Structural characteristics of arabinoxylans from barley, malt and wort

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Structural characteristics of arabinoxylans from barley, malt and wort

Structuurkenmerken van arabinoxylanen uit gerst, mout en wort

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Stellingen

bij het proefschrift 'Structural characteristics of arabinoxylans from barley, malt and wort'.

1. De primaire structuur van polysachariden kan alleen statistisch worden beschreven.

2. Het gebruik van Mw/Mn als maat voor het aggregatienummer van pectine gaat voorbij aan het polydisperse karakter van polysachariden.
   (R.H. Walter & H.L. Matias, *Carbohydr. Polym.* **15** (1991) 33-40)

3. Invoering van de zogenaamde tempobeurs leidt tot een verlaging van de kwaliteit van de afgestudeerden.

4. Het is naief te verwachten dat poly-etheen door toevoeging van zetmeel biologisch afbreekbaar wordt.
   (L.R. Krupp & W.J. Jewell, *Environm. Sci. Technol.* **26** (1992) 193-198)

5. ß-Glucanen zijn niet van belang voor de integriteit van de endosperm celwanden in granen.

6. De conclusie van Bengtsson et al. dat de water-oplosbare arabinoxylanen uit roggemeel bestaan uit twee typen ketens is onvoldoende onderbouwd.
   (S. Bengtsson, P. Åman & R.E. Anderson, *Carbohydr. Polym.* **17** (1992) 277-284)

7. Het effect van arabinoxylanen op wort viscositeit kan worden veroorzaakt door interacties tussen de wort arabinoxylanen en de wort ß-glucanen.
   (Hoofdstuk 6 van dit proefschrift)

8. De huidige Nederlandse regering bevordert het auto-gebruik.

9. Een te groot aandeel van onderzoek, gefinancieerd uit de derde geldstroom, kan leiden tot vermindering van de diepgang van het onderzoek en tot vermindering van de expertise bij de betrokken vakgroepen.
   Daarnaast bestaat het gevaar dat de reeds bestaande achterstand bij de aanschaf en vervanging van dure, geavanceerde apparatuur toeneemt.

R.J. Viëtor, Wageningen, 15 april 1992.

aan mijn ouders
aan Catherine

40951

Het in dit proefschrift beschreven onderzoek werd uitgevoerd op de sectie Levensmiddelenchemie en -microbiologie van de vakgroep Levensmiddelentechnologie aan de Landbouwuniversiteit Wageningen in samenwerking met de sectie AGRO-NIBEM, Instituut voor Biotechnologie en Chemie TNO te Zeist, met financiële steun van het Ministerie van Economische Zaken en van het Ministerie van Landbouw, Milieubeheer en Visserij, in het kader van het Innovatie-gericht Onderzoeksprogramma Koolhydraten (IOP-k).

Voorwoord

Het onderzoek, dat is beschreven in dit proefschrift, werd uitgevoerd op de sectie levensmiddelenchemie en -microbiologie van de vakgroep levensmiddelentechnologie aan de Landbouwuniversiteit te Wageningen. Ik wil alle medewerkers van de sectie bedanken voor hun ondersteuning in deze periode, ieder op hun eigen gebied, en voor de prettige samenwerking.

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BIBLIOTHEEK
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Abstract

Flours from dehusked barley and malt were fractionated to obtain water-insoluble cell wall material (WIS). A mass balance of these fractionations was determined. Arabinoxylans were extracted from this WIS in high purity and yield with $Ba(OH)_2$, and subfractionated with graded ethanol precipitation.

The structural elements present in these arabinoxylans and in arabinoxylans isolated from wort were determined. These arabinoxylans all consisted of a backbone of (1→4)-linked ß-D-xylopyranose units (Xyl*p*), a proportion of which were substituted with α-L-arabinofuranose (Ara*f*) at O-2 and/or O-3 of the Xyl*p* units. A new feature was the presence in barley and malt arabinoxylans of a large amount of Xyl*p* units carrying a single Ara*f* substituent at O-2. The amounts of Xyl*p* substituted at O-2 or at both O-2 and O-3 increased with increasing substitution of the xylan backbone. The wort arabinoxylans were found to be exceptionally rich in O-2,3-disubstituted Xyl*p*.

A number of fragments could be isolated after degradation of barley and malt arabinoxylans with endoxylanase 1 from *Aspergillus awamori*. The structures of the isolated fragments were determined. From the structures found, it could be shown that the position of Ara*f* substituents on the xylose residues of the arabinoxylan influenced the extent of enzymic degradation of the xylan backbone, substituents at O-2 being more efficient than substituents at O-3 with the enzyme used. From these data and the linkage composition of undegradable arabinoxylan fractions, it was concluded that the distribution of Ara*f* substituents over the xylan chain was not random, but fairly regular.

The arabinoxylans extracted from barley and malt cell wall material appeared to be very similar in composition and structural features. This implies that changes during malting are small or extremely localized.

List of abreviations

α1, α2	- Soluble fractions obtained after incubation of barley or malt flour with α-amylase
ß-glucan	- (1→3,1→4)-ß-D-glucan
^{1}H-nmr	- Proton nuclear magnetic resonance
ara	- Arabinose
ara*f*	- α-L-arabinofuranose
ara:xyl ratio	- Ratio of arabinose to xylose
AS	- Material soluble in 70 % (v/v) ethanol
BE	- Barium hydroxide extract from WIS
BE-*nn*	- Fractions obtained from BE fraction by precipitation with ethanol, *nn* indicates ethanol concentration (% v/v) at which the fraction precipitated.
BN	- Fraction obtained by washing of the residue after extraction with $Ba(OH)_2$
BP	- Material precipitated from BE fraction after neutralization
DMSO	- Dimethyl sulphoxide
gal	- Galactose
GLC	- Gas-liquid chromatography
glc	- Glucose
GLC-MS	- Gas-liquid chromatography/mass spectrometry
HOHAHA	- Homonuclear Hartmann-Hahn spectroscopy
HPAEC	- High performance anion exchange chromatography
HPLC	- High performance liquid chromatography
HPSEC	- High performance size exclusion chromatography

L1E	- Extract from the residue of WIS, after extraction with $Ba(OH)_2$, obtained with 1 M NaOH
L1N	- Fraction obtained by washing of the residue after extraction with 1 M NaOH
L1P	- Material precipitated from L1E fraction after neutralization
L4E	- Extract from the residue of WIS, after extraction with 1 M NaOH, obtained with 4 M NaOH
L4N	- Fraction obtained by washing of the residue after extraction with 4 M NaOH
L4P	- Material precipitated from L4E fraction after neutralization
m/e	- Mass per unit of electrical charge
man	- Mannose
nmr.	- Nuclear magnetic resonance
nsp	- Non-starch polysaccharides
PED	- Pulsed electrochemical detector
ROESY	- Rotating frame nuclear Overhauser enhancement spectroscopy
ST	- Material passing a 75 µm sieve
TFA	- Trifluoroacetic acid
WIS	- Cell wall material from barley or malt flour, insoluble in water at 70 °C
WS	- Fraction from barley or malt flour, soluble in water at 70 °C
xyl	- Xylose
Xyl*p*	- ß-D-Xylopyranose

Contents

Abstract p. i

List of abbreviations iii

Contents v

Chapter 1. 1

Introduction

Chapter 2. 21

Non-starch polysaccharides in barley and malt: a mass balance of flour fractionation

J. Cereal Sci. **14** (1991) 73-83

Chapter 3. 39

Structural features of arabinoxylans from barley and malt cell wall material

J. Cereal Sci., in press

Chapter 4. 57

Structures of small oligomers liberated from barley arabinoxylans by endoxylanase from *Aspergillus awamori*

Chapter 5. 75

Substitution patterns of water-insoluble arabinoxylans from barley and malt

Chapter 6. 91
Nsp composition of wort and spent grain from brewing trials with a good malting quality barley and a feeding barley

Chapter 7. 109
Concluding remarks

Summary 117

Samenvatting 121

Curriculum Vitae 127

Chapter 1
INTRODUCTION

1.1 PRODUCTION AND USE OF BARLEY AND MALT

Barley has been used for thousands of years for food and as a raw material in brewing[1]. Nowadays it ranks fourth in grown production among cereals. Most of the produced barley is used for animal feed[2]. Barley is the only grain which is commercially malted on a large scale. Malted barley is the major raw material for beer production in Europe and the America's, for some special beers (Belgian 'Lambic', German 'Weissbier') malted wheat is used additionally. In the last few years (successful) attempts have been made to malt other cereals (mainly sorghum) for use as raw material for 'European' types of beers (lager or pilsner) in Africa[3]. This has been stimulated since many African governments limited the import of barley for brewing purposes. Barley malt is further used as a source of amylases and flavour components in other products and processes.

1.2 OUTLINES OF MALTING AND BREWING

Raw materials

The main raw materials for brewing include water, malted barley, hops and yeast. Of these, malt is used to provide starch, proteins, flavour components and yeast nutrients (e.g. minerals). In the brewing process starch is converted into fermentable sugars and dextrins by the action of amylases. During fermentation, ethanol and carbon dioxide are formed from the fermentable sugars. The proteins are important as nitrogen

source for the yeast (after degradation to amino acids and small peptides) and as a main component of beer foam[4]. However, too high a level of protein is undesirable, as it can lead to haze formation during storage of the final beer, particularly when the beer is subjected to sudden changes in temperature[5].

Part of the malt is often replaced by other sources of fermentable sugars (adjuncts). The adjuncts can be divided into two groups: solid and liquid adjuncts. Solid adjuncts are mostly cereals (corn, rice, sorghum, wheat) that are rich in starch. In the brewing process, this starch also has to be converted into fermentable sugars by amylases prior to fermentation. Cereals can be used in brewing either raw or after a pretreatment which gelatinises the starch (flaking, torrification) or removes unwanted parts of the cereal (e.g. sorghum: the husks, if not removed, would impart a bitter flavour to the beer[6]). Use of raw cereals as adjuncts normally requires separate cookers to gelatinise the cereal starch[5]. Liquid adjuncts comprise starch syrups from corn, wheat or rice and sugar syrups (cane sugar, molasse). Starch syrups are produced from cereal starches by treatment with enzyme (amylase) or acid.

Adjuncts can be used for a variety of reasons. Most important, adjuncts are (usually) cheaper than malt and allow a higher wort production with given equipment (thus lowering production costs) and improve the taste of the produced beers (which becomes lighter and fresher)[5]. Also, the use of adjuncts leads to a lower protein content of the wort, preventing the development of certain types of haze during storage of the beer[5].

The hops are used to provide a bitter flavour, to improve shelf life of the beer and to help in removing excess proteinaceous material[5]. The yeast is required for conversion of glucose, maltose and maltotriose (from starch) into ethanol and carbon dioxide[5]. Also, compounds produced by the yeast are major contributors to beer flavour[5].

Malting of barley

The basic processes of malting and brewing are fairly well known today and have been extensively described in several textbooks[5,6]. In essence, malting is a natural germination of the grain, stopped after a short time by forced drying of the grain to prevent excessive growth of the new plant. The aims of malting are (i) the production of large amounts of enzymes (e.g. α- and ß-amylases, proteases and cell wall degrading enzymes), (ii) the degradation of the endosperm cell walls in the grain and (iii) conversion of the raw barley grain into a stable product that can be stored. In addition, a range of flavour components is formed during the process. The amylases are required to convert starch (from malt and other sources) into fermentable sugars. A function of the other enzymes is the degradation of material enclosing the starch, required to allow the amylases access to the starch.

A schematic overview of the malting process is shown in Fig. 1.1. The malting of barley starts with steeping of the barley grain in water in order to increase its moisture content to about 45 % (w/w). Steeping is interrupted every 12-24 h by draining. The drained grain is allowed to stand for a few hours in order to remove excess carbon dioxide, which in high concentrations is toxic to the embryo and would lead to uneven germination in the batch of barley[7]. When the required moisture content is reached (after about 2 days), the water is drained off and the barley is transferred to the germination facility.

During germination, the temperature is maintained at about 15 °C. When the endosperm is modified to the desired extent, germination is stopped by drying of the malt in a stream of heated air (kilning). As one of the aims in malting is the production of enzymes, the temperature of the malt has to be kept quite low initially (below 38 °C) to minimise inactivation of those enzymes when the water content of the malt is

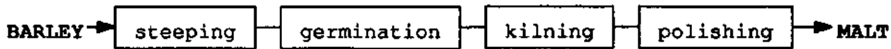

Figure 1.1. A schematic representation of the malting process.

high. Later in the process when the grain is partly dried, the temperature can be gradually raised to 80-100 °C in the final stage. Nevertheless, a large part of the enzymes is destroyed during kilning. Kilning is terminated at a moisture content of the malt of about 4 % w/w. Finally, the rootlets which emerged during germination are removed (polishing). Different time/temperature programs can be used during kilning to obtain different types of malts. Such alternative programmes generally employ higher temperatures than used in kilning for 'normal' pilsener malt (with high enzyme content and low colour). These special programmes usually lead to darker malts, which give a dark colour and a typical roasted flavour to the beer when used in brewing (e.g. 'Roasted', 'Chocolate' and 'Amber' malts). Also, part of the starch can be saccharified by 'stewing' (i.e. heating the wet green malt up to 100 °C) ('Crystal' malt, 'CaraPils')[8]. All these malts are added in small amounts to the grist in the brewing of some special beer types (e.g. 'Stout').

In order to malt properly, germination of the barley grain has to proceed easily and uniformly. Therefore, the barley grains have to be highly viable and of uniform size. Grain size also influences the maximal obtainable extract yield. Husk content of large grains is lower than that of small grains, and as a result large grains contain more starch. This results in a higher maximum extract yield from large kernels compared to small kernels. Therefore, in practice only barley with large kernels (> 2.5 mm) is used for malting and brewing. Furthermore, protein

content of the grain should be in the range of 9.5-11.5 % on dry matter. A final requirement is that the grain is not infested with insects or potentially harmful micro-organisms, such as *Fusarium* species. These micro-organisms may give rise to malting problems (e.g. retarded germination), or make the produced malt unfit for beer production[9].

The brewing process

A general schematic overview of the brewing process is shown in Fig. 1.2. The process starts with coarse milling of the malt to grist. The aim of milling is fragmentation of the endosperm to enable the extraction of starch during so-called mashing. At the same time, the husks have to be left intact as much as possible to form a good filter bed during wort filtration. A poor filter bed can either lead to very low filtration rates, lower extract yields or turbid worts with a high lipid content. Oxidation of these lipids in later stages of the process may cause off-flavours in the finished beer[10].

The aim of mashing is the enzymic conversion of starch from malt and solid adjuncts into the fermentable sugars glucose, maltose and maltotriose (saccharification). Also, further degradation of protein occurs. The amino acids and di- and tripeptides which are formed, are used by yeast as a nitrogen source in the fermentation. However, heat stabilities of the enzymes involved differ. As a result proteolysis cannot proceed at the optimum temperature for saccharification (65-70 °C), but requires a temperature in the range of 45-55 °C.

