16 h and the precipitate collected by centrifugation (50,000 *g*; 10 min). This resulted in the fractions BE1-U20, BE1-U30, BE1-U40, BE1-U50, BE1-U60 and weAX-20, weAX-30, weAX-40, weAX-50, weAX-60, weAX-70 for the BE1-U and weAX fraction, respectively. The last two digits refer to the ethanol concentration at which the precipitate was collected. The precipitates were dissolved in distilled water, and kept at -18°C. The final supernatants (70% ethanol for BE1-U, 80% ethanol for weAX) were dialysed against distilled water, concentrated and kept at -18 °C (BE1-U70, weAX-80). Aliquots were thawed or freeze-dried as required.

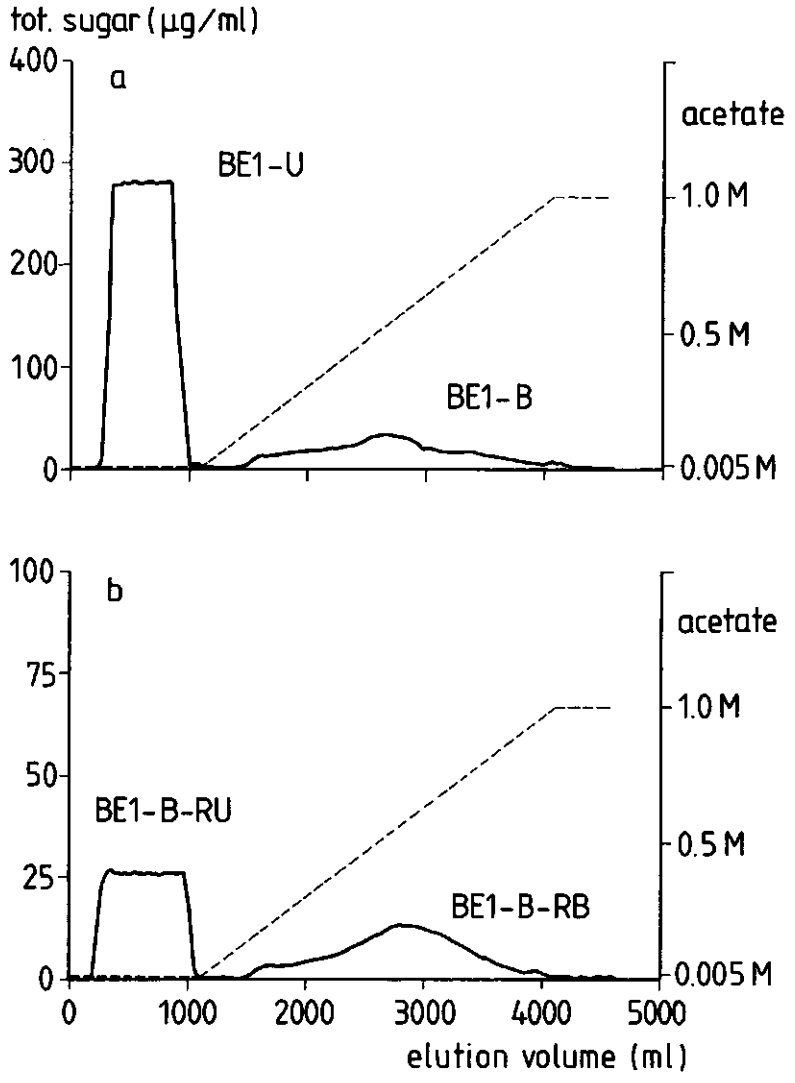

FIGURE 2. Fractionation of BE1 extract (a) and BE1-B fraction (b) on DEAE-Sepharose CL-6B. The total sugar content is expressed as arabinose equivalents, as determined by the orcinol method.

Analytical methods

Neutral sugar analysis was performed as described previously[1], after hydrolysis with 2M trifluoroacetic acid.

Methylation analysis was performed as described previously[1]. The BE1-B-RB fraction was also reduced prior to methylation according to Taylor and Conrad[9] in order to reveal the presence of uronic acids. The procedure was carried out twice.

High performance size exclusion chromatography (HPSEC) was performed with three Bio-Gel TSK columns linked in series (60XL-40XL-30XL) as described previously[10] using both 0.4M and 0.005M Na acetate buffer pH 3. The weight average molecular weight was determined using a DAWN-F multi angle laser light scattering detector[11] in combination with the HPSEC column set.

Uronic acids were determined as trimethylsilyl(TMS)-methylglycuronides as described previously[12].

Results

DEAE fractionation

The DEAE-Sepharose CL-6B elution pattern of the BE1 extract is given in Fig. 2a. Based on the mass of the composite sugar residues, 82% of the sugar material in the BE1 extract was eluted with the starting buffer (Table I). The bound material (18%) could be eluted from the column by increasing the buffer molarity to 1M. Unlike the other extracts, no material was eluted with NaOH for the BE1 extract. Neither the use of lower molarity buffers or water as starting eluent nor the use of a quaternary aminoethyl anion exchanger (QAE-Sephadex) resulted in any greater binding of BE1 to the column.

The DEAE-bound arabinoxylans (BE1-B) had a higher overall arabinose/xylose (Ara/Xyl) ratio (0.66) than those which were unbound (0.51). The BE1-B fraction was rechromatographed after being dialysed. This resulted in a second fractionation [Fig. 2(b)] in which 38% of the applied material was bound to the column and could be eluted using the buffer gradient (BE1-B-RB). The unbound material from the rechromatography experiment (BE1-B-RU) and the unbound fraction from the first chromatography step (BE1-U) had rather similar Ara/Xyl ratios of 0.54 and 0.51, respectively. The bound material (BE1-B-RB) had Ara/Xyl ratios that varied from 0.81 to 0.99. DEAE-bound arabinoxylans that were eluted at a higher buffer concentration had higher Ara/Xyl ratios than those eluted at a lower buffer concentration.

For the BE2 and 4M extracts almost 60% of the material was bound to the column. For the 1M extract this value was 46%. Rechromatography of the BE2-B, 1M-B and 4M-B fractions resulted again in complete binding of these fractions. Not all DEAE-bound material in these extracts could be eluted from the DEAE column using the buffer gradient only. For each of the extracts, elution with 1M buffer and 0.5M NaOH resulted in two major fractions (A-a3 and A-b, respectively). Cleaning the column with 1M NaOH resulted in the release of negligible amounts of carbohydrate material as determined with the automated neutral sugar assay[7].

From the data in Table I, it can be seen that, for the BE2, 1M and 4M extracts, the DEAE-bound fractions contained less glucose on a molar basis than the corresponding DEAE-unbound fractions. For each extract, the bound material had a higher overall Ara/Xyl ratio than the corresponding unbound fraction. For the DEAE-bound fractions it was found that, analogous to the BE1 extract, arabinoxylans with increasing Ara/Xyl ratio

TABLE I. Yields and neutral sugar compositions of fractions from WUS extracts obtained by DEAE chromatography

Fractions	Yield[a]	Molar proportion (%)[b]					Ara/Xyl
		ara	xyl	man	gal	glc	
BE1	100	34.3	65.4	0.1	0.1	0.3	0.53
BE1-U	82	33.5	65.8	0.1	0.3	0.2	0.51
BE1-B	18	39.5	60.0	0.1	0.1	0.3	0.66
BE1-B-RU[c]	62	34.8	64.7	0.1	0	0.3	0.54
BE1-B-RB[c]	38	45.5	52.2	0.3	0.9	1.2	0.87
RB-a1[c]	4	43.4	53.5	0.3	1.5	1.6	0.81
RB-a2[c]	19	45.5	52.9	0.1	0.7	0.8	0.86
RB-a3[c]	10	45.0	52.6	0.1	0.6	0.7	0.87
RB-a4[c]	7	48.2	48.8	0.4	1.0	1.6	0.99
RB-b	0						
BE2	100	32.8	48.7	0.9	0.7	17.0	0.67
BE2-U	43	22.5	39.5	1.4	0.4	36.2	0.57
BE2-B	57	42.7	40.3	2.3	1.2	13.5	1.06
B-a1	13	26.4	38.4	2.4	0.5	32.2	0.69
B-a2	15	42.0	44.5	1.3	1.1	11.1	0.94
B-a3	15	46.0	46.2	1.0	2.3	4.6	1.00
B-a4	8	49.3	45.2	1.0	0.8	3.6	1.09
B-b	6	37.9	44.6	2.2	0.5	14.7	0.85
1M	100	17.5	26.7	2.9	0.9	52.0	0.66
1M-U	54	7.9	19.4	0.9	0.8	71.0	0.40
1M-B	46	30.3	32.6	4.4	1.5	31.2	0.93
B-a1	10	12.1	19.9	4.4	0.5	63.1	0.61
B-a2	13	29.8	36.2	6.4	0.7	26.8	0.82
B-a3	7	36.2	39.4	4.0	1.5	18.9	0.92
B-b	16	32.7	40.8	3.0	3.0	20.5	0.80
4M	100	14.1	17.2	26.1	1.7	41.1	0.82
4M-U	42	15.6	21.1	19.0	1.8	42.5	0.74
4M-B	58	16.2	16.2	28.6	2.0	37.0	1.00
4M-B-a1	7	22.3	21.5	24.0	1.9	30.3	1.04
B-a2	8	22.4	17.1	24.7	2.3	33.6	1.31
B-a3	9	41.8	27.8	5.5	1.4	23.5	1.50
B-b	34	10.5	15.6	32.5	2.0	39.4	0.67

[a] The yield is expressed as weight percentage (as is basis) of BE1, BE2, 1M or 4M, based on the mass of the composite sugar residues.

[b] Abbreviations: ara = arabinose; xyl = xylose; man = mannose; gal = galactose; glc = glucose.

[c] Expressed as weight percentage of BE1-B.

TABLE II. Neutral sugar compositions and yields of subfractions from water-extractable and alkali-extractable arabinoxylans obtained by graded ethanol precipitation

Fractions	Yield[a]	Total sugar content[b]	Molar composition (%)[c]					
			ara	xyl	man	gal	glc	Ara/Xyl
BE1-U	100	91	33.5	65.8	0.1	0.2	0.3	0.51
BE1-U20	19.7	89	26.3	73.7	0	0	0	0.36
BE1-U30	33.2	90	31.4	68.6	0	0	0	0.46
BE1-U40	17.7	87	35.3	64.6	0	0	0	0.55
BE1-U50	16.0	90	40.0	59.1	0	0.2	0.7	0.68
BE1-U60	9.8	93	43.8	55.0	0	0	1.2	0.80
BE1-U70	3.6	79	48.0	44.6	0	0.6	6.5	1.08
weAX	100	87	34.8	62.9	0.1	0.1	2.2	0.55
weAX-20	5.4	85	24.8	57.9	0.1	2.2	15.2	0.43
weAX-30	28.5	82	27.7	70.6	0	0.2	1.5	0.39
weAX-40	19.8	83	33.7	64.6	0	0.3	1.4	0.52
weAX-50	18.4	83	39.7	59.6	0	0	0.8	0.67
weAX-60	15.9	84	45.5	53.9	0	0	0.6	0.84
weAX-70	2.2	79	49.1	48.4	0.2	0.4	1.9	1.02
weAX-80	9.8	9	44.3	25.8	0	15.8	14.2	1.72

[a] The yield is expressed as weight percentage (as is basis) of weAX or BE1-U.
[b] Expressed as weight percentage (as is basis) of each extract.
[c] Abbreviations: ara = arabinose; xyl = xylose; man = mannose; gal = galactose; glc = glucose.

were eluted with increasing sodium acetate buffer concentration. The fractions obtained with the final elution step with 0.5M NaOH had a lower Ara/Xyl ratio than those eluted with buffer.

The water-extractable arabinoxylan (weAX) did not bind to the DEAE column using low buffer molarities (no further data shown). For all alkaline extracts no uronide material could be detected in the bound fractions using either automated uronic acid analysis[7] or analysis of TMS derivatives[12]. E_{280} analysis showed no significant presence of UV absorbing materials.

Ethanol precipitation

Since the BE1-U fraction represented the major part of the arabinoxylans present in the WUS, ethanol precipitation was carried out with this alkali-extractable fraction only. The weAX fraction was also fractionated with ethanol. The yields, total sugar contents and molar neutral sugar compositions of each of the ethanol-precipitated fractions are shown in Table II. For both samples the first precipitate was obtained at 20% (v/v) ethanol

TABLE III. Glycosidic linkage composition of fractions

Alditol acetate of	Amount (mole %)[a]												
		BE1-B		BE1-U					weAX				
	BE1-U	RU	RB	20	30	40	50	60	20	30	40	50	60
2,3,5-Me_3-Ara [b]	33.3	35.1	37.7	26.4	29.8	33.9	39.1	41.1	26.5	26.9	32.7	39.3	43.2
3,5-Me_2-Ara	1.0	0.9	1.6	0.6	0.6	1.1	1.2	2.9	0.4	0.2	0.5	0.9	1.9
2,5-Me_2-Ara	0	0.3		0	0	0.3	0.1	0.2	0.4	0.1	0.1	0.2	
2,3-Me_2-Ara	0	0.1	1.2	0.1	0.1	0.1	1.0	0.2	0.6	0.7	0.2	0.2	0.2
2,3,4-Me_3-Xyl	0.6	0.8	0.6	0.5	0.5	0.7	1.0	1.0	0.3	0.3	0.4	0.7	0.9
2,3-Me_2-Xyl	42.3	39.8	33.4	51.2	46.6	42.1	34.8	27.8	36.3	48.6	41.2	36.1	28.0
2[3]-Me-Xyl [c]	13.2	11.6	11.9	16.3	14.1	11.4	8.6	7.3	12.5	15.4	13.5	8.8	7.1
	(8.8)	(6.7)	(5.4)	(26.8)	(18.5)	(8.5)	(3.9)	(1.8)	(17.6)	(25.1)	(14.5)	(5.3)	(1.8)
Xyl	9.5	11.5	12.8	4.8	8.3	10.8	14.9	17.6	8.4	5.4	10.0	14.0	17.9
2,4,6-Me_3-Glc	0	0	0.1	0	0	0	0	0.4	0.7	0.3	0.1	0	0.2
2,3,6-Me_3-Glc	0.1	0	0.4	0	0	0	0	1.4	14.0	1.8	1.3	0	0.5
2,3,6-Me_3-Gal	0	0	0	0	0	0	0	0.2	0	0	0	0	0
$\frac{\text{2,3-Me}_2\text{-Xyl}}{\text{2(3)-Me-Xyl + Xyl}}$	1.8	1.7	1.4	2.4	2.1	1.9	1.5	1.1	1.7	2.3	1.8	1.6	1.1
$\frac{\text{Xyl}}{\text{2(3)-Me-Xyl}}$	0.9	1.0	1.1	0.3	0.6	0.9	1.7	2.4	0.7	0.3	0.7	1.6	2.5

[a] Expressed as percentage (mol per 100 mol) of all partially methylated alditol acetates present.

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

[c] Sum of 2-Me-Xyl and 3-Me-Xyl as determined by GC-FID; the figures in parentheses represent the ratio 2-Me-Xyl/3-Me-Xyl as determined by GC-MS.

concentration. For the BE1-U and weAX fractions no further precipitation was observed at concentrations above 60% and 70% ethanol, respectively. Sugar analyses revealed that, with increasing ethanol concentration arabinoxylans were precipitated with increasing Ara/Xyl ratio. The weAX-20 fraction was an exception, it also contained 15% glucose on a molar basis. The Ara/Xyl ratio increased from 0.36 to 1.08 and 0.39 to 1.72 for the BE1-U and weAX subfractions, respectively. The weAX-80 fraction contained only 8% neutral sugars. The high Ara/Xyl ratio observed for this fraction is likely to be due to the presence of other arabinose-containing polysaccharides, such as arabinogalactans[13].

Glycosidic linkage analysis.

Methylation analysis was performed to determine the nature of the glycosidic linkages present in the BE1 subfractions. The results are given in Table III. The BE1-U fraction had a ratio of unsubstituted (2,3-Me_2-Xyl) to substituted xylose (2[3]-Me-Xyl + Xyl) of 1.8. For the BE1-B-RU and BE1-B-RB fractions, ratios of 1.7 and 1.4, respectively, were observed. The ratio of double substituted (Xyl) to single substituted xylose (2[3]-Me-Xyl) was 0.9 for the BE1-U extract. The BE1-B-RU and BE1-B-RB fractions had ratios of 1.0 and 1.1, respectively. The BE1-B-RB fraction was also reduced[9] prior to methylation analysis. Significant amounts of 2,3,4,6-Me_4-Glc could not be detected as a result of the reduction.

The ratio of unsubstituted to substituted xylose as determined in the BE1-U ethanol precipitates, decreased from 2.4 to 1.1 as the Ara/Xyl ratio increased, whereas the ratio of double substituted to single substituted xylose increased from 0.3 to 2.4. With the exception of the weAX-20 fraction, similar results were found for the water-extractable arabinoxylan ethanolic precipitates. In Fig. 3 the relative proportions of the partially methylated xylose residues present in each ethanol precipitate are expressed versus the Ara/Xyl ratio. It can be seen from these data that, with increasing Ara/Xyl ratio, the relative amounts of 3-Me-Xyl and Xyl increased also, whereas 2-Me-Xyl and 2,3-Me_2-Xyl decreased. For the BE1-U, BE1-B-RU and BE1-B-RB fraction, ratios of 2-Me-Xyl to 3-Me-Xyl of 8.8, 6.7 and 5.4 respectively, were found. For the weAX-20 fraction, the Ara/Xyl ratio and the relative proportions of different types of methylated xylose residues were inconsistent with the trends noted for the other ethanol precipitates. We have no explanation for this phenomenon. Since the weAX-20 precipitate accounted for only 5% of the weAX fraction, it was not investigated further.

Molecular size distribution

The HPSEC distribution patterns of the various BE1 fractions using a 0.4M buffer as eluant are given in Fig. 4. The BE1 subfractions had greater retention times than the BE1 fraction itself. For the ethanol precipitates it was observed that arabinoxylans with higher Ara/Xyl ratios had lower retention times. The weight-average molecular weight (M_w) data obtained by in-line light scattering analysis are shown in Table IV. The BE1 extract had a weight-average molecular weight of 850,000. The various subfractions exhibited weight-

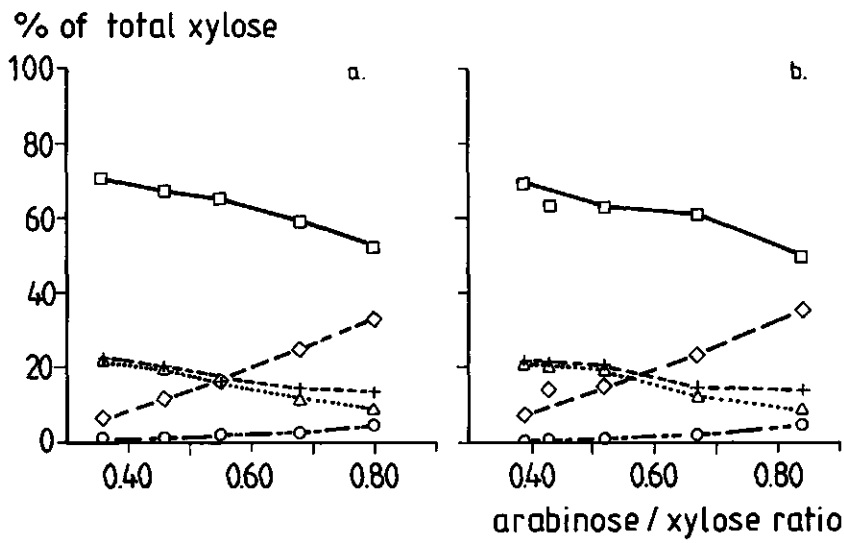

FIGURE 3. Relative amounts of partially methylated xylose residues in BE1-U (a) and weAX (b) subfractions; □ 2,3-Me_2-Xyl; + 2(3)-Me-Xyl; ◇ Xyl; Δ 2-Me-Xyl; ○ 3-Me-Xyl.

TABLE IV. Average molecular weights of various fractions

Fraction	M_w[a]	M_w/M_n[b]	Fraction	M_w	M_w/M_n
BE1	$8.5x10^5$	1.5	BE1-U20	$2.6x10^5$	1.3
			BE1-U30	$3.4x10^5$	1.3
BE1-U	$4.4x10^5$	1.8	BE1-U40	$3.6x10^5$	1.4
BE1-B-RU	$3.6x10^5$	2.5	BE1-U50	$4.8x10^5$	2.0
BE1-B-RB	$6.4x10^5$	10	BE1-U60	$5.3x10^5$	1.3

[a] M_w: weight average molecular weight.
[b] M_n: number average molecular weight.

average molecular weights varying between 260,000 and 650,000. For most of the fractions the ratio of weight average molecular weight to number average molecular weight (M_w/M_n) varied from 1.3 to 2.5.

In order to investigate the presence of acidic groups as constituents of the polymers, HPSEC analysis was also performed with 0.005M buffer molarity (Fig. 5). In the 0.4M buffer polysaccharides are separated by differences in hydrodynamic volume. In the 0.005M buffer charged groups will not be shielded by buffer ions. As a result, ionic polysaccharides will exhibit increased exclusion because of swelling of the polysaccharides[14] or repulsion of the polysaccharides by column material[15]. Comparison of Figs. 4 and 5 indicates that, for the BE1-B fraction, only a proportion of the material was eluted with a lower retention time under the low ionic strength conditions, indicating that

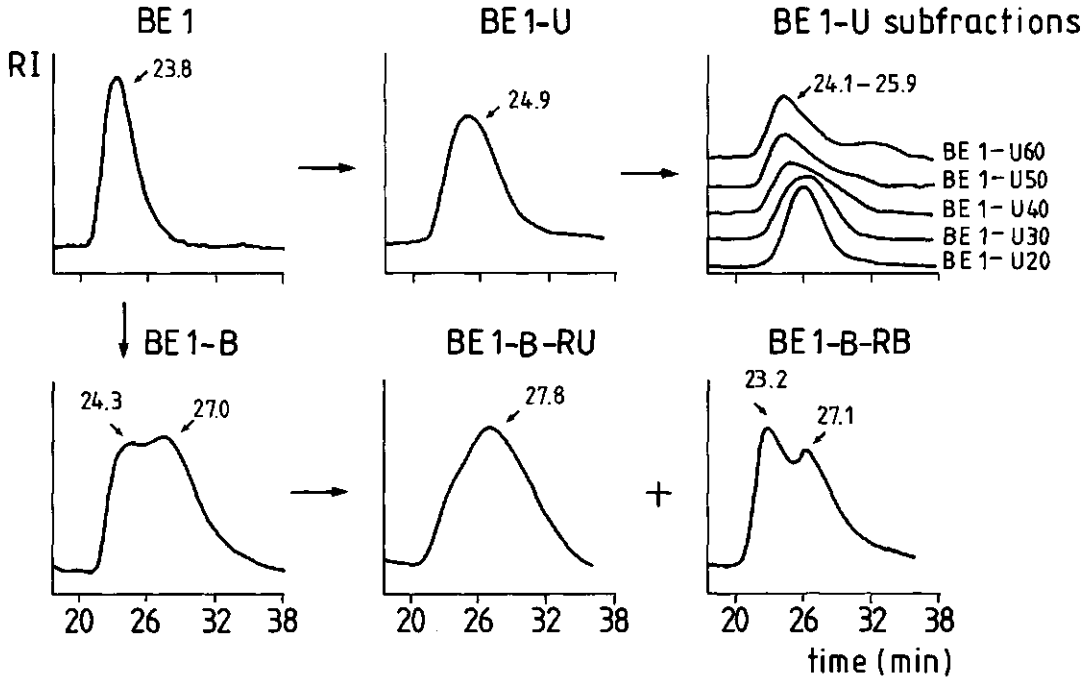

FIGURE 4. Elution pattern (HPSEC) of BE1 subfractions using 0.4M Na acetate buffer, pH 3.

non-acidic polysaccharides were still present in this DEAE-bound fraction, whereas, for the BE1-B-RB fraction, all the material was now eluted at the void volume. No effect on retention time was observed for the BE1-U and BE1-B-RU fractions, indicating their non-acidic characteristics. HPSEC analysis was not carried out with the other fractions.

Discussion

DEAE fractionation

DEAE chromatography showed that substantial proportions of all the water-unextractable fractions from wheat flour cell walls had acidic characteristics, whereas the weAX showed no acidic behaviour. The latter is in agreement with previous studies[16] on water-extractable arabinoxylans, which could not be fractionated on DEAE-cellulose at pH 8. Fractionation of both water-extractable and water-unextractable arabinoxylans has been achieved successfully with DEAE-cellulose in the borate form[16-19]. However, as the DEAE groups will be completely uncharged at the pH used, it has been suggested[20] that, for chromatography in this mode, fractionation is probably due to adsorption of arabinoxylans to the cellulose material rather than to an ion-exchange mechanism. Our results are in agreement with those found for beeswing wheat bran using DEAE-Sephacel chromato-

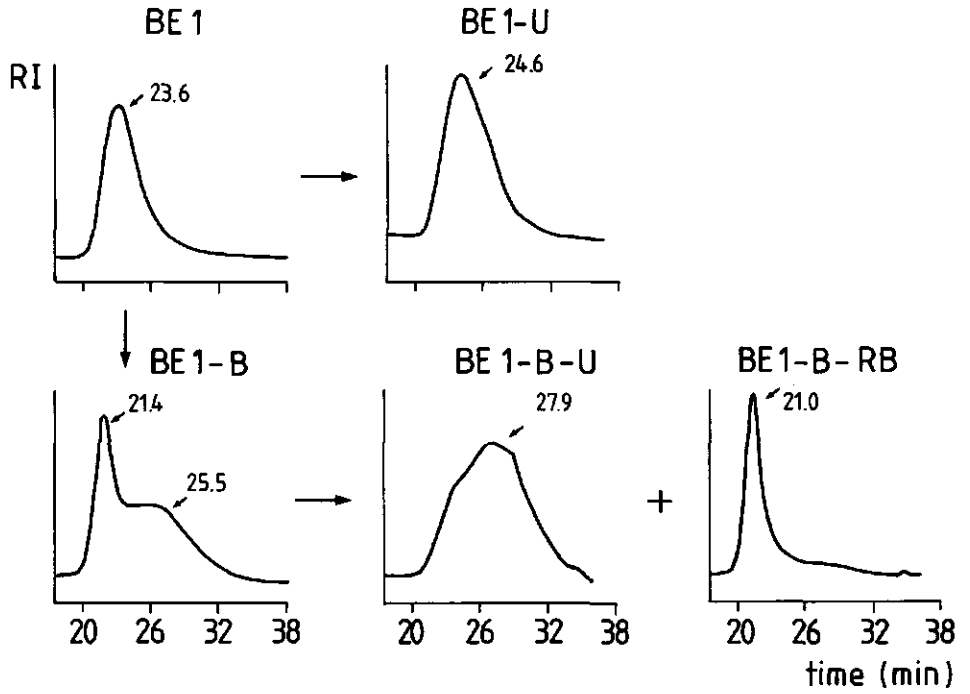

FIGURE 5. Elution pattern (HPSEC) of BE1 subfractions using 0.005M Na acetate buffer, pH 3.

graphy[2], in terms of both Ara/Xyl ratios observed at the buffer concentration to elute the polymers, as well as the sugar compositions of the fractions.

The mechanism of binding of arabinoxylans to the DEAE-column is not clear. The behaviour of the BE1 fraction upon rechromatography may be explained by aggregation. The results obtained by HPSEC analysis showed that the BE1-U fraction had about half the molecular weight of the BE1 fraction itself. Since molecular weight determination by laser light scattering analysis is independent of the molecular size of the molecule[11,21], the difference in molecular weight apparently indicates aggregation of the arabinoxylans. As high salt concentrations influence the conformation of polysaccharides[22] the associations within the BE1 fraction may be diminished during the gradient elution. This was examined by dissolving the BE1 in 1M sodium acetate buffer, pH 5, prior to dialysis and DEAE chromatography. After this pretreatment the BE1-U fraction accounted for 85% of the BE1 extraction versus 82% without pretreatment. The difference was more pronounced during the rechromatography step. Now the BE1-B-RU accounted for 50% of the BE1-A subfraction, whereas without this pretreatment, this value was 62% (Table I). This is in agreement with the above hypothesis regarding aggregation of the arabinoxylans. Because freeze-thaw treatments of polysaccharides can lead to irreversible aggregation we have also used unfrozen BE1 samples. DEAE-chromatography showed, however, no difference between the frozen and unfrozen BE1 samples, indicating that the aggregation was not an

artefact of the freeze-thaw treatment. Although this behaviour on rechromatography on DEAE-Sepharose was observed only for the BE1 extract, it does not seem to be related to presence of Ba^{2+} ions. The same phenomenon was observed for NaOH extracted lucerne xylans[23], and we have observed similar behaviour of an 1M NaOH extract of wheat flour WUS[10], although to a lesser extent (data not shown). For grasses it has been reported that GluA is a constituent of arabinoxylans[24]. Whereas GluA residues have been identified in wheat bran materials[25], its presence in wheat flour or endosperm material has only be indicated indirectly[26,27]. The presence of GluA residues in arabinoxylans might explain the binding to the DEAE-Sepharose CL-6B column. In this study, however, GluA residues could not be detected in significant amounts either colorimetrically, or as TMS derivatives, or with methylation analysis. Proteins seems to be involved in the binding of the material to the DEAE column. When the BE1-B-RB fraction was treated with a protease (Sigma, P5147), rechromatography showed that 40% of the material was now not bound to the column (results not shown). The protease preparation showed no polysaccharidase activity on the BE1-U and weAX fractions, as measured by HPSEC analysis. The unbound fraction from the protease treated BE1-B-RB fraction had an Ara/Xyl ratio of 0.55 whereas that for the bound fraction was 0.89. Surprisingly, no major difference in amino acid composition between the fractions was observed (results not shown).

