GLUCURONOARABINOXYLANS FROM SORGHUM GRAIN

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GLUCURONOARABINOXYLANS FROM SORGHUM GRAIN

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STELLINGEN

1. De speurtocht naar de rol van (glucurono-)arabinoxylanen in brouwproeven, is vergelijkbaar met het zoeken van een speld in een hooiberg.
   (hoofdstuk 6 van dit proefschrift)

2. De verhouding tussen de hoeveelheid arabinose en xylose als maat voor de substitutiegraad van (glucurono-)arabinoxylanen geldt niet voor dit type polysacharide in het algemeen.
   (hoofdstuk 4 van dit proefschrift)

3. Het uitblijven van de ontwikkeling van een blauwe kleur na een I_2-kleuring van een brouwmonster geeft geen garantie voor de afwezigheid van zetmeel. Deze methode zou daarom niet gebruikt moeten worden voor het aantonen van een volledige versuikering tijdens het brouwen.
   (hoofdstuk 6 van dit proefschrift)

4. Sommige parameters die in de brouwindustrie alom geaccepteerd zijn (diastatisch vermogen, HWE, FAN, etc.), worden opgenomen in wetenschappelijke publicaties, maar zijn wetenschappelijk gezien vaak nauwelijks van betekenis.
   (AOAC Official Methods of Analysis, 1990)

5. Zolang er onduidelijkheid heerst in de Nederlandse naslagwerken of sorghum en gierst al dan niet onder één noemer vallen, zullen deze gewassen onbelangrijk blijven in Nederland.
   (Grote Winkler Prins Encyclopedie, Winkler Prins van het Plantenrijk, Oosthoek's Encyclopedie, Spectrum Natuur Encyclopedie)

6. De door Saulnier *et al.* voorgestelde schematische structuur van heteroxylanen uit mais-zemelen is onvoldoende onderbouwd.
   (Saulnier L., Marot, C., Chanliaud, E. and Thibault, J.-F., *Carbohydr. Polym.* **26** (1995) 279-287).

7. De naamgeving, acetyl xylaan esterase, voor enzymen die acetylxylanen ontesteren, is volgens de internationale regels omtrent naamgeving van enzymen onjuist.
   (Anonymous, 'Enzyme Nomenclature', Academic Press, Inc., San Diego, etc., 1992).

8. De wettelijke definitie van bier als "gehopte drank die wordt verkregen door alcoholische gisting van wort", zou aangescherpt moeten worden met een waarde voor minimale gisting, om vast te kunnen stellen of alcoholvrij bier nog wel onder de naam bier verkocht zou mogen worden.

(Warenwet, Bierverordening 1984 II; Iserentant, D., in 'Fermented Beverage Production' (A.G.H. Lea and J.R. Piggott, eds.) Blackie Academic & Professional, Glasgow, U.K., 1995, pp 45-65).

9. De veelvuldige vertragingen bij de Nederlandse Spoorwegen doen vermoeden dat de (hoge) prijs voor een treinkaartje vastgesteld wordt op basis van reistijd in plaats van het aantal te bereizen kilometers.

10. Het Nederlandse onderwijs kent een vreemd gebruik: hoe hoger het niveau van een opleiding, des te minder eisen worden er gesteld aan het didaktisch kunnen van de docenten.

11. Een typerend verschil tussen studenten aan een HBO-opleiding en aan een universiteit zit in de vragen die ze zich stellen. De HBO-er vraagt zich in eerste instantie af wat de oplossing is, terwijl de universitaire student zich afvraagt wat het probleem is.

12. Met de opmars van tennisspelers die getypeerd kunnen worden als "servicekanonnen", zou het televisie-publiek gebaat zijn met een verplichte onderhandse opslag bij internationale tennistoernooien.

13. Te vaak lijken principe-akkoorden uit te lopen op niets meer dan "in principe"-akkoorden.

Stellingen behorende bij het proefschrift:
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Wageningen, 2 april 1996

VOORWOORD

Nogal wat later dan gepland, of misschien wel meer gehoopt, is dit proefschrift dan toch gereed gekomen. Zonder de hulp en steun van anderen was het nooit zover gekomen. Deze personen zou ik graag allemaal willen bedanken voor welke bijdrage dan ook. Enkele wil ik hier met name noemen, natuurlijk zonder anderen te kort te willen doen.

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Voor ons pap en ons mam

ABSTRACT

Water-unextractable cell wall materials (WUS) were prepared from raw, polished, and malted sorghum (*Sorghum vulgare* cv. Fara Fara). Except for the amounts, hardly any difference could be observed between the WUS of these three raw materials. This means that cell wall materials of the endosperm cell walls are basically the same as those of the outer endosperm and pericarp layers, and that the cell walls largely persist, during malting. These preparations were further fractionated by a sequential extraction procedure using aqueous solutions of saturated $Ba(OH)_2$, 1M KOH and 4M KOH. The WUS preparations were composed of glucuronoarabinoxylans (GAX), (1→3),(1→4)-ß-D-glucans, cellulose, and some protein. GAX was primarily extracted by $Ba(OH)_2$ solutions. All GAX fractions were composed of a highly substituted (1→4)-ß-D-xylan backbone, substituted by arabinose and uronic acid. It was concluded that sorghum GAX populations were characterized by a reasonable homogeneity, since they could not be separated further by several chromatographic and precipitation techniques.

Degradation studies using purified xylanases, arabinofuranosidases and a glucuronidase alone or in combination, showed that the GAX populations were hardly broken down. Some oligomers were formed by digesting $Ba(OH)_2$ extracted GAX with a combination of endo-xylanase I and (1→4)-ß-D-arabinoxylan arabinofuranohydrolase, both purified from *Aspergillus awamori*. These oligomers were found to have a main chain of three or four xylose units, and to contain α-glucuronic acid linked to *O*-2 of the non-reducing terminal xylose unit. Two oligomers were found to have a dimeric (1→2)-linked arabinose side chain linked at *O*-3 of an internal xylose unit. Also single arabinose substitution occured at *O*-3 of an internal xylose unit. There are strong indications that these side groups can also be linked at *O*-2 of an internal xylose residue. The reducing xylose units were unsubstituted. A model for the GAX populations from sorghum was proposed combining the results of the degradation studies, the identification of the oligomers, and knowledge about the mode of action of the enzymes used.

Finally, the developed techniques to investigate GAX in particular, were used to study the behaviour of GAX in the brewing process. Worts and spent grains of mashes, supplemented with commercial enzyme preparations containing xylanases among others were studied. Except for the amount of solubilized GAX, the GAX hardly changed with respect to the sugar composition and molecular weight distribution. A direct relationship between GAX, xylanases, and filtration behaviour of worts prepared from malted sorghum, could therefore not be established.

List of Abbreviations and Symbols

Ara	Arabinose
Ara*f*	α-L-Arabinofuranose
Ara/Xyl	Arabinose to xylose ratio
AraB	α-L-Arabinofuranosidase B from *Aspergillus niger*
AUA	Anhydro-uronic acid
AXH	(1→4)-ß-D-arabinoxylan arabinofuranohydrolase from *Aspergillus awamori* CMI 142717
BE1.1	First $Ba(OH)_2$ extract
BE1.2	Second $Ba(OH)_2$ extract
BE2.1	First washing with water of residue of $Ba(OH)_2$ extraction
BE2.2	Second washing with water
c.	Approximately
DEAE	Diethylaminoethyl
DP	Degree of polymerization
%ds	Percentage of dry matter
FAB-MS	Fast atom bombardment-mass spectrometry
Gal	Galactose
GalA	Galacturonic acid
GAX	Glucuronoarabinoxylan
GC-MS	Gas chromatography-mass spectrometry
Glc	Glucose
GlcA	Glucuronic acid
Glc*p*A	α-D-Glucuronopyranose
GXH	Glucuronoxylan xylanohydrolase from *Bacillus subtilis*
^{1}H NMR	Proton nuclear magnetic resonance
HOAc	Acetic acid
HPAEC	High-performance anion-exchange chromatography
HPSEC	High-performance size-exclusion chromatography
1K4	First 1M KOH extract, at 4°C
1K20	Second 1M KOH extract, at room temperature
4K	4M KOH extract
4KB	4M KOH + borate extract
LC-MS	Liquid chromatography-mass spectroscopy
Man	Mannose
MeOH	Methanol
M_w	Molecular weight
NaOAc	Sodium acetate
NSP	Non-starch polysaccharides
4OMeGlcA	4-*O*-methyl-D-glucuronic acid
PAD	Pulsed amperometric detection

Res	Unextractable residue after sequential extraction with $Ba(OH)_2$, and KOH solutions
RI	Refractive index
Rha	Rhamnose
RT	Retention time
TFA	Trifluoroacetic acid
WUS	Water-unextractable cell wall material
Xyl	Xylose
Xyl*p*	ß-D-Xylopyranose
XylI	Endo-(1→4)-ß-D-xylanase I from *Aspergillus awamori* CMI 142717
XylIII	Endo-(1→4)-ß-D-xylanase III from *Aspergillus awamori* CMI 142717
●	Xyl*p*
◇	α-Ara*f*
▲	α-GlcA*p*
●–●	ß-Xyl*p*-(1→4)-ß-Xyl*p*
	α-Ara*f*-(1→2)-ß-Xyl*p*
	α-Ara*f*-(1→3)-ß-Xyl*p*
	α-Ara*f*-(1→2)-α-Ara*f*
◇–◇	α-Ara*f*-(1→5)-α-Ara*f*
	α-GlcA*p*-(1→2)-ß-Xyl*p*

CONTENTS

CHAPTER 1

Introduction

BACKGROUND

Sorghum (*Sorghum bicolor* (L.) Moench or *Sorghum vulgare*) is an indigenous African cereal very well adapted to the semi-arid and sub-tropical conditions prevailing over much of the African continent. It can yield a crop under conditions of environmental stress, such as drought, where temperate cereals like barley and wheat fail. As a world food grain sorghum is ranked fifth after wheat, maize, rice and barley. Sorghum is the main food grain in parts of India and Africa, where it is mainly used in different types of breads, porridges and opaque beers[1-4]. The USA are, however, the largest producers of sorghum where it finds its application in animal feed[1].

In southern African countries, malted sorghum has been used since time immemorial to brew traditional (opaque) sorghum beer. During the twentieth century, sorghum beer brewing has developed into a major industry. Among other characteristics these types of beer differ from the European (lager) types in that, during the fermentation, lactic acid is formed as well as ethanol. The beers, which are consumed while still fermenting, contain large amounts of insoluble material[5]. Insoluble starch fragments and dextrins are the major contributors to the opaqueness of sorghum beer, and are not digested during fermentation[6].

In Nigeria, the lager type beer is the most common one produced industrially. Actually the only local raw material which is used for this, is water, whereas all others have to be imported. Recently, as a result of economic problems, Nigerian brewers of conventional lager or stout beers have been forced to develop technologies to enable them to brew with locally grown cereals such as maize or sorghum[7]. Preliminary trials, however, resulted in many problems in beer production, which will be discussed later in this chapter.

COMPOSITION OF SORGHUM

Kernel

Sorghum grains are round caryopses of variable sizes and colours, depending on variety and growth conditions[5]. Unlike *e.g.* barley, harvested sorghum does not contain a husk. The pericarp and testa (Fig 1) of sorghum have important properties in food processing because of their varietal differences in contents of polyphenols and thickness. Grains with thin pericarps are believed to be more susceptible to microbial infection[8]. Grain colour ranges from red, purple-black and brown to beige, yellow, white or cream. Some varieties do not retain their testa during development[5]. However, when present, the testa can contain high levels of phenolic materials which may not be as visible as in other grains where the corresponding pigments are clearly seen as anthocyanidin materials in the pericarp. These pigments are unpalatable to some birds, insects and other organisms. Therefore, this factor has a benificial effect in respect to crop yields[9].

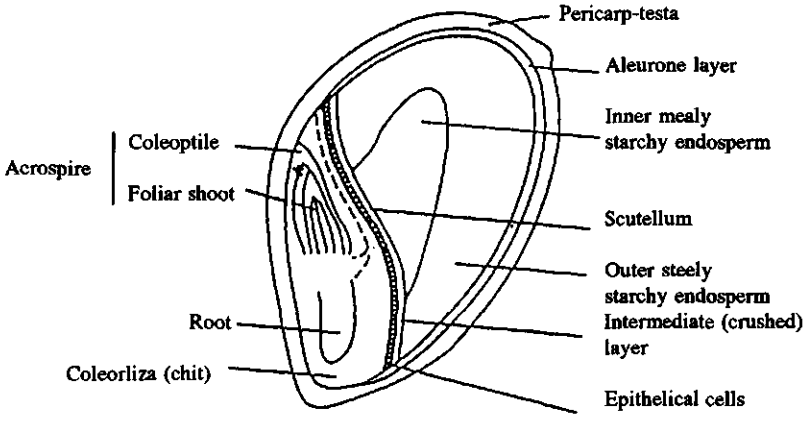


FIG 1. Longitudinal section of sorghum grain[10].

Like in all cereal grains, the embryo, the aleurone layer and the starchy endosperm constitute the internal structure of the kernel (Fig 1). The aleurone is composed of a single layer of thick walled cells which contain lipids and protein deposits. The starchy endosperm is the largest tissue of the grain and consists mainly of starch granules, storage proteins and the cell walls which surround the cells of the endosperm. The endosperm of sorghum can either be mainly floury (mealy), mainly corneous (steely), or intermediate (outer endosperm corneous-steely, inner endosperm floury-mealy) depending on the relative proportion of protein and starch. In the corneous endosperm, the protein forms a continuous interface between the starch granules with protein bodies embedded in the matrix, whereas the floury endosperm has a discontinuous protein network[5].

Starch

The major component of the endosperm is starch that is present in granules varying in size from 4 to 25μm (15μm average)[11]. The starch content of sorghum ranges from 61 to 74%[12,13]. The amylose content of sorghum starches is quite ordinary as it ranges between 21 and 30%[13], although varieties are known with less then 1% amylose (waxy)[8]. The gelatinization temperature of sorghum starch is quite high compared to those from other cereals. Most sorghum starches start to gelatinize at *c.* 70-75°C and reach maximum gelatinization far above these temperatures until about 90-97°C[14]. Gelatinization of starches can be influenced by time, temperature, moisture, ingredients and processing conditions[15]. It is very difficult to obtain sorghum starches devoid of protein. Wankhede *et al.*[13] have suggested that this phenomenon might be attributed to the presence of highly hydrated pentosans and insoluble protein in sorghum grains. Variations in the content of residual protein might be explained

by different isolation and purification methods.

Starches from floury and corneous endosperm fractions show different properties and characteristics. It was shown that corneous endosperm starches tended to exhibit smaller granule size, lower amylose content, slightly higher gelatinization temperature and higher intrinsic viscosity compared with the floury endosperm starches[16]. The smaller granule corneous endosperm starch seems to have a more crystalline structure and contain higher molecular weight components than the larger floury granules[16]. As a result, sorghum starches do not dissolve completely in either cold or boiling water[11,17,18]. The solubility can be increased by physically damaging the granules such as micronizing[17], pressure cooking or extrusion[19].

Proteins

The protein content of sorghum grains can vary from 6.8 to 19.6%[20-22], with an average value of *c.* 10.7%[20]. The crude protein content is generally calculated by multiplying the total nitrogen content, obtained by the micro-Kjeldahl method[23], by 6.25. This value however, is too high for sorghum and most other cereals. More correct would have been the conversion factor 5.65 determined by Mossé[24]. The protein distribution between the different morphological parts of the sorghum grains is uneven as is the case with other cereals. The germ is richest in protein content (*c.* 16%)[22,25]. The protein content of the endosperm is lower. Within the total endosperm, the outer layers are richer in proteins compared to the inner parts. The pericarp has the lowest protein content. *C.* 80% of the total protein of sorghum grain is located in the endosperm[22].

Based on their solubility, cereal proteins are fractionated into four main groups: albumins (water-extractable), globulins (extractable in salt solutions), prolamins (alcohol-extractable) and glutelins (insoluble in neutral aqueous solutions, saline solutions or alcohol, extractable with *e.g.* detergents)[26,27]. Albumins and globulins are considered to be the cytoplasmic, metabolic active, proteins[26] associated with the germ[25,28]. The prolamin fraction in sorghum is named kafirin, which is the storage protein deposited in the form of protein bodies (size 0.4-3μm across)[28,29] during kernel development[30,31]. For obtaining this important fraction (50-60% of the total sorgum protein) both the Landry-Moureaux[32] and the Osborne[33] fractionation schemes have been optimized by using 60%v/v *t*-butanol and 60%v/v *t*-butanol containing a reducing agent (for cross-linked kafirins) as extractants[28,31,34]. Similarly to the corresponding, so called, maize zeins, further classification into α-, ß- and γ-kafirins is based on extractability and molecular size differences[31,35]. The molecular weights were found to vary between 16 and 28kDa. The glutelins are the main components of the endosperm matrix proteins[28,29]. It is suggested that the glutelin matrix proteins could include certain enzymes involved in the hydrolysis of starch and protein reserves, rather than being solely a storage protein like the prolamins[28].

Sorghum usually has a high content of glutamic acid, leucine, alanine, proline and aspartic

acid. However, the nutritive value of the sorghum proteins is quite low, since sorghum is deficient in most essential amino acids, especially lysine[36]. However, these essential amino acids are present in relatively high amounts in the small albumin and globulin fractions[25]. Typically, kafirin is rich in glutamic acid and proline, but contains virtually no lysine[21,28,37].

Lipids

Lipids are one of the minor components of sorghum. Most papers mention that they only constitute *c.*3.6% (range 1.4-5.8%) of the sorghum grain kernel[5,20,38-41] and are mainy located in the germ, and aleurone layer[5,40]. A polishing procedure by which these tissues are being ground off, will therefore remove a substantial part of the fatty components[41]. However, alkali dehulling which removed only the pericarp, did not decrease the lipid content[42]. Palmitic (C16:0), oleic (C18:1) and linoleic (C18:2) acid are the most abundant fatty acids present in sorghum[5,38,40,43].

Non-starch polysaccharides

Another part of sorghum is formed by the so called non-starch polysaccharides (NSP) mainly located in the pericarp and endosperm cell walls. In cereals this group comprises cellulose and the hemicelluloses (1→3),(1→4)-ß-D-glucan and arabinoxylans[44]. There have been no reports about the presence of other hemicelluloses, like xyloglucans, arabinogalactans, or (hetero-)mannans. Only small quantities of lignin have been determined[45]. Information about the endosperm NSP of cereals like barley[46,47], wheat[48-50], rye[51-53], oats[54,55], and rice[56-60] are well documented. In this respect only little attention has been addressed toward sorghum[61].

The NSP are generally devided in water-soluble and water-insoluble components. This division is actually not correct. Components as such can be soluble but not directly be extracted with water from a sample, mostly a cell wall preparation. Therefore the terms WES (water-extractable) and WUS (water-unextractable) are favoured nowadays. The water-unextractable NSP are generally solubilized by an alkali treatment[44]. Once they are solubilized in alkali, they remain, although sometimes partly, soluble in water. This is the reason why in some papers "soluble" should be understood as "extractable". An attempt was made to summarize the contents of NSP reported for sorghum, sorghum endosperm and the crude composition of these NSP (Table I). The general terminology is very confusing and not well defined, since depending on the method used, the NSP are usually measured as crude or dietary fiber (including lignin) or as pentosan (without cellulose, or sometimes defined as arabinoxylans). Remarkably, there are no data reported about the cellulose content of sorghum grains.

In cereal research, the term pentosan is often used for the fraction determined as the total NSP minus the cellulose content, or Glc content[61]. The possible presence of other hemicelluloses, such as arabinogalactans or xyloglucans, are not taken into account. The

TABLE I. NSP content (%ds) and composition (%ds) of sorghum grain.

	Mean	Range	Reference
Total NSP	2.7[a]	0.4–7.3[a]	5
	6.7[a]	2.5–9.0[b]	45
	2.4[c]		41
	2.1[d]		62
	3.5[d]	2.5–5.6	63
Total endosperm NSP	0.9[a]		64
	0.7[c]		41
% NSP of sorghum grain:			
Water-extractable	0.9[d]		65
	0.5[b]		45
	0.1[d]		62
Water-unextractable[e]	0.4[d]		65
Crude composition of total NSP:			
Cellulose	–[f]	–[f]	
(1→3),(1→4)-ß-D-glucan	<0.3		66
		0.3–2.0	67
Arabinoxylan		3.5–4.9[d]	67

[a] Determined as NSP; [b] Determined as dietary fiber; [c] Determined as crude fiber; [d] Determined as pentosan; [e] Defined as being water-unextractable, however alkali-extractable; [f] No data reported.

term pentosan would literally suggest the total amount of pentose sugars, generally Ara and Xyl. This definition would therefore be identical to the one generally used for arabinoxylans. When the presence of uronic acid, generally (4OMe-)GlcA in the case of arabinoxylans, is confirmed as a structural element of the polysaccharides, these polymers should be named glucuronoarabinoxylans (GAX).

The term ß-glucan is often used in the brewing literature for (1→3),(1→4)-ß-D-glucan. ß-glucans include scientifically more polysaccharides, such as cellulose and callose. Although probably in very low amounts, especially cellulose may be present in cell walls of barley, its malt, and in other cereal grains used in brewing. It would therefore be recommendable to specify which ß-glucan is meant, and to use *e.g.* non-starch glucan when its origin is not exactly known.

In this chapter the terminology will be the same as the several authors have used in their articles. In the following chapters of this thesis, the polysaccharides will be named after their sugar composition in the case of arabinoxylans and glucuronoarabinoxylans. The glucans will

be termed as starch, cellulose and (1→3),(1→4)-ß-D-glucan, when determined as such.

According to Karim and Rooney[65] the major pentosan fraction of sorghum is water-extractable. The whole grain contained 0.9% water-extractable and 0.4% alkali-extractable pentosans. They, however, did not determine the total amount of pentosans, nor check for residual pentosans possibly present in the residue. Most of the sorghum pentosans are located in the pericarp[41].

Some of the polysaccharides have been further studied by Woolard and coworkers[67,68,69,70]. They isolated, purified and characterized several highly substituted (glucurono-)arabinoxylans (GAX) from sorghum pericarp, differing in degree of polymerization (DP), Ara/Xyl ratio, substitution by other components such as GlcA and its 4-*O*-methylated derivative 4OMeGlcA, Glc and Gal. All GAXs had a (1→4)-ß-D-linked xylan backbone in common with some Xyl units substituted with Ara, mainly as single unit non-reducing end-group, at either *O*-3 or both at *O*-2 and *O*-3. This basic structure for arabinoxylans is in general terms reported for all cereals. A profound presence of uronic acids has been noticed for GAX from the endosperms and outer layers from maize and rice kernels, however not for the endosperms from wheat, barley, rye and oats. The uronic acids are linked through *O*-2 of Xyl units, also as single unit non-reducing end-groups. In sorghum pericarp also Gal, when present, was located as non-reducing end-group[69-71].

From the water-extractable NSP from sorghum endosperm, the same authors[72,73] have identified several mixed-linked glucans. These differed somewhat in DP, but their linkage compositions were more distinct. A linear (1→3),(1→4)-ß-D-glucan was identified with a (1→3) to (1→4) ratio of 3/2, much higher than the 3/7 generally found for barley endosperm. Other slightly branched glucans, composed of both α- and ß-linked (1→4)- and (1→6)-Glc units in different ratios were identified. Although they could not be degraded by amylases nor stained with I_2, these structures resemble starch more than mixed-linked ß-glucans.

Both the (1→3),(1→4)-ß-D-glucans and the arabinoxylans are known to have strong water-holding properties, which can result in viscous solutions. Depending on the application this characteristic might be advantageous as in baking[74] or disadvantageous as in brewing[75]. Arabinoxylans can contain acetyl groups[44]. Moreover, they might be substituted with phenolic acids, such as *p*-coumaric acid and ferulic acid. Especially the latter one has the ability to couple oxidatively to another ferulic acid of an arabinoxylan or an aromatic amino acid moiety in a protein, by which gelation might occur[44].

Polyphenols

The testa and pericarp of sorghum, and especially the birdproof varieties, contain high concentrations of polyphenols[76]. The so called condensed tannins, which are flavonoid oligomers, also known as proanthocyanidins[77], form the most important group present, with respect to the amount as well as their functional properties, such as chemical defense agents against moulds, insects, and birds. Within birdproof varieties, the contents can vary between

0.36 and 3.48 catechin equivalents (CE; 1CE = 1mg catechine/100mg sample)[78]. In most white coloured varieties and obviously the varieties which do not have a testa, the tannin level has diminished to 0CE[76,78]. The condensed tannins show the ability to strongly interact with proteins[77]. This has implications for applications in human or animal nutrition, because digestive[79,80] or endogenous sorghum[81] enzymes can be inhibited. The polyphenols can be succesfully removed by polishing[82] and alkali extraction[83,84].

BREWING LAGER BEER FROM SORGHUM

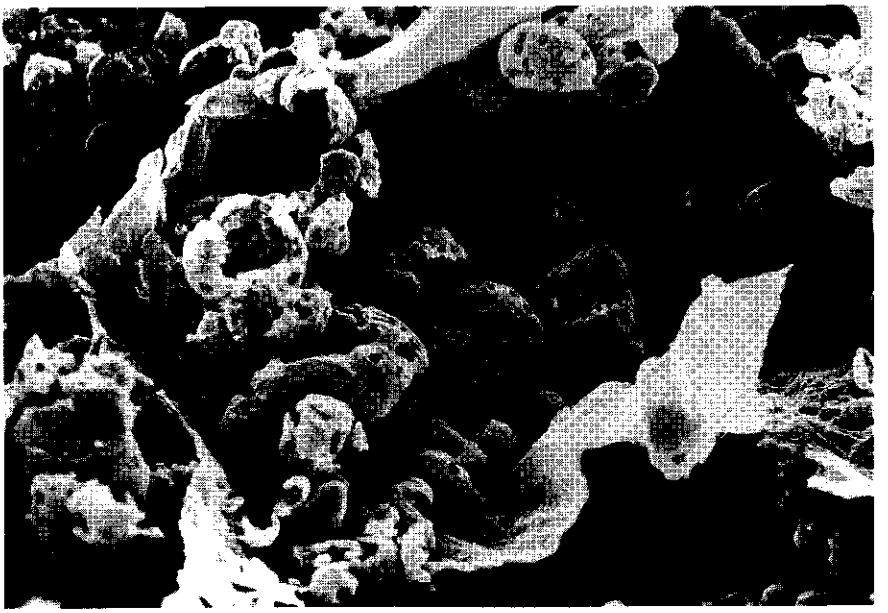
Brewing a lager type beer with sorghum can generally be done in two ways[9]. One way is to replace part of barley malt by cooked unmalted sorghum as an adjunct. The sorghum starch will be saccharified by the amylases developed in the barley malt or by exogenous supplemented enzymes like heat stable amylases, (1→3),(1→4)-ß-D-glucanases and proteases. In the other procedure only malted sorghum is used as the basis for brewing, with the same functions as barley malt.

Sorghum malt

The general purpose of malting is the synthesis and activation of enzymes, which is accompanied by a certain degradation of cell wall materials. The malting of sorghum has been an objective of many investigations. Sorghum malt shows some differences with barley malt, which are not beneficial for its use in lager beer brewing. Sorghum malt is characterized by high malting losses[85,86] and by having a low diastatic power[85-89]. Much controversy, however, surrounds the development of ß-amylase. It seems that some varieties show limited levels[14,90], whereas others contain reasonable amounts of this important maltose releasing enzyme[14,91,92]. The development of α-amylase[14,90,93-96], endo-ß-D-glucanase[14,90,96-99], endo-ß-(1→3)-glucanase[97,99], cellulase[100], pentosanase[99], limit dextrinase[96,98], carboxypeptidase[98,101,102] and endo-protease[96,98,101-104] has been found in germinated sorghum grains.

During germination, both the levels of starch and protein[105,106], more particularly the kafirins[30,107], are reported to decrease to a certain level.

During the malting process of barley, the cell walls are almost completely degraded. However, the malting of sorghum kernels is characterized by a persistance of the cell walls. As shown by electronmicroscopy[96,99,105,108,109] (Fig 2) and (bio-)chemical analysis[67,99,110] these cell walls seem to be resistant to degradation by endogenous enzymes. The reasons for this phenomenon still remains unclear. EtokAkpan[67] suggested that the cellulosic character of the cell walls may be of importance. Another factor might be the different pattern of enzyme synthesis and activation. In barley the major enzymes are produced under the influence of gibberellic acid in both the scutellum and aleurone layer[111,112], whereas the sorghum enzymes originate from the scutellum[105] and show no sensitiviy for gibberellic acid[100].



20μm

FIG 2. Cell walls of the endosperm of malted sorghum[99].

The presence of polyphenols, and in particular tannins, seems to have no effect on the malting of sorghum[113]. The production of enzymes is unaffected, but their activity can be decreased when enzymes and tannins can get in contact with each other as a result of *e.g.* milling of malt[81].

Mashing using sorghum malt

One of the characteristics of worts obtained from mashing trials with sorghum malt is the incomplete saccharification of the starch. A major cause for this phenomenon is the poor development of amylases in sorghum malt[14]. The insufficient ß-amylase activity is demonstrated by rather low concentrations of maltose in the worts. The relatively high levels of Glc originate from α-amylolytic and α-glucosidase activities[114]. Since the gelatinization temperatures of sorghum starches are higher than for barley starches, mashing with sorghum should be performed at elevated temperatures[9,115]. These temperatures are above the optimum, and sometimes inactivation temperatures of most amylases[14]. Special mashing schemes had to be developed to overcome this problem: for instance, boiling part of the mash and recombine that with the other unboiled enzymically active part[9,116]. However, irrespective of the type of mashing scheme, sorghum worts always show incomplete saccharification[114,115] and

thus low extract yields[9]. An acceptable lager beer can be produced from sorghum malt using sucrose (30%) as adjunct[117]. When sorghum is used in mashing programmes with barley malt, saccharification times have to be extended[118]. The supplementation of the mash with thermostable pullulanase can be a solution to further complete the saccharification of starch[119]. Bajomo and Young[120] used an amyloglucosidase in mashing trials with 100% raw sorghum supplemented with a heat stable α-amylase and proteolytic enzymes, and succeeded in converting the hot water extract (HWE) into fermentable sugars.

Mashing trials with sorghum result often in worts containing relatively low quantities of free α-amino nitrogen (FAN, amino acids and short peptides), compared to barley worts[114]. The actual FAN levels in the worts are dependent on the sorghum variety as they are directly propositional to the FAN levels in the malts[121]. The FAN quality is also important. Based on their rate of uptake by yeast during fermentation, the FANs are classified into 4 groups: A to D. The amino acids from group A are almost immediately absorbed from the wort whereas those from group D are only slightly absorbed under the anaerobic conditions during fermentation[122]. The quality of sorghum malt FAN is good since it does not contain high percentages of proline (group D)[121]. The quality of FAN from sorghum malt seems to be even better than from barley malt, since more group A amino acids are present[120]. The amount of 100mg FAN/l of wort is recommended for optimal yeast growth during the fermentation[123]. Limited yeast growth will result in incomplete fermentation of sugars and hence a beer with an alcohol level below the desired value. Acceptable FAN levels can be obtained by supplementing the mash with proteinases[120,124,125].

A very important practical obstacle in brewing with sorghum, and especially with sorghum malt, is the poor filterability of the mash and/or the final beer[115]. Poor filtration properties are characterized by a low filtration rate and/or a low filtration volume. One reason for this is that sorghum is a huskless grain. In brewing with barley malt, the husks form a natural filterbed in wort filtration. However, the absence of husks is not the only explanation. The poor wort filtration is often, but not necessarily, associated with higher wort viscosities[115]. However, also worts of low viscosity show impaired filtration rates and volumes[14]. The wort viscosity is determined by several high molecular weight compounds, which could be *e.g.* starch hydrolysis products[115,125], (1→3),(1→4)-ß-D-glucans[115,125], other hemicelluloses[125] and proteins[125]. Until now, these suggestions were substantiated by mashing experiments in which filtration performance improved after supplementing the mash with α-amylases, proteinases, (1→3),(1→4)-ß-D-glucanases, (1→4)-ß-glucanases and hemicellulases[115,120,124,125]. These enzyme preparations are named after their main activities. It can not be excluded that side activities present, or combinations of enzyme activities in the preparation, may be responsible for the observed effects. It remains unclear which compounds are the most responsible. Only the role of (1→3),(1→4)-ß-D-glucans has been investigated somewhat further.

Due to the insufficient development of (1→3),(1→4)-ß-D-glucanase in sorghum malt, (1→3),(1→4)-ß-D-glucan is released in the worts but is not further degraded[14,97]. However,

the quantities of these glucans are too low to cause a high wort viscosity[14]. It is hypothesized that, although the degree of polymerization of these partly degraded glucans is quite low (DP ~ 26), they are still large enough to get trapped in filterpores and cause difficulties with filtration[97]. Also Dufour *et al.*[14] concluded that, although low in quantity, these and other partly hydrolysed constituents (proteins, pentosans) could clog the filter bed. On the other hand, the influences of the addition of (1→3),(1→4)-ß-D-glucanases to the mash on filtration performance are contradicting, varying from improvement[125] to no influence[115] and even worsening[124].

AIM AND APPROACH OF THIS RESEARCH

It may be clear that explanations cited above for the poor malting and brewing properties of sorghum and sorghum malt give rise to much controversies. As shown in Fig 2, the cell walls are hardly broken down during malting. This can mean that the right enzymes are not produced nor activated during malting, or that the structure of the cell wall is too complex for a prosperous degradation. In brewing, high wort viscosities could retard filtration. However, Dufour *et al.*[14] stated that nor the solubilized high molecular starch nor the solubilized ß-glucans cause difficulties with respect to filtration rates. Also sorghum (malt) worts with the low viscosities similar to mashes obtained with barley malt show impaired filtration rates. The possible role of other cell wall components is not (yet) very well studied. These other cell wall components are cellulose, structural proteins and hemicelluloses.

Due to the low water holding capacity of native cellulose no effect on the filtration is expected. Enzymically obtained soluble cellulose fragments, are considered to be too small to clog the filterbed. A probable role of proteins is established, but not understood. In some cases it is noticed that filtration problems decrease when a protease is added to the mash. On the other hand also the reverse effect has been seen.

Since the Nigerian government restricted on the importation of barley and barley malt, a lot of research has started in the field of sorghum kernels and malt focussed on proteins, starch and the (1→3),(1→4)-ß-D-glucans. The aim of this work was the characterization of sorghum hemicelluloses, firstly because they may be important in explaining why sorghum cell walls are not broken down during malting, and secondly as they may have a retarding effect on filtration. Since the arabinoxylans were present in higher quantities than the (1→3),(1→4)-ß-D-glucans it was chosen to concentrate the work on the previous component. Therefore a method was developed to collect the cell wall polysaccharides as free from any contaminating proteins and lipids as practically possible (Chapter 2). The water-unextractable hemicelluloses were of major interest since they were considered to be the most important with respect to the poor filtration. A further isolation of the arabinoxylans from the water-unextractable cell wall preparation (WUS) was performed by a sequential alkali extraction

(Chapter 3). Some chemical characteristics and properties were established and a first tentative structure could be developed. Enzymic degradation (Chapter 5) of these isolated polysaccharide populations by specific purified enzymes and the determination of the primary structures of generated oligomers (Chapter 4) further substantiated the tentative structure of the major sorghum hemicellulose, GAX. Finally, mashing experiments were performed, using commercial enzyme preparations selected on their GAX degrading capacity. The influence on filtration is studied in Chapter 6.

REFERENCES

1. Rooney, L.W., Kirleis, A.W. and Murty, D.S., in 'Advances in Cereal Science and Technology', Vol 8 (Y. Pomeranz, ed.), Am. Assoc. Cereal Chem. Inc., St. Paul, USA (1986) pp 317-353.
2. Bello, A.B., Rooney, L.W. and Waniska, R.D.,'Factors affecting quality of sorghum tô, a thick porridge', *Cereal Chem.* **67** (1990) 20-25.
3. Mohamed, A.A., Hamaker, B.R. and Aboubacar, A.,'Effects of flour-to-water ratio and time of testing on sorghum porridge firmness as determined by an uniaxial compression test', *Cereal Chem.* **70** (1993) 739-743.
4. Serna-Saldivar, S.O., Knabe, D.A., Rooney, L.W., Tanksley Jr., T.D. and Sproule, A.M.,'Nutritional value of sorghum and maize tortillas', *J. Cereal Sci.* **7** (1988) 83-94.
5. Rooney, L.W. and Serna-Saldivar, S.O., in 'Handbook of Cereal Science and Technology' (K.L. Lorenz and K. Kulp, eds.), Marcel Dekker Inc., New York, USA (1991) pp 233-270.
6. Glennie, C.W. and Wight, A.W.,'Dextrins in sorghum beer', *J. Inst. Brew.* **92** (1986) 384-386.
7. Adejemilua, F.,'Trends in der Brauindustrie in Nigeria', *Brauwelt* **25** (1988) 1094-1097.
8. Doggett, H.,'Sorghum', Longman Scientific and Technical, Essex, U.K. (1988).
9. Palmer, G.H., Etokakpan, O.U. and Igyor, M.A.,'Sorghum as a brewing material', *MIRCEN Journal* **5** (1989) 265-275.
10. Palmer, G.H.,'Sorghum - food, beverage and brewing potentials', *Process Biochem.* **27** (1992) 145-153.
11. Stark, J.R., Aisien, A.O. and Palmer, G.H.,'Studies on starches from Nigerian sorghum', *Starch/Stärke* **35** (1983) 73-76.
12. Holdt, M.M. von and Brand, J.C.,'Kaffircorn malting and brewing studies. VI. Starch content of kaffir beer brewing materials', *J. Sci. Food Agric.* **11** (1960) 463-466.
13. Wankhede, D.B., Deshpande, H.W., Gunjal, B.B., Bhosale, M.B., Patil, H.B., Gahilod, A.T., Sawate, A.R. and Walde, S.G.,'Studies on the physicochemical, pasting characteristics and amylolytic susceptibility of starch from sorghum (*Sorghum bicolor* L. Moench) grains', *Starch/Stärke* **41** (1989) 123-127.
14. Dufour, J.P., Mélotte, L. and Srebrnik, S.,'Sorghum malts for the production of a lager beer', *ASBC Journal* **50** (1992) 110-119.
15. Lund, D.C.,'Influence of time, temperature, moisture, ingredients, and processing conditions on starch gelatinization', *CRC Critical Reviews in Food Science and Nutrition* **20** (1984) 249-273.
16. Cagampang, G.B. and Kirleis, A.W.,'Properties of starches isolated from sorghum floury and corneous endosperm', *Starch/Stärke* **37** (1985) 253-257.
17. Craig, S.S.A. and Stark, J.R.,'A comparison of the molecular properties of sorghum starches of different origins', *Starch/Stärke* **36** (1984) 127-131.
18. Jackson, D.S., Waniska, R.D. and Rooney, L.W.,'Differential water solubility of corn and sorghum starches as characterized by high-performance size-exclusion chromatography', *Cereal Chem.* **66** (1989) 228-232.
19. Glennie, C.W.,'Physico-chemical properties of sorghum starch thermally treated by different methods',

Starch/Stärke **39** (1987) 273-276.

20. Aucamp, M.C., Grieff, J.T., Novellie, L., Papendick, B., H.M. Schwartz and Steer, A.G.,'Kaffircorn malting and brewing studies. VIII. Nutritive value of some kaffircorn products', *J. Sci. Food Agric.* **12** (1961) 449-456.
21. Subramanian, V., Seetharama, N., Jambunathan, R and Rao, P.V.,'Evaluation of protein quality of sorghum (*Sorghum bicolor* (L.) Moench)', *J. Agric. Food Chem.* **38** (1990) 1344-1347.
22. Haikerwal, M. and Mathieson, A.R.,'The protein content and amino acid composition of sorghum grain', *Cereal Chem.* **48** (1971) 690-699.
23. Roozen, J.P. and Boxtel, L. van,'Half-automatische bepaling van stikstof in levensmiddelen', *De Ware(n) Chemicus* **9** (1979) 192-200.
24. Mossé, J.,'Nitrogen to protein conversion factor for ten cereals and six legumes or oilseeds. A reappraisal of its definition and determination. Variation according to species and to seed protein content', *J. Agric. Food Chem.* **38** (1990) 18-24.
25. Taylor, J.R.N. and Schüssler, L.,'The protein composition of the different anatomical parts of sorghum grain', *J. Cereal Sci.* **4** (1986) 361-369.
26. Lásztity, R., in 'The Chemistry of Cereal Proteins', CRC Press Inc., Boca Raton, USA (1984) pp 177-184.
27. Skerrit, J.H. in 'Advances in Cereal Science and Technology', Vol 9 (Y. Pomeranz, ed.), Am. Assoc. Cereal Chem. Inc., St. Paul, USA (1988) pp 263-338.
28. Taylor, J.R.N., Novellie, L. and Liebenberg, N.v.d.W.,'Sorghum protein body composition and ultrastructure', *Cereal Chem.* **61** (1984) 69-73.
29. Seckinger, H.L. and Wolf, M.J.,'Sorghum protein ultrastructure as it relates to composition', *Cereal Chem.* **50** (1973) 455-465.
30. Mazhar, H. and Chandrashekar, A.,'Differences in kafirin composition during endosperm development and germination in sorghum cultivars of varying hardness', *Cereal Chem.* **70** (1993) 667-671.
31. Mazhar, H., Chandrashekar, A. and Shetty, H.S.,'Isolation and immunochemical characterization of the alcohol-extractable proteins (kafirins) of *Sorghum bicolor* (L.) Moench', *J. Cereal Sci.* **17** (1993) 83-93.
32. Landry, J. and Moureaux, T.,'Hétérogénéité des glutélines du grain de maïs: extraction sélective et composition en acides aminés des trois fraction isolées', *Bull. Soc. Chim. Biol.* **52** (1970) 1021-1037.
33. Osborne, T.B.,'The Vegetable Proteins', Longmans, London, U.K., (1924).
34. Taylor, J.R.N., Schüssler, L. and Walt, W.H. van der,'Fractionation of proteins from low-tannin sorghum grain', *J. Agric. Food Chem.* **32** (1984) 149-154.
35. Shull, J.M., Watterson, J.J. and Kirleis, A.W.,'Proposed nomenclature for the alcohol-soluble proteins (kafirins) of *Sorghum bicolor* (L. Moench) based on molecular weight, solubility and structure', *J. Agric. Food Chem.* **39** (1991) 83-87.
36. Neucere, N.J. and Sumrell, G.,'Protein fractions from five varieties of grain sorghum: amino acid composition and solubility properties', *J. Agric. Food Chem.* **27** (1979) 809-812.
37. Wu, Y.V. and Wall, J.S.,'Lysine content of protein increased by germination of normal and high lysine sorghums', *J. Agric. Food Chem.* **28** (1980) 455-458.
38. Adeyeye, A. and Ajewole, K.,'Chemical composition and fatty acid profiles of cereals in Nigeria', *Food Chem.* **44** (1992) 41-44.
39. Caransa, A. and Bakker, W.G.M.,'Modern process for the production of sorghum starch', *Starch/Stärke* **39** (1987) 381-385.
40. Rooney, L.W.,'Sorghum and pearl millet lipids', *Cereal Chem.* **55** (1978) 584-590.
41. Reichert, R.D. and Youngs, C.G.,'Dehulling cereal grains and grain legumes for developing countries. II. Chemical composition of mechanically and traditionally dehulled sorghum and millet', *Cereal Chem.* **54** (1977) 174-178.
42. Blessin, C.W., Anderson, R.A., Deatherage, W.L. and Inglett, G.E.,'Effect of alkali dehulling on composition and wet-milling characteristics of sorghum grain', *Cereal Chem.* **48** (1971) 528-532.
43. Osagie, A.U.,'Total lipids of sorghum grain', *J. Agric. Food Chem.* **35** (1987) 601-604.
44. Fry, S.C.,'The growing Plant Cell Wall: Chemical and Metabolic Analysis', Longman Scientific and Technical, Harlow, U.K. (1988).