Traditionally, in several countries different temperature regimes are used during mashing, depending on the extent of modification of the malt available. These temperature regimes also influence the possibility to use adjuncts. The simplest and cheapest one regarding equipment and handling employs a constant temperature of about 65 °C during the

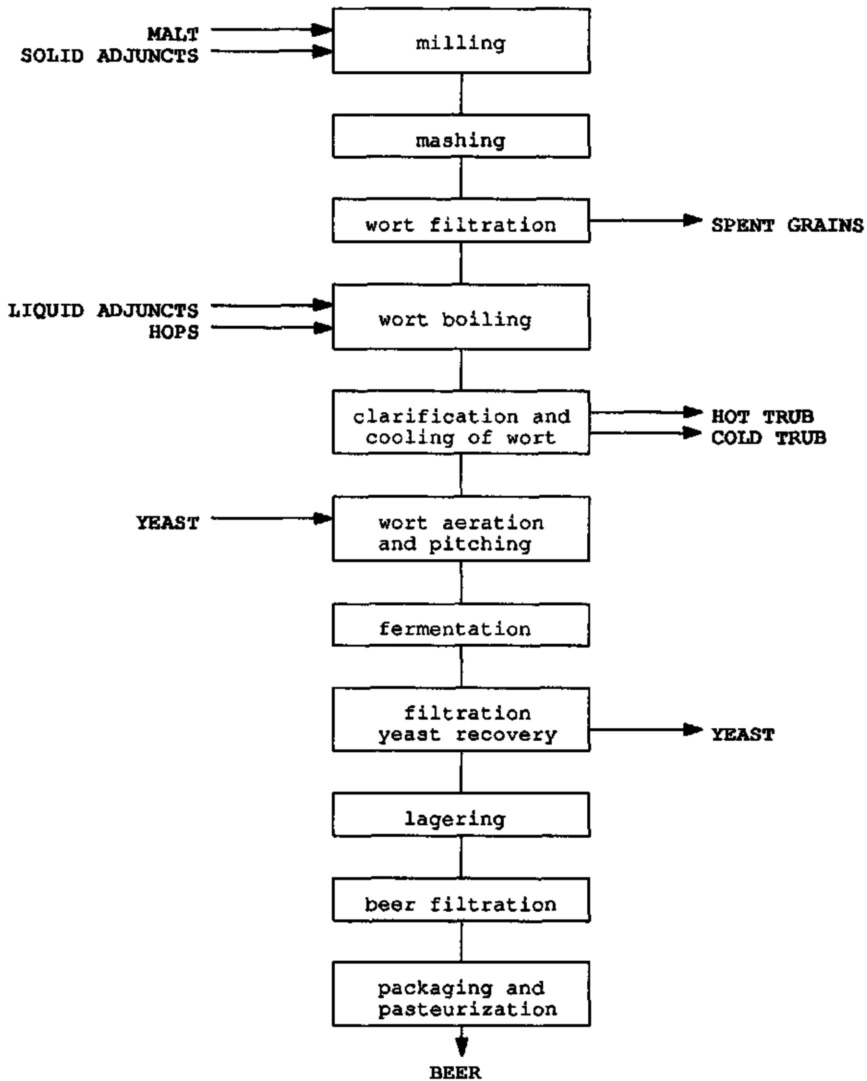

Figure 1.2. A schematic overview of the brewing process.

whole process (infusion mashing). As a result, proteases and cell wall degrading enzymes are rapidly inactivated. Therefore, well modified malt is required and the use of adjuncts is limited to small amounts (10 %)[5].

In the decoction process, mashing is started at a lower temperature, viz. 40-55 °C, which is more beneficial for proteolysis and degradation of residual cell wall polysaccharides. During the process the temperature of the mash is increased to 65 °C to allow conversion of starch into fermentable sugars. Such a temperature programme enables the brewer to use either less well-modified malts or larger amounts of adjuncts compared to infusion mashing. The disadvantage is that the required equipment and handling are more complicated and more expensive than in infusion mashing.

At the end of the mashing process, the temperature is increased to 75 °C to complete starch degradation by α-amylase and lower the viscosity of the liquid phase.

When mashing is completed, the liquid is separated from the remaining solids by filtration in which the malt husks provide the filter bed. After filtration of the mash, the filterbed is washed with water to optimise extract yield.

The filtered wort is then boiled with the addition of hops and liquid adjuncts (optionally). Hops (whole or pelleted) or hop extracts are added to provide a bitter flavour and some preserving components. The hop also helps in the precipitation of excess protein material by providing additional polyphenols. Boiling is required to sterilize and concentrate the wort and to remove unwanted volatile components and excess proteinaceous material and phenolics. Also, colour and flavour components develop during this step (Maillard products, isomerisation reactions).

When the wort is sufficiently concentrated, it is cooled and aerated. After cooling, brewers' yeast (usually a strain of *Saccharomyces cerevisiae* or *S. carlsbergensis*) is added and the fermentation of glucose and maltose to ethanol and carbon dioxide starts. In addition, the yeast

formes a range of metabolites (e.g. alcohols, esters and sulphur compounds) which are important for beer flavour[11].

When fermentation has completed, most of the yeast is removed and the green beer is stored for some time at low temperature (lagering) to allow the degradation of undesirable flavours (e.g. diacetyl and other diketones). The residual yeast plays an important role in this process.

After lagering, the beer is clarified, usually by filtration. Finally the finished beer is packed in bottles or kegs and pasteurized in order to prevent microbial spoilage. Stringent filtration can be applied instead of pasteurization to remove microbes from the beer.

The main solid byproducts of the brewing process are the spent grain (insoluble material obtained after wort filtration) and the hot and cold trub, obtained after wort boiling. These byproducts are relatively rich in nsp and protein. At the moment, the spent grain is mainly used as feed for ruminants. A possible future use of this byproduct is as a raw material for fermentation after enzymatic conversion of the polysaccharides into (fermentable) oligo- and monosaccharides. A problem might be that without pretreatment the enzymatic conversion of spent grain gives very poor yields[12].

The water content of the spent grain and trub is very high. This, in combination with the presence of residual fermentable sugars and peptides, makes them very susceptible to microbial infection, which is an additional complication for the industrial use of these materials.

1.3 COMPOSITION OF BARLEY GRAIN

Structure of the kernel

In the barley kernel several morphological elements can be distinguished (Fig. 1.3). The outermost layer, commonly called the husk, consists pri-

marily of cellulose, arabinoxylan and lignin. The next layers are the pericarp and the testa. Like the husk, they originate from maternal tissue and consist of dead cells.

Within the testa is the endosperm, which forms the largest part of the kernel. It can be subdivided in the aleurone and the starchy endosperm. The former is a living tissue, important in germination of the grain, and forms a layer around most of the grain. Where it covers the starchy endosperm the aleurone tissue is about three cell layers thick. It is a storage tissue for proteins and lipids and produces many enzymes during germination. The starchy endosperm consists of dead cells with thin walls, in which starch (as granules) and protein are stored.

Another part of the grain is formed by the embryo. This is the part of the grain from which the new plant develops. It is rich in lipids and protein. The scutellum produces a large amount of enzymes during germination and plays a role in the regulation of enzyme production by the aleurone. During malting the embryo starts growing, for which energy, i.e. starch, is needed. In order to limit losses of this valuable

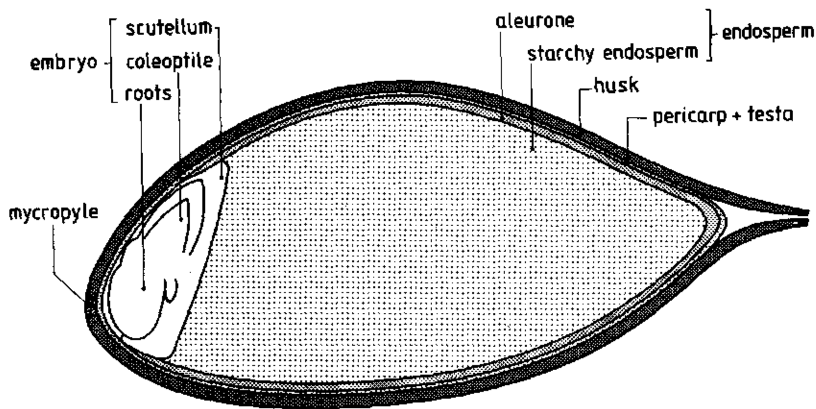

Figure 1.3. Longitudinal cross-section of a barley kernel.

component, the maltster will try to limit its growth as far as possible.

Composition of the grain

The main components of the kernel are starch (57-72 %), protein (8-11 %) and non-starch polysaccharides (nsp) (8-14 %)[13]. The starch is mainly present in the starchy endosperm. Chemically, starch consists of two main components, amylose and amylopectin. Both are build up from chains of $(1\rightarrow4)$-linked α-glucose, with branchpoints in which the chains are connected through an α-$(1\rightarrow6)$-link. Amylose and amylopectin differ in the amount of these branchpoints, amylopectin containing the larger proportion of branch-points (1 in 1000 (amylose)[14] respectively 1 in 25 (amylopectin)[15] glucose residues are branched).

Proteins are mainly present in the starchy endosperm surrounding the starch granules, and in the aleurone as granules. Degradation and solubilisation of proteins occurs during both malting and mashing. A large proportion of the proteinaceous material is precipitated during wort boiling by denaturation and coagulation with polyphenols derived from malt and hops[16]. The remaining degradation products are important as a nitrogen source for the yeast (see 1.2), as foam components[17] and as potential contributors to haze formation[18]. Protein material also has influence on wort filtration and beer taste[19].

The nsp are the major constituents of the cell walls of aleurone and endosperm. Barley contains several types of nsp, with $(1\rightarrow3),(1\rightarrow4)$-ß-D-glucans and arabinoxylans as the most prevalent cell wall carbohydrates[20]. Minor amounts of other polysaccharides are present as well[20,21]. The nsp will be discussed in more detail in paragraph 1.4.

Minor components further include lipids, phenolic compounds, lignin and minerals. Lipids (2-3 % of the grain) can be the cause of off-flavours[10] and diminish the stability of beer foam[22]. Polyphenols may

attribute to beer haze formation through interaction with protein during storage of the beer[23]. The lignin is found mostly in the husk. Minerals (especially zinc) can be important as nutrients for the yeast during fermentation.

1.4 THE STRUCTURE OF BARLEY NSP

(1→3),(1→4)-β-D-glucans

The (1→3),(1→4)-ß-D-glucans (ß-glucans) are the most abundant nsp in the endosperm of unmalted barley, comprising 3-6 % (w/w)[24,25] of the grain. They are mainly present in the cell walls of the starchy endosperm, of which they are the major component (70-75 % (w/w) of cell wall nsp[26,27]). The exact amount of ß-glucans found in the grain is dependent both on the variety of barley studied and on environmental conditions during growth and ripening[28].

The average molecular weight reported for the water-soluble ß-glucans is about 200 to 290 kDa[29]. In the cell wall, part of the ß-glucan is probably linked to proteins, forming aggregates with molecular weights of 10^6-10^7 Da[30]. The nature of this linkage to protein has not yet been determined. Solutions of ß-glucans have high viscosities[29], due to an asymmetric elongated shape of the molecules[31].

The ß-glucans consist of an unsubstituted, linear chain of ß-D-glucopyranose, linked by (1→3)- and (1→4)-bonds, which are found in the ratio of 3:7[27,32] (see Fig. 1.4). The (1→4)-linkages are present mainly in sequences of 3 or 4 adjacent bonds (95 %), longer sequences (of up to 10 bonds) are present in small amounts[32]. These longer sequences could be very important in determining the properties of the ß-glucans. Adjacent (1→3)-bonds are absent or very rare[32,33].

Arabinoxylans

The other major nsp in barley are the arabinoxylans. They constitute 4-10 % of the grain[25,34], with a large proportion of this amount being present in the husk (up to 75 % of total grain arabinoxylan)[35]. The remainder is found in the cell walls of the aleurone layer[36] and the starchy endosperm[26,27] (comprising 85 and 20-25 % (w/w) of the nsp in these cell walls, respectively). As with the ß-glucans, the exact amounts present in the grain vary with barley variety and environmental conditions during growth[25].

Arabinoxylans consist of a backbone of (1→4)-ß-D-xylan, substituted mainly with L-arabinofuranose residues at O-2, O-3 or both[36] (Fig. 1.4). The presence of substituents at O-2 has only been shown for aleurone arabinoxylans[36]. Other minor substituents include xylopyranosyl-arabinose[37], glucuronic acid[38] (the latter two mainly in husk arabinoxylans) and 5-O-feruloyl-arabinose[39]. The ratio of arabinose to xylose varies between arabinoxylans derived from different parts of the grain. Typical values for this ratio are 0.77 in the starchy endosperm[27], 0.58 in the

→4)-ß-D-Glc*p*-(1→3)-ß-D-Glc*p*-(1→4)-ß-D-Glc*p*-(1→4)-ß-D-Glc*p*-(1→3)-ß-D-Glc*p*-(1→

(A)

α-L-Ara*f* α-L-Ara*f*
1 1
↓ ↓
2 2
4)-ß-D-Xyl*p*-(1→4)-ß-D-Xyl*p*-(1→4)-ß-D-Xyl*p*-(1→4)-ß-D-Xyl*p*-(1→4)-ß-D-Xyl*p*-(1→
3 3
↑ ↑
1 1
α-L-Ara*f* α-L-Ara*f*

(B)

Fig. 1.4. Structures of the major nsp in endosperm cell walls of barley (A: ß-glucan; B: arabinoxylan, only arabinofuranose substituents are shown).

aleurone[36] and 0.20 in the husk[38].

It is very likely that the distribution of various substituents over the xylan backbone influences the properties and enzymic degradability of arabinoxylans. However, at the moment little is known about this distribution and its relation to arabinoxylan properties.

Other nsp

Residual minor nsp include cellulose, arabinogalactans and mannose-containing polysaccharides[20] (possibly glucomannans[21]). The presence of callose ((1→3)-ß-D-glucan) has been suggested[40,41] from the results of microscopy of barley kernel sections, stained with aniline blue.

All of these polysaccharides are as far as is known today of little importance in the brewing process.

1.5 CHANGES DURING MALTING AND BREWING

During malting a range of enzymes is formed. The most important of these are α- and ß-amylase[42], proteases[43], various endo- and exo-ß-glucanases[44-46] and arabinoxylan degrading enzymes (endo- and exo-xylanases, arabinofuranosidases)[47,48]. The synthesis of most of these enzymes is regulated by a group of plant hormones, the gibberellins[49]. Part of the enzymes formed during germination (except amylases) are rapidly inactivated at the temperatures commonly used in kilning and mashing[44,46]. A large part of the ß-glucanase activity may be of microbial rather than of plant origin[50]. The micro-organisms producing these enzymes are present on or in the outer layers of the grain[50,51]. Whether these micro-organisms can produce arabinoxylan degrading enzymes is unknown, but this seems feasible.

Further changes in the grain include the modification of the endosperm.

In this modification the cell walls are extensively degraded[52,53]. Especially the ß-glucans in the endosperm cell walls are degraded to a very large extent, amounts in the final malt being as low as 0.1 % (w/w) of the grain. The degradation of ß-glucans is thought to proceed in several steps. The first step is the degradation of the ß-glucan/protein complex by ß-glucan solubilase, a carboxypeptidase-like enzyme[30,54]. The liberated ß-glucan chains can then be degraded by endo- and exo-ß-glucanases[44]. The degradation of residual ß-glucans by ß-glucanases may continue during mashing[44], when the malt has been kilned at low temperature and the temperature during mashing is kept at about 50 °C for some time. However, when a well-modified malt is used, degradation of ß-glucans is already nearly completed during malting.

Rather less is known about the fate of arabinoxylans during malting and brewing, compared to that of the ß-glucans. Several enzymes involved in arabinoxylan degradation have been found in germinating barley grains[47,48,55], suggesting that arabinoxylan degradation is important in regular germination of barley. Like for the ß-glucans, degradation of arabinoxylans may continue during the low temperature stage of mashing.

Another major change during malting is the degradation of the protein matrix surrounding the starch granules by proteases[5]. This, in combination with the degradation of the endosperm cell walls, is important to make the starch accessible for amylases.