The acidic behaviour of the BE1-B-RB fraction was confirmed with HPSEC using different buffer concentrations. For the BE2, 1M and 4M fractions also, no acidic sugar residues could be detected as TMS derivatives. Considerably lower retention times were observed for the BE2, 1M and 4M extracts, however, when HPSEC analysis was carried out at lower ionic strength (results not shown), indicating their acidic characteristics. No further investigation on the nature of the DEAE-binding was performed.

Linkage analysis

The observation that the Ara/Xyl ratio increased for precipitates obtained with increasing ethanol concentration is consistent with the results of other studies on wheat endosperm arabinoxylans[3] and warm water-extractable wheat flour arabinoxylans[4]. For cold water-extractable wheat flour arabinoxylans[5], however, data have been published that show no such effect.

For the ethanol precipitates, the Ara/Xyl ratio seemed to be related to the proportions of 2,3-Me_2-Xyl, 2-Me-Xyl, 3-Me-Xyl and Xyl present, irrespective of whether they were obtained from the weAX or BE1-U fractions. The observed relationships between Ara/Xyl ratio and the ratio of double substituted to single substituted xylose are in agreement with those of warm water-extractable arabinoxylans from Kadet wheat flour[4], although, for cold water-extractable arabinoxylans from the same flour, this relationship was not observed[5]. Comparison of the above data on ethanol precipitates with those on the BE1-B-RU, BE1-B-RB (Table III) and unfractionated WUS extracts[1] showed, however, that similar Ara/Xyl

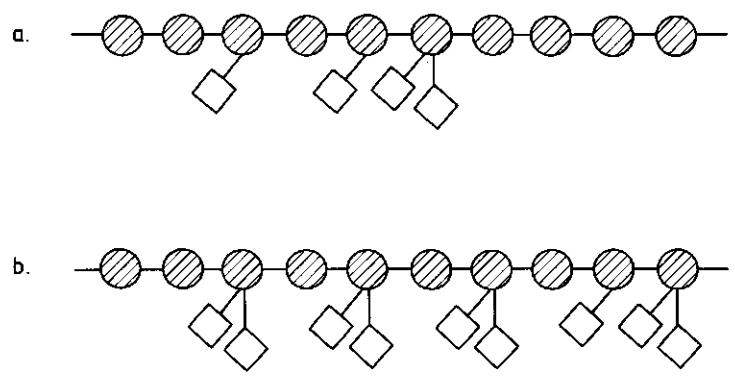

FIGURE 6. Schematic representations of the structures of arabinoxylans in BE1-U30 or weAX-30 (a) and BE1-U60 or weAX-60 (b) fraction, based on methylation data. Notation: ● : Xyl*p*; ◇ : α-Ara*f*; ●● : β-Xyl*p*-(1→4)-Xyl*p*; ⁂ : α-Ara*f*-(1→2)-β-Xyl*p*; ⁂ : α-Ara*f*-(1→3)-β-Xyl*p*

ratios were obtained even though the ratio of double substituted to single substituted xylose varied. This suggests that DEAE-bound and unbound arabinoxylans differ, not only in acidic character, but also in their substitution patterns.

The presence of substantial proportions of 3-Me-Xyl in the weAX-50 and weAX-60 fractions contrasts with results from the above mentioned study on water-extractable arabinoxylans from Kadet wheat flour[4,5], in which no 3-Me-Xyl was observed in any ethanol-precipitated fraction. The BE1-B-RB and the unfractionated WUS extracts[3] also contained substantial proportions of 3-Me-Xyl. However, for any given Ara/Xyl ratio, the ethanol precipitates had higher proportions of 3-Me-Xyl than the BE1-B-RB, BE2, 1M and 4M fractions. This is noteworthy since in glucuronoarabinoxylans from wheat bran, the GluA, which may be responsible for DEAE-binding, is linked to *O*-2 atoms of the xylose residues[2,28].

The results of the methylation analysis are summarized schematically in Fig. 6. These structures are not meant to imply the exact positions of the side chains along the xylan backbone. Based on methylation analysis, no difference between water-extractable arabinoxylans and the alkali-extractable arabinoxylans of the BE1-U fraction could be observed, the latter fraction representing 65% of all the arabinoxylans present in the WUS.

Molecular weight distribution

As indicated above, the observed difference in molecular weight between the BE1 and its subfractions may be due to aggregation. The molecular weight values for the subfractions measured by HPSEC/light scattering analysis are higher than those of wheat arabinoxylans determined by osmometry[18] or sedimentation analysis[19], but are lower than those obtained by gel permeation chromatography[3].

For the BE1-U ethanol precipitates it was shown that a higher Ara/Xyl ratio corresponded with both a higher hydrodynamic volume and a higher molecular weight. From the values for the M_w/M_n ratio it can be seen that all fractions were clearly polydisperse. Since the molar proportion of arabinose increases from 0.26 to 0.44, the doubling in molecular weight cannot be due solely to extra arabinose, but the data indicate also that there is an increase in the length of the backbone. Both are important in relation to the physical properties of the arabinoxylans since increased arabinose substitution 'stiffens' the (1→4)-ß-xylan backbone into a more extended conformation[30]. Extending the backbone will increase yet further the asymmetry of the molecule, which is the main factor responsible for the high viscosity of arabinoxylans[31].

Conclusions

When the alkali extracts of water-unextractable cell wall material from wheat flour contained a mixture of polysaccharides, the fractions that were bound to the DEAE-Sepharose column contained more arabinoxylans than the unbound fractions. For each extract, the DEAE-bound arabinoxylans had higher overall Ara/Xyl ratios than the corresponding unbound arabinoxylans. For the bound fractions it was found that, with increasing ionic strength, arabinoxylans with increasing Ara/Xyl ratios were eluted. Methylation analysis of the unbound BE1 fractions showed that, for these fractions an increase in Ara/Xyl ratio was apparently related to an increase in both the ratio of substituted xylose to unsubstituted xylose and the ratio of double substituted to single substituted xylose. The single substituted xylose was mainly *O*-3 substituted xylose, although, in fractions with high Ara/Xyl ratios, *O*-2 substituted xylose was present in substantial proportions. Based on data from methylation analysis, no difference in sugar linkage composition between alkali-extractable arabinoxylans of the BE1-U fraction, representing 65% of arabinoxylans present in the WUS, and water-extractable arabinoxylans could be observed.

HPSEC light scattering analysis showed that, with increasing Ara/Xyl ratio, the weight average molecular weights of BE1-U fractions increased from 260,000 to 640,000. All fractions were clearly polydisperse.

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CHAPTER 7

Characterisation by ^{1}H-NMR spectroscopy of enzymically-derived oligosaccharides from alkali-extractable wheat-flour arabinoxylan

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Summary

Oligosaccharides obtained from alkali-extractable wheat-flour arabinoxylans by digestion with endo-(1→4)-ß-D-xylanase from *Aspergillus awamori* were fractionated by size exclusion chromatography on Bio-Gel P-2 followed by high performance anion exchange chromatography, and subjected to monosaccharide analysis and ^{1}H-NMR spectroscopy. The results revealed (1→4)-linked ß-D-xylopyrano-oligosaccharides partly *O*-3 and/or *O*-2,3 substituted with single α-L-arabinofuranosyl groups. The structures of 12 such arabinoxylan oligosaccharides were established.

Introduction

Wheat flour arabinoxylans consist[1,2] of a linear backbone of (1→4)-ß-D-xylopyranosyl units, with mainly single α-L-arabinofuranosyl groups attached through positions 2 and 3 but little is known about the distribution of the sidechains. Periodate-oxidation studies[3,4] indicated the presence of clusters of 1 to 4 contiguous substituted ß-D-Xyl*p* residues. However, a study on water-extractable rye arabinoxylans[5] favours small isolated clusters of singly and doubly substituted residues. Enzymic hydrolysis of the cell wall material from bamboo and graminaceous plants yielded di-, tri-, and tetra-saccharides, some of which were feruloylated[6-11].

Detailed ^{1}H-NMR studies of arabinoxylan oligosaccharides, derived from a warm water-extractable arabinoxylan fraction by digestion with an *Aspergillus* endo-(1→4)-ß-D-xylanase, have been reported[12,13]. The structures of alkali-extractable arabinoxylans have been less well characterised, due to the difficulties in their purification. We have described the isolation[14] of highly-purified water-unextractable, alkali-extractable arabinoxylans from wheat flour, which were further characterised[15] and fractionated by anion-exchange chromatography[16]. We now report on the structures of oligosaccharides derived from these arabinoxylans by digestion with endo-(1→4)-ß-D-xylanase I from *Aspergillus awamori* CMI 142717, the mode of action of which is different from that of the endo-(1→4)-ß-D-xylanase previously used[12,13].

Experimental

Materials

Wheat alkali-extractable arabinoxylan (BE1-U) was prepared from water-unextractable cell wall material of the soft wheat variety Arminda[15,16]. Endo-(1→4)-ß-D-xylanase I was purified[17] from *Aspergillus awamori* CMI 142717.

Preparation of arabinoxylan oligosaccharides

A solution of wheat alkali-extractable arabinoxylan (80 mg) in 50mM sodium acetate buffer (80 ml, pH 5.0) was incubated with endo-(1→4)-ß-D-xylanase I (0.4 μg/ml) for 24 h at 30 °C. After inactivation of the enzyme (10 min, 100 °C), the solution was concentrated to 3 ml under reduced pressure, and applied to a column (100x2.6 cm) of Bio-Gel P-2 (200-400 mesh, Bio-Rad) at 60 °C and eluted with distilled water (17 ml/h). Fractions (2.4 ml) were assayed for total neutral sugar content[18]. Appropiate fractions were combined, designated ***1-13***, and each was concentrated to 1.5 ml under reduced pressure. The column was calibrated using a mixture of

xylose, maltose, raffinose, stachyose, and Dextran T150 (Pharmacia). The elution volumes of these compounds correspond with those of fraction ***1-4***, and ***13*** (void), respectively.

Fractions ***3-10*** were subjected to high performance anion exchange chromatography (HPAEC) using a Dionex Bio-LC GPM-II quaternary gradient module equipped with a Dionex CarboPac PA-1 column (250x9 mm). Samples (5x300 μl) were injected using a Spectra Physics SP8780 autosampler equipped with a Tefzel rotor seal in a 7010 Rheodyne injector valve. Elution (5 ml/min) involved linear gradients of sodium acetate in 0.1M NaOH 0-150mM during 10 min, then 150-500mM during 30 min at 20 °C. The solvents were degassed and stored under helium using a Dionex EDM module. The eluate was monitored using a Dionex PED detector in the pulsed-amperometric detection (PAD) mode. A reference Ag/AgCl electrode was used with a working gold electrode with 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. The eluate was neutralized with 1M acetic acid and the appropiate fractions (1.2 ml) were combined, desalted using columns (30x80 mm) of Dowex 50W X8 (H^+) and AG3 X4A (OH^-) resins (Bio-Rad) in series, concentrated under reduced pressure, and the residue was air dried.

Analysis

Monosaccharide analysis. Samples (30-70 μg) were hydrolysed by 2M trifluoroacetic acid for 1 h at 121 °C, the acid was evaporated in a stream of air at 40 °C. For Bio-Gel P-2 fractions, each monosaccharide mixture was reduced in 1.5M ammonia (0.2 ml), containing 75 mg $NaBH_4$/ml, the products were converted into their alditol acetates[19] and analysed[20]. myo-Inositol was used as internal standard. For Dionex fractions, each hydrolysate was dissolved in water and analysed on a CarboPac PA-1 column (250x4 mm), eluted (1 ml/min) with 0.1M NaOH, using PAD analysis (as described above).

FAB-MS Positive-ion fast atom bombardment (FAB)-mass spectra were recorded with a Jeol JMS AX 505 W spectrometer (Xe beam of 6 kV, acceleration potential of 3 kV) equipped with a HP9000 data system. Each sample was dispersed in a glycerol matrix, and the mass range was scanned at 10s/scan with a mass resolution of 1500.

^{1}H-NMR spectroscopy. Samples were repeatedly treated with D_2O (99.9 atom% D, MSD Isotopes), finally using 99.96 atom% D at pD $\geq$7. Resolution-enhanced 600-MHz ^{1}H-NMR spectra were recorded using a Bruker AM-600 (SON-hf-NMR facility, Department of Biophysical Chemistry, Nijmegen University, The Netherlands) spectrometer, operating at a probe temperature of 27 °C. Chemical shifts (δ) are expressed in p.p.m. and were measured by reference to internal acetone (δ 2.225 in D_2O at 27 °C)[21]. Full details of the HOHAHA spin-lock experiments and ROESY spectroscopy are described elsewhere[12].

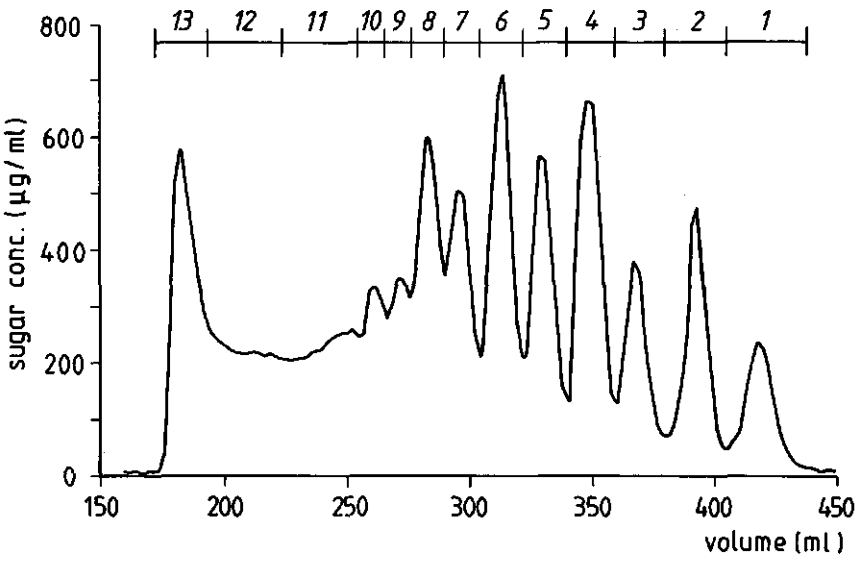


FIGURE 1. Elution profile of the arabinoxylan digest on Bio-Gel P-2.

Results and discussion

The fractionation of the arabinoxylan digest on Bio-Gel P-2 is shown in Fig. 1, and the monosaccharide composition and yield of each combined fraction are given in Table I. Of the 13 fractions, 11 (***1-10*** and ***13***) were observed as separate peaks, ***13*** was eluted in the void volume and ***1*** to ***10*** accounted for 72% of the arabinoxylan. Fractions ***1*** and ***2***, consisting of xylose only (Table I), showed both one peak with HPAEC, and are suggested to contain xylose and xylobiose, respectively.

Fractions ***3-10*** were subjected to HPAEC and the results are shown in Fig. 2. The monosaccharide compositions, molecular weights of the major components, and the recoveries are given in Table I. For fractions ***4*** and ***7***, no further fractionation was achieved, and fractions **4.1** and **7.1** represent 98 and 85% , respectively, of the total PAD responses. Fractions ***3***, ***5***, ***8***, and ***9*** each contained two major components, which represented >90% of the total PAD response of each fraction. Fraction ***6*** contained three oligosaccharides (**6.1**, **6.2**, and **6.3**) in the proportions 60, 27, and 12%, respectively. Fraction ***10*** contained one major component (47% of the total PAD response) and no attempt was made to isolate the minor components. The structures of the major oligosaccharides were elucidated by ^{1}H-NMR spectroscopy.

Fraction **3.1** The ^{1}H-NMR spectrum of **3.1** matched exactly that of the reference compound Xyl_3 [ß-D-Xyl*p*-(1→4)-ß-D-Xyl*p*-(1→4)-D-Xyl*p*][12] and the chemical shifts of the H-1 resonances are summarised in Table II.

TABLE I. Data on the fractions obtained by chromatography on Bio-Gel P-2 and Dionex of the products formed by the enzymic degradation of wheat arabinoxylan

Bio-Gel P-2 fractions	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	
Yield (%)[a]	4.4	6.9	6.6	10.6	8.4	11.1	7.7	8.9	4.1	3.9	8.8	8.3	10.9	
Ara (%)[b]	0	0	25	25	39	33	40	40	40	42	44	47	50	
Xyl (%)	100	100	75	75	61	67	60	60	60	58	56	53	50	
Dionex sub-fractions of ***3-10***	***3.1***	***3.2***	***4.1***	***5.1***	***5.2***	***6.1***	***6.2***	***6.3***	***7.1***	***8.1***	***8.2***	***9.1***	***9.2***	***10.1***
Recovery(%)[c]	31	68	98	48	44	60	27	12	85	53	28	64	27	47
Ara (%)	0	33	26	38	39	35	33	46	42	40	50	43	43	38
Xyl (%)	100	67	74	62	61	65	67	54	58	60	50	57	57	62
Mol. wt.[d]	414 (3)	414 (3)	546 (4)	678 (5)	678 (5)	810 (6)	810 (6)	810 (6)	942 (7)	1074 (8)	1074 (8)	1206 (9)	1074 1206 (8,9)	1338 (10)

[a] Percentage of total neutral sugars present in each fraction, based on spectrofotometric response of all sugars.

[b] Sugar composition of the material in each peak.

[c] Percentage of total PAD response from each Bio-Gel P-2 fraction.

[d] Determined by positive-ion FAB-MS (number of pentose units in brackets)

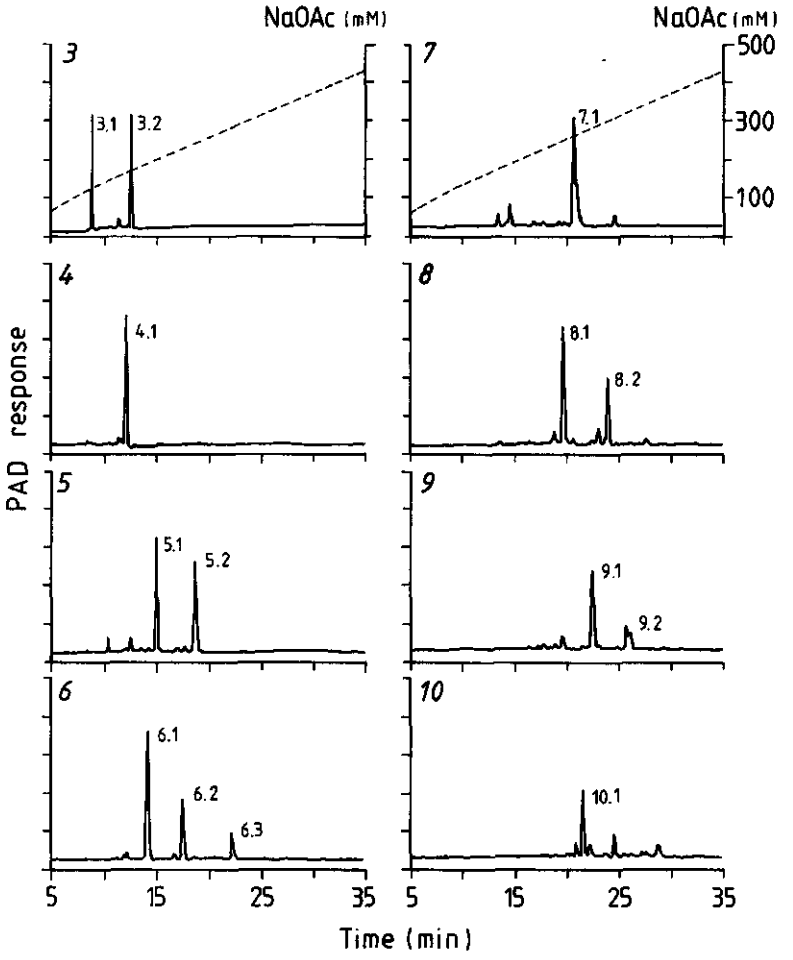


FIGURE 2. Elution profile on HPAEC of Bio-Gel P-2 fractions ***3-10*** in Fig. 1.

3 **2** **1**
ß-Xyl*p*-(1→4)-ß-Xyl*p*-(1→4)-Xyl*p*

3.1

Fraction **3.2** The intensities of the H-1 signals for **3.2** (Fig. 3) indicated an arabinosylxylobiose structure with the Xyl*p* units ß ($J_{1,2}$ 7-8 Hz) and the Ara*f* unit α ($J_{1,2}$ ~ 1.6 Hz)[6,22]. By comparison with published ^{1}H-NMR data for the related feruloylated compound [5-*O*-(*trans*-feruloyl)-α-L-Ara*f*]-(1→3)-ß-D-Xyl*p*-(1→4)-D-Xyl*p*)[9,10], the structure shown was assigned to ***3.2***. The ^{1}H-NMR data are recorded in Table III. Specific assignment of the α-Ara*f* H-5*proR*,5*proS* signals is based on their relative chemical shifts ($\delta_{5proR} > \delta_{5proS}$) supported by

TABLE II. Chemical shifts[a] of the H-1 resonances of fractions **3.1**, **6.1**, **8.1**, **9.1**, and **10.1** from Table I.

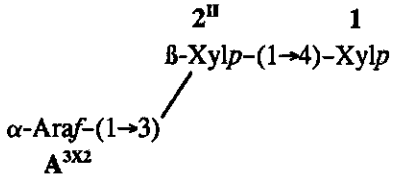
Residue[b]	 3.1[c]	 6.1	 8.1	 9.1	 10.1
α-Xyl*p*-**1**	5.184	5.184	5.184	5.183	5.184
ß-Xyl*p*-**1**	4.584	4.584	4.584	4.584	4.584
ß-Xyl*p*-2_{α}	4.475	4.465	4.465	4.464	4.465
ß-Xyl*p*-2_{β}	4.479	4.468	4.468	4.467	4.468
ß-Xyl*p*-**3**	4.461				
ß-Xyl*p*-3^{III}		4.639	4.637	4.628	4.639
ß-Xyl*p*-**4**		4.437			4.443
ß-Xyl*p*-4^{II}			4.480		
ß-Xyl*p*-4^{III}				4.578	
ß-Xyl*p*-**5**			4.435	4.428	
ß-Xyl*p*-5^{III}					4.628
ß-Xyl*p*-**6**					4.436
α-Ara*f*-A^{2X3}		5.225	5.225	5.222	5.224
α-Ara*f*-A^{3X3}		5.274	5.270	5.293	5.271
α-Ara*f*-A^{2X4}				5.242	
α-Ara*f*-A^{3X4}			5.402	5.281	
α-Ara*f*-A^{2X5}					5.220
α-Ara*f*-A^{3X5}					5.271

[a] Measured at 600 MHz on solutions in D_2O at 27 °C (internal acetone, δ 2.225).

[b] The Xyl*p* residue in the reducing position is denoted **1**, etc.; $\mathbf{2}_{\alpha}$ and $\mathbf{2}_{\beta}$ means that Xyl*p*-**1** is α or ß, Ara*f*-A^{2X3} means arabinofuranose linked to *O*-2 of Xyl*p*-**3**, etc., Xyl*p*-3^{I} means Xyl*p*-**3** substituted at *O*-2, Xyl*p*-3^{II} means Xyl*p*-**3** substituted at *O*-3, Xyl*p*-3^{III} means Xyl*p*-**3** substituted at *O*-2,3.

[c] Key: ● : Xyl*p*; ◇ : α-Ara*f*; ●● : β-Xyl*p*-(1→4)-Xyl*p*; : α-Ara*f*-(1→2)-β-Xyl*p*; : α-Ara*f*-(1→3)-β-Xyl*p*

the $J_{4,5}$ values ($J_{4,5proR} < J_{4,5proS}$)[23]. Owing to the presence of a reducing residue, there is an anomeric effect with doubling of the H-1,2,3,4 signals of ß-Xyl*p*-$\mathbf{2}^{II}$ and the H-1 of (1→3)-linked α-Ara*f*-A^{3X2}.



3.2

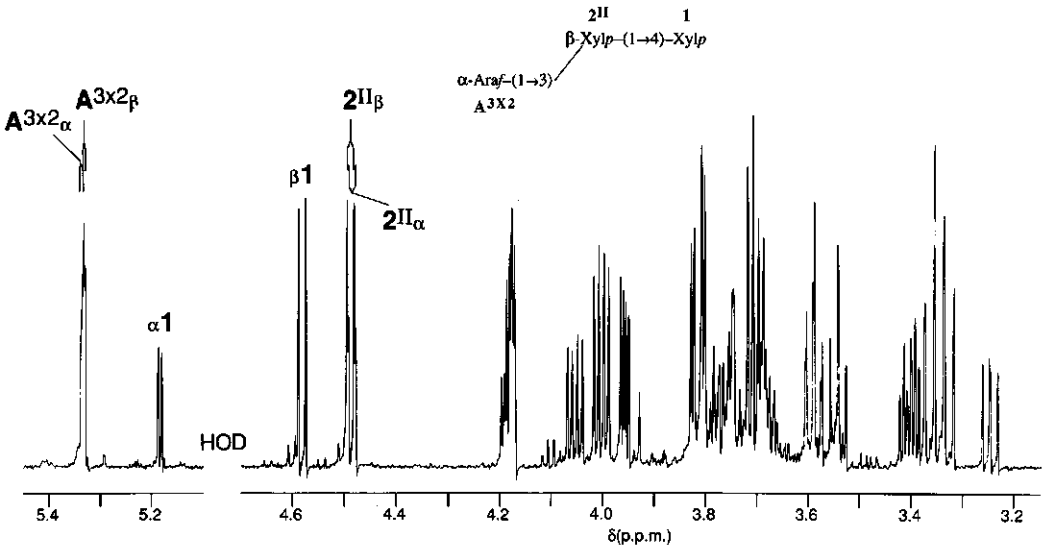
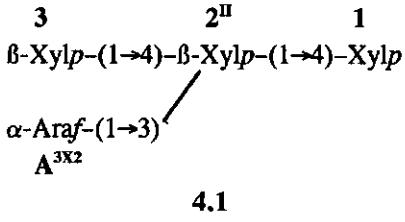


FIGURE 3. Resolution-enhanced 600-MHz ^{1}H-NMR spectrum of fraction **3.2**. The numbers and letters in the spectrum refer to the corresponding residues in the structure.

Fraction **4.1** The intensities of the H-1 signals for **4.1** (Fig. 4) indicated an arabinosylxylotriose with the Xyl*p* units ß ($J_{1,2}$ 7-8 Hz) and the Ara*f* unit α ($J_{1,2}$ ~1 Hz). Comparison of the ^{1}H-NMR data (Table III) with those[12] for **AX-31** [ß-Xyl*p*-4-(α-Ara*f*-**A**^{3X3})ß-Xyl*p*-**3**^{II}-ß-Xyl*p*-2-Xyl*p*-1] shows that the ß-Xyl*p*-4-(α-Ara*f*-**A**^{3X3})ß-Xyl*p*-**3**^{II} moiety is also part of **4.1**, denoted ß-Xyl*p*-**3**-(α-Ara*f*-**A**^{3X2})ß-Xyl*p*-**2**^{II}. However, the H-1 resonance (δ 4.509) of ß-Xyl*p*-**2**^{II} of **4.1** has shifted slightly upfield (0.005 p.p.m), and the resonances of H-1,2,3 of ß-Xyl*p*-**2**^{II} and H-1 of α-Ara*f*-**A**^{3X2} are double due to an anomeric effect, which accords with the linkage of the α-Ara*f*-(1→3)[ß-Xyl*p*-(1→4)]-ß-Xyl*p* group to a reducing xylose residue in **4.1**.



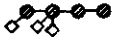
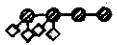
4.1

Comparison of the ^{1}H-NMR data (Table III) of **4.1** and **3.2** shows significant downfield shifts of the α-Ara*f*-**A**^{3X2} H-1,4,5*proS* signals and significant upfield shifts of the H-2,3,5*proR* signals, reflecting the effect of the change of the terminal α-Ara*f*-(1→3)-ß-Xyl*p* group to an α-Ara*f*-(1→3)[ß-Xyl*p*-(1→4)]-ß-Xyl*p* unit. The feruloylated compound ß-D-Xyl*p*-(1→4)-[5-*O*-(*trans*-feruloyl)-α-L-Ara*f*-(1→3)]-ß-D-Xyl*p*-(1→4)-D-Xyl*p* has been described[10,24], and the reported ^{1}H-NMR assignments accorded those of comparable residues in **4.1**.