45. Nyman, M., Siljestrõm, M., Pedersen, B., Bach Knudsen, K.E., Asp, N.-G., Johansson, C.-G. and Eggum, B.O.,'Dietary fiber content and composition in six cereals at different extraction rates', *Cereal Chem.* **61** (1984) 14-19.
46. Viëtor, R.J., Ph.D. Thesis, Wageningen Agricultural University, Wageningen, The Netherlands (1992).
47. Bengtsson, S., Ph.D. Thesis, Swedish University of Agricultural Sciences, Uppsala, Sweden (1991).
48. Gruppen, H., Ph.D. Thesis, Wageningen Agricultural University, Wageningen, The Netherlands (1992).
49. Hoffmann, R.A., Ph.D. Thesis, Utrecht University, Utrecht, The Netherlands (1991).
50. Andersson, R., Ph.D. Thesis, Swedish University of Agricultural Sciences, Uppsala, Sweden (1993).
51. Girhammar, U., Ph.D. Thesis, University of Lund, Lund, Sweden (1992).
52. Vinkx, C.J.A., Nieuwenhove, C.G. van and Delcour, J.A.,'Physicochemical and functional properties of rye nonstarch polysaccharides. III. Oxidative gelation of a fraction containing water-soluble pentosans and proteins', *Cereal Chem.* **68** (1991) 617-622.
53. Vinkx, C.J.A., Reynaert, H.R., Grobet, P.J. and Delcour, J.A.,'Physicochemical and functional properties of rye nonstarch polysaccharides. V. Variability in the structure of water-soluble arabinoxylans', *Cereal Chem.* **70** (1993) 311-317.
54. Westerlund, E., Andersson, R. and Åman, P.,'Isolation and chemical characterization of water-soluble mixed-linked ß-glucans, and arabinoxylans in oat milling fractions', *Carbohydr. Polym.* **20** (1993) 115-123.
55. Autio, K., Myllymäki, O., Suortti, T., Saastamoinen, M. and Poutanen, K.,'Physical properties of (1→3),(1→4)-ß-D-glucan preparates isolated from Finnish oat varieties', *Food Hydrocolloids* **5** (1992) 513-522.
56. Shibuya, N., in 'Plant Cell Wall Polymers: Biogenesis and Biodegradation' (N.G. Lewis and M.G. Paice, eds.), ACS Symposium Series no. 399, American Chemical Society, Washington, USA (1989) pp 333-344.
57. Shibuya, N., Misaki, A. and Iwasaki, T.,'The structure of arabinoxylan and arabino-glucuronoxylan isolated from rice endosperm cell wall', *Agric. Biol. Chem.* **47** (1983) 2223-2230.
58. Shibuya, N., Nakane, R. Yasui, A, Tanaka, K. and Iwasaki, T.,'Comparative studies on cell wall preparations from rice bran, germ and endosperm', *Cereal Chem.* **62** (1985) 252-258.
59. Shibuya, N. and Misaki, A.,'Structure of hemicellulose isolated from rice endosperm cell wall: mode of linkages and sequences in xyloglucan, ß-glucan and arabinoxylan', *Agric. Biol. Chem.* **42** (1978) 2267-2274.
60. Mod, R.R., Normand, F.L., Ory, R.L. and Conkerton, E.J.,'Effect of hemicellulose on viscosity of rice flour', *J. Food Sci.* **46** (1981) 571-573, 578.
61. EtokAkpan, O.U., Ph.D. Thesis, Heriot-Watt University, Edinburgh, U.K. (1988).
62. Hashimoto, S., Shogren, M.D., Bolte, L.C. and Pomeranz, Y.,'Cereal pentosans: their estimation and significance. III. Pentosans in abraded grains and milling by-products', *Cereal Chem.* **64** (1987) 39-41.
63. Karim, A. and Rooney, L.W.,'Pentosans in sorghum grain', *J. Food Sci.* **37** (1972) 365-368.
64. Kavitha, R and Chandrashekar, A.,'Content and composition of non-starchy polysaccharides in endosperms of sorghums varying in hardness', *Cereal Chem.* **69** (1992) 440-443.
65. Karim, A. and Rooney, L.W.,'Characterization of pentosans in sorghum grain', *J. Food Sci.* **37** (1972) 369-371.
66. Palmer, G.H. and MacKenzie, C.I.,'Levels of alkali-soluble ß-D-glucans in cereal grains', *J. Inst. Brew.* **92** (1986) 461-462.
67. EtokAkpan, O.U.,'Comparative studies of the degradation of non-starch polysaccharides by sorghums and barleys during malting', *J. Sci. Food Agric.* **58** (1992) 129-134.
68. Woolard, G.R., Rathbone, E.B. and Novellie, L.,'Structural studies on a glucurono arabinoxylan from the husk of sorghum grain', *Carbohydr. Res.* **51** (1976) 239-247.
69. Woolard, G.R., Rathbone, E.B. and Novellie, L.,'A heteroxylan from the husk of *Sorghum* grain', *Phytochem.* **16** (1977) 957-959.
70. Woolard, G.R., Rathbone, E.B. and Novellie, L.,'DMSO-soluble hemicelluloses from the husk of *Sorghum* grain', *Phytochem.* **16** (1977) 961-963.
71. Woolard, G.R., Rathbone, E.B. and Novellie, L.,'Structural studies on three hemicellulose B fractions

from the husk of sorghum grain', *Carbohydr. Res.* **59** (1977) 547-552.
72. Woolard, G.R., Rathbone, E.B. and Novellie, L.,'A hemicellulosic ß-D-glucan from the endosperm of sorghum grain', *Carbohydr. Res.* **51** (1976) 249-252.
73. Woolard, G.R., Rathbone, E.B., Novellie, L. and Ohlsson, J.T.,'Structural studies on the water-soluble gums from the endosperm of sorghum grain', *Carbohydr. Res.* **53** (1977) 95-100.
74. Rouau, X.,'Investigations into the effects of an enzyme preparation for baking on wheat dough pentosans', *J. Cereal Sci.* **18** (1993) 145-157.
75. Viëtor, R.J., Angelino, S.A.G.F. and Voragen, A.G.J.,'NSP composition of wort and spent grain from brewing trials with a good malting quality barley and a feeding barley', *J. Inst. brew.* **99** (1993) 243-248.
76. Hahn, D.H. and Rooney, L.W.,'Effect of genotype on tannins and phenols of sorghum', *Cereal Chem.* **63** (1986) 4-8.
77. Butler, L.G., Riedl, D.J., Lebryk, D.G. and Blytt, H.J.,'Interaction of proteins with sorghum tannin: mechanism, specificity and significance', *JAOCS* **61** (1985) 916-920.
78. Earp, C.F., Akingbala, J.O., Ring, S.H. and Rooney, L.W.,'Evaluation of several methods to determine tannins in sorghums with varying kernel characteristics', *Cereal Chem.* **58** (1981) 234-238.
79. Butler, L.G., Rogler, J.C., Mehansho, H. and Carlson, D.M., in 'Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological, and Structure-Activity Relationships' (V. Cody, E. Middleton and J.B. Harborne, eds.), Alan R. Liss, Inc., USA (1986) pp 141-157.
80. Price, M.L. and Butler, L.G.,'Tannins in nutrition', *Station Bulletin* **272**, Purdue University, West Lafayette, USA (1980) 1-37.
81. Daiber, K.H.,'Enzyme inhibition by polyphenols of sorghum grain and malt', *J. Sci. Food Agric.* **26** (1975) 1399-1411.
82. Jambunathan, R. and Mertz, E.T.,'Relationship between tannin levels, rat growth, and distribution of proteins in sorghum', *J. Agric. Food Chem.* **21** (1973) 692-696.
83. Kock, J.L.F., Groenewald, E.G., Krüger, G.H.J., Eloff, J.N. and Lategan, P.M.,'Extraction of polyphenols and hydrolysis of birdproof sorghum starch', *J. Sci. Food Agric.* **36** (1985) 1140-1144.
84. Chavan, J.K., Kadam, S.S., Ghonsikar, C.P. and Salunkhe, D.K.,'Removal of tannins and improvement of in vitro protein digestibility of sorghum seeds by soaking in alkali', *J. Food Sci.* **44** (1979) 1319-1321.
85. Novellie, L.,'Kaffircorn malting and brewing studies. XII. Effect of malting conditions on malting losses and total amylase activity', *J. Sci. Food Agric.* **13** (1962) 121-123.
86. Jayatissa, P.M., Pathirana, R.A. and Sivayogasunderam,'Malting quality of Sri Lankan varieties of sorghum', *J. Inst. Brew.* **86** (1980) 18-20.
87. Novellie, L.,'Kaffircorn malting and brewing studies. XI. Effect of malting conditions on the diastatic power of kaffircorn malt', *J. Sci. Food Agric.* **13** (1962) 115-120.
88. Novellie, L.,'Kaffircorn malting and brewing studies. XIII. Variation of diastatic power with variety, season, maturity and age of grain', *J. Sci. Food Agric.* **13** (1962) 124-126.
89. Swanston, J.S., Taylor, K., Rao, N.S., Subramanian, V. and Murty, D.S.,'The use of rapid screening tests to compare changes during malting in sorghum', *J. Inst. Brew.* **99** (1993) 483-486.
90. Nout, M.J.R. and Davies, B.J.,'Malting characteristics of finger millet, sorghum and barley', *J. Inst. Brew.* **88** (1982) 157-163.
91. Aniche, G.N. and Palmer, G.H.,'Development of amylolytic activities in sorghum and barley malt', *J. Inst. Brew.* **96** (1990) 377-379.
92. Taylor, J.R.N. and Robbins, D.J.,'Factors influencing beta-amylase activity in sorghum malt', *J. Inst. Brew.* **99** (1993) 413-416.
93. Rathnavati, C.V. and Bala Ravi, S.,'Effect of different durations of steeping and malting on the production of alpha-amylase in sorghum', *J. Cereal Sci.* **14** (1991) 287-296.
94. Malleshi, N.G. and Desikachar, H.S.R.,'Studies on comparative malting characteristics of some tropical cereals and millets', *J. Inst. Brew.* **92** (1986) 174-176.
95. Dyer, T.A. and Novellie, L.,'Kaffircorn malting and brewing studies. XVI. The distribution and activity of α- and ß-amylases in germinating kaffircorn. *J. Sci. Food Agric.* **17** (1966) 449-456.
96. Aisien, A.O.,'Enzymic modification of sorghum endosperm during seedling growth and malting', *J. Sci. Food Agric.* **33** (1982) 754-759.

97. EtokAkpan, O.U.,'Comparative studies of the ß-D-glucan released into sorghum and barley worts', *J. Inst. Brew.* **98** (1992) 301-304.
98. Aisien, A.O., Palmer, G.H. and Stark, J.R.,'The development of enzymes during germination and seedling growth in Nigerian sorghum', *Starch/Stärke* **35** (1983) 316-320.
99. EtokAkpan, O.U. and Palmer, G.H.,'Comparative studies of the development of endosperm-degrading enzymes in malting sorghum and barley', *World J. Microbiol. Biotechnol.* **6** (1990) 408-417.
100. Agu, R.C., Okeke, B.C., Nwufo, S.C., Ude, C.M. and Onwumelu, A.H.,'Influence of gibberellic acid (GA_3) on diastase and cellulase development of Nigerian millet (*Pennisetum maiwa*) and sorghum (*Sorghum bicolor*)', *Process Biochem.* **28** (1993) 105-108.
101. Evans, D.J. and Taylor, J.R.N.,'Influence of cultivar and germination conditions on proteolytic activities in sorghum malt', *J. Inst. Brew.* **96** (1990) 399-402.
102. Evans, D.J. and Taylor, J.R.N.,'Extraction and assay of proteolytic activities in sorghum malt', *J. Inst. Brew.* **96** (1990) 201-207.
103. Taylor, J.R.N. and Evans, D.J.,'Action of sorghum proteinases on the protein bodies of sorghum starchy endosperm', *J. Experimental Botany* **40** (1989) 763-768.
104. Garg, G.K. and Virupaksha, T.K.,'Acid protease from germinated sorghum. 1. Purification and characterization of the enzyme', *Eur. J. Biochem.* **17** (1970) 4-12.
105. Glennie, C.W., Harris, J. and Liebenberg, N.v.d.W.,'Endosperm modification in germinating sorghum grain', *Cereal Chem.* **60** (1983) 27-31.
106. Subramanian, V., Murty, D.S., Rao, N.S. and Jambunathan, R.,'Chemical changes and diastatic activity in grains of sorghum (*Sorghum bicolor*) cultivars during germination', *J. Sci. Food Agric.* **58** (1992) 35-40.
107. Okoh, P.N., Kubiczek, R.P., Njoku, P.C. and Iyeghe, G.T.,'Some compositional changes in malted sorghum (*Sorghum vulgare*) grain and its value in broiler chicken diet', *J. Sci. Food Agric.* **49** (1989) 271-279.
108. Palmer, G.H.,'Enzymic degradation of the endosperm cell walls of germinated sorghum', *World J. Microbiol. Biotechnol.* **7** (1991) 17-21.
109. Glennie, C.W.,'Endosperm cell wall modification in sorghum grain during germination', *Cereal Chem.* **61** (1984) 285-289.
110. EtokAkpan, O.U.,'Enzymic degradation and nature of the endosperm cell walls of germinating sorghums and barley', *J. Sci. Food Agric.* **61** (1993) 389-393.
111. Gibbons, G.C. and Nielsen, E.B.,'New analysis in malting and brewing', *J. Inst. Brew.* **89** (1983) 8-14.
112. Palmer, G.H.,'A reassessment of the pattern of the endosperm hydrolysis (modification) in germinated barley', *J. Inst. Brew.* **88** (1982) 145-153.
113. Glennie, C.W.,'Polyphenol changes in sorghum grain during malting', *J. Agric. Food Chem.* **31** (1983) 1295-1299.
114. Aniche, G.N. and Anih, A.C.,'Malting properties of two sorghum cultivars and the effect of mashing schemes on their wort properties', *Process Biochem.* **29** (1994) 369-371.
115. Aisien, A.O. and Muts, G.C.J.,'Micro-scale malting and brewing studies of some sorghum varieties', *J. Inst. Brew.* **93** (1987) 328-331.
116. Taylor, J.R.N.,'Mashing with malted grain sorghum', *ASBC Journal* **50** (1992) 13-18.
117. Okafor, N. and Aniche, G.N.,'Studies on the brewing of lager beer from Nigerian sorghum', *J. Food Sci. Technol.* **24** (1987) 131-134.
118. Ugboaja, F.C., Bednarski, W. and Babuchowski, A.,'The technology and properties of beer produced from unmalted sorghum or maize grains', *World J. Microbiol. Biotechnol.* **7** (1991) 225-230.
119. Odibo, F.J.C. and Obi, S.K.C.,'Mashing trials of two Nigerian sorghum varieties using thermostable microbial pullulanase', *MIRCEN Journal* **5** (1989) 187-192.
120. Bajomo, M.F. and Young T.W.,'The properties, composition and fermentabilities of worts made from 100% raw sorghum and commercial enzymes', *J. Inst. Brew.* **99** (1993) 153-158.
121. Taylor, J.R.N. and Boyd, H.K.,'Free α-amino nitrogen production in sorghum beer mashing', *J. Sci. Food Agric.* **37** (1986) 1109-1117.
122. Jones, M. and Pierce, J.S.,'Absorption of amino acids from wort by yeasts', *J. Inst. Brew.* **70** (1964)

307-315.

123. Pickerell, A.T.W.,'The influence of free alpha-amino nitrogen in sorghum beer fermentations', *J. Inst. Brew.* **92** (1986) 568-571.
124. Bajomo, M.F. and Young T.W.,'Development of a mashing profile for the use of microbial enzymes in brewing with raw sorghum (80%) and malted barley or sorghum malt (20%)', *J. Inst. Brew.* **98** (1992) 515-523.
125. Dale, C.J., Young, T.W. and Omole, A.T.,'Small scale mashing experiments with grits containing high proportions of raw sorghum', *J. Inst. Brew.* **96** (1990) 403-409.

CHAPTER 2

Water-unextractable Cell Wall Material from Sorghum: Isolation and Characterization

*A procedure for the isolation of water-unextractable cell wall material (WUS) from flours of sorghum and polished sorghum (*Sorghum vulgare *cv. Fara Fara) is described. The composition of all fractions obtained was determined and a mass balance made. For whole grain as well as for polished sorghum 87% (dry weight) of the flours was recovered in the fractions. The analysed components, being mainly starch, protein and non-starch polysaccharides (NSP), accounted for 84-100% (dry weight) of almost all fractions. For whole grain and polished sorghum respectively, the WUS fractions accounted for 5.3 and 1.05% of the flour and contained 2.8 and 1.9% starch, 6.8 and 13.9% protein and 73.6 and 61.0% NSP (dry weight). These NSP were composed mainly of arabinose, xylose, glucose and uronic acid. The polysaccharide composition of the outer layers is similar to that of the inner layers of the sorghum kernel as is seen by comparison of the neutral sugar composition in whole grain and polished sorghum and in the grindings obtained after polishing.*

Most of the non-starch glucose is present as cellulose and (1→3),(1→4)-β-D-glucan. The arabinoxylan present in sorghum is highly substituted (arabinose/xylose = 0.9) and contains uronic acids, acetyl and feruloyl substituents.

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INTRODUCTION

Sorghum is a major food staple in semi-arid areas. Due to its drought tolerance and adaptation to tropical conditions, sorghum can still be produced where agricultural and environmental conditions are unfavourable for the production of other crops. Sorghums are classified as high or low tannin types. The high tannin varieties, often brown or red coloured, have certain advantages involving resistance to birds, mould infestation and sprouting. An important disadvantage is their reduced nutritional value[1] in comparison with white (low tannin) varieties that are comparable in nutritional value to maize[2].

Sorghum is traditionally used in porridges and for the production of various kinds of African beers[3]. Amongst other characteristics these beers differ from European type lager beers in that, during the fermentation, lactic acid is formed as well as ethanol. The beers, which are consumed while still fermenting, contain large amounts of insoluble material. Sorghum can also be used for brewing European type lager beers[4]. Part of the malt can be replaced by sorghum grits as an inexpensive source of fermentable carbohydrate. Production of beer from unmalted sorghum is possible, but requires the addition of exogenous enzymes for optimal processing[5]. Another possibility is the production of a lager type beer from malted sorghum, but brewing trials with sorghum malt showed a slow and incomplete saccharification and difficult beer filtration[6,7]. These problems are probably caused by poor degradation of endosperm walls resulting in limited accessibility of aleurone derived amylases to the gelatinized starch. Palmer[8] showed by electron microscopy that sorghum endosperm cell walls persist during malting, although holes develop in the walls. In contrast, endosperm walls in barley malt are almost completely degraded[9]. Compared with the endosperm, elevated levels of non-cellulosic polysaccharides, lipids and polyphenols are present in the outer non-endospermic layers of sorghum kernels. These compounds can cause problems during the brewing process. Sorghum is therefore often polished before use.

Etokakpan[10] isolated walls from sorghum endosperm by milling, dry and wet sieving of crude endosperm wall fragments in 70% ethanol, which were composed of 28% (1→3),(1→4)-ß-D-glucan, 4.3% arabinoxylan and 62% protein (dry weight). However, he showed no structural analyses of the component polysaccharides. Neither did Glennie[11], who succeeded in preparing walls which contained 46% protein. These proteins however, were presumably of cytoplasmic origin. Detailed studies on the isolation and structural characteristics of non-cellulosic polysaccharides in sorghum pericarp and endosperm were reported by Woolard and co-workers[12,13]. They identified a water-unextractable, alkali-extractable polysaccharide composed of L-Ara, D-Xyl, GlcA and 4OMeGlcA from the pericarp. They also isolated two low molecular weight, water-extractable glucans from sorghum endosperm containing (1→4)- and (1→6)-linked D-Glc residues in different ratios.

In this study, we describe the isolation of water-unextractable residues from flours of whole grain and polished sorghum that are expected to contain water-unextractable cell wall polysaccharides. The fractions obtained were characterized and mass balances were calculated. The NSP rich fractions will be the base for future studies of the detailed chemical structure and enzymic degradability of the non-cellulosic polysaccharides.

EXPERIMENTAL

Materials

Sorghum grain (*Sorghum vulgare* cv. Fara Fara, Nigeria, 1988 harvest) was polished at the Institute for Cereals, Flour and Bread TNO (Wageningen, The Netherlands), using an Olmia laboratory scale polishing machine (Vercelli, Italy). The germ and bran layers were satisfactorily removed when 20.3% of the sorghum kernel was removed.

Flours were prepared separately from both the original and the polished grains using a Fritsch pulverisette (Marius Instrumenten, Utrecht, The Netherlands), equiped with a 0.5mm sieve.

Isolation of the WUS

Fig 1 shows a schematic diagram of the fractionation procedure developed for the isolation of water-unextractable cell wall material from sorghum flour. *C.*400g of flour was extracted with *n*-hexane (3.5l) in a Soxhlet apparatus (refluxing for 6h). The free lipid fraction was obtained by vacuum evaporation of the hexane. The residue was extracted with 1.5%w/v sodium dodecylsulphate (SDS, 1l) containing 0.05%v/v 2-mercaptoethanol, under continuous stirring for 1h and collected by centrifuging for 15min at 25,000*g*. This procedure was repeated twice. The final pellet was washed twice with 1l distilled water. The combined supernatants were freeze-dried (code SDSS). The residue was filtered over a 45μm sieve by washing with distilled water (9l). The filtrate was centrifuged for 15min at 25,000*g*. The pellet (code starch) and the supernatant (code RCWS) were freeze-dried.

The residue which retained on the sieve was suspended in buffer solution (2l, pH6.5) containing 10mM maleic acid, 10mM NaCl, 1mM $CaCl_2$ and 0.05% NaN_3. The suspension was heated to 85°C for 1.5h. After cooling to 30°C, 2mg (300U) porcine pancreatic α-amylase (Merck art. 16312) was added and the mixture incubated at 30°C for 20h. The α-amylase digestion was repeated twice. After each digestion and centrifugation (15min, 25,000*g*) the residues were washed with hot distilled water (65°C) and centrifuged again. The remaining unextractable residues (code WUS) and the combined supernatants (code HWS) were freeze-dried.

Analytical methods

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

Amino acid composition was determined using a Biotronik LC 6000 E automatic amino acid analyser, after hydrolysis of the samples with 6M HCl for 21h at 110°C under N_2. *Protein content* (5.8 x N) was determined by a semi-automated micro-Kjeldahl method[14]. The conversion factor 5.8 was calculated from the amino acid analysis.

Starch content was determined enzymically using the test kit supplied by Boehringer (Mannheim, Germany).

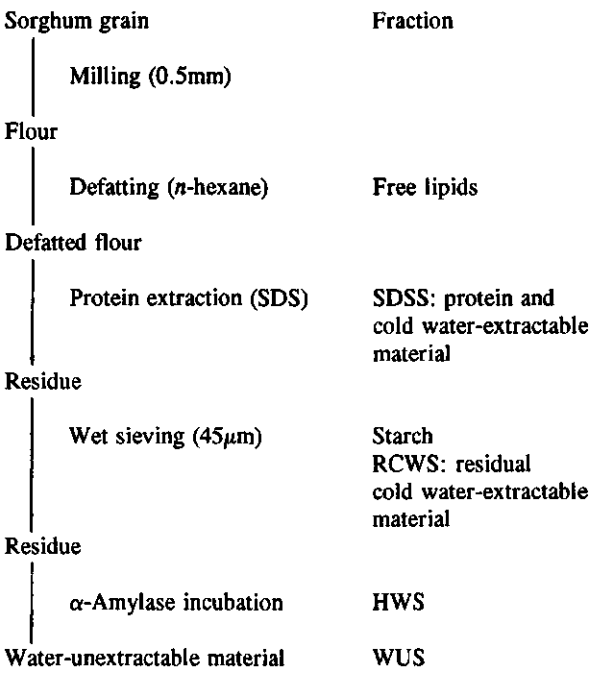

FIG 1. Fractionation procedure for the isolation of WUS from sorghum flour.

Neutral sugars in the fractions were analysed as alditol acetates by gas chromatography according to Englyst and Cummings[15], using myo-inositol as internal standard. The flours, starch, RCWS and HWS fractions were treated, prior to hydrolysis, with DMSO and incubated with α-amylase (Merck, art. 16312) and pullulanase (Sigma, P 5420) to digest the starch. The residues from these materials were treated with 72%w/w H_2SO_4 (1h, 30°C) prior to hydrolysis with 1M H_2SO_4 for 3h at 100°C and the constituent sugars released were analysed as their alditol acetates. Alditol acetates were separated on a 3m x 2mm i.d. glass column, packed with Chrom WAW 80-100 mesh coated with 3% OV 275, in a Carlo Erba Fractovap 2300 GC. The oven temperature was set at 200°C, the F.I.D. detector at 270°C. H_2 was used as carrier gas. Non-starch Glc in the WUS fractions was calculated as the difference between the Glc content determined as its alditol acetate and Glc determined with the Boehringer starch test kit. Cellulosic Glc was calculated as the difference between the content of Glc found with and without prehydrolysis step.

Uronic acids were determined as anhydro-uronic acid (AUA) by the *m*-hydroxydiphenyl assay[16] using an auto-analyser (Skalar Analytical B.V., Breda, The Netherlands). GalA could be distinguished from GlcA by running the test with and without the addition of sodium tetraborate to the H_2SO_4. Corrections were made for interference by neutral sugars present in the samples.

(1→3),(1→4)-β-D-Glucans were determined enzymically using the test kit supplied by Biocon (Victoria, Australia). A Glc test kit from Boehringer was used for the analysis of liberated Glc.

Lipid contents in the flours were determined gravimetrically after extraction with *n*-hexane and vacuum evaporation.

Phenolic acids were determined in the fractions and in the flours as follows: Samples were treated with 5ml 0.5M KOH (flushed with N_2) for 24h in the dark at room temperature under N_2. 6M HCl was added and the phenolic components were recovered by extraction with 4ml ethyl acetate (twice). The combined extracts were dried in a Christ Alpha rotational vacuum concentrator (I.K.S., Leerdam, The Netherlands). The residues obtained were dissolved in 1 ml MeOH and 20μl aliquots were used for analysis by HPLC. A Spectra Physics SP 8000 HPLC was used, equipped with a reversed phase Spherisorb 10 ODS column (Merck, 250mm x 4.6mm). The eluent was a gradient mixture of 4%v/v HOAc in MeOH (A)/4%v/v HOAc in H_2O (B), with a flow of 1.5ml/min. The starting conditions were 10% A/90% B which were kept constant for 9min. Then the gradient changed linearly to 50% A/50% B in 15min and was kept in this ratio for the following 6min. *p*-Hydroxybenzoic acid was used as internal standard. The eluent was monitored using a Kratos spectroflow 773 UV detector set at 278nm.

Degree of methylation and acetylation of WUS fractions was determined by HPLC after saponification with 0.4M NaOH[17].

Results and Discussion

Yield and composition of the flour fractions

Tables I and II present the yield and composition of fractions obtained from whole sorghum grain and polished sorghum. The results are shown as g ds/100g ds flour to give a mass balance of the fractionation. The distribution of components within the fractions, are also given as % dry weight of the flour. For both whole grain and polished sorghum flours, 87% of the original material was recovered in the fractions.

Whole grain flour contained *c.*10% protein, 67% starch, 4% lipids and 5% NSP based on dry weight. These amounts agree well with results published earlier on the composition of sorghum. Aucamp et al.[18] compared some characteristics of eight sorghum varieties and found the protein content to vary from 8.5 to 11.9% with an average of 10.7%. The mean lipid content was 3.6% (range 1.6 - 4.6%) and the fibre content, which may be equated with NSP, averaged at 4.7% with a range of 3.4 to 7.3%. The starch content of five sorghum varieties examined by Von Holdt and Brand[19] varied from 61 to 69%. None of these studies included *Sorghum vulgare* cv. Fara Fara.

Tannins are believed to have an inhibitory effect on enzymes, for instance during malting, by forming protein-tannin complexes[20,21]. However, no tannins were detected in this white sorghum variety using the modified vanillin-HCl test[22].

The WUS fraction contained 78% of the NSP of the flour. This fraction also contained most of the ferulic acid and *p*-coumaric acid. These phenolic acids are considered to be involved in the coupling of polysaccharides to one another or to proteins[23].

The WUS fraction still contained *c.*3% starch, even after prolonged incubation with α-amylase. This may be attributed to certain physical changes in the starch component introduced by the isolation procedure wich may cause resistance to degradation by α-amy-

TABLE I. Yield and composition of fractions from sorghum flours prepared from whole sorghum grain.

A. Yield of flour fractions and distribution of flour components between fractions[a].

	Free lipids	SDSS	Starch	RCWS	HWS	WUS	Total recovered	Flour
Yield	4.3	7.9	64.8	0.5	3.6	5.3	86.4	100
Protein		7.4		0.03	0.06	0.4	7.9	9.9
NSP		0.5	4.8	0.03	0.09	3.9	9.3	5.0
Starch		0.3	60.0	0.02	3.5	0.2	64.0	66.9
Lipids								3.9
Ferulic acid		tr[b]	0.01	tr	tr	0.04	0.03	0.05
p-Coumaric acid		tr	tr	tr	tr	0.01	0.01	0.02
Uronic acid		0.02	0.1	0.002	0.004	0.3	0.43	0.5
Total		8.2	64.9	0.08	3.7	4.8		86.3

B. Composition of flour fractions calculated as %ds.

	SDSS	Starch	RCWS	HWS	WUS
Protein	93.1		6.9	1.8	6.8
NSP	6.3	7.4	5.0	2.5	73.6
Starch	3.7	92.6	3.0	97.6	2.8
Ferulic acid	0.03	0.02	0.03	0.01	0.6
p-Coumaric acid	0.004	0.001	0.003	0.01	0.2
Uronic acid	0.2	0.2	0.4	0.1	6.5
Total	103.3	100.2	15.3	102.0	90.5

[a] Calculated as g ds/100 g ds flour; [b] Trace amount.

lases[24]. In addition, the WUS fraction contained 7% of protein. Beside its presence as structural proteins, some of this material could be glycoprotein in origin or being present as intracellular protein[23].

The protein recovery from the fractionation was 80% and only 5% of total recovered protein was found in the WUS fraction. Although the WUS material had been treated with SDS in an attempt to remove intracellular protein, it still conbtained 7% protein of undetermined origin. However, this is significantly lower than was found in material isolated in the 70% ethanol medium of Mares and Stone[25] which yielded a WUS fraction containing over 30% protein (unpublished observations). It was also considerably lower than the 46% protein obtained by Glennie[11] for a sorghum endosperm wall preparation. In this case *t*-butanol containing dithiothreitol extraction of ball milled flour wet sieved in

ethanol, was used.

Beside proteins, the SDSS fraction also contained other water-extractable materials such as extractable NSP, which represented *c.*10% of the total NSP present, and minor amounts of starch. Karim and Rooney[26] stated that the water-extractable NSP is the major arabinoxylan containing fraction. However, they only compared the amount and composition of polysaccharides extracted with water with those extracted with alkali. Although presumably part of the water-unextractable NSP will be extracted by alkali, they did not determine the unextractable nor total NSP content as such. Their results show that 0.9% of the polymeric material of whole grain sorghum was extractable with water.

In both SDSS and HWS fractions considerable amounts of buffer salts were present. Since we did not want to introduce any loss of material by dialysis, no attempts were made to remove these salts. Therefore, yields were determined by correcting absolute weights with the amounts of buffer salts calculated from the volumes and concentrations of the buffers used.

The NSP content in the starch fraction seemed to be very high. Moreover, the sum of the NSP contents analysed in the fractions (9.3%) is 186% of the NSP initially analysed in the sorghum flour (5.0%). Apparently the determination of NSP according to Englyst and Cummings[15] from the starch fraction was not adequate with respect to the complete removal of starch. The total yield of starch was 96% of which 94% was collected in the starch fraction. The remaining starch was recovered mainly in the HWS fraction and represented more than 97% of this fraction.

The smallest fraction, the RCWS fraction consisted of residual water-extractable material. Its constituents were mainly proteins, NSP and starch. In total they accounted for only 15.3% of the fraction. It is assumed that the missing part of this fraction is made up by residual SDS.

Polished sorghum flours: As can be seen from Table II, the fat content in flour decreased by 44% to 2.2%ds as a result of the polishing treatment of the sorghum kernels. There was a slightly higher protein level and the starch content in the flour of polished sorghum was increased. Apparently, elevated levels of lipids are present in the outer layers of sorghum kernels. Our results agree in part with studies of Reichert and Youngs[27] who reported that the oil level decreased by 36% when 20%w/w of the kernel of a red-branned sorghum variety (*Sorghum bicolor* (L.) Moench) was removed mechanically. However, they also found that the protein level in the polished flour diminished by 8%.

The NSP content in the flour of the polished sorghum was significantly lower than flour from whole grain. The outer kernels layers, corresponding to about 20% dry grain weight, contained 70% of the NSP. The uronic acid and phenolic acid contents decreased proportionally with the NSP level. Blessin et al.[28] reported similar results for alkali dehulled sorghum. The fibre content of these dehulled products, which made up 8%w/w

TABLE II. Yield and composition of fractions from polished sorghum flours.

A. Yield of flour fractions and distribution of flour components between fractions[a].

	Free lipids	SDSS	Starch	RCWS	HWS	WUS	Total recovered	Flour
Yield	1.6	9.5	71.0	0.16	3.7	1.05	87.0	100
Protein		9.0		0.01	0.05	0.15	9.2	10.5
NSP		0.3	0.2	0.002	0.02	0.64	1.16	1.5
Starch		0.1	70.0	0.002	3.6	0.02	73.7	73.1
Lipids								2.2
Ferulic acid		0.003	0.002	tr[b]	tr	0.01	0.01	0.03
p-Coumaric acid		tr	tr	tr	tr	0.002	0.002	0.005
Uronic acid		0.01	0.07	0.0003	0.007	0.06	0.15	0.2
Total		9.4	70.3	0.014	3.7	0.88		87.5

B. Composition of flour fractions calculated as %ds.

	SDSS	Starch	RCWS	HWS	WUS
Protein	95.4		9.3	1.3	13.9
NSP	3.1	0.3	1.3	0.5	61.0
Starch	1.0	98.6	1.3	98.1	1.9
Ferulic acid	0.03	0.003		0.01	0.7
p-Coumaric acid	0.002	0.001		0.01	0.2
Uronic acid	0.1	0.1	0.2	0.2	5.6
Total	99.6	99.0	12.1	100.1	83.3

[a] Calculated as g ds/100 g ds flour; [b] Trace amount.

of the kernel material, was *c.*50% of that of the original grain. The yield of WUS from polished sorghum was five times lower compared with the yield from whole grain sorghum. NSP comprised 61% of the WUS fraction. The ratio NSP/uronic acid (AUA) was similar (ratio value ≈ 10), which supports the idea that the uronic acids are an integral part of wall polysaccharides. The reduction of protein in the WUS fraction was again successful. Although it still contained 14% protein, this accounted for only 1.6% of the total recovered protein.

The fractionation of polished sorghum showed similar results as for the whole grain sorghum. About 88% of the proteins were recovered during the fractionation. By treatment with SDS 98% from these proteins were removed from the defatted residue. Starch was 100% recovered during the fractionation, of which 95% was collected in the starch fraction. An additional 3.6% of starch was collected in the HWS fraction, after

incubation with α-amylase. The protein content of the RCWS fraction was somewhat higher compared with the corresponding fraction of the unpolished sorghum, whereas the NSP and starch contents were lower. The phenolic acids were not determined because the size of the fraction was too small.

The grindings, obtained after polishing, were also analysed and found to contain 18.6% NSP and 1.9% AUA (results not shown). The mass balances for NSP and AUA over the polishing treatment are fitting very well as can be concluded from the following calculations: The whole grain flour contained 5.0% NSP. This amount equals the amounts of NSP in the grindings and polished sorghum grain together. As 20% from the kernels was removed, 0.8 x 1.5 + 0.2 x 18.6 = 4.9% NSP. Using the same calculation for the uronic acid content, the addition of the uronic acid contents of polished sorghum flour and grindings (0.5%) equals the uronic acid content in the whole grain sorghum flour.

The amino acid composition

The amino acid composition was determined for the protein rich SDSS fractions, the WUS and the complete flours. As can be seen from Table III, sorghum proteins contain high amounts of glutamic acid/glutamine, leucine, alanine, proline and aspartic acid/asparagine. The content of the nutritionally essential amino acid lysine in sorghum is relatively

TABLE III. Molar amino acid composition of sorghum flour fractions.

	Sorghum			Polished sorghum		
	Flour	WUS	SDSS	Flour	WUS	SDSS
Asx	6.6	6.1	6.2	6.4	4.4	5.9
Thr	4.5	4.7	3.3	4.5	4.0	3.3
Ser	4.5	5.3	5.0	4.6	5.0	4.9
Glx	19.4	15.5	19.5	21.7	18.9	19.7
Gly	5.3	9.5	4.9	4.8	7.5	4.9
Ala	13.4	12.0	13.8	13.7	10.7	13.6
Val	5.5	5.9	5.5	5.5	5.5	5.5
Met	1.4	0.8	1.6	1.3	1.0	1.5
Ile	3.7	3.6	3.6	3.7	3.3	3.6
Leu	12.8	11.0	13.2	14.2	12.2	13.6
Tyr	2.0	2.2	3.0	1.3	2.3	3.1
Phe	4.0	3.7	4.0	4.3	3.5	4.1
Lys	2.1	2.0	1.8	1.6	0.9	1.6
His	1.9	2.8	1.7	1.8	3.5	1.7
Arg	3.2	3.3	3.3	2.9	2.7	2.8
Hyp	0.0	0.0	0.0	0.0	0.0	0.0
Pro	9.7	11.6	9.6	7.7	14.6	10.2

TABLE IV. Sugar composition and mass balance for sugars in the sorghum flour fractions.
A. Molar sugar composition.

	SDSS	Starch	RCWS	HWS	WUS	Flour
Rha	1.0	0.0	0.0	0.1	0.3	<0.1
Ara	5.8	0.4	4.8	0.4	25.1	2.2
Xyl	4.3	tr[a]	3.2	0.3	26.6	2.4
Man	1.4	0.0	3.3	0.1	1.4	0.1
Gal	8.2	0.0	5.1	0.1	2.1	0.2
Glc[b]	37.4	6.9	46.5	2.0	33.8	2.7
Starch	32.5	91.3	27.3	96.7	3.1	92.3
Uronic acid	9.4	1.3	9.9	0.4	7.7	
Ara/Xyl	1.3	nc[c]	1.5	1.3	0.9	0.9

B. Mass balance for neutral sugars (g ds/100 g ds flour).

	SDSS	Starch	RCWS	HWS	WUS	Total	Flour
Rha	0.01	0.00	0.00	<0.01	0.01	0.02	0.01
Ara	0.04	0.22	<0.01	0.01	0.97	1.24	1.31
Xyl	0.03	tr	<0.01	0.01	1.11	1.15	1.46
Man	0.01	0.00	<0.01	<0.01	0.06	0.07	0.10
Gal	0.07	0.00	<0.01	<0.01	0.10	0.17	0.16
Glc[d]	0.58	64.57	0.03	3.52	1.81	70.53	68.89
Starch	0.27	60.04	0.01	3.45	0.15	63.92	66.90
Cellulose	0.00	0.26	0.00	0.00	0.66	0.92	1.20
ß-Glucan	nd[e]	nd	nd	nd	0.14	0.14	0.30
Other	0.31	4.27	0.02	0.07	0.86	5.55	0.49
Total	0.74	64.79	0.03	3.54	4.06	73.18	71.93
Total NSP	0.47	4.75	0.02	0.09	3.91	9.26	5.03

[a] Trace amount; [b] Non-starch Glc; [c] Not calculated; [d] Total Glc content; [e] Not determined.

low. The amino acid composition found for unpolished sorghum flour agrees well with earlier published results[29,30]. The polishing treatment hardly had any effect on the amino acid composition. Taylor and Schüssler[30] have reported that the germ and pericarp are richer in glycine, lysine and arginine whereas relatively more glutamic acid, proline, alanine and leucine was present in the endosperm. However, our results do not confirm that particular anatomical parts of the sorghum kernels are richer in certain amino acids. From these analyses we calculated a conversion factor of 5.8 to express the protein content from the determination of total nitrogen. However, tryptophan, cystein and amide nitrogen were not determined, which could result in an overestimation of the conversion factor. Mossé[30], calculated 5.64 as the conversion factor for sorghum protein. The

generally used factor 6.25 is obviously too high for sorghum proteins.

Carbohydrate composition

*C.*50% of the polysaccharides in the WUS is accounted for by Ara and Xyl, presumably present as arabinoxylans[32] (Tables IV and V). These arabinoxylans are highly substituted as expressed by the Ara/Xyl ratio. The Ara/Xyl ratio, 0.94, indicates that these arabinoxylans are more highly substituted than arabionoxylans in WUS material from wheat[33] (0.6) and barley[34] (0.7).

The WUS fractions contain quite high amounts of uronic acids, mainly GlcA, which may be linked to Xyl residues to form a GAX[12]. Acetyl groups but no methyl groups were detected in the WUS fractions. Acetyl groups have been found in pectins or linked to Xyl units in heteroxylans. Pectin can hardly be present in this fraction, because only very small amounts of GalA were found. Assuming all acetyl groups are bound to the Xyl residues, the degree of acetylation in WUS obtained from both whole grain and polished sorghum flour is *c.*26mol%, equivalent to one acetyl substituent for every four Xyl units.

A major part of the Glc is present as cellulose (20.3 and 14.7mol% for unpolished and polished sorghum, respectively). The (1→3),(1→4)-ß-D-glucan content, 2.9% and 1.6% for WUS of unpolished and polished sorghum, respectively, is very low compared with barley. WUS NSP from barley is composed of *c.*31mol% (1→3),(1→4)-ß-D-glucan[34].

The neutral sugar composition of the grindings is similar to the composition in the flours or WUS fractions. The grindings were composed of 24.1mol% Ara, 24.1mol% Xyl and 41.2mol% Glc, with minor amounts of Man and Gal. Also, the mass balance for neutral sugars over the polishing treatment fits well.

The water-extractable part of the sorghum flour polysaccharides was recovered in the SDSS and RCWS fractions. As can be seen from Tables IV and V, a major part of the total amount of the Gal residues was present in these fractions. Considering the relatively high amount of Ara in the same fractions, this polysaccharide may be a soluble arabinogalactan possibly associated with proteins as found in wheat flour[32,33]. The uronic acid content of the SDSS fractions was relatively high, suggesting that there might be a soluble pectin-like polysaccharide present in these fractions. The actual amount of starch in the SDSS and the RCWS fractions will be higher than the values presented in Tables IV and V. Starch was determined using the Boehringer test kit in which the solubilization of starch in DMSO/HCl (80/20) is followed by an enzymic analysis. Although the material is highly diluted, residual SDS may diminish enzymic action by denaturing the amyloglucosidase. The same problem may arise for the determination of (1→3),(1→4)-ß-D-glucan in these fractions. It is possible that the SDS can to a certain extent, denature the (1→3),(1→4)-ß-D-glucanase and ß-glucosidase used in the test.

The starch, RCWS and HWS fractions consisted mainly of starch Glc. Hardly any non-cellulosic polysaccharides were extracted during the wet-sieving step and the washings

with hot water.

TABLE V. Sugar composition and mass balance for sugars in the polished sorghum flour fractions.
A. Molar sugar composition.

	SDSS	Starch	RCWS	HWS	WUS	Flour
Rha	1.4	0.0	0.0	0.1	0.6	0.0
Ara	6.6	0.2	tr[a]	0.1	21.0	0.7
Xyl	5.0	tr	tr	0.3	24.7	0.8
Man	2.6	0.0	tr	0.2	2.0	0.1
Gal	10.2	0.0	tr	0.1	1.9	0.1
Glc[b]	40.6	0.1	54.4	0.1	39.0	1.1
Starch	21.5	98.4	37.8	99.0	2.6	97.3
Uronic acid	12.1	1.3	7.8	0.2	8.1	
Ara/Xyl	1.3	nc[c]	nc	0.3	0.9	0.9

B. Mass balance for neutral sugars (g ds/100 g ds flour).

	SDSS	Starch	RCWS	HWS	WUS	Total	Flour
Rha	0.01	0.00	0.00	<0.01	<0.01	0.01	0.00
Ara	0.02	0.12	tr	<0.01	0.14	0.28	0.34
Xyl	0.02	tr	tr	0.01	0.16	0.19	0.39
Man	0.01	0.00	tr	0.01	0.02	0.04	0.04
Gal	0.04	0.00	tr	<0.01	0.02	0.06	0.05
Glc[d]	0.26	70.06	0.004	3.63	0.32	74.58	73.77
Starch	0.09	69.97	0.002	3.63	0.02	74.02	73.10
Cellulose	0.00	0.00	0.00	0.00	0.14	0.14	0.40
ß-Glucan	nd[e]	nd	nd	nd	0.01	0.01	0.10
Other	0.17	0.09	0.002	0.00	0.15	0.41	0.17
Total	0.36	70.18	0.004	3.65	0.66	75.16	74.59
Total NSP	0.27	0.21	0.002	0.02	0.64	1.14	1.49

[a] Trace amount; [b] Non-starch Glc; [c] Not calculated; [d] Total Glc content; [e] Not determined.

CONCLUSIONS

With the isolation method described here, WUS materials could be obtained from flours of whole grain sorghum and polished sorghum. These two sorghum flours differed mainly in their lipid and NSP content. The major part of these components are located in the pericarp and germ of the sorghum kernel. No tannins were detected in this white sorghum variety.

The flours contained 5.3 and 1.05% WUS materials for whole grain sorghum and polished sorghum, respectively. The WUS fractions contained 73.6 and 61.0% NSP. The sugar compositions of these NSP were similar which means that the sugar composition of pericarp NSP hardly differs from endosperm NSP.

Highly substituted arabinoxylans carrying considerable amounts of uronic acids and acetyl groups, form over 50%w/w of the NSP. The analytical results show that the Glc residues arise mainly from cellulose, but small amounts of (1-3),(1-4)-ß-D-glucan are also present in these fractions.

The WUS fractions contained small amounts of amylase resistant starch. After SDS extraction of proteins, considerable amounts of protein still remained in the WUS preparation. SDS did not selectively extract specific protein fractions.