1.6 ROLE OF NSP IN THE MALTING AND BREWING PROCESS

The quality of barley malt for brewing is inversely correlated with the ß-glucan levels in malt, lower levels resulting in a better performance in brewing[56]. The ß-glucan content of a malt depends on both the ß-glucan content of the raw barley and its degradation by endo-ß-glucan-

ase during malting[57]. No relationship has been established between ß-glucan levels in barley itself and brewing quality. Arabinoxylan and starch contents of the malt also play a role in determining the brewing performance of a malt, although correlation between these variables and brewing performance is lower than the correlation between malt ß-glucan and brewing properties[58]. In accordance with these results, it has been suggested[59] that differences in malting potential are linked with physical and chemical properties of the intact endosperm cell walls rather than with the levels of individual cell wall components (ß-glucan, arabinoxylans, proteins).

Excessive amounts of nsp in brewing raw materials may cause problems during this process. Amongst these are clogging of the mash filter bed[60,61], increase of wort and beer viscosities[62] and formation of haze or precipitates in bottled beer after prolonged storage[62-64]. On the other hand, certain amounts of nsp in the beer could improve the sensoric properties of the beer ('mouthfeel') and the stability of beer foam[65]. The main nsp said to be involved in these phenomena are the ß-glucans[60,64,65], but ß-glucan content alone can not always explain problems encountered during processing[66].

In general, the arabinoxylans have received little attention in studies on effects of nsp on processing except for one report, describing the role of arabinoxylans in haze formation[67]. Arabinoxylans, like ß-glucans, can form highly viscous solutions[20] and are still present in the malt at rather high levels. Therefore, the problems usually attributed to ß-glucans could in fact also be caused by arabinoxylans, especially when wheat or maize are used as adjuncts[20]. Considering that arabinoxylans are the major component of aleurone cell walls, it might be expected that in these cell walls they have to be degraded in order to enable transport of enzymes formed in the aleurone into the starchy endosperm[49]. Also, degradation

of arabinoxylans from the endosperm cell walls could be required for optimal accessibility of the starch granules during mashing.
The use of various ß-glucanases and xylanases to alleviate such problems as mentioned above, has been described[68-70]. For instance, addition of ß-glucanase during mashing can improve wort filtration rate and extract yield by lowering wort viscosity and by improving the structure of the filter bed[71].
One point mostly overlooked in discussing the effects of ß-glucanases in the brewery is that quite a few of these enzymes, including ß-glucanases from the malt itself[72,73], have been reported to show activity on arabinoxylans[74]. The presence of xylan degrading activities is even more likely in the crude enzyme preparations normally used when enzymes are applied in the brewery. For efficient application of exogeneous glycanases a detailed knowledge of the structure of the target polysaccharides is essential. This knowledge is available to a large extent for the ß-glucans but not for the arabinoxylans.

1.7 AIM AND APPROACH OF THIS INVESTIGATION

The aim of this research was the elucidation of the structural features of nsp from barley and malt and their relationship to the enzymic degradability of these nsp, with special emphasis on endosperm and aleurone arabinoxylans. Also, the changes in arabinoxylan structures during malting and brewing were studied. Most of the barley and malt arabinoxylans are water-insoluble, but as malt contains a range of arabinoxylan-degrading enzymes[47,48], the arabinoxylans will be solubilized during germination and possibly during mashing, thereby contributing to the haze and viscosity problems described before (see 1.6).
The relevant sources of nsp for problems encountered in the brewing

process are the endosperm and aleurone cell walls. The husks also contain significant amounts of nsp, but these are not significantly solubilized during malting and mashing. Therefore, it was decided to isolate these polysaccharides from dehusked material and more specifically from the water-insoluble fraction of the dehusked grain.

In order to draw valid conclusions about the nsp in the original material while obtaining results with purified fractions, it is important to minimize losses during isolation. Care should be taken to prevent such losses and to obtain representative nsp fractions. Therefore, mass balances of the isolation of water-insoluble material from barley and malt and of the subsequent extraction of this material were determined.

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Chapter 2

Non starch polysaccharides in barley and malt: a mass balance of flour fractionation

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SUMMARY

Flours from dehusked barley and malt were fractionated to obtain water-insoluble cell wall material for structural studies of cell wall polysaccharides and study of their enzymic degradation. Composition was determined for all the fractions obtained. Mass balances were calculated to quantify losses of water-soluble material and to monitor major changes due to malting of the grain. Both in barley and malt, more than 95 % (w/w) of the flour was recovered after fractionation. In the isolated fractions 80-100 % of the mass could be accounted for by the analysed components (neutral sugars, proteins, lipids). Main components were starch, protein and non-starch polysaccharides (nsp). The nsp consisted predominantly of arabinose, xylose and glucose. In barley, most of the non-starch glucose was present as (1→3),(1→4)-ß-D-glucan (ß-glucan). Small amounts of feruloyl and acetyl groups (less than 0.1 % w/w) were also present. After malting, ß-glucan and water-soluble arabinoxylans were almost totally degraded. Water-insoluble arabinoxylans were partly degraded as indicated by recovery of a large amount of arabinose- and xylose-containing material in the malt AS fraction.

2.1 INTRODUCTION

In barley, non-starch polysaccharides (nsp) are important in determining the malting and brewing qualities of the grain. Insufficient degradation of nsp during malting can have adverse effects on the subsequent mashing process. It is known that high levels of high molecular weight $(1\rightarrow3),(1\rightarrow4)$-ß-D-glucan (ß-glucan) may lead to low extract yields, high wort viscosities resulting in filtration problems[1,2] and haze formation in the finished beer[3]. Although evidence exists that ß-glucan is not the only component involved in these problems, most research efforts have been dedicated to this component. Among other components involved in the phenomena mentioned, might be arabinoxylans, which are found in high concentrations in the cell walls of the aleurone layer[4]. As the aleurone layer is an important source for cell wall degrading enzymes in germinating barley, a slow breakdown of its cell walls may result in an impaired release of enzymes into the endosperm. This in turn can lead to inadequate breakdown of endosperm nsp and starch[4].

Knowledge of the degradation of barley cell wall polysaccharides by well defined enzyme preparations can help in understanding the role of various polysaccharides and polysaccharide-degrading enzymes in the malting process. Furthermore, insight into the nature of residual polysaccharides left after malting will help to understand the action pattern of malt enzymes.

The aim of the work presented here was the preparation and characterization of water-insoluble cell wall material (WIS) from decorticated barley and barley malt in quantities sufficient for studies of chemical structure and enzymic degradability. The material obtained should comprise as much as possible of the cell wall material present in endosperm and aleurone tissue, as these grain parts will primarily contribute to

the nsp solubilised during mashing. In preliminary experiments, a very elegant method suitable for the preparation of pure endosperm cell walls from wheat[5], based on washing of cell wall fragments from a dough, was tested. This method appeared to be unsuitable for use with barley flour, as barley failed to develope a dough on kneading of the flour with water. Therefore, a large scale method was applied based on the method of Mares and Stone[6] for the preparation of cell walls from wheat endosperm. Their method was modified to increase the yield of cell wall material and to allow quantitative determination of the distribution of various components across the fractions obtained.

2.2 EXPERIMENTAL

Materials

Barley (cv. Triumph, 1984 harvest) grown in the Wieringermeer (The Netherlands) was used.
All chemicals applied were analytical grade or best available.

Determination of neutral sugars

Neutral sugar composition of samples was determined by GLC of their alditol acetates, using a 3 m x 2 mm i.d. glass column packed with 3 % OV-275 on Chromosorb W-AW 80-100 mesh at 212 °C. Nitrogen was used as carrier gas.
Alditol acetates were prepared according to Englyst and Cummings[7]. In this method starch is removed prior to hydrolysis by solubilisation with DMSO and subsequent enzymic degradation. After enzymic treatment, solubilised non-starch polysaccharides are precipitated by addition of ethanol to a concentration of 80 % (v/v). Dextrins are removed by washing the residue with aqueous ethanol (80 %, v/v). The steps to

remove starch were omitted for samples containing less than 40 % (w/w) starch.
Hydrolysis of samples was performed in 1 M sulphuric acid (3 h at 100 °C) after pre-incubation in 72 % (w/w) sulphuric acid (1 h at 30 °C). Inositol was used as internal standard. This was possible as earlier experiments had shown that no endogenous inositol could be measured (data not shown). Losses due to degradation of monosaccharides were countered by giving standards the same treatment as samples. Reproducibility of this method was estimated at 5%.
For detection of total sugars in extracts the phenol-sulphuric acid test according to Dubois[8] was used.

Protein determination

Total nitrogen was determined by a semi-automated micro-Kjeldahl method, using a Cu/Ti catalyst. Protein content was estimated as total nitrogen x 6.25.

Determination of phenolic acids

Phenolic acids (e.g. ferulic and diferulic acid) were determined by HPLC as described by Gruppen et al[5]. Recovery of standard ferulic acid was more than 90 % and the coefficient of variation for this method was about 5 % (data not shown).

Determination of acetyl groups

Bound acetyl groups were liberated by saponification of samples with 0.4 M NaOH in water/isopropanol 1:1 (v/v)[9]. Liberated acetic acid was determined by HPLC using the conditions described[9]. Loss of acetic acid by evaporation was prevented by keeping the sample solutions alkaline.

Determination of fructose

Fructose (present as free fructose, sucrose or fructans) was determined by HPLC after enzymic hydrolysis of fructans with an inulinase (Novozym 230, NOVO Ferment). An Aminex HPX-87P column (25 cm, 4 mm i.d., BioRad), operated at 85 °C and eluted with water at a flow rate of 0.5 ml/min, was used for the analysis of free and liberated fructose.

Determination of lipids

Lipids were determined as methyl esters of fatty acids after saponification[10]. Methyl esters were prepared according to IUPAC[11].

Determination of ß-glucan

ß-Glucan was determined using a test kit from Biocon Ltd., according to the suppliers' instructions[12]. For the analysis of liberated glucose, however, a glucose test kit from Boehringer was employed, using hexokinase/glucose-6-P-oxidase in the final enzymic reaction.

Determination of apparent starch

Apparent starch (defined here as the total of 'true' starch, dextrins and maltose) was determined using a test kit from Boehringer according to the suppliers' instructions.

Preparation of barley flour

Barley was decorticated using 50 % (v/v) sulphuric acid[13]. After drying the decorticated barley was milled and passed through a 0.5-mm sieve. The resulting flour was defatted by Soxhlet extraction with n-hexane (7.5 l n-hexane per kg flour) for an 8-hour period. 1.5 kg of barley grain gave about 1 kg of defatted flour. The material lost consists of

husk and damaged kernels which dissolved during sulphuric acid treatment.

Preparation of malt flour

Barley was malted according to a standard micro-malting method[14]. The finished malt was roller-milled and sieved to remove part of the husk. Further removal of husk particles was achieved by suspending the grist in chloroform containing 5 % (v/v) ethanol (Figure 2.1). The mixture was stirred (1 min) and left to settle for ½ h at ambient temperature. The husk particles floated and were removed by decanting. Extra chloroform (without ethanol) was added and after stirring and resting as before the floating material was again removed. This procedure was repeated until the husk had been removed as far as possible (3-4 times).

In husk fractions obtained after roller-milling and sieving (HUSK1) and after flotation (HUSK2) endosperm particles were present. In the

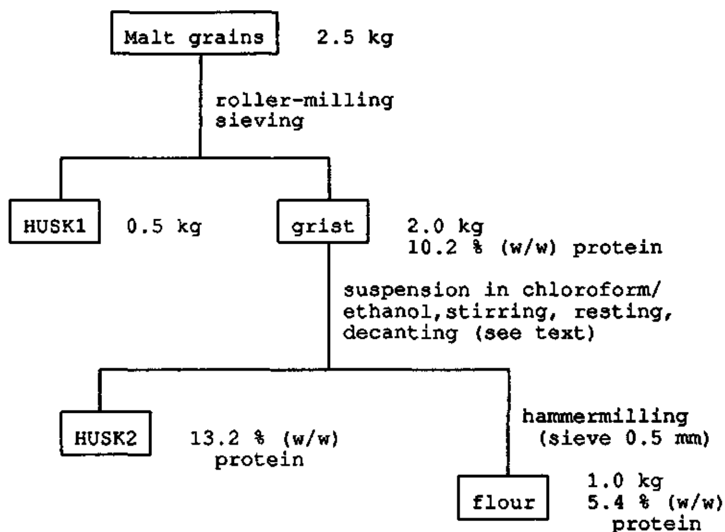

Fig. 2.1. Removal of husk from barley malt. (See text for experimental details)

HUSK1 fraction mainly unmodified kernel material was present (H.A. Vermeire, personal communication), which was not relevant to the comparison of barley and malt in this research. After milling 2.5 kg of malt gave 1 kg of defatted malt flour.

Fractionation of flour

The method used for fractionation of barley and malt flour was based on the procedure published by Mares and Stone[6]. To optimize removal of starch and WIS (hot water insoluble material) yield, several modifications had to be made. The final method is described in Figure 2.2.

First, flour was repeatedly extracted with aqueous ethanol (70 % v/v), until sugars could no longer be detected in the extract using the phenol-sulphuric acid test. The extract (the AS fraction) was concentrated under vacuum and freeze-dried. Next, the larger part of starch was removed by wet sieving of the residue in aqueous ethanol (70 % v/v) over a 75-µm stainless steel sieve. Material passing the sieve (the ST fraction) was collected and air-dried. The residue after wet sieving was suspended in 0.01 M sodium maleate buffer (pH 6.5) containing 0.5 g/l sodium azide and heated for 1 h at 70 °C to gelatinize residual starch. After cooling, purified α-amylase (porcine pancreatic α-amylase, Sigma) was added (10 units per g residue) and the mixture was incubated with shaking at 35 °C for 2½ days. After removal of liquid (the α1-extract) by centrifugation the residue was suspended in fresh maleate buffer, and heated and incubated as before with incubation time reduced to 24 h. The supernatant obtained after this incubation (α2-extract) and the α1-extract were concentrated and freeze-dried separately.

The residue after α-amylase treatment was suspended in water and extracted at 70 °C for 1 h. The suspension was centrifuged and water extraction was repeated until no sugars could be detected in the extract

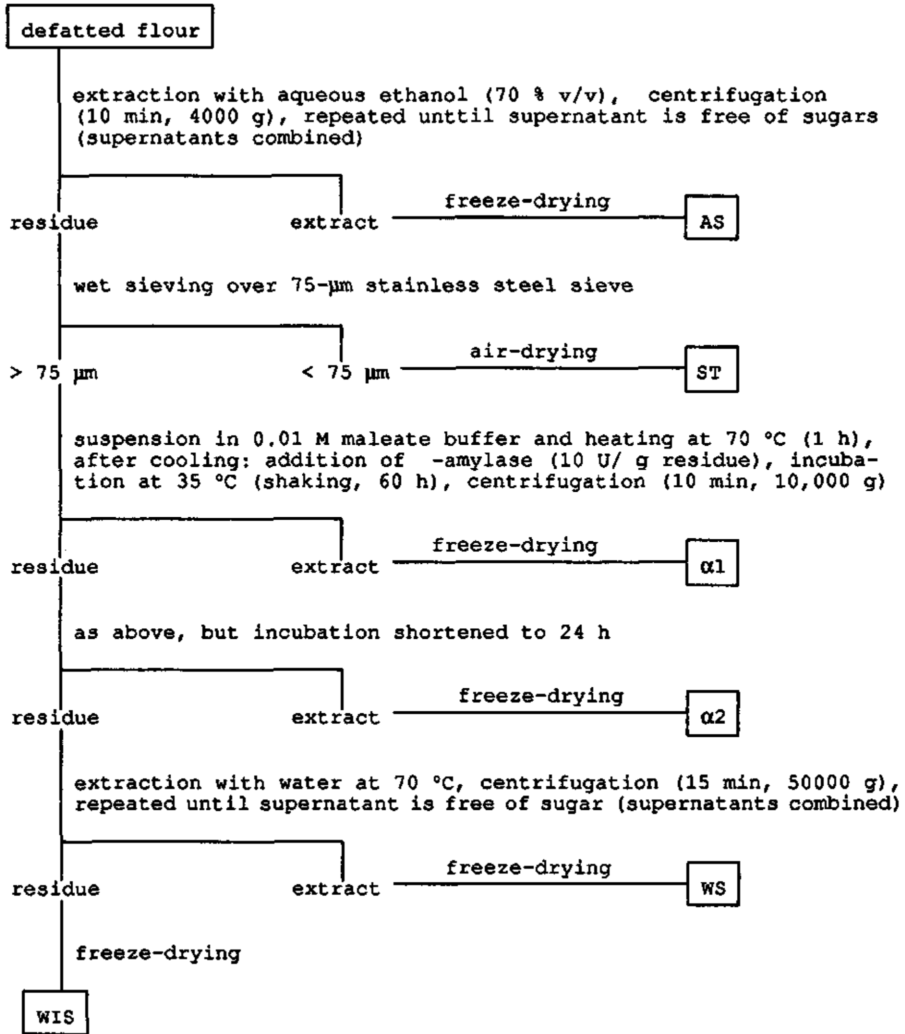

Fig. 2.2. Procedure used to fractionate defatted flour from barley and malt (see text for experimental details).

using the phenol/sulphuric acid test. The combined water extracts (WS fraction) were concentrated and freeze-dried. The residue (WIS fraction) was also freeze-dried.