TABLE III. ^{1}H-NMR data on fractions from Table I

Compound[a]	Residue[a]	Chemical shift[b]					
		H-1	H-2	H-3	H-4	H-5*eq*/H-5*proR*	H-5*ax*/H-5*proS*
	α-Xyl*p*-**1**	5.185	3.545		— 3.73 — 3.82 —		
	ß-Xyl*p*-1	4.584	3.250	3.545	3.781	4.055	3.377
3.2	ß-Xyl*p*-2^{II}_{α}	4.487	3.413	3.594	3.692		
	ß-Xyl*p*-$2^{II}_{ß}$	4.490	3.403	3.591	3.690	4.004	3.340
	α-Ara*f*-A^{3X2}_{α}	5.335					
	α-Ara*f*-$A^{3X2}_{ß}$	5.332	4.175	3.959	4.185	3.817	3.705
	α-Xyl*p*-**1**	5.185	3.545		— 3.73 — 3.82 —		
	ß-Xyl*p*-1	4.583	3.249	3.544	3.771	4.054	3.375
4.1	ß-Xyl*p*-2^{II}_{α}	4.507	3.348	3.749			
	ß-Xyl*p*-$2^{II}_{ß}$	4.509	3.440	3.746	3.829	4.124	3.400
	ß-Xyl*p*-3	4.442	3.245	3.414	3.595	3.912	3.278
	α-Ara*f*-A^{3X2}_{α}	5.400					
	α-Ara*f*-$A^{3X2}_{ß}$	5.395	4.158	3.903	4.272	3.796	3.714
	α-Xyl*p*-**1**	5.183	3.546		— 3.73 — 3.82 —		
	ß-Xyl*p*-1	4.584	3.249	3.546	3.771	4.050	3.376
5.1	ß-Xyl*p*-2_{α}	4.465	3.298				
	ß-Xyl*p*-$2_{ß}$	4.467	3.290	3.558	3.792	4.140	3.416
	ß-Xyl*p*-3^{III}	4.596	3.539	3.689	3.724	4.023	3.344
	α-Ara*f*-A^{2X3}	5.238	4.151	3.956	4.132	3.816	3.720
	α-Ara*f*-A^{3X3}	5.246	4.175	3.973	4.198	3.813	3.706

	α-Xyl*p*-1	5.186	3.547		— 3.73 — 3.82 —		
	ß-Xyl*p*-1	4.584	3.251	3.546	3.781	4.055	3.377
5.2	ß-Xyl*p*-2$^{II}_{\alpha}$	4.507	3.451				
	ß-Xyl*p*-2$^{II}_{ß}$	4.510	3.443	3.745	3.837	4.126	3.401
	ß-Xyl*p*-3^{II}	4.475	3.400	3.581	3.663	3.948	3.312
	α-Ara*f*-A^{3X2}	5.396					
	α-Ara*f*-A$^{3X2}_{ß}$	5.391	4.159	3.902	4.270	3.796	3.715
	α-Ara*f*-A^{3X3}	5.328	4.175	3.959	4.182	3.817	3.704
	α-Xyl*p*-1	5.186	3.547		— 3.73 — 3.82 —		
	ß-Xyl*p*-1	4.584	3.251	3.545	3.781	4.054	3.377
6.2	ß-Xyl*p*-2$^{II}_{\alpha}$	4.507	3.448				
	ß-Xyl*p*-2$^{II}_{ß}$	4.510	3.440	3.740	3.836	4.123	3.399
	ß-Xyl*p*-3^{II}	4.489	3.430	3.735	3.793	4.068	3.366
	ß-Xyl*p*-4	4.432	3.237	3.409	3.591	3.907	3.269
	α-Ara*f*-A^{3X2}	5.391					
	α-Ara*f*-A$^{3X2}_{ß}$	5.386	4.161	3.914	4.272	3.800	3.719
	α-Ara*f*-A^{3X3}	5.398	4.157	3.900	4.272	3.794	3.715
	α-Xyl*p*-1	5.184	3.545		— 3.73 — 3.82 —		
	ß-Xyl*p*-1	4.584	3.249	3.544	3.770	4.049	3.373
6.3	ß-Xyl*p*-2$^{II}_{\alpha}$	4.494	3.448	3.764			
	ß-Xyl*p*-2$^{II}_{ß}$	4.496	3.440	3.761	3.830	4.136	3.455
	ß-Xyl*p*-3^{III}	4.563	3.536	3.67	3.68	4.056	3.309
	α-Ara*f*-A^{3X2}	5.427					
	α-Ara*f*-A$^{3X2}_{ß}$	5.422	4.158	3.911	4.307	3.816	3.728
	α-Ara*f*-A^{2X3}	5.244	4.150	3.973	4.15	3.839	3.745
	α-Ara*f*-A^{3X3}	5.244	4.174	3.973	4.196	3.811	3.704

	α-Xyl*p*-1	5.184	3.545		— 3.73 — 3.82 —		
	ß-Xyl*p*-1	4.584	3.248	3.546	3.772	4.051	3.376
7.1	ß-Xyl*p*-2_{α}	4.467	3.298				
	ß-Xyl*p*-$2_{ß}$	4.467	3.290	3.561	3.792	4.143	3.417
	ß-Xyl*p*-3^{III}	4.639	3.572	3.828	3.878	4.146	3.431
	ß-Xyl*p*-4^{II}	4.467	3.409	3.582	3.667	3.956	3.307
	α-Ara*f*-A^{2X3}	5.225	4.148	3.958	4.127	3.817	3.720
	α-Ara*f*-A^{3X3}	5.271	4.164	3.932	4.301	3.796	3.720
	α-Ara*f*-A^{3X4}	5.331	4.175	3.958	4.186	3.815	3.704
	α-Xyl*p*-1	5.184	3.544		— 3.73 — 3.82 —		
	ß-Xyl*p*-1	4.584	3.247	3.545	3.771	4.051	3.376
8.2	ß-Xyl*p*-2_{α}	4.464	3.297				
	ß-Xyl*p*-$2_{ß}$	4.466	3.288	3.558	3.782	4.139	3.415
	ß-Xyl*p*-3^{III}	4.626	3.565	3.85	3.86	4.139	3.491
	ß-Xyl*p*-4^{III}	4.548	3.546	3.669	3.689	3.959	3.302
	α-Ara*f*-A^{2X3}	5.221	4.152	3.957	4.125	3.816	3.720
	α-Ara*f*-A^{3X3}	5.293	4.163	3.939	4.338	3.826	3.737
	α-Ara*f*-A^{2X4}	5.254	4.149	3.973	4.16	3.838	3.737
	α-Ara*f*-A^{3X4}	5.246	4.175	3.973	4.198	3.811	3.704

[a] See Table II for the key.

[b] In p.p.m. relative to the signal of internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (using internal acetone at δ 2.225) in D_2O at 27 °C, acquired at 600 MHz.

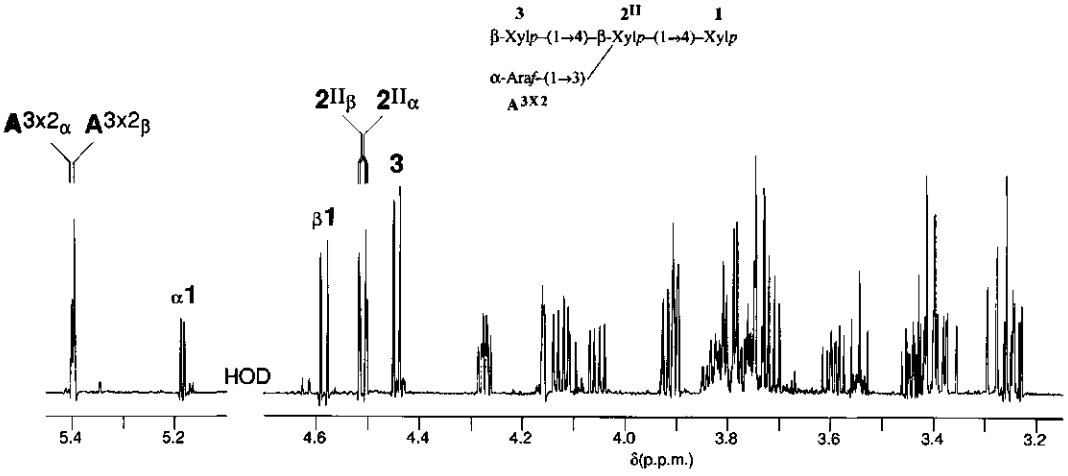
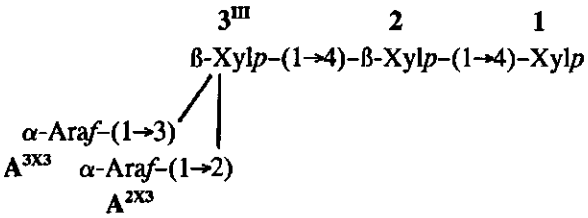


FIGURE 4. Resolution-enhanced 600-MHz ^{1}H-NMR spectrum of fraction **4.1**. The numbers and letters in the spectrum refer to the corresponding residues in the structure.

Fraction **5.1** The intensities of the H-1 signals for **5.1** (Fig. 5) indicated a single diarabinosylxylotriose with the Xyl*p* units ß ($J_{1,2}$ 7-8 Hz) and the Ara*f* units α ($J_{1,2}$ 1-1.5 Hz). On the various H-1 tracks of the constituent monosaccharides in the 2D HOHAHA spectrum, the total scalar-coupled network for each residue was observed, and the data obtained are summarised in Table III. The observed ROE's along the H-1 tracks in the ROESY spectrum are compiled in Table IV. The ROE's between H-1 of ß-Xyl*p*-(**n**) and H-4,5*eq* of ß-Xyl*p*-(**n-1**), together with the connectivities α-Ara*f*-$\mathbf{A^{2X3}}$ H-1,ß-Xyl*p*-$\mathbf{3^{III}}$ H-2 and α-Ara*f*-$\mathbf{A^{3X3}}$ H-1,ß-Xyl*p*-$\mathbf{3^{III}}$ H-3 established the sequence in **5.1**.



5.1

Comparison of the ^{1}H-NMR data for **5.1** with those[12] of **AX-33** [ß-Xyl*p*-**4**-(α-Ara*f*-$\mathbf{A^{2X3}}$)(α-Ara*f*-$\mathbf{A^{3X3}}$)ß-Xyl*p*-$\mathbf{3^{III}}$-ß-Xyl*p*-**2**-Xyl*p*-**1**] shows significant upfield shifts of all the signals of ß-Xyl*p*-$\mathbf{3^{III}}$ in **5.1** and there are differences in chemical shifts of the signals of the α-Ara*f* residues. The signals of α-Ara*f*-$\mathbf{A^{3X3}}$ are shifted in the same manner as found for α-Ara*f*-$\mathbf{A^{3X2}}$ on going from **4.1** to **3.2**. For α-Ara*f*-$\mathbf{A^{2X3}}$, only the H-1 resonance shows a small downfield shift (0.014 p.p.m.), as compared to the same residue in **AX-33**. The inter-residual connectivities $\mathbf{A^{2X3}}$-H-1,$\mathbf{A^{3X3}}$-H-2 and $\mathbf{A^{3X3}}$-H-1, $\mathbf{A^{2X3}}$-H-2, found[12] for **AX-33**, were also

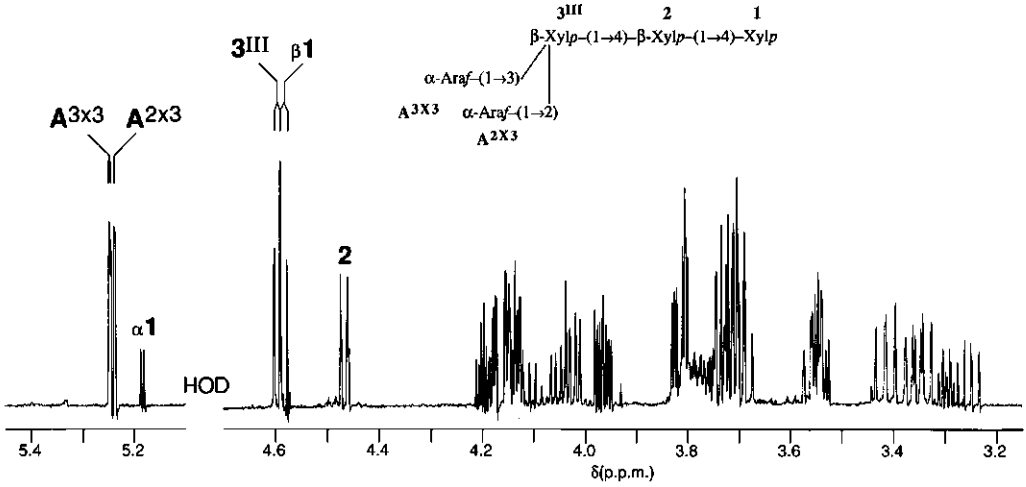
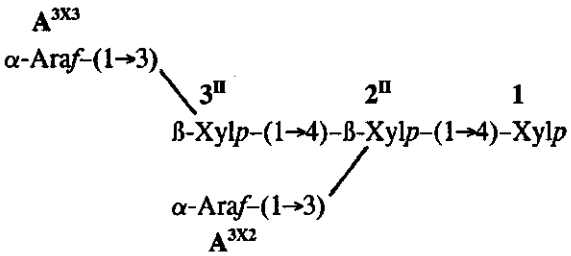


FIGURE 5. Resolution-enhanced 600-MHz ^{1}H-NMR spectrum of fraction **5.1**. The numbers and letters in the spectrum refer to the corresponding residues in the structure.

present in the ROESY spectrum of **5.1**, but with lower intensities.

Fraction **5.2** The intensities of the H-1 signals for **5.2** (Fig. 6) indicated a diarabinosylxylotriose. The connectivities were determined as for **5.1** and the data are summarised in Tables III and IV. The ROE's between H-1 of ß-Xyl*p*-(**n**) and H-4,5*eq* of ß-Xyl*p*-(**n-1**), together with the connectivities α-Ara*f*-$\mathbf{A^{3X2}}$ H-1,ß-Xyl*p*-$\mathbf{2^{II}}$ H-3 and α-Ara*f*-$\mathbf{A^{3X3}}$ H-1,ß-Xyl*p*-$\mathbf{3^{II}}$ H-3 established the sequence in **5.2**. Comparison of the ^{1}H-NMR data for **5.2** with those[13] of



5.2

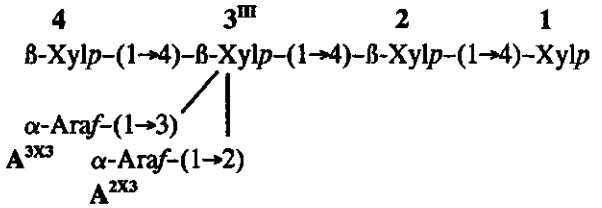
AX-41 [ß-Xyl*p*-**5**-(α-Ara*f*-$\mathbf{A^{3X4}}$)ß-Xyl*p*-$\mathbf{4^{II}}$-(α-Ara*f*-$\mathbf{A^{3X3}}$)ß-Xyl*p*-$\mathbf{3^{II}}$-ß-Xyl*p*-**2**-Xyl*p*-**1**] shows large upfield shifts for all signals derived from ß-Xyl*p*-$\mathbf{3^{II}}$ relative to those of ß-Xyl*p*-$\mathbf{4^{II}}$ of **AX-41**, as expected on going from an internal to a terminal residue. The significant upfield shifts of the α-Ara*f*-$\mathbf{A^{3X3}}$ H-1,4,5*proS* signals and downfield shifts of the H-2,3,5*proR* signals in **5.2**, relative to the same signals of α-Ara*f*-$\mathbf{A^{3X4}}$ in **AX-41**, is noteworthy. Similar differences in chemical shifts were found for α-Ara*f*-$\mathbf{A^{3X2}}$ in **3.2** and **4.1** (see above). The ^{1}H-NMR data of α-Ara*f*-$\mathbf{A^{3X3}}$ in **5.2** and of α-Ara*f*-$\mathbf{A^{3X2}}$ß in **3.2**. are (almost) identical.

TABLE IV. Cross-peaks observed at the H-1 tracks in the ROESY spectra of arabinoxylan oligosaccharides, measured with a mixing time of 200 ms

Compound	Residue	ROE effect
5.1	Xyl-2 H-1	Xyl-2 H-3,5*ax*, Xyl-1ß H-4,5*eq*, Xyl-1α H-4,5
	Xyl-3^{III} H-1	Xyl-3^{III} H-3,5*ax*, Xyl-2 H-4,5*eq*
	Ara-A^{2X3} H-1	Ara-A^{2X3} H-2, Ara-A^{3X3} H-2, Xyl-3^{III} H-2
	Ara-A^{3X3} H-1	Ara-A^{3X3} H-2, Ara-A^{2X3} H-2, Xyl-3^{III} H-3
5.2	Xyl-2^{II} H-1	Xyl-2^{II} H-3,5*ax*, Xyl- 1ß H-4
	Xyl-3^{II} H-1	Xyl-3^{II} H-3,5*ax*, Xyl-2^{II} H-4,5*eq*
	Ara-A^{3X2} H-1	Ara-A^{3X2} H-2, Xyl-2^{II} H-3
	Ara-A^{3X3} H-1	Ara-A^{3X3} H-2, Xyl-3^{II} H-3
6.3	Xyl-2^{II} H-1	Xyl-2^{II} H-3,5*ax*, Xyl-1ß H-4,5*eq*, Xyl-1α H-5
	Xyl-3^{III} H-1	Xyl-3^{III} H-3,5*ax*, Xyl-2^{II} H-4,5*eq*
	Ara-A^{3X2} H-1	Ara-A^{3X2} H-2, Xyl-2^{II} H-3
	Ara-A^{2X3} H-1	Ara-A^{2X3} H-2, Ara-A^{3X3} H-2^{a}, Xyl-3^{III} H-2
	Ara-A^{3X3} H-1	Ara-A^{3X3} H-2, Ara-A^{2X3} H-2^{a}, Xyl-3^{III} H-3
7.1	Xyl-2 H-1	Xyl-2 H-3,5*ax*, Xyl-1ß H-4, Xyl-1α H-4,5
	Xyl-3^{III} H-1	Xyl-3^{III} H-3(weak),5*ax*, Xyl-2 H-4,5*eq*
	Xyl-4^{II} H-1	Xyl-4^{II} H-3,5*ax*, Xyl-3^{III} H-4,5*eq*
	Ara-A^{2X3} H-1	Ara-A^{2X3} H-2, Ara-A^{3X3} H-2, Xyl-3^{III} H-2
	Ara-A^{3X3} H-1	Ara-A^{3X3} H-2, Ara-A^{2X3} H-2, Xyl-3^{III} H-3
	Ara-A^{3X4} H-1	Ara-A^{3X4} H-2(very weak), Xyl-4^{II} H-3
8.2	Xyl-2 H-1	Xyl-2 H-3(weak),5*ax*, Xyl-1ß H-4,5*eq*, Xyl-1α H-5
	Xyl-3^{III} H-1	Xyl-3^{III} H-3,5*ax*, Xyl-2 H-4,5*eq*
	Xyl-4^{III} H-1	Xyl-4^{III} H-3,5*ax*, Xyl-3^{III} H-4,5*eq*
	Ara-A^{2X3} H-1	Ara-A^{2X3} H-2, Ara-A^{3X3} H-2, Xyl-3^{III} H-2
	Ara-A^{3X3} H-1	Ara-A^{3X3} H-2(weak), Ara-A^{2X3} H-2, Xyl-3^{III} H-3
	Ara-A^{2X4} H-1	Ara-A^{2X4} H-2, Ara-A^{3X4} H-2, Xyl-4^{III} H-2
	Ara-A^{3X4} H-1	Ara-A^{3X4} H-2(weak), Ara-A^{2X4} H-2, Xyl-4^{III} H-3

[a] Due to the overlap of the H-1 resonances of A^{2X4} and A^{3X4} the presence of these inter-residual ROE contacts could not be proved unambiquously, but were assumed to be present according to the structure **5.1**.

Fraction **6.1** The intensities of the H-1 signals for **6.1** indicated a diarabinosylxylotetraose. The chemical shift data matched exactly those[12] of **AX-33** and the chemical shifts of the H-1



6.1

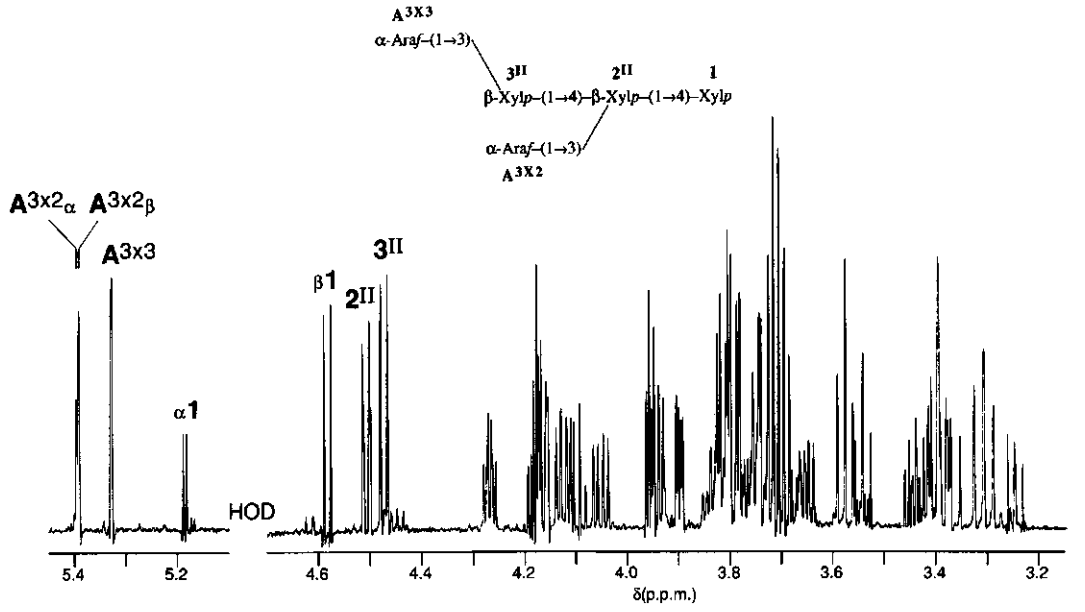


FIGURE 6. Resolution-enhanced 600-MHz ^{1}H-NMR spectrum of fraction **5.2**. The numbers and letters in the spectrum refer to the corresponding residues in the structure.

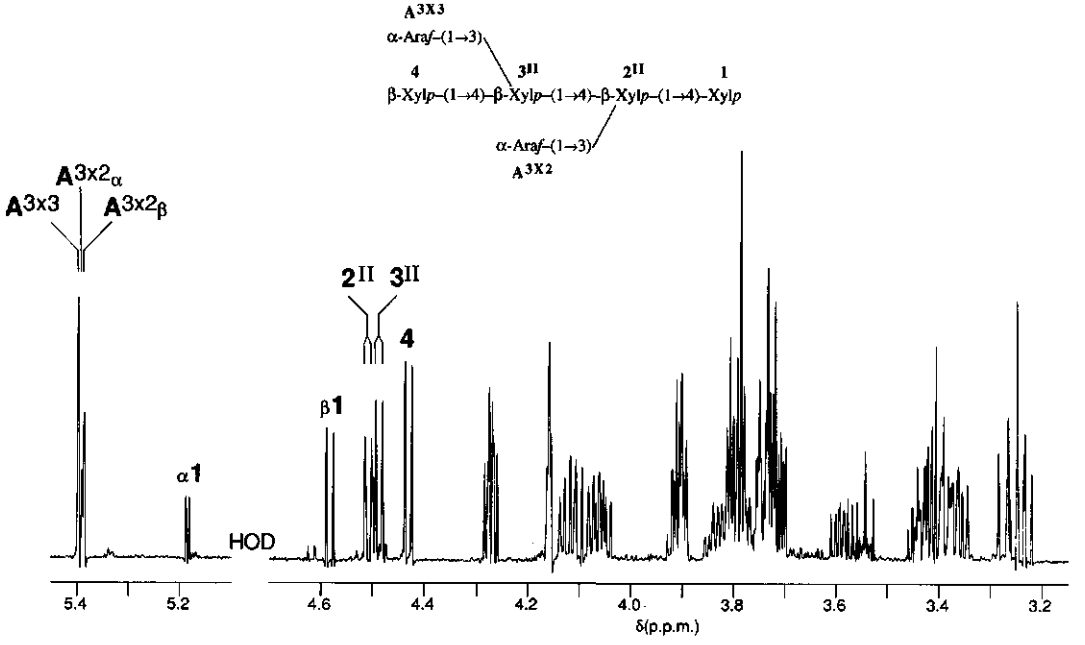
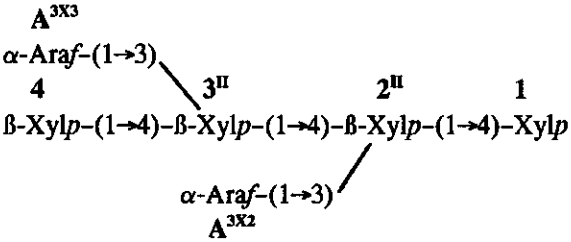


FIGURE 7. Resolution-enhanced 600-MHz ^{1}H-NMR spectrum of fraction **6.2**. The numbers and letters in the spectrum refer to the corresponding residues in the structure.

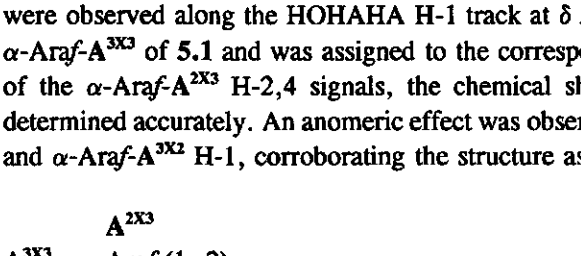
resonances are summarised in Table II. Compound **6.1** was also formed on enzymic treatment of barley flour[11].

Fraction **6.2** The intensities of the H-1 signals for **6.2** (Fig. 7) indicated a diarabinosylxylotetraose. The connectivities were determined as for **5.1** and the data obtained are summarised in Table III. The ^{1}H-NMR data that reflect[13] the presence of the terminal ß-Xyl*p*-**5**-(α-Ara*f*-**A**^{3X4})ß-Xyl*p*-**4**^{II}-(α-Ara*f*-**A**^{3X3})ß-Xyl*p*-**3**^{II} moiety in **AX-41** are also found for **6.2** and are assigned to ß-Xyl*p*-**4**-(α-Ara*f*-**A**^{3X3})ß-Xyl*p*-**3**^{II}-(α-Ara*f*-**A**^{3X2})ß-Xyl*p*-**2**^{II} and a reducing xylose residue. The finding of anomeric effects on the resonance of α-Ara*f*-**A**^{3X2} H-1 and ß-Xyl*p*-**2**^{II} H-1,2 and a small upfield shift (δ 4.510) of the resonance of ß-Xyl*p*-**2**$^{II}_{\beta}$-H-1 compared to that (δ 4.514) of the ß-Xyl*p*-**3**^{II} H-1 resonance in **AX-41**, supports this conclusion (see also ß-Xyl*p*-**2**^{II} of **4.1** and **5.2**) and the structure assigned.



6.2

Fraction **6.3** The intensities of the H-1 signals for **6.3** (Fig. 8) indicated a triarabinosylxylotriose. The connectivities were established as for **5.1** and the data obtained are summarised in Tables III and IV. The ROE's between H-1 of ß-Xyl*p*-(**n**) and H-4,5*eq* of ß-Xyl*p*-(**n-1**), together with the connectivities α-Ara*f*-**A**^{3X2} H-1,ß-Xyl*p*-**2**^{II} H-3, α-Ara*f*-**A**^{2X3} H-1,ß-Xyl*p*-**3**^{III} H-2, and α-Ara*f*-**A**^{3X3} H-1,ß-Xyl*p*-**3**^{III} H-3 established the sequence in **6.3**. Owing to overlap of the H-1 resonances of α-Ara*f*-**A**^{2X3} and α-Ara*f*-**A**^{3X3}, two sets of signals were observed along the HOHAHA H-1 track at δ 5.244. One set of signals match those of α-Ara*f*-**A**^{3X3} of **5.1** and was assigned to the corresponding residue in **6.3**. Owing to overlap of the α-Ara*f*-**A**^{2X3} H-2,4 signals, the chemical shift of the H-4 resonance could not be determined accurately. An anomeric effect was observed on the signals for ß-Xyl*p*-**2**^{II} H-1,2,3 and α-Ara*f*-**A**^{3X2} H-1, corroborating the structure assigned.