REFERENCES

1. Bach-Knudsen, K.E., Munck, L. and Eggum, B.O.,'Effect of cooking, pH and polyphenol level on carbohydrate composition and nutritional quality of a sorghum (*Sorghum bicolor* (L.) Moench) food, ugali', *British J. Nutr.* **59** (1988) 31-47.
2. Serna-Saldivar, S.O., Knabe, D.A., Rooney, L.W., Tanksley Jr., T.D. and Sproule, A.M.,'Nutritional value of sorghum and maize tortillas', *J. Cereal Sci.* **7** (1988) 83-94.
3. Bello, A.B., Rooney, L.W. and Waniska, R.D.,'Factors affecting quality of sorghum tô, a thick porridge', *Cereal Chem.* **67** (1990) 20-25.
4. Rooney, L.W. and Serna-Saldivar, S.O., in 'Handbook of Cereal Science and Technology' (K.J. Lorenz and K. Kulp, eds.), Marcel Dekker Inc., New York, USA (1991) pp 233-270.
5. MacFadden, D.P. and Clayton, M.,'Brewing with sorghum, use of exogenous enzymes', *Brewing and Beverage Industry International* (1989) 77-81.
6. Okafor, N. and Aniche, G.N.,'Studies on the brewing of lager beer from Nigerian sorghum', *J. Food Sci. Technol.* **24** (1987) 131-134.
7. Aisien, A.O. and Muts, G.C.J.,'Micro-scale malting and brewing studies of some sorghum varieties', *J. Inst. Brew.* **93** (1987) 328-331.
8. Palmer, G.H.,'Enzymic degradation of the endosperm cell walls of germinated sorghum', *World J. Microbiol. Biotechnol.* **7** (1991) 17-21.
9. Palmer, G.H.,'Influence of cell wall structure in enzymic breakdown of the endosperm of germinated barley', *J. Inst. Brew.* **93** (1987) 105-107.
10. Etokakpan, O.U. Ph.D. Thesis, Heriot-Watt University, Edinburgh, U.K. (1988).
11. Glennie, C.W.,'Endosperm cell wall modification in sorghum grain during germination', *Cereal Chem.* **61** (1984) 285-289.
12. Woolard, G.R., Rathbone, E.B. and Novellie, L.,'Structural studies on a glucurono arabinoxylan from the husk of sorghum grain', *Carbohydr. Res.* **51** (1976) 239-247.
13. Woolard, G.R., Rathbone, E.B., Novellie, L. and Ohlsson, J.T.,'Structural studies on the water-soluble gums from the endosperm of sorghum grain', *Carbohydr. Res.* **53** (1977) 95-100.
14. Roozen, J.P. and Boxtel, L. van,'Half-automatische bepaling van stikstof in levensmiddelen', *De Ware(n) Chemicus* **9** (1979) 192-200.
15. Englyst, H.N. and Cummings, J.H.,'Simplified method for the measurement of total non-starch polysaccharides by gas-liquid chromatography of constituent sugars as alditol acetates', *Analyst* **109** (1984) 937-942.
16. Blumenkrantz, N. and Asboe-Hansen, G.,'New method for quantitative determination of uronic

acids', *Anal. Biochem.* **54** (1973) 484-489.
17. Voragen, A.G.J., Schols, H.A. and Pilnik, W.,'Determination of the degree of methylation and acetylation of pectins by HPLC', *Food hydrocolloids* **1** (1986) 65-70.
18. Aucamp, M.C., Grieff, J.T., Novellie, L., Papendick, B., Schwartz, H.M. and Steer, A.G.,'Kaffircorn malting and brewing studies. VIII. Nutritive value of some kaffircorn products', *J. Sci. Food Agric.* **12** (1961) 449-456.
19. Holdt, M.M. von and Brand, J.C.,'Kaffircorn malting and brewing studies. VI. Starch content of kaffir beer brewing materials', *J. Sci. Food Agric.* **11** (1960) 463-466.
20. Daiber, K.H.,'Enzyme inhibition by polyphenols of sorghum grain and malt', *J. Sci. Food Agric.* **26** (1975) 1399-1411.
21. Price, M.L. and Butler, L.G.,'Tannins and nutrition', *Station bulletin* **272**, Purdue University, West-Lafayette, USA (1980) 1-37.
22. Price, M.L., Scoyoc, S. van and Butler, L.G.,'A critical evaluation of the vanillin reaction as an assay for tannin in sorghum grain', *J. Agric. Food Chem.* **26** (1978) 1214-1218.
23. Fry, S.C.,'The growing plant cell wall: Chemical and metabolic analysis', Longman Scientific and Technical, Harlow, U.K. (1988).
24. Englyst, H.N. and Kingman, S.M., in 'Dietary Fiber' (D. Kritchevsky, C. Bonfield and J.W. Anderson, eds.), Plenum Publishing Corporation, USA (1990) pp 49-65.
25. Mares, D.J. and Stone, B.A.,'Studies on wheat endosperm. II. Properties of the wall components and studies on their organization in the wall', *Aust. J. Biol. Sci.* **26** (1973) 813-830.
26. Karim, A. and Rooney, L.W.,'Characterization of pentosans in sorghum grain', *J. Food Sci.* **37** (1972) 369-371.
27. Reichert, R.D. and Youngs, C.G.,'Dehulling cereal grains and grain legumes for developing countries. II. Chemical composition of mechanically and traditionally dehulled sorghum and millet', *Cereal Chem.* **54** (1977) 174-178.
28. Blessin, C.W., Anderson, R.A., Deatherage, W.L. and Inglett, G.E.,'Effect of alkali dehulling on composition and wet-milling characteristics of sorghum grain', *Cereal Chem.* **48** (1971) 528-532.
29. Jones, R.W. and Beckwith, A.C.,'Proximate composition and proteins of three grain sorghum hybrids and their dry-mill fractions', *J. Agric. Food Chem.* **18** (1970) 33-36.
30. Taylor, J.R.N. and Schüssler, L.,'The protein composition of the different anatomical parts of sorghum grain', *J. Cereal Sci.* **4** (1986) 361-369.
31. Mossé, J.,'Nitrogen to protein conversion factor for ten cereals and six legumes or oilseeds. A reappraisal of its definition and determination. Variation according to species and to seed protein content', *J. Agric. Food Chem.* **38** (1990) 18-24.
32. Fincher, G.B. and Stone, B.A., in 'Advances in Cereal Science and Technology' Vol 8 (Y. Pomeranz, ed.), Am. Assoc. Cereal Chem. Inc., St. Paul, USA (1986) pp 207-295.
33. Gruppen, H., Marseille, J.P., Voragen, A.G.J., Hamer, R.J. and Pilnik, W.,'Mild isolation of water-unextractable cell wall material from wheat flour: Composition of fractions obtained with emphasis on non-starch polysaccharides', *J. Cereal Sci.* **9** (1989) 247-260.
34. Viëtor, R.J., Voragen, A.G.J., Angelino, S.A.G.F. and Pilnik, W.,'Non-starch polysaccharides in barley and malt: a mass balance of flour fractionation', *J. Cereal Sci.* **14** (1991) 73-83.

CHAPTER 3

The Selective Extraction of Glucuronoarabinoxylans from Sorghum Endosperm Cell Walls using Barium and Potassium Hydroxide Solutions

Various populations of hemicellulosic material were solubilized from water-unextractable cell wall material (WUS) of sorghum (Sorghum vulgare *cv. Fara Fara) by sequential extractions with alkali. Saturated $Ba(OH)_2$-solutions, followed by distilled water, 1M KOH, 4M KOH and 4M KOH containing 4%(w/v) H_3BO_3 were used to extract primarily glucuronoarabinoxylans (GAX) from sorghum WUS. Cellulose remained in the residue. In total over 90% of all GAX originally present in the WUS were recovered, particularly in the saturated $Ba(OH)_2$, 1M KOH and 4M KOH extracts. Saturated $Ba(OH)_2$ was found to be the most selective of the extractants tested for GAX. (1→3),(1→4)-β-D-Glucans were found predominantly in the fraction obtained by washing with water after the extraction with saturated $Ba(OH)_2$. All extracted arabinoxylans were highly substituted (arabinose/xylose > 1) and contained, besides L-arabinose and D-xylose, the acidic sugars D-glucuronic, 4-O-methyl-D-glucuronic and D-galacturonic acid. The average molecular weight ranged from 210,000 - 1,300,000, which corresponds with DPs of c.1,500 to 9,300. The selectivity of the extractant was apparently enhanced by the presence of a bivalent cation.*

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INTRODUCTION

Sorghum is now being used increasingly in the brewing of lager-type beers. Sorghum flour contains 5-6%(w/w) cell wall material, which consists largely of non-starch polysaccharides (NSP), although 6.5% of protein is also present[1]. The cell walls appear to be resistant to degradation during malting[2]. This may explain the limited accessibility of the gelatinized starch for α-amylases at later stages of the brewing process. Moreover, excessive binding of water by insoluble arabinoxylans in the spent grains could be a cause of impaired wort filtration[3].

Cereal cell wall polysaccharides comprise mainly arabinoxylans, (1→3),(1→4)-ß-D-glucans and cellulose. Generally, only a portion of the NSP is extractable in water directly. The structures of the water-extractable polysaccharides have been studied for several cereals[4-6], but the structure of water-extractable hemicelluloses from sorghum endosperm has received little attention. Only a small portion of the NSP is extractable in water and is composed of arabinogalactan, (1→3),(1→4)-ß-D-glucan and arabinoxylan populations[1]. A water-extractable endosperm (1→3),(1→4)-ß-D-glucan from sorghum was isolated and characterized by Woolard *et al.*[7]. This glucan, with a DP of 26 (M_w = 4,230) was composed of ß-(1→3)- and ß-(1→4)-linked D-Glc units in a molar ratio of 3/2. They also isolated two extractable (1→4),(1→6)-α-D-glucans with DPs of 23 and 20 that differed in the ratio of (1→4)-/(1→6)-linkages and distribution of branch points[8].

Regarding the water-unextractable cell wall material (WUS), Woolard and co-workers isolated water-unextractable hemicellulosic materials from sorghum pericarp. They described a glucuronoarabinoxylan composed of Ara, Xyl, GlcA and 4OMeGlcA in a ratio of 15/18/2/1[9]. Four other heteroxylans composed of these sugars and Glc and Gal, in different ratios and with varying DP and degree of substitution have also been described[10,11]. All five xylans had a backbone of (1→4)-ß-linked Xyl*p* residues in common, with single Ara*f* units attached at *O*-3 or at both *O*-2 and *O*-3 of certain Xyl units and GlcA and 4OMeGlcA attached through *O*-2 of Xyl. According to results obtained by permethylation analysis, some of the Ara units are substituted at either *O*-2 or *O*-3. When present, Gal and Glc mainly occur as non-reducing end-groups[10,11]. The presence of linear side chains of about 10 to 12 (1→4)-ß- and (1→3)-ß-linked Glc units has also been described, however[10]. As far as we are aware, no research has been dedicated toward the isolation and characterization of the water-unextractable hemicelluloses from sorghum endosperm.

The main aim of this study was to determine some structural features of water-unextractable sorghum cell wall hemicelluloses and the arabinoxylans in particular.

EXPERIMENTAL

Materials

Water-unextractable cell wall material (WUS) was obtained from flour (milled through a 0.5mm sieve) from *Sorghum vulgare* cv. Fara Fara as described previously[1]. In brief, this procedure consisted of collecting endosperm cell wall material after removing lipids, proteins and starch as completely as practically possible by extraction with *n*-hexane, sodium dodecyl sulphate and hot water after a wet sieving step and incubation with α-amylase.

Extraction procedure

The procedure used to extract hemicellulosic polymers from WUS was based on a method described by Gruppen *et al.*[12], but extended with subsequent KOH extractions. Cell wall material (2g) was suspended in a saturated $Ba(OH)_2$ solution (400ml) containing 1%(w/v) (0.26M) $NaBH_4$ to prevent alkaline degradation, and stirred for 16h at room temperature (RT; *c.*20°C). The suspension was centrifuged at 17,700*g* for 45min. The residue was re-extracted twice with the same solvent (200ml, 7h and 16h, respectively, at RT). The three extracts were neutralized separately using HOAc and dialysed extensively against 0.2M NaOAc buffer, pH5 at 4°C and distilled water. The resultant extracts are referred to as BE1.1, BE1.2 and BE1.3, respectively. The residue was resuspended in distilled water (200ml) and neutralized using HOAc. After stirring for 20h (RT) the suspension was centrifuged (45min, 17,700*g*) and re-extracted using distilled water (200ml) for 7h at RT. The extracts were dialysed extensively against distilled water, and the resultant extracts are referred to as BE2.1 and BE2.2. Next, extract 1K4 was obtained by suspending the residue in 1M KOH (250ml), containing 1%(w/v) $NaBH_4$, stirring for 16h at 4°C, centrifugation (45min, 17,700*g*), neutralization with HOAc and dialysis against distilled water. Similarly, extracts 1K20, 4K and 4KB were prepared, using 1M KOH for 7h at RT, 4M KOH for 16h at RT and 4M KOH containing 4%(w/v) H_3BO_3 for 7h at RT (250ml, all containing 1%(w/v) $NaBH_4$), respectively. The H_3BO_3 in the latter extractant favours the extraction of glucomannans, that may be present[13]. The final residue (RES) was neutralized and dialysed against distilled water. The extracts and the residue were stored at -20°C and thawed or freeze-dried before use, depending on further investigations.

For comparison, WUS was also extracted by saturated $Ca(OH)_2$ containing 1%(w/v) $NaBH_4$ by a procedure similar to that described above for BE1.1.

The solubility behaviour of barley (1→3),(1→4)-ß-D-glucan was investigated by dissolving this glucan (1mg/ml solvent, RT, stirring continuously until 4h) in distilled water, saturated $Ba(OH)_2$ and $Ca(OH)_2$, and KOH solutions (0.02M, 0.2M, 0.33M, 1M and 4M). All solvents were tested with and without the presence of 1%(w/v) $NaBH_4$. The presence of undissolved materials was determined after centrifugation, before and after neutralization (pH5, HOAc).

Graded ethanol precipitation was performed as described by Gruppen *et al.*[14]. The extracts were dissolved in 0.1M sodium succinate buffer, pH4, prior to the addition of ethanol, to suppress the charge on the uronic acid side groups.

Analytical methods

Neutral sugars were analysed as alditol acetates by gas chromatography according to Englyst and Cummings[15], using myo-inositol as internal standard. Extracts were treated with 72%(w/w) H_2SO_4 (1h, 30°C) prior to hydrolysis with 1M H_2SO_4 for 3h at 100°C and the released constituent sugars were analysed as their alditol acetates. Also *4-O-methyl-D-glucuronic acid* was determined

as its alditol acetate. In this case the carboxyl groups were reduced using $NaBH_4$ prior to hydrolysis[16]. Alditol acetates were separated on a 15m x 0.53mm i.d. DB 225 column (J&W Scientific, Folsom, California, USA) using a Carlo Erba 4200 GC. The oven temperature was set at 230°C and the F.I.D. detector at 260°C. H_2 was used as carrier gas.

Total uronic acid content was determined as anhydro-glucuronic acid (AUA) by the *m*-hydroxydiphenyl assay[17] using an auto-analyser (Skalar Analytical B.V., Breda, The Netherlands)[18]. Sodium tetraborate was added to the H_2SO_4 to minimize differences in response between GalA and GlcA. Corrections were made for interference of neutral sugars present in the samples.

Glucuronic and galacturonic acid were quantified by high-performance anion-exchange chromatography (HPAEC) using a Dionex BioLC GPM-II quaternary gradient module (Sunnyvale, CA, USA) equipped with a CarboPac PA-100 column (250 x 4mm) in combination with a CarboPac PA guard column (25 x 3mm). H_2SO_4 hydrolysed samples (20μl) were injected using a Spectra Physics SP8780 autosampler equipped with Tefzel rotor seal in a 7010 Rheodyne injector valve. The column was eluted (20°C, 1ml/min) with a linear gradient of NaOAc and NaOH in which the concentrations of both solutes increased simultaneously (NaOAc 0→0.25M and NaOH 0→0.025M) over a period of 25min, followed by 0.25M NaOAc in 0.025M NaOH isocratic for 5min. After each analysis the column was rinsed for 5min with 1M NaOAc in 0.1M NaOH, and equilibrated in 0.1M NaOH for 15min. 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 (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.

Protein contents of the extracts were determined by a modification of the Lowry-method[19] using bovine serum albumin as standard.

Methylation analysis Carboxyl groups in all fractions were reduced according to Taylor and Conrad[16] using sodium borodeuteride, and the reduced fractions were subsequently methylated by a modification of the Hakomori method[20], dialysed and dried in a stream of air. The reduction and methylation steps were repeated in order to improve the completeness of both reactions. Glycitol acetates were prepared as described above except that samples were hydrolysed using 2M TFA (1h, 121°C). After derivatisation, the samples were dried in a stream of air (at <10°C to prevent the loss of pentitol acetates). The partially methylated glycitol acetates were dissolved in ethyl acetate and separated and quantified on a fused silica capillary column (30m x 0.32mm; wall coated with DB1701; 0.25μm; J&W Scientific, Folsom, California, USA) using a Carlo Erba Fractovap 4160 GC equipped with a F.I.D. detector set at 280°C. Helium was used as carrier gas. The temperature program was set as follows; 80→180°C at 20°C/min, 180→230°C at 2°C/min and 230°C isothermal for 3min. The derivatives were quantified according to their effective carbon response (ECR) factors[21]. The identity of the compounds was confirmed by gas chromatography-mass spectrometry (GC-MS, Hewlett Packard, USA) using a mass selective detector (MSD) 5970B coupled to a HP 5890 equipped with a fused silica column (CPSIL 19CB, 26m x 0.22mm; 0.18μm; Chrompack Nederland B.V., Middelburg, The Netherlands). The temperature program was 160→185°C at 0.5°C/min, 185→230°C at 10°C/min and 230°C for 5.5min. The 3,4- and 2,3-*O*-methylated xylitol acetates were co-eluted, as were the 2- and 3-*O*-methylated xylitol acetates, and their relative amounts were estimated from the relative abundance of the ions at m/z 117 and m/z 118, and m/z 118 and m/z 129+130, respectively.

Ion-exchange chromatography Freeze-dried extracts (20mg) were dissolved in 5mM NaOAc buffer pH5.0 (2ml) and were applied to a DEAE-Sepharose CL-6B column (20cm x 2cm; Pharmacia) equilibrated with the same buffer. After loading with sample, the column was washed with the same buffer (50ml) and then eluted successively with linear gradients of 0.005→1.0M

NaOAc buffer, pH5.0 (130ml), and 1.0→2.0M NaOAc buffer, pH5.0 (50ml) followed by 2.0M NaOAc buffer (25ml) and 0.5M NaOH (50ml) at 0.75ml/min. Fractions (2.5ml) were assayed for uronic acids as described above, and for neutral sugars using the orcinol method[22]. The neutral sugar compositions of pooled fractions were determined as described above.

Size-exclusion chromatography Freeze-dried BE1.1 extract (20mg) was dissolved in 0.1M NaOAc buffer pH5.0 (2ml) and applied to a Sephacryl S1000 column (115cm x 1.4cm; Pharmacia; separation range for dextrans: M_w $5x10^5$ - est. $> 10^8$), which was eluted with 0.1M NaOAc buffer pH5.0. Fractions (2ml) were assayed for total neutral sugar[22] and uronic acid[18] contents, using Ara and GlcA, respectively, as standards. Every fraction was analysed for neutral sugar composition as described above. The void and total volumes of the column were measured from the elution volumes of *E. coli* cells and Glc, respectively.

High-performance size-exclusion chromatography (HPSEC) was performed using a SP 8700 HPLC (Spectra Physics) equipped with three Bio-Gel TSK columns (each 300x7.5mm; Bio-Rad Labs) in series: 60XL (M_w exclusion limit for dextrans of $5x10^7$); 40XL (M_w exclusion limit for dextrans of 10^6) 30XL (M_w exclusion limit for dextrans of $2x10^5$) in combination with a TSK XL guard column (40 x 6mm) and eluted at 30°C with 0.4M NaOAc buffer (pH3) at a flow rate of 0.8ml/min. The column effluent was monitored using a Shodex SE-61 refractive index detector. The weight average molecular weight was determined using a Dawn-F/HT multi-angle laser light scattering detector and an ERMA refractive index detector connected in series. Separation was performed with the HPSEC column set, run under the same conditions.

Results and Discussion

Extraction and sugar composition of polysaccharides

As with other cereals[23], a substantial proportion of the polysaccharides in the cell walls of sorghum kernels consists of arabinoxylans[1]. Other commonly present polysaccharides are (1→3),(1→4)-ß-D-glucans and cellulose. An attempt was made to isolate the arabinoxylans and to determine some of their structural characteristics. The arabinoxylan complex is held in the cereal cell walls by alkali-labile linkages. WUS from sorghum is devoid of lignin, but contains phenolic acids, such as coumaric acid (0.2%,w/w) and ferulic acid (0.6%,w/w)[1], which may be involved in oxidative cross-linking of polysaccharides and other cell wall components. Acetyl groups have also been determined in sorghum WUS[1]. Since pectin is absent, it was assumed that these acetyl groups were attached to the xylan backbone. Treatment of the cell walls with alkali will saponify these, and other ester-linkages, causes cellulose to swell and disrupts the hydrogen bonds between hemicelluloses and cellulose, resulting in the solubilization of hemicelluloses. Nine fractions containing hemicellulosic material were obtained by sequential extractions of sorghum WUS by saturated $Ba(OH)_2$ solutions (BE1.1, BE1.2, BE1.3), distilled water (at pH5; BE2.1, BE2.2), 1M KOH at 4°C (1K4), 1M KOH at 20°C (1K20), 4M KOH (4K) and 4M KOH containing 4% H_3BO_3 (4KB). A water- and alkali-unextractable cellulose-rich residue (Res) remained (Table I). Our extraction procedure yielded over 90% of the GAX originally present in the WUS, expressed as the sum of Ara, Xyl and AUA (Table I). A

TABLE I. Molar sugar compositions of fractions of sorghum WUS obtained by sequential alkaline extraction[a].

	BE1.1	BE1.2	BE1.3	BE2.1	BE2.2	1K4	1K20	4K	4KB	Res	WUS
Rha	0.2	0.3	0.5	0.8	0.9	0.7	0.4	0.3	0.3	0	0.3
Ara	42.4	44.1	42.5	31.5	32.7	24.8	30.5	26.0	22.9	8.8	25.1
Xyl	42.2	38.7	36.6	27.6	29.9	29.9	33.8	33.7	22.6	8.5	26.6
Man	0.3	0.6	0.8	1.3	2.4	0.0	1.5	0.9	15.2	3.4	1.4
Gal	2.4	2.3	2.6	3.2	2.8	2.7	3.1	3.9	2.6	0.9	2.1
Glc	2.8	3.5	7.2	27.1	21.8	32.2	19.5	27.3	31.0	74.0	36.9
AUA	9.8	9.9	9.8	8.5	9.5	9.5	11.3	7.8	5.4	4.0	7.7
Ara/Xyl	1.01	1.14	1.16	1.14	1.09	0.83	0.90	0.77	1.01	1.04	0.94
Yield											
% WUS[b]	15.1	4.2	2.5	3.1	0.5	11.1	1.8	9.3	1.5	26.5	
% GAX[c]	37.7	10.1	5.3	4.7	0.6	16.3	2.5	13.0	0.6	9.1	

[a] See *Experimental* for a description of the extraction procedure and an explanation of the extract codes;
[b] Total sugar recovered (mg)/total sugar present in WUS (mg) x 100;
[c] (Ara+Xyl+AUA,mg) /(Ara+Xyl+AUA) totally recovered in WUS (mg) x 100.

major part, *c.*53%, was obtained by extraction with saturated $Ba(OH)_2$ solutions. A further 29% was obtained in the 1K4 and 4K extracts.

BE1.1 contained a rather pure arabinoxylan population with a very high degree of substitution as indicated by the high Ara to Xyl ratio (Ara/Xyl). Only minor amounts of Rha, Man, Gal and Glc were present in this fraction. Moreover, the molar uronic acid/Xyl ratio was *c.*0.23. The stuctures of these polysaccharides showed considerable similarities to GAX from maize coleoptiles[24], but they are much more complex than those found in wheat and barley. The degree of substitution is lower for the arabinoxylans in these cereals (Ara/Xyl $\approx$ 0.6 and 0.7 for wheat[25] and barley[26], respectively), and uronic acids are almost absent.

The uronic acid (determined as anhydro-uronic acid (AUA)) present in the BE1.1 fraction comprised GlcA, GalA and 4OMeGlcA presumably linked to the xylan backbone. Hydrolysis of the uronic acid-Xyl glycosidic linkages cannot be achieved without some degradation of sugars. For calculating the relative amounts of the constituent uronic acids, it was assumed that the degree of incompleteness of hydrolysis was the same for GlcA-Xyl, 4OMeGlcA-Xyl and GalA-Xyl glycosidic linkages. Based on this assumption, GlcA accounted for 90% (on molar basis) of the uronic acids, whereas both 4OMeGlcA and GalA accounted for only 5%. Therefore the polysaccharide was termed a glucuronoarabinoxylan (GAX). Fractions BE1.2 and BE1.3 were composed of polymers with similar characteristics with a slightly higher degree of substitution (Ara/Xyl). The amounts of uro-

TABLE II. Solubility of barley (1→3),(1→4)-ß-D-glucan (1mg/ml).

Solution	[OH⁻]	0.26M $NaBH_4$ added		Without $NaBH_4$	
		as is[a]	pH5[b]	as is	pH5
Distilled water		±	±	±	±
Saturated $Ba(OH)_2$	0.33	−	+	−	+
Saturated $Ca(OH)_2$	0.02	±	±	±	±
0.02M KOH	0.02	±	±	±	±
0.2M KOH	0.20	+	+	nd[c]	nd
0.33M KOH	0.33	+	+	+	+
1M KOH	1.0	+	+	nd	nd
4M KOH	4.0	+	+	nd	nd

[a] Under alkaline conditions; [b] After neutralization with HOAc; [c] Not determined;
+ Completely dissolved; ± Some undissolved material remains; − Undissolved.

nic acids were similar, although the ratios of the constituent uronic acids were somewhat different (GlcA/4OMeGlcA/GalA ≈ 80/4/16). In addition, higher levels of Glc were detected. It was decided not to combine these fractions, therefore.

Saturated $Ca(OH)_2$ solutions containing 1% $NaBH_4$ solubilized GAX with a purity similar to that solubilized by the saturated $Ba(OH)_2$ solutions (results not shown). The sugar compositions of the two extracts were identical, but the yields were lower in the case of $Ca(OH)_2$, which may be explained by the lower hydroxyl ion concentration (0.02M *vs.* 0.33M for $Ba(OH)_2$ solutions). Only 28% of all arabinoxylans present in WUS could be solubilized by $Ca(OH)_2$. It is asssumed that both Ba^{2+} and Ca^{2+} can form insoluble complexes with glucomannans[27] and (1→3),(1→4)-ß-D-glucans[25]. Table II shows the solubility of barley (1→3),(1→4)-ß-D-glucan in several alkaline solutions. The glucan was soluble in KOH ≥ 0.2M but not in saturated $Ba(OH)_2$. Obviously the Ba^{2+} prevented the solubilization of the polysaccharide. In the case of saturated $Ca(OH)_2$ the hydroxyl ion concentration did not meet the requirements for dissolving the polysaccharide. The (1→3),(1→4)-ß-D-glucan could be precipitated from a solution in 0.5M KOH by addition of saturated $Ca(OH)_2$, however. In both cases, the polysaccharide could be solubilized by neutralizing the solutions. It was concluded, therefore, that the presence of a bivalent cation may be of importance for the selectivity of extraction.

Treatment with saturated $Ba(OH)_2$ apparently modified the residual 'WUS' in such a way that polysaccharides remaining after $Ba(OH)_2$ extraction, especially Glc containing polysaccharides, could be extracted subsequently using distilled water (pH5). The yields of these fractions (3% and 0.5% of WUS for BE2.1 and BE2.2, respectively) were quite low. It is assumed that the bulk of the GAX in these BE2 fractions was obtained by

extracting these polysaccharides, together with residual $Ba(OH)_2$ from the residue. The GAX composition (Ara/Xyl/uronic acids and uronic acid composition) was similar to the GAX in BE1.3.

By treatment with KOH solutions containing 1% $NaBH_4$, less substituted GAX could be solubilized and again more glucan was co-extracted. Extraction with 1M KOH at 4°C solubilized another 16% of the arabinoxylans and 4M KOH (20°C) another 13%. The uronic acids in these fractions comprised mainly GlcA (85%) and GalA (15%). Only traces of 4OMeGlcA were detected. Addition of 4%(w/v) H_3BO_3 to 4M KOH resulted in high amounts of Man in fraction 4KB. Presumably this Man is present in glucomannans as described for other cereals, such as wheat[12,28] and barley[26]. Only 0.6% of the arabinoxylans were recovered in this fraction. The bulk of the Glc remained in the final residue (Res) presumably as cellulose. Other polysaccharides present in Res were GAX and Man containing polysaccharides, presumably glucomannans.

Apart from polysaccharides, all the fractions obtained by $Ba(OH)_2$ extraction contained *c.*3% protein (3.8%, 3.0% and 2.9%(w/w), for BE1.1, BE1.2 and BE1.3, respectively). The fractions obtained with distilled water after $Ba(OH)_2$ extraction contained the lowest amounts of protein (1.6% and 0.9%(w/w), for BE2.1 and BE2.2, respectively). The protein contents of 1K4, 1K20, 4K and 4KB were somewhat higher, 5.1%, 6.0%, 3.1% and 7.1%(w/w), respectively. The WUS contained 6.5%(w/w) protein, of which 42% was extracted by the alkaline extraction sequence. Some of this protein may have been covalently linked to the hemicelluloses, in particular to arabinoxylans, although the alkaline conditions may also have had solubilizing effects on the proteins that were not attached to polysaccharides.

Linkage composition

Methylation analysis confirmed that the alkali extracted polysaccharides comprised mainly highly substituted arabinoxylans (Table III). In BE1.1 *c.*45% of the Xyl residues were monosubstituted, with branch points mainly at the *O*-3-position. The polysaccharide also contained a large amount of *O*-2, *O*-3-disubstituted Xyl (17% of the Xyl units). A further 28% of the Xyl units in the (1→4)-xylan backbone was unsubstituted. Of the Ara units, *c.*88% was present as terminal furanosyl units, presumably linked to the *O*-3-mono-, and *O*-2, *O*-3-disubstituted Xyl. The remainder being 2- and 5-linked Ara residues, may be present in short side-chains attached to the (1→4)-linked Xyl backbone as has been described for rice bran arabinoxylans[29]. The linkage composition of BE1.2 was similar to that of BE1.1, as expected, although some differences were noticed, especially in the ratio unsubstituted/*O*-3-monosubstituted/*O*-2-monosubstituted/disubstituted Xyl which was 6/12/4/5 in BE1.2. In these studies, uronic acids were reduced with $NaBD_4$ prior to methylation, hydrolysis and derivatisation, and therefore, were determined as deuterated Glc and Gal. Samples BE1.1 and BE1.2 contained predominantly deuterated terminal Glc

TABLE III. Methylation analysis (mol%) of $NaBD_4$ reduced alkaline extracts of sorghum WUS[a].

Component	Linkage type	BE1.1	BE1.2	BE2.1	1K4	1K20	4K	4KB	Res	WUS
2,3,5-Me_3-Ara	(Ara*f*)1→	34.9	37.5	24.6	19.8	25.7	25.1	15.3	6.1	19.7
3,5-Me_2-Ara	→2(Ara*f*)1→	2.1	1.9	1.5	1.3	1.6	1.5	0.7	0.4	1.2
2,3-Me_2-Ara	→5(Ara*f*)1→	2.6	3.1	5.4	2.6	3.0	2.7	1.8	0.6	1.6
2,3,4-Me_3-Xyl	(Xyl*p*)1→	0.9	0.9	0.7	2.7	1.1	3.6	1.2	1.8	1.7
2,3-Me_2-Xyl	→4(Xyl*p*)1→	12.1	9.1	5.8	15.5	10.9	13.0	9.6	3.7	11.6
2-Me-Xyl[b]	→4(Xyl*p*)1→ 3↑	19.7	17.1	12.6	10.1	13.0	11.7	9.8	2.6	9.5
3-Me-Xyl[b]	→4(Xyl*p*)1→ ↑2	3.6	5.7	3.7	3.2	4.4	4.0	–[c]	1.0	3.6
Xyl	→4(Xyl*p*)1→ 3↑ ↑2	7.5	7.1	4.4	3.4	5.8	5.0	3.5	1.5	3.6
2,3,4,6-Me_4-Glc	(Glc*p*)1→	6.9	7.6	3.3	5.6	7.0	6.4	3.7	1.8	2.9
2,3,6-Me_3-Glc	→4(Glc*p*)1→	2.3	4.3	22.7	24.9	15.7	18.2	33.4	73.0	36.2
2,4,6-Me_3-Glc	→3(Glc*p*)1→	–	0.3	10.9	4.6	1.5	0.5	–	–	1.8
Glc	→4(Glc*p*)1→ 3↑ ↑6	5.7	3.4	2.2	3.0	7.0	4.4	7.2	4.0	3.7
2,3,4,6-Me_4-Gal	(Gal*p*)1→	1.4	1.4	1.3	2.3	2.3	3.4	2.2	0.9	1.5
2,3,6-Me_3-Gal	→4(Gal*p*)1→	0.3	0.6	0.8	1.2	1.0	0.6	–	–	–
2,3,6-Me_3-Man	→4(Man*p*)1→	–	–	–	–	–	–	11.7	2.8	–

[a] See *Experimental* for a description of the extraction procedure and an explanation of the extract codes; [b] Calculated from the FID response and the ratio 2-Me-Xyl/3-Me-Xyl determined by GC-MS[12]; [c] Absent.

and Gal units, and only traces of their hydrated forms. This means that the originally extracted polysaccharide had hardly any terminal Glc and Gal residues. The uronic acids are assumed to be attached to the (1→4)-xylan backbone at the *O*-2 position of Xyl[23], although our data are not entirely consistent with this view since more uronic acid groups were found than *O*-2-substituted Xyl.

Neutral sugar analysis of BE2.1 (Table I) suggested the presence of a glucan that was co-extracted with some of the GAX, probably a (1→3),(1→4)-ß-D-glucan. This was confirmed by the methylation analysis: Glc was linked predominantly at both the 1 and 3 and the 1 and 4 positions. Assuming that the Glc units in BE2.1 are all present in this type of glucan, the ratio of (1→3)- to (1→4)-linked Glc in sorghum (1→3),(1→4)-ß-D-glucan is *c.*1/2. This value is different from that of 3/2 for a water-extractable mixed linkage ß-D-glucan from sorghum reported previously[7], but similar to that of barley (1→3),(1→4)-ß-D-glucan[30]. The composition of the GAX fraction in BE2.1 was similar to the previous extracts.

The 1M and 4M KOH extracts contained GAX, together with considerable amounts of

glucan with a lower ratio of (1→3)- to (1→4)-linked Glc. These glucans could be linked covalently to the GAX as described by Woolard *et al.*[10]. Because of the presence of both (1→3)- and (1→4)-linked Glc, however, it is more probable that these were simply co-extracted (1→3),(1→4)-ß-D-linked glucans. The large amount of 2,3,6-Me_3-Man in 4KB indicated the presence of glucomannans, which have been found in various cereal cell walls[23]. Some residual glucomannans also occurred in the residue fraction (Res). The majority of this fraction consisted of cellulose, as indicated by the large amounts of (1→4)-linked Glc together with the insolubility of the residue in either water or alkali.

Comparison of the results of the methylation analysis with the sugar compositions (Table I) shows that the amounts of terminal Glc (upon reduction) were lower than the amounts of uronic acid determined directly in the fractions in all cases. This suggests a substantial under-estimation of GlcA content in the former analysis, even though the reduction and methylation was repeated.

Homogeneity

The fractions obtained in the highest yields and containing the bulk of the GAX (BE1.1, BE2.1, 1K4 and 4K) were studied for homogeneity by anion-exchange chromatography, size-exclusion chromatography and graded ethanol precipitation. Fig 1 shows the elution pattern (sugar content) from a DEAE-Sepharose CL-6B-column. Yields and sugar compositions have been estimated in several fractions, which were pooled based on their elution behaviour (Table IV).

BE1.1 was the most homogeneous fraction. Almost all the material was bound to the column, probably due to the presence of uronic acids in all the arabinoxylan molecules, although some protein was present. The uronic acids are probably evenly distributed over the polysaccharides, since the material eluted in one peak with similar sugar compositions and uronic acid/Xyl ratios in earlier and later eluted material. Only a small proportion (6% of the total fraction), which was quite rich in Glc, did not bind. Another 5.4% of the fraction, which again contained relatively more Glc, was eluted with 0.5M NaOH. The homogeneity of BE1.1 was further studied by size-exclusion chromatography on a Sephacryl S1000 column, the sugar composition being determined in every fraction. As can be seen from the elution pattern (Fig 2), BE1.1 comprised a population of polysaccharides that varied widely in hydrodynamic volume. The sugar compositions of all the fractions were similar, however, although slightly more Glc and Gal was present in the fractions of lower hydrodynamic volume. Thus, the relative abundances of Ara and uronic acid side groups were not related to the hydrodynamic volume. For arabinoxylans extracted from wheat it was shown[14] that the Ara/Xyl ratio of material that was eluted early from a Sephacryl S1000 column, which had a greater hydrodynamic volume, was greater than that of material that was eluted later. The hydrodynamic volume of an arabinoxylan has generally been associated with the degree of substitution and distribution of the substitu-

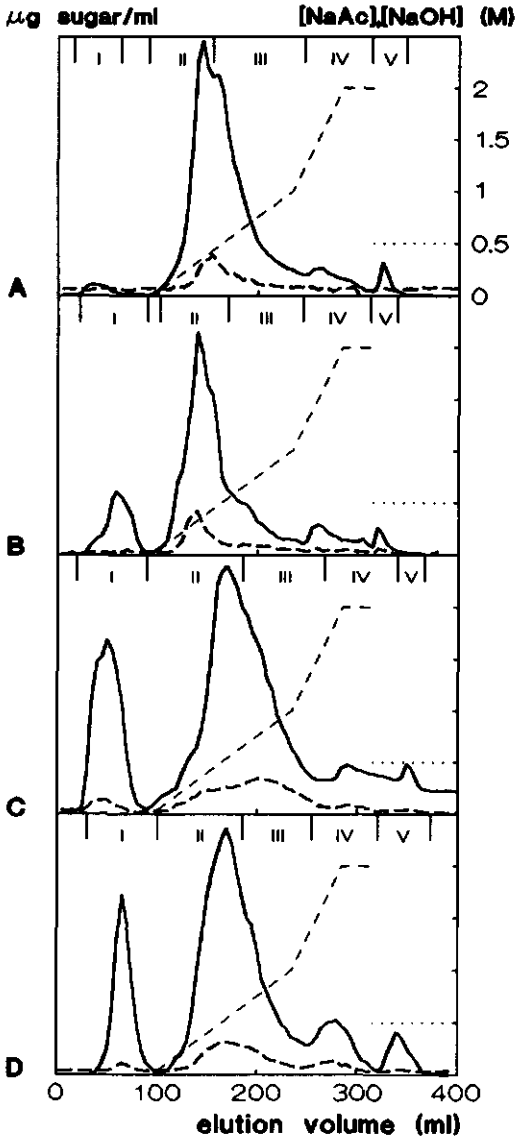

FIG 1. Fractionation of extracts on DEAE-Sepharose Cl-6B eluted with a linear gradient of NaOAc buffer, pH5.0, followed by 0.5M NaOH; A, BE1.1; B, BE2.1; C, 1K4; D, 4K (See *Experimental* for an explanation of the extract codes). The total sugar content (—) is expressed as Ara equivalents, as determined by the orcinol method. The total uronic acid content (— —) is presented as GlcA equivalents, as determined by the *m*-hydroxydiphenyl assay. (--- NaOAc-concentration; ···· NaOH-concentration).

TABLE IV. Molar sugar compositions and yields[a] of pools obtained by chromatography of extracts on DEAE-Sepharose CL-6B.

	I	II	III	IV	V
BE1.1[b] pool					
Rha	0.0	0.0	0.4	0.0	0.0
Ara	8.2	40.7	40.4	42.2	14.2
Xyl	3.8	41.5	41.8	39.7	14.2
Man	4.0	0.5	0.8	1.1	6.3
Gal	5.0	4.5	2.8	2.8	4.7
Glc	76.7	2.0	3.5	3.8	50.1
AUA	2.3	10.8	10.3	10.5	10.5
Yield	6.0	39.3	40.8	8.5	5.4
BE2.1 pool					
Rha	0.0	0.8	2.0	0.7	0.8
Ara	5.6	41.3	38.9	42.6	37.9
Xyl	5.4	36.3	34.6	33.8	32.6
Man	5.1	1.7	2.0	3.0	4.4
Gal	1.3	4.7	7.1	4.2	5.1
Glc	81.5	2.3	3.1	10.7	12.2
AUA	1.1	12.9	12.3	5.1	7.0
Yield	16.2	46.8	25.0	7.8	4.2
1K4 pool					
Rha	0.4	0.6	2.4	0.0	0.0
Ara	3.9	38.6	34.0	24.3	17.8
Xyl	5.5	34.7	32.6	32.5	55.9
Man	2.0	1.2	3.1	2.0	2.1
Gal	0.6	3.4	4.4	1.7	2.1
Glc	85.6	6.3	9.0	33.9	15.2
AUA	2.0	15.2	14.5	5.6	6.9
Yield	29.0	36.7	26.0	5.3	3.0
4K pool					
Rha	0.0	0.7	0.7	0.4	0.6
Ara	16.4	40.3	39.1	20.2	16.8
Xyl	22.5	40.6	40.4	33.7	42.5
Man	7.5	1.4	1.6	1.2	3.2
Gal	5.5	4.7	5.3	5.6	3.8
Glc	46.5	3.2	3.6	32.2	25.9
AUA	1.6	9.1	9.3	6.6	7.2
Yield	19.0	37.2	28.9	8.8	6.1

[a] Yields are expressed as % of total recovered; [b] See *Experimental* for an explanation of the extract codes.

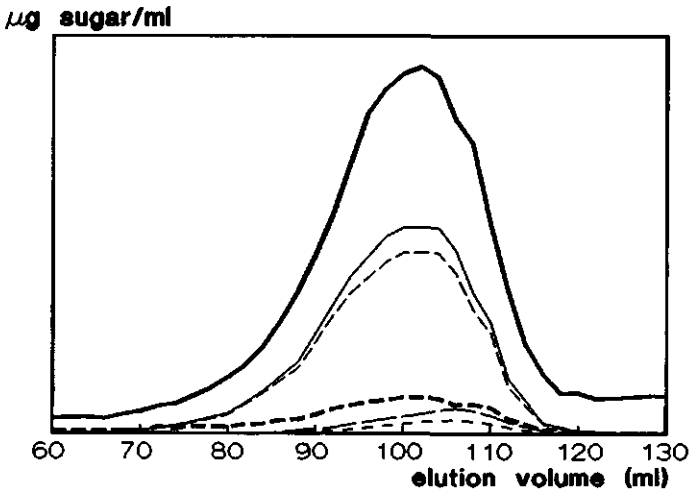

FIG 2. Chromatography of the BE1.1 extract on Sephacryl S1000 in 0.1M NaOAc buffer, pH5.0, and the distribution of the various sugars in the column effluent. The total sugar content is expressed as Ara equivalents as determined by the orcinol method. The total uronic acid content is presented as GlcA equivalents, as determined by the *m*-hydroxydiphenyl assay. The individual sugars were analysed as their alditol acetates by GLC. (— total sugar; — AUA; — Ara; — Xyl; — — Glc; - - Gal; — - Ara/Xyl).

ents. In the case of sorghum GAX, it is likely that the backbone length plays an important role, as well as an uneven distribution of Ara side groups and the charge density.

Another indication of the uniformity of the $Ba(OH)_2$ extracted arabinoxylans was their behaviour upon graded ethanol precipitation. Only with 70%(v/v) ethanol the bulk of the GAX material (80%) precipitated. Very small amounts were precipitated with 60%(v/v) and 80%(v/v) ethanol (11.5 and 8.7%, respectively; results not shown). Similar results were obtained for GAX from wheat grain pericarp tissue[31]. The presence of uronic acid side groups does not appear to account for the inability to fractionate the sorghum GAX by ethanol precipitation since similar results were obtained when the charge on the uronic acid side groups was suppressed by dissolving the GAX in 0.1M sodium succinate buffer (pH4) before the addition of ethanol. Methylation analysis of the three ethanol precipitated fractions did not show significant differences with respect to their constituent sugars or glycosidic linkage compositions (results not shown). For barley and wheat it was possible to fractionate arabinoxylans according to their Ara/Xyl ratio by graded ethanol precipitation[27,14]. In both cases, arabinoxylan populations with Ara/Xyl ratios of *c.*1.0-1.1 were precipitated with 70%(v/v) ethanol, and the more lowly substituted populations were precipitated with lower ethanol concentrations.

All the extracts examined had similar elution behaviours on DEAE-Sepharose CL-6B (Fig 1). The unbound fraction was enriched in Glc compared with the bound material, but

the amounts of unbound material were much higher in the later extracts. The bound fractions were eluted predominantly in a single peak, starting as soon as the NaOAc gradient was applied. These fractions consisted of rather pure GAX. Ara, Xyl and uronic acid accounted for *c.*89% (81-93%) of the bound fractions. Minor amounts of Rha, Man, Gal and Glc were also present. Some material, which generally contained higher levels of Glc but much lower levels of uronic acids, was eluted when higher concentrations of NaOAc (>1M) were used as eluant (fractions IV). Interestingly, the Ara/Xyl-ratios of the fractions IV of 1K4 and 4K were lower than those of the corresponding fractions IV of BE1.1 and BE2.1. The observation that material containing fewer charged groups was eluted later from the ion-exchange column may be due to an uneven distribution of uronic acid side groups in these fractions. The uronic acid residues in the polymers present in fractions IV may be distributed in a more block-wise way than those in the polymers in the main GAX populations (fractions II and III). It is presumed that all the remaining hemicelluloses (fractions V) were removed from the column by elution with 0.5M NaOH since the recoveries were 96.2, 97.6, 92.4 and 86.9% for BE1.1, BE2.1, 1K4 and 4K, respectively. Only very small amounts of material were obtained in the fractions V; all of them contained some GAX and some glucan. The amounts of material that remained bound to the column after the complete elution procedure were negligible.