2.3 RESULTS

Fractions of barley flour

Results of the fractionation of barley flour are summarized in Table 2.I. All reported values are expressed as g per 100 g of dry solids in the original flour, unless stated otherwise.

Total recovery of all fractions together was 95 g. Total nsp in the flour fractions (defined as the total amount of neutral sugars, excluding starch and fructose) amounted to 11.3 g when the nsp present in the AS fraction (1.4 g) is included. The amount of nsp measured in the flour as such was 8.2 g. Part of the difference between nsp measured in flour and total nsp recovered in the fractions is due to the alcohol washing step of the flour samples in the Englyst method (Experimental section). In the WIS fraction, 5.1 g nsp was recovered and in the fractions containing soluble material (α1, α2 and WS) 2.9 g. The ST fraction contained 2.0 g of nsp, together with most of the starch (57 g). Most of the remaining starch (7.9 g) was recovered as maltodextrins in the fractions obtained after enzymic degradation (α1 and α2). Fructose was recovered predominantly in the AS fraction (1.0 g), smaller amounts were found in the fractions α1, α2 and WS (0.04, 0.02 and 0.02 g respectively).

Total recovery of protein in the isolated fractions was 10.4 g. Most of the protein was found in the AS and the ST fractions (3.7 and 3.5 g respectively). The WIS fraction also contained a substantial amount of protein (2.4 g).

Only a minor amount of lipid was found in defatted flour (0.29 g), indicating that defatting has been efficient. These lipids were predominantly recovered in the AS fraction.

Ferulic acid and acetic acid were present in very low quantities and

Table 2.I. Composition of fractions from defatted barley and malt flour.

Fraction	flour	AS	ST	α1	α2	WS	WIS	total in fractions
	g dry matter/ 100 g flour dry matter (% w/w recovery)							
Barley fractions								
protein	11.4	3.7 (32)	3.5 (31)	0.4 (4)	0.2 (2)	0.2 (2)	2.4 (21)	10.4 (91)
total nsp[a]	8.2	1.4 (17)	2.0 (24)	2.0 (24)	0.3 (4)	0.6 (7)	5.1 (61)	11.3 (138)
starch	72	---	57 (78)	6.8 (9)	1.1 (2)	0.6 (1)	0.3 (0.4)	66 (92)
fructan	2.1	1.0 (48)	tr[c]	0.04 (2)	0.02 (1)	0.02 (1)	0.0	1.1 (52)
lipid	0.29	0.17 (59)	0.10 (34)	---	---	---	---	0.27 (93)
ferulic acid	0.05	tr	0.01 (20)	---	---	tr	0.03 (60)	0.04 (80)
acetyl groups	0.08	0.03 (38)	0.02 (25)	tr	tr	tr	0.04 (50)	0.09 (113)
total determined	94	6.2 (7)	63 (67)	9.1 (10)	1.7 (2)	1.5 (2)	7.9 (8)	88 (94)
total mass	100.0	6.7	66.2	10.5[b]	2.4[b]	1.6	7.6	95.0
Malt fractions								
protein	5.4	2.3 (43)	1.9 (35)	0.4 (7)	0.2 (4)	0.1 (2)	0.2 (4)	5.1 (94)
total nsp[a]	3.2	0.9 (28)	2.0 (63)	0.1 (3)	0.01 (0.3)	0.01 (0.3)	0.7 (22)	3.7 (116)
starch	82	4.2 (5)	71 (86)	5.2 (6)	0.4 (1)	0.05 (0.1)	0.03 (0)	81 (99)
fructan	1.6	1.4 (88)	0.2 (13)	0.1 (6)	tr	tr	tr	1.7 (106)
lipid	0.4	0.2 (50)	---	---	---	---	---	0.2 (50)
ferulic acid	0.02	tr	tr	---	---	---	0.01 (50)	0.01 (50)
acetyl groups	0.07	0.01 (14)	0.03 (43)	tr	tr	tr	0.01 (14)	0.05 (71)
total determined	93	9.0 (10)	75 (81)	5.8 (6)	0.6 (1)	0.2 (0)	1.0 (1)	92 (99)
total mass	100.0	9.9	75.9	7.2[b]	1.2[b]	0.2	1.2	95.6

[a]: Nsp determined by Englyst's method[7] except for AS, WS and WIS, where sugar content minus starch content was used for calculation.
[b]: Including salts from maleate buffer (see experimental).
[c]: Tr: trace amount detected

were retained predominantly in the WIS fraction. Diferulic acid was not detected in any of the fractions.

The main constituent sugars of nsp of all fractions were glucose, arabinose and xylose. Galactose and mannose were present in smaller amounts (Table 2.II). In flour, arabinose and xylose accounted for 36 % of the composite sugars of nsp, glucose analyzed as ß-glucan for 54 % and other sugars (galactose, mannose and non-ß-glucan glucose) for 10 % (on a molar base). Nsp in the WIS fraction consisted for 49 % of arabinose and xylose, for 31 % of ß-glucan glucose and for 19 % of other sugars. Nsp in the soluble fractions (α1, α2 and WS) consisted mainly of glucose present as ß-glucan.

Table 2.II. Sugar composition of nsp in fractions from defatted barley and malt flour.

Component	flour	AS	ST	α1	α2	WS	WIS
				mol %			
Barley							
arabinose	15	2	12	7	3	8	20
xylose	21	1	15	9	4	12	29
mannose	3	6*	3	2	1	3	5
galactose	1	19	1	1	0	0	1
glucose							
ß-glucan	54	---	25	82	91	74	31
other	6	71	44	0	0	3	13
arabinose/xylose	0.71	2	0.80	0.78	0.75	0.67	0.68
Malt							
arabinose	20	17	14	32	31	25	31
xylose	25	28	15	37	38	25	47
mannose	1	12*	1	0.7	2	4	2
galactose	1	2	1	4	2	0	1
glucose							
ß-glucan	3	---	2	22	20	7	1
other	50	40	66	5	7	39	19
arabinose/xylose	0.80	0.64	0.93	0.86	0.82	1.0	0.66

*: Possibly formed from fructose during reduction for alditol acetates.

The soluble fractions together comprised 25 % (w/w) of total nsp, 48 % (w/w) of total ß-glucan glucose and 10 % (w/w) of total arabinose and xylose (Table 2.III). In the WIS fraction, 45 % (w/w) of total nsp was recovered. This fraction contained 40 % (w/w) of total ß-glucan glucose and 73 % (w/w) of total arabinose and xylose. A large proportion of the mannose (64 % w/w) was also recovered in this fraction. Galactose was mainly retrieved in the AS fraction (79 % (w/w) of total galactose).

Fractions of malt flour

The total recovery of malt flour in the various fractions obtained by the fractionation was 95.6 g on dry matter base (Table 2.I). Most of this material was found in the ST fraction (75.9 g).

Table 2.III. Distribution of nsp monosaccharides over barley and malt fractions.

Fraction	AS	ST	α1	α2	WS	WIS	Total nsp (g/100 g flour)
	% of total in fractions						
Barley							
arabinose	2	16	7	1	2	73	1.2
xylose	1	16	6	1	3	73	1.8
mannose	16*	10	4	0	4	64	0.5
galactose	79	8	3	0	0	11	0.4
glucose:							
ß-glucan	--	12	29	7	12	40	4.2
other	31	28	19	0	0	22	3.2
total	13	17	17	3	5	45	11.3
Malt							
arabinose	32	32	5	tr	tr	32	0.6
xylose	38	25	4	tr	tr	36	0.8
mannose	81*	6	tr	tr	tr	12	0.2
galactose	57	14	tr	tr	tr	29	0.1
glucose:							
ß-glucan	--	71	29	tr	tr	tr	0.1
other	21	68	tr	tr	1	8	1.9
total	24	54	2	tr	0	19	3.7

*: Possibly formed from fructose during reduction for alditol acetates.

The flour contained 3.2 g nsp as determined with Englyst's method (see Experimental). The total amount of nsp isolated in the fractions was 3.7 g, of which 0.9 g was found in the AS fraction. The WIS fraction comprised 0.7 g, the ST fraction 2.0 g and the water-soluble fractions (α1, α2 and WS) 0.12 g of nsp. Since the nsp soluble in 70 % (v/v) aqueous ethanol was not determined in the Englyst method used, the total amount of nsp found in the fractions is higher than the amount found in flour.

Malt flour contained 5.4 g of protein. In the recovered fractions a total amount of 5.1 g of protein was found, mainly in the AS fraction (2.3 g) and the ST fraction (1.9 g). The WIS fraction contained 0.2 g and the water-soluble fractions together 0.7 g of protein.

Small amounts of lipid were detected as well, predominantly in the AS fraction. The amounts of acetyl groups and phenolic acids were low but still measurable. Diferulic acid was not detected.

Nsp in malt fractions consisted primarily of arabinose, xylose and glucose (Table 2.II). Glucose accounted for 53 mol% of the nsp sugars in malt flour, arabinose and xylose for 45 mol%.

The nsp in the WIS fraction consisted predominantly of arabinose and xylose. These sugars comprised 78 mol% of total nsp in this fraction, glucose accounted for 20 mol% and mannose and galactose for 3 mol% of nsp sugars.

Nsp in the WIS accounted for 19 % (w/w) of total recovered nsp. Arabinose in WIS comprised 32 % (w/w) of total arabinose and xylose 36 % (w/w) of total xylose (Table 2.III). The AS fraction also contained large amounts of arabinose and xylose, accounting for about 32 % (w/w) of total arabinose and 38 % (w/w) of total xylose. In all fractions only traces of ß-glucan was present (total less than 0.1 g). ß-Glucan could not be determined in the AS fraction due to the large amounts of

maltose and maltodextrins present. These starch degradation products could not be removed prior to ß-glucan determination, since ß-glucan in the AS fraction is present in an oligomeric form.

2.4 DISCUSSION

Fractionation methods

Barley grain was dehusked using 50 % (v/v) sulphuric acid[13]. After dehusking, more than 90 % of the grains germinated within 48 hours. This indicates that the treatment with 50 % (v/v) sulphuric acid, used in dehusking of barley, did not damage the germs. The material obtained after dehusking consisted of the endosperm, aleurone and germ.

Dehusking with sulphuric acid was not feasible for malt, due to the increased solubility and porosity of the malt. This would cause severe dissolution of the malt grains, leading to unacceptable modification and loss of material. Therefore, we developed a method for the removal of husk from coarsely ground malt, based on the difference in specific gravity of husk and endosperm particles in chloroform/ethanol mixtures, containing up to 5 % (v/v) ethanol. The endosperm material obtained with this method contained aleurone tissue and some residual husk, but was practically devoid of germ tissue. Visual inspection of the removed husk material (fraction HUSK2 in Figure 2.1) showed that this material contained some endosperm, aleurone and germ tissue. The method applied for fractionation of the barley and malt flour was optimised for the production of large amounts of water-insoluble cell wall material. This material is currently being used for studies of the structure and degradability of arabinoxylans. According to recent literature, these polysaccharides play a more important role in the brewing process than hitherto recognized[15].

Barley and malt fractions

With the fractionation method described here a large proportion of the water-insoluble nsp could be isolated. Furthermore, losses could be accounted for, as we recovered more than 90 % (w/w) of the original flour.

The main nsp sugars present were arabinose, xylose and glucose. Galactose and mannose were present in small amounts. These sugars are said to be present in arabinoxylans, ß-glucans and cellulose[4]. Minor amounts of (gluco-)mannans and arabinogalactans are also present[4].

For barley, most of the arabinoxylans and mannans were water-insoluble. A large part of the ß-glucans was soluble in (hot) water. Malt arabinoxylans were either soluble in 70 % (v/v) ethanol or insoluble. In both barley and malt, the arabinogalactans were mostly soluble in 70 % (v/v) ethanol (Table 2.III).

Due to differences in the composition of the starting materials (see above), detailed comparison of the fractionations of barley flour and of malt flour is not possible. Some of the differences in results obtained for these two fractionations, however, cannot be attributed to or induced by our isolation methods, but must be caused by the malting process.

Most obvious of these differences is the absence of ß-glucan in malt flour and its fractions, whereas barley flour and fractions thereof contain significant amounts of ß-glucan (table 2.III). This indicates that ß-glucan is degraded almost completely during malting.

Furthermore, arabinoxylans are at least partly degraded during malting, as indicated by the absence of water-soluble arabinoxylans in malt fractions $\alpha 1$, $\alpha 2$ and WS and the presence of relatively large amounts of arabinose and xylose in the malt AS fraction.

Comparison of our results for barley with previously reported data for mixed cell wall preparations[14,16] shows a large similarity in neutral sugar

composition. The high glucose content found here, in comparison to the results previously reported by Voragen et al.[14] (who used a comparable procedure to prepare and fractionate barley flour), can be explained in part by the different hydrolysis conditions applied in the neutral sugar determination. In this study, prehydrolysis with 72 % (w/w) sulphuric acid was employed for insoluble samples to solubilise cellulose, whereas Voragen et al.[14] used 2 M trifluoroacetic acid, which causes underestimation of cellulose. When comparing the composition of our WIS with the compositions given for pure endosperm cell wall fractions[17-19], the main differences are found in the relative amount of glucose.

In our studies we were able to analyze and characterize fractions obtained with excellent recoveries from barley and malt flour. This opens up possibilities to assess differences in composition between various barley varieties, both before and after malting.

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Chapter 3

Structural features of arabinoxylans from barley and malt cell wall material

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SUMMARY

Arabinoxylans were extracted from water-insoluble cell wall material (WIS) of barley and malt with saturated barium hydroxide solution containing sodium borohydride (5 g/l). The residue obtained after this extraction was further extracted with 1 M and 4 M sodium hydroxide. The barium hydroxide extracts (BE fractions) were subfractionated by graded ethanol precipitation. Both the BE fractions obtained, comsisted for more than 90 % of arabinoxylan with arabinose:xylose ratios of 0.72 and 0.68 for barley and malt respectively. The remaining 10 % was predominantly glucan. The BE fractions accounted for 63 % (in barley) and 61 % (in malt) of the arabinoxylans originally present in the WIS. Ethanol precipitation yielded a range of arabinoxylan fractions, in which the ratio of arabinose to xylose increased with ascending ethanol concentration. Methylation analysis showed that the main sugar residue found in all analysed fractions was 1,4-linked xylopyranose, in part substituted with single unit arabinofuranose groups at positions 2, 3 or both. A novel feature was the presence of large amounts of xylopyranose, substituted at O-2 (and not at O-3). Substitution of the xylopyranose backbone in the BE fractions of barley and malt and in the ethanol precipitates varied between 28 % and 62 % for barley fractions and between 22 % and 56 % for malt fractions. Most of the variation was due to differing levels of double substitution and single substitution at O-2. No significant differences were observed between arabinoxylans extracted from barley and malt WIS.