6.3

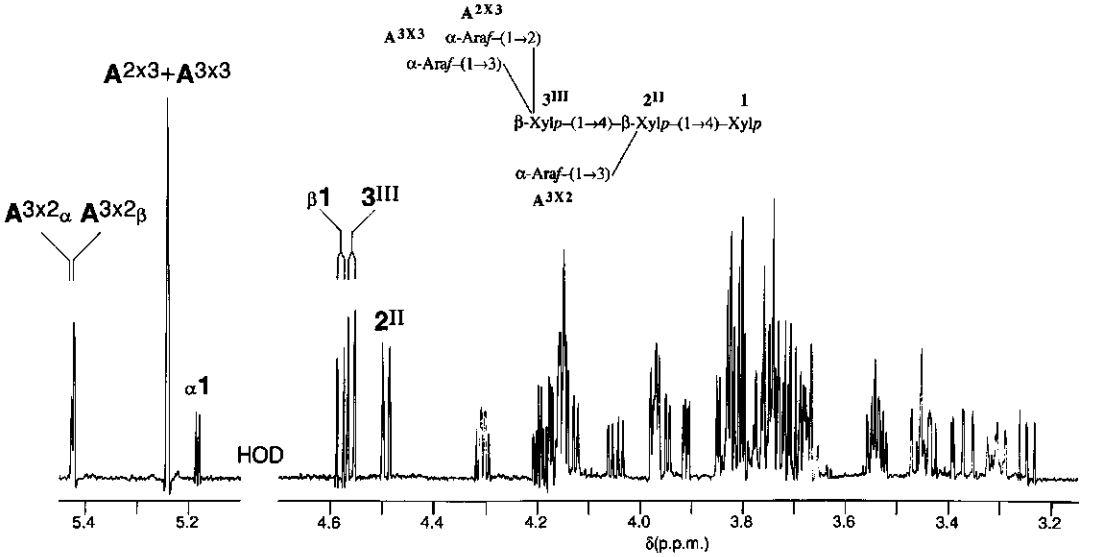
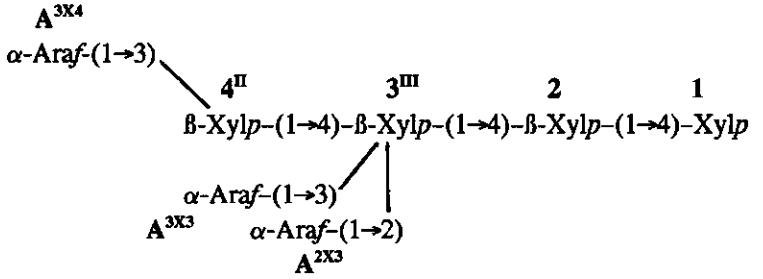


FIGURE 8. Resolution-enhanced 600-MHz ^{1}H-NMR spectrum of fraction **6.3**. The numbers and letters in the spectrum refer to the corresponding residues in the structure.

Fraction **7.1** The intensities of the H-1 signals for **7.1** (Fig. 9) indicated a triarabinosylxylotetraose as the major compound. The connectivities were established as for **5.1** and the data obtained are summarised in Tables III and IV. The ROE's between H-1 of ß-Xyl*p*-(**n**) and H-4,5*eq* of ß-Xyl*p*-(**n-1**), together with the connectivities α-Ara*f*-A^{2X3}H-1,ß-Xyl*p*-3^{III} H-2, α-Ara*f*-A^{3X3} H-1,ß-Xyl*p*-3^{III} H-3, and α-Ara*f*-A^{3X4} H-1,ß-Xyl*p*-4^{II} H-3 established the sequence in **7.1**.



7.1

Owing to the overlap of the H-1 signals of ß-Xyl*p*-**2** and ß-Xyl*p*-4^{II}, two sets of signals were observed along the HOHAHA H-1 track at δ 4.467. Besides the ROE's mentioned above, the ROESY spectrum shows also the inter-residual connectivities A^{2X3}-H-1,A^{3X3}-H-2 and A^{3X3}-H-1,A^{2X3}-H-2, characteristic for the 2,3-substitution of a (1→4)-linked ß-Xyl*p* residue by two terminal α-Ara*f* groups[12,13]. Comparison of the ^{1}H-NMR data of **7.1** with those[13] of **AX-54c** [ß-Xyl*p*-**5**-(α-Ara*f*-A^{3X4})ß-Xyl*p*-4^{II}-(α-Ara*f*-A^{2X3})(α-Ara*f*-A^{3X3})ß-Xyl*p*-3^{III}-ß-Xyl*p*-**2**-Xyl*p*-**1**] shows a good match for the resonances of ß-Xyl*p*-**1,2,**3^{III} and α-Ara*f*-A^{2X3} and only

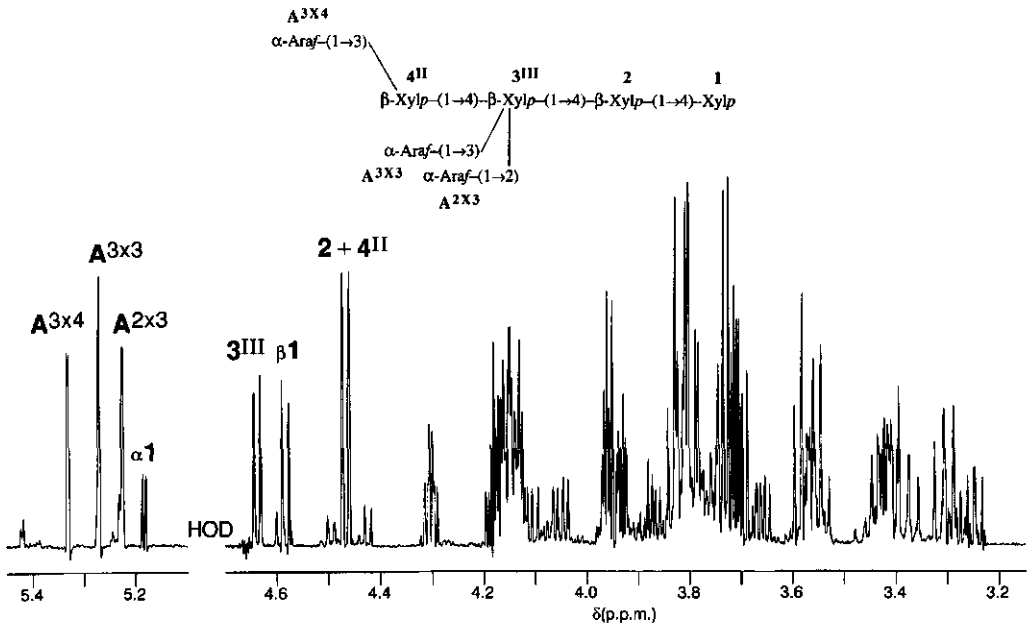


FIGURE 9. Resolution-enhanced 600-MHz ^{1}H-NMR spectrum of fraction **7.1**. The numbers and letters in the spectrum refer to the corresponding residues in the structure.

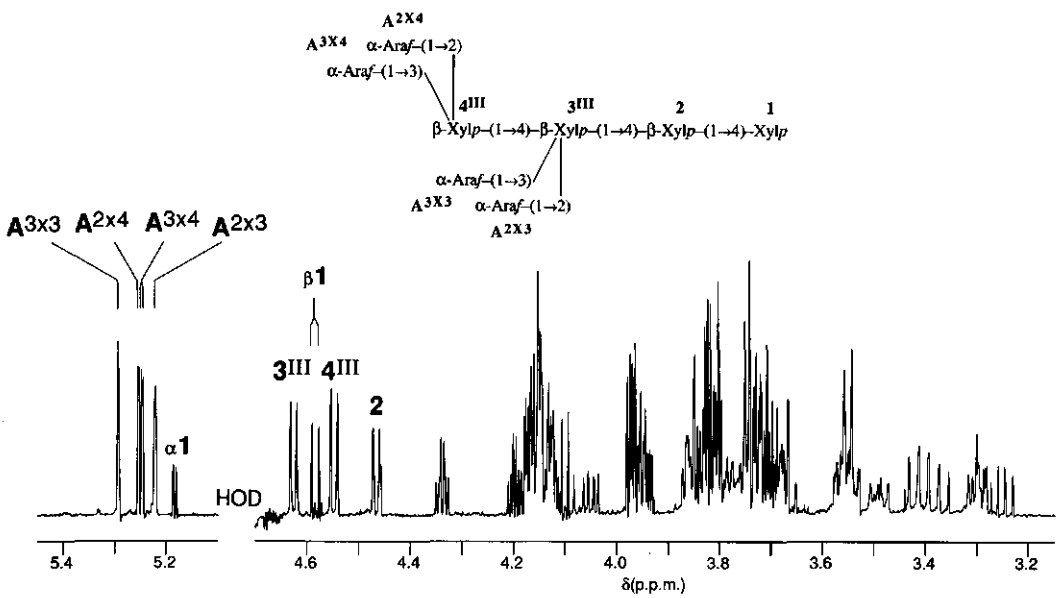
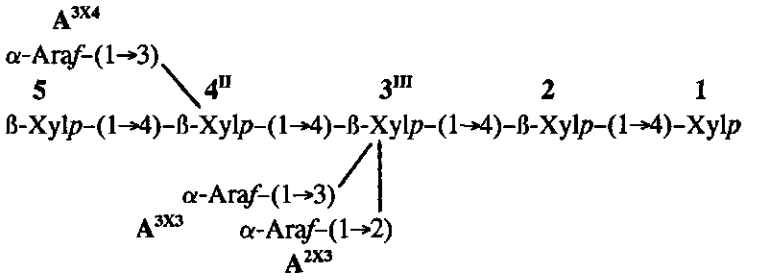


FIGURE 10. Resolution-enhanced 600-MHz ^{1}H-NMR spectrum of fraction **8.2**. The numbers and letters in the spectrum refer to the corresponding residues in the structure.

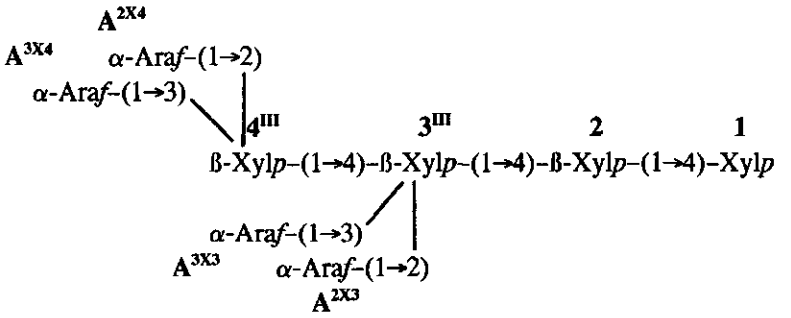
small deviations for those of α-Ara*f*-A^{3X3}, in particular H-3,5*proR*,5*proS*. The structural change in the non-reducing terminal part on going from **7.1** to **AX-54c** is reflected by the large upfield shifts for all signals of ß-Xyl*p*-4^{II}. The ^{1}H-NMR data of α-Ara*f*-A^{3X4} in **7.1** match those for α-Ara*f*-$A^{3X2}{}_{ß}$ in **3.2** and α-Ara*f*-A^{3X3} in **5.2**, and support the structure assigned.

Fraction **8.1** The intensities of the H-1 signals for **8.1** indicated a triarabinosylxylopentaose. The chemical shift data match exactly those[13] of **AX-54c**, and the chemical shifts of the H-1 resonances are summarised in Table II.



8.1

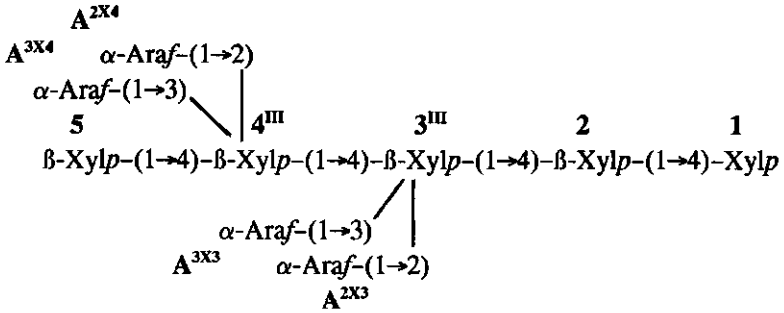
Fraction **8.2** The intensities of the H-1 signals for **8.2** (Fig. 10) indicated a tetraarabinosylxylotetraose. The connectivities were established as for **5.1** and the data are summarised in Tables III and IV. The ROE's between H-1 of ß-Xyl*p*-(**n**) and H-4,5*eq* of ß-Xyl*p*-(**n-1**), together with the connectivities α-Ara*f*-A^{2X3} H-1,ß-Xyl*p*-3^{III} H-2, α-Ara*f*-A^{3X3} H-1,ß-Xyl*p*-3^{III} H-3, α-Ara*f*-A^{2X4} H-1,ß-Xyl*p*-4^{III} H-2, and α-Ara*f*-A^{3X4} H-1,ß-Xyl*p*-4^{III} H-3, establish the sequence in **8.2**.



8.2

The presence of the tetraarabinosylxylobiose unit in **8.2** is reflected by a characteristic set of four α-Ara*f* H-1 signals (Table III). The inter-residual α-Ara*f* H-1,2 connectivities between α-Ara*f*-A^{2X3} and α-Ara*f*-A^{3X3} and between α-Ara*f*-A^{2X4} and α-Ara*f*-A^{3X4} support the presence of two 2,3-substituted ß-Xyl*p* residues.

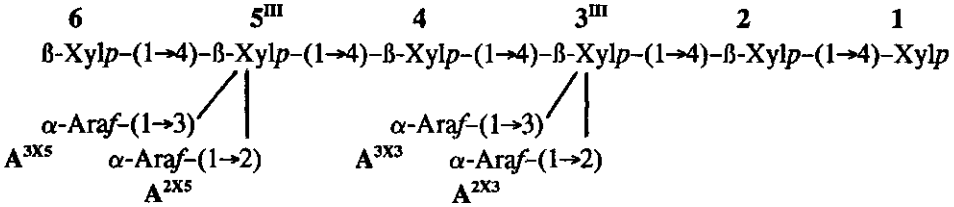
Fraction **9.1** The intensities of the H-1 signals for **9.1** indicated a tetra-arabinosyl-xylopentaose. The chemical shift data match exactly those[13] of **AX-59** and the chemical shifts of the H-1 resonances are summarised in Table II. The characteristic set of four α-Ara*f* H-1 and two ß-Xyl*p* H-1 signals, which reflects the presence of an internal (1→4)-linked xylobiose unit composed of two 2,3-substituted ß-Xyl*p* residues with four α-Ara*f* residues, differs significantly from the set for **8.2**.



9.1

Fraction **9.2** The ^{1}H-NMR spectrum of **9.2** which indicated the presence of at least two components, could not be unravelled due to its complexity. The presence of a mixture was reflected by the HPAEC chromatogram (Fig. 2) for fraction *9*.

Fraction **10.1** The intensities of the H-1 signals for **10.1** indicated a tetra-arabinosylxylohexaose. The chemical shift data match exactly those[13] of **AX-57^{II}** and the chemical shifts of the H-1 resonances are summarised in Table II.



10.1

Conclusions

Thus, of the arabinosylxylose oligosaccharides present in the enzymic digest of an alkali-extractable arabinoxylan from the soft wheat variety Arminda, four (**6.1**, **8.1**, **9.1**, and **10.1**) have been identified also in an enzymic digest of a warm water-extractable arabinoxylan from Kadet wheat flour[12,13]. Comparison of the oligosaccharides obtained in this and the previous studies[12,13] showed remarkable differences. Since, for Arminda wheat flour, based

on methylation analysis, no major differences in substitution pattern between water-extractable and alkali-extractable arabinoxylans were found[16], the differences in the oligosaccharides obtained are likely to reflect differences in the mode of action of the enzymes rather than differences in arabinoxylan structure.

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CHAPTER 8

Water-unextractable cell wall material from wheat flour. III. A structural model for arabinoxylans

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Summary

In order to elucidate their structural features, water-unextractable wheat flour arabinoxylans were degraded with purified endo-(1→4)-ß-D-xylanases and a (1→4)-ß-D-arabinoxylan arabinofuranohydrolase from *Aspergillus awamori*. The arabinoxylan fragments obtained were characterized and quantified using Bio-Gel P-2 chromatography in combination with high performance anion exchange chromatography. From these results a model is proposed showing that the arabinoxylan contains highly branched regions, mostly consisting of tetrameric repeating units of an unsubstituted and a double arabinofuranosylated xylose residue, interlinked with less branched regions which include subregions of unsubstituted xylose up to 7 contiguous residues. The strongly branched regions are enriched in both *O*-2,3 as well as *O*-2 substituted xylose. The latter are absent in the less branched regions. Variation in arabinose/xylose ratio between different arabinoxylan populations is due to variation in the relative proportion as well as composition of the less branched regions. In general, water-unextractable arabinoxylans are degraded to a lesser extent than water-extractable arabinoxylans with similar arabinose/xylose ratios and glycosidic linkage compositions.

Introduction

Arabinoxylans represent the major part of the cell wall components from wheat flour. The arabinoxylans can be divided in a water-extractable and a water-unextractable part which represent 25-30% and 70-75% of the total arabinoxylans, respectively[1,2]. Almost all water-unextractable arabinoxylans can be solubilised using alkali[3]. Both water- and alkali-extracted arabinoxylans can be separated into fractions having different arabinose/xylose (Ara/Xyl) ratios using ethanol or ammoniumsulphate precipitation[4-6], anion-exchange chromatography[5-7] or DEAE-borate chromatography[8,9]. The presence of arabinose side groups is important for the physical properties (e.g. solubility) of the arabinoxylans[10,11].

In previous papers[3,6] we reported on the sugar composition, linkage analysis and molecular weight distribution of the different arabinoxylans from water-unextractable cell wall material (WUS) which were compared to the water-extractable arabinoxylans. So far, little information is available on the α-L-Ara*f* distribution along the xylan backbone. Periodate-oxidation studies[12,13] on water-extractable wheat flour arabinoxylans indicated the presence of clusters of 1 to 4 contiguous substituted xylose residues. Based on enzymic degradation of water-extractable arabinoxylans a model has been proposed[14] in which highly branched regions, containing isolated unsubstituted xylose residues separated by one or two substituted xylose residues, are interlinked by sequences of contiguous unsubstituted xylose with a DP of at least 2 to 5. Recently, in analogy to studies on water-extractable wheat flour arabinoxylans[15,16], the structural elements of different oligosaccharides derived from digestion of water-unextractable arabinoxylans from Arminda wheat flour with purified endoxylanases[17] from *Aspergillus awamori*, were identified using ^{1}H-NMR[18,19]. In the present study we report on the abundance of different arabinoxylan oligomers obtained after enzymic degradation of alkali-extractable wheat flour arabinoxylans with different Ara/Xyl ratios. These results are compared to those of water-extractable arabinoxylans of the same wheat flour. From these data a structural model for wheat flour arabinoxylans is proposed.

Experimental

Materials

The preparation and characterization of DEAE-unbound alkali-extractable arabinoxylans, (BE1-U), and water-extractable arabinoxylans (weAX) from Arminda wheat flour and subfractions thereof obtained by graded ethanol precipitation (BE1-U20 - BE1-U60; weAX30 - weAX-60) is described elsewhere[3,6]. Endo-(1→4)-ß-D-xylanase I (endoI), endo-(1→4)-ß-D-xylanase III (endoIII) and (1→4)-ß-D-arabinoxylan arabinofuranohydrolase (AXH) were purified[17,20] from *Aspergillus awamori* CMI 142717. In order to split glycosidic linkages in arabinoxylans endoI needs 1 unsubstituted xylose

residue when this is adjacent to an *O*-3 substituted xylose residue and 2 contiguous unsubstituted xylose residues when these are adjacent to either an *O*-2 or an *O*-2,3 substituted xylose residue. It is able to split the glycosidic linkage at the non-reducing site of a single or double substituted xylose residue. EndoIII needs at least 3 contiguous unsubstituted xylose residues and is unable to split the glycosidic linkage at the non-reducing site of a single or double substituted xylose residue[21]. AXH is able to remove *O*-3 substituted arabinose residues from polymeric arabinoxylan as well as from arabinoxylan oligosaccharides[22,23].

Preparation of oligomers

Arabinoxylan preparations (20 mg) were dissolved in 50mM sodium acetate buffer (20 ml, pH 5.0) and incubated with endoI or endoIII (0.4 μg/ml) for 24 h at 30 °C. BE1-U (20 mg) was also incubated with AXH (0.4 μg/ml) in the same buffer (20 ml) for 8 h at 30 °C, followed by incubation with endoI or endoIII (0.4 μg/ml) for 24 h at 30 °C. After inactivation of the enzyme(s) (10 min; 100 °C), the solutions were concentrated to 2 ml under reduced pressure. The AXH/xylanase digests needed centrifugation (18,000 *g*; 10 min) to remove a precipitate formed. The resulting supernatant was concentrated and the residue was freeze-dried.

Analytical methods

Bio-Gel P-2 chromatography Concentrated digests were applied to columns (100x2.6 cm) of Bio-Gel P-2 (200-400 mesh, Bio-Rad Labs, Richmond, CA., USA) thermostated at 60 °C and eluted with water (17 ml/h). Fractions (2.4 ml) were assayed for total neutral sugar content[24]. Appropriate fractions were pooled as indicated in Fig. 1 and each pool was concentrated to 1.5 ml under reduced pressure. The Bio-Gel P-2 column was calibrated using a mixture of xylose, maltose, raffinose, stachyose and Dextran T150 (Pharmacia). The elution volumes of these compounds corresponded with those of fraction ***1-4*** and ***13*** (void), respectively. In all experiments almost complete recoveries (>95%) were obtained.

High performance anion exchange chromatography (HPAEC) Bio-Gel P-2 fractions were subjected to HPAEC using a Dionex BioLC GPM-II quaternary gradient module (Sunnyvale, CA., U.S.A.) equipped with a CarboPac PA-1 column (250x4 mm) in combination with a CarboPac PA guard column (25x3 mm). Samples (20 μl) were injected using a Spectra Physics SP8780 autosampler equipped with Tefzel rotor seal in a 7010 Rheodyne injector valve. Elutions (1 ml/min) involved linear gradients of sodium acetate in 0.1M NaOH 0-100mM during 10 min, then 100-400mM during 35 min at 20 °C. The solvents were degassed and stored under helium using a Dionex EDM module. The effluent was monitored using a Dionex PED detector in the pulsed amperometric detection

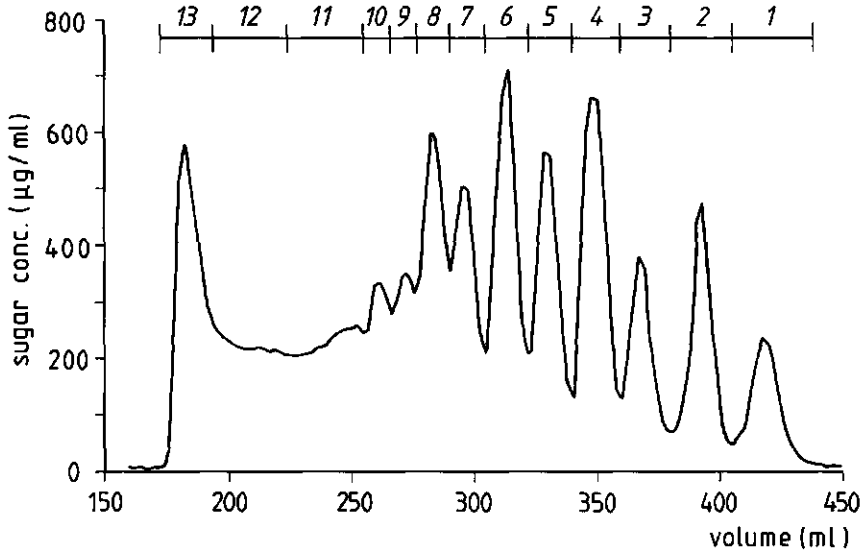


FIGURE 1. Typical Bio-Gel P-2 chromatogram of arabinoxylan digest (from Gruppen *et al*[18]).

(PAD) mode. A reference Ag/AgCl electrode was used with a working gold electrode with the following pulse potentials and durations: E_1 0.1V and 0.5s, E_2 0.6V and 0.1s, E_3 -0.6V and 0.1s. The identification of the oligosaccharides is described elsewhere[18,19]. The relative amounts of identified oligosaccharides in each digest were calculated from the total neutral sugar content[24] of each Bio-Gel P-2 peak and the distribution of the isomers within each peak as calculated by their respective PAD responses combined with the measured molar PAD responses of the identified oligosaccharides. Next to the identified oligosaccharides, part of the Bio-Gel P-2 fractions contained PAD responding carbohydrate materials which were not further identified.

High performance size exclusion chromatography (HPSEC) was performed with three Bio-Gel TSK columns in series (40XL-30XL-20XL) as described previously[25] using 0.4M sodium acetate buffer pH 3 as eluent. The system was calibrated with dextrans (Pharmacia) having mol.wts. in the range of 500 - 500,000.

Neutral sugar analysis using 2M trifluoroacetic acid for hydrolysis was performed as described previously[3].

Methylation analysis was performed as described previously[3].

Periodate oxidation was performed by the in-sequence oxidation/reduction procedure, followed by mild hydrolysis (20 min; 0.02M HCL; 100 °C), according to the method of Åman and Bengtsson[26]. The hydrolysate was applied to columns (60x1.0 cm) of Fractogel TSK HW-40(S) (25-40 µm, Merck) thermostated at 50 °C and eluted with water (0.6

ml/min). The effluent was monitored using a Shodex SE-61 refractive index detector.

Simulation of arabinoxylan chains and their degradation was performed according to Viëtor[27]. Arabinoxylan chain simulation was based on the random assignment of un-, 2-, 3- and 2,3-substituted xylose to each of the xylose positions in the chain. Chain lengths of 3000 xylose residues were used and calculations (for both chain simulation and degradation) were repeated 20 times and results were averaged. The probabilities for selecting each of the four possible units were set equal to the molar proportions of the corresponding xylose residues in the actual arabinoxylan fraction as determined previously[6]. The amounts of un-, *O*-2 and *O*-2,3 substituted xylose for the AXH-pretreated arabinoxylan were calculated from the data of the untreated arabinoxylan by eliminating all *O*-3 substituted xylose residues and adding this percentage to the amount of unsubstituted xylose, in accordance with the established specificity of AXH[23]. The simulation of the degradation by endoI and endoIII was based on the reported mode of action of these enzymes[21].

Results

Distribution of oligosaccharides

The amounts of the individual oligosaccharides with DP ≤ 10 and the Bio-Gel P-2 fractions (***11***, ***12***, and ***13***), containing carbohydrate material with DP > 10, in the endoI and endoI/AXH digests of BE1-U, obtained after 24 h incubation, are given in Table I. Incubation with endoI alone resulted in a digest in which *c.* 72% of the material was present as oligosaccharides with DP ≤ 10. The digest contained relatively high proportions of oligosaccharides in which one xylose residue is either *O*-3 or *O*-2,3 substituted and oligosaccharides with both an *O*-3 and *O*-2,3 substituted xylose residue.

Pretreatment with AXH in combination with incubation with endoI yielded a soluble digest and a precipitate which made up c. 4% of the parental material having an Ara/Xyl ratio of 0.18, which is resistant to hydrolysis with endoI. The formation of a precipitate from wheat arabinoxylan upon hydrolysis with an α-L-arabinofuranosidase has been reported previously[28] but only traces of arabinose were found in the precipitate upon analysis. Analysis of the soluble digest (Table I) showed the presence of 11.5% monomeric arabinose, expressed as percentage of total carbohydrate material present in the parental material. The amount of liberated arabinose almost equals the theoretical value of 11.9%, which can be calculated from the amount of arabinose substituted at position *O*-3[6], taking into account the amount of arabinose present in the precipitate formed. When compared to the endoI digest an increase in the amounts of xylose and xylobiose and oligosaccharides with *O*-2,3 substituted xylose residues was observed. This was accompanied with the complete absence of oligosaccharides with *O*-3 substituted

TABLE I. Abundance[a] of identified mono- and oligomers in endoI and endoI/AXH digests of BE1-U.