The homogeneity of the extracted polymers was also studied by HPSEC (Fig 3). In general, the peak retention times decreased in going from BE1.1 to BE2.1. Thus, the molecular weight distribution shifted towards higher molecular weight ranges, or at least towards higher hydrodynamic volumes. Although the same extractant was used for the first three fractions, the molecular weight distributions shifted towards the higher molecular weight ranges from BE1.1 to BE1.2. The fractions BE2.1 and BE2.2, obtained by washing with water (after neutralization) showed clearly the existence of more than one population of polysaccharides. The population that was eluted at a relatively low retention time (24.2min) apparently had a higher hydrodynamic volume than the population in the $Ba(OH)_2$ extracts. Also fractions with much lower molecular weights were present in the BE2.1 and BE2.2 extracts (RT=28.3min). This might indicate that a lower molecular weight (1→3),(1→4)-ß-D-glucan was co-eluted with high molecular weight GAX, whereas in the $Ba(OH)_2$ extracts only high molecular weight GAX was present. The elution profiles for the fractions extracted with KOH solutions on this HPSEC system showed no further increase in hydrodynamic volume. Since the elution of excluded molecules occurs at a retention time of about 22min, the hydrodynamic volumes of the polymers in these fractions were too large for fractionation in the chromatographic system used. These elution profiles also indicate the presence of more than one population of polysaccharides as evidenced by shoulders on the main peaks.

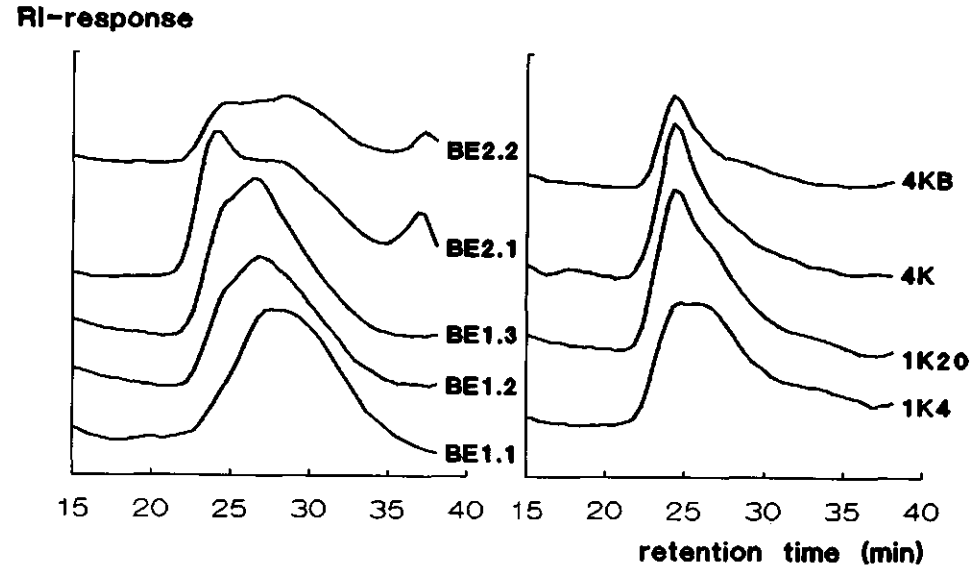

FIG 3. HPSEC of extracts in 0.4M NaOAc buffer, pH3.0. (See *Experimental* for an explanation of the extract codes).

The weight-average molecular weight data obtained by in-line light scattering analysis (Table V) confirmed that the molecular weight increased with the subsequent extractions as was indicated by HPSEC. The M_w of the BE1.1 extract was 210,000 which corresponds to a DP of *c.*1,500, assuming no aggregation occurred. The highest M_w (1,277,000) was found for 4KB suggesting a DP of *c.*9,300. All fractions were clearly polydisperse as indicated by the M_w/M_N ratio.

CONCLUSIONS

Using a sequential alkali extraction method, over 90% of all arabinoxylans present in sorghum cell wall material could be solubilized. The use of saturated $Ba(OH)_2$ solutions was of considerable value in this respect, since it selectively extracted more than 50% of the glucuronoarabinoxylans (GAX). The $Ba(OH)_2$ extracted GAX were quite homogeneous. The GAX populations were essentially similar, although they differed in terms of molecular size, and hence in terms of the degree of polymerization. All the GAX populations were very highly substituted and all contained considerable amounts of GlcA and minor amounts of GalA and 4OMeGlcA. The uniformity of the polysaccharides was illustrated by their behaviour on anion-exchange and size-exclusion columns. The degree of substitution for the $Ba(OH)_2$ extracted GAX was extremely high, with Ara/Xyl ratios

TABLE V. Average molecular weights of the extracts.

Extract[a]	M_w[b]	M_w/M_N[c]	Extract	M_w	M_w/M_N
BE1.1	210,000	1.82	1K4	917,000	1.61
BE1.2	324,000	1.88	1K20	1,149,000	1.50
BE1.3	539,000	1.73	4K	1,167,000	1.50
BE2.1	730,000	2.06	4KB	1,277,000	1.51
BE2.2	701,000	2.25			

[a] See *Experimental* for an explanation of the extract codes; [b] Weight average molecular weight; [c] Number average molecular weight.

from 1.0 to 1.2. For KOH extracted GAX the degree of Ara substitution was lower, but still quite high. These degrees of substitution probably result in very rigid structures, especially when the presence of uronic acid groups is also taken into account. This may be one of the reasons for the poor degradability of sorghum endosperm cell walls.

REFERENCES

1. Verbruggen, M.A., Beldman, G., Voragen, A.G.J. and Hollemans, M.,'Water-unextractable cell wall material from sorghum: isolation and characterization', *J. Cereal Sci.* **17** (1993) 71-82. (Chapter 2 in this thesis)
2. Palmer, G.H.,'Enzymic degradation of the endosperm cell walls of germinating sorghum', *World J. Microbiol. Biotechnol.* **24** (1991) 17-21.
3. Aisien, A.O. and Muts, G.C.J.,'Micro-scale malting and brewing studies of some sorghum varieties', *J. Inst. Brew.* **93** (1987) 328-331.
4. Hoffmann, R.A., Roza, M., Maat, J., Kamerling, J.P. and Vliegenthart, J.F.G.,'Structural characteristics of the cold-water-soluble arabinoxylans from the white flour of the soft wheat variety Kadet', *Carbohydr. Polym.* **15** (1991) 415-430.
5. Bengtsson, S. and Åman, P.,'Isolation and chemical characterization of water-soluble arabinoxylans in rye grain', *Carbohydr. Polym.* **12** (1990) 267-277.
6. Girhammar, U. and Nair, B.M.,'Isolation, separation and characterization of water soluble non-starch polysaccharides from wheat and rye', *Food Hydrocolloids* **6** (1992) 285-299.
7. Woolard, G.R., Rathbone, E.B. and Novellie, L.,'A hemicellulosic ß-D-glucan from the endosperm of sorghum grain', *Carbohydr. Res.* **51** (1976) 249-252.
8. Woolard, G.R., Rathbone, E.B., Novellie, L. and Ohlsson, J.T., 'Structural sudies on the water-soluble gums from the endosperm of sorghum grain', *Carbohydr. Res.* **53** (1977) 95-100.
9. Woolard, G.R., Rathbone, E.B. and Novellie, L.,'Structural studies on a glucurono arabinoxylan from the husk of sorghum grain', *Carbohydr. Res.* **51** (1976) 239-247.
10. Woolard, G.R., Rathbone, E.B. and Novellie, L.,'A heteroxylan from the husk of sorghum grain', *Phytochem.* **16** (1977) 957-959.
11. Woolard, G.R., Rathbone, E.B. and Novellie, L.,'Structural studies on three hemicellulose B fractions from the husk of sorghum grain', *Carbohydr. Res.* **59** (1977) 547-552.
12. Gruppen, H., Hamer, R.J. and Voragen, A.G.J.,'Water-unextractable cell wall material from wheat flour. I. Extraction of polymers with alkali', *J. Cereal Sci.* **16** (1992) 41-51.
13. Fry, S.C.,'The Growing Plant Cell Wall: Chemical and Metabolic Analysis', Longman Scientific and

Technical, Harlow, U.K. (1988).
14. Gruppen, H., Hamer, R.J. and Voragen, A.G.J.,'Water-unextractable cell wall material from wheat flour. II. Fractionation of alkali-extracted polymers and comparison with water-extractable arabinoxylans', *J. Cereal Sci.* **16** (1992) 53-67.
15. Englyst, H.N. and Cummings, J.H.,'Simplified method for the measurement of total non-starch polysaccharides by gas-liquid chromatography of constituent sugars as alditol acetates', *Analyst* **109** (1984) 937-942.
16. Taylor, R.L. and Conrad, H.E.,'Stoichiometric depolymerization of polyuronides and glycosaminoglycuronans to monosaccharides following reduction of their carbodiimide-activated carboxyl groups', *Biochem.* **11** (1972) 1383-1388.
17. Blumenkranz, N. and Asboe-Hansen, G.,'New method for quantitative determination of uronic acids', *Anal. Biochem.* **54** (1973) 484-489.
18. Thibault, J.-F.,'Automatisation du dosage des substances pectiques par la méthode au méta-hydroxydiphenyl', *Lebensm.-Wiss. Technol.* **12** (1979) 247-251.
19. Bensadoun, A. and Winterstein, D.,'Assay of proteins in the presence of interfering materials', *Anal. Biochem.* **70** (1976) 241-250.
20. Sandford, P.A. and Conrad, H.E.,'The structure of the *Aerobacter aerogenes* A3(SI) polysaccharide. I. A reexamination using improved procedures for methylation analysis', *Biochem.* **5** (1966) 1508-1517.
21. Sweet, D.P., Shapiro, R.H. and Albersheim, P.,'Quantitative analysis by various GLC response-factor theories for partially methylated and partially ethylated alditol acetates', *Carbohydr. Res.* **40** (1975) 217-225.
22. Tollier, M.-T. and Robin, J.-P.,'Adaptation de la méthode à l'orcinol-sulfurique au dosage automatique des glucides neutres totaux: conditions d'application aux extraits d'origine végétale', *Ann. Technol. Agric.* **28** (1979) 1-15.
23. Fincher, G.B. and Stone, B.A., in 'Advances in Cereal Science and Technology,' Vol 8 (Y. Pomeranz, ed.), Am. Assoc. Cereal Chem., St. Paul, USA (1986) pp 207-295.
24. Carpita, N.C.,'Fractionation of hemicelluloses from maize cell walls with increasing concentrations of alkali', *Phytochem.* **23** (1984) 1089-1093.
25. Gruppen, H., Hamer, R.J. and Voragen, A.G.J.,'Barium hydroxide as a tool to extract pure arabinoxylans from water-unextractable cell wall material from wheat flour', *J. Cereal Sci.* **13** (1991) 275-290.
26. Viëtor, R.J., Angelino, S.A.G.F. and Voragen, A.G.J.,'Structural features of arabinoxylans from barley and malt cel wall material', *J. Cereal Sci.* **15** (1992) 213-222.
27. Meier, H.,'Fractionation and precipitation with barium hydroxide', *Meth. Carbohydr. Chem.* **5** (1965) 45-46.
28. Mares, D.J. and Stone, B.A.,'Studies on wheat endosperm. II. Properties of the wall components and studies on their organization in the wall', *Aust. J. Biol. Sci.* **26** (1973) 813-830.
29. Shibuya, N. and Iwasaki, T.,'Structural features of rice bran hemicelluloses', *Phytochem.* **24** (1985) 285-289.
30. Viëtor, R.J., Voragen, A.G.J., Angelino, S.A.G.F. and Pilnik, W.,'Non-starch polysaccharides in barley and malt: a mass balance of flour fractionation', *J. Cereal Sci.* **14** (1991) 73-83.
31. Brillouet, J.-M. and Joseleau, J.-P.,'Investigation of the structure of a heteroxylan from the outer pericarp (beeswing bran) of wheat kernel', *Carbohydr. Res.* **159** (1987) 109-126.

CHAPTER 4

Structures of Enzymically Derived Oligosaccharides from Sorghum Glucuronoarabinoxylan

Oligosaccharides derived from alkali-extracted sorghum glucuronoarabinoxylan (GAX) by digestion with a combination of (1→4)-β-D-arabinoxylan arabinofuranohydrolase (AXH) and endo-(1→4)-β-D-xylanase (XylI), both from Aspergillus awamori, *were purified by size-exclusion chromatography followed by preparative high-performance anion-exchange chromatography. Combining structural information obtained by ^1H NMR spectroscopy, methylation analysis and mass spectrometry with knowledge of the mode of action of the enzymes used, resulted in the identification of four novel oligosaccharides. A tetrasaccharide, composed of (1→4)-β-D-xylotriose substituted with α-D-glucuronopyranosyl at* O-2 *of the non-reducing terminal xylose unit, and a pentasaccharide containing an additional α-L-arabinofuranosyl group at* O-3 *of the vicinal xylose unit, have been identified. Two other oligosaccharides were (1→4)-linked β-D-xylose oligosaccharides carrying α-D-glucuronopyranosyl units (1→2)-linked at the non reducing terminal xylose unit and α-L-arabinofuranosyl groups as mono- and dimeric side chains (DP7 and DP8). Three other oligosaccharides were isolated but not fully identified. These seem to differ only in α-L-arabinofuranosyl substitution at* O-3 *or* O-2 *of the backbone xylose units.*

The seven isolated structures provided additional insights on the structure of sorghum glucuronoarabinoxylan and the mode of action of XylI and AXH towards glucuronoarabinoxylans.

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INTRODUCTION

Arabinoxylans are components widely found in cereal endosperm cell wall materials. These polysaccharides consist of a backbone of (1→4)-ß-linked D-Xyl*p* residues substituted with primarily single Ara*f* units at *O*-3 or at both *O*-2 and *O*-3 of certain Xyl units. Single Ara*f* substitution at *O*-2 does occur, but is quite rare. The arabinoxylans of the kernel endosperms from wheat[1,2], barley[3], rye[4,5] and oat[6] differ in their degree and pattern of substitution and molecular weight[7]. The arabinoxylans from the grains of sorghum[8], maize[9] and some rice[10] varieties are more complex. Their Ara/Xyl ratio is generally much higher (~0.9-1.1), which means that they have a higher level of substitution. Moreover, they contain considerable amounts of uronic acid groups, predominantly as D-Glc*p*A attached through *O*-2 of Xyl residues[10-12].

Detailed structural information of GAXs has never been reported before, although their isolation, fractionation and rough characterization have been published[9,10,13-15]. However, these first characterizations are not enough to explain their functional properties and behaviour. Further knowledge can be obtained by degradation studies of defined polysaccharide fractions, followed by the identification of the formed oligosaccharides. Arabinoxylans can be fragmented by chemical and enzymic hydrolysis. Dilute acid can be used to remove the Ara*f* groups in a rather a-specific way. As a result of this treatment, some degradation in the Xyl backbone will easily occur. The GlcA-Xyl linkage, however, is much more stable to acid conditions[11]. Mixed Ara-Xyl oligosaccharides can be prepared treating GAX with endo-(1→4)-ß-D-xylanases (EC 3.2.1.8). Each xylanase is characterized by its substrate specificity and mode of action, and will lead to specific oligomers. In this respect, highly purified, well characterized enzymes are very valuable, since they have a certain predictive value. Advanced analytical techniques, such as NMR spectroscopy, will be necessary for the identification of the oligosaccharides obtained. These types of studies have been published recently for neutral arabinoxylans extracted from wheat[16,17], barley[18] and rye[19], resulting in detailed information about the chemical structure of the polysaccharides as well as the mode of action of enzymes[20].

Degradation of sorghum GAX by xylanases is limited, due to the high degree of substitution. To create more sites of attack for these xylanases, the use of α-L-arabinofuranosidases is very helpful. The selective release of certain (*O*-3 linked) Ara*f* groups results in more unsubstituted Xyl units, and so in more sites of attack for the xylanase.

The identification of almost all possible neutral arabinoxylan oligomers derived from wheat and barley arabinoxylans by an endo-(1→4)-ß-D-xylanase[21] (XylI) have been reported[22,23]. We will now focus on the identification of some acidic oligomeric structures derived from sorghum GAX by digestion with the same xylanase in combination with arabinoxylan arabinofuranohydrolase[24]. Both enzymes were isolated from *Aspergillus awamori* CMI 142717. As far as we know, acidic arabinoxylan oligosaccharides have

never been purified and identified before. New insights in the mode of action of the used enzymes, and in the structure of GAX will be discussed.

EXPERIMENTAL

Materials

Glucuronoarabinoxylan (GAX) has been extracted from water-unextractable cell wall material from sorghum[25] (*Sorghum vulgare* cv. Fara Fara) by saturated $Ba(OH)_2$ solutions[8]. Endo-(1→4)-ß-D-xylanase I[21] (XylI, EC 3.2.1.8) and arabinoxylan arabinofuranohydrolase[24] (AXH, EC 3.2.1.55) were purified from *Aspergillus awamori* CMI 142717.

Preparation of GAX oligosaccharides

A solution of sorghum GAX (250mg) in 50mM NaOAc buffer (100ml, pH5.0) was digested with a combination of XylI (0.65μg protein/ml) and AXH (0.30μg protein/ml) for 24h at 50°C, continuously mixed head-over-tail. The incubation was stopped by heating (15min, 100°C).

The molecular weight distribution of the generated mixture was determined by high-performance size-exclusion chromatography (HPSEC) using a SP 8700 HPLC (Spectra Physics) equipped with three Bio-Gel TSK columns (each 300 x 7.5mm; Bio-Rad Labs, Richmond, CA, USA) in series: 60XL (M_w exclusion limit for dextrans of $5x10^7$); 40XL (M_w exclusion limit for dextrans of 10^6) and 30XL (M_w exclusion limit for dextrans of $2x10^5$) in combination with a TSK XL guard column (40 x 6mm). Samples (20μl) were eluted at 30°C with 0.4M NaOAc solution (pH3) at a flow rate of 0.8ml/min. The eluate was monitored using a Shodex SE-61 refractive index detector (Showa Denko K.K., Tokyo, Japan).

Analytical high-performance anion-exchange chromatography (HPAEC) was performed using a Dionex BioLC GPM-II quaternary gradient module (Sunnyvale, CA, USA) equipped with a CarboPac PA-100 column (250 x 4mm) in combination with a CarboPac PA guard column (25 x 3mm). Samples (20μl) were injected using a Spectra Physics SP8780 autosampler equipped with Tefzel rotor seal in a 7010 Rheodyne injector valve. Elutions (20°C, 1ml/min) involved linear gradients of NaOAc in 0.1M NaOH from 0→0.2M during 30min, then increasing the NaOAc concentration to 0.6M in 0.1M NaOH during 15min, followed by an increase to 1M NaOAc in 0.1M NaOH in 5min. This step was again followed by elution with 0.1M NaOH for 15min without NaOAc. 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 (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.

For preparative work-ups, digests were concentrated by reduced pressure and fractionated on two columns (600 x 26mm) in series packed with Fractogel TSK HW-40(S) (25-40μm, Merck, Darmstadt, Germany) thermostated at 60°C, using a Spectra Physics SP8800-ternary HPLC-pump. Samples (1ml) were injected (several repetitions) using a Spectra Physics SP8880 autosampler and eluted with 0.1M NaOAc (pH5, 2.5ml/min). The effluent was recorded using a Shodex RI-SE-61 detector. Fractions (1.67ml) were collected starting from 65min. They were assayed for total neutral sugar[26] and uronic acid[27] content using an autoanalyzer (Skalar Analytical BV, Breda, The Netherlands), pooled accordingly and concentrated by reduced pressure. NaN_3 was added as preservative (0.01%w/v).

Preparative HPAEC was performed using a Spectra Physics P4000 pump equipped with a

CarboPac PA-100 column (250 x 22mm). A Spectra Physics AS3000 autosampler was used to inject 900μl samples (several repetitions). The flow rate was 25ml/min, and the gradients were optimized for each sample using 0.2M NaOH, 2M NaOAc in 0.2M NaOH and millipore water as eluents. A Dionex PED detector was used for detection. The effluent actually passing the detector was reduced to 1ml by splitting the effluent post-column. Fractions of 10ml were collected and immediately neutralized by on-line addition of 1M HOAc. They were pooled according to the recorded chromatogram. The pooled fractions were concentrated and desalted on a Sephadex G10 column (600 x 50mm). Samples up to 50ml were applied and eluted with distilled water (5ml/min) using a Pharmacia Hiload system equipped with a Pharmacia P50 pump. A Shodex RI-72 detector was used to monitor the refractive index. Due to the low amounts of oligomers present, fractions of 7.5ml were also checked for their conductivity and pooled accordingly (free, poor or rich in salt) and concentrated by reduced pressure. The oligomers were present in the pools free or poor in salt. In the latter case the desalting procedure had to be repeated. Purity of the oligosaccharides was again checked by analytical HPAEC. Samples were stored frozen.

Analytical methods

NMR Spectroscopy. Prior to ^{1}H NMR analysis, GAX oligomers were exchanged twice in D_2O (99.9atom% D_2O, MSD Isotopes) with intermediate freeze-drying. Finally, samples were dissolved in 99.96% D_2O (MSD Isotopes). ^{1}H NMR spectra were recorded at 500MHz on a Bruker AMX-500, or at 600MHz on an AMX/2-600 spectrometer, at a probe temperature of 27°C. Chemical shifts (δ) are expressed in ppm relative to internal acetone (δ 2.225). Typically, one dimensional (1D) spectra were recorded with a spectral width of 5000Hz at 500MHz, collecting 80-1000 free induction decays of 8K or 16K complex data points. Suppression of HOD was achieved by applying the WEFT pulse sequence as described[29]. The resolution of the 1D spectra was enhanced by Lorentzian-to-Gaussian transformation and the final spectra were baseline corrected with a polynomial function when necessary.

2D nuclear Overhauser enhancement spectroscopy (NOESY) was carried out with a 250ms mixing time. 2D Hartmann-Hahn (HOHAHA) measurements were performed using a MLEV-17 mixing sequence of 100-120ms. For the HOHAHA and NOESY spectra 512 measurements of 2K data points were recorded. The spectral width was 4032Hz or 4500Hz in each dimension.

2D rotating frame nuclear Overhauser enhancement spectroscopy (ROESY) was carried out using a spin-lock mixing pulse of 250ms at a field strength corresponding to a 90° pulse-width between 100-110ms. The carrier frequency was placed at the left side of the spectrum at δ 5.9 in order to minimize HOHAHA-type magnetisation transfer. The spectral width was 5500Hz in each dimension, and 512 experiments of 4K data points were recorded.

The NOESY, HOHAHA, and ROESY experiments were performed using the time-proportional phase-increment method to create t_1 amplitude modulation. The HOD signal was suppressed by presaturation for 1.0s. 2D NMR data were processed on Silicon Graphics Iris Indigo or 4D/35 stations, using Triton software.

Methylation analysis. Oligosaccharides were permethylated[29,30], and then converted into mixtures of partially methylated alditol acetates by hydrolysis with 2M TFA (1h, 120°C), reduction with $NaBD_4$, and acetylation with acetic anhydride (3h, 120°C)[31]. The samples were analysed by GC-MS[32] using a Fisons GC8060 gas chromatograph, equipped with a DB-1 capillary column (30m x 0.32mm; J&W Scientific), coupled to a Fisons MD800 mass spectrometer.

Monosaccharide analysis. Oligosaccharides were converted into a mixture of (methyl ester) methyl glycosides by treatment with 1M methanolic HCl (18h, 85°C)[33]. Methyl glycosides were trimethylsilylated using a mixture of pyridine, hexamethyldisilazane, and trimethylchlorosilane (10/2/1, v/v/v)[34], and analysed directly by GC-MS as described for the methylation analysis.

Fast atom bombardment mass spectrometry. The molecular masses of the underivatised oligosaccharides were determined using fast atom bombardment mass spectrometry. Positive-ion fast atom bombardment mass spectra were obtained using MS1 of a JEOL JMS-SX/SX102A tandem mass spectrometer operated at accelerating voltages of 10kV (sample ***F3.2***) or 9kV (sample ***F3.1***). The FAB gun was operated at 6kV accelerating voltage with an emission current of 10mA and using xenon as the bombarding gas. Spectra were scanned at a speed of 30s for the full mass range specified by the accelerating voltage used, and were recorded and averaged on a Hewlett Packard HP9000 data system running JEOL COMPLEMENT software.

Notation. Notation of the side groups was done similarly to the notations used before[22,23,35-37]. These notations, however, needed extension for the new oligomers described in this paper. The Xyl residues were numbered (**1** to **4**), starting from the reducing side. **X**, **A**, and **GA** represent Xyl*p*, Ara*f*, and Glc*p*A, respectively. Their type of linkage and position at the backbone is indicated in the superscripts. As an example, $\mathbf{A}^{2A(3X2)}$ means that the Ara*f* unit is linked to *O*-2 of another Ara residue, which again is linked at *O*-3 of the second Xyl residue in the xylan backbone from the reducing side.

Results and Discussion

A GAX population, obtained by $Ba(OH)_2$ extraction of water-unextractable cell wall material from sorghum, was digested with enzymes. Degradation by XylI only, resulted in a rather slight shift in molecular weight distribution on HPSEC (results not shown), whereas supplementing the reaction mixture with AXH showed a reasonable shift in molecular weight distribution (Fig 1A). Analytical HPAEC of the latter digest gave rise to a complex chromatogram (Fig 1B). The digest turned out to be composed of both neutral

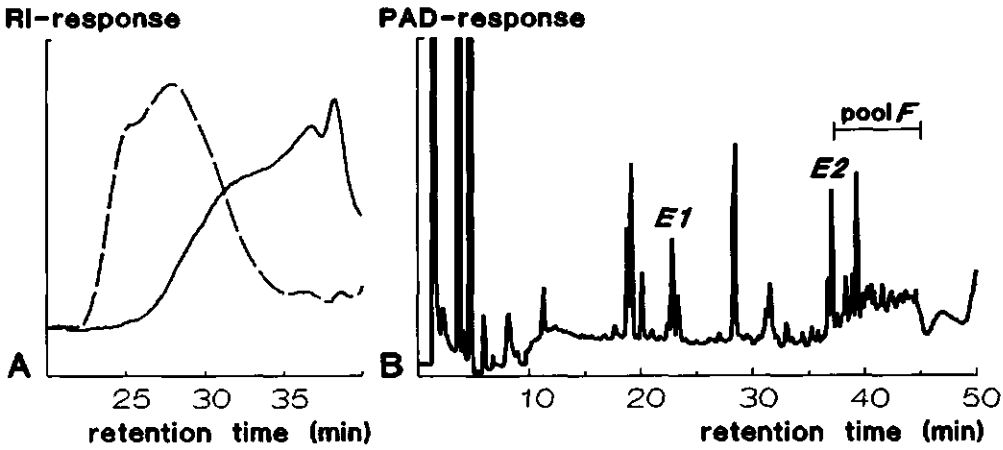


FIG 1. Elution pattern of GAX digested with a combination of AXH and XylI on HPSEC (A) (---- blank; —— digest) and HPAEC (B).

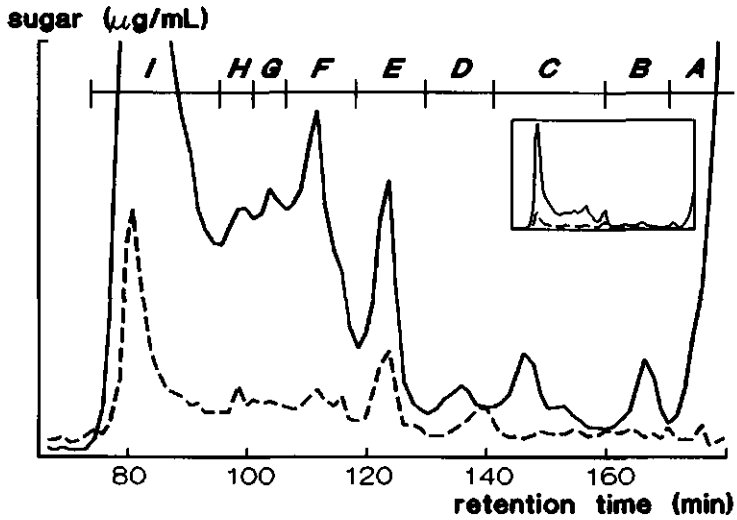


FIG 2. Fractionation of GAX digest on Fractogel TSK (—— total neutral sugar; ---- uronic acid). The pools of interest are indicated by *E* and *F*. A complete picture of the elution profile is shown in upper right corner.

and acidic oligosaccharides. The analysis of the neutral oligosaccharides will be described elsewhere[38], whereas the acidic oligosaccharides are the subject of this study.

The first step in isolating the acidic oligosaccharides was fractionating the digest on Fractogel TSK (Fig 2). The eluate was pooled into nine fractions denoted *A* - *I* (*A*, monomers; *I*, void volume). The polymeric degradation products in fraction *I* (DP > 10) were not seen by HPAEC. The compounds, which are observed by HPAEC, were present in the fractions *A* - *F*. Fraction *A* is mainly composed of Ara and Xyl. Oligomers in the fractions *B* to *F* were present in relatively low amounts when compared to the polymeric fraction *I* or the monomeric fraction *A*. Fractions *F*, *G* and *H* were not well separated, and contained oligomers with an estimated DP > 6. Fraction *E* clearly contained acidic oligosaccharides, since the uronic acid signal nicely followed the pattern of the neutral sugar signal in this fraction. Re-analysing the desalted fraction *E* on HPAEC showed that this fraction contained two compounds *E1* and *E2* (Fig 3A; adjusted gradient). These compounds are indicated in the original chromatogram of the total digest (Fig 1B). Fraction *F* indicated in Fig 1B, contained a mixture of oligomers of an approximate DP8, which could be further fractionated by HPAEC, yielding four major compounds, indicated by *F1* to *F4* (Fig 3B). However, when "compound *F3*" was re-chromatographed by HPAEC with an adjusted NaOAc gradient (flatter) after desalting, it was observed that this fraction was still not pure, and that two peaks were present (results not shown). This fraction was once more fractionated by preparative HPAEC and desalted, resulting in the

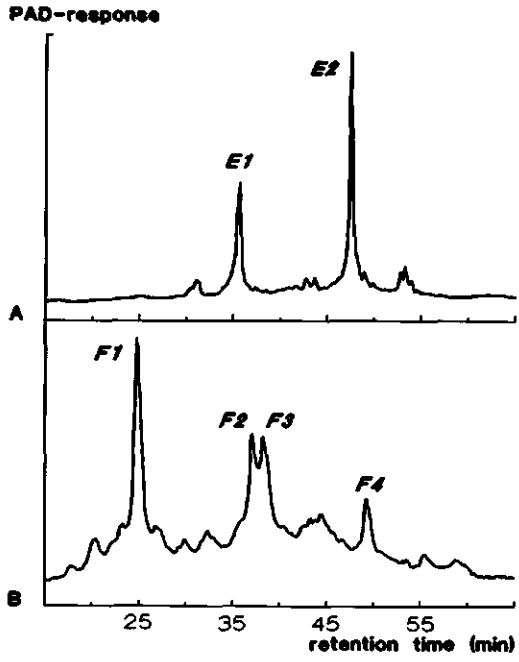


FIG 3. Elution profile (HPAEC) of fractions obtained after fractionation on Fractogel TSK (A: fraction ***E***; B: fraction ***F*** (Fig 2)).

compounds ***F3.1*** and ***F3.2***. Fractions eluting earlier than fraction ***F*** contained materials which were too large to be fractionated on HPAEC with the used gradients. In Fig 1B, a relatively large peak can be seen at *c.*39min. The component responsible for this peak was not one of the components isolated in pool ***F***.

The desalting method, using Sephadex G10, was quite impractical. During this treatment, the samples were deluted too far, to obtain a clear RI-response. Therefore, it was decided to pool the fractions according to the measured conductivity into three pools: no, low and high salt concentrations. Then the volumes were reduced, and the samples were re-chromatographed by HPAEC to determine which pool contained the oligosaccharide. In most cases this desalting step had to be repeated. Beforehand, two other methods were investigated. Both methods used a cation-exchanger (Dowex 50W X8, at 4°C) to exchange the sodium ions. In one method the preceeding was followed by using an anion-exchanger (Dowex AG3 X4A) in sequence[22,23,35]. In the other method, the cation-exchanger was followed by removing HOAc by co-evaporation[39]. However, in both cases a severe degradation of the oligosaccharides, probably because of the development of acid conditions during desalting, was observed. Due to this instability, we chose for the milder Sephadex G10 gel-permeation method.

Fractions *E1*, *E2*, *F1*, *F2*, *F3.1*, *F3.2*, and *F4* were subjected to ^{1}H NMR spectroscopy. In most cases monosaccharide, methylation, and FAB-MS analysis were also performed. However, for some oligosaccharides we had to restrict our experiments, due to the very low amounts of the oligosaccharides obtained. The monosaccharide compositions, and linkage patterns are summarized in Table I. All compounds were composed of Xyl, Ara and GlcA, but in different ratios. The non-reducing Xyl terminus in the backbone, was in every case substituted at *O*-2, whereas the reducing Xyl was unsubstituted in every case. Presumably, the GlcA units will be linked at the *O*-2 positions of the non-reducing Xyl units. The Ara units were present as terminal units, or as part of chains.

As will be shown below, in some cases knowledge about the mode of action of the enzymes used, was very valuable in the final structure elucidation.

Compound E1 — The monosaccharide composition (Table I) indicated the presence of three Xyl and one GlcA units, together with an Ara. Methylation analysis, however, showed only the presence of Xyl units, (1→4)-linked forming a xylotriose, with a substituent at *O*-2 of the non-reducing Xyl terminus. Also the ^{1}H NMR spectrum (Fig 4) could not confirm the presence of Ara. Noting the high values obtained in the monosaccharide analysis, it would be hard to speak of an impurity. However, considering the amounts of Ara in the other oligosaccharides which will be discussed later, all the obtained data for the amounts of Ara in the monosaccharide analyses seemed to be overestimated.

On the H-1 tracks of the constituent monosaccharides in the 2D HOHAHA spectrum the scalar coupled networks of the separate residues are observed. The chemical shift

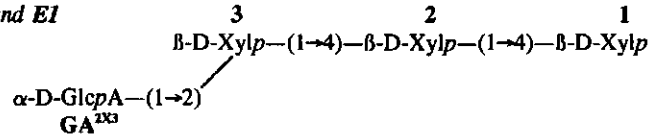
TABLE I. Molar ratios of constituent monosaccharides of the isolated oligosaccharides, and the relative amounts of the present types of linkages of Ara*f* and Xyl*p* residues.

Compound	*E1*	*F1*	*F3.1*	*F3.2*
Ara/Xyl/GlcA	0.6/3.0/1.2	2.4/4.0/0.8	3.5/5.3/0.5	3.6/4.0/0.8
Ara*f*(1→	—[a]	1	2	2
→2)Ara*f*(1→	—	1	1	1
→5)Ara*f*(1→	—	—	—	+[b]
→4)Xyl*p*	1	1	1	1
→4)Xyl*p*(1→	1	1	1	—
→2)Xyl*p*(1→	1	1	1	1
→3,4)Xyl*p*(1→	—	1	1	2
→2,4)Xyl*p*(1→	—	—	2	—

[a] — Absent; [b] Trace.

values are summarized in Table II. The track starting at 5.323ppm is very typical for a GlcA of which the H-5 proton is shifted outside the bulk and has a large coupling. The $J_{1,2}$ value of 3.9Hz for the Glc*p*A H-1 signal indicates the α-configuration. The $J_{1,2}$ value of 7.8Hz for the Xyl*p* H-1 signals indicates the ß-configuration. The relative positions of the different Xyl residues in the backbone were characterized by interresidual cross-peaks between H-1 of Xyl_x and H-4, H-5eq of Xyl_{x-1}[35]. The presence of a ROESY cross-peak from GlcA H-1 to the overlapping terminal Xyl **3** H-2, H-3 was established (Table III). The methylation analysis, however, excluded the possibility of a (1→3)-linkage (Table I). Comparing the ^{1}H NMR data of ***EI*** to xylotriose, shows that Xyl **2** H-1 and Xyl **1** H-1 α,β exhibit nearly identical chemical shift values whereas Xyl **3** H-1 shifts downfield from 4.460ppm in xylotriose to 4.617ppm[35] confirming the substitution on the terminal Xyl residue. 2D ROESY cross-peaks for this fraction are summarized in Table III. This compound was concluded to be

*Compound **EI***



The 4-*O*-methylated Glc*p*A derivative of the identified compound has been described previously[39].

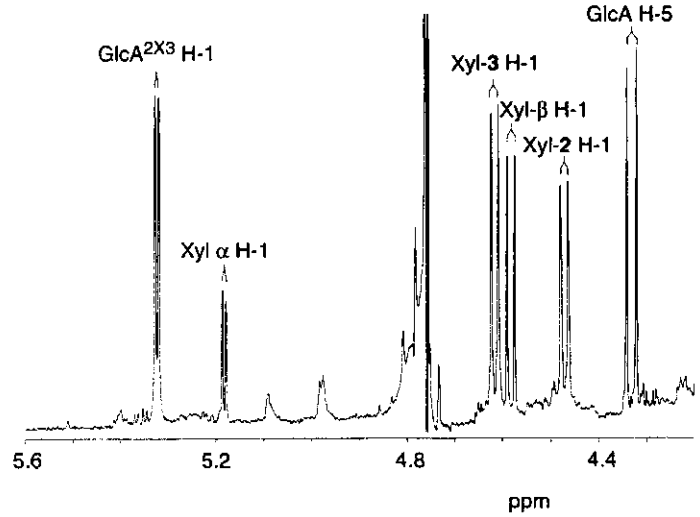


FIG 4. 500MHz ^{1}H NMR spectrum of compound ***EI***. The numbers in the spectrum refer to the corresponding residues in the structure.

TABLE II. ^{1}H NMR chemical shifts of the GAX oligosaccharides, enzymically derived from water-unextractable sorghum cell wall material.

Residue	Group	Chemical shifts (ppm) in compound					
		E1	*E2*	*F1*	*F2*	*F3.1*	*F3.2*
Xyl **1**	H-1α	5.183	5.185	5.183			5.185
	H-1ß	4.583	4.582	4.583	4.586	4.583	4.584
	H-2	3.24		3.24			
	H-3	3.54		3.53			
	H-4	3.78		3.76			
	H-5eq	4.04		4.04			
	H-5ax	3.37		3.36			
Xyl **2**	H-1	4.473	4.504	4.494	4.493	*c.*4.482	4.484
	H-2	3.27		3.28			
	H-3	3.56		3.72			
	H-4	3.76		3.82			
	H-5eq	4.14		4.11			
	H-5ax	3.44		3.44			
Xyl **3**	H-1	4.617	4.597	4.455	4.493	*c.*4.482	4.578
	H-2	3.46[a]		3.24			
	H-3	3.46[a]		3.55			
	H-4	3.65		3.75			
	H-5eq	3.97		4.10			
	H-5ax	3.30		3.40			
Xyl **4**	H-1			4.603	4.586	4.583	4.609
	H-2			3.43			
	H-3			3.47			
	H-4			3.63			
	H-5eq			3.97			
	H-5ax			3.30			
GA^{2X}	H-1	5.323	5.366	5.314	5.356	5.355	5.356
	H-2	3.54		3.54			
	H-3	3.73		3.72			
	H-4	3.46		3.46			
	H-5	4.332	4.323	4.330	4.320	4.318	4.320
A^{3X}	H-1		5.401	5.509	5.509	5.507	5.509
	H-2		4.153	4.172			
	H-3			4.06			
	H-4		4.274	4.272			

Table II. Continued

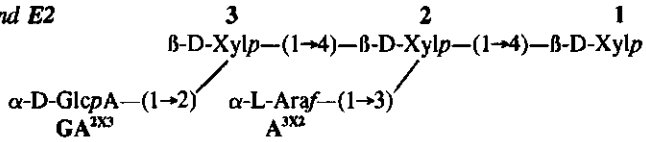
A^{3X2}	H-1	5.192	5.193	5.192	5.193
	H-2	4.10			
$A^{2A(3X2)}$	H-1		5.401	5.400	5.401

[a] These protons overlap; [b] The chemical shift values determined from the 2D spectrum are given in two digits.

Compound E2 — The ^{1}H NMR spectrum showed the presence of an Ara residue, in addition to the constituent monosaccharides of the previous compound. This Ara unit has an α-configuration as was shown by a small $J_{1,2}$ coupling of ~1Hz.

Comparing the chemical shift values of this compound to ***E1***, to ●●●[22] and to xylotriose, the chemical shifts for Xyl **1** H-1 did not shift in all cases. Comparing xylotriose to ●●● , the substitution of Xyl **2** *O*-3 with Ara caused a downfield shift for Xyl **2** H-1 at 4.479ppm in xylotriose to 4.509ppm in ●●● ($\Delta\delta$ +0.030), and an upfield shift for Xyl **3** H-1 at 4.460 to 4.442ppm ($\Delta\delta$ -0.018). Going from ***E1*** to ***E2*** similar $\Delta\delta$ values are observed, namely, a downfield shift for Xyl **2** H-1 from 4.473ppm to 4.504 ppm ($\Delta\delta$ +0.031) and an upfield shift for Xyl **3** H-1 from 4.617 to 4.597ppm ($\Delta\delta$ -0.020). In compound ●●[22] the chemical shift for the (1→3)-linked Ara to Xyl **2** is 5.395ppm and in the present compound the Ara signal resonates at 5.399ppm. This gives a strong evidence for the presence of a (1→3)-linked Ara to Xyl **2**. The GlcA H-1 signal shifts from 5.323ppm to 5.366ppm going from ***E1*** to ***E2***, probably under influence of the close proximity of the neighbouring Ara residue. The chemical shift values are summarized in Table II. The structure of compound ***E2*** was concluded to be

Compound E2



Compound ***E2*** was shown[38] to be degradable by an α-glucuronidase[40]. This enzyme specifically releases *O*-2 linked Glc*p*A from a non-reducing Xyl terminus. The resulting substance co-eluted with ●●● on HPAEC. Furthermore, the latter compound could be degraded by AXH, which typically removes Ara, (1→3)-linked to the xylan backbone (results not shown). Apparently, AXH action is restricted when the Ara is linked at the Xyl residue adjacent to a GlcA-substituted non-reducing Xyl terminus.

Compound F1 — Monosaccharide analysis and methylation analysis suggest that this compound was built up from one GlcA, four Xyl and two Ara units (Table I). Again the

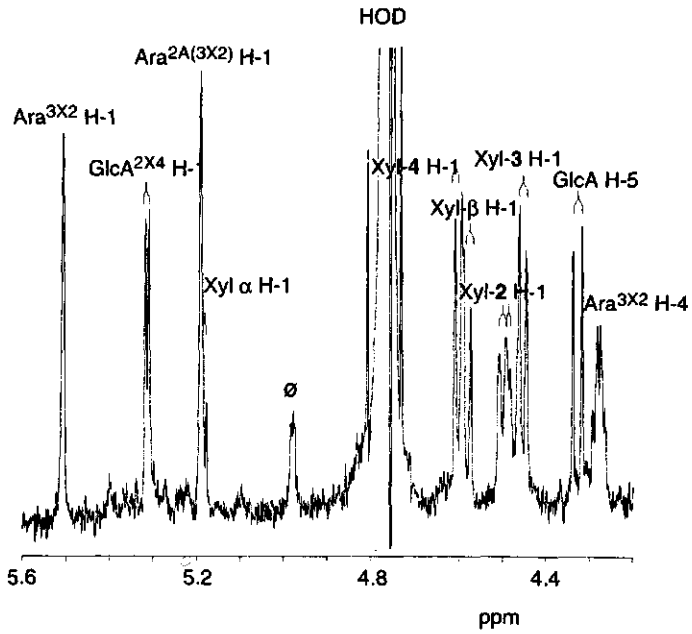


FIG 5. 500MHz ^{1}H NMR spectrum of compound ***F1***. The numbers in the spectrum refer to the corresponding residues in the structure.

Ara content seems to be overestimated. The Xyl units form a (1→4)-linked backbone, of which the non-reducing terminal is substituted at *O*-2, and one of the two internal Xyl units at *O*-3. Only one terminal Ara unit could be distinguished, suggesting that a short Ara side chain could be present.

The ^{1}H NMR spectrum of this compound is shown in Fig 5. Four Xyl, one GlcA and two Ara signals were distinguished. A $J_{1,2}$ coupling smaller than 1Hz for the Ara H-1 signals again indicated the α-configuration. On the various H-1 tracks of the constituent monosaccharides in the 2D HOHAHA spectrum (Fig 6) the scalar coupled networks of the separate residues could be characterized (Table II). As has been reported earlier[35], when two Ara residues are linked to the same Xyl residue like in ●◇●● [35], interresidual ROE contacts are expected between the (1→2)-linked Ara H-1 and the (1→3)-linked Ara H-2 and the H-2 atom of the Xyl it is linked to. For the (1→3)-linked Ara H-1 ROE contacts are expected to the (1→2)-linked Ara H-2 and the H-3, H-4 of the Xyl it is linked to. For the present compound the interresidual ROE contacts are presented in Table III and Fig 7. The Ara units only show interresidual ROE contacts between $A^{2A(3X2)}$ H-1 and A^{3X2} H-2, and between A^{3X2} H-1 and Xyl H-3. These ROE contacts together with the methylation analysis data establish an Ara—(1→2)—Ara—(1→3)—Xyl branch. The involved Xyl residue, being Xyl **2**, and position of the GlcA unit (at Xyl **4**) are evident

TABLE III. Cross-peaks observed at the H-1 tracks in the 2D ROESY spectra, measured with a mixing time of 250ms.