3.1 INTRODUCTION

Arabinoxylans are widespread in plant cell walls of monocotylodons. In cereal endosperm they comprise between 20 % (w/w) (in barley)[1,2] and 72 % (w/w) (in wheat)[3,4] of the cell wall. Although the absolute amounts of pentosans in the grains are small, they are of great technological importance with regard to their contribution to the the baking quality of wheat and rye[5] and the brewing quality of barley and adjuncts[5].

The composition and structural features of wheat arabinoxylans are now fairly well known. They consist of a backbone of (1→4)-linked ß-D-xylopyranosyl residues, a proportion of which are substituted on O-3 or both O-2 and O-3 with α-L-arabinofuranosyl residues[5]. The presence of dimeric sidechains, for example 2-O-(ß-xylopyranosyl)-arabinofuranoside, has also been demonstrated[6,7]. Also glucuronic acid, 4-O-methyl-glucuronic acid and feruloyl groups (attached to arabinofuranosyl residues) can be present as substituents[5]. The arabinose:xylose ratio (ara:xyl ratio) is about 0.5 and the glucuronic acid content is about 1 %[8].

Barley arabinoxylans are less well characterized. They have a higher ara:xyl ratio[9,10] than wheat pentosans. In addition to the linkages found in wheat arabinoxylans, 2-substituted xylose has been found in barley aleurone pentosans[11].

Total barley grain and barley endosperm contain 6.6 % and 1.4 % (w/w) arabinoxylan, respectively[12]. Preliminary estimates[a] indicate that about 46 % of the total grain arabinoxylan is present in the husk, 6 % in the pericarp, 24 % in the aleurone and subaleurone layers and 24 % in the endosperm. The polysaccharides in the aleurone layer in barley

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[a] Angelino, S.A.G.F. (1989) in: 53rd NIBEM Annual Report, NIBEM Foundation, Zeist, The Netherlands, pp. 51-58 (in Dutch).

comprise about 85 % pentose[11]. The other major non-starch polysaccharide (nsp) in barley is (1→3),(1→4)-ß-D-glucan (ß-glucan). This polysaccharide accounts for 4.2 % (w/w) of the grain and 4.1 % (w/w) of the endosperm[12].

During the malting of barley, a proportion of the nsp is degraded. The main changes occur in the endosperm and the aleurone layer. The ß-glucan is degraded to a very large extent, whereas the pentosans are more resistant to degradation[13]. The undegraded, solubilized pentosans could be a cause of haze formation or high viscosity during subsequent processing of the malt[14], problems which are often solely attributed to soluble high molecular weight ß-glucans[15]. Excessive binding of water by insoluble arabinoxylans in the spent grains could also impair wort run-off during lautering.

Until now, most research on barley nsp has concerned ß-glucan, which is present in the endosperm in much larger amounts than pentosan[12]. Therefore, the aim of this research was to elucidate the structural features of arabinoxylans isolated from barley and malt water-insoluble cell wall material (WIS). To extract the arabinoxylans from WIS, a recently developed method for the specific extraction of arabinoxylans was applied, which is based on the use of $Ba(OH)_2$ in combination with $NaBH_4$[16]. WIS was used as the source of arabinoxylans since most of the arabinoxylans in barley and malt endosperm and aleurone are insoluble in water[13].

3.2 EXPERIMENTAL

General

The procedures used for isolation and characterization of the WIS used in this study have been described elsewhere[13].

In brief, WIS was prepared from flours of dehusked barley and malt. First the flour was extracted with 70 % (v/v) ethanol. The residue was then sieved in 70 % (v/v) ethanol over a 70-µm sieve and the material passing the sieve (mostly starch) discarded. The material remaining on top of the sieve was heated to 70 °C and incubated with α-amylase (at 35 °C) to remove the remaining starch. The insoluble material was then extracted with water at 70 °C until no more sugars could be detected in the extract. The remaining residue was freeze-dried and stored dry over silica gel.
Chemicals used were analytical grade or best available. All determinations were done at least in duplicate.

Fractionation of WIS

The scheme describing the extraction of WIS is shown in Fig. 3.1. This method was based on a method published recently by Gruppen *et al.*[16]. WIS was suspended in saturated $Ba(OH)_2$ solution containing 0.13 M $NaBH_4$ (100 ml/g WIS) and stirred overnight at room temperature. The suspension was centrifuged (15 min at 5000 *g*) and the residue was washed with saturated $Ba(OH)_2$ solution (50 ml/g WIS, 1 h). After centrifugation (15 min at 5000 *g*), the supernatant was added to the first extract. The residue was washed subsequently with water (50 ml/g WIS, 1 h). During this step, the suspension was neutralized with acetic acid. The combined $Ba(OH)_2$ extracts were neutralized with glacial acetic acid and dialyzed extensively against water (24 h against running tap-water, 2 x 24 h against deionized water). Precipitates that formed during dialysis were separated by centrifugation (15 min at 5000 *g*) and freeze-dried (BP fraction). The clear retentate was concentrated, made up to a final volume equivalent to 50 ml/g WIS with distilled water and stored at -18 °C (BE fraction). The water washing fraction was treated and

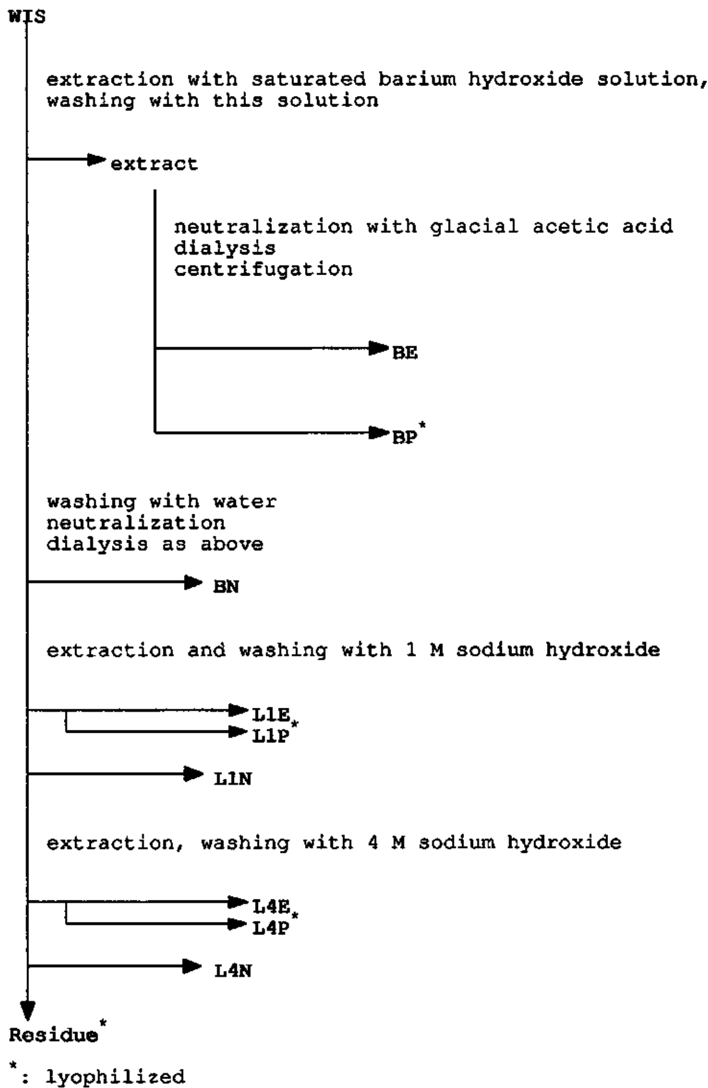

Figure 3.1. Extraction of barley and malt WIS (all alkaline solutions contained sodium borohydride (5 g/l).

stored identically (BN fraction).

The residue after washing with water was subsequently extracted with 1 M and 4 M NaOH according to the same procedure, yielding the

fractions L1E (alkaline extracted material soluble after dialysis), L1P (material precipitated during dialysis of the alkaline extract), L1N (material extracted during washing with water), L4E, L4P and L4N (analogous fractions obtained with 4 M NaOH). All extracts were stored frozen and aliquots were thawed when required. The precipitates and the residue after extraction with 4 M NaOH were freeze-dried and stored in a dessicator.

Subfractionation by ethanol precipitation

To thawed BE extract (containing about 2 mg/ml of polysaccharide) absolute ethanol was slowly added at room temperature under constant stirring, until a concentration of 20 % (v/v) was reached. The mixture was stored at 4 °C for at least 16 h. After centrifugation (15 min at 5000 *g*) the residue was freeze-dried, and ethanol was added to the supernatant at room temperature until the ethanol concentration had increased by 10 % (v/v). This stepwise procedure was repeated until an ethanol concentration of 70 % (v/v) was reached. The final supernatant was concentrated under vacuum in order to remove ethanol and was then freeze-dried.

Determination of neutral sugars

Neutral sugar composition and content of fractions were determined by gas-liquid chromatography (GLC) of alditol acetates[13]. Samples were hydrolyzed either with 2 M trifluoroacetic acid (TFA) (1 h at 121 °C) for water-soluble samples or by treatment with 72 % (w/w) H_2SO_4 (1 h at 30 °C), followed by 1 M H_2SO_4 (3 h at 120 °C) for water-insoluble samples. Inositol was used as internal standard. In order to correct for losses of monosaccharides due to acid hydrolysis, monosaccharide standards were included in each series of samples. These standards were

treated identically to the samples and recoveries of these standards were used for quantification of the sample results.

Methylation analysis

The method used for methylation of polysaccharide samples was based on the procedure described by Hakamori[17]. Samples (containing less than 10 mg of sugars) were dried overnight under vacuum over phosphorus pentoxide. To each sample, dry dimethylsulphoxide (DMSO) (1 ml) was added and the mixture was placed in an ultrasonic bath to dissolve the polysaccharide. Next, dimsyl anion solution in DMSO (1 ml) was added (prepared by addition of 314 mg NaH to 10 ml dry DMSO and sonication of the mixture at 60 °C for 1.5 h). The solution was left overnight at room temperature. The mixture was subsequently cooled on ice and three aliquots (0.35 ml each) of CH_3I were added dropwise (15 min intervals between additions). The resulting solution was placed in an ultrasonic bath for 1 h. Surplus CH_3I was removed under a stream of air, and the samples were dialysed for at least 48 h. The retentate was dried in a stream of air.

The methylated polysaccharides were hydrolysed with 2 M TFA, converted into alditol acetates as described above and analysed by capillary GLC (column: fused silica, liquid phase DB-1701) and by coupled gas-liquid chromatography-mass spectrometry (GC-MS) (Hewlett-Packard Corp., GLC: column: fused silica, 26 m x 0.22 mm i.d., liquid phase CP Sil 19 CB, film thickness 0.18 µm, MS: HP 5970 MSD, ionisation: e.i., 70 eV, mass separation by quadrupole). Effective carbon response factors as given by Sweet *et al.*[18] were used for calculation of molar quantities from peak areas as determined by GLC. As 2-O-methyl-xylitol and 3-O-methyl-xylitol differ only slightly in GLC retention times under the conditions used, the amounts of these components could not be

determined directly. Their ratio could be estimated from MS results from integration of signals for m/e values 117 (specific for 2-O-methyl-xylitol)[19] and 129 (specific for 3-O-methyl-xylitol)[19] over the combined peak. As the two components had slightly differing retention times, it was possible to obtain spectra containing signals at either m/e 117 or m/e 129 but not both. These spectra were representative for 2-O-methyl- and 3-O-methyl-xylitol respectively. From these spectra, the intensities of the signals at m/e 117 or m/e 129 were estimated at 20 % and 25 % of the total ion current for the corresponding component, respectively (data not shown).

3.3 RESULTS AND DISCUSSION

Extraction

The results of the extraction of barley and malt WIS are shown in Table 3.I. After complete extraction of barley WIS, 11 % of the WIS carbohydrates remained in the residue. Most of the extracted material was recovered in the BE fraction (28 %), in the BN fraction (36 %) and in the L1E fraction (14 %).

About 15 % of the malt WIS polysaccharides remained in the residue after extraction. The other main carbohydrate-containing fractions were the fractions BE (44 %), BN (11 %) and L1E (10 %) (Table 3.I).

For barley WIS, 63 % of total arabinoxylan (sum of arabinose and xylose) was recovered in the BE fraction, 14 % in fraction BN, 12 % in fraction L1E and 2 % remained in the residue.

Glucose was mainly recovered in the BN fraction (54 %), in the residue (19 %) and in the L1E extract (13 %). The fractions L1E, BN and L4E contained most of the mannose (27, 17 and 17 % of original in WIS, respectively (Table 3.I)).

From malt WIS 61 % of the total arabinoxylan was extracted in the BE fraction, 11 % was recovered in each of the fractions BN and L1E. The residue contained 6 % of the arabinoxylan originally present in WIS (Table 3.I). Glucose was mainly recovered in the final residue (41 %)

Table 3.I. Mass balance for neutral sugars in fractions from barley and malt WIS (average of two extractions).

Fraction	Ara		Xyl		Man		Glc		Total	
					mg/g WIS d.m.[a]		(% w/w)[b]			
Barley										
BE	59	(64)	82	(62)	1	(3)	14	(5)	158	(28)
BN	15	(16)	17	(13)	5	(17)	168	(54)	207	(36)
BP	1	(1)	1	(1)	0	(0)	2	(1)	4	(1)
L1E	11	(12)	17	(13)	8	(27)	41	(13)	78	(14)
L1N	0	(0)	0	(0)	1	(3)	9	(3)	10	(2)
L1P	0	(0)	1	(1)	0	(0)	2	(1)	3	(1)
L4E	3	(3)	5	(4)	5	(17)	8	(3)	21	(4)
L4N	0	(0)	0	(0)	0	(0)	1	(0)	1	(0)
L4P	0	(0)	0	(0)	1	(3)	12	(4)	12	(2)
Residue	2	(2)	2	(2)	1	(3)	60	(19)	65	(11)
Sum of fractions	91	(98)	125	(96)	22	(73)	317	(103)	560	(99)
WIS	92		132		30		310		569	
Malt										
BE	112	(62)	166	(60)	1	(7)	7	(5)	289	(44)
BN	23	(13)	25	(9)	2	(14)	19	(13)	72	(11)
BP	1	(1)	2	(1)	0	(0)	1	(1)	4	(1)
L1E	19	(10)	30	(11)	3	(21)	12	(8)	65	(10)
L1N	2	(1)	1	(0)	0	(0)	1	(1)	4	(1)
L1P	1	(1)	2	(1)	0	(0)	2	(1)	5	(1)
L4E	9	(5)	17	(6)	5	(36)	9	(6)	41	(6)
L4N	1	(1)	1	(0)	0	(0)	1	(1)	3	(0)
L4P	1	(1)	1	(0)	1	(7)	11	(7)	14	(2)
Residu	12	(7)	16	(6)	0	(0)	62	(41)	99	(15)
Sum of fractions	190	(102)	261	(94)	12	(85)	125	(84)	596	(91)
WIS	182		275		14		151		661	

[a]: Expressed as mg of anhydro-sugar per g of WIS on dry matter.
[b]: Expressed as % w/w of anhydro-sugar present in WIS.

and in the fractions BN and L1E (13 % and 8 %, respectively). The fractions L1E and L4E contained most of the mannose (21 and 36 %, respectively), comparable to the corresponding barley WIS fractions.