Monomer/oligomer[b]		Experiment endoI	Experiment endoI/AXH	Simulation endoI	Simulation endoI/AXH
●		4.5	7.4	7.8	11.5
◇		0	11.5	0	11.9
●–●		8.6	13.8	12.6	25.0
●–●–●		1.6	1.4	0	0
●–●◇	[*3.2*]	6.3	0	9.5	0
●–●–●◇	[*4.1*]	10.1	0	4.6	0
	other[c]	0	0.2	1.1	1.9
●–●–●◇	[*5.1*]	4.1	6.6	7.7	16.1
●–●–●◇	[*5.2*]	3.5	0	2.9	0
	other	0	0	1.2	1.0
●–●–●◇◇	[*6.3*]	1.1	0	2.9	0
●–●–●–●◇	[*6.1*]	7.1	15.1	3.6	8.9
●–●–●–●◇	[*6-2*]	2.5	0	1.2	0
	other	0	5.1	0.7	0
●–●–●–●◇◇	[*7-1*]	6.2	0	1.8	0
	other	0	1.4	1.9	1.1
●–●–●–●◇◇	[*8.2*]	2.6	3.1	2.0	4.3
●–●–●–●–●◇	[*8.1*]	5.4	0	0.7	0
	other	0	1.6	3.6	1.4
●–●–●–●–●◇	[*9.1*]	2.9	2.1	1.0	1.9
	other	1.3	2.2	4.8	4.1
●–●–●–●–●–●◇	[*10.1*]	1.9	1.9	0.6	2.0
	other	2.0	3.1	4.2	0.3
DP> 10		27.6	22.6	21.1	8.6
fraction *11*[d]		8.6 (*0.79*)	8.2 (*0.78*)	-	-
fraction *12*		8.3 (*0.87*)	7.8 (*0.76*)	-	-
fraction *13*		10.7 (*0.98*)	6.6 (*0.86*)	-	-

[a] Expressed as percentage (w/w) of arabinoxylan.

[b] Notation: ●: Xyl*p*;◇:Ara*f*; ●–●: ß-Xyl*p*-(1→4)-Xyl*p*; ●◇: α-Ara*f*-(1→2)-ß-Xyl*p*; ●◇: α-Ara*f*-(1→3)-ß-Xyl*p*; [ ]: code according to Gruppen *et al*[18]; the reducing glycoside is positioned at the right.

[c] Other refers to either unidentified oligosaccharides (experiment) or to oligosaccharides generated in the simulation other than those identified experimentally (simulation).

[d] The figures in parentheses represent the arabinose/xylose ratio.

TABLE II. Abundance[a] of identified mono- and oligomers in endoIII and endoIII/AXH digests of BE1-U.

Monomer/oligomer[b]		Experiment endoIII	Experiment endoIII/AXH	Simulation endoIII	Simulation endoIII/AXH
⊘		1.0	3.5	4.0	9.2
◇		0	11.8	0	11.9
⊘-⊘		1.9	12.3	6.4	20.0
⊘-⊘-⊘		0.4	4.8	0	0
⊘-⊘-⊘-⊘		0.8	1.1	0	0
⊘-⊘-⊘-⊘ (◇)	[*5.4*]	7.0	0	3.6	0
	other[c]	0	0	0.5	1.7
⊘-⊘-⊘-⊘-⊘ (◇)	[*6.4*]	4.8	0	1.3	0
⊘-⊘-⊘-⊘ (◇◇)	[*6.1*]	5.4	10.7	3.1	14.3
	other	0	0	0	0
⊘-⊘-⊘-⊘-⊘ (◇)	[*7.2*]	3.6	9.4	1.3	7.6
⊘-⊘-⊘-⊘-⊘ (◇◇)	[*7.3*]	4.2	0	0.9	0
	other	0	0	0.2	0.1
⊘-⊘-⊘-⊘-⊘-⊘ (◇◇)	[*8.3*^{I}]			0.4	0
⊘-⊘-⊘-⊘-⊘-⊘ (◇◇)	[*8.3*^{II}]	2.8	0	0.7	0
⊘-⊘-⊘-⊘-⊘ (◇◇)	[*8.1*]	4.1	0	0.7	0
⊘-⊘-⊘-⊘-⊘ (◇◇)	[*8.4*]	0.9	0	0.9	0
	other	0	2.3	0.4	0.8
⊘-⊘-⊘-⊘-⊘-⊘-⊘ (◇◇)	[*9.3*^{I}]			0.4	0
⊘-⊘-⊘-⊘-⊘-⊘-⊘ (◇◇)	[*9.3*^{II}]	1.6	0	0.7	0
⊘-⊘-⊘-⊘-⊘ (◇◇)	[*9.4*^{I}]			0.2	0
⊘-⊘-⊘-⊘-⊘ (◇◇)	[*9.4*^{II}]	3.1	0	0	0
⊘-⊘-⊘-⊘ (◇◇)	[*9.1*]	2.0	3.0	0.9	3.1
	other	1.3	0	2.5	1.0
⊘-⊘-⊘-⊘-⊘ (◇◇)	[*10.1*]	1.0	2.3	0.5	2.4
	other	2.5	1.0	3.3	2.3
DP > 10		51.3	36.4	68.3	25.6
fraction *11*[d]		13.1 (*0.51*)	11.2 (*0.58*)	-	-
fraction *12*		18.4 (*0.59*)	11.5 (*0.71*)	-	-
fraction *13*		20.2 (*0.76*)	13.7 (*0.87*)	-	-

[a] Expressed as percentage (w/w) of arabinoxylan.

[b] ●: Xyl*p*; ◇: Ara*f*; ●-●: ß-Xyl*p*-(1→4)-Xyl*p*; ◇ (●): α-Ara*f*-(1→2)-ß-Xyl*p*; ◇ (●): α-Ara*f*-(1→3)-ß-Xyl*p*; [ ]: code according to Kormelink *et al*[19] and Gruppen *et al*[18]; The reducing glycoside is positioned at the right.

[c] Other refers to either unidentified oligosaccharides (experiment) or to oligosaccharides generated in the simulation other than those identified experimentally (simulation).

[d] The figures in parentheses represent the arabinose/xylose ratio.

xylose residues. The fractions with DP >10 decreased from 27.6 to 22.6%. From these fractions the void fraction (*13*) was decreased from 10.7 to 6.6%.

The corresponding data for the endoIII digestion are given in Table II. In contrast to the endoI digest only small amounts of low oligomeric materials were obtained by incubation with endoIII. This is accompanied by a large amount of material with DP >10 (51%) relative to endoI. Similar to endoI incubation, relatively high proportions of oligosaccharides with either one *O*-3 or *O*-2,3 substituted xylose residue and oligosaccharides with both an *O*-3 and an *O*-2,3 substituted xylose residue were found. Pretreatment with AXH resulted in an increase in the amounts of xylose, xylobiose and xylotriose next to the formation of a precipitate similar to the one in the endoI/AXH experiment, both in yield and Ara/Xyl ratio. The presence of xylotriose in the digests can be explained by the reported slow hydrolysis of xylotriose by the endoxylanases[23]. Under the conditions used in this study, this might also be the case for xylotetraose. The void fraction (*13*) decreased from 20 to 14% whereas the sum of fractions with DP >10 decreased from 51 to 36%. For the endoIII incubations not all oligomeric material could be assigned[19] to a specific oligosaccharide structure but some were identified as mixtures of related oligosaccharides (i.e. structures $8.3^{I,II}$, $9.3^{I,II}$, and $9.4^{I,II}$.)

In Tables I and II also the Ara/Xyl ratios of fractions *11*, *12*, and *13* for each digest are given. With increasing molecular weight of the reaction products (i.e. increasing fraction number) increasing Ara/Xyl ratios were found. For endoI incubations the fractions *11*, *12*, and *13* obtained after pre-incubation with AXH exhibited lower Ara/Xyl ratios relative to those obtained without pretreatment, whereas for endoIII the results were opposite. Also, with one exception, fractions *11*, *12*, and *13* obtained with endoIII or endoIII/AXH had lower Ara/Xyl ratios than the corresponding fractions obtained with endoI or endoI/AXH incubation. The void fraction (*13*) of the endoI digest was analysed for glycosidic linkage composition. Arabinose was predominantly present as terminal furanosyl residues. In Table III the relative amounts of the different xylose residues are given together with those of the non-digested BE1-U and BE1-U60 fractions, the latter also having a high Ara/Xyl ratio (0.80)[6]. The void fraction was *c.* 3 times enriched in *O*-2 and *O*-2,3 substituted xylose residues relative to the parental BE1-U fraction. The linkage

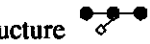
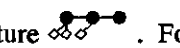
TABLE III. Relative amounts of different xylose residues[a] in BE1-U fractions

linkage type	BE1-U[b]	BE1-U60[b]	EndoI-digest BE1-U: fraction 13
1,4-linked	65	53	39
1,3,4-linked	18	9	10
1,2,4-linked	2	5	7
1,2,3,4-linked	15	34	43

[a] Expressed as molar percent of xylose.

[b] Data adapted from Gruppen *et al*[18].

composition data showed that the relative proportions of *O*-2 and *O*-3 substituted xylose residues of the void fraction resemble those of the BE1-U60 fraction.

In order to study the influence of the Ara/Xyl ratio of the arabinoxylans on the formation of the different oligosaccharides, incubations of subfractions of BE1-U having different Ara/Xyl ratios with endoI and endoIII were performed and the digests were analyzed for oligosaccharide composition. The results for incubation with endoI are shown in Table IV. It can be seen that for fraction BE1-U20, having the lowest Ara/Xyl ratio (0.36), 91% of the material is present as oligosaccharides with a DP ≤ 10. A relative high amount is present as xylobiose (11%) and structure  (19.8%). With increasing Ara/Xyl ratios of the polymeric material the amounts of oligosaccharides having either *O*-3 or both a *O*-3 and *O*-2,3 substituted xylose residue decrease whereas the amounts of oligosaccharides with *O*-2,3 substituted xylose residues first show an increase and then a decrease, an exception being formed by structure  . For the BE1-U60 (Ara/Xyl= 0.80) almost all material is present as fragments with DP> 10. Table IV shows that with increasing Ara/Xyl ratio of the parental material, in general, also the Ara/Xyl ratios of the fractions ***11***, ***12***, and ***13*** increase. Table IV also shows the results for the water-extractable arabinoxylans with different Ara/Xyl ratios. In general, these results showed great similarities with the BE1-U subfractions with respect to the effect of increasing Ara/Xyl ratio on the amounts of the different types of oligosaccharides. However, when comparing fractions with corresponding Ara/Xyl ratios, it can be seen that, in general, water-extractable arabinoxylans are degraded to a larger extent than alkali-extractable arabinoxylans. In Table V the corresponding results for the BE1-U subfractions with endoIII are given. In this case also the amounts of oligosaccharides having *O*-3 substituted xylose residues decrease with increasing Ara/Xyl ratio of the parental material whereas for most of the oligosaccharides having *O*-2,3 substituted xylose residues or both an *O*-3 and *O*-2,3 substituted xylose residue the amounts analyzed first show an increase and then a decrease. For all fractions the amounts of xylose oligomers up to DP 4 were small.

Periodate analysis

Table VI shows the relative amounts of glycerolxylosides obtained after periodate oxidation and subsequent mild hydrolysis. The TSK-40 HW fractionation revealed 5 separate peaks. The last two fractions to elute had elution times larger than xylose. In accordance with Åman and Bengtsson[26], they were ascribed to low-molecular weight degradation products, and not further studied. In order of elution the other three peaks had retention times smaller than xylotriose, xylobiose and xylose, respectively. The fractions were considered to be the glycerol derivatives of xylotriose, xylobiose and xylose, respectively, but not further identified. The relative amounts were calculated from the refractive index value assuming equal molar responses for the glycerolxylosides. The results show that for each arabinoxylan most of the branched material is present as

TABLE IV. Abundance[a] of identified oligomers in endoI digests of alkali- and water-extractable arabinoxylans having different Ara/Xyl ratios

Oligomer[b]	BE1-U					weAX			
	20(*0.36*)[c]	30(*0.46*)	40(*0.55*)	50(*0.68*)	60(*0.80*)	30(*0.39*)	40(*0.52*)	50(*0.67*)	60(*0.84*)
⊘	7.0	4.0	2.6	1.2	0.1	6.8	5.2	3.4	1.7
⊘–⊘	11.0	5.6	3.4	1.4	0.3	10.6	7.0	3.9	1.6
◇⊘–⊘	8.7	4.2	2.5	1.0	0.3	9.0	5.6	2.5	1.8
⊘–⊘–⊘	2.3	1.1	0.8	0.2	0.1	2.1	1.0	0.8	0.9
⊘(◇)–⊘–⊘	19.8	9.2	5.3	1.7	0.4	18.9	11.8	5.0	1.9
◇⊘–⊘–⊘	2.7	4.8	5.1	3.6	0.6	2.6	4.1	4.3	3.2
◇⊘(◇)–⊘–⊘	8.1	3.9	2.2	0.9	0.2	8.8	5.6	2.9	0.7
◇⊘(◇)–⊘–⊘	0.7	1.3	1.3	1.0	0.1	0.6	1.2	1.3	0.7
⊘(◇)–⊘–⊘–⊘	7.1	9.2	7.5	4.5	0.4	4.9	8.3	6.6	2.8
⊘(◇)–⊘(◇)–⊘–⊘	4.6	2.9	1.5	0.5	0.1	5.6	4.0	1.5	0.6
◇⊘–⊘(◇)–⊘–⊘	9.2	8.3	6.7	3.6	0.4	7.5	8.9	6.4	2.6
◇⊘(◇)–⊘(◇)–⊘–⊘	0.6	2.5	4.5	4.9	0.8	1.3	2.3	4.9	5.5
⊘(◇)–⊘–⊘–⊘–⊘	6.1	5.8	4.6	2.0	0.2	5.0	7.1	4.1	1.5
⊘(◇)–⊘(◇)–⊘–⊘–⊘	1.2	4.0	5.1	4.9	0.7	1.2	3.1	6.1	3.9
DP 9+10[d]	1.6	3.7	4.2	0.7	0.1	1.6	1.3	1.1	0.3
◇⊘(◇)–⊘–⊘(◇)–⊘–⊘	1.1	3.3	3.1	2.4	0.4	1.4	2.4	3.1	1.6
DP > 10	9	26	40	66	95	12	21	42	69
fraction ***11***[c]	5(*0.79*)	11(*0.67*)	15(*0.82*)	12(*0.93*)	2(*0.93*)	6(*0.79*)	9(*0.82*)	15(*0.82*)	13(*0.90*)
fraction ***12***	2(*0.83*)	9(*0.77*)	16(*0.90*)	17(*1.01*)	9(*1.03*)	3(*0.84*)	6(*0.84*)	16(*0.90*)	17(*0.97*)
fraction ***13***	1(*0.85*)	6(*0.90*)	9(*0.97*)	36(*1.02*)	84(*1.07*)	3(*0.90*)	6(*0.93*)	11(*0.98*)	38(*1.01*)

[a] Expressed as percentage (w/w) of arabinoxylan.

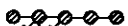
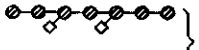
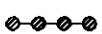
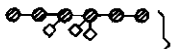
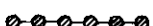
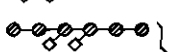
[b] ⊘:Xyl*p*; ◇:Ara*f*; ⊘–⊘ : ß-Xyl*p*-(1→4)-Xyl*p*; ⊘(●)–⊘: α-Ara*f*-(1→2)-ß-Xyl*p*; ⊘(◇)–⊘ : α-Ara*f*-(1→3)-ß-Xyl*p*.

[c] The figures in parentheses represent the arabinose/xylose ratio.

[d] Unidentified

isolated units (*c.* 50%) or blocks of two contiguous substituted xylose residues (*c.* 40%). Since no methylation analysis was carried out in order to verify whether hemiacetal linkages have been formed, the amounts of dimer and trimer may be overestimated. Nevertheless, from the data it can be concluded that most of the substituted xylose is present in small clusters of single and double substituted residues.

TABLE V. Abundance[a] of identified oligomers in endoIII digests of alkali-extractable arabinoxylans with different Ara/Xyl ratios.

Oligomer[b]	BE1-U				Oligomer[a]	BE1-U			
	20(*0.36*)[c]	30(*0.46*)	40(*0.55*)	50(*0.68*)		20(*0.36*)	30(*0.46*)	40(*0.55*)	50(*0.68*)
	2.4	0.9	0.7	0.2		1.3	1.1	0.9	0.7
	3.4	1.4	1.3	0.3					
	0.9	0.2	0.5	0		2.2	1.5	0.6	0.2
	1.1	0.5	0.4	0.1					
	12.9	5.5	2.8	0.8		2.2	3.7	2.3	0.9
	8.4	3.4	1.7	0.5		0.9	1.9	2.4	2.5
	5.0	5.4	4.6	2.5		0.7	1.1	1.2	0.8
	3.6	4.2	3.3	1.6	unidentified (DP=10)	3.5	2.4	2.2	1.2
	6.4	3.3	1.7	0.5	**DP >10**	36	56	69	85
	4.5	1.9	0.9	0.3	fraction *11*[c]	14(*0.52*)	16(*0.57*)	17(*0.62*)	9(*0.65*)
					fraction *12*	12(*0.47*)	20(*0.64*)	28(*0.68*)	15(*0.74*)
	4.7	5.2	3.6	2.0	fraction *13*	6(*0.65*)	20(*0.75*)	24(*0.80*)	61(*0.88*)

[a] Expressed as percentage (w/w) of arabinoxylan.

[b] Notation: ●:Xyl*p*; ◇:Ara*f*; ●—● : ß-Xyl*p*-(1→4)-Xyl*p*;  : α-Ara*f*-(1→2)-ß-Xyl*p*; : α-Ara*f*-(1→3)-ß-Xyl*p*.

[c] The figures in parentheses represent the arabinose/xylose ratio.

TABLE VI. Relative distribution[a] of glycerolxylosides of different alkali-extractable arabinoxylans after periodate oxidation.

fraction	monomer	dimer	trimer
BE1-U	53	38	9
BE1-U20	50	41	9
BE1-U30	56	40	5
BE1-U40	54	39	7
BE1-U50	50	40	9
BE1-U60	54	36	10
weAX	45	47	8
weAX30	44	49	7
weAX40	50	42	8
weAX50	47	44	9
weAX60	36	41	11

[a] Expressed as percentage of total refractive index response.

HPSEC

The molecular size distributions of the void fractions (***13***) from the different digests of BE1-U on Bio-Gel TSK are shown in Fig 2, in which equal amounts of void material were applied to the column. The void fraction obtained by endoIII eluted within a more narrow range than the corresponding endoI fraction. Based on dextran standards the weight-average molecular weights of the void fractions of endoI and endoIII are *c.* 40,000 and 30,000, respectively. Taken into account the Ara/Xyl ratio of 0.8-1.0 (Tables I and II), the average chain length would be 180 and 120 xylose residues, respectively. For fractions ***11*** and ***12*** of both the endoI and endoIII digest the weight-average molecular weights were *c.* 2000 and 3000, respectively (results not shown). Pretreatment with AXH clearly increases the retention time. The weight-average molecular weights for endoI/AXH and endoIII/AXH void fractions are now 15,000 and 13,000, corresponding with a xylan backbone length of 61 and 53 xylose residues, respectively. Pretreatment of the BE1-U fraction with AXH showed no effect on the retention time of fractions ***11*** whereas for fractions ***12*** a small decrease in retention time was noticed (results not shown). In Fig. 3 the molecular size distributions of the Bio-Gel P-2 void fractions (***13***) of each endoI digest from the ethanol fractionated arabinoxylans are given. Except for the BE1-U60 fraction, the bulk of the material from the different digests peaked at the same retention time (30 min). With increasing Ara/Xyl ratio more material eluted at lower retention time, whereas for the BE1-U60 fraction most material was polymeric. The curves also reflect the quantity of the void fraction after enzymic treatment of equal amounts of each fraction.

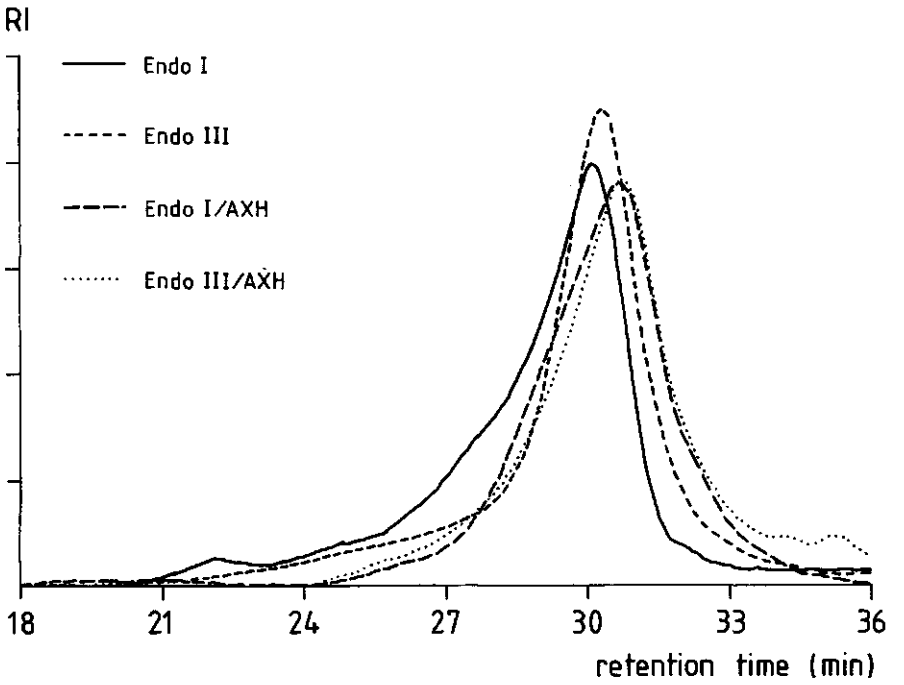


FIGURE 2. Elution pattern (HPSEC) of Bio-Gel P-2 void fractions from BE1-U after digestion with different enzymes.

Simulation of arabinoxylan degradation

In order to investigate whether the arabinose side groups are distributed randomly along the xylan backbone, a simulation experiment was performed based on the random assignment of the different xylose residues in the xylan backbone and the specificity of the enzymes. These data are also given in Tables I and II. The simulation program did not distinguish between the specificity of endoI to split at either the non-reducing end of a substituted xylose or at the non-reducing end of an adjacent unsubstituted xylose residue when the enzyme has these two possibilities. Therefore, in comparing the experimental and simulation values the sum of the corresponding oligosaccharides, corrected for the additional unsubstituted xylose, should be used rather than the values of the individual oligosaccharides. When calculated (results not shown), it was found that for both the non- and AXH-pretreated BE1-U fraction the measured amounts of various fragments clearly differ from the theoretical values obtained by the simulation experiment. Whereas AXH is reported to remove *O*-3 linked Ara*f* groups from single substituted xylose residues of both polymeric wheat flour arabinoxylans and oligosaccharides[22,23] the removal of *O*-2 linked Ara*f* from single substituted xylose has only been reported for one oligosaccharide[23] and not for the polymeric arabinoxylan. Due to the low amounts of *O*-2 substituted xylose in

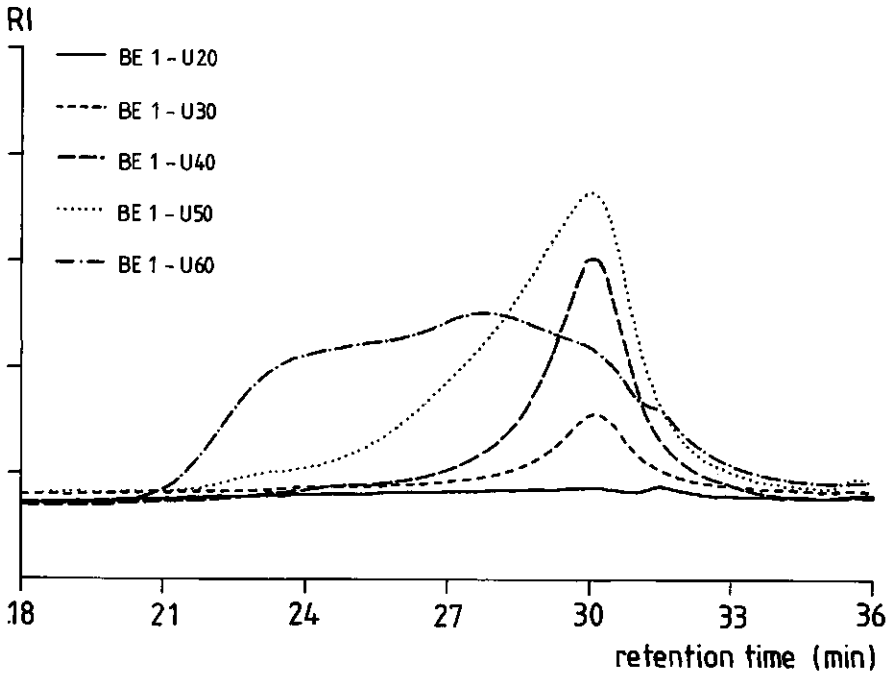


FIGURE 3. Elution pattern (HPSEC) of Bio-Gel P-2 void fractions from BE1-U subfractions after digestion with endoI.

the parental material[6], the hydrolysis of *O*-2 linked arabinofuranosyl groups was difficult to establish with the NMR analysis used[22], and therefore the possibility of removing *O*-2 linked Ara*f* from polymeric arabinoxylans can not be excluded. When this possibility is taken into account in the simulation program the predicted compositions (data not given) differed only slightly from the ones based on the removal of *O*-3 linked arabinose by AXH, as given in Tables I and II. For incubation with endoIII the differences between simulated and experimentally found values were more pronounced than with endoI, when for this enzyme also, the sum of corresponding oligosaccharides are used for the comparison (see above). The amounts of xylose, xylobiose, and xylotriose were clearly lower than the theoretical values. Also, the experimental values of fractions with DP >10 differed markedly with those found by simulation. For the endoIII digest the simulated amounts of fragments with DP >10 were higher than the experimental values, whereas for the other digests the predicted values were lower.

Discussion

From the results of the simulation experiment it can be concluded that both single as well as double substituted xylose residues are not randomly distributed along the xylan

backbone. Based on the abundance of the identified oligosaccharides and the composition of the void fractions a structural model can be composed.

Oligosaccharides

Given the mode of action of endoIII[21] it can be concluded that the oligosaccharides in Table II are preceeded in the xylan chain by at least two contiguous unsubstituted xylose residues. When in the oligosaccharide one or two unsubstituted xylose residues are present at the non-reducing end of the substituted xylose residue at least 3 or 4 contiguous unsubstituted xylose residues must have been present in the arabinoxylan, respectively. The presence of xylobiose, xylotriose and xylotetraose reveal the presence of at least 5, 6, and 7 contiguous unsubstituted xylose residues in the parental arabinoxylan. Based on the abundance of the different oligosaccharides in Table II, together with the relative proportion of the unsubstituted xylose residue(s) at the nonreducing site in each oligosaccharide, the Ara/Xyl ratio and sugar linkage composition of BE1-U[6], it can be calculated that for the BE1-U fraction 32 and 25% of all unsubstituted xylose is present as clusters of at least 3 and 4 contiguous residues, respectively, whereas at least another 4, 1 and 2% is present as at least 5, 6 and 7 contiguous residues, respectively.

For the BE1-U20 fraction, having the lowest Ara/Xyl ratio (Table V), 40 and 39% of all unsubstituted xylose is present as clusters of at least 3 and 4 contiguous residues, respectively with another 7, 2 and 2% present as at least 5, 6 and 7 contiguous residues, respectively. For the fraction BE1-U50 11 and 7% of all unsubstituted xylose is present as clusters of at least 3 and 4 contiguous residues, respectively, and hardly any longer unsubstituted xylosyl homologues were present. The absence of oligosaccharides with single *O*-2 substituted xylose residues indicates that these are prevailing in the polymeric fraction.

Polymeric material

From the mode of action of endoI[21], as summarized in the experimental section, it can be concluded that fraction ***13*** in Table I must be composed of the elements  , and  . From the linkage composition analysis data (Table III) it can be calculated that the xylose present in element  accounts for 10% of all xylose present in fraction ***13***. The xylose in elements and together make up 7-14% of all xylose. When arabinose is also taken into account the following weight distribution for the elements in the void can be calculated: : 5-16%, : 64-78%, : 10%, : 0-7%, and : 0-10%.

Pretreatment with AXH prior to incubation with endoI resulted in a decrease in polymeric material from 10.7 to 6.6%. The material released comprised 1.9% xylose and 2.3% arabinose, expressed as per cent of the BE1-U. From the methylation analysis and the amount of arabinose present in the polymeric fraction it can be calculated that the

removal of *O*-3 linked arabinose by AXH accounted for a decrease of 0.5%. To account for the release of the remaining 1.8% arabinose and 1.9% xylose, taken into account the distribution of structural elements given above, the region in the endoI void from which the material is released upon AXH pretreatment must contain relatively many repeating elements.

From Table IV it can be seen that the void fraction of BE1-U (Table I) in fact is made up of different populations with Ara/Xyl ratios varying from 0.85 to 1.07 (c.q. void fractions of BE1-U20 to BE1-U60). Since the identified oligomers do not contain any *O*-2 linked xylose and the relative amount of *O*-2 linked xylose increases with increasing Ara/Xyl ratio[6] the compositions of the void fractions differ in the amounts of  and  , but are also likely to differ in the other constituent elements. Given the facts that for the different arabinoxylans no void fractions were found with Ara/Xyl ratios much larger than 1 and that with increasing Ara/Xyl ratio the relative amount of *O*-2 substituted xylose increased from only 0.8 to 4.9%, the increase of Ara/Xyl ratio must be due to isolated *O*-2,3 substituted xylose residues rather than regions of contiguous substituted xylose residues.