Compound	Residue	N.O.e effect
E1	Xyl **2** H-1	Xyl **1** H-5eq, Xyl **1** H-4, Xyl **2** H-5ax, Xyl **2** H-3
	Xyl **3** H-1	Xyl **2** H-5eq, Xyl **2** H-4, Xyl **3** H-5ax, Xyl **3** H-3
	GA^{2X3} H-1	Xyl **3** H-3[a], Xyl **3** H-2[a]
F1	Xyl **2** H-1	Xyl **1** H-4, Xyl **1** H-5ax, Xyl **2** H-3
	Xyl **3** H-1	Xyl **2** H-5eq, Xyl **2** H-4, Xyl **3** H-5ax, Xyl **3** H-3
	Xyl **4** H-1	Xyl **3** H-5eq, Xyl **3** H-4, Xyl **4** H-5ax, Xyl **4** H-3
	GA^{2X4} H-1	Xyl **4** H-2, Xyl **4** H-3 (weak), GA^{2X4} H-2
	A^{3X2} H-1	Xyl **2** H-3, A^{3X2} H-2, A^{3X2} H-3
	$A^{2A(3X2)}$ H-1	A^{3X2} H-2, $A^{2A(3X2)}$ H-2, $A^{2A(3X2)}$ H-3

[a] These chemical shift values overlap.

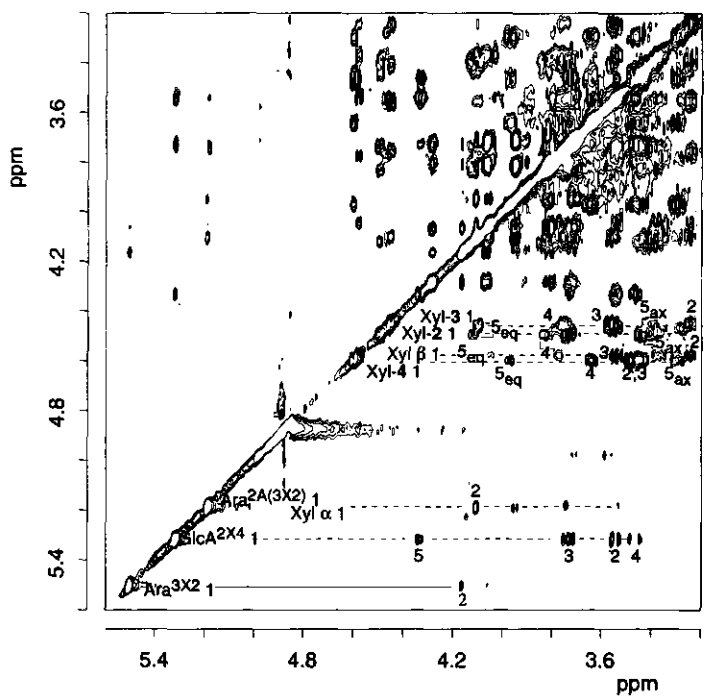
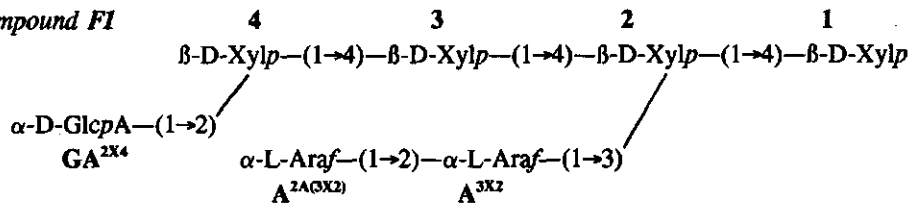


FIG 6. 500MHz 2D HOHAHA spectrum of compound ***F1***. In the spectrum the diagonal peaks of the protons in the anomeric region are indicated. The numbers near cross-peaks in the spectrum, refer to the protons of the scalar coupling network to a diagonal peak.

from the ROE contacts. This compound is concluded to have the following sequence

Compound F1



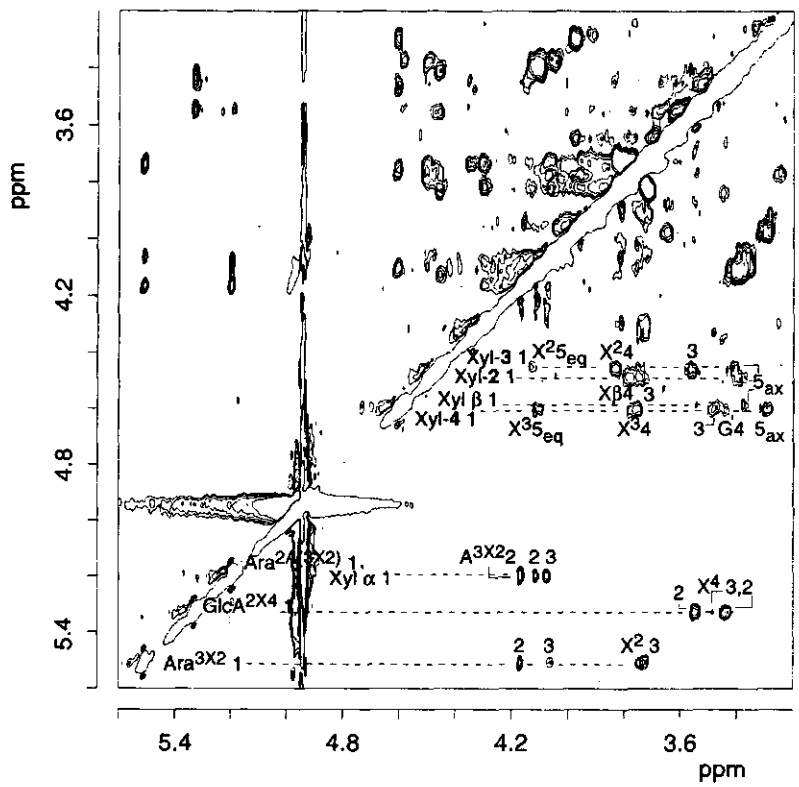


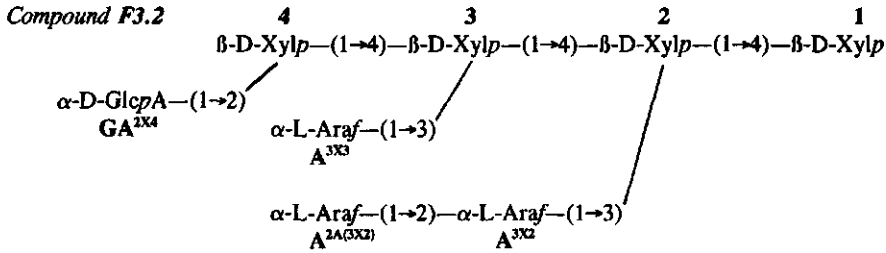
FIG 7. 500MHz 2D ROESY spectrum of compound *F1*. The n.O.e. connectivities along the H-1 tracks are denoted. For notations see *Experimental*.

Compound F3.2 — Using FAB-MS, the molecular mass of the compound present was determined to be m/z 1163, corresponding with a structure consisting of seven pentose units and one uronic acid residue. This finding was consistent with the methylation analysis and monosaccharide composition (Table I), except for the overestimated Ara.

Furthermore, methylation analysis showed the presence of two terminal Ara units, and only *O*-3 substitution at the internal Xyl residues.

The 1D ^{1}H NMR spectrum of this compound, showed four Xyl, one GlcA and three Ara H-1 signals. When comparing this spectrum to that of compound ***F1*** three major differences were observed (Table II). Firstly, an extra Ara signal was detected at δ 5.401, the other two Ara H-1 signals exhibited the same chemical shift values as for the α-L-Ara*f*—(1→2)—α-L-Ara*f*—(1→3) branch in compound ***F1***. The chemical shift value of the extra Ara H-1 resonance correlated very nicely whith the chemical shift value of the (1→3)-linked Ara H-1 signal in ***E2*** (5.399ppm). The second difference refers to the Xyl residues, where the chemical shifts of the H-1 resonances of Xyl **2**, **3** and **4** were shifted markedly. The third difference was the chemical shift position of GlcA H-1, which shifted from δ 5.314 in ***F1*** to δ 5.356 in this fraction. This again compared very well to the difference in chemical shift values for GlcA H-1 in compounds ***E1*** and ***E2***. When the GlcA—(1→2)—Xyl element is neighboured by an unbrached Xyl residue, the chemical shift values were 5.323ppm in ***E1*** and 5.314ppm in ***F1***. When the neighbouring Xyl unit was substituted at *O*-3 by Ara than the chemical shift values were 5.366ppm in ***E2*** and 5.356ppm in ***F3.2***.

Considering the NMR results and the methylation analysis data, it was not expected that the third Ara residue was part of the oligo-Ara branch, but that it was linked directly to a bare Xyl unit in the backbone. This explains also that the internal Xyl residues showed major chemical shift differences when comparing to compound ***F1***. The structure proposed for this compound is



Considering the mode of action of AXH, known so far, exchanging the two Ara branches would probably result in a compound degradable by AXH[41]. It can be expected that in this case, the single (1→3)-linked Ara at Xyl **2** would have been cleaved off.

*Compounds **F2**, **F3.1**, and **F4*** — FAB-MS showed that the mass of the major compound in ***F3.1*** was identical to the mass of compound ***F3.2*** (m/z 1163). However, also considerable amounts of a compound with m/z 1295 were found, suggesting impurities composed

of eight pentose units and one uronic acid unit. Apparently, the major compound was also constituted of one GlcA and seven pentose residues. However, monosaccharide analysis would suggest one, and methylation analysis would suggest two Xyl units more. Interestingly, fraction ***F3.1*** contained internal Xyl units substituted at *O*-2. Assuming that GlcA is attached at the non-reducing Xyl terminus, this would mean that at least one Ara side group (monomeric or dimeric) is substituted through *O*-2 at a Xyl unit.

Also compounds ***F2*** and ***F4*** were isolated from one pool (***F***, Fig 2) eluted from TSK. Therefore we do not expect a large difference in DP. Typically, when the ^{1}H NMR spectra of these compounds are compared to each other and to that of compound ***F3.2*** the only differences in chemical shift values were found on the Xyl residues (Table II). Apparently, only the linkage type at the internal Xyl units was changed.

The structures of the GAX oligosaccharides proposed in this study differ from those described in literature, in that they all contain GlcA units linked at *O*-2 of the non-reducing Xyl terminus, and that they contain small Ara side chains, apart from single Ara substituents. Apparently, assuming that no undermethylation occurred, the Ara residues in sorghum GAX, single or as chains, might be substituted either at *O*-3 or *O*-2 of a Xyl residue in the xylan backbone. Translating these results to the original GAX polysaccharide, it may be concluded that sorghum GAX is rather complex. Disubstituted Xyl units were not found in the present study. These kind of structures, however, are present indeed in sorghum GAX, as could be demonstrated by methylation analyses in previous studies[8]. Oligosaccharides, containing these disubstituted Xyl units, will probably be found in the larger oligosaccharides (DP > 8) in the pools ***G*** - ***I*** (Fig 2).

The majority of the *O*-3 linked single Ara residues has been removed from the polymeric GAX by AXH, prior to the purification of the oligosaccharides. Apparently, AXH was not able to remove the Ara which is linked to the Xyl residue right at the reducing side of a GlcA substituted Xyl. Also, the short Ara side chains, and the probably present *O*-2 linked single Ara units, were not removed.

The elucidation of the structure of GAX oligosaccharides also resulted in some new insights about the mode of action of XylI of *Aspergillus awamori*. XylI is able to split the glycosidic linkage at the non-reducing site of a GlcA substituted Xyl residue. Our results confirm the generalized mode of action of XylI[20]. Glycosidic linkages involving *C*-1 of a Xyl residue can only be cleaved when the Xyl residue is unsubstituted and adjacent to an unsubstituted or single substituted Xyl residue.

CONCLUSIONS

Sorghum GAX was shown to be a rather complex polysaccharide. Until now only the structure of neutral arabinoxylans of wheat[16], barley[18] and rye[19] have been described in detail. For other cereals, such as maize, rice and sorghum, the presence of GAX has been described, but its structure has never been studied in detail. In this study we have shown, that the xylan backbone in sorghum GAX is substituted, next to monomeric Ara units at *O*-3 or at both *O*-2 and *O*-3 of the Xyl units, with short Ara*f* side chains and Glc*p*A residues, which has never been proven before. There are indications that the Ara residues or side chains are linked either at *O*-3 or *O*-2 of a Xyl unit. Chapter 5 will be directed towards translating the presented results into a tentative structural model for sorghum GAX. Apart from the exceptionally high degree of substitution, this and the presence of GlcA residues might explain the poor enzymic degradability of the sorghum cell wall hemicelluloses. Since we have shown the presence of short Ara containing side chains, the Ara/Xyl ratio, which is generally used to indicate the degree of substitution, is not suitable for this type of GAX.

In this study, knowledge about the mode of action of XylI and AXH has proven to be very valuable. Without these enzymes we would not have been able to obtain oligomers, representing small parts of GAX. Unfortunately, some information was lost, because a second enzyme, AXH, was needed to obtain enough oligomeric material. The mode of action of XylI towards GAX was shown to be similar as towards neutral arabinoxylans. The enzyme "treats" the GlcA substitution in the same way as a disubstituted Xyl residue[20]. AXH action is limited by GlcA substitution.

REFERENCES

1. Gruppen, H., Hamer, R.J. and Voragen, A.G.J.,'Water-unextractable cell wall material from wheat flour. II. Fractionation of alkali-extracted polymers and comparison with water-extractable arabinoxylans', *J. Cereal Sci.* **16** (1992) 53-67.
2. Izydorczyk, M.S. and Biliaderis, C.G.,'Studies on the structure of wheat-endosperm arabinoxylans', *Carbohydr. Polym.* **24** (1994) 61-71.
3. Viëtor, R.J., Angelino, S.A.G.F. and Voragen, A.G.J.,'Structural features of arabinoxylans from barley and malt cell wall material', *J. Cereal Sci.* **15** (1992) 213-222.
4. Bengtsson, S. and Åman, P.,'Isolation and chemical characterization of water-soluble arabinoxylans in rye grain', *Carbohydr. Polym.* **12** (1990) 267-277.
5. Vinkx, C.J.A., Reynaert, H.R., Grobet, P.J. and Delcour, J.A.,'Physicochemical and functional properties of rye nonstarch polysaccharides. V. Variability in the structure of water-soluble arabinoxylans', *Cereal Chem.* **70** (1993) 311-317.
6. Aspinall, G.O. and Carpenter, R.C.,'Structural investigations on the non-starchy polysaccharides of oat bran', *Carbohydr. Polym.* **4** (1984) 271-282.
7. Girhammar, U. and Nair, B.M.,'Isolation, separation and characterization of water soluble non-starch polysaccharides from wheat and rye', *Food Hydrocolloids* **6** (1992) 285-299.

8. Verbruggen, M.A., Beldman, G. and Voragen, A.G.J.,'The selective extraction of glucuronoarabinoxylans from sorghum endosperm cell walls using barium and potassium hydroxide solutions', *J. Cereal Sci.* **21** (1995) 271-282. (Chapter 3 in this thesis)
9. Carpita, N.C.,'Fractionation of hemicelluloses from maize cell walls with increasing concentrations of alkali', *Phytochem.* **23** (1984) 1089-1893.
10. Shibuya, N., Misaki, A. and Iwasaki, T.,'The structure of arabinoxylan and arabino-glucuronoxylan isolated from rice endosperm cell wall', *Agric. Biol. Chem.* **47** (1983) 2223-2230.
11. Carpita, N.C. and Wittern, D.,'A highly substituted glucuronoarabinoxylan from developing maize coleoptiles', *Carbohydr. Res.* **146** (1986) 129-140.
12. Nevins, D.J. and Kato, Y., in 'New Approaches to Research on Cereal Carbohydrates' (R.D. Hill and L. Munck, eds.), Elseviers Science Publishers B.V., Amsterdam, The Netherlands (1985) pp 253-260.
13. Woolard, G.R., Rathbone, E.B. and Novellie, L.,'A hemicellulosic ß-D-glucan from the endosperm of sorghum grain', *Carbohydr. Res.* **51** (1976) 249-252.
14. Woolard, G.R., Rathbone, E.B. and Novellie, L.,'A heteroxylan from the husk of *Sorghum* grain', *Phytochem.* **16** (1977) 957-959.
15. Woolard, G.R., Rathbone, E.B. and Novellie, L.,'Structural studies on three hemicellulose B fractions from the husk of sorghum grain', *Carbohydr. Res.* **59** (1977) 547-552.
16. Gruppen, H., Kormelink, F.J.M. and Voragen, A.G.J.,'Water-unextractable cell wall material from wheat flour. III. A structural model for arabinoxylans', *J. Cereal Sci.* **18** (1993) 111-128.
17. Hoffmann, R.A., Kamerling, J.P. and Vliegenthart, J.F.G.,'Structural features of a water-soluble arabinoxylan from the endosperm of wheat', *Carbohydr. Res.* **226** (1992) 303-311.
18. Viëtor, R.J., Kormelink, F.J.M., Angelino, S.A.G.F. and Voragen, A.G.J.,'Substitution patterns of water-insoluble arabinoxylans from barley and malt', *Carbohydr. Polym.* **24** (1994) 113-118.
19. Bengtsson, S., Åman, P. and Andersson, R.E.,'Structural studies on water-soluble arabinoxylans in rye grain using enzymatic hydrolysis', *Carbohydr. Polym.* **17** (1992) 277-284.
20. Kormelink, F.J.M., Gruppen, H., Viëtor, R.J. and Voragen, A.G.J.,'Mode of action of the xylan-degrading enzymes from *Aspergillus awamori* on alkali-extractable cereal arabinoxylans', *Carbohydr. Res.* **249** (1993) 355-367.
21. Kormelink, F.J.M., Searle-van Leeuwen, M.J.F. Wood, T.M. and Voragen, A.G.J.,'Purification and characterization of three endo-(1,4)-ß-xylanases and one ß-xylosidase from *Aspergillus awamori*', *J. Biotechnol.* **27** (1993) 249-265.
22. Gruppen, H., Hoffmann, R.A., Kormelink, F.J.M., Voragen, A.G.J., Kamerling, J.P. and Vliegenthart, J.F.G.,'Characterisation by ^{1}H-NMR spectroscopy of enzymically-derived oligosaccharides from alkali-extractable wheat flour arabinoxylan', *Carbohydr. Res.* **233** (1992) 45-64.
23. Viëtor, R.J., Angelino, S.A.G.F., Hoffmann, R.A., Kamerling, J.P., Vliegenthart, J.F.G. and Voragen, A.G.J.,'Structures of small oligomers liberated from barley arabinoxylans by endoxylanase from *Aspergillus awamori*', *Carbohydr. Res.* **254** (1994) 245-255.
24. Kormelink, F.J.M., Searle-van Leeuwen, M.J.F., Wood, T.M. and Voragen, A.G.J.,'Purification and characterization of an (1,4)-ß-D-arabinoxylan arabinofuranohydrolase from *Aspergillus awamori*', *Appl. Microbiol. Biotechnol.* **35** (1991) 753-758.
25. Verbruggen, M.A., Beldman, G., Voragen, A.G.J. and Hollemans, M.,'Water-unextractable cell wall material from sorghum: isolation and characterization', *J. Cereal Sci.* **17** (1993) 71-82. (Chapter 2 in this thesis)
26. Tollier, M.-T. and Robin, J.-P.,'Adaptation de la méthode à l'orcinol-sulfurique au dosage automatique des glucides neutres totaux: conditions d'application aux extraits d'origine végétale', *Ann. Technol. Agric.* **28** (1979) 1-15.
27. Blumenkranz, N. and Asboe-Hansen, G.,'New method for quantitative determination of uronic acids', *Anal. Biochem.* **54** (1973) 484-489.
28. Hård, K., Zadelhoff, G. van, Moonen, P., Kamerling, J.P. and Vliegenthart, J.F.G.,'The Asn-linked carbohydrate chains of human Tamm-Horsfall glycoprotein of one male. Novel sulfated and N-acetylgalactosamine-containing N-linked chains', *Eur. J. Biochem.* **209** (1992) 895-915.

29. Ciucanu, I. and Kerek, A.F.,'A simple and rapid method for the permethylation of carbohydrates', *Carbohydr. Res.* **131** (1984) 209-217.
30. Needs, P.W. and Selvendran, R.R.,'Avoiding oxidative degradation during sodium hydroxide/methyl iodide-mediated carbohydrate methylation in dimethyl sulfoxide', *Carbohydr. Res.* **245** (1993) 1-10.
31. York, W.S., Darvill, A.G., McNeil, M., Stevenson, T.T. and Albersheim, P.,'Isolation and characterization of plant cell walls and cell wall components', *Methods Enzymol.* **118** (1985) 3-40.
32. Björndal, H., Lindberg, B. and Svensson, S.,'Mass spectrometry of partially methylated alditol acetates', *Carbohydr. Res.* **5** (1967) 433-440.
33. Chambers, R.E. and Clamp, J.R.,'An assessment of methanolysis and other factors used in the analysis of carbohydrate-containing materials', *Biochem. J.* **125** (1971) 1009-1018.
34. Kamerling, J.P. and Vliegenthart, J.F.G., in 'Clinical Biochemistry - Principles, Methods, Applications, Vol 1, Mass spectrometry' (A.M. Lawson, ed.), Walter de Gruyter, Berlin, Germany (1989) pp 176-263.
35. Hoffmann, R.A., Leeflang, B.R., Barse, M.M.J. de, Kamerling, J.P. and Vliegenthart, J.F.G.,'Characterisation by ^{1}H-n.m.r. spectroscopy of oligosaccharides, derived from arabinoxylans of white endosperm of wheat, that contain the elements →4)[α-L-Ara*f*-(1→3)]-ß-D-Xyl*p*-(1→ or →4)[α-L-Ara*f*-(1→2)][α-L-Ara*f*-(1→3)]-ß-D-Xyl*p*-(1→', *Carbohydr. Res.* **221** (1991) 63-81.
36. Hoffmann, R.A., Geijtenbeek, T., Kamerling, J.P. and Vliegenthart, J.F.G.,'^{1}H-N.m.r. study of enzymically generated wheat-endosperm arabinoxylan oligosaccharides: Structures of hepta- to tetradeca-saccharide containing two or three branched xylose residues', *Carbohydr. Res.* **223** (1992) 19-44.
37. Kormelink, F.J.M., Hoffmann, R.A., Gruppen, H., Voragen, A.G.J., Kamerling, J.P. and Vliegenthart, J.F.G.,'Characterization by ^{1}H-n.m.r. spectroscopy of oligosaccharides derived from alkali-extractable wheat-flour arabinoxylan by digestion with endo-(1,4)-ß-D-xylanase III from *Aspergillus awamori*', *Carbohydr. Res.* **249** (1993) 369-382.
38. Verbruggen, M.A., Beldman, G. and Voragen, A.G.J.,'Enzymic degradation of sorghum glucuronoarabinoxylans leading to tentative structures', to be submitted for publication in *Carbohydr. Res.* (1995). (Chapter 5 in this thesis)
39. Düsterhöft, E.-M., Bonte, A.W., Venekamp, J.C. and Voragen, A.G.J.,'The role of fungal polysaccharidases in the hydrolysis of cell wall materials from sunflower and palm-kernel meals', *World J. Microbiol. Biotechnol.* **9** (1993) 544-554.
40. Kroef, C.H.M., Beldman, G. and Voragen, A.G.J., in 'Cellulose Hydrolysis and Fermentation' (J. Coombs and G. Grassi, eds.), Proceedings of a CEC Workshop, CPL-Press-Newbury, Brussels, Belgium (1992) pp 94-102.
41. Kormelink, F.J.M., Gruppen, H. and Voragen, A.G.J.,'Mode of action of (1,4)-ß-D-arabinoxylan arabinofuranohydrolase (AXH) and α-L-arabinofuranosidases on alkali-extractable wheat-flour arabinoxylan', *Carbohydr. Res.* **249** (1993) 345-353.

CHAPTER 5

Enzymic Degradation of Sorghum Glucurono-arabinoxylans Leading to Tentative Structures

Three glucuronoarabinoxylan (GAX) populations, obtained from water-unextractable cell wall material from sorghum by different alkali extractants, were digested by combinations of endo-xylanases (XylI, XylIII and GXH), arabinofuranosidases (AXH and AraB) and an α-glucuronidase (GlcAase). All three GAX populations were shown to be rather poorly degradable, due to the very high degree of substitution, as well as the substitution pattern.

The $Ba(OH)_2$ extracted GAX showed a maximum degree of degradation of 11.7%, using XylI combined with GXH and AXH. The GAX population extracted by 4M KOH was hardly degraded by any of the tested combinations. In all cases XylIII showed lowest activity upon the three extracts. Synergistic effects were observed between XylI and AXH. Both neutral and acidic arabinoxylan oligomers were formed. The GlcAase acted only upon oligomeric material released by XylI. No synergistic effects were observed between the GXH and AXH.

Combining the patterns of degradation with the modes of action of the enzymes, structures were proposed for sorghum GAX. Evidence was obtained that the xylan backbone of especially the GAX extracted by 4M KOH, is substituted by arabinose and glucuronic acid according to a strict pattern, which hinders the enzymes to act.

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INTRODUCTION

Arabinoxylan, being one of the major non-starch polysaccharides (NSP) present in cereal cell walls, plays an important role in the processing of cereals, such as baking[1] and brewing[2]. In brewing they are associated with processing problems like poor wort and beer filtration rates[3,4] or the formation of beer hazes and precipitates[5]. A rather new development is the use of sorghum malt as a raw material for brewing lager type beers. EtokAkpan and Palmer[6] visualized, by scanning electron microscopy, the difference between germinating sorghum and barley grains. The cell wall structures of barley almost completely disappeared, whereas almost intact cell walls remained in the case of sorghum malt. Biochemical studies on barley malt have shown a partial degradation of arabinoxylans during malting[7]. This result seems to be in contradiction with the stable or increased amounts of arabinoxylans in malt described by EtokAkpan[8]. Research on sorghum also gave some contradicting results. EtokAkpan[8] found that the arabinoxylan content of sorghum decreased during malting, whereas unpublished results from our lab showed no change in arabinoxylan content nor composition.

Degradation of polysaccharides during malting can only be explained by action of newly synthesized and activated endogenous enzymes during the malting process. Apart from α- and ß-amylases, ß-glucanases and proteases also xylanases[9,10,11], xylosidases and arabinofuranosidases[12,13] have been identified, (partially) purified and characterized from barley malt. The malt from sorghum has been less investigated. Despite reports about some varieties lacking ß-amylase[3], this enzyme[14,15], as well as α-amylases[16,17], ß-glucanases[18,19] and proteases[20] have been determined, sometimes (partially) purified and characterized.

Although xylanase activity is present in sorghum malt[6], the arabinoxylans are hardly degraded. A possible reason for this may be the structure of these arabinoxylans. Previous work has shown that the major part of the NSP is composed of highly substituted glucuronoarabinoxylans (GAX)[21,22,23]. In the intact cell wall these components are esterified with acetic acid en ferulic acid; the latter may be involved in cross-linking polysaccharides and/or proteins.

The objective of this research was to obtain a better understanding of the chemical structure of GAX from sorghum kernels. This knowledge may result in an explanation for the poor malting and brewing characteristics. An elegant way to obtain this information in detail is by using enzymes. Previous reports have dealt with the chemical characterization of sorghum GAX[21,22]. Here, we will report on their enzymic degradability with purified enzymes.

EXPERIMENTAL

Materials

Three GAX populations were extracted from water-unextractable cell wall material (WUS) prepared from whole sorghum kernels[21] (*Sorghum vulgare* cv. Fara Fara) by saturated $Ba(OH)_2$, 1M KOH and 4M KOH solutions in sequence. The populations were named accordingly: BE1.1, 1K4 and 4K, and were the three main fractions, which together accounted for 67% of the total GAX present in the sorghum WUS[22]. They differ in molecular weight distribution and in the ratios Ara/Xyl/GlcA and un-/mono-/disubstitution at Xyl with Ara[22].

Endo-(1→4)-ß-D-xylanase I and III[24] (XylI, XylIII), and (1→4)-ß-D-arabinoxylan arabinofuranohydrolase[25] (AXH), were purified from *Aspergillus awamori* CMI 142717. α-L-Arabinofuranosidase B (AraB) was purified from *Aspergillus niger* by Rombouts *et al.*[26] Glucuronoxylan xylanohydrolase[27] (GXH) was a kind gift from dr. D.J. Nevins (UCD, Davis, USA), and purified from *Bacillus subtilis*. The α-glucuronidase (GlcAase) was purified from *Trichoderma viride*[28].

GAX digest

A solution of sorghum GAX (1mg/ml) in 0.05M NaOAc buffer (1ml total incubation volume, pH5.0) was digested with several combinations of XylI (0.22μg protein/ml), XylIII (0.26μg protein/ml), AXH (0.06μg protein/ml), AraB (3.27μg protein/ml), GlcAase (2.60μg protein/ml), and GXH (0.14μg protein/ml) for 24h at 50°C, mixed head-over-tail continuously, and inactivated (10min, 100°C). The degree of degradation was determined by estimating the release of reducing end-groups according to Somogyi[29]. The three GAX populations were all buffer-soluble when used in the indicated concentrations. For each enzyme it was checked that, at the concentration used, the limit of digestion was reached after 24h, meaning that additional enzyme gave no further degradation.

The change in molecular weight distribution was determined by high-performance size-exclusion chromatography (HPSEC) as described elsewhere[22]. The release of oligomeric endproducts was studied by high-performance anion-exchange chromatography (HPAEC) as described elsewhere[30].

Periodate oxidation of the BE1.1 fraction was performed by the in sequence oxidation/reduction procedure, followed by mild hydrolysis (20min, 0.02M HCl, 100°C) according to the method of Åman and Bengtsson[31]. The hydrolysates were fractionated on two columns (600 x 26mm) in series filled with Fractogel TSK HW-40(S) (25-40μm, Merck) thermostated at 60°C, using a Pharmacia LKB-controller LCC-501 Plus system equipped with a Pharmacia P50 pump. Samples (1ml) were eluted with millipore water (2.5ml/min). The effluent was recorded using a Shodex RI-72 detector.

Methylation analysis was performed as described previously[22]. Samples were carboxyl reduced[32] using $NaBD_4$, prior to methylation by a modified Hakomori method[33].

On line *liquid chromatography-mass spectrometry* (LC-MS) was performed as described by Niessen *et al.*[34], using a HPAEC system run under the same conditions as described before[30] coupled to a Finnigan MAT (San Jose, CA, USA) thermospray interface fitted on to a Finnigan MAT TSQ-70 tandem mass spectrometer.

RESULTS AND DISCUSSION

Digestion by purified enzymes

Cereal cell wall polysaccharides mainly consist of (1→3),(1→4)-ß-D-glucan, cellulose, and arabinoxylan. In sorghum, the latter is composed of Ara, Xyl and uronic acid (mainly GlcA) building units, although minor amounts of other sugars occur. Xyl is the building unit of the (1→4)-ß-linked backbone and is substituted at either *O*-3, *O*-2 or both *O*-2 and *O*-3 with α-L-Ara and at *O*-2 with α-D-GlcA. Some of the side groups are composed of short Ara-chains (Chapter 4). The GAX fractions isolated from sorghum appeared to be poorly xylanase degradable (Table I). It was tried to increase the degree of degradation by supplementing the endo-xylanases with AXH, an Ara releasing enzyme. Synergistic effects were observed between AXH and XylI. AXH was capable to promote xylanase activity by creating new cleaving sites for this endo-xylanase. The degree of degradation increased two to five times compared to XylI alone, depending on the substrate. This effect was less pronounced in the combination experiments with XylIII or GXH. Similar effects for AXH and XylI or XylIII were noticed in rice GAX[35], although a synergistic effect between AXH and XylIII was more pronounced in that case. In every combination of xylanase plus accessory enzymes, it was noticed that XylI showed the highest degree of degradation of sorghum GAX followed by GXH, and XylIII the lowest. From the three GAX populations tested, BE1.1 was digested to a higher extent than 1K4, followed by 4K with every combination of enzymes (Table I). Maximum degree of degradation was observed with BE1.1 digested by a combination of XylI, GXH and AXH (11.7%), 4K was degraded very poorly by any of the enzyme combinations. Degradation of 1K4 was intermediate with a degradation maximum of 9.2%.

Another Ara releasing enzyme, AraB, was also tested in sorghum GAX degradation. Interestingly AraB could remove some Ara, although less than AXH did. These Ara units, however, were different than the ones split off by AXH. This was observed in studies in which AraB and AXH were combined. The total amount of Ara was exactly the same as the sum of the Ara released by the two enzymes separately (results not shown).

The mode of action of XylI and XylIII upon wheat and barley arabinoxylans has been established[36]. XylI can split the glycosidic linkage at the non-reducing side of a Xyl residue, independent of the presence of an Ara substituent. XylIII requires at least one unsubstituted Xyl residue at the non-reducing side of an Ara substituted Xyl residue. To be able to split the glycosidic linkage at the reducing side of a Xyl residue substituted with one Ara unit, XylI needs at least one, and XylIII at least two unsubstituted Xyl units towards the reducing end. For a disubstituted Xyl, both enzymes require at least two unsubstituted Xyl residues towards the reducing end. Thus, Ara substitution restricts the action of both xylanases, although XylI is affected less than XylIII. These differences explain why XylI gives a higher degree of degradation of such a highly substituted

TABLE I. Degradation (%) of three populations of sorghum GAX by combinations of XylI[a], XylIII[b], GXH[c], AXH[d], and GlcAase[e] (Nelson-Somogyi assay, standard Ara).

	BE1.1[f]	1K4[g]	4K[h]
XylI	1.6	3.0	0.4
XylIII	0.3	0.4	0.6
GXH	1.4	0.0	0.0
AXH	3.3	1.7	1.1
GlcAase	0.0	0.0	0.0
XylI+GXH	3.5	4.8	1.0
XylI+AXH	7.9	6.4	2.1
XylI+GlcAase	2.0	3.9	0.3
XylI+GXH+AXH	11.7	9.2	3.5
XylI+AXH+GlcAase	9.3	8.0	2.9
XylIII+AXH	4.2	3.1	1.1
XylIII+GlcAase	0.3	0.7	0.9
XylIII+AXH+GlcAase	4.8	3.9	1.4
GXH+AXH	7.2	3.1	1.5
GXH+GlcAase	3.0	0.5	0.3
AXH+GlcAase	4.1	1.8	0.9

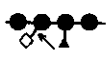
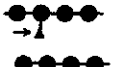
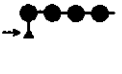
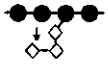
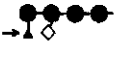
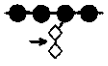
[a] Endo-(1→4)-ß-D-xylanase I; [b] Endo-(1→4)-ß-D-xylanase III; [c] Glucuronoxylan xylanohydrolase; [d] (1→4)-ß-D-Arabinoxylan arabinofuranohydrolase; [e] α-Glucuronidase; [f] Saturated $Ba(OH)_2$ extracted GAX; [g] GAX extracted with 1M KOH; [h] GAX extracted with 4M KOH.

arabinoxylan like sorghum GAX than XylIII. XylIII hardly acts upon the GAX populations. This means that these substrates essentially do not possess the three required adjacent unsubstituted Xyl residues. The influence of a GlcA substituent on the mode of action of XylI and XylIII is unknown. Endo-xylanases from several origins are reported to be able to split the xylan backbone right next to the (4OMe)GlcA substituted Xyl toward the non-reducing side, others need at least one or two unsubstituted Xyl units[37,38,39]. Toward the reducing side most endo-xylanases need at least two unsubstituted Xyl residues[37,38], although some need only one unsubstituted Xyl between the (4OMe)GlcA substituted Xyl and site of attack[37], or split directly next to the (4OMe)GlcA substituted Xyl[39].

In the following, the new insights obtained in this study on the mode of action of the enzymes used, will be discussed. For a better understanding, these results are beforehand summarized and visualized in Table II. The data, on which the conclusions are based will be explained later.

GXH is only active on BE1.1. The GXH recognizes the presence of GlcA as single unit side chain and hydrolyses the ß-(1→4)-Xyl backbone in such a way that the GlcA

TABLE II. Summary of the most important new insights, obtained in the present study, on the mode of action of XylI, GXH, AXH, AraB and GlcAase on some glucuronoarabinoxylan structures.

Linkage[a]	Enzyme	Activity	Linkage	Enzyme	Activity
	XylI	⊕		GXH	⊖
	XylI	⊖		GXH	⊖/+
	XylI	⊕		AXH	⊕/−
	GlcAase	⊖		AXH	⊖
	GlcAase	⊕		AraB	⊕/−
	GlcAase	⊕		AraB	⊖/+

[a] Linkage to be cleaved is indicated by the arrows; ⊕ Activity; ⊖ No activity; ⊖/+ Doubtful activity; ⊕/- Expected activity; ● : ß-Xyl*p*; ◇ : α-Ara*f*; ▲ : α-GlcA; ●● : ß-Xyl*p*-(1→4)-ß-Xyl*p*; ● : α-Ara*f*-(1→3)-ß-Xyl*p*; ● : α-GlcA-(1→2)-ß-Xyl*p*; ◇ : α-Ara*f*-(1→2)-α-Ara*f*; ◇◇ : α-Ara*f*-(1→5)-α-Ara*f*.

substituent is situated at the penultimate Xyl residue from the reducing end[27]. Preliminary results from our lab have shown that substitution by 4OMeGlcA was not recognized by this enzyme (results not shown). The GXH preparation was free from arabinofuranosidase, xylosidase and GlcAase active on sorghum GAX, since no Ara nor Xyl nor GlcA were released. The question rises, why BE1.1 is degraded whereas 1K4 and 4K are not, since all substrates meet the requirement of carrying GlcA[22]. The Ara substitution in 1K4 and in 4K is less than in BE1.1 although still very high (Ara/Xyl≈0.8). A typical substitution pattern might be the cause for this resistance to enzyme attack. It can be hypothesized that *e.g.* arabinosylated Xyl residues near the cleaving site for GXH inhibit degradation (Table II). Another possibility is that *O*-3- and *O*-2-mono-arabinosylated Xyl at certain essential positions, probably near the GlcA substituted Xyl residue towards the reducing terminus, may play a role. The activity of GXH is not influenced by AXH treatment. AXH on its turn might be hindered by the presence of GlcA substitution or oligomeric Ara side chains in the vicinity of mono-arabinosylated Xyl residues, and will not split off these Aras. This needs further clarification.

AXH acts specifically upon (glucurono-)arabinoxylans, not on arabinans or arabinogalactans[25], and is capable of releasing Ara from *O*-3-monosubstituted Xyl[40]. The Ara releasing capacity of AXH in the case of sorghum GAX is presented in Table III. Only

TABLE III. Release of Ara (% of Ara present) from sorghum GAX by combinations of XylI[a], XylIII[b], AXH[c], and GlcAase[d] (HPAEC).

	BE1.1[e]	1K4[f]	4K[g]
XylI	0.0	0.0	0.1
XylIII	0.2	0.0	0.1
AXH	9.5	5.5	2.7
GlcAase	0.0	0.0	0.0
XylI + AXH	9.3	4.9	3.3
XylI + GlcAase	0.0	0.0	0.8
XylI + AXH + GlcAase	9.5	5.6	3.2
XylIII + AXH	10.1	6.0	3.2
XylIII + GlcAase	0.1	0.0	0.1
XylIII + AXH + GlcAase	10.4	6.3	3.1
AXH + GlcAase	10.3	5.6	2.8

[a] Endo-(1→4)-ß-D-xylanase I; [b] Endo-(1→4)-ß-D-xylanase III;
[c] (1→4)-ß-D-Arabinoxylan arabinofuranohydrolase; [d] α-Glucuronidase;
[e] Saturated $Ba(OH)_2$ extracted GAX; [f] GAX extracted with 1M KOH;
[g] GAX extracted with 4M KOH.

small amounts of the total Ara units present were released by using the specified enzyme-combinations. In incubations with BE1.1 ~10% of the Ara originally present was released. Relatively less Ara was released in incubations with 1K4 (~6% of total Ara) and 4K (~3% of total Ara) as substrates, variations in the data in Table III are due to methodology. Methylation analysis indicated that AXH alone released ~50% of all Ara-residues substituted at the *O*-3-monosubstituted Xyl units in the (1→4)-ß-D-xylan backbone of BE1.1 (results not shown). For wheat arabinoxylan this value was close to 100%[25], and this amount corresponds to 33% of the total amount of Ara present. AXH is inhibited apparently, to remove the remaining Ara from the mono-arabinosylated Xyl residues in sorghum GAX, probably by the reasons mentioned previously.

AraB is reported to cleave the linkage between an Ara unit substituted at *O*-3 of a non-reducing terminal Xyl unit[40], and to split preferentially the α-(1→5)-linked Ara in arabinans[26]. Sorghum GAX does contain small Ara chains as substituents[30] (Chapter 4). Although only indicated by methylation analysis, these could be like α-Ara*f*-(1→5)-α-Ara*f*-(1→2)-α-Ara*f*-(1→3)-ß-Xyl*p*, Xyl being part of the backbone. It is thought that AraB can cleave the α-(1→5)-linkage in the side chain, but not the other linkages present (see Table II). This explains why AraB does not act synergistically with AXH, XylI and GXH.

Endo-type degradation of GAX was followed by studying shifts in molecular weight distribution by HPSEC. The shifts in molecular weight distribution of incubations of BE1.1, 1K4 and 4K with XylI or XylIII alone and in combination with AXH and GlcAase are summarized in Fig 1. Graph A shows that XylI shifts the molecular weight distribution

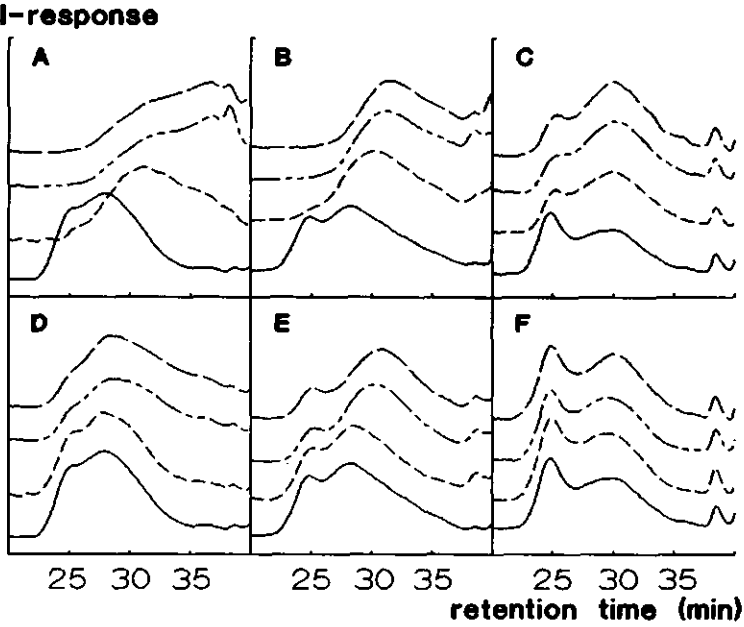


FIG 1. HPSEC analysis of the incubation mixtures of GAX with combinations of XylI, XylIII, AXH, and GlcAase. A and D substrate BE1.1; B and E substrate 1K4; C and F substrate 4K; A,B and C digested with XylI combined with accessory enzymes; D,E and F digested with XylIII combined with accessory enzymes; —— substrate blank; -- +Xyl; — - - — Xyl+AXH; — — +Xyl+AXH+GlcAase.

of BE1.1 toward the lower molecular weight ranges. Its average M_w shifted from 210,000[22] to ~45,000, thus from DP ~1,500 to ~300. These calculations were done using 140 as the average molecular weight per sugar unit, since the average Xyl/GlcA ratio ≈ 4/1). The downward shift of the molecular weight of BE1.1 is enhanced by supplementation with AXH. GlcAase had no effect on the molecular weight distribution. Similar effects were seen for 1K4 (B), although the shift in retention time was not as extensive as for BE1.1. The average M_w of the 1K4 population decreased from 917,000[22] to ~160,000, a five to six-fold reduction of the DP from 6,500 to ~1,100. Incubations of 4K with combinations of XylI and the accessory enzymes (C) showed no obvious shift in molecular weight distribution, confirming the data in Table I. It was noticed again that 4K was the most xylanase resistant GAX population. Comparison of graphs D, E and F with the corresponding graphs A, B and C shows that the action of XylIII was very limited on sorghum GAX. For BE1 and 1K4 only a slight decrease in molecular weight distribution could be observed, whereas for 4K no change was measured for any combination of enzymes. A decrease in molecular weight distribution by GXH was only seen with BE1.1 and neither with 1K4 nor 4K (Fig 2). The effects of supplementing the incubation

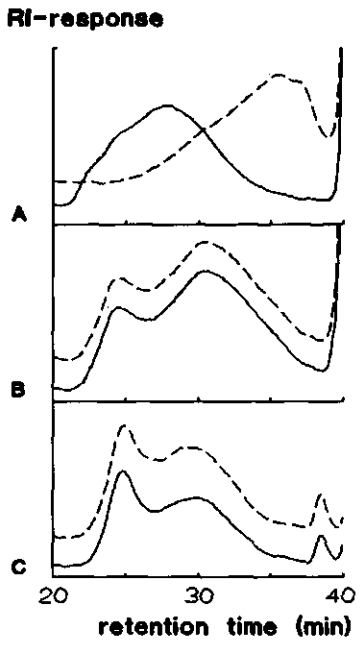


FIG 2. HPSEC analysis of BE1.1 (A), 1K4 (B) and 4K (C) digested by GXH; —— substrate blank; --- +GXH.

mixtures with the accessory enzymes was negligible for all the substrates.