On a molar basis, the polysaccharides in the barley BE fraction comprised 38 % arabinose (ara), 53 % xylose (xyl), 8 % glucose (glc) and 1 % other monosaccharides. The malt BE fraction comprised 39 % ara, 57 % xyl, 2 % glc and traces of other monosaccharides (Table 3.II).

Extraction with barium hydroxide yielded extracts containing mainly arabinoxylans with only a small amount of glucan. Thus, there was no

Table 3.II. Neutral sugar compositions of fractions from barley and malt WIS (average of two extractions).

Fraction	Ara	Xyl	Man	Glc	Ara/Xyl Ratio
	mol% of anhydro-sugars				
Barley					
BE	38	53	1	8	0.72
BN	6	7	2	84	0.86
BP	29	41	4	26	0.71
L1E	16	24	9	50	0.67
L1N	7	6	13	75	1.17
L1P	5	12	2	82	0.42
L4E	18	30	23	30	0.60
L4N	20	17	0	64	1.18
L4P	2	7	6	85	0.29
Residue	2	2	1	95	1.00
Malt					
BE	39	57	0	2	0.68
BN	35	37	2	22	0.95
BP	26	41	3	30	0.63
L1E	28	50	4	15	0.56
L1N	38	31	1	29	1.23
L1P	13	32	3	52	0.41
L4E	24	42	12	20	0.57
L4N	39	34	6	19	1.15
L4P	4	8	6	82	0.50
Residue	27	22	0	50	1.23

need for further purification of these extracts, avoiding the possibility of degradation or loss of arabinoxylan.
The specific retention of ß-glucan in the residue during $Ba(OH)_2/NaBH_4$ extraction was observed first in wheat nsp fractionation[8]. Both the barium ions and the borohydride were shown to be necessary for this retention of glucan[8], but the mechanism is as yet unknown.

Ethanol precipitation

Only the BE fractions were used for graded ethanol precipitation, as these extracts comprised most of the WIS arabinoxylan with little contamination of other polysaccharide material. The results of ethanol precipitation are shown in Table 3.III. Both from the barley and the malt BE fraction major precipitation of arabinoxylan was achieved at an ethanol concentration of 60 % (v/v). At this concentration, 55 % of the barley arabinoxylan and 44 % of the malt arabinoxylan precipitated. The main difference between the distribution of arabinoxylans in the barley and malt fractions was found in the 20 and 30 % precipitates. More material precipitated at these concentrations for malt than for barley (Table 3.III).
The ara:xyl ratio of the 60 % precipitates was comparable with that in the original extract both for barley and for malt (Table 3.III). For the other fractions this ratio was in general higher for the barley precipitates than for the corresponding malt precipitates. The distribution of glucose in the barley fractions was fairly even, whereas, for malt, glucose was concentrated in both the first precipitates and the supernatant.
The results of graded ethanol precipitation show that both barley and malt arabinoxylans consist of a population of polysaccharides differing in ara:xyl ratio. The fractions with the lowest ara:xyl ratio precipitated at the lowest ethanol concentration. The malt arabinoxylans tended to

Table 3.III. Compositions of ethanol precipitates from $Ba(OH)_2$ extracts (average of two precipitations).

% ethanol	Ara	Xyl	Glc	Ara/Xyl Ratio	% (w/w) of total sugars
	mol% of anhydro-sugars				
Barley					
20	29	67	4	0.43	6
30	30	68	3	0.44	10
40	33	63	4	0.52	2
50	32	62	6	0.52	10
60	39	55	6	0.71	55
70	46	43	9	1.07	15
sup	30	23	37	1.30	3
Malt					
20	26	65	5	0.40	20
30	26	70	4	0.37	8
40	32	68	0	0.47	2
50	35	65	0	0.54	3
60	37	61	1	0.61	44
70	46	51	1	0.90	19
sup	46	45	9	1.02	5

precipitate at a somewhat lower ethanol concentration than the barley arabinoxylans.

Another factor which could influence arabinoxylan precipitation in ethanol is the molecular weight of these polysaccharides. However, high performance size exclusion chromatography did not show significant differences in molecular weight distribution for the fractions obtained here (data not shown).

Methylation analysis

The results of methylation analysis (summarized in Table 3.IV) confirmed that the main residues present in the arabinoxylan fractions analysed were terminal arabinofuranosyl residues and unsubstituted (1→4)-linked xylopyranosyl residues.

The composition of the arabinoxylans from the barley and malt BE fractions were almost identical, with about 42 % of the xylose residues from the backbone substituted. In the barley BE fraction 43 % of these residues carried a substituent at both O-2 and O-3, 22 % at O-2 and 34 % at O-3. For malt BE these values were 39 %, 28 % and 33 % respectively (calculated from table 3.IV). The presence of (unspecified amounts of) O-2-substituted xylose has previously only been reported for arabinoxylan from barley aleurone[11]. Substituted arabino-furanosyl residues appeared to be present in small quantities, as were terminal xylose residues. The presence of small quantities of substituted arabinofuranose indicates that short oligomeric side chains might be present in barley and malt arabinoxylans. As some terminal xylose was found, these oligomeric sidechains would partly consist of xylopyranosyl-arabinofuranose.

Comparison with earlier results for barley arabinoxylan[9-11] shows that in previous work much lower amounts of double substituted xylose were found for arabinoxylan fractions with comparable or higher ara:xyl ratios than reported here.

The linkage composition of arabinoxylan in the BN and L1E fractions is similar to the linkage composition of the BE fractions. This indicates that the degree of substitution is not the only factor influencing the solubility of arabinoxylan. Another factor that could play a role may be the distribution of (arabinosyl) substituents over the xylan backbone. For instance the L1E fractions may have a less regular distribution than the BE fractions, with longer sequences of unsubstituted xylose residues. These longer sequences could interact more strongly with cellulose or other xylan chains.

Glucose was present in both 3-linked and 4-linked forms. In all samples,

Table 3.IV. Linkage composition of selected arabinoxylan fractions.
A. Barley.

Sugar	Substituents	Fraction						
		BE	BE-20	BE-30	BE-60	BE-70	BN	L1E
Araf	t[a]	38	26	31	40	47	7	14
	2	1	0	0	1	1	0	1
	3	1	0	0	1	2	0	1
	5	0	0	2	1	1	1	1
Xylp	t	1	1	0	2	2	1	2
	4	30	49	42	28	13	5	14
	2,4+3,4[b]	13 (1.5)	13 (4.0)	13 (2.2)	12 (1.5)	13 (1.1)	2 (1.9)	6 (2.7)
	2,3,4	10	6	10	11	13	2	5
Glcp	3	1	0	0	1	1	12	8
	4	3	1	3	3	5	57	36
	4,6	0	0	0	0	0	1	1
Manp	4	0	0	0	1	0	1	4
	2,3,4,6	0	0	0	0	0	1	2
total Ara		40	26	33	43	51	8	17
total Xyl		54	69	65	53	41	10	27
total Man		0	0	0	1	0	2	6
total Glc		4	1	3	4	6	70	45
branch/Xyl[c]		0.61	0.36	0.51	0.64	0.95	0.60	0.59
% subst. Xyl[d]		43	28	35	43	63	40	41

Table 3.IV (Continued). B: Malt.

Sugar	Substituents	Fraction						
		BE	BE-20	BE-30	BE-60	BE-70	BN	L1E
Araf	t[a]	36	23	25	38	45	26	18
	2	1	0	1	1	2	2	3
	3	2	0	1	1	3	3	2
	5	1	0	1	1	3	3	2
	2,3	1	0	0	0	1	1	2
	2,3,5	0	0	0	0	0	1	1
Xylp	t	2	1	1	3	3	6	4
	4	31	58	48	31	15	14	31
	2,4+3,4[b]	14 (1.2)	13 (5.3)	13 (ND)[e]	12 (1.4)	14 (0.9)	9 (2.6)	11 (2.9)
	2,3,4	9	3	5	8	13	7	6
Glcp	3	0	0	0	0	0	3	1
	4	1	1	1	1	1	21	13
	4,6	0	0	1	0	0	0	1
Manp	4	1	0	1	3	0	1	4
total Ara		41	23	28	41	54	36	28
total Xyl		56	75	67	54	45	36	52
total Man		1	0	1	3	0	1	4
total Glc		1	1	2	1	1	24	15
branch/Xyl[c]		0.57	0.25	0.34	0.52	0.89	0.64	0.44
% subst. Xyl[d]		41	21	27	37	60	44	33

[a]: t = terminal residue. [b]: Between brackets: ratio of 3- to 2-substituted xylopyranose. [c]: Calculated from (2,4-Xyl + 3,4-Xyl + 2 * 2,3,4-Xyl)/(total Xyl). [d]: Calculated from (2,4- + 3,4- + 2,3,4-Xyl)/(total Xyl) * 100%. [e]: ND = not determined;

the ratio of these two forms was much lower than 0.5, the value expected if all glucose was present as ß-glucan. This was in agreement with the presence of starch (and possily cellulose) in the WIS[13], besides the expected (1→3),(1→4)-ß-D-glucans. As expected from the much lower ß-glucan content of malt WIS[13], the ratio of 3- to 4-linked glucose is much lower in the malt samples than in the barley samples.
The results from methylation analyses of the BE and L1E fractions and the ethanol precipitates indicate that the amounts of O-2- and O-2,3-substituted xylose increased linearly with ara:xyl ratio (Fig. 3.2). For all the plots shown, correlation coefficients are above 0.9. The amount of O-3-substituted xylopyranose remains constant at 15 ± 1 % of total xylopyranose for barley and malt samples (calculated from Table 3.IV). These results support the idea that arabinofuranose substituents are not randomly distributed over the xylopyranose backbone.

3.4 CONCLUSIONS

The extracted arabinoxylans from barley and malt WIS, representing more than 60 % of the arabinoxylans present in WIS, apparently consist of a family of polysaccharides with a poly-(1→4)-ß-D-xylopyranose backbone, in which 21 to 63 % of the xylose residues are substituted with arabinofuranosyl residues at O-2, O-3 or at both O-2 and O-3. Most of the arabinose is present as monomeric sidechains while a small proportion of it appeared to be present as short oligomeric sidechains, comprising 2 (or more) arabinofuranosyl residues or arabinofuranosyl residues carrying a terminal xylopyranosyl residue. The relative proportion of O-3-substituted xylose was found to be independent of the ara:xyl ratio of the fraction. Variations in ara:xyl ratio are due to differences in the relative proportions of O-2- and O-2,3-substituted xylose. The

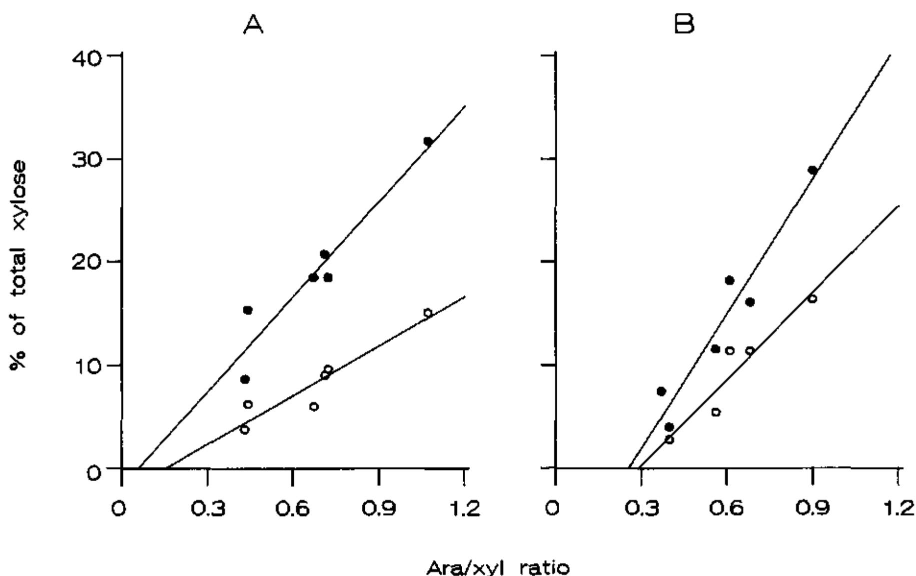

Figure 3.2. Relative amounts of substituted xylose versus ara/xyl ratio for $Ba(OH)_2$-extracted arabinoxylans from barley (A) and malt (B) (: 2-substituted xylose; : 2,3-disubstituted xylose; total xylose in fraction = 100 %).

distribution of arabinose side chains over the xylose residues of the backbone appeared to be non-random. Further work on these fractions, e.g. by degradation studies of the polysaccharide with specific enzymes, might reveal more about the distribution of substituents over the xylan backbone.

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Chapter 4

Structures of small oligomers liberated from barley arabinoxylans by endoxylanase from *Aspergillus awamori*

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SUMMARY

Arabinoxylans containing less than 10 % other sugars were extracted from water-insoluble cell wall material of dehusked barley grain, and degraded with a purified endo-(1→4)-ß-D-xylanase from *Aspergillus awamori*. Twelve of the oligosaccharide fragments released were isolated by a combination of size-exclusion and anion-exchange chromatography, and their structures were determined by ^{1}H-n.m.r. spectroscopy. The identified oligosaccharides consisted of (1→4)-linked ß-D-xylopyranose residues, some of which were substituted at O-2, O-3 or both O-2 and O-3 with α-L-arabinofuranose groups at O-3 or at O-2 and O-3. A structure not reported before and consisting of a xylotriose backbone, solely 2-substituted at the non-reducing terminal xylopyranose residue with an α-arabinofuranose group was described.

4.1 INTRODUCTION

Barley arabinoxylans consist of a backbone of (1→4)-linked ß-D-xylopyranose residues. Part of these residues are substituted at O-2, O-3, or both O-2 and O-3 with mainly single unit α-L-arabinofuranosyl groups[1,2]. (4-O-Methyl-)glucuronic acid[3], 3-O-xylopyranosyl-arabinofuranose[4] and 5-O-feruloyl-arabinofuranose[5] have also been found in small amounts in barley arabinoxylans, depending on the tissues the arabinoxylans were extracted from.

Enzymes with different specificities are required for complete degradation of these arabinoxylans. Major enzymes in this respect are endo- and exo-ß-(1→4)-xylanase, xylosidase and arabinofuranosidase. The nature and distribution of these substituents along the xylan backbone will determine the extent to which these enzymes can degrade the arabinoxylan.

In a previous report, we described the isolation and linkage composition of arabinoxylan fractions extracted from water-insoluble barley and malt cell wall material with a barium hydroxide/sodium borohydride solution[2]. These arabinoxylan fractions consisted for more than 90 % of arabinose and xylose, even without any purification after extraction. A feature unique to arabinoxylans from barley and malt was the presence of large quantities of (1→4)-linked xylopyranose bearing a single arabinofuranose substituent at O-2[2].

Those results, however, gave no information about the distribution of the arabinose residues over the xylan backbone. As a first step in resolving this distribution of arabinose substituents over the xylan backbone, we studied the structures of small oligomeric fragments liberated by enzymic degradation of arabinoxylans isolated from water-insoluble cell wall material from barley[2]. The structures of such oligosaccharides can give information on the structure of the polysaccharide itself as

well as information about the xylan structures recognized by the endo-xylanase used.

4.2 EXPERIMENTAL

General

An arabinoxylan fraction was extracted from barley cell wall material with a saturated solution of barium hydroxide containing 0.13 M sodium borohydride. Isolation and chemical composition of this extract (BE fraction) is described elsewhere[2]. The material consisted for 38 % of arabinose, 53 % of xylose and 8 % of glucose, with trace amounts of mannose and galactose. Methylation analysis showed arabinose to be present mainly as terminal arabinofuranose; xylose was present as xylo-pyranose, 56 % of which was 1,4-linked, 10 % 1,2,4-linked, 14 % 1,3,4-linked, and 19 % 1,2,3,4-linked, with a trace of terminal xylopyranose[2]. Purified endoxylanase 1 from *Aspergillus awamori*[6] was a gift from F. Kormelink (Agricultural University, Department of Food Science). Preliminary tests had shown that this enzyme was the most active of the endoxylanase preparations available that did not release arabinose from the substrate (data not shown).