A structural model for arabinoxylans

Based on the above results, in Fig. 4 a model for arabinoxylans from wheat is proposed in which part of the material is present in a configuration as in region A, which is presumed to have a rather constant structure. The other part is present in region B which varies in composition depending on the type of arabinoxylan (Ara/Xyl ratio). Region A is highly branched and can be considered to be composed of repeating units of 1 to 3 substituted xylose residues followed by an unsubstituted xylose residue. In this repeating unit the unsubstituted xylose residue is never preceeded by an *O*-3 substituted xylose residue. The number of repetitions (N) can not be determined unambiguously in this model since the reported DP of the void arabinoxylans may be overestimated, due to the use of dextran standards[29]. In addition, the length of the repeating unit may vary from two to four. Region B is lowly branched and includes contiguous unsubstituted xylose residues. The substituted xylose is present either isolated or in pairs. Variation in Ara/Xyl ratio between arabinoxylans is due to variation in ratio region A/region B as well as the composition of region B. From region B the identified oligomers of DP 1-10 are released whereas in the model proposed region A corresponds with the void fraction of the endoI digest. The structure of arabinoxylans in fractions 11 and 12 of the endoI digest might also be described as region A, in which N has a low value. For each arabinoxylan, the weighted Ara/Xyl ratio average of all oligosaccharides with DP 1-10 and the Ara/Xyl ratio of the void fraction (Tables I and IV) provide boundary values for the sum of $P1i_n, P2j_n, Qk_n, Rl_n$ in the tentative model.

The model proposed shows close resemblance to the model proposed by Goldschmid and Perlin for water-extractable wheat arabinoxylans[14]. In this model[14] the highly branched

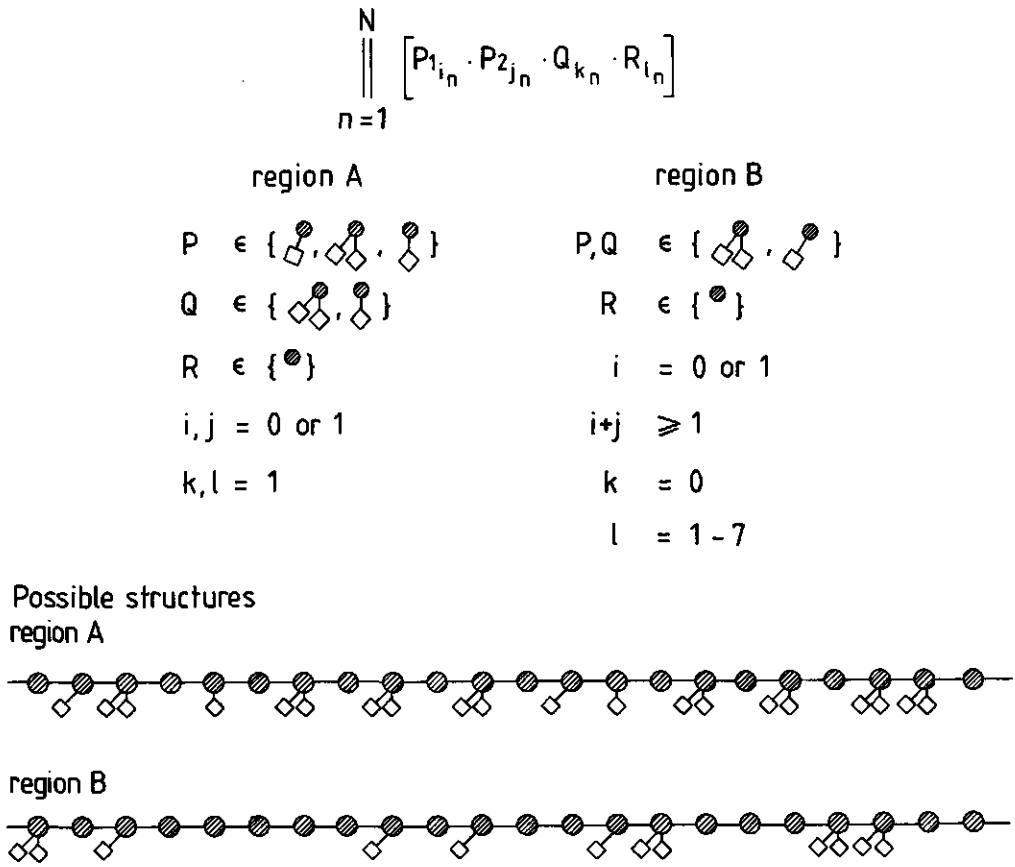


FIGURE 4. Tentative model for wheat flour arabinoxylans.

regions, interlinked with regions of up to 5 contiguous unsubstituted xylose residues, have a DP of 20-25. In our model the DP of the branched regions seems higher, although the calculated value 180, may be overestimated[29]. In both their as well as our model the presence of 3 contiguous substituted xylose residues is not excluded.

The model proposed differed from those for alkali-extractable arabinoxylans from barley[27] and water-extractable arabinoxylans[30] from rye. In the latter model the occurrence of two types of arabinoxylans or the occurrence of two types of regions in the arabinoxylan molecule are proposed, one containing contiguous double substituted xylose residues and the other consisting of unsubstituted and *O*-3 substituted xylose residues. Despite these differences, from the results of the periodate analysis it can be seen that for wheat as well as rye[30] arabinoxylans most of the substituted xylose residues are present in small isolated clusters of singly and doubly substituted residues. In the model for barley arabinoxylans[27], regions of enzyme-resistant polymeric fragments are separated by regions of contiguous unsubstituted xylose residues. Our model for wheat arabinoxylans, however, proposes an alternation of highly branched regions (A) with regions of less dense branching (B), including subregions of unsubstituted xylose up to at least 7 contiguous

residues. In addition, for barley arabinoxylans[27], using endoI, it was found that the relative distribution of the fragments with DP ≤ 10 were similar for digests obtained from arabinoxylans with different Ara/Xyl ratios. In order to verify whether this was also the case for wheat arabinoxylans, the relative composition of all oligomers with DP ≤ 10 were calculated from the data in Table IV. The results (not given) showed that with increasing Ara/Xyl ratio the composition of the digests changes in favour of oligosaccharides with *O*-2,3 substituted xylose accompanied by lower amounts of oligosaccharides with *O*-2 substituted xylose residues whereas the amounts of oligosaccharides having both an *O*-3 and an *O*-2,3 substituted xylose residue stays relatively constant. This indicates a different substitution pattern of the arabinoxylans in barley compared to those in wheat. Whereas for barley arabinoxylans the difference in Ara/Xyl ratio is ascribed to the variation in the relative proportion of unbranched regions, for wheat this variation is also due to variation in substitution pattern of the less branched regions (B).

The observation that the total amount of clusters of two contiguous substituted xylose residues were more abundant than predicted by the random simulation (Table I and II) was also found for galactomannans[31]. These authors found a second order Markov chain[32] simulation to be more in agreement with the results found. In the second order Markov chain the probability of a given residue being substituted is dependant on the nature of substitution of the previous two residues. Because the xylan backbone consists of four different units in stead of two for galactomannans, such a Markov chain simulation would require four times more parameters than used in the model for galactomannans, and was therefore not used here.

Arabinoxylan populations

The HPSEC data show that incubation of BE1-U with endoIII resulted in a void fraction having a similar Ara/Xyl ratio to that of endoI, but was eluted with a longer retention time than the endoI void fraction. Based on its specificity however, one would expect that incubation with endoIII would lead to a void fraction with a larger molecular weight compared to endoI. Although at an equal Ara/Xyl ratio possible differences in the substitution pattern of arabinose along the xylan backbone might influence the elution volume, the higher retention times strongly indicate a lower molecular weight of the endoIII void fraction. This points to the presence of different populations in the BE1-U fraction. This is further corroborated by the HPSEC profiles of the void fraction of the ethanol fractionated arabinoxylans which exhibit a broadening in the elution profile rather than a complete shift in retention time (Fig. 3). The calculated apparent molecular weights of the void fractions are probably overestimated because of the high asymmetrical shape of the arabinoxylans compared to dextrans[11,29], which were used for calibration. Since the intact arabinoxylans exhibit molecular weights of 260,000 - 640,000, as analyzed by laser

light scattering[11], the results indicate at least a 15-fold decrease in molecular weight upon enzymic treatment. Although fraction BE1-U60 was not degraded to oligosaccharides it is susceptible to xylanase hydrolysis since the apparent molecular weight was decreased upon enzymolysis.

In a previous study it was shown that the water-extractable arabinoxylans and alkali-extractable arabinoxylans in the BE1-U subfractions which had similar Ara/Xyl ratios also had similar relative amounts of un-, *O*-2, *O*-3, and *O*-2,3 substituted xylose residues. The data given in Table IV, showing an extended degradation of water-extractable arabinoxylans, indicate a different type of substitution. With similar relative amounts of un-, *O*-2, *O*-3, and *O*-2,3 substituted xylose residues this may be explained by the presence of more contiguous substituted xylose residues and less isolated substituted xylose residues in the water-extractable arabinoxylans relative to the alkali-extractable arabinoxylans. The results from the periodate analysis (Table VI) seems to corroborate these results but in order to substantiate the above hypothesis a more quantitative analysis should be performed using total-neutral sugar response instead of refractive index in the size exclusion chromatography of the hydrolysis products. The results also indicate that the presence of ferulic acid which was determined by HPLC[2] to be 0.03, 0.09, 0.16 and 0.04% (w/w) for the weAX20, weAX30, weAX40 and weAX50 fraction, respectively (no further data given), does not hinder an extensive enzymic degradation of the water-extractable arabinoxylans. The presumed difference in substitution might be related to difference in water-extractability, although also other factors like covalent interactions and physical entanglement have been mentioned[29,33].

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CHAPTER 9

Enzymic degradation of water-unextractable cell wall material and arabinoxylans from wheat flour

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Summary

In order to study differences in enzymic breakdown, water-unextractable cell wall material (WUS) of wheat flour was incubated with endoxylanase I (endoI) and endoxylanase III (endoIII) purified from *Aspergillus awamori*. Also water-extracted and alkali-extracted arabinoxylans were degraded by these enzymes. Both enzymes released in total 90% of the arabinoxylans present in the WUS, without solubilization of glucomannans, (1→3,1→4)-ß-glucans and cellulose. Both poly- and oligosaccharides were released during the incubation. Arabinoxylans with similar arabinose/xylose ratios, obtained from different fractions of the WUS, showed different degrees of degradation. No differences could be observed in enzymic solubilization and degradation between WUS and alkali-extracted arabinoxylans from different wheat varieties.

Compared to endoI, incubation with endoIII resulted into a more rapid solubilization and/or degradation of WUS and extracted arabinoxylans. EndoIII digestion resulted also in a faster decrease of the total water holding capacity of the residual WUS. However, the water holding capacity per gram of residual WUS showed a twofold increase as a result of enzyme incubation. Application of endoIII in baking trials yielded a 14% increase in loaf volume together with the improvement of crumb structure and 'break and shread', whereas endoI yielded 4% increase of loaf volume with no improvement of other baking characteristics.

Introduction

Wheat flour contains 2-3% (w/w) of cell wall material, which consists largely of non-starch polysaccharides although minor amounts of protein are also present[1,2]. This cell wall material partly determines the dough characteristics and baking properties of wheat flours, effects that have been ascribed to its high water absorbing capacity[3,4] and which can be modified by enzymic degradation. Only a proportion of the cell wall material is extractable in water[2,5]. In previous studies[2,6-9] we reported on the isolation of water-unextractable cell wall material (WUS) and the extraction/characterization of arabinoxylans thereof. It was found that in Arminda wheat flour different arabinoxylans are present varying in structural features. In earlier studies[10] it was observed that the addition of xylanase to wheat dough resulted in increased loaf volumes but, with a loss in loaf height and quality. A recent publication[11] on the effect of xylanase addition mentions the same increase in loaf volume but no or only small detrimental effects to other loaf characteristics were found. In both studies[10,11] only the effect of xylanase addition on the baking performance was given. The biochemical breakdown of the cell wall material was not studied. In the present study we report on the enzymic degradation of different water-unextractable wheat flour arabinoxylans from Arminda flour as well as corresponding soluble arabinoxylans extracted from different wheat varieties, using two endoxylanases from *Aspergillus awamori* having different modes of action[12]. The results obtained are compared with the effects of both enzymes on baking performance and water holding capacity.

Experimental

Materials

The preparation and characterization of water-unextractable cell wall material (WUS) and $Ba(OH)_2$-extractable arabinoxylans (BE1) from Arminda, Granta, Okapi, Taurus, and Camp Remy wheat flour has been published previously[6,13]. The preparation and characterization of DEAE-bound (BE1-B-RB) and DEAE-unbound (BE1-U; BE1-B-RU) and ethanol precipitated fractions thereof (BE1-U50; BE1-U60) from the BE1 extract of Arminda wheat flour is described elsewhere[8]. The extraction and characterization of the subsequent water extract (BE2) and 1M KOH extract (1M) after $Ba(OH)_2$ extraction together with that of water-extractable arabinoxylans (weAX) from Arminda flour is described previously[7]. Endo-(1→4)-ß-D-xylanase I (endoI) and endo-(1→4)-ß-D-xylanase III (endoIII), were purified[14] from *Aspergillus awamori* CMI 142717. In order to split glycosidic linkages in arabinoxylans endo I needs 1 unsubstituted xylose residue when this is adjacent to an *O*-3 substituted xylose residue and 2 contiguous unsubstituted xylose residues when these are adjacent to either an *O*-2 or an *O*-2,3 substituted xylose residue.

It is also able to split the glycosidic linkage at the non-reducing site of a single or double substituted xylose residue. Endo III needs at least 3 contiguous unsubstituted xylose residues and is unable to split the glycosidic linkage at the non-reducing site of a single or double substituted xylose residue[12].

Enzyme incubations

For determination of the K_m and V_{max} of endoI and endoIII, BE1-U, weAX and WUS, in various concentrations (0.5-8.0 mg/ml), were incubated with either enzyme in 250 μl 50mM sodium acetate buffer pH 5.0 for 1 h at 30 °C (0.2 μg protein/ml; Sedmak assay[15]), and analysed for reducing sugars (Nelson-Somogyi)[16] using micro-titer plates. Linewaever-Burk plots were used for the calculation of K_m and V_{max}. The molar activity was determined at 0.1 mg/ml substrate concentration under the conditions described above.

For the other enzyme incubations, arabinoxylan preparations were dissolved in 50mM sodium acetate buffer pH 5.0 (0.1% w/v) and incubated with endoI or endoIII (4 ng protein/ml for WUS incubations and 4 μg protein/ml for incubation with weAX and BE1-U). Incubation mixtures were held at 30 °C for 0-24 h, inactivated at 100 °C for 10 min, stored for 24 h at room temperature, and subsequently centrifuged for 10 min at 2,600 *g*. The supernatants were analysed for total sugar content, neutral sugar composition, and molecular size distribution. The resulting residues were washed three times with water (5 ml) and centrifuged (10 min; 2,600 *g*). The final residue was freeze-dried and analysed for neutral sugar composition.

Baking experiments

The influence of xylanases was tested in a puploaf baking test similar to the one described by Finney[17]. Puploaves were baked from 150 g dough pieces obtained by mixing 200 g (100%) of a dutch flour for breadmaking (protein content of 11.8% based on dry matter, ash content 0.51%, moisture content 14.4%), 1.2 g (0.6%) Fermipan™ instant dry yeast (Gist-brocades, Delft, The Netherlands), 4 g (2%) salt, 3 g (1.5%) sugar, 400 mg (0.2%) $CaCl_2$, 5 mg (1125 SKB/kg flour) fungal *alpha*-amylase (Fermizyme P200™; Gist-brocades, Delft, The Netherlands), 110 ml (55%) water and 25 μg purified xylanase. The latter was added in solution and the amount of water added was corrected for. The dry ingredients were blended through the flour and mixing was started immediately after addition of the water and the purified xylanase. After mixing for 6.25 min (which was optimal to both machinability as well as loaf volume obtained) at 52 r.p.m. in a pin mixer (National Mfg, Co. type) the dough, having a temperature of 27 °C, was divided into two pieces, proofed for 45 min at 31 °C/85% R.H., punched, proofed for another 25 min, moulded and panned. After a final proof for 70 min at 31 °C/85% R.H., the dough was baked for 20 min in a National Mfg., Co. (Lincoln, Nebraska, USA) rotary oven at 250 °C. Loaf volume was determined by a rapeseed displacement method for puploaves.

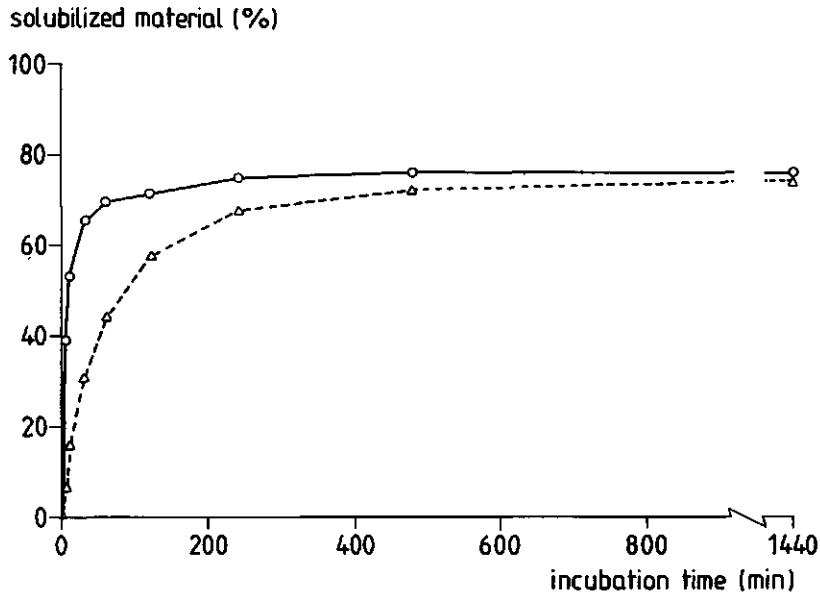


FIGURE 1. Time course of solubilization of polysaccharide material from water-unextractable cell wall material by endoI (---) and endoIII (—), expressed as percentage of total polysaccharide material present in WUS

Break and shread and crumb structure of the baked products were scored one day after baking. The *alpha*-amylase preparation used (Fermizyme P200) showed no endo-xylanase side activity on BE1-U material, as analysed by either HPSEC or reducing endgroup analysis[16], using a 50 fold higher enzyme-substrate ratio as in the baking experiment. With the baking procedure described in general a standard deviation of 2% in loaf volume determination is obtained in the pilot plant bakery.

Analytical methods

Total neutral sugar content of the supernatants was determined by an automated orcinol assay[18], using arabinose as standard.

Neutral sugar composition Neutral sugars in the residues were released by pretreatment with 72% (w/w) H_2SO_4 for 1 h at 30 °C followed by hydrolysis with 1M H_2SO_4 for 3 h at 100 °C. Next, sugars were converted to their alditol acetates[19] and analysed as described previously[2]. Neutral sugars in the extracts were analysed using 2M trifluoroacetic acid for hydrolysis as described previously[7].

High performance anion exchange chromatography (HPAEC) was performed as described previously[9]. The identification of the oligosaccharides is described elsewhere[20,21].

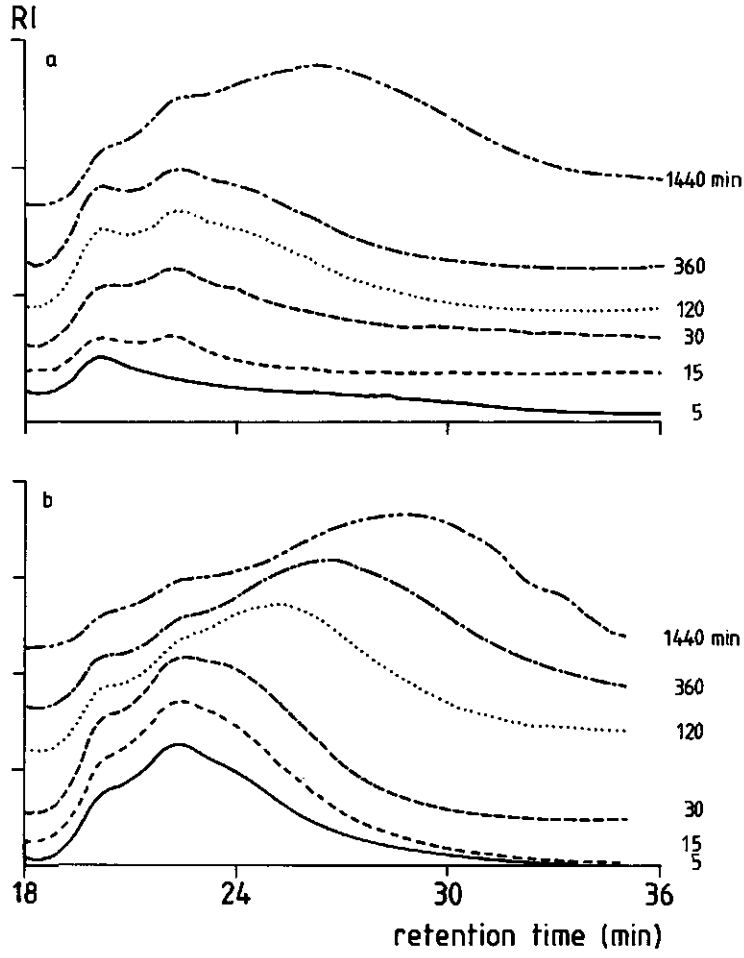


FIGURE 2. Elution pattern (HPSEC) of enzymic solubilized material from WUS by endoI (a) and endoIII (b) after incubation times varying from 5 to 1440 min.

High performance size exclusion chromatography (HPSEC) was performed with three Bio-Gel TSK columns in series (40XL-30XL-20XL) as described previously[13] using 0.4M sodium acetate buffer pH 3 as eluent. The system was calibrated with dextrans (Pharmacia) having mol.wts. in the range of 500-500,000.

Swelling power was determined qualitatively by comparing the volumes of the WUS residues upon enzymic degradation with those of the enzyme untreated WUS. The swelling volumes were compared after 24 h of storage of the samples at room temperature.

Total water-holding capacity was determined by a modification of the method of

Robertson and Eastwood[22] by measuring the wet weight (g) of the final residue after washing.

Water-holding capacity (g water/g residue) was calculated as the quotient of the total water-holding capacity and the weight (dry matter) of the residue. The latter was calculated from the difference between the original dry weight of the WUS and the amount of sugars (arabinose plus xylose) solubilized as determined by the orcinol assay.

Results

Solubilization of water-unextractable cell wall material

In Fig. 1 the percentage of solubilized polysaccharide cell wall material as a result of incubation with endoI and endoIII is given for different incubations times. Incubation with endoIII resulted in a faster solubilization of cell wall material than incubation with endoI. For endoIII the maximum solubilization was reached after 1 h whereas for endoI the maximum was reached after 4 h under the conditions used. For both incubations, based on orcinol response, 75% of all polysaccharides present in the WUS could be solubilized

TABLE I. Neutral sugar composition of WUS residues after enzyme treatment

Time (min)	Molar proportion[a]					
	Ara	Xyl	Man	Gal	Glc	Ara/Xyl
EndoI						
0	30.7	54.6	1.9	0.6	12.3	0.56
5	30.6	53.8	2.0	0.6	13.0	0.57
10	30.2	53.8	2.0	0.6	13.3	0.56
30	29.9	53.0	2.2	0.6	14.3	0.56
60	28.6	50.9	2.7	0.8	17.2	0.56
120	27.1	47.7	3.4	0.9	21.0	0.57
240	25.2	42.9	4.3	1.2	26.5	0.59
480	15.4	17.4	9.6	2.2	55.5	0.89
1440	13.3	14.5	11.1	2.2	58.9	0.92
EndoIII						
0	30.7	54.6	1.9	0.6	12.3	0.56
5	27.8	42.9	4.0	1.1	24.3	0.65
10	25.5	35.7	5.4	1.4	31.9	0.71
30	23.9	31.4	6.7	1.6	36.5	0.76
60	21.2	27.2	7.3	1.7	42.7	0.78
120	18.5	21.8	8.4	2.1	49.3	0.85
240	16.0	18.3	9.3	2.2	54.3	0.87
480	14.7	16.0	9.9	2.2	57.2	0.92
1440	14.4	15.8	9.8	2.2	57.8	0.91

[a] Abbreviations used: ara = arabinose; xyl = xylose; man = mannose; gal = galactose; glc = glucose.

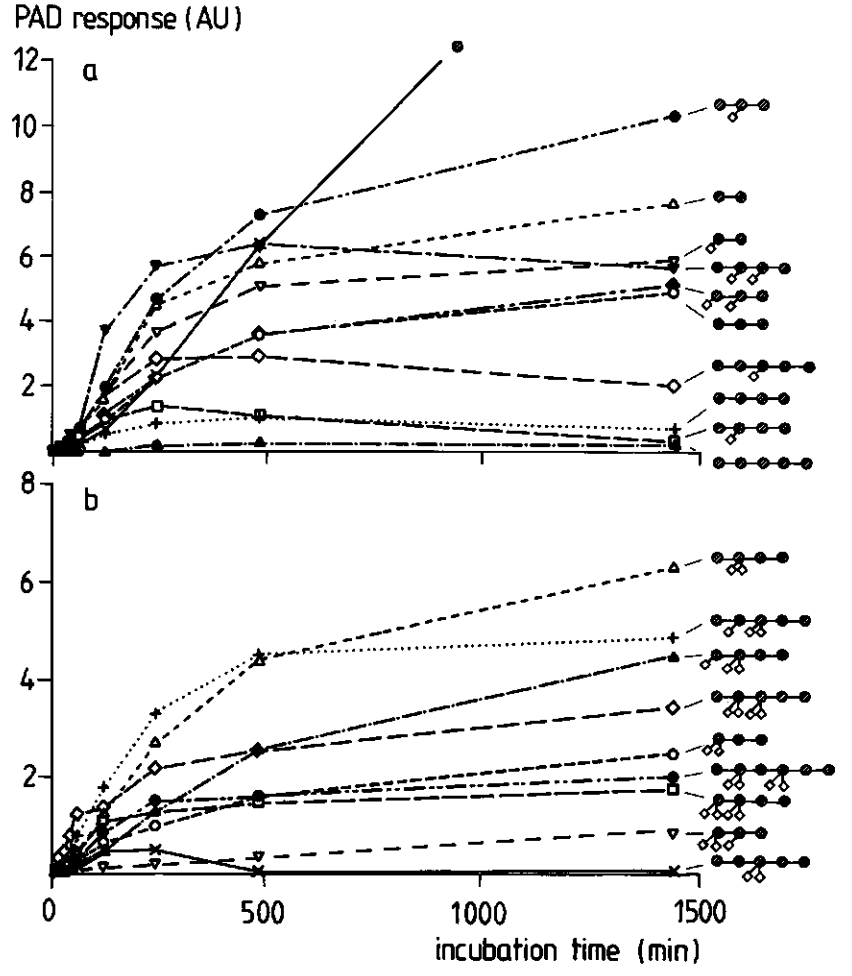


FIGURE 3. Release of arabinoxylan oligosaccharides by incubation of water-unextractable cell wall material by endoI as a function of incubation time; (a) un- and *O*-3 substituted oligosaccharides and (b) *O*-2,3 and *O*-3/*O*-2,3 substituted oligosaccharides

after prolonged incubation. In Fig. 2 the molecular weight distribution of the solubilized material after different incubations times is given. It shows that for each incubation time the bulk of the material solubilized by endoIII has a lower molecular weight than the material released by endoI. With increasing incubation time a decrease in molecular size is observed.