The analysis of the reaction products formed was performed by HPAEC. Fig 3 shows the elution patterns for BE1.1 digested with XylI, XylI + AXH and XylI + AXH + GlcAase. Incubation with only XylI (A) resulted in small amounts of oligomeric reaction products. The concentration of most of these products could be increased by addition of AXH (B) to the reaction mixture. Taking the PAD response factors into account, both Ara (RT=3.3min) and Xyl (RT=4.3min) were released in relatively high amounts by this enzyme combination, whereas xylobiose (RT=7.8min) and xylotriose (RT=11.7min) occurred in only low amounts. The identity of the neutral compounds indicated was revealed by using purified, ^1H NMR identified arabinoxylan oligomers[41] as standards. They were eluted in one peak with the corresponding indicated peaks. Further confirmation was performed by LC-MS (data not shown). Moreover LC-MS indicated that the component with RT=22.8min was composed of three pentoses and one GlcA unit. ^1H NMR confirmed that this compound is  [30] (see legend at Fig 3 for the characters used). This information leads to some clues about the mode of action of the enzymes. XylI can cleave the glycosidic linkage toward the non-reducing side of a polymer, right next to a GlcA substituted Xyl residue (Table II).

Digestion of the GAX populations by XylI, or even better by XylI + AXH resulted in

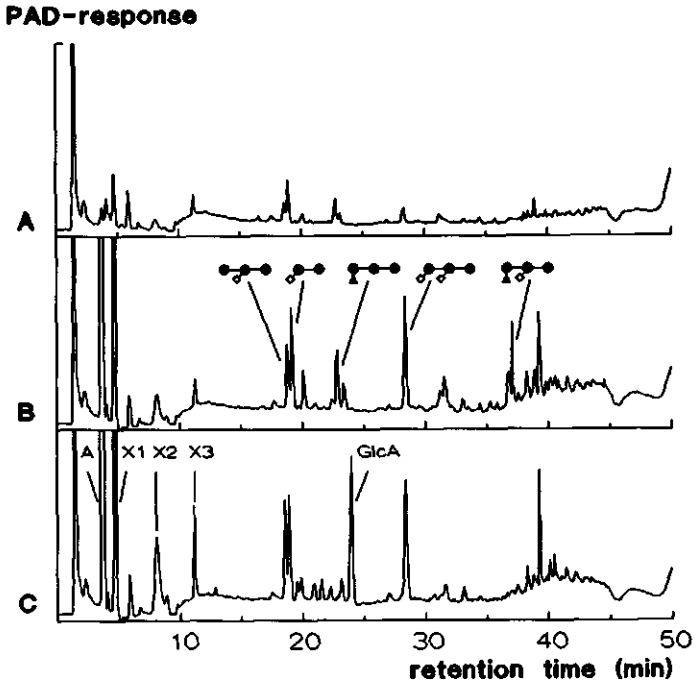


FIG 3. HPAEC patterns of BE1.1 digested by A, XylI; B, XylI+AXH, and C, XylI+AXH+GlcAase. ● : ß-Xyl*p*; ◇ : α-Ara*f*; ▲ : α-GlcA; ●● : ß-Xyl*p*-(1→4)-ß-Xyl*p*; ◇● : α-Ara*f*-(1→3)-ß-Xyl*p*; ▲● : α-GlcA-(1→2)-ß-Xyl*p*.

some oligomers which served as substrates for GlcAase (Fig 3C). GlcA was clearly detected as degradation product by HPAEC, and no other uronic acids could be identified.

The identity of the oligomer which was eluted at RT=37min was ◇●●● [30] (see legend at Fig 3 for the characters used). The addition of GlcAase to the reaction mixture resulted in the disappearance of ◇●●● . GlcA was released from this oligomer, and resulted in the formation of ◇●● , since its concentration tended to increase relatively to ◇●● , and no other peaks were formed. Also ▲●● disapperared upon GlcAase treatment, leading to the formation of xylotriose. The GlcAase can split off a GlcA unit from a Xyl unit at the non-reducing side (Table II). Care should be taken with further conclusions since this enzyme preparation contained traces of ß-xylosidase activity.

The degradation patterns of incubations of 1K4 with these enzymes showed similar reaction products, although in very low amounts. Products were hardly detected in digests of 4K (results not shown).

Digestion of BE1.1 with GXH resulted in larger oligomeric reaction products (Fig 4, RT>37min). The main component eluted at RT=40.2min, and contained Ara since this peak disappeared when AXH was added to the reaction mixture. In this case a newly

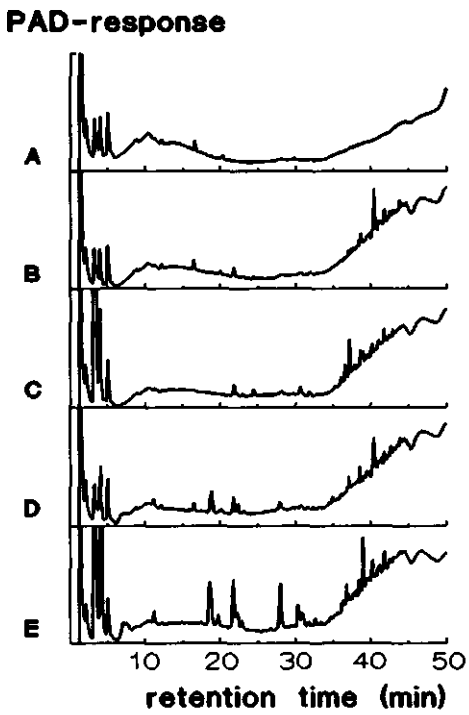


FIG 4. HPAEC pattern of BE1.1 (A, blank) digested by GXH (B); GXH+AXH (C); GXH+XylI (D), and GXH+XylI+AXH (E).

formed peak at RT=37min, and an Ara peak were noticed. The oligomer eluting at RT=40.2min was not a substrate for XylI. Only extra oligomers, typical for XylI degradation (Fig 3) occurred in the mixture, which were intensified by AXH supplementation. Interestingly, AXH and GXH showed no synergism, and GXH degradation products were not degraded by XylI, and vice versa.

The GAX populations were not digested by GlcAase, and also the oligomers originating from GXH digestion were not degraded by GlcAase (results not shown). GXH hydrolyses the glycosidic linkage between the second and third Xyl residue towards the reducing side, calculated from a GlcA substituted Xyl unit (which is the first Xyl residue in this calculation). It can be concluded that the GlcAase is not capable of splitting off the GlcA, α-(1→2)-linked to a Xyl residue adjacent to the reducing Xyl residue.

This research has been carried out on alkali-extracted GAX populations. In the intact cell walls, these polysaccharides are much more complex, because they contain acetyl groups, probably linked to Xyl residues and feruloyl groups linked to the Ara side groups[21]. Moreover the accessibility of the polymers for enzyme action will further be reduced by their 'fixation' in the cell wall matrix by hydrogen bonds and probably

covalent and/or ionic bonds. These factors, together with the poor degradability of the polymers might give clues to explain part of the malting characteristics of sorghum, that is the intact cell walls during malting.

Structural features of sorghum GAX

Previous chemical analyses showed only minor differences between the three GAX populations[22]. The Ara substitution of BE1.1 was higher than of 4K followed by 1K4. The uronic acid substitution of BE1.1 was similar to 4K, but less than of 1K4. The latter two contained however, relatively more GalA than BE1.1. The average molecular weight increased in the order BE1.1, 1K4 and 4K. BE1.1 was a pure GAX population, whereas 1K4 and 4K contained some contaminating populations of (1→3),(1→4)-ß-D-glucan.

The patterns of enzymic degradation allow us to speculate on the structure of sorghum GAX. Favourable for this purpose is the 4K fraction, which was not degraded by XylI at all. Although supplementing the incubation mixture by AXH showed release of some Ara, the molecular weight distribution remained unchanged. Apparently, action of AXH did not introduce (new) cleaving sites for XylI.

Taking in consideration the chemical and structural features of the sorghum GAX populations (sugar and glycosidic linkage composition[22]) and the modes of action of XylI[36] and AXH[40], and also the new insights summarized in Table II, we propose an 'overall' sorghum GAX structure composed of structural units presented in Fig 5. It is thought that the structure of 4K GAX is quite strictly built up by the structural units E1 to E5. The backbone of the formed structure will not be degraded by XylI and AXH. In addition, these can theoretically not be degraded by GXH either[27], which is supported by the results. The Ara present as single substituent at *O*-3 of Xyl can theoretically be released by AXH. Most probably however, the Ara at an *O*-3-monosubstituted Xyl adjacent to the reducing side of the GlcA substituted Xyl unit, will not be removed by AXH (Table II). In the presented model AXH activity will not create cleavage sites for XylI.

Although strong indications were obtained, that Ara units and short side chains linked at *O*-2 of Xyl in the bachbone were present, these structures were not integrated in the overall model for sorghum GAX. From the methylation analyses of the GAX populations[22] it can be expected that these structures are present in rather low amounts.

Additional chemical information about the distribution of side groups was obtained by periodate oxidation followed by mild hydrolysis. Table IV shows the relative amounts of glycerolxylosides separated on TSK HW-40(S) for the three substrates. A broad peak eluted shortly after Xyl. Åman and Bengtsson[31] suggested, that this is ascribed to low molecular weight degradation products, and was therefore not further studied. The relative amounts were calculated from the RI-response. The results show that in BE1.1 ~35% of the substituted Xyl*p* units are present as isolated units and another 35% as blocks of two contiguous units. Also larger blocks of three, four or more units occur. The relative

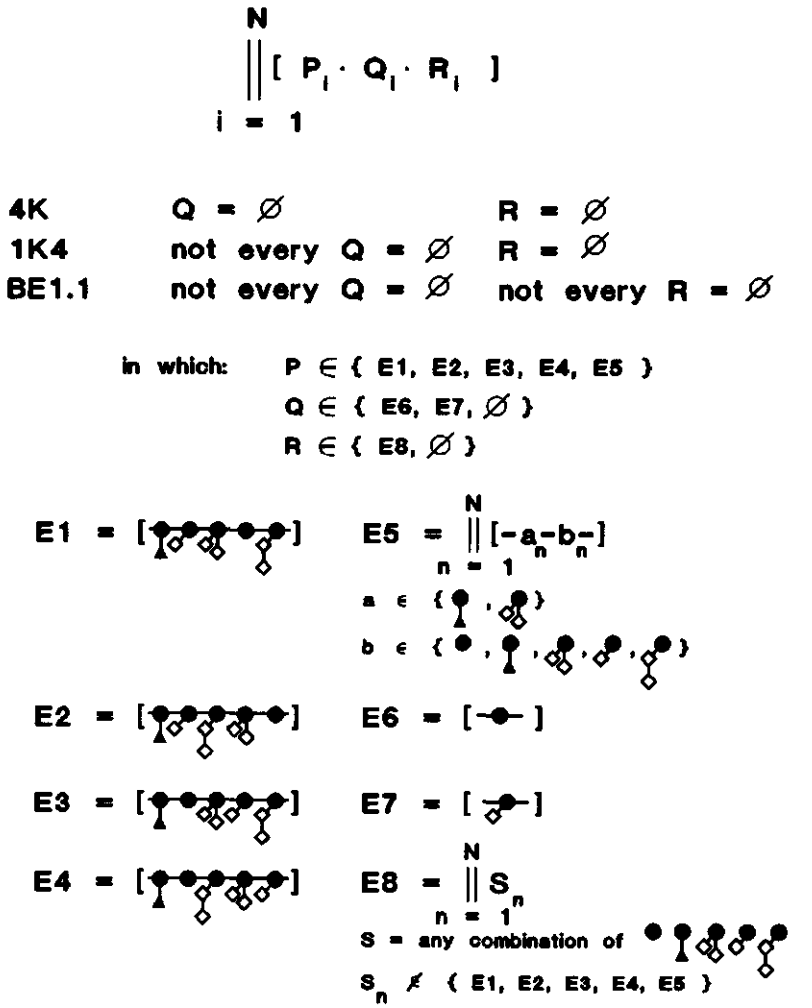


FIG 5. Tentative model for sorghum glucuronoarabinoxylans built of 8 elements (E1 to E8). E1 to E5 represent undegradable combinations of substituted and unsubstituted Xyl units. E6 and E7 represent interrupting elements. E8 represents any degradable combination of Xyl units, either substituted or not. These elements are collected in P (responsible for an undegradable structure), Q (responsible for a degradable structure, however, hardly resulting in small oligomers) and R (responsible for a degradable structure, resulting in small oligomers). (See *Structural features of sorghum GAX* for further explanation).

∅ : Empty set; N : Number of repetitions starting from 1 (n, i);

● : ß-Xyl*p*; ◇ : α-Ara*f*; ▲ : α-GlcA; ●● : ß-Xyl*p*-(1→4)-ß-Xyl*p*; : α-Ara*f*-(1→2)-ß-Xyl*p*;

: α-Ara*f*-(1→3)-ß-Xyl*p*; : α-GlcA-(1→2)-ß-Xyl*p*; : α-Ara*f*-(1→2)-α-Ara*f*;

TABLE IV. Relative distribution[a] of glycerolxylosides of different GAX populations after periodate oxidation.

	Monomer	Dimer	Trimer	Tetramer	DP>4
BE1.1[b]	35	35	7	16	7
1K4[c]	30	32	10	20	8
4K[d]	27	28	12	23	10

[a] Expressed as percentage of total RI-response; [b] Saturated $Ba(OH)_2$ extracted GAX; [c] GAX extracted with 1M KOH; [d] GAX extracted with 4M KOH.

amounts of these larger blocks was higher in 1K4 and 4K. The proposed structural elements (Fig 5) are not fully supported by the results of the periodate oxidation, although the relative contribution of tri- and tetrameric arrangements of contiguous substituted Xyls was more prevalent. The periodate oxidation method seems to have some drawbacks. Although the improved method of Åman and Bengtsson[31] was used, full oxidation can not be guaranteed. These data are therefore not considered as proof, but only as an indication.

The structures of BE1.1 and 1K4 are probably (small) modifications of the 4K model. It is believed that 1K4 is built by the same structural elements as 4K. This '4K like polymer' however, is probably interrupted by an introduction of an un- or *O*-3-substituted Xyl unit (E6 and E7) between the elements E1 to E5. This may explain the certain extent of degradation (shift in molecular weight distribution) although small oligomers were hardly formed, which was noticed. Degradation of the BE1.1 fraction resulted in the release of small oligomers. Therefore it is believed that, additionally to the 1K4 structure, interruptions also occur within the elements E1 to E5. These smaller degradable parts are represented by E8 in Fig 5: any combination of Xyl units, substituted or not, other than E1 to E5, will result in degradable parts.

Although these degradation data give quite some information about the GAX structure, proposing a well underpinned model as has been done for wheat[42], barley[43] and rye[44] can not be done because of the too low degree of degradation by the purified, and characterized xylanases.

CONCLUSIONS

The enzymic degradability of sorghum GAX with endo-xylanases is very limited due to the high degree of Ara substitution and the presence of considerable amounts of GlcA. Application of accessory enzymes, especially AXH, increases the degree of degradation by xylanases. By removing part of the Ara units new sites can be created for xylanases to act

on. GlcAase acted only upon oligomeric material produced by XylI, but only when the GlcA unit was linked at a non-reducing Xyl residue. XylIII showed hardly or only very low activity on sorghum GAX. The influence of uronic acids on the action of all enzymes mentioned above is unknown. For GXH action the presence of GlcA is obligatory.

GAX in the 4K fraction was not degradable by a combination of XylI and AXH. Knowing the Ara/Xyl ratio and linkage composition, only a very few structures can meet these requirements of being not degradable. Therefore, a structure has been proposed for GAX in the 4K fraction (Fig 5). The other GAX populations are thought to be slight modifications of the 4K structure.

With this knowledge about the structure of sorghum GAX it can be understood, that sorghum cell walls are hard to degrade when the right enzymes are not present. The enzymes used in the present study failed in degrading extracted GAX. Other enzymes are necessary, such as a glucuronidase active on polymeric substrates or arabinofuranosidases which can cleave Ara units from *O*-2-substituted, or disubstituted Xyl residues. For the degradation crude cell wall material also acetyl- or feruloyl-esterases might be important. These enzymes are probably not synthesized or activated sufficiently in sorghum during malting, since the cell walls are poorly broken down.

REFERENCES

1. Rouau, X.,'Investigations into the effects of an enzyme preparation for baking on wheat flour dough pentosans', *J. Cereal Sci.* **18** (1993) 145-157.
2. Letters, R.,'Origin of carbohydrates in beer sediments', *J. Inst. Brew.* **75** (1969) 54-60.
3. Dufour, J.P., Mélotte, L. and Srebrnik, S.,'Sorghum malts for the production of a lager beer', *ASBC Journal*, **50** (1992) 110-119.
4. Aisien, A.O. and Muts, G.C.J.,'Micro-scale malting and brewing studies of some sorghum varieties', *J. Inst. Brew.* **93** (1987) 328-331.
5. Coote, N. and Kirsop, B.H.,'A haze consisting largely of pentosan', *J. Inst. Brew.* **82** (1976) 34.
6. EtokAkpan, O.U. and Palmer, G.H.,'Comparative studies of the development of endosperm-degrading enzymes in malting sorghum and barley', *World J. Microbiol. Biotechnol.* **6** (1990) 408-417.
7. Viëtor, R.J., Voragen, A.G.J., Angelino, S.A.G.F. and Pilnik, W.,'Non-starch polysaccharides in barley and malt: a mass balance of flour fractionation', *J. Cereal Sci.* **14** (1991) 73-83.
8. EtokAkpan, O.U.,'Comparative studies of the degradation of non-starchy polysaccharides by sorghums and barleys during malting', *J. Sci. Food Agric.* **58** (1992) 129-134.
9. Slade, A.M., Høj, P.B., Morrice, N.A. and Fincher, G.B.,'Purification and characterization of three (1→4)-ß-D-xylan endohydrolases from germinated barley', *Eur. J. Biochem.* **185** (1989) 533-539.
10. Benjavongkulchai, E. and Spencer, M.S.,'Purification and characterization of barley-aleurone xylanase', *Planta* **169** (1986) 415-419.
11. Dashek, W.V. and Chrispeels, M.J.,'Gibberellic-acid-induced synthesis and release of cell-wall-degrading endoxylanase by isolated aleurone layers of barley', *Planta* **134** (1977) 251-256.
12. Preece, I.A. and MacDougall, M.,'Enzymic degradation of cereal hemicelluloses. II. Pattern of pentosan degradation', *J. Inst. Brew.* **64** (1958) 489-500.
13. Taiz, L. and Honigman, W.A.,'Production of cell wall hydrolyzing enzymes by barley aleurone

layers in response to gibberillic acid', *Plant Physiol.* **58** (1976) 380-386.
14. Novellie, L.,'Kaffircorn malting and brewing studies. V. Occurrence of ß-amylase in kaffircorn malts', *J. Sci. Food Agric.* **11** (1960) 457-463.
15. Botes, D.P., Joubert, F.J. and Novellie, L.,'Kaffircorn malting and brewing studies. XVII. Purification and properties of sorghum malt α-amylase', *J. Sci. Food Agric.* **18** (1967) 409-415.
16. Lecommandeur, D. and Daussant, J.,'Polymorphism in maize, oats and sorghum α-amylases', *Phytochem.* **28** (1989) 2921-2925.
17. Botes, D.P., Joubert, F.J. and Novellie, L.,'Kaffircorn malting and brewing studies. XVIII. Purification and properties of sorghum malt ß-amylase', *J. Sci. Food Agric.* **18** (1967) 415-419.
18. Stuart, I.M., Loi, L. and Fincher, G.B.,'Immunological comparison of (1→3,1→4)-ß-glucan endohydrolases in germinating cereals', *J. Cereal Sci.* **6** (1987) 45-52.
19. Kavitha, R., Ph.D. Thesis, University of Mysore, Mysore, India (1994).
20. Garg, G.K. and Virupaksha, T.K.,'Acid protease from germinated sorghum. I. Purification and characterization of the enzyme', *Eur. J. Biochem.* **17** (1970) 4-12.
21. Verbruggen, M.A., Beldman, G., Voragen, A.G.J. and Hollemans, M.,'Water-unextractable cell wall material from sorghum: isolation and characterization', *J. Cereal Sci.* **17** (1993) 71-82. (Chapter 2 in this thesis)
22. Verbruggen, M.A., Beldman, G. and Voragen, A.G.J.,'The selective extraction of glucuronoarabinoxylans from sorghum endosperm cell walls using barium and potassium hydroxide solutions', *J. Cereal Sci.* **21** (1995) 271-282. (Chapter 3 in this thesis)
23. Woolard, G.R., Rathbone, E.B. and Novellie, L.,'Structural studies on three hemicellulose B fractions from the husk of sorghum grain', *Carbohydr. Res.* **59** (1977) 547-552.
24. Kormelink, F.J.M., Searle-van Leeuwen, M.J.F., Wood, T.M. and Voragen, A.G.J.,'Purification and characterization of three endo-(1,4)-ß-xylanases and one ß-xylosidase from *Aspergillus awamori*', *J. Biotechnol.* **27** (1993) 249-265.
25. Kormelink, F.J.M., Searle-van Leeuwen, M.J.F., Wood, T.M. and Voragen, A.G.J.,'Purification and characterization of an (1,4)-ß-D-arabinoxylan arabinofuranohydrolase from *Aspergillus awamori*', *Appl. Microbiol. Biotechnol.* **35** (1991) 753-758.
26. Rombouts, F.M., Voragen, A.G.J., Searle-van Leeuwen, M.J.F., Gereads, C.C.J.M., Schols, H.A. and Pilnik, W.,'The arabinanases of *Aspergillus niger* — purification and characterisation of two α-L-arabinofuranosidases and an endo-1,5-α-L-arabinanase', *Carbohydr. Polym.* **8** (1988) 25-47.
27. Nishitani, K. and Nevins, D.J.,'Glucuronoxylan xylanohydrolase: a unique xylanase with the requirement for appendant glucuronosyl units', *J. Biol. Chem.* **266** (1991) 6539-6543.
28. Kroef, C.H.M., Beldman, G. and Voragen, A.G.J., in 'Cellulose Hydrolysis and Fermentation' (J. Coombs and G. Grassi, eds.), Proceedings of a CEC Workshop, CPL-Press-Newbury, Brussels, Belgium (1992) pp 94-102.
29. Somogyi, M.,'Notes on sugar determination', *J. Biol. Chem.* **195** (1952) 19-25.
30. Verbruggen, M.A., Schols, H.A., Beldman, G., Voragen, A.G.J., Spronk, T., Thomas, J., Kamerling, J.P. and Vliegenthart, J.F.G.,'Structures of enzymically derived oligomers from sorghum glucuronoarabinoxylan', to be submitted for publication in *Carbohydr. Res.* (1995). (Chapter 4 in this thesis)
31. Åman, P. and Bengtsson, S.,'Periodate oxidation and degradation studies on the major water-soluble arabinoxylan in rye grain', *Carbohydr. Polym.* **15** (1991) 405-414.
32. Taylor, R.L. and Conrad, H.E.,'Stoichiometric depolymerization of polyuronides and glycosaminoglycuronans to monosaccharides following reduction of their carbodiimide-activated carboxyl groups', *Biochem.* **11** (1972) 1383-1388.
33. Sandford, P.A. and Conrad, H.E.,'The structure of the *Aerobacter aerogenes* A3(SI) polysaccharide. I. A reexamination using improved procedures for methylation analysis', *Biochem.* **5** (1966) 1508-1517.
34. Niessen, W.M.A., Hoeven, R.A.M. van der, Greef, J. van der, Schols, H.A., Voragen, A.G.J. and Bruggink, C.,'Recent progress in high-performance anion-exchange chromatography-thermospray mass spectrometry of oligosaccharides', *J. Chrom.* **647** (1993) 319-327.

35. Kormelink, F.J.M. and Voragen, A.G.J.,'Degradation of different [(glucurono)arabino]xylans by combination of purified xylan-degrading enzymes', *Appl. Microbiol. Biotechnol.* **38** (1993) 688-695.
36. Kormelink, F.J.M., Gruppen, H., Viëtor, R.J. and Voragen, A.G.J.,'Mode of action of the xylan-degrading enzymes from *Aspergillus awamori* on alkali-extractable cereal arabinoxylans', *Carbohydr. Res.* **249** (1993) 355-367.
37. Kormelink, F.J.M., Ph.D. Thesis, Wageningen Agricultural University, Wageningen, The Netherlands (1992) pp 1-44.
38. Biely, P., Vrsanska, M., Kremnicky, L., Tenkanen, M., Poutanen, K. and Hayn, M., in '*Trichoderma reesei* cellulases and other hydrolases' (P. Suominen and T. Reinikainen, eds.), Proceedings of the Tricel symposium, Foundation for Biotechnical and Industrial Fermentation Research, Helsinki, Finland (1993) pp 125-135.
39. Debeire, P., Priem, B., Strecker, G. and Vignon, M.,'Purification and properties of an endo-1,4-xylanase excreted by a hydrolytic thermophilic anaerobe *Clostridium thermolacticum*. A proposal for its action mechanism on larchwood 4-*O*-methylglucuronoxylan', *Eur. J. Biochem.* **187** (1990) 573-580.
40. Kormelink, F.J.M., Gruppen, H. and Voragen, A.G.J.,'Mode of action of (1,4)-ß-D-arabinoxylan arabinofuranohydrolase (AXH) and α-L-arabinofuranosidases on alkali-extractable wheat-flour arabinoxylan', *Carbohydr. Res.* **249** (1993) 345-353.
41. Gruppen, H., Hoffmann, R.A., Kormelink, F.J.M., Voragen, A.G.J., Kamerling, J.P. and Vliegenthart, J.F.G.,'Characterisation by ^{1}H-NMR spectroscopy of enzymically-derived oligosaccharides from alkali-extractable wheat flour arabinoxylan', *Carbohydr. Res.* **233** (1992) 45-64.
42. Gruppen, H., Kormelink, F.J.M. and Voragen, A.G.J.,'Water-unextractable cell wall material from wheat flour. III. A structural model for arabinoxylans', *J. Cereal Sci.* **18** (1993) 111-128.
43. Viëtor, R.J., Kormelink, F.J.M., Angelino, S.A.G.F. and Voragen, A.G.J.,'Substitution patterns of water-insoluble arabinoxylans from barley and malt', *Carbohydr. Polym.* **24** (1994) 113-118.
44. Bengtsson, S., Åman, P. and Andersson, R.E.,'Structural studies on water-soluble arabinoxylans in rye grain using enzymatic hydrolysis', *Carbohydr. Polym.* **17** (1992) 277-284.

CHAPTER 6

Brewing with 100% Sorghum Malt: NSP Composition of Wort and Spent Grains in Relation to Wort Filtration

To investigate the influence of cell wall polysaccharides on filtration characteristics in sorghum beer brewing, mashing trials with 100% sorghum malt were performed, in which the mashes were supplemented with two commercial enzyme preparations (A and B). Preparation A was high in (1→3),(1→4)-β-D-glucan and cellulose degrading activities; preparation B was strong in xylan degrading activity. Wort filtration, wort volume and extract yield were evaluated. The non-starch polysaccharide (NSP) contents and compositions were determined in the worts and spent grains. Preparation A showed better filtration improving properties whereas preparation B seemed to have variable effects on filtration, depending on enzyme dose. With increasing filtration rates, wort volumes and extract yields also increased, whereas the amounts of spent grains decreased. The spent grains of preparation A supplemented mashes showed a clear decrease in NSP and protein content. In the case of preparation B, less starch was solubilized. Structural characteristics of the (glucurono-) arabinoxylans (GAX) could not be correlated with filtration rate. Alkali extracted GAX from the spent grains did not differ in sugar composition nor molecular weight distribution from the GAX similarly extracted from water-unextractable materials from malted or raw sorghum. Some polymeric carbohydrates were present in the worts. The wort viscosities were similar in all mashes. The GAX from the malt is solubilized during mashing, and degraded only partly into small oligomers and monomers.

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INTRODUCTION

Sorghum is one of the cereals that grow well in semi-arid environments, and has found many applications in foods. Sorghum malt has found its use in the brewing of South-African kaffir beer and similar products[1]. A rather recent development is the use of sorghum and its malt in European type lager beer, as a substitute for barley malt. Reports often show contradictory results, which can be explained by differences in sorghum varieties and mashing programmes. The variable and high gelatinization temperatures (65-97°C[2]) of sorghum starches and the different levels of polyphenols[3], especially the tannins[4], might play important roles. Malted sorghum exhibits high malting losses[5,6], low values of diastatic power[2,5,7,8] and limited endosperm cell wall degradation[7,9-13]. often associated with low ß-glucanase[2,7,11,14], pentosanase[11] and proteolytic[11] acitivities. The cell walls in sorghum malt can still be observed using electron microscopy, and show a strongly pitted structure, whereas the cell walls in barley malt have been degraded almost completely[15]. Comparison of more than sixty sorghum malts in laboratory scale brewing experiments has shown clearly that the choice of the cultivar has an effect on the final saccharification as a result of large variabilities in starch gelatinization temperatures, the filtration rates, diastatic powers and wort amino acid contents[2]. In most cultivars ß-amylase was low or absent.

One of the major reasons for using sorghum malt instead of unmalted sorghum in lager beer brewing, is that the endogenous proteolytic enzymes produce free α-amino nitrogen (FAN) levels in the wort, sufficient for yeast growth. Nevertheless, some other difficulties might occur. Due to the high starch gelatinization temperatures, special mashing programmes had to be developed[16-19]. However, brewing trials still showed a slow and incomplete saccharification and poor filtration characteristics. Filtration rates could be improved significantly by enzyme supplementation. It has been stated that starch and cell wall material ((1→3),(1→4)-ß-D-glucan) are the main source for viscous, filtration inhibiting materials in the wort[20]. Higher levels of (1→3),(1→4)-ß-D-glucans were released into worts compared to worts obtained from barley malt, which means that (1→3),(1→4)-ß-D-glucans are indeed solubilized but not further degraded[14,21]. Mashing trials using pullulanase resulted in higher yields of fermentable sugars[22].

Mashing experiments have been performed using raw sorghum with the supplementation of exogenous enzymes[23]. Commercially acceptable hot water extracts (HWE), although low in FAN, were obtained using α-amylase and proteolytic enzymes[24]. Amyloglucosidase was needed to convert the hot water extract into a fermentable wort.

When using sorghum as unmalted adjunct, problems may arise concerning slow and incomplete saccharification and poor wort and beer filtration[23,25]. Others have reported, however, that they obtained mashes with acceptable filtration rates and extract recoveries but lower levels of total and free amino nitrogen[26]. Addition of preparations containing α-

amylase and ß-glucanase resulted in higher extract recoveries. Cellulase and ß-glucanase supplementation decreased wort viscosities. A protease (neutral) increased the levels of total and free amino nitrogen in the worts. Satisfactory FAN and HWE levels[27] were obtained using a preparation containing amylolytic and proteolytic activities[28].

Until now it is still unclear which factor in the sorghum malt and unmalted sorghum causes the filtration characteristics of worts and beer. The amount of (1→3),(1→4)-ß-D-glucans in sorghum cell walls is very low[2] compared to barley. The (1→3),(1→4)-ß-D-glucan content is also low relatively to the amount of (glucurono-)arabinoxylans in sorghum cell walls[29]. Therefore, it is not likely that only the (1→3),(1→4)-ß-D-glucans can cause these problems. Other constituents such as the glucuronoarabinoxylans, cellulose or proteins might also be of importance. The aim of the present study was to investigate which factor determines the filtration characteristics. Therefore, two commercial enzyme preparations were used to study the degradation of sorghum cell walls during mashing. An attempt was made to correlate changes in filtration rate with the degradation of cell wall components, especially the glucuronoarabinoxylans (GAX).

EXPERIMENTAL

Sorghum malt

Sorghum malt (*Sorghum vulgare* cv. Fara Fara, Nigeria, 1992 harvest, 6 days malt) was kindly supplied by Heineken Technical Services (Zoeterwoude, The Netherlands). Sorghum malt and isolated endosperm cell wall material (WUS)[29] thereof, were analysed for starch content (test kit supplied by Boehringer, Mannheim, Germany), protein content[30] (5.8xN)[29], (1→3),(1→4)-ß-D-glucan (test kit supplied by Megazyme, Sydney, Australia)[29], cellulose[29], non-starch polysaccharide (NSP) content, sugar composition[29,31], and uronic acid content[29,32]. The activities of α- and ß-amylase, and (1→3),(1→4)-ß-D-glucanase in the malt were determined using the corresponding Megazyme assays.

Enzyme preparations

Two commercial available enzyme preparations were used in this study; preparation A, originating from *Trichoderma viride* and preparation B, originating from *Humicola insolens*. Both were dialysed extensively before use against 0.05M NaOAc buffer containing 0.01%w/v NaN_3, pH5.4 (tubings were replaced every 2h to prevent possible cellulase degradation). These two preparations were able to degrade sorghum cell wall material (WUS), whereas other preparations could not or only to a minor extent (preliminary research).

Both preparations were analysed for activities of α- and ß-amylase, (1→3),(1→4)-ß-D-glucanase (test kits supplied by Megazyme), xylanase (oat spelt xylan, Sigma), cellulase (CMCase: Akucell, AF 0305, AKZO, Arnhem, The Netherlands); Avicelase (Avicel, type SF, Serva, Heidelberg, FRG) and α-arabinofuranosidase (*p*-Nitrophenyl-arabinofuranoside). Xylanase and cellulase activities were measured by the release of reducing sugars from oat spelt xylan, Akucell and Avicel, as determined by the Nelson-Somogyi assay[33] (standard sugars were Xyl and Glc, accordingly). Substrates (1%w/v) were dissolved/suspended in 50mM NaOAc buffer, pH5.4.

Enzyme dilutions appropriate to achieve a good measurable absorption reading were used, incubated for 1h at 45°C, and inactivated subsequently at 100°C for 10min. The arabinosidase activity was determined by determining the release of *p*-nitrophenol at 30°C, spectrophotometrically at 400nm. One unit (U) was defined as the amount of enzyme needed to release 1 μmol of reducing endgroups from its substrate per minute under reaction conditions.

Preparation A was separated further into three fractions by anion-exchange chromatography[34]. For this purpose 20g was dissolved in 60ml 0.01M NaOAc buffer pH5.0. After centrifugation to remove solids, the supernatant was desalted by extensive dialysis against 0.01M NaOAc buffer, pH5.0 at 4°C (degradation of the tubing was prevented as described above). Column chromatography was carried out on DEAE-Bio-Gel A (200 x 40mm, Bio-rad Laboratories, Richmond, USA) at 4°C. A gradient of 0→0.1M NaCl in 0.01M NaOAc, pH5.0, was applied to elute bound material. All buffers contained 0.01M NaN_3 as preserving agent. Fractions were pooled based on the protein profile, detected at 280nm. Three pools were obtained. They were concentrated by ultrafiltration (cut-off 10,000) and analysed for the following activities (substrate; method): α-amylase (Megazyme), ß-amylase (Megazyme), (1→3),(1→4)-ß-D-glucanase (Megazyme), cellulase (Avicel, Akucell; Somogyi[33]), xylanase (azo-xylan; Megazyme), protease (azo-casein; A440nm), glycosidases such as α-L-arabinofuranosidase, ß-D-glucosidase, and ß-D-xylosidase (their corresponding *p*NP-substrates; A400nm) and activity towards alkali extracted sorghum GAX[35] (Somogyi[33]). All activities were measured in 0.05M NaOAc, pH5.0 at 45°C.

The protein contents of enzyme preparations and fractions thereof were determined by a modification of the Lowry-method[36]. Bovine serum albumin was used as standard.

Small scale mashes

Sorghum malt was milled in a Bühler Miag DLFU mill (gap setting 0.2). Mashing occurred in a mashing bath (BRF-type), stirring 50g sorghum malt with 200ml water constantly (pH was *c.*5.4). The amount of added enzyme preparation, based on xylanase activity (U), is specified in the results. A mashing scheme (starting at 45°C) was used in which both the endogenous and exogenous enzymes were able to act. After settling, part of the mash (100ml) was kept at 4°C (2ml was taken for amylase determinations), while the solids containing fraction was heated to gelatinize the starch. The two fractions were pooled, and heated to 70°C. After cooling down to 20°C and equalizing the weights of the mashes (450g) with water, these mashes were reheated to 60°C prior to filtration. Saccharification of starch was examined by staining an aliquot of each mash with I_2/KI. Filtration occurred at 60°C through filter paper (Schleicher & Schull, 589²). Filtration rates were determined by measuring the time for 100ml to pass the filter after the first turbid 50ml. Also the total filtration volume was measured.

Analyses of worts

The worts were analysed for extract yield[37] (Density Meter DMA 48, Paar, Australia), and total sugar composition (by gas chromatography after derivatisation of the monomeric sugars, obtained by acid hydrolysis, into their alditol acetates[29]). The viscosity of the wort was measured using a Micro Falling Ball Viscometer[38] (Haake, type 001 1926) at 4°C. High molecular weight material was separated from low molecular weight material by ethanol precipitation of worts, with and without a pretreatment with amyloglucosidase (Boehringer) to remove maltodextrins. Ethanol (96%v/v) was added slowly to 100ml wort, while stirred extensively, until a final ethanol concentration of 80%v/v was reached. Samples were left overnight at 4°C to settle. The precipitates (AIS), obtained after centrifugation (15min, 17,700*g*), were washed with 80%v/v ethanol. The AIS and combined supernatants were dried (air-dried followed by freeze-drying) for further use.

Sugar compositions were determined as mentioned above. Molecular weight distributions were

determined by high-performance size-exclusion chromatography (HPSEC) as described elsewhere[35]. Oligomers and monomers in the ethanol soluble fractions were analysed by high-performance anion-exchange chromatography (HPAEC)[35]. Elutions (20°C, 1ml/min) involved linear gradients of NaOAc in 0.1M NaOH from 0→0.2M during 30min, then increasing the NaOAc concentration to 0.6M in 0.1M NaOH during 15min, followed by an increase to 1M NaOAc in 0.1M NaOH in 5min. The column was equilibrated by eluting 0.1M NaOH for 15min.

Analyses of spent grains

NSP, starch, (1→3),(1→4)-ß-D-glucan, protein and cellulose were determined as described above. Since it was practically impossible to collect the spent grains quantitatively, yields of spent grains were calculated as follows: the amount of solubilized material (calculated from extract yield and wort volume) was subtracted from the original amount of malt.

Hemicellulosic polymers from spent grains and WUS were extracted by a slight modification of the method described by Verbruggen *et al*[5]. In principle the procedure was as follows: eight fractions were obtained by extracting WUS or spent grains subsequently, twice with saturated $Ba(OH)_2$ solutions (2g/400ml; BE1.1, BE1.2), twice with distilled water (200ml) after neutralization (pH5, HOAc; extracts were combined; BE2), 1M KOH at 4°C (200ml; 1K4), 1M KOH at RT (200ml; 1K20), 4M KOH at RT (200ml; 4K) and 4M KOH containing 4%w/v H_3BO_3 at RT (200ml; 4KB), respectively. All alkali solutions contained 1%w/v $NaBH_4$ to prevent alkaline degradation. The BE1.1 and BE1.2 fractions were neutralized (HOAc) and dialysed firstly against 0.2M NaOAc buffer, pH5 at 4°C, followed by distilled water. The other extracts and the final residue (Res) were neutralized, and also dialysed extensively against distilled water (at 4°C). All extracts were made up to 25ml. The extracts and the residues were stored at -20°C and thawed or freeze-dried before use, depending on application. Sugar compositions and the eluting behaviour on HPSEC after a pretreatment with amyloglucosidase, were determined for all extracts, as mentioned above.

The BE1.1 fractions were fractionated further by ion-exchange chromatography. Extracts (5ml) were applied to a DEAE-Sepharose CL-6B column (26cm x 1.8cm; Pharmacia) equilibrated with 0.005M NaOAc buffer, pH5.0. After application of the sample, the column was washed with the same buffer (60ml) and then eluted successively with a linear gradient of 0.005→2.0M NaOAc buffer, pH5.0 (500ml) followed by 2.0M NaOAc buffer (60ml) at 1.0ml/min. Fractions (5ml) were assayed for uronic acids, as described above, and neutral sugars[39]. Fractions forming a peak were pooled. The sugar compositions of the pooled fractions were determined as described above. The bound fractions were digested (1mg/ml) in 50mM NaOAc buffer (1ml total incubation volume, pH5.0) with a combination of endo-(1→4)-ß-D-xylanase I[40] (XylI; 0.22µg protein/ml) with (1→4)-ß-D-arabinoxylan arabinofuranohydrolase[41] (AXH; 0.06µg protein/ml) for 24h at 50°C, under continuous mixing "head-over-tail". Both enzymes were purified from *Aspergillus awamori* CMI 142717[40,41]. The incubation was stopped by heat (10min, 100°C). The change in molecular weight distribution was determined by HPSEC, and the release of oligomeric end-products by HPAEC as described above.

RESULTS

Characterization of malt

The white sorghum variety (*Sorghum vulgare* cv. Fara Fara) used in this study was

TABLE I. Composition of sorghum malt and isolated water-unextractable cell wall material (WUS) thereof (%w/w).

	Malt	WUS
Protein	9.9	9.5
Lipids	3.0	nd[a]
Starch	67.0	9.0
NSP[b]	5.3	76.6
of which:		
Ara	1.0	20.0
Xyl	1.0	19.2
Man	0.1	1.5
Gal	0.3	2.6
Glc	2.7	28.2
of which:		
Cellulose	1.1	10.8
ß-Glucan[c]	0.1	2.4
Uronic acid	0.2	5.1
Total declared	85.2	95.1

[a] Not determined; [b] Non-starch polysaccharides;
[c] (1→3),(1→4)-ß-D-glucan.

malted during six days and kilned. The activities of α-amylase, ß-amylase and (1→3), (1→4)-ß-D-glucanase were respectively 114, 187 and 0.1 U/g. The compositions of the malt and isolated water-unextractable cell wall material (WUS) thereof are presented in Table I. WUS was prepared to obtain a suitable model for sorghum malt cell walls. Malt was composed of *c.*10% protein, 3% hexane extractable lipids, 67% starch and 5% non-starch polysaccharides (NSP). The water content of the kilned malt was 5%. The WUS was composed of 76.6% NSP. It still contained 9.5% protein; over 95% of the proteins originally present in the malt was removed. Although 99% of the original starch was removed, the remaining 1% still represented 9% of WUS.

The NSP are mainly composed of Ara, Xyl, Glc and uronic acids. The arabinoxylans which contain considerable amounts of predominantly GlcA[35], were the group of polysaccharides which occur most abundantly in sorghum cell walls. In barley, these are the (1→3),(1→4)-ß-D-glucans. Based on the Ara/Xyl ratio, the (1→4)-ß-D-xylan backbone in sorghum malt arabinoxylans is heavily substituted by arabinofuranose groups, relative to barley[42]. The Ara units are presumably linked at mostly *O*-3 or both *O*-2 and *O*-3 of Xyl as is described for GAX from raw sorghum WUS[35]. The molar NSP composition of the WUS was similar to the malt (results not shown). This indicates that the WUS preparation may serve well as a model for the polysaccharide part of the cell wall.

TABLE II. Enzyme activities in preparation A and B (U/mg protein).

Activity	A	B
Xylanase	2.61	5.34
α-Amylase	tr[a]	tr
ß-Amylase	tr	0
ß-Glucanase[b]	3.13	0.94
Cellulase (Akucell)	55.77	0.04
Cellulase (Avicel)	2.08	0.03
α-Arabinofuranosidase	tr	0.12

[a] Trace amounts; [b] (1→3),(1→4)-ß-D-glucanase.

Characterization of commercial enzyme preparations

Two commercially available enzyme preparations were tested for starch and cell wall degrading activities (Table II). Amylase activity was very low in both preparations. Traces of both α- and ß-amylase were found in preparation A, whereas preparation B did contain only some α-amylase. Preparation A showed relatively high cellulase activity, whereas preparation B showed relatively high xylanase activity.

Because of the predominant presence of GAX in NSP, it was calculated from the xylanase activities that, theoretically, 2.14U xylanase should be sufficient to degrade all the xylan present in 100mg WUS to monomer in 1h (45°C, 0.05M NaOAc, pH5.4). Dosage of 2.14U xylanase activity of preparation A and B means that *c.*45.7U cellulase and 2.6U (1→3),(1→4)-ß-D-glucanase were added in the case of preparation A, whereas only 0.02U cellulase and 0.4U (1→3),(1→4)-ß-D-glucanase were added in the case of preparation B. In preliminary experiments, testing these doses in incubations with WUS, it was shown that hardly any degradation took place (0.04% and 0.1% for preparation A and B, respectively), probably due to a poor availability and complexity (*e.g.* degree and pattern of substitution) of the arabinoxylans. Multiple doses (preparation A 150x, preparation B 250x) were needed to increase the degree of degradation to 7.1% and 5.3%, respectively. Reaction products, analysed by HPAEC, consisted of mainly monomeric Ara, Xyl and Glc for both enzyme preparations. In addition, preparation A predominantly formed cellobiose next to other oligomers which were present only in low quantities. Cellobiose was hardly formed by digestion with preparation B. However, other oligomers than in the preparation A digest, were formed in higher quantities. As expected, the enzyme preparations have different patterns of action.