Total sugar content in column eluent fractions was determined with orci-nol-sulphuric acid reagent using a Skalar autoanalyzer. Methylation analysis[2] of oligosaccharides, using partially methylated alditol acetates, was carrried out after reduction of the oligosaccharide with sodium borodeuteride[7].

Enzyme incubations

For enzymic degradation of arabinoxylan fractions, 100 mg substrate and 20 µg endoxylanase 1 in 100 ml buffer (50 mM sodium acetate,

pH 5.0, containing 1.5 mM sodium azide) was incubated for 16 h at 40 °C. Enzyme activity was terminated by heating the mixture 10 min in a boiling water bath.

HPAEC analysis

Analytical and preparative high performance anion-exchange chromatography (HPAEC) were performed on CarboPac PA-1 columns (Dionex) (250 x 4.5 mm and 250 x 9 mm i.d., respectively, and a corresponding guard column was used with the analytical column) using electrochemical detection (PED-detector, Dionex). For analytical runs a linear gradient of 0-150 mM NaOAc in 100 mM NaOH in 10 min, followed by 150-500 mM NaOAc in 100 mM NaOH in 35 min was used at a flow rate of 1 ml/min. For preparative work, the flow rate was 5 ml/min and the gradients were optimized for each sample. The eluent was immediately neutralised by on-line addition of 1 M acetic acid and fractions of 1.25 ml were collected. Total sugars in the collected fractions were determined and fractions were pooled according to sugar distribution. The pooled fractions were desalted using columns filled with Dowex 50WX8 (H^+) (100-200 mesh, BioRad) and AG-3 (OH^-) (200-400 mesh, BioRad) resins, consecutively[7].

Monosaccharide composition

Oligosaccharides were hydrolysed in 2 M trifluoroacetic acid for 1h at 121 °C. The hydrolysates were dried under a stream of air and analysed by HPAEC on a CarboPac PA-1 column (see above). Elutions were carried out isocratically with 100 mM sodium hydroxide and a runtime of 10 min. Calibration curves were used for quantification of arabinose and xylose contents.

Purification of degradation products

Arabinoxylan oligosaccharides were fractionated according to size on a column (100 cm x 1.6 cm i.d.) of Bio-Gel P-2 (200-400 mesh, BioRad) and eluted at 65 °C with water containing 0.15 mM sodium azide. The eluent was collected in fractions of 1.3 ml and analysed for total sugar contents. Fractions were pooled and, if necessary, further fractionated by HPAEC.

^{1}H-N.m.r. spectroscopy

Samples were repeatedly treated with D_2O (99.9 atom% D, MSD Isotopes), finally using 99.96 atom% D at pD ≥ 7. Resolution-enhanced 600 MHz ^{1}H-n.m.r. spectra were recorded using a Bruker AM-600 spectrometer (SON-hf-NMR facility, Department of Biophysical Chemistry, Nijmegen University), operating at a probe temperature of 27 °C. Chemical shifts (δ) are expressed in p.p.m. and were measured by reference to internal acetone (δ 2.225 in D_2O at 27 °C)[8].

Homonuclear Hartmann-Hahn (HOHAHA) spin-lock experiments were recorded using the pulse sequence 90°-t_1-SL-acq[9-11], where SL stands for a multiple of the MLEV-17 sequence. The spin-lock field strength corresponded to a 90° pulse width of 28 μs and a total spin-lock mixing time of 105 ms. The spectral width was 3205 Hz in each dimension.

Rotating-frame n.O.e. spectroscopy (ROESY) involved the pulse sequence 90_ϕ°-t_1-SL-acq[12], where SL stands for a continuous spin-lock pulse of 200 ms at a field strength corresponding to a 90° pulse width at 114 μs. The carrier frequency was placed at the left side of the spectrum at 5.6 p.p.m. in order to minimise HOHAHA-type magnetisation transfer. The HOD signal was suppressed by presaturation during 1.0 s. The spectral width was 4201 Hz in each dimension.

For both HOHAHA and ROESY spectra 256 experiments of 4K data

points were recorded. The time-proportional phase-increment method (TPPI)[13] was used to create t_1 amplitude modulation. The data matrices were zero-filled to 512 x 8K and multiplied in each time domain with a phase-shifted sine function, shifted $\pi/3$ for the HOHAHA and $\pi/2$ for the ROESY, prior to phase-sensitive F.t..

4.3 RESULTS AND DISCUSSION

Isolation of arabinoxylan oligosaccharides

The mixture of oligosaccharides, obtained by degradation of barley arabinoxylan BE fraction with endoxylanase 1, was fractionated on Bio-Gel P-2 to yield 7 fractions (fractions **1-6** and **void** fraction, Fig. 4.1). Incubation of the **void** fraction with fresh endoxylanase 1 did not result in any further degradation, showing that degradation had proceeded as far as possible with the enzyme used (data not shown). Investigation of **1** and **2** by HPAEC in combination with monosaccharide analysis showed that these fractions consisted of one component each, xylose and xylobiose, respectively.

Fractions **3-6** contained 2 or 3 major components, consisting of ara-

Table 4.I. Distribution and monosaccharide composition of fractions from arabinoxylans digested with endoxylanase 1 from *Aspergillus awamori.*

Fractions from Bio-Gel P-2						
Fraction	**1**	**2**	**3**	**4**	**5**	**6**
% of total	1.8	4.0	4.5	5.7	4.5	3.9
Ara/Xyl ratio[a]	0.00	0.00	0.17	0.37	0.46	0.52

Fractions from HPIEC										
Fraction[b]	**31**	**32**	**41**	**42**	**51**	**52**	**53**	**61**	**62**	**63**
% of pool	59	41	40	60	57	12	30	69	21	10
Ara/Xyl ratio	0.00	0.50	0.39	0.36	0.69	0.74	0.66	0.51	0.49	0.74

[a]: ara:xyl ratio = ratio of arabinose to xylose; other monosaccharides were not found in any of the fractions.
[b]: fractions **1** and **2** consisted only of xylose.

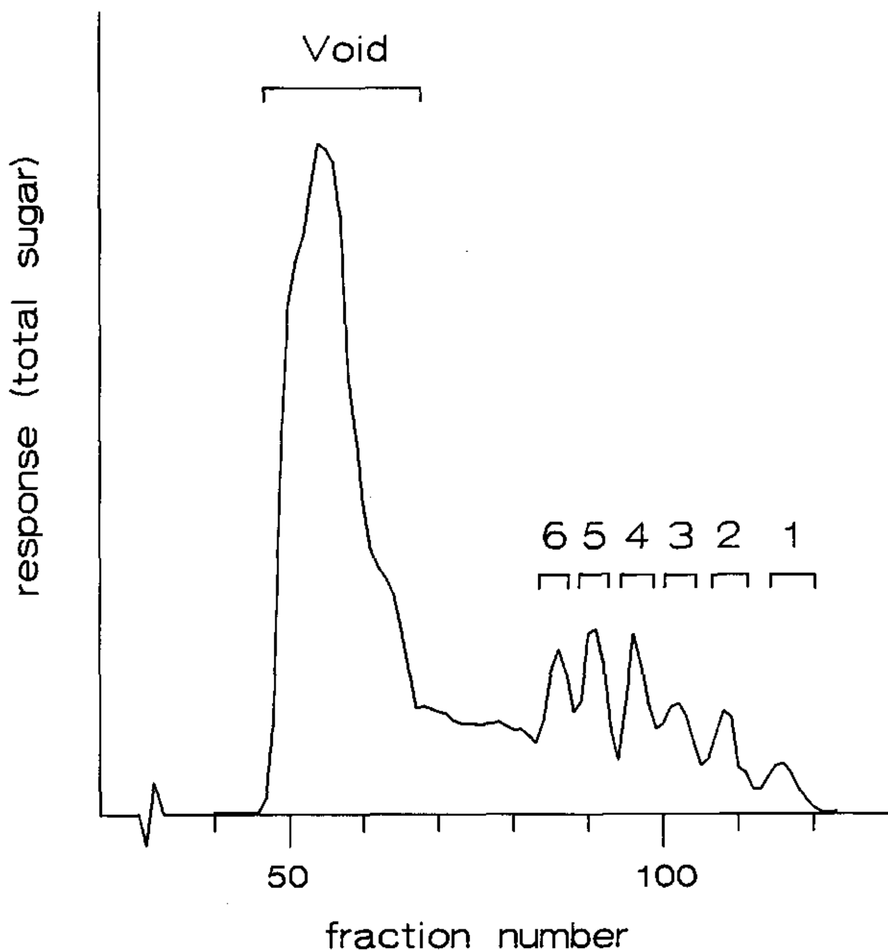


Figure 4.1. Separation on Bio-Gel P-2 of fragments in digest obtained from barley arabinoxylans with endoxylanase 1.

binose and xylose (Fig. 4.2 and Table 4.I). These components were isolated by preparative HPAEC.

[1]H-n.m.r. analysis of oligosaccharides

Primary structures of the major oligosaccharides present in the isolated HPAEC fractions were further elucidated by [1]H-n.m.r. spectroscopy.

All isolated fractions, except **41** and **52**, contained compounds having

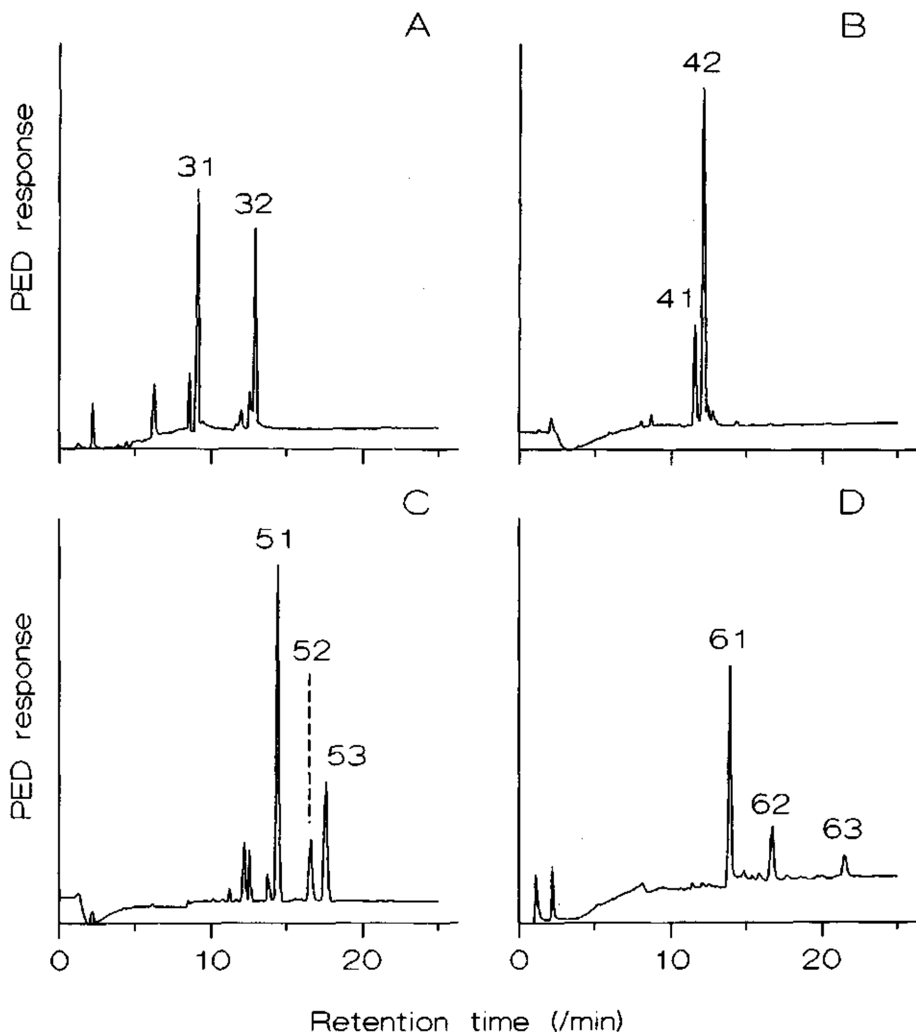


Figure 4.2. HPAEC fractionation patterns of Bio-Gel P-2 fractions 3-6 (A-D, respectively). The column was eluted with a gradient of sodium acetate in 100 mM sodium hydroxide (start at 0 mM acetate, linear increase to 150 mM acetate in 10 min, further linear increase to 500 mM in 35 min).

structures identical to recently published reference compounds[7,14,15], and the H-1 chemical shifts and structures are given in Table 4.II and Fig. 4.3, respectively. The monosaccharide composition of these oligosaccharides was in agreement with the structures assigned (Table 4.I).

Table 4.II. ^{1}H-n.m.r. chemical shifts of the anomeric protons of the constituent monosaccharides for the oligosaccharides **31-63**, derived by enzymic degradation of barley cell wall arabinoxylans.

Proton[a]	Compound									
	RV-31	**RV-32**	**RV-41**	**RV-42**	**RV-51**	**RV-52**[b]	**RV-53**	**RV-61**	**RV-62**	**RV-63**
α-ara*f* $^{3X2\alpha}$	--[c]	5.335	--	5.401	--	5.426	5.396	--	5.391	5.427
α-ara*f* $^{3X2\beta}$	--	5.331	--	5.396	--	5.421	5.391	--	5.387	5.422
α-ara*f* ^{2X3}	--	--	5.280	--	5.237	5.293	--	5.226	--	5.244
α-ara*f* ^{3X3}	--	--	--	--	5.246	--	5.329	5.274	5.398	5.244
α-xyl*p*-**1**	5.184	5.185	5.183	5.185	5.183	5.183	5.185	5.183	5.186	5.185
ß-xyl*p*-**1**	4.584	4.584	4.583	4.584	4.584	4.583	4.584	4.584	4.584	4.584
ß-xyl*p*-**2α**	4.478	4.487	4.462	4.508	4.465	--	--	4.466	--	--
ß-xyl*p*-**2ß**	4.475	4.490	4.466	4.510	4.468	4.494	4.510	4.468	4.510	4.495
ß-xyl*p*-**3**	4.461	--	4.555	4.443	4.597	4.527	4.475	4.640	4.489	4.562
ß-xyl*p*-**4**	--	--	--	--	--	--	--	4.437	4.432	--

[a]: The Xyl*p* residue *in the reducing position is denoted* **1**, *etc.*; $2_{\alpha/\beta}$ means reducing Xyl*p*-**1** residue in α/ß-configuration (anomerisation effect). Ara*f*-A^{2X3} means arabinofuranose linked to *O*-2 of Xyl*p*-3, etc.; Xyl*p*-3^{I} means Xyl*p*-3 branched at *O*-2; Xyl*p*-3^{II} means Xyl*p*-3 branched at *O*-3; Xyl*p*-3^{III} means Xyl*p*-3 branched at *O*-2,3.
[b]: Assignments for this fragment are tentative (see text).
[c]: -- = corresponding proton is not present in the oligosaccharide.