For each incubation time the neutral sugar composition of both residue and supernatant was determined. The supernatants contained mainly arabinose (36 mole%), xylose (62 mole%) and minor amounts of glucose and mannose. The supernatant composition was similar for the different incubation times (no further data shown). The results for the remaining residues are given in Table I. For both enzyme incubations the sum of the molar proportions of arabinose plus xylose decreased from 85% to *c.* 30%, whereas the

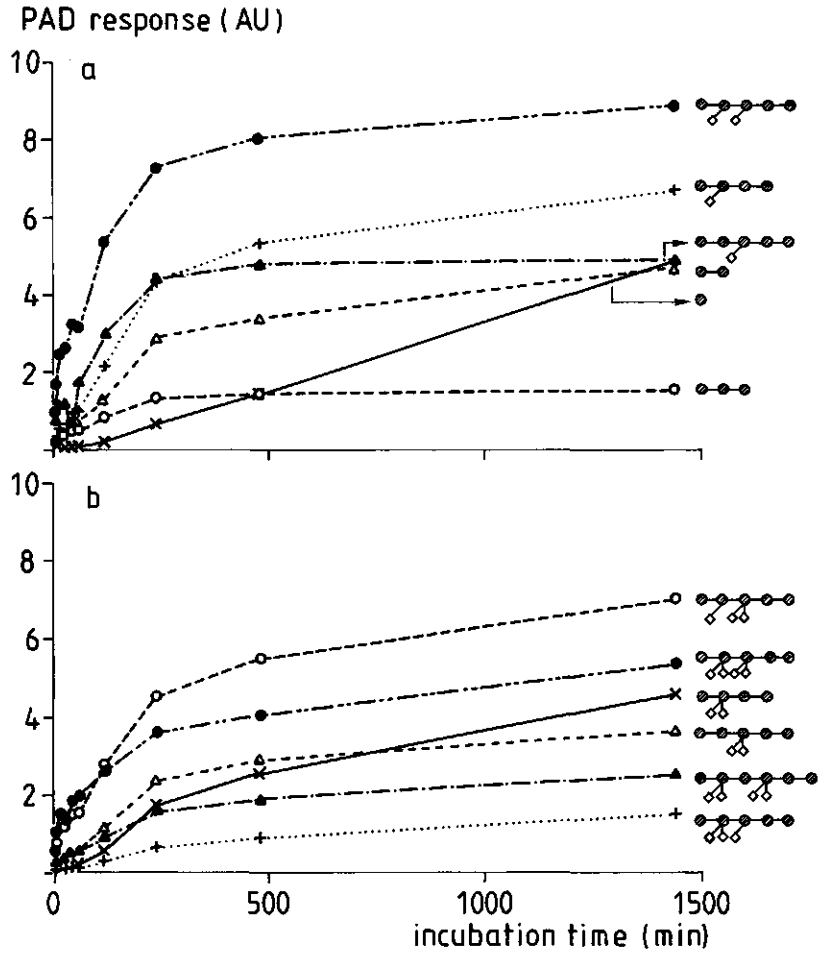


FIGURE 4. Release of arabinoxylan oligosaccharides by incubation of water-unextractable cell wall material by endoIII as a function of incubation time; (a and b, see Fig. 3).

molar proportions of galactose, mannose and galactose each increased *c.* 4 times. It can be seen that with increasing incubation times the Ara/Xyl ratio of the residue increases. This increase was much quicker for endoIII than for endoI.

The availability of purified arabinoxylan oligosaccharides[20,21] as reference compounds made it possible to study the action of endoI and endoIII by monitoring the release of oligosaccharides from WUS at different incubation times. (Fig. 3 and 4, respectively). Whereas for endoIII the amounts of the different oligosaccharides either increased continuously or remained at a certain level, for endoI also a decrease in the amount of specific oligosaccharides was observed. For endoIII a faster initial release of the oligosaccharides , , and was observed compared to

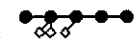
the other oligosaccharides present in both endoI and endoIII digests. With the exception of oligosaccharide  , there seems to be a faster release of oligosaccharides having two contiguous substituted xylose residues, which could indicate a preference for splitting glycosidic bonds near substituted xylose residues. For endoI no specific release was shown. Throughout the incubation, the rate of xylose release was higher than that for the oligosaccharides. It is suggested that next to direct release from polymeric material xylose is also released from oligosaccharides. In Table II the V_{max} and K_m of endoI and endoIII are given for BE1-U and weAX, together with the molar activity on these substrates and WUS. The molar activity, measured at a substrate concentration of 1 g/l and an incubation period of 1 h, was higher for endoI than for endoIII. For both substrates endoIII showed both a higher V_{max} and K_m than endoI. Because part of the WUS is solubilised upon enzymic treatment, the Michaelis-Menten theory does not allow a determination of K_m and V_{max} on this substrate, under the conditions measured. Therefore apparent V_{max} and K_m, designated in parentheses, are given for this substrate.

TABLE II. Properties of endoI and endoIII

		BE1-U	weAX	WUS
Molar activity[a,b]				
	EndoI	1700	1100	1100
	EndoIII	440	340	330
V_{max}[b]				
	EndoI	7400	7300	(9100)
	EndoIII	11100	12300	(12800)
K_m (g/l)				
	EndoI	3	5	(7)
	EndoIII	18	27	(29)

[a] Measured at 0.1% substrate concentration using 1 h incubation.

[b] Values represent moles of reducing groups released/mol enzyme/min, using M_r values of enzymes[14].

Comparison of the degradation of different arabinoxylans

In a previous paper[9] the oligosaccharide composition of the various endoI digests of ethanol subfractionated arabinoxylans from both the major, DEAE-unbound, alkaline extract of WUS and water-extractable arabinoxylan fraction were given. The results revealed that despite similar Ara/Xyl ratios and relative proportions of un-, *O*-2, *O*-3 and *O*-2,3 substituted xylose residues the water-extractable arabinoxylans were degraded to a larger extent than the alkali-extractable arabinoxylans. In addition to the arabinoxylan fractions described, representing 65% of the WUS, wheat flour WUS also contains other

TABLE III. Relative abundance[a] of identified oligosaccharides in endoI digests of different arabinoxylans

	Fraction								
	weAX	BE1-U[b]	BE1-B-RU	BE2	1M	BE1-U50[b]	BE1-B-RB	BE1-U60[b]	WUS
Ara/Xyl[c]	0.55	0.51	0.54	0.67	0.66	0.68	0.87	0.80	0.57
Relative arabino-xylan abundance[d]	-	65	9	10	4	11	6	6	100
Oligomer[e]									
●	6.1	6.4	6.7	6.3	5.8	3.5	9.4	2.9	6.7
●-●	11.8	12.2	11.2	9.1	9.9	4.1	10.6	5.7	8.2
◇-●-●	8.6	8.9	6.4	4.6	4.6	2.9	2.9	5.7	6.3
●-●-●	1.5	2.3	1.5	2.8	3.8	0.6	1.0	1.9	3.3
●●-●	15.1	14.3	16.9	13.3	15.7	4.9	6.2	7.6	16.3
◇◇-●-●	6.0	5.8	6.4	6.9	5.2	10.4	2.6	11.5	6.0
◇◇-●-●	6.0	5.0	4.5	5.5	7.5	2.6	2.6	3.8	7.5
◇◇-●-●	1.3	1.6	1.3	2.2	1.2	2.9	1.0	1.9	1.6
●●-●-●	9.6	10.1	10.4	10.5	9.7	13.0	4.8	7.6	8.0
●●-●-●	4.2	3.5	4.2	3.8	4.4	1.4	1.9	1.9	5.3
◇◇-●-●-●	8.8	8.8	9.4	9.9	9.1	10.4	15.3	7.6	10.3
◇◇-●-●-●	4.1	3.7	4.6	6.3	5.6	14.2	11.3	15.3	5.5
●●-●-●-●	7.9	8.8	8.1	8.1	6.9	5.8	12.2	3.8	6.7
●●-●-●-●	4.4	4.1	4.6	5.5	5.0	14.2	8.2	13.4	3.9
unidentified (DP=9)	1.6	1.8	1.0	1.8	2.8	2.0	2.9	1.5	2.0
◇◇-●-●-●-●	2.9	2.7	2.7	3.4	2.6	7.0	7.2	7.6	2.4
total DP 1-10[f]	70	72	71	55	62	35	36	5	64

[a] Expressed as percentage (w/w) of all oligosaccharides with DP ≤ 10.

[b] Data from Gruppen *et al*[7,8].

[c] Data from Gruppen *et al*[9].

[d] Relative amount of WUS arabinoxylan present in each fraction, expressed as percentage (w/w) of total arabinoxylan present in WUS, data from Gruppen *et al*[7,8].

[e] ● : Xyl*p*; ◇ : α-Ara*f*; ●● : β-Xyl*p*-(1→4)-Xyl*p*; ◇● : α-Ara*f*-(1→2)-β-Xyl*p*; ◇● : α-Ara*f*-(1→3)-β-Xyl*p*

[f] Expressed as percentage (w/w) of total arabinoxylan in each fraction.

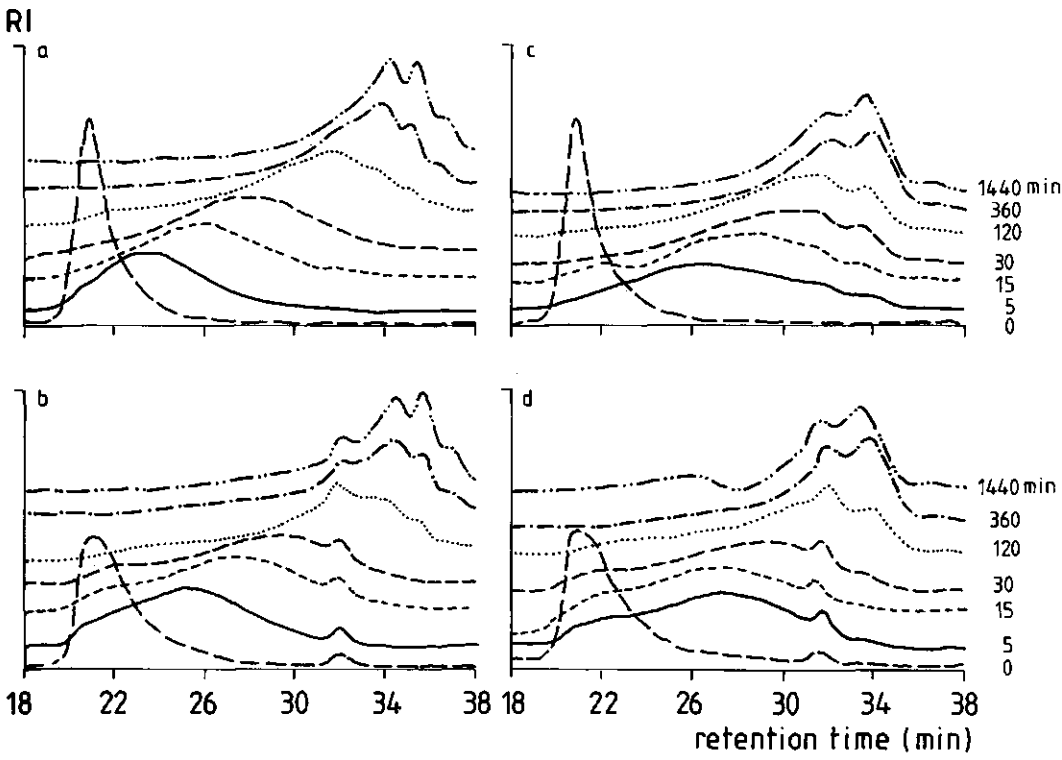


FIGURE 5. Elution pattern (HPSEC) of enzymic degradation of alkali-extractable (a: endoI; c:endoIII) and water-extractable arabinoxylans (b: endoI; d: endoIII).

arabinoxylans, which vary in Ara/Xyl ratio[7,8]. In order to see whether these alkali-extractable arabinoxylans exhibit differences in their degree of degradation they were also degraded by endoI. In Table III the results for these alkaline extracts together with those of WUS, weAX and some of the previous analysed[9] fractions (BE1-U, BE1-U50, and BE1-U60) are given, expressed as proportions of the total identified oligosaccharides (DP 1-10) present. For each fraction both the Ara/Xyl ratio and the relative amount of WUS arabinoxylan present in the fraction is given. In this table also the total amount of oligosaccharides with DP 1-10 is given, expressed as percentage (w/w) of the total amount arabinoxylan present in the parental material. It can be seen that arabinoxylans which have similar Ara/Xyl ratios differ in the composition of their digest. The BE2 and 1M fraction contain relatively less arabinoxylan oligosaccharides with *O*-2,3 substituted xylose residues and more arabinoxylan oligosaccharides with *O*-3 substituted xylose residues than the DEAE unbound subfraction with similar Ara/Xyl ratio (BE1-U50). When comparing the BE1-B-RB fraction (DEAE-bound material) to the BE1-U60 fraction it can be seen that the latter contains more oligosaccharides with only *O*-3 substituted xylose residues and more oligosaccharides with both an *O*-3 substituted and an O-2,3 substituted xylose residue. Also, the total amount of oligosaccharides with DP 1-10 is larger for the BE-2,

TABLE IV. Water-holding capacity of WUS residues after enzymic treatment

	EndoI		EndoIII	
Time (min)	Tot.WHC[a]	WHC[b]	Tot.WHC[a]	WHC[b]
0	2.0	26	2.2	28
5	1.9	26	1.5	32
10	1.9	28	1.5	42
30	1.9	35	1.5	55
60	1.5	35	1.2	51
120	1.4	44	1.2	52
480	1.2	48	1.1	58
1440	1.2	63	1.1	60

[a] Tot. WHC: Total water holding capacity; expressed as total amount of water (g) bound by the residue.

[b] WHC: Water holding capacity; expressed as amount of water bound per g residue (g/g).

1M and BE1-B-RB digests than for the BE1-U50 and BE1-U60 digests, having a corresponding parental Ara/Xyl ratio.

The enzymic degradation of weAX and BE1-U with both endoI and endoIII was followed by the analysis of the molecular size distribution (Fig 5.). For both endoI and endoIII the weAX fraction seemed to be faster degraded than the BE1-U fraction. This effect is more pronounced for endoI than for endoIII. For both substrates incubation with endoIII resulted in a more rapid downward shift in molecular size distribution than incubation with endoI.

Whereas the above results with endoI were obtained from incubations with different arabinoxylan fractions from Arminda flour, similar experiments were performed with WUS and BE1 fractions from the wheat varieties Okapi, Taurus, Granta and Camp Remy[13]. The results revealed that the HPSEC elution patterns of both the solubilization of WUS as well as the degradation of the BE1 extracts were almost identical to those of Arminda flour (no further data given).

Effects on water holding capacity and baking characteristics

In order to see whether the observed changes in chemical composition and molecular size distributions of the arabinoxylans effected by the endo-xylanase treatments had also effects on functional properties of WUS and wheat flour, the influence of enzyme addition on both swelling properties and water-holding capacity of the WUS as well as on baking characteristics of a commercial wheat flour was determined.

The effect of the enzyme treatments on the swelling of the residue from WUS is shown in Fig. 6. For endoIII a more rapid increase in swelling can be observed than for endoI. A 30 min incubation with endoIII resulted into a maximum swelling followed by a decrease at longer incubations times. Incubation with endoI had a slower effect: after 4 h

TABLE V. Baking characteristics of endoxylanase treated dough and bread

	Stickiness	Volume (ml)	Break & Shread[a]	Crumb structure[a]
Blank	no	489	6.5	6.5
EndoI	no	511	6.5	6.5
EndoIII	slightly	557	7.0	7.3

[a] Rating on a scale of 0-10.

a maximum was reached. This maximum is higher for the endoIII incubation than for endoI. In Table IV the total water-holding capacity of the insoluble residue (g water) together with the calculated water-holding capacity (g water/g residue) after enzyme incubation are given. It can be seen that for both enzymes the total water-holding capacity of the residue decreased from *c.* 2.1 (average) to *c.* 1.1 g water during enzyme treatment. However, per gram of residual material the water-holding capacity was increased from *c.* 27 (average) to *c.* 62.

The effect of enzyme addition on the baking characteristics of wheat flour is given in Table V. As with the other experiments equal amounts of protein (25 μg) were added rather than equal xylanase units. It can be estimated[2,5] that the enzyme/substrate ratio in the baking trials is 2.10^{-6} to 4.10^{-6} (g enzyme/g WUS) which parallels that in the solubilization experiments (4.10^{-6}). Both with endoI as well as endoIII an increase in loaf volume is measured. However, the increase was more pronounced for endoIII (14%) than for endoI (4%). The endoIII treated loaf was also superior to both the endoI treated loaf as well as the blank with respect to break and shread and crumb structure. Although the endoIII treated dough was slightly more sticky than either the blank or the endoI treated dough no problems were met during the handling of the dough.

Discussion

EndoIII is able to hydrolyse both water-extractable and alkali-extractable arabinoxylans more rapidly into smaller polymeric fragments than endoI. It also exhibited a faster release of specific oligosaccharides from WUS in comparison to endoI. EndoIII formed smaller amounts of oligosaccharides compared to endoI which is in accordance with the differences in mode of action[12]. After the initial stage of WUS hydrolysis by endoIII no differences (Fig. 4) in increase between oligosaccharides with *O*-3, *O*-2,3 substituted xylose residues or both *O*-3 and *O*-2,3 substituted xylose residues was observed. EndoI shows a higher affinity and a lower V_{max} for the substrates than endoIII. In the enzyme experiments the substrate concentration is 1 g/l which is smaller than both K_m values. The

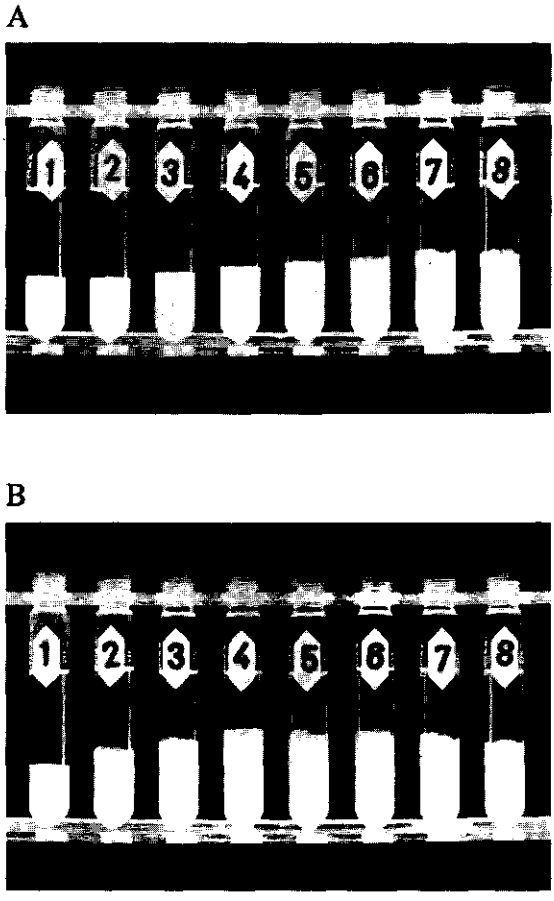


FIGURE 6. Swelling of non-solubilized material from WUS as a result of incubation with endoI (A) and endoIII (B). The numbers refer to the different times of incubation: 1 = blank; 2 = 5 min; 3 = 10 min; 4 = 30 min; 5 = 60 min; 6 = 2 h; 7 = 4 h; 8 = 24 h.

higher molar activity found for endoI can be explained by a higher affinity for the substrates, despite the lower V_{max} relative to endoIII. The faster downward shift in molecular size distribution obtained with endoIII can therefore only be ascribed to a higher degree in randomness in endoIII action relative to endoI. Such a difference in degree of randomness has been previously described for cellulases[23-25]. Given the molar activity and the apparent V_{max} and K_m values for WUS, compared to those on BE1 and weAX, the difference in randomness is also likely to be responsible for both the faster solubilization of WUS (Fig 1.) and the lower molecular size of arabinoxylans upon solubilization from WUS (Fig. 2).

No difference in pattern of degradation could be observed between alkali-extracted arabinoxylans and the arabinoxylans present in the WUS. At each time of incubation the soluble arabinoxylans obtained by endoIII incubation exhibited lower average molecular

sizes than those obtained by endoI. This may be the result of the release of smaller arabinoxylan fragments from the WUS directly, but it may also be due to a more rapid degradation of high molecular weight arabinoxylans after their solubilization by endoIII, as observed for weAX. The more rapid downward shift in molecular size distribution of weAX compared to BE1-U by the enzymes indicates that the presence of ferulic acid substituents, which amount was determined to be 0.12% (w/w) using HPLC[2] (no further data given), does not result in a slower or limited degradation of the arabinoxylans.

The comparison of the WUS and BE1 fractions of different wheat varieties showed that, despite differences in Ara/Xyl ratios[13], no differences in degradation or solubilization could be found. The observed differences in the composition of the digests and the amount of degradation for the 1M and BE1-B-RB fractions may be due to the lower relative proportions of *O*-2,3 substituted xylose residues relative to BE1-U50 and BE1-U60[7,8], which limits enzymic breakdown[12]. Since the BE2 and BE1-U50 fractions have quite similar molar proportions of the different xylose residues[7,8], the difference in enzymic degradation between these fractions can only be explained by a different distribution of the substituted xylose residues along the xylan backbones. Comparison of the degradation of BE1-B-RB and BE1-U60 fractions shows that DEAE-bound arabinoxylans are further degraded by endoI than DEAE-unbound arabinoxylans, despite equal Ara/Xyl ratios.

Since in a study on water-extractable wheat flour arabinoxylans it was found that the degree of hydration of arabinoxylans was not correlated with the extent of arabinose substitution[26], it is unlikely that the increase in water holding capacity as a result of enzyme treatment can be explained by a high water holding capacity of the remaining arabinoxylans, having a high Ara/Xyl ratio. Therefore, the results suggest that arabinoxylans hinder the swelling of the other components present in the WUS, like cellulose, (1→3,1→4)-ß-glucans and galactomannans[1,8]. Since the released material consisted only of arabinose and xylose it can be calculated from the orcinol response that 90% of all arabinose and xylose is solubilised. The observed increase in water holding capacity per gram of residual material also implicates that the use of xylanases in order to decrease the water-binding properties is less effective than might be expected from the amount of arabinoxylans solubilized. The remaining insoluble arabinoxylans had a high Ara/Xyl ratio (0.91) compared to the parental material (0.56). This is noteworthy since for gramineae a reinforced gel model has been proposed consisting of a gel matrix composed mainly of arabinoxylans which interact, via lowly substituted regions, with cellulose microfibrils surfaces[27]. The fact that the non-solubilized arabinoxylans have high Ara/Xyl ratios indicate that a high degree of substitution does not hinder binding to cellulose. This is in contrast with the hypothesis[28] that steric hindrance due to a high percentage of arabinose is responsible for the inability of oat spelts arabinoxylans to bind to (1→4)-ß-D-glucans[29]. Therefore other factors (e.g. physical entanglement[30]) must be responsible for the un-extractability of part of the arabinoxylans in water.

With respect to the effect of xylanase addition to doughs it has been postulated that the resulting improvement of loaf volume and dough characteristics is due to a redistribution of water from the hemicellulose to the gluten[11]. Also the breakdown of cell wall components themselves has been related to improved baking characteristics[31] as it has been proposed that these cell wall components are responsible for hindrance of the protein network formation[31]. The fact that the endoIII treated dough is slightly more sticky than the endoI treated dough indicates that during dough formation more WUS and water-extractable arabinoxylans had been degraded by endoIII relative to endoI, resulting in the release of water. As both enzymes have a temperature optimum of *c.* 50 °C[14], degradation will continue during the initial phase of baking before both enzymes are rapidly inactivated at temperatures exceeding 61 °C[14]. In order to explain the observed differences in baking performance between endoI and endoIII on the basis of the hypotheses above, one might speculate that the observed differences are due to the fact that endoI, because of its lower degree of randomness, is not able to degrade WUS or water-extractable arabinoxylans fast enough into fragments of lower molecular weight. However, this hypothesis should be confirmed by studies following the rate of hydrolysis of WUS and arabinoxylans in dough systems, together with the effect on dough rheology.

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CHAPTER 10

Concluding remarks

Aim of the research

When we started our research back in 1987 a lot of data had been published on NSP, also less appropriate addressed to as pentosans and/or hemicellulose. Most of the research had been focused on the water-extractable NSP, as reviewed by several authors[1-4]. The water-extractable NSP had been successfully fractionated into arabinogalactans and arabinoxylans[5]. Little attention had been given towards the elucidation of the structural characteristics and the enzymic degradation of water-unextractable NSP, although their influence on dough handling and baking performance had been widely recognized, but not understood[6]. The reason for the latter are insufficient knowledge of their structure and incorrect isolation methods leading to undefined preparations in which either large amounts of intracellular material or irreversible modified NSP or cell wall material were present. This bias in attention towards water-extractable NSP still persists in recent years. In addition to comparisons of the sugar composition and functional properties of water-extractable NSP from different varieties[7-10], recent publications directed towards water-extractable NSP now include detailed glycosidic linkage analysis, NMR studies, and identification of reaction products formed by endoxylanase[11-14]. The rare reports[15,16] on water-unextractable NSP deal mostly with their functional aspects without attention for the structural characteristics.

The aim of the research presented here was therefore (i) the development of a technique for the large-scale isolation of highly purified WUS (ii) the elucidation of the structural characteristics of the arabinoxylans present in wheat flour with emphasis on the water-unextractable arabinoxylans, and (iii) determination of the enzymic degradability of WUS and isolated arabinoxylans.

Isolation of water-unextractable NSP.

The isolation of water-unextractable NSP fractions was conducted via the isolation of water-unextractable cell wall material followed by alkaline extraction. Whereas for fruits and vegetables the residue obtained after aqueous extraction predominantly consists of cell wall material, a direct application of this method towards cereals would end up in a preparation mainly consisting of starch and proteins. For wheat different methods had been developed based on centrifuging of flour or gluten-free flour/water suspensions at either low or high speed, removal of protein and starch by specific solvents and suspending wheat flour in ethanol followed by wet-sieving. However, where the first methods resulted in WUS preparations having still relatively high starch and protein contents, the second resulted in the removal of specific cell wall polymers. As a preliminary study (data not shown) we compared the ethanol-sieving method, extended with a pre-extraction of the

flour with aqueous sodium dodecyl sulphate (SDS) with the method described in chapter 2. Despite the fact that the ethanol-sieving method was applied successfully to different wheat varieties[17] our results showed that this method resulted in preparations high in starch and protein. Using the ethanol-sieving method, Viëtor *et al*[18] also found large amounts of proteins in WUS prepared from barley flour. This is probably caused by the denaturation of protein which hinders sieving and the subsequent removal of residual adherent starch. Pretreatment with aqueous SDS avoided this problem and together with the dough kneading method the ethanol-sieving method resulted into highly purified WUS. The use of the dough-kneading method (chapter 2) was favoured since SDS-extraction might remove cell wall protein, which in our later studies seems to be involved with DEAE-binding of arabinoxylans (chapter 6). The method developed could be easily scaled up to yield 100 g quantities of WUS material devoid of intracellular starch (chapter 3). Its neutral sugar composition revealed arabinose and xylose being the predominant cell wall constituents with also glucose being prominent present (14%). The presence of substantial amounts of cell wall derived glucose was also shown for other wheat varieties (chapter 4). Large amounts of glucose present in WUS had been previously published, but either no distinction between glucose derived from residual starch or NSP was made or the amount of NSP glucose was not taken into account. Our studies show that ß-glucans are part of the WUS and might play a role in water-holding capacity of WUS (chapter 9). Therefore, the first conclusion must be that dough kneading in combination with wet sieving is a good method to obtain highly purified water-unextractable cell wall material from wheat flour in large amounts.

A sequential procedure (chapter 5) was used to extract the NSP from the WUS. The use of $Ba(OH)_2$ was included (chapter 4), because this extractant was found to extract most of the arabinoxylans as a pure fraction. This method is superior to the generally used sequential extraction with NaOH or KOH only, because the latter extractants yield a primary extract containing a mixture of ß-glucans and arabinoxylans, which are difficult to separate. The molecular size distribution showed that the arabinoxylans in the different extracts had rather similar molecular size distributions. The alkali-extractable (1→3,1→4)-ß-glucans and (gluco)mannans clearly had lower apparent molecular weights than the arabinoxylans. With light scattering analysis the weight average molecular weight of the arabinoxylans in the major alkaline extract (BE1) was determined to range from 260.000 and 640.000, depending on the Ara/Xyl ratio. Although the molecular weight of the arabinoxylans in the other fractions was not determined, the results of the molecular size measurements do not indicate large differences in molecular weight between the different wheat flour arabinoxylans.

Structural characteristics of arabinoxylans

The second conclusion is that the bulk of the water-unextractable arabinoxylans, which were shown to have glycosidic linkage compositions similar to the water-extractable arabinoxylans (chapter 6) are degraded both faster (chapter 9) and to a larger extent (chapter 8) than the water-extractable arabinoxylans. Also, the alkali-extractable

arabinoxylans had slightly higher apparent molecular weights than water-extractable arabinoxylans.

From methylation and enzymic degradation data (chapters 5+6 and 9, respectively) it can be seen that arabinoxylans which had similar Ara/Xyl ratios but differed in DEAE binding clearly differed in their structural characteristics. It would be interesting to see whether, in analogy to graded ethanol precipitation, the DEAE-bound arabinoxylans which are gradually released also show a gradual shift in the composition of the basic elements of the xylan backbone. The separation obtained with DEAE chromatography also implicates that the use of DEAE-borate chromatography of water-unextractable NSP[19] will result in mixtures of arabinoxylan populations. Based on the above mentioned results, the third conclusion is that DEAE chromatography is an important fractionation tool despite of the fact that the mechanism of DEAE-binding of wheat flour arabinoxylans is not yet understood.