Mashing trials

Based on the previous experiments, enzyme doses were chosen in the mashing trials. Mashes (50g) were supplemented with 50 to 325U xylanase activity, originating from

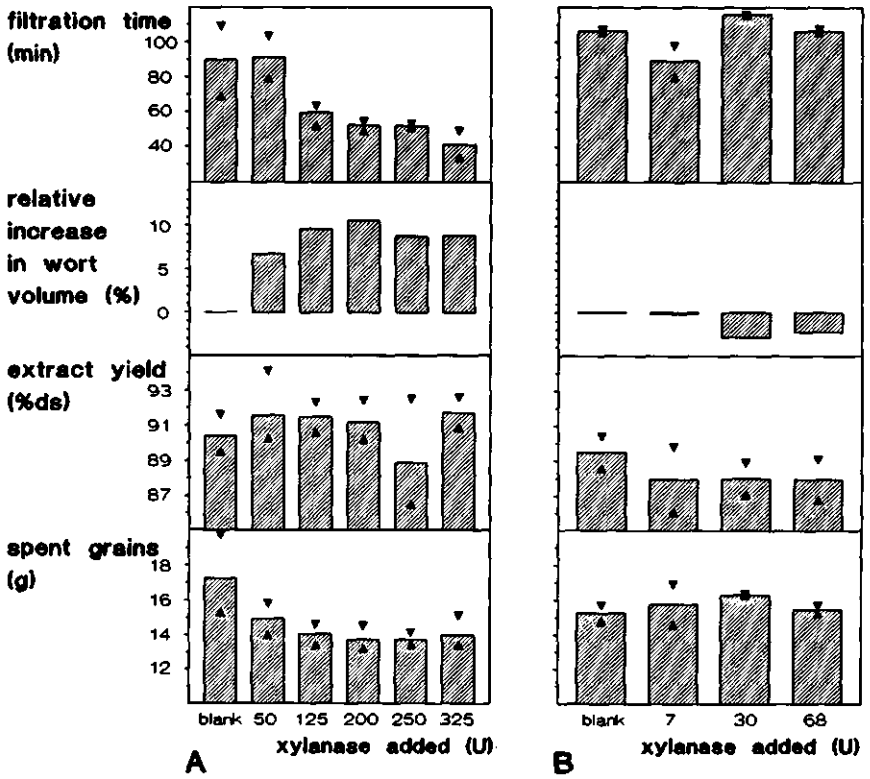


FIG 1. Mashing trials supplemented with various doses of preparation A (A) and preparation B (B). Doses are expressed as xylanase units. The effects of enzyme treatment on filtration time, relative increase in wort volumes, extract yields, and the amount of spent grains are presented (averages of 4 determinations, including the lowest (▲) and highest values (▼)).

preparation A or 7 to 68U xylanase from preparation B. The mashing performance was studied by determining the filtration time of 100ml wort as the most important parameter. Also the total wort volume and extract yield were determined. Theoretical weights of spent grains were calculated from these two parameters by subtracting the total amount of solubilized material from the 50g starting malt. The results are presented in Fig 1. The reproducibility within one mashing experiment (12 mashes) was satisfactory. The reproducibility between mashing trials, however, was rather poor. This is the reason for the large differences between the lowest and highest values presented in the figures (Fig 1). Although the absolute values differed quite a lot, similar trends were demonstrated. Absolute comparisons will therefore only be made within one mashing trial.

Clear dose-effect relationships were shown for preparation A for all measured parame-

TABLE III. Composition of spent grains calculated as g out of 50g malt.

A.	Brewing trials supplemented with preparation A.	Blank	125U[a]	325U
	Protein	4.88 (24.8)[b]	3.01 (20.6)	2.77 (20.5)
	Starch	6.09 (30.9)	6.43 (44.0)	5.74 (42.5)
	NSP	3.93 (19.9)	2.51 (17.2)	2.47 (18.3)
	of which:			
	Ara	1.05	0.69	0.68
	Xyl	1.07	0.67	0.67
	uronic acid[c]	0.21	0.15	0.15
	Glc[d]	0.95	0.33	0.43
	Cellulose	0.55	0.59	0.50
	ß-Glucan[e]	0.10	0.08	0.04
	Total declared[f]	75.6	81.8	81.3
	Spent grains (g)[g]	19.7	14.6	13.5
B.	Brewing trials supplemented with preparation B.	Blank	7U	30U
	Protein	3.14 (20.0)	3.51 (20.8)	2.70 (16.6)
	Starch	7.11 (45.3)	8.15 (48.2)	9.73 (59.7)
	NSP	2.50 (15.9)	3.38 (20.0)	2.31 (14.2)
	of which:			
	Ara	0.65	0.85	0.56
	Xyl	0.64	0.83	0.53
	Uronic acid[c]	0.14	0.18	0.15
	Glc[d]	0.42	0.84	0.35
	Cellulose	0.54	0.62	0.59
	ß-Glucan[e]	0.11	0.06	0.13
	Total declared[f]	81.2	89.0	90.5
	Spent grains (g)[g]	15.7	16.9	16.3

[a] Units xylanase activity; [b] % of spent grains in parenthesis; [c] Determined as anhydro-uronic acids; [d] Non cellulose, non (1→3),(1→4)-ß-D-glucan, non starch; [e] (1→3),(1→4)-ß-D-glucan; [f] As % of spent grains; [g] Calculated by subtracting the amount of solubilized material from 50g malt, see also *Experimental.*

ters. Filtration times decreased by *c.*30% by adding 125U xylanase from preparation A compared to control mashes. Higher doses resulted finally in a maximum decrease of *c.*45%. Accordingly, the wort volumes and the extract yields increased clearly as a function of the enzyme dose. The maximum reduction of the amount of spent grains (*c.*20%) was reached when 200U xylanase or more of preparation A was used in the mashing trial.

Mashing trials with preparation B supplementation did not show a clear pattern. There seemed to be an optimum for the amount of preparation B added during mashing. Too high levels of this enzyme showed none or even retarding effects on filtration. The improvement of the filtration time using relatively low levels of preparation B was not as prominent as for preparation A (only *c.*15%). Wort volumes hardly changed by the treatment with preparation B. A decrease of the extract yields, and a small increase in the amount of spent grains were found, using preparation B.

No relation was found between the effects on filtration and the α- and ß-amylase activities, which predominantly originated from the malt. The α-amylase activity varied from 60 to 80U/ml and the ß-amylase activity from 120 to 160U/ml wort.

Analysis of the spent grains

The compositions of the spent grains after freeze-drying are presented in Table III. To be able to make objective comparisons, the results were calculated for 50g malt. As already shown in Fig 1A, the total amount of spent grains decreased when preparation A was added during mashing. Predominantly the lower levels of remaining proteins and NSP accounted for this decrease. The amount of starch changed only slightly. Supplementation with preparation B resulted in higher levels of starch remaining in the spent grains. This explains the lower extract yields in Fig 1B.

Also the NSP composition changed in the spent grains (Table III). Lower levels of GAX, represented as the sum of Ara, Xyl and uronic acid, remained in the spent grains of preparation A supplemented mashes. Since the ratios of Ara/Xyl, and of uronic acid/Xyl, both as a measure for the degree of Xyl substitution, hardly changed, it could be concluded that the GAX composition was not changed during mashing. Although preparation A contained high cellulase activities, the cellulose content hardly changed.

It is well known that solubilized arabinoxylans can bind high amounts of water, causing viscous solutions[43] and may thereby cause filtration problems. The viscosity is determined by the hydrodynamic volume of the polymers, which is dependent on degree and pattern of substitution[44], as well as substitition with uronic acids, degree of polymerization, backbone length, and size of the side chains. On the other hand, unsolubilized GAX, in the spent grains may clog the filter, because they may bind water and swell. To study this, GAX populations were extracted from sorghum malt WUS, and from spent grains by alkaline solutions, and compared with eachother. The extracts obtained from the malt WUS were similar to those extracted from WUS from raw sorghum flour[35] with respect to yields, sugar composition and molecular weight distribution measured by HPSEC (results not shown). Apparently, the GAX populations in sorghum cell walls have not changed during malting. These data confirm the poor degradation of sorghum cell walls during germination as observed by means of electron microscopy by EtokAkpan and Palmer[15].

The sugar compositions of the GAX populations extracted from the spent grains were

TABLE IV. Molar sugar compositions of fractions of spent grains[a] obtained by sequential alkaline extraction[b].

	Spent grains	BE1.1	BE1.2	BE2	1K4	1K20	4K	4KB	Res
Rha	0.4	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ara	8.8	35.0	38.5	37.4	27.8	36.5	32.0	28.5	7.5
Xyl	8.5	32.0	35.2	33.2	28.1	34.1	33.2	24.7	6.9
Man	0.6	0.1	0.1	0.1	0.0	0.0	0.0	6.4	1.2
Gal	0.8	0.5	0.4	0.4	0.3	0.0	0.5	4.5	0.1
Glc	76.5	23.8	17.4	19.5	35.5	18.8	26.5	30.3	82.2
AUA	2.3	8.2	8.4	9.4	8.3	10.7	7.8	5.6	2.0

[a] Obtained from mash supplemented with 125U xylanase activity from preparation A; [b] See *Experimental* for a description of the extraction procedure and an explanation of the extract codes.

also similar. Table IV shows the molar sugar composition of the GAX populations extracted from the spent grains obtained after mashing sorghum malt supplemented with 125U xylanase from preparation A. Comparable sugar compositions were found in corresponding extracts from the other spent grains. The major part of the GAX present were extracted with saturated $Ba(OH)_2$ solutions (BE1.1; 20 to 25% of GAX present in the spent grains), 1M KOH (1K4; 15 to 20%) and 4M KOH (4K; 10 to 15%). The extraction by saturated $Ba(OH)_2$ has been proven before to be highly selective for (glucurono-)arabinoxylans from WUS[35,45]. Compared to fractions from WUS, however, relatively high amounts of glucans were co-extracted from the spent grains. These were probably starch degradation products which could not pass the dialysis tubes. The (1→3),(1→4)-ß-D-glucans were reported not to be solubilized by saturated $Ba(OH)_2$ solutions[35,45]. The major part of these glucans could be separated from the GAX fractions by anion-exchange chromatography (DEAE-Sepharose CL-6B). The unbound fractions were highly enriched in Glc, the bound fractions in GAX. The first extractant (saturated $Ba(OH)_2$) also extracted a number of low molecular weight glucans, which were again lost during dialysis. A mass balance could therefore not be made. These glucans were probably maltodextrins, which were left in the spent grains. Washing the spent grains will probably remove these compounds. The starch in all mashes was 'fully' saccharified, checked by the absence of blue color. The starch fragments were probably too small to stain with I_2. This method can apparently only be used as an indication for the saccharification of starch during mashing.

The molecular weight distributions of the extracts were investigated by HPSEC. As an example, Fig 2 shows the molecular weight distributions of the quantitatively most important extracts obtained from the spent grains of mashes supplemented with 125U xylanase from preparation A. To identify the nature of the peaks, the extracts were

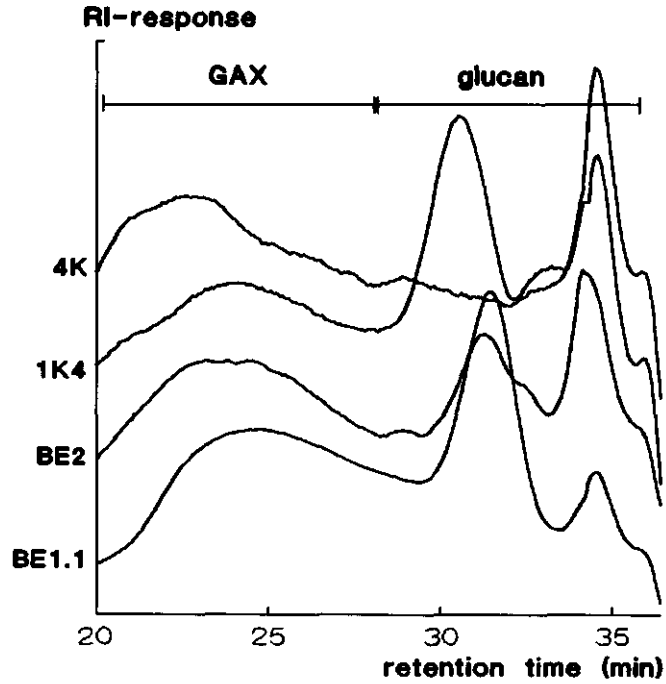


FIG 2. HPSEC of alkali extracted material from spent grains. Mash was supplemented with preparation A (125U of xylanase activity); see *Experimental* for the codes of the extracts.

fractionated by anion-exchange chromatography (DEAE-Sepharose CL-6B). The unbound and bound fractions were re-analysed on HPSEC, and their sugar compositions were determined. The components which were eluted at higher retention times (lower molecular weight) were composed mainly of Glc. Apparently, residual starch degradation products were not fully degraded further, although these samples were pretreated with amyloglucosidase. The high molecular weight materials were predominantly composed of Ara, Xyl and uronic acid. Similarly with the extracts from WUS from malt or raw sorghum flour[35], the molecular weight tended somewhat to shift toward the higher molcular weight ranges (decrease in retention time), when stronger alkaline solutions were used for the extractions. Again, the extracts of the various spent grains showed similar patterns (results not shown).

The bound fractions on DEAE-Sepharose CL-6B of the BE1.1 extracts were digested with a xylanase combined with an arabinoxylan specific arabinofuranosidase (AXH), similarly as described for GAX isolated from raw sorghum WUS[35]. The patterns of degradation of the fractionated BE1.1 extracts, analysed by HPAEC and HPSEC were identical to the one of raw sorghum GAX. The same oligomers were formed (HPAEC)

and a similar shift in molecular weight distribution (HPSEC) was shown (results not shown)[35].

Analysis of the worts

The sugar compositions of the carbohydrate material solubilized in the worts were very similar to each other, as expected >95% of the total sugar content was composed of Glc. By HPAEC it was observed that almost all components were starch degradation products like Glc, maltose, and maltodextrins (DP < 10). Oligomers originating from arabinoxylans could be observed in very low levels.

High molecular weight material was isolated from the worts by ethanol precipitation after treating these with amyloglucosidase. All ethanol soluble fractions were composed of Glc (*c.*96.5% of all monomeric sugars), Xyl (*c.*3%) and Ara (*c.*0.5%), and low levels of small oligomers. The ethanol insoluble fractions (AIS) contained still high amounts of glucan (*c.*40% of sugars in fraction), which was apparently not degraded by amyloglucosidase, presumably (1→3),(1→4)-ß-D-glucan. According to EtokAkpan[14], sorghum contains glucanases which can solubilize (1→3),(1→4)-ß-D-glucan from the cell wall, but lacks the enzymes which can further degrade its degradation products. Our results imply that preparation A as well as B, lack these enzymes as well. The AIS further contained *c.*15% Ara and *c.*12% Xyl, with slightly higher values for the preparation A supplemented samples. This indicates that indeed some more NSP was solubilized into the wort by this supplementation. HPSEC showed, however, that no clear differences could be observed between the several AIS fractions, which means that the released NSP were of similar molecular size and were hardly broken down further.

Mashing with fractions from preparation A

From the presented results above it was very difficult to determine which enzyme activity (or which combination of activities) in preparation A caused the filtration improving effect. Therefore preparation A was fractionated by anion-exchange chromatography, into three fractions (1 to 3) which were used in mashing trials. Fraction 1, containing the enzymes which did not bind to the column, showed the highest activity towards soluble oat spelt xylan and alkali extracted sorghum GAX. Also the activity towards CMC was highest in this fraction, although fraction 2 (eluting with 0.26M NaCl) also contained considerable activities towards these substrates. The avicelase activity was present in the first fraction together with the major part of α-arabinofuranosidase and proteolytic activities. The major part of the (1→3),(1→4)-ß-D-glucanases was found in fraction 2, however, this activity was also important in fraction 1. Fraction 3 eluted with 0.67M NaCl. Beldman *et al.*[34], who fractionated the same enzyme preparation in the same way, showed that in this fraction cellobiohydrolase activity accumulated. This was confirmed in our experiments by SDS-PAGE (results not shown). Also some avicelase

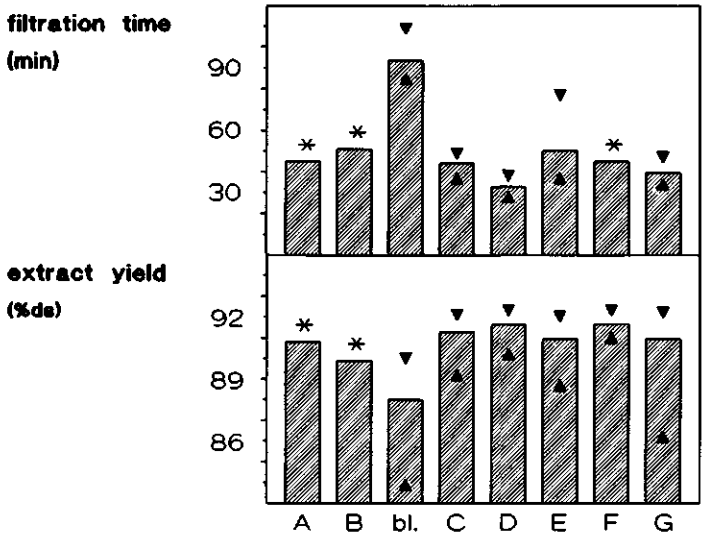


FIG 3. Mashing trials with supplementation with fractions of preparation A (averages of 5 determinations, including the lowest (▲) and highest values (▼) except * single determination). A, fr.1; B, fr.3; bl., blank; C, 250U of xylanase activity from preparation A; D, A+fr.1; E, A+fr.2; F, A+fr.1+2; G, A+fr.3. The effects of enzyme treatment on filtration time and extract yield are presented.

activity was present in this fraction. The enzyme dose used in the mashing trials was calculated based on the protein recovery in the three enzyme fractions.

Mashing trials were performed using the enzyme fractions alone, or in combination with preparation A (250U xylanase) to see whether certain enzyme activities are limiting in the crude enzyme preparation. Improvement of the performance by a certain combination would point to such activities. Fig 3 shows that fraction 1 could reduce the filtration time by another 20% when supplemented to preparation A (C). Also the extract yield was a little higher. Fraction 2 showed opposite effects. The filtration time was increased and extract yield decreased. The combination of enzyme fractions 1 and 2, showed no extra effect on filtration time, however, the extract yield had increased as much as for fraction 1 alone. Fraction 3 did not show any effect on filtration rate or extract yield when used in combination with preparation A (G). The supplementation with only fraction 3 (B) however showed a decrease in filtration time, but not as much as fraction 1 did alone (A). Also clear increases in extract yields were observed for both enzyme fractions. Fraction 1 could almost reach the same positive effects on filtration time and extract yield as the crude preparation A.

DISCUSSION

One of the major reasons to use malted sorghum instead of raw sorghum in brewing lager types of beer is the production of sufficient FAN by endogenous proteolytic enzymes. The production and activation of amylolytic and cell wall degrading enzymes during malting is generally rather low. In particular the low production of the latter may be part of the explanation for the often reported retention of the cell wall structures in malted sorghum[7,9-13]. This was confirmed by comparison of the cell wall polysaccharides. The NSP content and composition of the malt was comparable to the values for raw sorghum kernels[29]. The isolated WUS fractions and extracted arabinoxylan populations thereof were similar[35]. Contrary to our results, it has recently been reported, that the levels of insoluble arabinoxylans decrease during malting[46].

Compared to barley starches, sorghum starches are characterized by relatively high gelatinization temperatures. This may be a drawback for its use, but is very dependent on the variety used. This factor may require special adaptations for mashing programmes. Moreover, the level of polyphenols is also a factor of consideration for the choice of variety. In the present research the white variety *Sorghum vulgare* cv. Fara Fara was used. Its gelatinization temperature is *c.*73°C (Brabender amylograph, preliminary research) and tannins were not detected[29].

The results have shown that preparation A improved mashing performance: filtration times and amounts of spent grains decreased, whereas total wort volumes and extract yields increased. Preparation A contained primarily cellulose degrading activities. Some xylanase and (1→3),(1→4)-ß-D-glucanase activity was found and only traces of arabinofuranosidase activity. Apparently, the poor sorghum malt enzyme balance can (partly) be overcome by supplementing mashes with exogenous enzymes. Some reports about studies using exogenous enzymes in mashing and brwing trials with sorghum malt have appeared recently. Supplementation with preparations which contain α-amylase, ß-amylase, proteolytic, and (1→3),(1→4)-ß-D-glucanase activities have been studied. Commercial xylanase preparations have hardly been studied in relation to mashing or brewing performance in spite of the fact that the cell walls of sorghum do contain considerable amounts of xylans. Also cellulolytic enzyme preparations have not had much attention, probably because it is not logical to assume that cellulose degradation will have a direct influence on filtration or other parameters. By degrading cellulose, however, other polysaccharides and/or proteins may become accessible for enzymic degradation and solubilization.

The filtration improving effects were found in a fraction of preparation A which did not bind to DEAE-Bio-Gel A. This fraction contained almost all the xylanase activity originally present in the crude preparation. The major part of the (1→3),(1→4)-ß-D-glucanase activity was recovered in the second fraction, which actually retarded filtration. Other important activities present in the first fractions were cellulase and protease.

Proteolytic acitivities can be associated with both filtration improving[20] and retarding effects (preliminary results, not shown). It has been stated before[9] that the fungal cellulases from *Trichoderma viride* and *Penicillum funiculosum* can attack sorghum cell walls to release Glc. Pentose residues were also released by these enzymes. In brewing with sorghum malt, the major effect of the cellulases may be the loosening of the structure of the cell walls. This may cause solubilization of more NSP components, and enlarging the pore sizes in the filterbed. Supplementation with the proper xylanases may increase this effect. These xylanases should be able to hydrolyse highly substituted xylan backbones. The model experiments on WUS from sorghum malt has demonstrated that the xylanases, probably in combination with accessory enzymes such as arabinofuranosidases or glucuronidases, in preparation A were able to degrade the highly substituted xylans (results not shown). In mashing experiments, however, this type of degradation hardly occurred, probably due to the poor availability of the GAX.

The effect of preparation B was much less clear. Although this preparation contained the enzymes considered favourable for brewing, brewing performance declined. Unsurprisingly, in this case, the cellulose network seems to remain intact. The role of the xylanases remains unclear. Speculating further, partly degraded or modified components originating from *e.g.* GAX, (1→3),(1→4)-ß-D-glucans or proteins, could be retained in the spent grains, and act as filtration retarding agents as a result of their swelling and water binding properties. A similar proposition was suggested previously for partly degraded (1→3),(1→4)-ß-D-glucans in sorghum malt spent grains[12], but can probably be expanded for viscous substances in general.

Poor filtration characteristics have been associated with viscous substances in the worts[20], such as starch and cell wall material. The arabinoxylans in wort from barley malt do play a role in determining the viscosity[48]. The viscosities of the worts that were produced, were essentially the same in every mashing trial (*c.*3mPa·s), and too low to explain filtration problems.

The present research has not shown definitely, whether or not GAX in sorghum cell walls do have an effect on brewing performance. We were able to demonstrate that more GAX was released into the wort, with unchanged structural characteristics. Any relationship with the filtration rate could, however, not be proven. Several methods were used to investigate the NSP in the spent grains and the worts. The major problem in investigating this type of problem is that it is very difficult to remove all the starch, without loosing other interesting sugars. Even digestion with amyloglucosidase results in so much Glc, that accurate measurements the other sugars is difficult.

The NSP, and especially GAX, were emphasized during these studies. The compositional analysis of the spent grains from mashes supplemented with preparation A, however, has also shown a decrease in protein content. As others have indicated[20,24] the sorghum proteins might be of interest too with respect to brewing performance studies.

REFERENCES

1. Rooney, L.W. and Serna-Saldivar, S.O., in 'Handbook of Cereal Science and Technology' (K.L. Lorenz and K. Kulp, eds.), Marcel Dekker Inc., New York, USA (1991) pp 233-270.
2. Dufour, J.P., Mélotte, L. and Srebrnik, S.,'Sorghum malts for the production of a lager beer', *ASBC Journal* **50** (1992) 110-119.
3. Daiber, K.H.,'Enzyme inhibition by polyphenols of sorghum grain and malt', *J. Sci. Food Agric.* **26** (1975) 1399-1411.
4. Butler, L.G., Riedl, D.J., Lebryk, D.G. and Blytt, H.J.,'Interaction of proteins with sorghum tannin: mechanism, specificity and significance', *JAOCS* **61** (1984) 916-920.
5. Morrall, P., Boyd, H.K., Taylor, J.R.N. and Van Der Walt, W.H.,'Effect of germination time, temperature and moisture on malting of sorghum', *J. Inst. Brew.* **92** (1986) 439-445.
6. Lasekan, O.O.,'A preliminary study of the comparative malting qualities of *Sorghum bicolor* and *Sorghum guineensis*', *Food Chem.* **39** (1991) 241-247.
7. Aisien, A.O.,'Enzymic modification of sorghum endosperm during seedling growth and malting', *J. Sci. Food Agric.* **33** (1982) 754-759.
8. Olaniyi, O.A. and Akinrele, A.I.,'Screening Nigerian sorghum for malting characteristics', *J. Food Agric.* **2** (1987) 91-94.
9. EtokAkpan, O.U.,'Enzymic degradation and nature of the endosperm cell walls of germinating sorghums and barley', *J. Sci. Food Agric.* **61** (1993) 389-393.
10. Glennie, C.W.,'Endosperm cell wall modification in sorghum grain during germination', *Cereal Chem.* **61** (1984) 285-289.
11. Palmer, G.H.,'Enzymic degradation of the endosperm cell walls of germinated sorghum', *World J. Microbiol. Biotechnol.* **7** (1991) 17-21.
12. EtokAkpan, O.U.,'Comparative studies of the degradation of non-starchy polysaccharides by sorghums and barleys during malting', *J. Sci. Food Agric.* **58** (1992) 129-134.
13. Glennie, C.W., Harris, J. and Liebenberg, N.v.d.W.,'Endosperm modification in germinating sorghum grain', *Cereal Chem.* **60** (1983) 27-31.
14. EtokAkpan, O.U.,'Comparative studies of the ß-D-glucan released into sorghum and barley worts', *J. Inst. Brew.* **98** (1992) 301-304.
15. EtokAkpan, O.U. and Palmer, G.H.,'Comparative studies of the development of endosperm-degrading enzymes in malting sorghum and barley', *World J. Microbiol. Biotechnol.* **6** (1990) 408-417.
16. Taylor, J.R.N.,'Mashing with malted sorghum grain', *ASBC Journal* **50** (1992) 13-18.
17. Okafor, N. and Aniche, G.N.,'Brewing a lager beer from Nigerian sorghum', *Brewing and Distilling International* **11** (1980) 32-34.
18. Okafor, N. and Aniche, G.N.,'Studies on the brewing of lager beer from Nigerian sorghum', *J. Food Sci. Technol.* **24** (1987) 131-134.
19. Ogbonna, A.C.,'Developments in the malting and brewing trials with sorghum', *World J. Microbiol. Biotechnol.* **8** (1992) 87-91.
20. Aisien, A.O. and Muts, G.C.J.,'Micro-scale malting and brewing studies of some sorghum varieties', *J. Inst. Brew.* **93** (1987) 328-331.
21. Palmer, G.H., EtokAkpan, O.U. and Igyor, M.A.,'Sorghum as a brewing material', *MIRCEN Journal* **5** (1989) 265-275.
22. Odibo, F.J.C. and Obi, S.K.C.,'Mashing trials of two Nigerian sorghum varieties using thermostable microbial pullulanase', *MIRCEN Journal* **5** (1989) 187-192.
23. Aisien, A.O.,'Sorghum: a suitable source for brewing beer?', *Brewing and Distilling International* **19** (1988) 20-22,31.
24. Bajomo, M.F. and Young, T.W.,'The properties, composition and fermentabilities of worts made from 100% raw sorghum and commercial enzymes', *J. Inst. Brew.* **99** (1993) 153-158.
25. Ugboaja, F.C., Bednarski, W. and Babuchowski, A.,'The technology and properties of beer

produced from unmalted sorghum or maize grains', *World J. Microbiol. Biotechnol.* **7** (1991) 225-230.

26. Dale, C.J., Young, T.W. and Omole, A.T.,'Small scale mashing experiments with grits containing high proportions of raw sorghum', *J. Inst. Brew.* **96** (1990) 403-409.
27. Pickerell, A.T.W.,'The influence of free *alpha*-amino nitrogen in sorghum beer fermentations', *J. Inst. Brew.* **92** (1986) 568-571.
28. Bajomo, M.F. and Young, T.W.,'Development of a mashing profile for the use of microbial enzymes in brewing with raw sorghum (80%) and malted barley or sorghum malt (20%)', *J. Inst. Brew.* **98** (1992) 515-523.
29. Verbruggen, M.A., Beldman, G., Voragen, A.G.J. and Hollemans, M.,'Water-unextractable material from sorghum: isolation and characterization', *J. Cereal Sci.* **17** (1993) 71-82. (Chapter 2 in this thesis)
30. Roozen, J.P. and Boxtel, L. van,'Half-automatische bepaling van stikstof in levensmiddelen', *De Ware(n) Chemicus* **9** (1979) 192-200.
31. Englyst, H.N. and Cummings, J.H.,'Simplified method for the measurement of total non-starch polysaccharides by gas-liquid chromatography of constituent sugars as alditol acetates', *Analyst* **109** (1984) 937-942.
32. Blumenkrantz, N. and Asboe-Hansen, G.,'New method for quantitative determination of uronic acids', *Anal. Biochem.* **54** (1973) 484-489.
33. Somogyi, M.,'Notes on sugar determination', *J. Biol. Chem.* **195** (1952) 19-25.
34. Beldman, G., Searle-van Leeuwen, M.J.F., Rombouts, F.M. and Voragen, A.G.J.,'The cellulase of *Trichoderma viride*. Purification, characterization and comparison of all detectable endoglucanases, exoglucanases and ß-glucosidases', *Eur. J. Biochem.* **146** (1985) 301-308.
35. Verbruggen, M.A., Beldman, G. and Voragen, A.G.J.,'The selective extraction of glucuronoarabinoxylans from sorghum endosperm cell walls using barium and potassium hydroxide solutions', *J. Cereal Sci.* **21** (1995) 271-282. (Chapter 3 in this thesis)
36. Bensadoun A. and Winterstein, D.,'Assay of proteins in the presence of interfering materials', *Anal. Biochem.* **70** (1976) 241-250.
37. Official Method 988.06, *ASBC Journal* **36** (1978) 118.
38. Official Method 974.07, *ASBC Proceedings* **86** (1975).
39. Tollier, M.-T. and Robin, J.-P.,'Adaptation de la méthode à l'orcinol-sulfurique au dosage automatique des glucides neutres totaux: conditions d'application aux extraits d'origine végétale', *Ann. Technol. Agric.* **28** (1979) 1-15.
40. Kormelink, F.J.M., Searle-van Leeuwen, M.J.F., Wood, T.M. and Voragen, A.G.J.,'Purification and characterization of three endo-(1,4)-ß-xylanases and one ß-xylosidase from *Aspergillus awamori*', *J. Biotechnol.* **27** (1993) 249-265.
41. Kormelink, F.J.M., Searle-van Leeuwen, M.J.F., Wood, T.M. and Voragen, A.G.J.,'Purification and characterization of an (1,4)-ß-D-arabinoxylan arabinofuranohydrolase from *Aspergillus awamori*', *Appl. Microbiol. Biotechnol.* **35** (1991) 753-758.
42. Viëtor, R.J., Angelino, S.A.G.F. and Voragen, A.G.J.,'Structural features of arabinoxylans from barley and malt cell wall material', *J. Cereal Sci.* **15** (1992) 213-222.
43. D'Appolonia, B.L. and MacArthur, L.A.,'Comparison of starch, pentosans and sugars of some conventional height and semidwarf hard red spring wheat flours', *Cereal Chem.* **52** (1975) 230-239.
44. Andrewartha, K.A., Phillips, D.R. and Stone, B.A.,'Solution properties of wheat-flour arabinoxylans and enzymically modified arabinoxylans', *Carbohydr. Res.* **77** (1979) 191-204.
45. Gruppen, H., Hamer, R.J. and Voragen, A.G.J.,'Barium hydroxide as a tool to extract pure arabinoxylans from water-unextractable cell wall material from wheat flour', *J. Cereal Sci.* **13** (1991) 275-290.
46. EtokAkpan, O.U. and Palmer, G.H.,'Properties of endospem cell walls isolated from unmalted and malted grains of barley and sorghum', *Process Biochem.* **29** (1994) 559-563.
47. Verbruggen, M.A., Beldman, G. and Voragen, A.G.J.,'Enzymic degradation of sorghum glucuronoarabinoxylans leading to tentative structures', to be submitted for publication in *Carbohydr. Res.*

(1995). (Chapter 5 in this thesis)

48. Viëtor, R.J., Angelino, S.A.G.F. and Voragen, A.G.J.,'NSP composition of wort and spent grain from brewing trials with a good malting quality barley and a feeding barley', *J. Inst. Brew.* **99** (1993) 243-248.

CHAPTER 7

Concluding Remarks

AIM OF THIS RESEARCH

Since the Nigerian government restricted the import of brewhouse raw materials such as barley and malt in 1988, quite some research has been initiated on indigenous cereals suitable for to the production of lager type beers. Sorghum has been used traditionally in opaque beers; fermented beverages prepared from sorghum malt, containing insoluble materials. Therefore, a quite logical continuation, is the investigation of the performance of sorghum (malt) in lager beer brewing. The first mashing trials have shown that several problems may occur when sorghum malt is used[1]. Often reported problems are poor filtration characteristics (rates and volumes), low extract yields, low hot water extracts, and low levels of free amino nitrogen. It has become clear that the malting characteristics of sorghum varieties have a large impact on brewing performance. Certain varieties do not develop *e.g.* ß-amylase during malting, while others produce even more ß-amylase than α-amylase[2]. Sorghum malt is reported to develop only (1→3)-ß-D-glucanase resulting in a partial degradation of (1→3),(1→4)-ß-D-glucans to larger oligomers of DP ~ 26[3]. The sorghum variety *Sorghum vulgare* cv. Fara Fara, is considered to have good malting characteristics. Brewing performance, however, was rather poor. The worts obtained filtered very slowly.

The exact reasons for poor brewing performance are still unknown. Some authors suggest important roles for starch[1], viscous non-starch polysaccharides[1] such as (1→3),(1→4)-ß-D-glucans[1,3,4], or proteins[5]. In brewing, the arabinoxylans have been found to be related to wort and beer viscosity[6]. Investigations, in which components of crops like sorghum are studied in detail, are rather limited. Our studies have been directed towards a group of viscous non-starch polysaccharides, the arabinoxylans. These polysaccharides are reported to have a large impact on dough formation and baking performance of wheat flour[7], due to their high water holding capacity[8], demonstrating that a minor constituent can have a large impact on processing. As our laboratory has accumulated quite some experience in structural research on arabinoxylans from wheat[9] and barley[10], and on their enzymic degradation[11], we engaged in this study to sorghum arabinoxylans. The objectives for the research presented here were (i) to isolate these hemicelluloses, (ii) to elucidate the structural characteristics of water-unextractable (glucurono-) arabinoxylans from flours of sorghum and sorghum malt, (iii) to determine the enzymic degradability of these (glucurono-)arabinoxylans, and (iiii) to investigate the changes in their structural characteristics during malting and brewing.

WATER-UNEXTRACTABLE NSP

This research was started with the isolation of water-unextractable cell wall materials (WUS) from flour of sorghum, polished sorghum (Chapter 2), and in a later stage sorghum malt (Chapter 6), satisfactory free of lipids, starch and proteins. It may be concluded that the

developed method for the isolation of WUS fulfils its purpose (Chapter 2). WUS from maize and rice were prepared satisfactory in a similar way (unpublished results). This method might have a drawback when one is interested in both the water-extractable and the water-unextractable NSP. The water-extractable NSP were recovered in the protein fraction, in which very high amounts of SDS remained, and which might be difficult to remove. The sorghum variety used in this study contained only a very small amount of water-extractable NSP (Chapter 2). Therefore, other techniques like preparing alcohol insoluble solids were not further investigated.

Other investigators used other methods for the isolation of sorghum cell walls (WUS). EtokAkpan[12] used a 50% H_2SO_4 treatment to remove the testa, followed by a milling and several sieving steps to obtain a crude cell wall preparation. Final sieving of this fraction in 70% ethanol through 75 μm nylon resulted in a starch free cell wall preparation. This preparation was composed of *c.*28% ß-glucan, 62% protein and only 4.3% arabinoxylan. It can be expected that a certain part of the linkages between Ara and Xyl were hydrolysed by the acid treatment of the intact kernels. The ß-glucan content was determined after hydrolysis and determination of Glc. The presence of starch was excluded as proved by failure of blue color development upon I_2-staining; however, no further proof was presented. Because it has been shown in Chapter 6, that I_2-staining is no guarantee for the absence of starch, the data for ß-glucans from EtokAkpan are questionable. In another method[13], 60% *t*-butanol containing dithiothreitol was used to remove prolamin proteins, followed by similar sieving steps to remove starch. Still 42% protein was present in the preparation, which was found not to be an integral part of the cell walls, but associated with them, indeed.

The sugar compositions of the WUS (Chapter 2) showed that the levels of (1→3),(1→4)-ß-D-glucan were rather low, and that quite some cellulose was present. The arabinoxylans were highly substituted with mainly terminal Ara units (Chapter 3). This was expressed by the high Ara/Xyl ratio (*c.*0.9, Chapter 2). A small amount of arabinogalactans was recovered in the water-extractable SDSS fraction (Chapter 2). The composite polysaccharides were extracted from the WUS by a sequential alkaline extraction procedure (Chapter 3). Arabinoxylans are considered to be H-bonded to cellulose, and oxidatively linked to *e.g.* other arabinoxylans, proteins or lignin. These cellulose-bound arabinoxylans can be situated on the surface of, or between the cellulose fibrils. To extract the latter mentioned hemicelluloses, higher hydroxyl concentrations up to 6M are necessary to cause extensive swelling of the cellulose[14]. In the present research, particularly the first extractant (saturated $Ba(OH)_2$), resulted in a rather pure glucuronoarabinoxylan (GAX) population. Also saturated $Ca(OH)_2$ solutions could be used to extract GAX of similar purity. It is discussed that in the latter extracts, yields were lower because of a lower hydroxyl concentration. A certain hydroxyl concentration is essential for the release of certain cell wall polysaccharides[15], by saponifying ester linkages. Probably these GAX have originated from the surface of the cellulose fibrils. The presence of a bivalent, rather than a monovalent cation, is considered to be responsible for the selectivity of

extraction, by keeping other polysaccharides such as the (1→3),(1→4)-ß-D-glucans insoluble by complexation (Chapter 3). The swelling of cellulose by treatments with 1M and 4M KOH resulted in the extraction of more GAX. In similar sequential extractions of WUS from wheat[16] it was noticed that the Ara/Xyl ratios increased with increasing hydroxyl concentrations in the extractant. This was not the case for sorghum GAX, the Ara/Xyl ratios decreased a little. It was observed in all extracts, that every arabinoxylan population contained uronic acids. The majority of the uronic acids were GlcA, however, small quantities of 4OMeGlcA and GalA could be detected. Therefore it was concluded that all arabinoxylan populations were actually composed of highly substituted GAX.

Combining these results with those from other studies in our laboratory, it is concluded that the specific extraction of (glucurono-)arabinoxylans by saturated $Ba(OH)_2$ (and probably also $Ca(OH)_2$ solutions) may work universally for all cereals and all types of (glucurono-)-arabinoxylans. Gruppen *et al.*[17] introduced this method, which proofed to be extremely selective to extract arabinoxylans from WUS of wheat flour and wheat bran[18]. These first findings were followed by extracting rather pure arabinoxylan populations from WUS of barley and barley malt[19], maize and rice (unpublished results). Table I shows the molar sugar compositions of the $Ba(OH)_2$ extracts of all these WUS preparations.

TABLE I. Molar sugar compositions of saturated $Ba(OH)_2$ extracted material from several cereal WUS preparations.

	Sorghum[20]	Rice	Maize	Wheat flour[17]	Wheat bran[18]	Barley[19]	Barley malt[19]
Ara	42	38	39	34	41	38	39
Xyl	42	48	51	65	53	53	57
Man	0	0	0	0	0	1	0
Gal	2	2	4	0	1	0	0
Glc	3	4	1	0	1	8	2
Uronic acid	10	9	6	0	4	0	0
Ara/Xyl	1	0.8	0.8	0.5	0.8	0.7	0.7

The Ara/Xyl ratio is the highest for sorghum, rice and maize. These populations also contain the highest levels of uronic acids. Since these cereals can be grown in (semi-)tropical environments, it might be that the GAX in the cell walls may have a function in protecting the grains against heat and/or drought, maybe by strongly retaining water in the outer layers of the grains, but also in the endosperm cell walls. Another posibility is that due to the very high degree of substitution, the GAX molecules show reduced flexibility. This may have a hardening effect on the GAX containing tissues. Further speculating, highly substituted GAX could also be involved in the resistance to microbial infection, although such role has also

been ascribed to proteins[21,22]. The GAX has proven to be poorly degradable by xylanases (Chapter 5), due to its very high degree of substitution, and pattern of substitution. As presented in Table I, the outer layers of wheat kernels (the bran fraction, which has a protective function) also show a slightly higher Ara/Xyl ratio and contains uronic acid. The generally accepted assumption that water solubility would improve with an increasing degree of substitution, is not confirmed by our results. Of course, other factors may play a role in the solubility of polysaccharides, such as DP, the distribution of side groups and possible interactions or associations with other cell wall components. The average molecular weight of sorghum GAX is higher, compared to wheat arabinoxylans[23]. Once the highly substituted GAX populations are solubilized by alkali, they are soluble in water only in low amounts.

Structural Characteristics of Sorghum GAX

Further fractionation of the isolated GAX populations was difficult to accomplish (Chapter 3). Contaminating glucans could be removed by anion-exchange chromatography, which, unlike glucuronoarabinoxylans, did not bind to this anion-exchanger. No further separation in sub-populations could be performed by size-exclusion chromatography. The molecular weight distributions of the populations were very broad, not showing peaks. No further fractionation was accomplished with graded ethanol precipitation. All material precipitated at 70% ethanol. Arabinoxylan populations from wheat and barley could be fractionated further according their Ara/Xyl ratio by graded ethanol precipitation. Typically, the 70% ethanol fractions of these arabinoxylans were composed of arabinoxylans with high Ara/Xyl ratios (1.08 and 1.07 for respectively wheat[23] and barley[19]) as found for sorghum. Apparently, the sorghum GAX populations are rather homogeneous, or their structural characteristics make further fractionation difficult. The effects of the high levels of GlcA as terminal single unit side group are not well understood in this fractionation procedure.

The GAX populations were degraded by purified xylanases (XylI, XylIII, GXH), arabinofuranosidases (AraB, AXH) and an α-glucuronidase (GlcAase) (Chapter 5). Just recently, the purification and characterization of a similar α-glucuronidase has been reported by Siikaaho *et al*[24]. This enzyme was capable of splitting off (4OMe)GlcA from the terminal non-reducing Xyl, as the glcAase which was used in this study. Clear differences between the three GAX populations were shown with respect to their degradability by the xylanolytic enzymes.

The GAX extracted with $Ba(OH)_2$, the purest GAX fraction, was the only population which could be degraded to a certain extent. Several GlcA containing oligomeric degradation products have been purified and identified (Chapter 4). Two of them were unique in that they were composed of an oligomeric (1→4)-ß-xylan backbone substituted with GlcA (α-(1→2)), and arabinosyl side chains, composed of an Ara dimeric unit, attached at *O*-3 of Xyl. Many

authors indicated the presence of these side chains for arabinoxylans from different origins based on methylation analysis[19,25-30]. The availability of well defined purified enzymes was indispendable for the preparation of the GAX specific oligomers. Furthermore, the knowledge about their modes of action was very valuable in the elucidation of the structures. Prefractionation on Fractogel TSK HW-40(S) and purification by preparative HPAEC (CarboPac PA-100) have shown to be powerful techniques for the separation and purification of such compounds (Chapter 4). The more or less 'traditional' chromatographic techniques would not have been suitable for this purpose.

Oligomers isolated by HPAEC, contained quite some NaOAc, which had to be removed prior to further elucidation by ^{1}H NMR. During desalting it was noticed that the small arabinosyl side chains are rather acid labile, or that the presence of GlcA causes an increased sensitivity of the other groups in the molecule. Degradation of GAX oligoaccharides was observed using similar desalting methods as have been used succesfully for the desalting of neutral arabinoxylan oligosaccharides. The arabinoxylan oligosaccharides apparently tolerated the acid conditions which occured, whereas the GAX oligosaccharides did not. Three desalting methods were tried, two of them were successfully used for desalting 4-*O*-methylglucuronoxylan oligomers[31], and neutral arabinoxylans[32-34]. Desalting oligomers composed of Xyl and 4OMeGlcA[31], was performed using a column of Dowex 50W X8 (H^+) resin (Bio-Rad), followed by evaporation of HOAc (co-evaporation using MeOH). For GAX oligomers this method failed because, although desalting was performed at 4°C, Ara was easily released from the oligomers. Neutral arabinoxylan oligomers were easily desalted using the previously mentioned Dowex column in combination with Dowex AG3 X4A (OH^-) resin in series[32-34]. Again, Ara was released from GAX. In our case using size-exclusion appeared to be the best method; salt levels were reduced satisfactory for ^{1}H NMR analysis. A complete removal, however, could not be accomplished, probably because the negative charge of GlcA units resulted in co-elution of cations other than H^+.

The population extracted with 4M KOH was not degraded at all by endo-xylanase I (XylI), also not when AXH was added to introduce cleaving sites by removing Ara units. This important information enabled us to propose a tentative model for sorghum GAX (Chapter 4). Based on this structural information together with our knowledge about the mode of action of the enzymes, five structural elements could be designed which backbones are undegradable by these enzymes. The undegradable population (4K) is thought to be strictly composed of these five elements. The other populations (BE1.1 and 1K4) are believed to be small modifications of the undegradable one. The GAX extracted by 1M KOH is similar to the 4M KOH extracted one, however some insertions of *O*-3- or unsubstituted Xyl units occur to interrupt the undegradable sequences. This results in some degradation, essentially without the formation of small oligomeric products. The $Ba(OH)_2$ extract again differs from the 1M KOH extract in that *O*-3- or unsubstituted Xyl are also introduced within the basic structural elements. It is quite remarkable that with increasing strength of the extraction medium,

populations were obtained with only very small structural differences, but with large different degradation characteristics. Interestingly, the patterns of degradation of BE1.1 from WUS obtained from rice and maize by the same set of enzymes, were similar to the degradation patterns of sorghum BE1.1 (unpublished results), suggesting a similar structure. This was further substantiated by comparable results in the methylation analyses (unpublished results).