The bulk of the water-unextractable arabinoxylans and the water-extractable arabinoxylans show great similarity in chemical composition. However, the results from the enzymic degradation (chapter 8) indicate a different substitution of the arabinose sidegroups present. This raises the question of the reason(s) of the solubility of the latter. Hydrogen bonding between arabinoxylans itself and other polysaccharides has been proposed. This can be concluded from the result that upon acetylation water-unextractable cell wall material is partly brought into solution[20], as acetylation of polysaccharides leads to diminishing H-bonding between hydroxyl groups from neighbouring sugar chains. It is also supposed that a higher degree of arabinosylation decreases association between arabinoxylans and ß-glucans[21]. However, the results we obtained upon sequential extraction (chapter 5) showed that the highest substituted arabinoxylans are the most difficult to extract which points at a different mechanism. Also the involvement of covalent ester linkages has been proposed with emphasis on ferulic acid derivatives. Because of the potential of the formation of diferulic bridges upon oxidation it has been speculated that, due to the presence of oxidases in wheat endosperm[22], part of the soluble arabinoxylans become insoluble upon ripening of the wheat grain[23]. This could indeed apply for only a part of the WUS since the OH^- concentration in saturated $Ba(OH)_2$ would be sufficient to break all ester linkages and a complete ester-based solubilization does not concur with the presence of $Ba(OH)_2$ unextractable material (chapters 4,5). Greater physical entanglement as a results of a higher degree of branching has been proposed[24]. Although in our studies similar Ara/Xyl were found for water-extractable and water-unextractable arabinoxylans the high Ara/Xyl ratio found in the residue after enzymic degradation might endorse this hypothesis.

The fourth important conclusion is that the major part of the alkali-extractable arabinoxylans can be described in a model in which arabinoxylans are composed of highly branched regions, mostly consisting of tetrameric repeating units of an unsubstituted and a double arabinofuranosylated xylose residue, interlinked with less branched regions which include subregions of unsubstituted xylose up to 7 contiguous residues. The fact that our model deviates from the previous reported one and being more complicated, is probably due to differences in techniques used rather than differences in wheat variety. This may

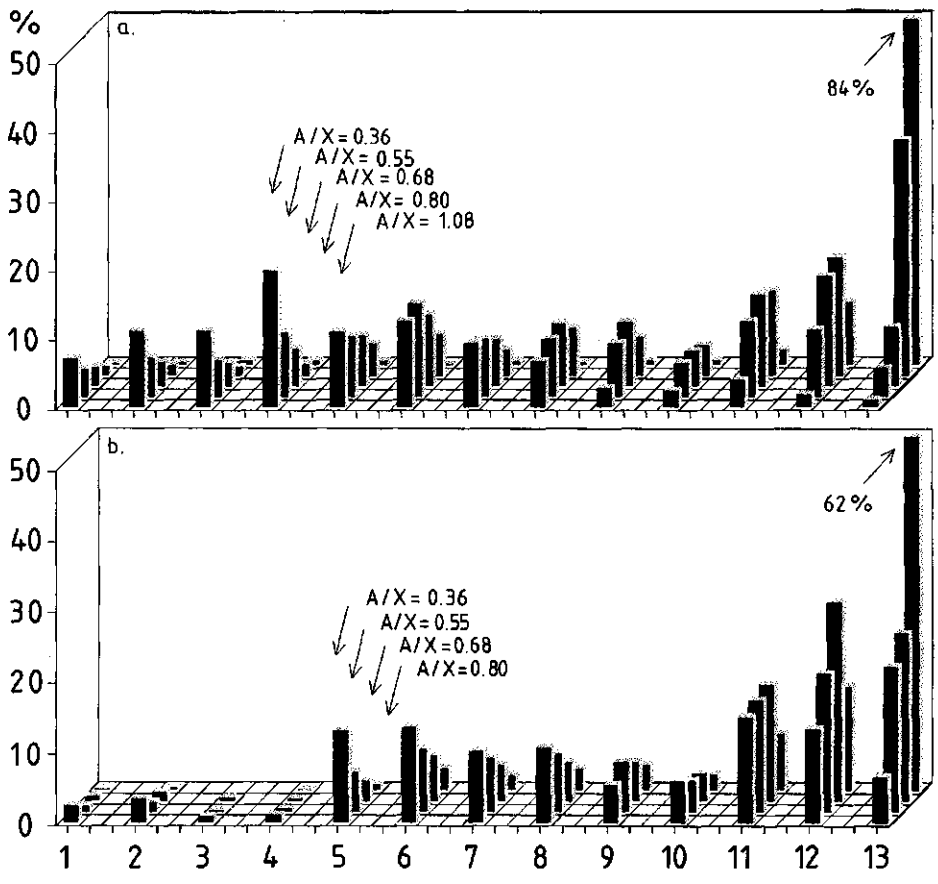

FIGURE 1 Distribution of oligomers (1-12) and polymeric material (13) on Bio-Gel P-2 from endoxylanase I (a) and endoxylanase III (b) digests of different alkali-extractable arabinoxylans (from Voragen *et al*[25]).

also be partly the case for the difference with the model proposed for barley. But the differences observed in the relative abundance of the various oligosaccharides in the enzyme digests from arabinoxylans with different Ara/Xyl ratios between wheat (chapter 8) and barley[26] allow a different model[26]. From the data obtained for rye[27] it is evident that rye arabinoxylans differ markedly from those of wheat. In order to be able to further substantiate the model proposed the enzymic degradation experiments should be extended to combinations of endoI and endoIII together with prehydrolysis with AXH or a purified arabinofuranosidase capable of removing arabinose from double substituted xylose residues which is indicated to be present in a semi-purified xylanase preparation from a *Trichoderma reesei* preparation[27]. The characterization of the various polymeric fractions should include periodate oxidation analysis[28] and molecular weight analysis, next to neutral sugar and glycosidic linkage analysis.

The fifth conclusion is that the degree of substitution greatly influences the extent of enzymic breakdown. The effect of arabinosylation upon enzymic degradation can be

interpreted from the amounts of different oligosaccharides present in the digests of the arabinoxylans with varying Ara/Xyl ratio as presented in tables V and VI in chapter 8. A more schematic depiction of this effect is given in Fig. 1, in which fractions 1 and 2-10 corresponds with xylose and oligosaccharides with DP 2-10, respectively. For incubation with endoI it can be seen that with increasing Ara/Xyl ratio of the parental material the concentration of xylose and small oligosaccharides rapidly decrease while the concentration of larger (DP>6) oligosaccharides first increase and then show a decrease. In addition, the polymeric void fraction ('13' in Fig.1) rapidly increases when arabinoxylans with increasing Ara/Xyl ratio are used as substrate. For incubation with endoIII a similar effect is observed although xylose and oligosaccharides with DP 2-4 are almost absent. In general, water-unextractable arabinoxylans with similar Ara/Xyl ratios and glycosidic linkage compositions are degraded to a lesser extent than the corresponding water-extractable arabinoxylans.

Whereas the extent of degradation is important in conversion of biomass, effects like the decrease of water-binding and the reduction of viscosity may be more important, in other applications. Therefore, the amounts and molecular size distributions of the void fractions, as given in Fig. 2 and 3 in chapter 8, together with the rate of hydrolysis (chapter 9) seem to be more important parameters. The results show that both the amount as well as the molecular weight of the void fractions are increased with increasing Ara/Xyl ratio (chapter 8). The rate of initial hydrolysis depends on the degree of randomness of the enzyme used rather than on its mode of action (chapter 9). The differences in baking performance effected by the enzymes may be related to the differences in degree of randomness yielding a faster solubilization and degradation of WUS arabinoxylans by endoIII compared to endoI, resulting in the desired distribution of water from the cell wall material to the gluten phase[29] or softening of the cell wall particles[30]. The latter are considered being responsible for a discontinuous gluten film[30] or hindrance of the protein network formation[31]. The above described hypotheses would allow the conclusion that an increased dosage of endoI would overcome this shortcoming in baking improvement. However, also other factors, like the moment of water release have been proposed to be important[32]. It is clear that further studies addressing these aspects should be conducted in order to reveal the underlying mechanisms. This thesis provides analytical tools and data to study these effects on a molecular level. The results may also be applicable for studies outside this field, like the influence of arabinoxylans on ice-crystal formation[33], an important factor in winter survival of cereals.

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SUMMARY

The research described in this thesis was focused on three targets: (i) the development of a method for the large scale isolation of highly purified water-unextractable cell wall material (WUS), (ii) the elucidation of the structural features of the arabinoxylans present in wheat flour with emphasis on the water-unextractable arabinoxylans, and (iii) the determination of the enzymic degradability of WUS and isolated arabinoxylans.

Chapter 1 describes the chemical composition of wheat flour with emphasis on the non-starch polysaccharides (NSP) present. The baking process is outlined and the literature concerning the influence of cell wall material on dough handling and baking performance is briefly discussed.

In chapter 2 a method is described for the mild isolation of WUS from wheat flour based on dough-kneading and wet-sieving. The WUS was reclaimed in two fractions which differed in size of the particles obtained. The NSP present in these fractions together made up *c.* 50% of the total NSP and *c.* 68% of all water-unextractable NSP present in the wheat flour. The fraction containing the largest particles ($\geq$ 32 μm) consisted of approximately 93% NSP, 2% residual starch, and 2% protein. The NSP was mainly composed of arabinose (30%), xylose (52%) and glucose (15%) with minor amounts of mannose (2%) and galactose (1%). The protein present in the WUS fractions contained significantly more glycine than the protein in the other fractions. Besides NSP, protein and starch, the WUS fractions contained small amounts of ferulic acid and lipids. The upscaling of the isolation procedure for the WUS reclaimed on the 32 μm sieve is described in chapter 3. With the method described, 100 g quantities of WUS could be obtained without the need of the labour-intensive centrifugation of large amounts of wheat flour suspensions. The results showed that the large-scale isolated WUS is similar in both NSP and protein composition to the small-scale isolated WUS, but had a lower residual starch content.

In chapter 4 various extractants for arabinoxylan isolation from WUS were compared in terms of yield, sugar composition, amino acid composition and molecular weight distribution of the extracts obtained. Extraction with saturated $Ba(OH)_2$ containing 260mM $NaBH_4$ yielded a pure arabinoxylan fraction (BE1) which represented *c.* 80% of all the arabinoxylan present in the WUS and a second fraction containing arabinoxylans and glucans whereas other extractants yielded mixtures of polysaccharides or extracted only limited amounts of arabinoxylans. Application of $Ba(OH)_2$ extraction to wheat flour WUS from other wheat varieties, wheat bran, oat spelt xylan and starch-containing wheat flour WUS also resulted in selective extraction of arabinoxylans. The presence of $NaBH_4$ in the saturated $Ba(OH)_2$ solution appeared to be important for the selective extraction.

The sequential extraction of WUS using $Ba(OH)_2$ as a primary extractant in combination with 1M KOH and 4M NaOH is given in chapter 5. The resultant extracts, which together contained 96% of the total WUS in the flour, were enriched in

arabinoxylans, (1→3,1→4)-ß-glucans, and glucomannans, respectively. The remaining 4% of the wheat flour WUS was mainly of cellulosic nature. Methylation analysis of the arabinoxylans showed that the xylopyranosyl residues are substituted at *O*-3 or *O*-2,3 with mainly terminal arabinofuranosyl residues. Only minor amounts of *O*-2 substituted xylose were found. The degree of substitution of xylopyranosyl residues was highest in the NaOH extract and lowest in the first $Ba(OH)_2$ extract (BE1), with that in the second $Ba(OH)_2$ extract and the KOH extract being intermediate. Alkali-extractable arabinoxylans had slightly higher apparent molecular weights than water-extractable arabinoxylans. The alkali-extractable (1→3,1→4)-ß-glucans and (gluco)mannans clearly had lower apparent molecular weights than the arabinoxylans.

In chapter 6 the fractionation of the alkaline extracts using DEAE anion-exchange chromatography is described. Neutral sugar analysis showed that, for each extract, the arabinoxylans that did not bind to the ion exchanger had lower overall arabinose/xylose (Ara/Xyl) ratios than those that did bind. When the extract contained a mixture of polysaccharides, the fractions that were bound to the DEAE column contained more arabinoxylans than the unbound fractions. With increasing buffer strength, arabinoxylans with increasing Ara/Xyl ratios were eluted. The unbound DEAE fraction (BE1-U) of the major alkaline extract (BE1) was further fractionated by graded ethanol precipitation. This resulted in arabinoxylan fractions with Ara/Xyl ratios increasing from *c.* 0.4 to 1.1. Glycosidic linkage analysis showed that as the Ara/Xyl ratio increased, so too did the ratio of total substituted to unsubstituted xylose and the ratio of double substituted to single substituted xylose. The single substituted xylose was mainly *O*-3 substituted, although in fractions with high Ara/Xyl ratios, substantial proportions of *O*-2 substituted xylose were present. No difference in linkage composition between arabinoxylans from the BE1-U fraction, representing 65% of arabinoxylans present in the WUS, and water-extractable arabinoxylans from the same wheat flour was observed. HPSEC/light scattering analysis showed that, with increasing Ara/Xyl ratio the weight average molecular weights of BE1-U fractions increased from 260,000 to 640,000.

In chapter 7 the structures of 12 arabinoxylan oligosaccharides derived from the BE1-U extract by digestion with endo-(1→4)-ß-D-xylanase I from *Aspergillus awamori* are elucidated. The digest obtained was fractionated by size exclusion chromatography on Bio-Gel P-2 followed by high-performance anion-exchange chromatography, and subjected to monosaccharide analysis and ^{1}H-NMR spectroscopy. The results revealed (1→4)-linked -ß-D-xylopyrano-oligosaccharides partly *O*-3 and/or *O*-2,3-substituted with single α-L-arabinofuranosyl groups. Next to oligosaccharides reported, also the presence of xylose, xylobiose and xylotriose in the digest was established.

In chapter 8 a model is proposed for the water-unextractable arabinoxylans present in the BE1-U fraction. This model is based on the glycosidic linkage composition of the arabinoxylan, the distribution of the oligosaccharides obtained after incubation with the purified endo-(1→4)-ß-D-xylanases I and III (endo I and III) from *Aspergillus awamori* in combination with pre-incubation with an (1→4)-ß-D-arabinoxylan arabinofuranohydrolase

from the same micro organism, and the mode of action of enzymes used. The results showed that the substituted xylose residues were not randomly distributed in the xylan chain. It is proposed that the arabinoxylan contains highly branched regions, mostly consisting of tetrameric repeating units of an unsubstituted and a double arabinofuranosylated xylose residue, interlinked with less branched regions which include subregions of unsubstituted xylose up to 7 contiguous residues. The strongly branched regions are enriched in both *O*-2,3 as well as *O*-2 substituted xylose, the latter being absent in the less branched regions. Variation in arabinose/xylose ratio between different arabinoxylan populations is due to variation in both relative amount as well as in composition of the less branched regions. In general, water-unextractable arabinoxylans with similar arabinose/xylose ratios and glycosidic linkage compositions are degraded to a lesser extent than the corresponding water-extractable arabinoxylans.

In chapter 9 the degradation of WUS, water-extracted and alkali-extracted arabinoxylans with endoI and endoIII was studied. In total 90% of the arabinoxylans present in the WUS were released by either enzyme, without solubilization of glucomannans, (1→3,1→4)-ß-glucans and cellulose. Both poly- as well as oligosaccharides were present during the incubation. Arabinoxylans with similar Ara/Xyl ratios obtained from different fractions of the WUS showed different degrees of degradation. No differences in solubilization and degradation could be observed between WUS and alkali-extracted arabinoxylans from different wheat varieties.

Incubation with endoIII resulted into a more rapid solubilization and degradation of WUS and extracted arabinoxylans, respectively, when compared to endoI. EndoIII digestion also resulted into a more rapid decrease of the total water holding capacity of the residual WUS. However, for both endoI and endoIIII incubation of WUS, the water holding capacity per gram of residual WUS showed a twofold increase as a result of enzyme incubation. Application of endoIII in baking trials yielded a 14% increase in loaf volume together with the improvement of crumb structure and 'break and shread', whereas endoI yielded a 4% increase of loaf volume with no improvement of other baking characteristics.

Summarizing, it can be concluded (chapter 10) that dough kneading in combination with wet sieving is a good method to obtain highly pure water-unextractable cell wall material from wheat flour in large amounts. The major part of the water-unextractable arabinoxylans, being extracted with saturated $Ba(OH)_2$, had glycosidic linkage compositions similar to those of water-extractable arabinoxylans, but seem to have a different substitution pattern. The alkali-extractable arabinoxylans mentioned are built up from highly branched regions, interlinked with less branched regions. The extent of enzymic breakdown depends on both the type of substitution of the arabinoxylans as well as the mode of action of the enzyme whereas the initial rate of degradation seemed to be more dependent on the degree of randomness of the enzyme used.

SAMENVATTING

Het onderzoek beschreven in dit proefschrift had als doel: (1) het ontwikkelen van een methode voor isolatie van zeer zuiver niet-water-extraheerbaar celwandmateriaal (WUS), (2) het ophelderen van de fijnstructuur van arabinoxylanen, aanwezig in tarwebloem, met nadruk op de niet-water-extraheerbare arabinoxylanen, (3) het bepalen van de enzymatische afbreekbaarheid van WUS en geïsoleerde arabinoxylanen.

Hoofdstuk 1 beschrijft de chemische samenstelling van tarwebloem met nadruk op de aanwezige niet-zetmeel polysacchariden (NSP). Het bakproces wordt beschreven en literatuur betreffende de invloed van celwandmateriaal/NSP op de deeg- en bak-eigenschappen is kort bediscussieerd.

In hoofdstuk 2 wordt een methode beschreven voor de milde isolatie van WUS uit tarwebloem. Deze methode is gebaseerd op de vorming van een deeg waaruit vervolgens zetmeel en WUS werd uitgewassen en welke dan verder gescheiden werden via zeven. De WUS werd verkregen in twee fracties die verschilden qua grootte van de celwand-deeltjes. De NSP aanwezig in deze twee fracties maakte *ca.* 50% van de totale NSP en *ca.* 68% van de niet-water-extraheerbare NSP uit. De fractie die de grootste celwand-deeltjes bevatte ($\geq$ 32 μm) bestond uit *ca.* 93% NSP, 2% rest-zetmeel and 2% eiwit. De NSP bestond voornamelijk uit xylose (52%), arabinose (30%) en glucose (15%) tezamen met kleinere hoeveelheden mannose (2%) en galactose (1%). Het in de WUS aanwezige eiwit bevatte duidelijk meer glycine dan het eiwit in de andere fracties. Naast NSP, eiwit en zetmeel bevatten de WUS fracties kleine hoeveelheden ferulazuur en vetten. De opschaling van de isolatieprocedure voor de WUS fractie met deeltjes groter dan 32 μm is beschreven in hoofdstuk 3. Met deze methode kon 100 g WUS worden verkregen zonder de noodzaak van arbeidsintensief centrifugeren van grote hoeveelheden bloem/water suspensie. De op grote schaal bereide WUS was qua NSP- en eiwitsamenstelling gelijk aan de op kleine schaal geïsoleerde WUS, maar had een lager rest-zetmeel gehalte.

In hoofdstuk 4 zijn verschillende extractiemiddelen vergeleken op basis van opbrengst, neutrale suikersamenstelling, aminozuursamenstelling en molekuulgewichts verdelingen van de verkregen extracten. Extractie met verzadigde $Ba(OH)_2$, bevattende 260mM $NaBH_4$, resulteerde in zowel een puur arabinoxylaan extract (BE1) welke *ca.* 80% van al het arabinoxylaan aanwezig in de WUS bevatte, als een tweede extract dat zowel arabinoxylanen als glucanen bevatte. De andere extractiemiddelen extraheerden of mengsels van polysacchariden of maar een klein gedeelte van de arabinoxylanen. Toepassing van $Ba(OH)_2$ extractie op WUS afkomstig uit bloem van andere tarwerassen, tarwezemelen, haver en natief zetmeel-bevattende WUS resulteerde ook in de selectieve extractie van arabinoxylanen. Het bleek dat de aanwezigheid van $NaBH_4$ belangrijk is voor de selectieve extractie van arabinoxylanen.

De achtereenvolgende extractie van WUS met $Ba(OH)_2$, 1M KOH en 4M NaOH is weergegeven in hoofdstuk 5. De verkregen extracten, welke tezamen 96% van de WUS

uitmaken, waren verrijkt in achtereenvolgens arabinoxylanen, (1→3,1→4)-ß-glucanen en glucomannanen. De niet-geëxtraheerde 4% van de WUS bevatte grotendeels cellulose. Methyleringsanalyse van de arabinoxylanen gaf te zien dat de xylopyranosyl bouwstenen hoofdzakelijk gesubstitueerd waren op *O*-3 of *O*-2,3 met voornamelijk eindstandige arabinofuranosyl eenheden. Slechts kleine hoeveelheden *O*-2 gesubstitueerde xylose werd gevonden. De mate van substitutie van de xylose bouwstenen was het hoogst in het NaOH extract en het laagst in het eerste $Ba(OH)_2$ met daartussen in de waarden van het tweede $Ba(OH)_2$ extract en het KOH extract. Loog-extraheerbare arabinoxylanen hadden hogere schijnbare molecuulgewichten dan water-extraheerbare arabinoxylanen. De loog-extraheerbare (1→3,1→4)-ß-glucanen en (gluco)mannanen hadden lagere schijnbare molecuulgewichten dan zowel de water- als loog-extraheerbare arabinoxylanen.

In hoofdstuk 6 is de fractionering van de loog extracten beschreven, welke is uitgevoerd met behulp van DEAE chromatografie. Suiker analyse liet zien dat voor elk extrac, de arabinoxylanen die niet aan de ionwisselaar bonden een lagere totale arabinose/xylose (Ara/Xyl) ratio hadden dan de arabinoxylanen die wel bonden. Wanneer in het extract meerdere polysacchariden aanwezig waren dan bevatten de fracties de gebonden werden aan de DEAE kolom meer arabinoxylanen dan de niet-gebonden fracties. Met oplopende buffersterkte werden arabinoxylanen met toenemende Ara/Xyl ratio van de kolom geëlueerd. De fractie van het eerste $Ba(OH)_2$ extract die niet aan de DEAE kolom bond (BE1-U), werd verder gefractioneerd m.b.v. stapsgewijze ethanol precipitatie. De ethanol fractionering resulteerde in arabinoxylaan fracties met Ara/Xyl ratio's oplopend van 0.4 tot 1.1. Analyse van de het suiker-bindingstype gaf te zien dat wanneer de Ara/Xyl ratio toenam ook de ratio's (enkel+dubbel) gesubstitueerd/ ongesubstitueerd en dubbel gesubstitueerd/enkel gesubstitueerd toenam. De enkel gesubstitueerde xylose was hoofdzakelijk *O*-3 gesubstitueerd, hoewel in fracties met een hoge Ara/Xyl ratio behoorlijke hoeveelheden *O*-2 gesubstitueerd xylose voorkwamen. Qua bindingstypesamenstelling was er geen verschil tussen arabinoxylanen in de BE1-U fractie, welke 65% van alle in de WUS aanwezige arabinoxylanen uitmaken, en de water-extraheerbare arabinoxylanen geëxtraheerd uit dezelfde tarwebloem.

In hoofdstuk 7 is de opheldering van de structuur van 12 arabinoxylaan oligosaccharides, afkomstig van een met endo-(1→4)-ß-D-xylanase I van *Aspergillus awamori* verkregen hydrolysaat van BE1-U, beschreven. Het verkregen hydrolysaat werd gefractioneerd met behulp van Bio-Gel P-2 gelpermeatie chromatografie, gevolgd door 'high performance' anionenwisselings chromatografie. De fracties zijn vervolgens met behulp van suiker analyse, FAB-MS en ^{1}H-NMR spectroscopie geanalyseerd. De resultaten gaven (1→4)-gebonden ß-D-xylopyrano-oligosacchariden te zien die gedeeltelijk via *O*-3 of *O*-2,3 gesubstitueerd zijn met enkelstandige α-L-arabinofuranosyl groepen. Behalve de oligosacchariden bevatte het hydrolysaat ook xylose, xylobiose en xylotriose.

In hoofdstuk 8 is een model opgesteld voor de alkali-extraheerbare arabinoxylanen aanwezig in de BE1-U fractie. Dit model is gebaseerd op de verdeling van de verschillende oligosacchariden verkregen na incubatie met de gezuiverde endo-(1→4)-ß-D-

xylanase I en III (endoI en endoIII) van *Aspergillus awamori* in combinatie met een voor-incubatie met een (1→4)-ß-D-arabinoxylaan arabinofuranohydrolase van hetzelfde microorganisme en methyleringsanalyse. De resultaten gaven te zien dat de gesubstitueerde xylose eenheden niet random verdeeld zijn in de xylaan keten. In het opgestelde model geldt dat de arabinoxylanen hoog-vertakte gedeelten bevat, welke voornamelijk bestaan uit repeterende tetramere eenheden van een ongesubstitueerd xylose en een dubbel, met arabinose, gesubstitueerd xylose molecuul, verbonden via laag vertakte gedeelten welke zelf subgedeelten bevatten met tot 7 achter elkaar geplaatste ongesubstitueerde xylose residuen. De sterk vertakte gebieden zijn verrijkt met *O*-2,3 en *O*-2 gesubstitueerde xylose. De *O*-2 vertakte xylose is afwezig in de minder vertakte gebieden. Variatie in Ara/Xyl ratio tussen verschillende arabinoxylanen is een gevolg van zowel verschillen in hoeveelheid als samenstelling van de laag vertakte gebieden. In het algemeen geldt dat niet-water-extraheerbare arabinoxylanen minder ver worden afgebroken dan water-extraheerbare arabinoxylanen met overeenkomstige Ara/Xyl ratio en suikerbindingstype samenstelling.

In hoofdstuk 9 is de enzymatische afbraak van WUS, water-extraheerbare en loog-geëxtraheerde arabinoxylanen bepaald. In totaal kon 90% van de in de WUS aanwezige arabinoxylanen vrijgemaakt worden zonder dat glucomannanen, (1→3,1→4)-ß-glucanen en cellulose werden opgelost. Tijdens de hydrolyse waren zowel poly- als oligosacchariden aanwezig. Arabinoxylanen met verschillende Ara/Xyl ratio afkomstig van verschillende WUS extracten gaven een verschillende mate van afbraak te zien. Er kon geen verschil in afbraak tussen WUS en alkali-geëxtraheerde arabinoxylanen uit verschillende tarwerassen worden waargenomen. Vergeleken met endoI, resulteerde incubatie met endoIII in een snellere solubilisatie en afbraak van WUS en geëxtraheerde arabinoxylanen. Afbraak van de WUS met endoIII resulteerde ook in een snellere afname van het totale waterhoudend vermogen van het niet-geëxtraheerde residu. Voor zowel endoI als endoIII nam het waterhoudend vermogen per gram niet-geëxtraheerd residu ruim tweemaal toe als een gevolg van enzymatische afbraak. Toepassing van endoIII in een bakproef gaf een verhoging van het broodvolume met 14% te zien tezamen met een verbetering van de bakaard en kruimstructuur, terwijl endoI een broodvolume verbetering van 4% gaf zonder een verbeteringen van de andere broodeigenschappen.

Samenvattend kan worden geconcludeerd (hoofdstuk 10) dat de in hoofdstuk 2 en 3 beschreven werkwijze een goede methode is om grote hoeveelheden niet-water-extraheerbaar celwandmateriaal in grote hoeveelheden uit tarwebloem te isoleren. Het grootste gedeelte van de niet-water extraheerbare arabinoxylanen komen qua suikerbouwstenen overeen met de water extraheerbare arabinoxylanen maar hebben een andere verdeling van de bouwstenen. De arabinoxylanen bestaan uit hoog-vertakte gebieden afgewisseld met gebieden die laag of niet vertakt zijn. De mate van afbraak van arabinoxylanen is afhankelijk van het substitutiepatroon van de arabinoxylanen en het werkingsmechanisme van de enzymen, terwijl de initiële snelheid van afbraak meer afhankelijk lijkt van de mate van 'randomness' in de werking van de gebruikte enzymen.

CURRICULUM VITAE

Harry (Harm) Gruppen werd op 17 september 1960 geboren te Hoogeveen. In 1978 behaalde hij het VWO-diploma aan het Menso Alting College te Hoogeveen. In datzelfde jaar werd begonnen met de studie Levensmiddelentechnologie aan de toenmalige Landbouwhogeschool te Wageningen. In januari 1985 slaagde hij voor het doctoraal examen met als hoofdvakken levensmiddelenchemie en proceskunde en als bijvak organische chemie. Daarna werkte hij tot 1 januari 1987 als produktontwikkelaar Food & Fine chemicals bij AVEBE b.a. te Foxhol (Gr.).

Vanaf 1 januari 1987 tot 1 juli 1990 is de auteur als onderzoeks-assistent en toegevoegd docent werkzaam geweest bij de sectie Levensmiddelenchemie en -microbiologie van de vakgroep Levensmiddelentechnologie van de Landbouwuniversiteit Wageningen. In deze periode werd het in dit proefschrift beschreven onderzoek verricht.

Sedert 1 september 1990 is hij als universitair docent aan bovengenoemde sectie verbonden.