Fig 1 shows a compilation of proposed models for arabinoxylans of several origins. The major resemblance is the recognition of regions which are less easily degraded by xylanases than other regions. This feature is translated in models with regions, characterized by highly and lower substituted xylan backbones, respectively. Neither rye, wheat, nor barley contained GlcA or short side chains. The structural model for barly arabinoxylans was not as well underpinned as the ones for rye and wheat. Barley arabinoxylans contain more Ara*f* units linked at *O*-2 of Xyl residues in the backbone compared to wheat arabinoxylans. Unfortunately, no models are proposed for maize or rice GAX. A good comparison with those polysaccharides is therefore not possible. Until now there are no reasons to believe that also in sorghum regions occur, which are more degradable than others. Disubstituted Xyl units were not present in the elucidated oligomers. Apparently, these were mainly present in the undegradable parts. More research will be needed to study this further.

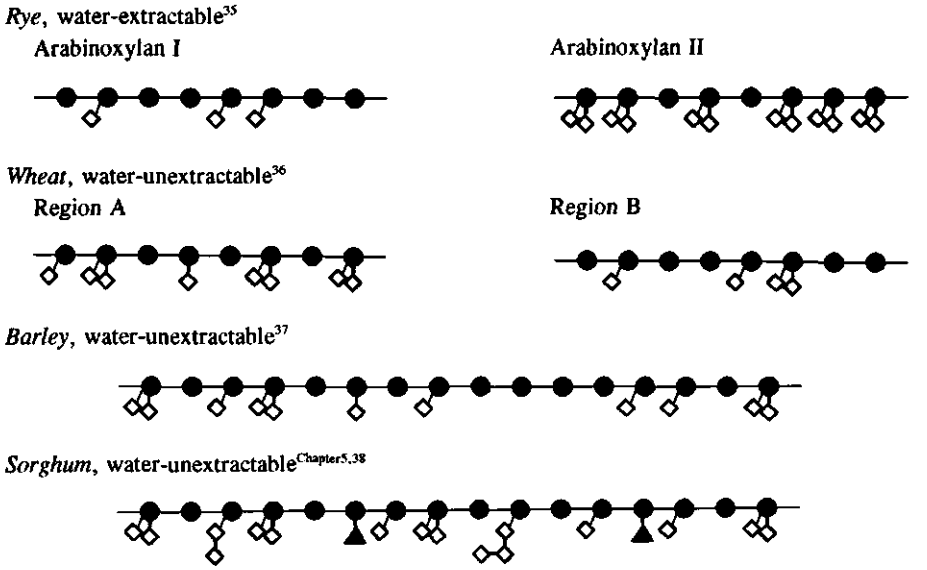
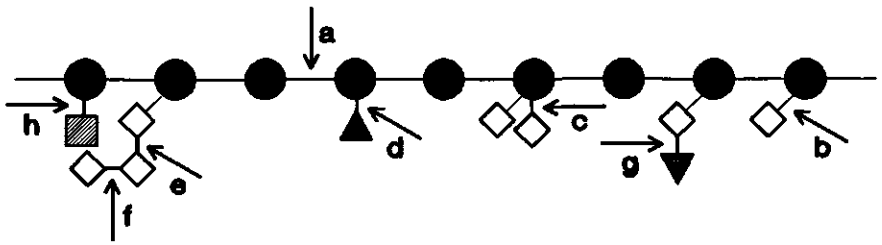


FIG 1. Compilation of structural models for cereal (glucurono-)arabinoxylans.

The mode of action of XylI, XylIII, AXH and AraB on neutral arabinoxylans is known[39,40]. The identification of GlcA containing degradation products (Chapter 4), revealed some

additional information on the mode of action of the enzymes toward GlcA substituted arabinoxylans, which are summarized in Table II of Chapter 5. Still these enzymes were not capable of degrading the sorghum GAX extensively. Obviously other enzymes are needed such as arabinofuranosidases which split off Ara from *O*-2-monosubstituted or disubstituted Xyl, or glucuronidases active on polymeric substrates. Enzymes active towards the short Ara side chains have to be considered too. Probably we will have to search for the opposite of the very specific AXH; an extremely a-specific arabinofuranosidase, which would be able to cleave off both *O*-2- or *O*-3-linked Ara residues. Other arabinofuranosidases which would be able to attack the Ara units of the disubstituted Xyl residues, or the short chain Aras, would be helpful. The GAX in sorghum is, when incorporated in the cell wall in native form, esterified with ferulic and coumaric acid and acetyl groups (Chapter 2). Particularly the ferulic acids are known to be able to link polysaccharides with each other or with proteins[41] and lignin, and herewith influence the degradability[42]. Alkali, as has been used, will of course destroy these bridges[15]. Feruloyl-, coumaroyl- and acetylesterases will therefore be needed to release GAX from cell wall materials. In Fig 2 a xylan backbone is drawn, with all the substituents present in the case of sorghum GAX. All possible sites of enzyme attack are indicated by the arrows, with an indication of which enzyme would be capable of hydrolysing the indicated linkage. The figure summarizes what is mentioned above.



a. Endo-(1,4)-ß-D-xylanase
b. (1,3)-α-L-Arabinofuranosidase
c. (1,2)-α-L-Arabinofuranosidase
d. (1,2)-α-D-Glucuronidase
e. (1,2)-α-L-Arabinofuranosidase
f. (1,5)-α-L-Arabinofuranosidase
g. Ferulic acid or coumaric acid esterase
h. Acetyl xylan esterase

FIG 2. Sites of attack in sorghum GAX for xylanolytic and accessory enzymes.

BREWING WITH SORGHUM MALT

As visualized by electron microscopy[43,44], endosperm cell walls from sorghum were hardly degraded during malting, although so-called portals in the cell walls were noticed. The starch granules were present with pitted surfaces due to amylolytic degradation. These holes were

however also present in raw sorghum starches[45]. They are considered to be the sites of initial enzyme attack, or to be related to control of starch conversion during germination. The structure of the starch at these spots is considered to be more accessible for enzymes. The formation of portals in the cell walls suggests a certain degree of degradation. The composition of sorghum malt and WUS thereof, however, was very similar to raw sorghum (Chapter 6). On the other hand, it has been reported that the amount of 'pentosans', determined as total sugar content minus ß-glucans (=cellulose), decreases whereas the (1→3),(1→4)-ß-D-glucan content increases in sorghum[46-48], during malting. Based on only these observations, it has been speculated that (1→3),(1→4)-ß-D-linked glucans are being synthesized during malting[48]. The data however are presented as % of the malt, not taking the malting losses into account. On the other hand, others reported, a decrease in (1→3),(1→4)-ß-D-glucan during malting[4]. Varieties with low (1→3),(1→4)-ß-D-glucan levels, showed better performance with respect to filtration and wort volumes, than malts with higher (1→3),(1→4)-ß-D-glucan levels.

It has been demonstrated (Chapter 5) that the GAX from sorghum cell walls are difficult to degrade, due to their highly substituted structure. During mashing with sorghum malt some of these GAX are being released into the wort (Chapter 6). This implies that in the sorghum malt particular enzymes are synthesized or activated which can attack such complicated GAX structures. Next to endogenous xylanases, accessory enzymes like α-glucuronidases, arabinofuranosidases, but also esterases like feruloyl-, or acetylesterases have to be considered, in this respect. Solubilization of GAX could also be a secundary result of degradation of other cell wall components.

The exact origin for filtration problems remains unknown, although it is generally accepted that during malting, sorghum is not producing enough of the favourable enzymes recognized for the reduction of viscosity, and water-holding capacity of the wort and spent grains respectively. It even has been stated that it is not beneficial to use sorghum malt to produce beer. Raw sorghum with exogenous enzymes would give more acceptable worts and beers[49]. Barley malt can be a source for additional enzymes[50]. Barley malt, as such or with additional commercial enzymes, has also been added in brewing trials using 80% raw sorghum[51]. As an extra advantage, barley malt will also provide a filter bed. Others used an amylolytic enzyme mixture from *Bacillus* sp. and *Aspergillus* sp. to improve filtration rate[52]. These crude enzyme preparations may contain, besides amylolytic activities, also cell wall degrading activities. Also the potential production of enzymes by the microorganisms *e.g.* out of sorghum silos[53] or from the seed microflora[54-56] need to be studied.

Two commercial enzyme preparations were chosen in the presented work. They were first tested towards WUS from sorghum malt. A certain degree (*c.*6%) of degradation could be observed. Preparation A, the cellulolytic preparation, released only small amounts of oligomeric arabinoxylan products, whereas digestion by preparation B, containing xylanases and (1→3),(1→4)-ß-D-glucanases at relatively higher levels, resulted in higher amounts of

arabinoxylan degradation products. It has been reported by Bajomo and Young[51] that the use of xylanase reduces the wort viscosities. The same authors actually found that with any of the tested enzyme preparations (proteolytic, amylolytic, xylan, and ß-glucan degrading), increased wort filtration volumes were found. However, they did not present any clues about the exact enzyme activities necessary. Mashing trials supplemented with preparation A improved filtration characteristics. Unfortunately, we can only speculate on explanations for this result. A certain degree of cellulose degradation might have loosened the cell wall. This may result in improved solubilization of starch, GAX, (1→3),(1→4)-ß-D-glucans, and proteins by an improved accessibility of these substrates for endogenous malt enzymes and the enzymes present in preparation A. Cellulases are lacking in preparation B. EtokAkpan[46] suggested that fuco-xyloglucan may contribute to the resistance to enzymic attack. Until recently it was considered that the xyloglucans from graminaceous monocotyledons were free of fucose. McDougall and Fry[57] revised this idea by proving the existence of a fucosylated xyloglucan in tall fescue grass. To our knowledge, the presence of such xyloglucans in grains have never been reported. The fucose, which EtokAkpan[46] has found, could have originated from glycoproteins. The used cell wall preparation contained a lot of protein. Fucose has, however, never been determined in the present study. There is also no evidence obtained for the presence of a fucose-free xyloglucan.

Only a few research groups are tackling the problems of sorghum beer brewing in a biochemical way. Other recent studies focus mainly on the breeding of new sorghum varieties with improved malting characteristics[2,58,59], and an improved brewing performance[2,4,59]. Furthermore, improved malting conditions[60,61] and mashing processes[51,62,63] are being developed. Recent investigations have shown that barley varieties with poor malting characteristics (low in ß-glucanases) can be improved by genetic techniques[64]. Extending this approach to sorghum, better sorghum malts would become available. To realize such an approach, much more knowledge about sorghum (malt) is necessary.

In conclusion it seems that the problems with brewing a lager type beer from sorghum malt are still far from being solved. Successful brewing trials, however, have been reported. Recently, acceptable beers have been produced from a certain sorghum variety (SK 5912) without the aid of exogenous enzymes, according to a patented brewing method[65].

REFERENCES

1. Aisien, A.O. and Muts, G.C.J.,'Micro-scale malting and brewing studies of some sorghum varieties', *J. Inst. Brew.* **93** (1987) 328-331.
2. Dufour, J.P., Mélotte, L. and Srebrnik, S.,'Sorghum malts for the production of a lager beer', *ASBC Journal* **50** (1992) 110-119.
3. EtokAkpan, O.U.,'Comparative studies of the ß-D-glucan released into sorghum and barley worts', *J. Inst. Brew.* **98** (1992) 301-304.

4. Ogbonna,A.C. and Egunwu, A.L.,'ß-Glucan degradation in malting sorghum', *World J. Microbiol. Biotechnol.* **10** (1994) 595-596.
5. Bajomo, M.F. and Young, T.W.,'The properties, composition and fermentabilities of worts made from 100% raw sorghum and commercial enzymes', *J. Inst. Brew.* **99** (1993) 153-158.
6. Viëtor, R.J., Angelino, S.A.G.F. and Voragen, A.G.J.,'Nsp composition of wort and spent grain from brewing trials with a good malting quality barley and a feeding barley', *J. Inst. Brew.* **99** (1993) 243-248.
7. Gruppen, H., Kormelink, F.J.M. and Voragen, A.G.J.,'Enzymic degradation of wheat flour arabinoxylans', *J. Cereal Sci.* **18** (1993) 129-143.
8. Rouau, X. and Moreau, D.,'Modification of some physicochemical properties of wheat flour pentosans by an enzyme complex recommended for baking', *Cereal Chem.* **70** (1993) 626-632.
9. Gruppen, H., Ph.D. Thesis, Wageningen Agricultural University, Wageningen, The Netherlands (1992).
10. Viëtor, R.J., Ph.D. Thesis, Wageningen Agricultural University, Wageningen, The Netherlands (1992).
11. Kormelink, F.J.M., Ph.D. Thesis, Wageningen Agricultural University, Wageningen, The Netherlands (1992).
12. EtokAkpan, O.U., Ph.D. Thesis, Heriot-Watt University, Edinburg, U.K. (1988).
13. Glennie, C.W.,'Endosperm cell wall modification in sorghum grain during germination', *Cereal Chem.* **61** (1984) 285-289.
14. Edelmann, H.G. and Fry, S.C.,'Factors that affect the extraction of xyloglucan from the primary cell walls of suspension-cultured rose cells', *Carbohydr. Res.* **228** (1992) 423-431.
15. Fry, S.C.,'The Growing Plant Cell Wall: Chemical and Metabolic Analysis', Longman Scientific and Technical, Harlow, U.K. (1988).
16. Gruppen, H., Hamer, R.J. and Voragen, A.G.J.,'Water-unextractable cell wall material from wheat flour. I. Extraction of polymers with alkali', *J. Cereal Sci.* **16** (1992) 41-51.
17. Gruppen, H., Hamer, R.J. and Voragen, A.G.J.,'Barium hydroxide as a tool to extract pure arabinoxylans from water-unextractable cell wall material of wheat flour', *J. Cereal Sci.* **13** (1991) 275-290.
18. Bergmans, M., Beldman, G., Gruppen, H. and Voragen, A.G.J.,'Optimisation of the selective extraction of arabinoxylans from wheat bran: use of barium and calcium hydroxide solutions at elevated temperatures', accepted for publication in *J. Cereal Sci.* (1996).
19. Viëtor, R.J., Angelino, S.A.G.F. and Voragen, A.G.J.,' Structural features of arabinoxylans from barley and malt cell wall material', *J. Cereal Sci.* **15** (1992) 213-222.
20. Verbruggen, M.A., Beldman, G. and Voragen A.G.J.,'The selective extraction of glucuronoarabinoxylans from sorghum endosperm cell walls using barium and potassium hydroxide solutions', *J. Cereal Sci.* **21** (1995) 271-282. (Chapter 3 in this thesis)
21. Kumari, S.R. and Chandrashekar, A.,'Relationships between grain hardness and the contents of prolamin and three anti-fungal proteins in sorghum', *J. Cereal Sci.* **20** (1994) 93-99.
22. Kumari, S.R. and Chandrashekar, A.,'Isolation and purification of three antifungal proteins from sorghum endosperm', *J. Sci. Food Agric.* **64** (1994) 357-364.
23. Gruppen, H., Hamer, R.J. and Voragen, A.G.J.,'Water-unextractable cell wall material from wheat flour. II. Fractionation of alkali-extracted polymers and comparison with water-extractable arabinoxylans', *J. Cereal Sci.* **16** (1992) 53-67.
24. Siika-aho, M., Tenkanen, M., Buchert, J., Puls, J. and Viikari, L.,'An alpha-glucuronidase from *Trichoderma reseei* RUT C-30', *Enz. Microb. Technol.* **16** (1994) 813-819.
25. Kato, Y and Nevins, D.J.,'Enzymic dissociation of *Zea* shoot cell wall polysaccharides I. Preliminary characterization of the water-insoluble fraction of *Zea* shoot cell walls', *Plant Physiol.* **75** (1984) 740-744.
26. Darvill, J.E., McNeil, M., Darvill, A.G. and Albersheim, P.,'Structure of plant cell walls XI. Glucuronoarabinoxylan, a second hemicellulose in the primary cell walls of suspension-cultured sycamore cells', *Plant Physiol.* **66** (1980) 1135-1139.
27. Nishitani, K. and Nevins, D.J.,'Enzymic analysis of feruloylated arabinoxylans (Feraxan) derived from *Zea mays* cell walls I. Purification of novel enzymes capable of dissociating Feraxan fragments from *Zea mays* coleoptile cell wall', *Plant Physiol.* **87** (1988) 883-890.
28. Shibuya, N. and Iwasaki, T.,'Structural features of rice bran hemicellulose', *Phytochem.* **24** (1985) 285-

289.
29. Shibuya, N., Misaki, A. and Iwasaki, T.,'The structure of arabinoxylan and arabinoglucuronoxylan isolated from rice endosperm cell wall', *Agric. Biol. Chem.* **47** (1983) 2223-2230.
30. Gowda, D.C., Gowda, J.P. and Anjaneyalu, Y.V.,'Structure of an arabinoxylan from the bark of *Persea macrantha* (Lauraceae)', *Carbohydr. Res.* **108** (1982) 261-267.
31. Düsterhöft, E.-M., Bonte, A.W., Venekamp. J.C. and Voragen, A.G.J.,'The role of fungal polysaccharidases in the hydrolysis of cell wall materials from sunflower and palm-kernel meals', *World J. Microbiol. Biotechnol.* **9** (1993) 544-554.
32. Gruppen, H., Hoffmann, R.A., Kormelink, F.J.M., Voragen, A.G.J., Kamerling, J.P. and Vliegenthart, J.F.G.,'Characterisation by ^{1}H-NMR spectroscopy of enzymically-derived oligosaccharides from alkali-extractable wheat-flour arabinoxylan', *Carbohydr. Res.* **233** (1992) 45-64.
33. Kormelink, F.J.M., Hoffmann, R.A., Gruppen, H., Voragen, A.G.J., Kamerling, J.P. and Vliegenthart, J.F.G.,'Characterization by ^{1}H-n.m.r. spectroscopy of oligosaccharides derived from alkali-extractable wheat-flour arabinoxylan by digestion with endo-(1,4)-ß-xylanase III from *Aspergillus awamori*', *Carbohydr. Res.* **249** (1993) 369-382.
34. Viëtor, R.J., Angelino, S.A.G.F., Hoffmann, R.A., Kamerling, J.P., Vliegenthart, J.F.G. and Voragen, A.G.J.,'Structures of small oligomers liberated from barley arabinoxylans by endoxylanase from *Aspergillus awamori*', *Carbohydr. Res.* **254** (1994) 245-255.
35. Bengtsson, S., Åman, P. and Andersson, R.E.,'Structural studies on water-soluble arabinoxylans in rye grain using enzymatic hydrolysis', *Carbohydr. Polym.* **17** (1992) 277-284.
36. Gruppen, H., Kormelink, F.J.M. and Voragen, A.G.J.,'Water-unextractable cell wall material from wheat flour. III. A structural model for arabinoxylans', *J. Cereal Sci.* **18** (1993) 111-128.
37. Viëtor, R..J., Kormelink, F.J.M., Angelino, S.A.G.F. and Voragen, A.G.J.,'Substitution patterns of water-insoluble arabinoxylans from barley and malt', *Carbohydr. Polym.* **24** (1994) 113-118.
38. Verbruggen, M.A., Beldman, G. and Voragen, A.G.J.,'Enzymic degradation of sorghum glucuronoarabinoxylans leading to tentative structures', to be submitted for publication in *Carbohydr. Res.* (1996). (Chapter 5 in this thesis)
39. Kormelink, F.J.M., Gruppen, H., Viëtor, R.J. and Voragen, A.G.J.,'Mode of action of the xylan-degrading enzymes from *Aspergillus awamori* on alkali-extractable cereal arabinoxylans', *Carbohydr. Res.* **249** (1993) 355-367.
40. Kormelink, F.J.M., Gruppen, H. and Voragen, A.G.J.,'Mode of action of (1,4)-ß-D-arabinoxylan arabinofuranohydrolase (AXH) and α-L-arabinofuranosidases on alkali-extractable wheat-flour arabinoxylan', *Carbohydr. Res.* **249** (1993) 345-353.
41. Iiyama, K., Lam, T.B.-T. and Stone, B.A.,'Covalent cross-links in the cell wall', *Plant Physiol.* **104** (1994) 315-320.
42. Eraso, F. and Hartley, R.D.,'Monomeric and dimeric phenolic constituents of plant cell walls – possible factors influencing wall biodegradability', *J. Sci. Food Agric.* **51** (1990) 163-170.
43. Palmer, G.H.,'Enzymic degradation of the endosperm cell walls of germinated sorghum', *World J. Microbiol. Biotechnol.* **7** (1991) 17-21.
44. EtokAkpan, O.U. and Palmer, G.H.,'Comparative studies of the development of endosperm-degrading enzymes in malting sorghum and barley', *World J. Microbiol. Biotechnol.* **6** (1990) 408-417.
45. Fannon, J.E., Hauber, R.J. and BeMiller J.N.,'Surface pores of starch granules', *Cereal Chem.* **69** (1992) 284-288.
46. EtokAkpan, O.U.,'Enzymic degradation and nature of the endosperm cell walls of germinating sorghums and barley', *J. Sci. Food Agric.* **61** (1993) 389-393.
47. EtokAkpan, O.U. and Palmer, G.H.,'Properties of endosperm cell walls isolated from unmalted and malted grains of barley and sorghum', *Process Biochem.* **29** (1994) 559-563.
48. EtokAkpan, O.U.,'Comparative studies of the degradation of non-starchy polysaccharides by sorghums and barleys during malting', *J. Sci. Food Agric.* **58** (1992) 129-134.
49. Bajomo, M.F. Ph.D thesis, University of Birmingham, Birmingham, U.K. (1991).
50. Demuyakor, B., Ohta, Y., Nakatani, K., Fukui, N. and Kanagawa, K.,'Brewing of beer with *Sorghum vulgare* malt with minimal barley malt blending', *ASBC Journal* **52** (1994) 111-115.

51. Bajomo, M.F. and Young, T.W.,'Development of a mashing profile for the use of microbial enzymes in brewing with raw sorghum (80%) and malted barley or sorghum malt (20%)', *J. Inst. Brew.* **98** (1992) 515-523.
52. Agu, R.C., Okeke, B.C. and Chibuko, C.C.,'Application of microbial enzymes in the mashing of Nigerian millet and sorghum malts', *Bioresource Technol.* **44** (1993) 53-56.
53. Tjandraatmadja, M., Norton, B.W. and MacRae, I.C.,'Fermentation patterns of forage sorghum ensiled under different environmental conditions', *World J. Microbiol. Biotechnol.* **7** (1991) 206-218.
54. Satish Kumar, L., Prakash, H.S., Shetty, H.S. and Malleshi, N.G.,'Influence of seed mycoflora and harvesting conditions on milling, popping and malting qualities of sorghum (*Sorghum bicolor*)', *J. Sci. Food Agric.* **55** (1991) 617-625.
55. Satish Kumar, L., Daodu, M.A., Shetty, H.S. and Malleshi, N.G.,'Seed mycoflora and malting characteristics of some sorghum cultivars', *J. Cereal Sci.* **15** (1992) 203-209.
56. Boboye, B.E. and Adetuyi, F.C.,'Fungal populations associated with raw materials and intermediate products of lager beers produced from Nigerian sorghum grains', *J. Food Sci. Technol.* **31** (1994) 148-150.
57. McDougall, G.J. and Fry, S.C.,'Fucosylated xyloglucan in suspension-cultured cells of the graminaceous monocotyledon', *Festuca arundinacea*', *J. Plant Phys.* **143** (1994) 591-595.
58. Demuyakor, B. and Ohta, Y.,'Comparison of the malt characteristics of traditional and improved varieties of *Sorghum vulgare* from Ghana', *J. Inst. Brew.* **99** (1993) 227-230.
59. Swanston, J.S., Rao, N.S., Subramanian, V. and Taylor, K.,'The influence of some aspects of grain quality on malting potential in sorghum', *J. Cereal Sci.* **19** (1994) 91-97.
60. Owuama, C.I. and Asheno, I.,'Studies on malting conditions for sorghum', *Food Chem.* **49** (1994) 257-260.
61. Rathnavati, C.V. and Bala Ravi, S.,'Effect of different durations of steeping and malting on the production of alpha-amylase in sorghum', *J. Cereal Sci.* **14** (1991) 287-296.
62. Aniche, G.N. and Anih, A.C.,'Malting properies of two sorghum cultivars and the effect of mashing schemes on their wort properties', *Process Biochem.* **29** (1994) 369-371.
63. Taylor, J.R.N. and Dewar, J.,'Role of alpha-glucosidase in the fermentable sugar composition of sorghum malt mashes', *J. Inst. Brew.* **100** (1994) 417-419.
64. Phillipson, B.A.,'Expression of a hybrid (1-3,1-4)-ß-glucanase in barley protoplasts', *Plant Sci.* **91** (1993) 195-206.
65. Ogundiwin, J.O., Ilori, M.O. and Okelaye, A., in 'Industrial Utilisation of Sorghum', Summary Proceedings of a Symposium on the Current Status and Potential of Industrial Uses of Sorghum in Nigeria (1990) pp 31.

SUMMARY

The subject of this thesis was the characterization of glucuronoarabinoxylans (GAX) from sorghum. Sorghum grain is a very important food grain in the semi-arid regions of the world, where it is used in traditional breads, porridges and beers. As a food grain, sorghum is ranked fifth after wheat, maize, rice and barley.

A rather recent development is the production of a lager type beer from sorghum and its malt. During this process some problems occur, such as an incomplete saccharification of the starch, and a poor filterability of the mash and final beer. Specifically the filtration problems were the background of this research. The reasons for this typical behaviour are still not known, although some speculations about the possible roles of proteins, starch, and hemicelluloses are published. An extensive description of the general background is presented in Chapter 1. In the presented thesis, it was tried to answer the question if certain hemicellulosic components present in cereal cell walls, called glucuronoarabinoxylans, play a role in filtration. To do this, it was necessary to obtain detailed information about the structural characteristics of these components of sorghum.

First of all, a fraction rich in cell wall materials was prepared from sorghum flour (Chapter 2). The sorghum used, was predominantly composed of starch (*c.*67%), protein (*c.*10%), lipids (*c.*4%) and non-starch polysaccharides (NSP, *c.*5%). The free lipids could be removed from the sorghum flour by extraction with *n*-hexane. This step was followed by the removal of proteins. This was done by extraction with a sodium dodecylsulphate solution, containing mercaptoethanol as a reducing agent. The majority of the present starch could be removed by wet sieving through a 45μm sieve. Residual starch was degraded and solubilized by amylases, and washed from the cell wall preparation by hot water. The final residu was called water-unextractable solids (WUS), representing the cell wall materials of sorghum. The mass balances which were determined were satisfactory, losses were kept to a minimum. As expected the NSP had concentrated in the WUS (74% of the WUS). They were primarily composed of cellulose (*c.*12%), glucuronoarabinoxylan (*c.*42%), and (1→3),(1→4)-ß-D-glucan (*c.*3%).

WUS was also prepared from polished sorghum (Chapter 2) and malted sorghum (Chapter 6) according the same isolation procedure. All three WUS preparations were compared, leading to the following conclusions: (i) the NSP content of the outer layers of the sorghum kernels was much higher than of the endosperm, (ii) the composition, however, of the NSP from the outer layers and endosperm was comparable, and (iii) neither the NSP content nor its composition changed markedly during the malting of sorghum.

The next step was to isolate the glucuronoarabioxylans from the sorghum WUS (Chapter 3). Saturated $Ba(OH)_2$ solution has been proven before to be a highly selective extractant of neutral arabinoxylans from WUS from wheat and barley. Since only *c.*30% of the GAX could be extracted from sorghum WUS in this way, the method was extended with several subsequent extractions using KOH solutions of increasing molarity. Alkaline degradation of the polysaccharides was prevented by the addition of $NaBH_4$ to the extractants. Mass balances

were again calculated. A rather pure polysaccharide population was obtained by extraction with saturated $Ba(OH)_2$ solutions. This population was predominantly (90%) composed of arabinose, xylose and uronic acids. The other extracts obtained by 1M KOH and 4M KOH contained more glucose. The most prevalent uronic acid was glucuronic acid. Small amounts of 4-*O*-methyl-glucuronic acid and galacturonic acid were present as well. The polysaccharides were therefore called glucuronoarabinoxylans (GAX). The arabinose to xylose ratio indicated a very highly substituted xylan backbone. As in arabinoxylans of other cereals the arabinose units are generally substituted at *O*-3 or at both *O*-2 and *O*-3 of a xylose unit in the backbone. Methylation analysis showed the presence of *O*-2 monosubstituted xylose. Glucuronic acid units were linked at this position. The $Ba(OH)_2$ extracted GAX seemed to be quite homogeneous, since they could hardly be further fractionated by several chromatographic and precipitation techniques (Chapter 3). The glucans present in the 1M KOH, and 4M KOH extracted fraction could be separated from the GAX by anion-exchange chromatography. The residue which remained after the extraction procedure was largely composed of cellulose.

Three extracts were selected to study their enzymic degradation. These were the fractions extracted by saturated $Ba(OH)_2$ (BE1.1), 1M KOH (1K4), and 4M KOH (4K) solutions. These fractions together comprised 67% of the GAX originally present in the WUS. Differences between the populations were found in the arabinose to xylose to glucuronic acid ratio, and the molecular weight distributions. BE1.1 contained relatively more arabinose and glucuronic acid, and showed the lowest apparent molecular weight (Chapter 3).

These three substrates appeared to be poorly degradable by any combination of xylanases, arabinofuranosidases and an α-glucuronidase (Chapter 5). In these studies three xylanases were used (XylI, XylIII and GXH). The modes of action of these enzymes were largely known, and of course very specific for each enzyme. XylIII showed only very small activities on all three substrates, hardly any decrease in molecular weight distribution was observed. Even a supplementation with an arabinoxylan specific arabinofuranosidase (AXH) to create more cleaving sites for the xylanase to act, did not help. Only in the case of BE1.1, incubation resulted in some shift in molecular weight distribution. BE1.1 also was the only substrate which could be degraded by GXH. Apart from the shift in apparent molecular weight, the formation of some typical oligosaccharides was noticed. The 1K4 and 4K fractions were not degradable by this enzyme. XylI was the most active xylanase on at least two of the three substrates tested. The 4K fraction again remained difficult to degrade, even when the incubation mixture was supplemented with AXH. Decreases in apparent molecular weights became very clear in the digests of XylI with BE1.1 or 1K4. The molecular weight distributions shifted further toward the lower molecular weight ranges when AXH was added. Furthermore, only in the case of BE1.1, the formation of oligosaccharides by XylI became distinct, and even more when AXH was supplemented to the incubation mixture. Several of these oligosaccharides could be degraded further by α-glucuronidase, indicating the presence

of glucuronic acid residues. Also neutral arabinoxylan oligosaccharides, which have been described before by others, could be recognized.

Apparently, BE1.1 was the only substrate which released oligosaccharides upon treatment with XylI. AXH was needed to reach levels of oligosaccharides high enough to purify and identify them (Chapter 4). Four new oligosaccharides were elucidated by combining analytical techniques, such as ^{1}H NMR, FAB-MS and GC-MS, with knowledge of the modes of action of the used enzymes. The oligosaccharides had a (1→4)-ß-D-xylan main chain in common, composed of three or four xylose units. The *O*-2-position of the non-reducing terminal xylose was substituted with an α-glucuronic acid unit, in all purified oligosaccharides. The reducing xylose units were unsubstituted. The differences were observed in the substitution patterns of the one or two internal xylose units. These were substituted at *O*-3 with mono- or dimeric arabinose groups. The terminal arabinose unit in the dimeric arabinose side chain was linked at *O*-2 of the arabinose unit linked to the xylose residue of the backbone. In three other oligosaccharides the presence of *O*-2 linked arabinose containing side groups was indicated. Disubstituted xylose units were not found in any of the oligosaccharides.

A tentative structure for sorghum GAX was proposed (Chapter 5), based on the degradation studies and the identified oligosaccharides. The foundations for the model were the sugar composition of the undegradable 4K fraction, and the knowledge about the properties of the enzymes. 4K GAX is now thought to be constructed out of five structural elements which backbones will not be degradable by XylI, XylIII, GXH, and AXH. 1K4 GAX would contain un- or monosubstituted xylose units between some of the undegradable structural elements. As a result, this polysaccharide would be partly degradable, however, without a distinct release of oligosaccharides. The proposed model for BE1.1 GAX, is again a slight modification. Apart from the elements mentioned before, this GAX also contains degradable elements. These will be responsible for the formation of oligomers.

Micro-scale mashing experiments were performed, to investigate the role of GAX in especially wort filtration. Mashes from sorghum malt were supplemented with several dosages of commercial enzyme preparations, selected on their capability to degrade isolated GAX and WUS (Chapter 6). Wort filtration characteristics were improved with one of the enzymes (preparation A). The worts and spent grains were examined extensively. More GAX was solubilized upon enzyme supplementation, however, the structural characteristics of the GAX which remained in the spent grains were not changed. One of the main activities in preparation A was cellulase activity, next to xylanase activity. It is speculated, that cellulose in the sorghum malt is modified in such a way, that GAX which is located between the cellulose fibrils, is more easily solubilized. Herewith, the role of GAX in wort filtration still remains unclear.

SAMENVATTING

Het in dit proefschrift beschreven onderzoek betrof de karakterisatie van glucuronoarabinoxylanen (GAX) uit sorghum. Sorghum is in Nederland een onbekende graansoort. Echter, wanneer de mondiale produktiecijfers van granen als voedingsgewas in ogenschouw genomen worden, neemt sorghum een vijfde plaats in, na tarwe, mais, rijst en gerst. Bepaalde volkeren uit bv. Afrika, zuid-Azië en zuid- en midden-Amerika verwerken dit graan in o.a. traditionele broden, pappen en bieren.

Een recente ontwikkeling is de produktie van een lager bier type, zoals dat in Europa bekend is. Karakteristieke problemen die bij het vervaardigen van dit bier uit sorghum om de hoek komen kijken, zijn o.a.: een slechte versuikering van zetmeel wat noodzakelijk is voor een optimale alcoholproduktie, en slechte filtratie-eigenschappen. Met name de slechte filtratie gold als achtergrond voor het hier beschreven onderzoek. De oorzaak van dit probleem is niet bekend. De achtergrond van dit onderzoek wordt uitgebreid toegelicht in Hoofdstuk 1. In dit proefschrift is onderzocht of bepaalde bestanddelen uit de celwanden, de glucuronoarabinoxylanen, een rol spelen bij de filtratie, en welke strukturele eigenschappen deze componenten hebben.

Allereerst werd het celwandmateriaal geïsoleerd uit de sorghumkorrels (Hoofdstuk 2). Sorghum bestaat voornamelijk uit zetmeel (ca.67%), eiwit (ca.10%), lipiden (ca.4%) en polysachariden anders dan zetmeel (NSP, ca.5%). Na malen, werd het aanwezige vrije vet verwijderd m.b.v. *n*-hexaan. Vervolgens werden de eiwitten zoveel mogelijk verwijderd met natrium dodecylsulfaat, waarin zich mercapto-ethanol bevond als reduktiemiddel. Het grootste deel van het zetmeel kon verwijderd worden door nat te zeven over een 45μm zeef. Het resterende zetmeel werd vervolgens afgebroken en opgelost door α-amylase, en verwijderd d.m.v. heet water extrakties. Het residu dat na deze behandelingen overbleef, werd celwand materiaal genoemd (WUS). Van deze isolatiemethode werd een redelijk kloppende massabalans verkregen. In de WUS bevond zich vooral de NSP (ca.74% van WUS), bestaande uit voornamelijk cellulose (ca.12%), glucuronoarabinoxylaan (ca.42%) en (1→3),(1→4)-ß-D-glucaan (ca.3%).

Dezelfde fraktioneringsmethode werd gebruikt om WUS te isoleren uit geslepen sorghum (Hoofdstuk 2) en sorghummout (Hoofdstuk 6). De belangrijkste konklusies die vergelijking van de samenstellingen van deze twee WUS frakties met die van de eerste WUS opleverden, waren (i) dat het gehalte aan NSP in de buitenste lagen van de sorghumkorrel veel hoger was dan in het endosperm (binnenste deel), (ii) dat de NSP samenstelling van de buitenste lagen vergelijkbaar was met die van het endosperm, en (iii) dat noch de NSP samenstelling noch de NSP hoeveelheid noemenswaardig veranderden door te mouten.

Een volgende stap was het isoleren van de glucuronoarabinoxylanen uit de sorghum-WUS (Hoofdstuk 3). Hiervoor werd een eerder beschreven methode gebruikt, waarbij arabinoxylanen geëxtraheerd konden worden uit tarwe-WUS m.b.v. verzadigde $Ba(OH)_2$-oplossingen. De methode werd uitgebreid met vervolgextrakties m.b.v. diverse KOH-oplossingen. Alkalische afbraak werd voorkomen door de toevoeging van $NaBH_4$ aan de

extraktiemiddelen. Over deze fraktioneringen werd wederom een vrij kloppende massabalans verkregen. Met $Ba(OH)_2$ kon een vrij zuivere populatie polysachariden verkregen worden die voor 90% bestond uit arabinose, xylose en uronzuren. In de overige extrakten bleek meer glucose voor te komen. In alle extrakten was het meest voorkomende uronzuur glucuronzuur, maar 4-*O*-methyl-glucuronzuur en galacturonzuur kwamen eveneens voor. Daarom werden deze polysachariden glucuronoarabinoxylanen genoemd. De verhouding arabinose/xylose duidde op een xylaanketen die in hoge mate gesubstitueerd was met arabinose. In arabinoxylanen komt arabinose vooral voor als enkelvoudige zijgroepen die op de *O*-3-plaats van een xylose-eenheid zit, of op zowel de *O*-2- en *O*-3-plaats van een xylose-eenheid. Methyleringsanalyse liet zien dat ook *O*-2-gesubstitueerd xylose voorkwam. Deze plaats werd voornamelijk bezet door de uronzuren. Fraktionering van de $Ba(OH)_2$-geëxtraheerde GAX-populatie in subpopulaties bleek nauwelijks mogelijk. Diverse chromatografische technieken alsmede stapsgewijse alcohol precipitatie resulteerden niet in een verdere scheiding. In de overige extrakten bleek een glucose-houdende fraktie van de GAX-populatie gescheiden te kunnen worden d.m.v. anionen-uitwisselingschromatografie. Het residu wat overbleef na de extrakties bleek voornamelijk uit cellulose te bestaan.

Om de afbreekbaarheid van sorghum-GAX te bestuderen werden drie extrakten geselekteerd, en wel de frakties geëxtraheerd met verzadigde $Ba(OH)_2$-, 1M KOH- en 4M KOH-oplossingen. De extrakten werden respektievelijk BE1.1, 1K4 en 4K genoemd. Tesamen bevatten deze frakties 67% van het in WUS aanwezige GAX. Ze verschilden in arabinose/xylose/glucuronzuur-verhoudingen, met BE1.1 relatief meer arabinose en glucuronzuur t.o.v. de overige twee. 4K had het hoogste schijnbare molekuulgewicht, BE1.1 het laagste van de drie extrakten (Hoofdstuk 3).

Alle drie extrakten bleken zeer matig tot niet afbreekbaar met verschillende combinaties van xylanases, arabinofuranosidases en een α-glucuronidase (Hoofdstuk 5). Drie xylanases werden getest (XylI, XylIII en GXH), ieder met een verschillend werkingsmechanisme. XylIII gaf bij alle substraten geen enkele mate van afname in molekuulgewichtsverdeling te zien. Toevoeging van een arabinoxylaan-specifiek arabinofuranosidase mocht niet baten. Alleen in het geval van BE1.1 was een zeer lichte verschuiving waarneembaar. Ook GXH kon alleen BE1.1 afbreken. Dit leidde tot een afname in schijnbaar molekuulgewicht en de vorming van enkele oligomeren. XylI bleek het meeste effekt te hebben op tenminste twee van de drie substraten. 4K bleek nog steeds nauwelijks afbreekbaar, ook wanneer AXH aan het reaktiemengsel toegevoegd werd. Voor BE1.1 en 1K4 verschoven de molekuulgewichtsverdelingen aanmerkelijk onder inwerking van XylI, en in het geval van BE1.1 kon de vorming van oligomeren waargenomen worden. Deze vorming werd versterkt door toevoeging van AXH. Diverse van de gevormde oligomeren werden afgebroken door α-glucuronidase, wat wees op de aanwezigheid van glucuronzuur. Een aantal neutrale, al beschreven oligomeren, werden herkend.

BE1.1 bleek als enig substraat m.b.v. XylI en AXH afbreekbaar tot oligomeren, in

hoeveelheden die voldoende waren om opgezuiverd te kunnen worden (Hoofdstuk 4). Er werden vier nog niet eerder beschreven oligomeren geïsoleerd, opgezuiverd en geïdentificeerd. De combinatie van analysetechnieken zoals ^{1}H NMR, FAB-MS en GC-MS, en de kennis van de werkingsmechanismen van de gebruikte enzymen bleek daarbij zeer waardevol. De opgehelderde oligomeren hadden een (1→4)-ß-D-xylaan-hoofdketen gemeen, die bestond uit drie of vier xylose-eenheden. In alle oligomeren was aan de *O*-2-plaats van het niet-reducerende eindstandige xylose een α-glucuronzuur-groep gebonden, de reducerende xylose-eenheden waren ongesubstitueerd. De tussenliggende xylose-eenheden waren gesubstitueerd op diverse wijzen. Substitutie op *O*-3-plaats kwam voor met monomere of dimere arabinose-eenheden. In deze laatste gevallen was het eindstandige arabinose gebonden aan *O*-2 van de tweede arabinose-eenheid. Voor drie andere oligosachariden werden sterke indicaties gevonden voor substitutie op de *O*-2-plaats van interne xylose-eenheden. Substitutie op zowel de *O*-2- als de *O*-3-plaats van een xylose-eenheid werd niet aangetroffen in deze oligomeren.

Uit het voorgaande kon uiteindelijk een model samengesteld worden van sorghum-GAX (Hoofdstuk 5). De suikersamenstelling en de samenstelling van glycosidische bindingen van de onafbreekbare 4K-fraktie diende daarbij als basis, samen met de kennis over het werkingsmechanisme van de enzymen. 4K zou bestaan uit een aaneenschakeling van vijf strukturele elementen, resulterende in een door XylI, XylIII, GXH en AXH onafbreekbare struktuur. In het geval van 1K4 zouden zich op enkele plaatsen, tussen de onafbreekbare elementen, enkel- of ongesubstitueerde xylose-eenheden bevinden. Dit levert een enigzins afbreekbaar polysacharide op. Hierbij zullen echter nauwelijks oligosachariden vrijkomen. BE1.1 is samengesteld zoals 4K en 1K4, maar in deze struktuur bevinden zich verder ook nog afbreekbare elementen, die uiteindelijk tot de vorming van oligosachariden zullen leiden onder inwerking van XylI en AXH.

De link tussen de sorghum-GAX en het brouwproces met sorghummout werd gelegd in Hoofdstuk 6. Gezuiverde enzymen konden, vanwege de benodigde hoeveelheden niet gebruikt worden in maische-proeven. Daarom werden voorafgaand enkele commerciële enzympreparaten getest op o.a. xylanase-aktiviteit en aktiviteit op sorghum-GAX en sorghum-WUS. Twee preparaten waarin deze aktiviteiten aanwezig waren, werden in verschillende doseringen toegepast tijdens het maischen, de eerste stap in het brouwproces. Deze fase werd afgesloten met de wortfiltratie, die in het geval van een van de twee enzympreparaten (preparaat A) veel voorspoediger verliep, dan zonder enzymtoevoeging. Er werd een duidelijke dosis-effekt-relatie gevonden. Het tweede enzym bleek de filtratie te vertragen. De ontstane worten en bostels werden uitvoerig onderzocht. In de bostels van de beter filtrerende monsters bleek dat meer GAX in oplossing was gegaan. De struktuur van het achterblijvende GAX was nauwelijks veranderd. Preparaat A bevatte naast xylanase- ook cellulase-aktiviteit. Het zou mogelijk kunnen zijn dat met name de cellulases, de struktuur van cellulose in sorghum zodanig hebben gemodificeerd, dat de GAX die zich tussen de cellulose-fibrillen

bevinden, gemakkelijker in oplossing gaan. Helaas kon nog niet eenduidig vastgesteld worden of sorghum-GAX nu wel of geen rol in de filtratie-eigenschappen van sorghumbier heeft.

CURRICULUM VITAE

Maria Anna (Marian) Verbruggen werd geboren op 16 maart 1965, te Boxmeer. In 1983 behaalde zij het VWO-diploma aan het Elzendaal College te Boxmeer. In datzelfde jaar is zij met de studie levensmiddelentechnologie begonnen aan de Landbouwuniversiteit te Wageningen. De accenten gedurende de studie lagen op het gebied van levensmiddelenchemie en -toxicologie.

Het afstuderen in 1989 werd direkt vervolgd met een promotieonderzoek bij de sectie levensmiddelenchemie en -microbiologie van de vakgroep levensmiddelentechnologie aan de Landbouwuniversiteit te Wageningen, waarvan de resultaten in dit proefschrift beschreven zijn. Het praktisch werk werd eind 1993 afgerond.

Sedert maart 1994 is Marian werkzaam als docent bij de opleiding levensmiddelentechnologie van de Hogeschool Delft.