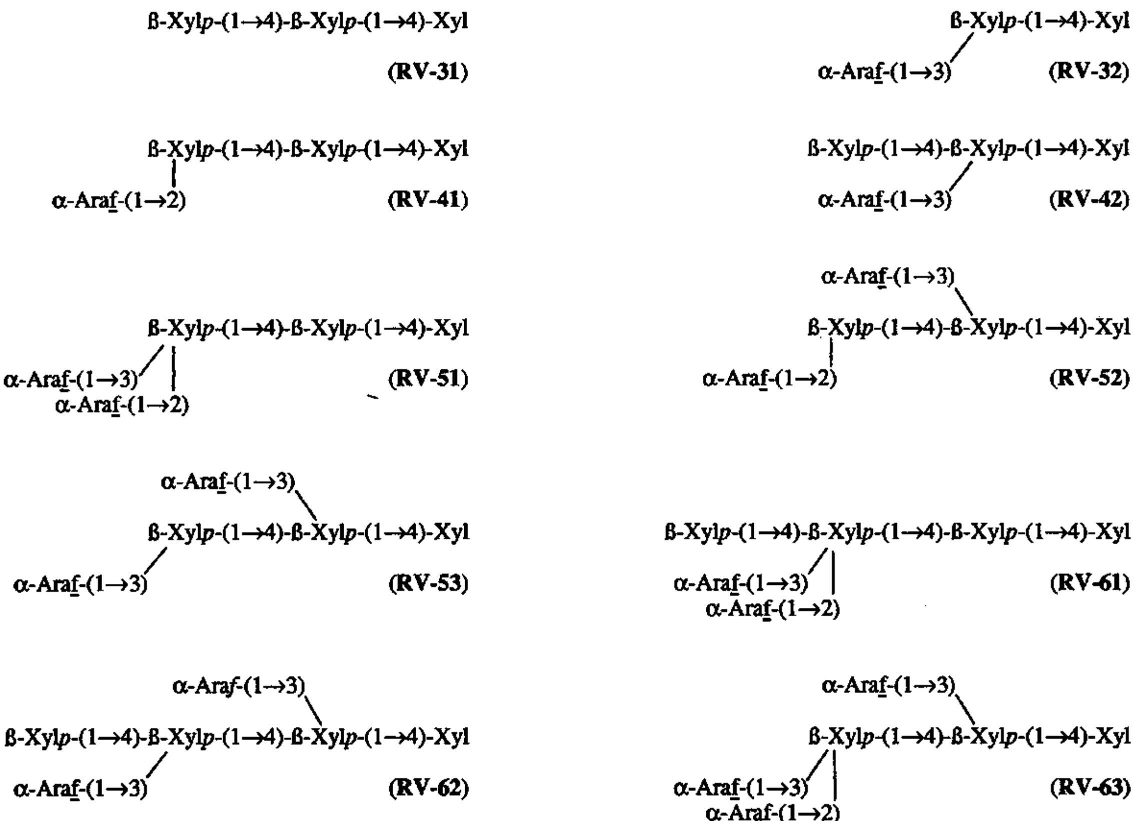
Table 4.III. ¹H-N.m.r. data for the arabinoxylan oligosaccharide **RV-41**, together with those of reference compounds **HG-32** and **HG-51**, derived from wheat arabinoxylans[15].

Compound	Residue[a]	H-1	H-2	H-3	H-4	H-5*eq*/H-5proR	H-5*ax*/H-5proS
		Chemical shift[b]					
	α-Xyl*p*-**1**	5.185	3.545	—	3.73	3.82	—
	ß-Xyl*p*-**1**	4.584	3.250	3.545	3.781	4.055	3.377
	ß-Xyl*p*-**2**$^{II}_{\alpha}$	4.487	3.413	3.594	3.692 ┬	4.004	3.340
HG-32	ß-Xyl*p*-**2**$^{II}_{ß}$	4.490	3.403	3.591	3.690 ┘		
	α-Ara*f*-A$^{3X2\alpha}$	5.335 ┬	4.175	3.959	4.185	3.817	3.705
	α-Ara*f*-A^{3X2ß}	5.332 ┘					
	α-xyl*p*-**1**	5.183	3.543	—	3.73	3.82	—
	ß-xyl*p*-**1**	4.583	3.247	3.544	3.770	4.050	3.375
	ß-xyl*p*-**2**$_{\alpha}$	4.462	3.295 ┬	3.556	3.785	4.134	3.409
	ß-xyl*p*-**2**$_{ß}$	4.466	3.287 ┘				
RV-41	ß-xyl*p*-**3**^{I}	4.555	3.411	3.560	3.660	3.988	3.308
	α-ara*f*-A^{2X3}	5.280	4.166	3.948	4.140	3.814	3.716
	α-Xyl*p*-**1**	5.183	3.546	—	3.73	3.82	—
	ß-Xyl*p*-**1**	4.584	3.249	3.546	3.771	4.050	3.376
	ß-Xyl*p*-**2**$_{\alpha}$	4.465	3.298 ┬	3.558	3.792	4.140	3.416
	ß-Xyl*p*-**2**$_{ß}$	4.467	3.290 ┘				
HG-51	ß-Xyl*p*-**3**^{III}	4.596	3.539	3.689	3.724	4.023	3.344
	α-Ara*f*-A^{2X3}	5.238	4.151	3.956	4.132	3.816	3.720
	α-Ara*f*-A^{3X3}	5.246	4.175	3.973	4.198	3.813	3.706

[a]: The Xyl*p* residue in the reducing position is denoted **1**, etc.; $2_{\alpha ß}$ means reducing Xyl*p*-**1** residue in α/ß-configuration (anomerisation effect). Ara*f*-A^{2X3} means arabinofuranose linked to *O*-2 of Xyl*p*-**3**, etc.; Xyl*p*-**3**^{I} means Xyl*p*-**3** branched at *O*-2; Xyl*p*-**3**^{II} means Xyl*p*-**3** branched at *O*-3; Xyl*p*-**3**^{III} means Xyl*p*-**3** branched at *O*-2,3.

[b]: In p.p.m. relative to the signal of internal acetone at δ 2.225 in D_2O at 27 °C, acquired at 600 MHz.

Figure 4.3. Structures determined for fractions **31-63**.



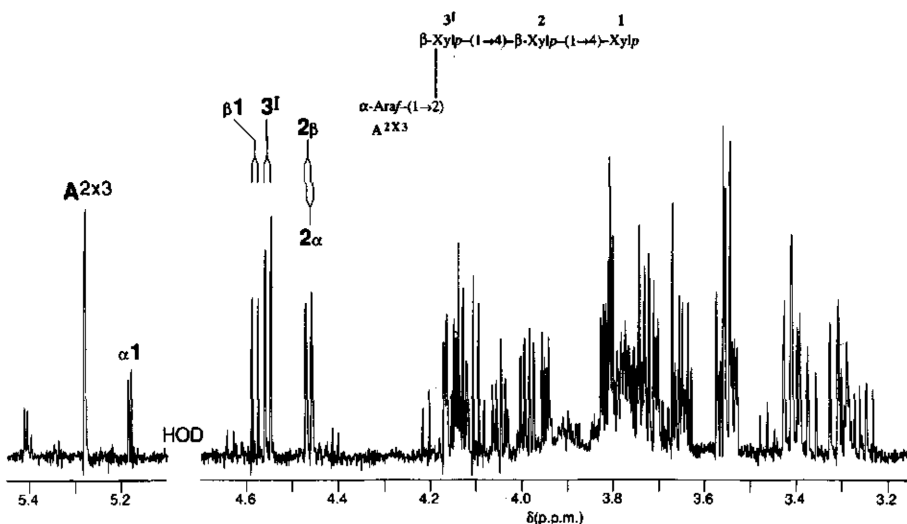


Figure 4.4. Resolution-enhanced 600 MHz ^{1}H-n.m.r. spectrum of oligosaccharide **41**. The numbers and letters in the spectrum refer to the corresponding residues in the structure.

The ^{1}H-n.m.r. spectra of oligosaccharides **41** and **52** did not occur in the reference library, and will be discussed below.

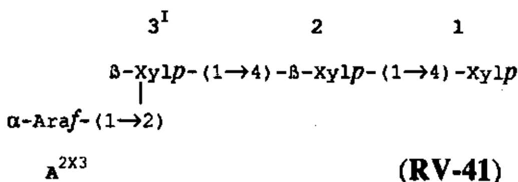
Fraction 41

The intensities of the signals for anomeric protons in the ^{1}H-n.m.r. spectrum of **41** (Fig. 4.4) indicated the presence of an arabinosyl-xylotriose, **RV-41**, as the major compound with the Xyl*p* units in the ß-configuration ($J_{1,2}$ = 7-8 Hz) and the Ara*f* unit in the α-configuration ($J_{1,2} \approx$ 1 Hz)[16].

On the various H-1 tracks of the constituent monosaccharides in the 2D HOHAHA spectrum (Fig. 4.5), the total scalar-coupled networks of each residue were observed, and the data obtained are summarized in Table 4.III. Specific assignment of the α-Ara*f* H-5*proR*,5*proS* signals is based on their relative chemical shifts ($\delta_{5proR} > \delta_{5proS}$), supported by the $J_{4,5}$

Figure 4.5. 600 MHz HOHAHA spectrum of fraction **41**. Diagonal peaks of the anomeric protons are indicated. The numbers near cross-peaks refer to the protons of

values ($J_{4,5proR} < J_{4,5proS}$)[17]. Part of the ROESY spectrum is presented in Fig. 4.6. The observed R.O.e.s. between H-1 of ß-Xyl*p*-(**n**) and H-4,5*eq* of ß-Xyl*p*-(**n-1**), together with the α-Ara*f*-A^{2X3} H-1, ß-Xyl*p*-3^{I} H-2 connectivity, established the sequence of **RV-41**.



(RV-41)

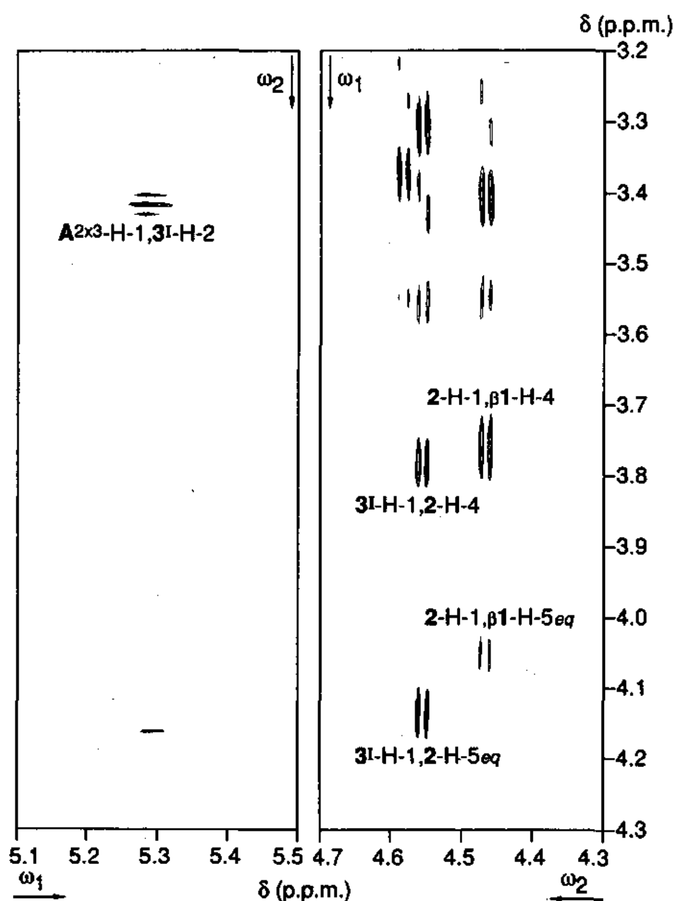


Figure 4.6. 600 MHz ROESY spectrum of fraction **41**. R.O.e. connectivities are given along the H-1 tracks in the ω_1-dimension for the ß-Xyl*p* residues, and in the ω_2-dimension for the α-Ara*f* residue. Only the inter-residue r.O.e. connectivities are denoted and the negative levels given. A^{2X3}-H-1,3^I-H-2 means the cross-peak between H-1 of α-Ara*f*-A^{2X3} and H-2 of ß-Xyl*p*-3^I, etc.

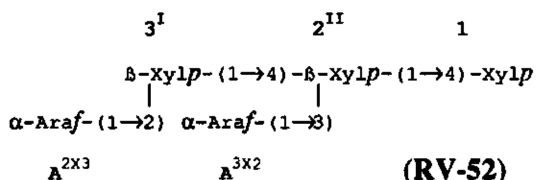
Comparison of the ^{1}H-n.m.r. data of **RV-41** with those of reference compounds **HG-32** and **HG-51**[15] (identical to **RV-32** and **RV-51**, respectively; Table 4.III) shows an intermediate chemical shift position of the ß-Xyl*p*-3^I H-1 signal of [α-Ara*f*-(1→2)-ß-Xyl*p*-(1→] relative to that of the

ß-Xyl*p*-**2**^{II} H-1 signal of [α-Ara*f*-(1→3)-ß-Xyl*p*-(1→] and the ß-Xyl*p*-**3**^{III} H-1 signal of the **HG-51** {α-Ara*f*-(1→2)-[α-Ara*f*-(1→3)]-ß-Xyl*p*-(1→}. The same was observed for the α-Ara*f*-**A**^{2X3} H-1 signal, relative to the α-Ara*f*-**A**^{3X2} H-1 signal of **HG-32** and the α-Ara*f*-A^{2X3} and α-Ara*f*-A^{3X3} H-1 signals of **HG-51**, respectively. Owing to the presence of a reducing residue, an anomerisation effect occurs, doubling the H-1,2 signals of ß-Xyl*p*-**2**[7,18].

The structure for **RV-41** was confirmed by methylation analysis of the borodeuteride-reduced oligosaccharide, showing the presence of terminal arabinofuranose, 4-linked xylopyranose and 2-linked xylopyranose in a molar ratio of 1:0.9:0.9. The 4-linked xylitol could not be detected by methylation analysis, due to its high volatility.

Fraction 52

Based on P-2 chromatography fraction **52** contained fragments consisting of five pentose residues. This was confirmed by the arabinose to xylose ratio (0.74, Table 4.II) and by 1D ^{1}H-n.m.r. spectroscopy, showing 5 main signals in the region for anomeric protons (Table 4.II). Too little material of fraction **52** was available for a complete 2D n.m.r. analysis. The observed H-1 signals of xyl*p*-1, ß-xyl*p*-2^{II} and α-Ara*f*-A^{3X2} of the major component of **RV-52** (Table 4.II) resonate at the same chemical shift position as those of the corresponding residues in **RV-63**. The terminal arabinosyl-xylose unit of **RV-52** was characterized by the ß-Xyl*p*-3^{I} and α-Ara*f*-A^{2X3} H-1 signals at δ 4.527 and 5.293, respectively. Both H-1 signals resonate at an intermediate chemical shift position relative to the ß-Xyl*p* and α-Ara*f* H-1 signals of the terminal arabinosyl-xylose unit of **RV-53** and the di-arabinosyl-xylose unit of **RV-63**, respectively. Based on these data the major component of oligosaccharide **52** is proposed to be:



4.4 CONCLUSIONS

The endoxylanase 1 used for the degradation of the arabinoxylans from barley WIS could only degrade these substrates to a limited extent, compared with results obtained for wheat arabinoxylans[15]. Although the differences in substitutions in the substrate can explain part of the differences in degradability, the distribution of substituted and unsubstituted regions over the xylan backbone could also be of importance. Further work is in progress to gain insight in the distribution of substituents over the xylan backbone.

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Chapter 5

Substitution patterns of water-insoluble arabinoxylans from barley and malt

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SUMMARY

Intact arabinoxylan polymer fractions from barley and malt differing in their ratio of arabinose to xylose (ara:xyl ratio) were degraded with a purified endoxylanase (E.C. 3.2.1.8) from Aspergillus awamori. Enzymic degradability decreased with increasing ara:xyl ratio. No differences in degradability between similar fractions from barley and malt could be observed. The fragments liberated by enzymic action were characterized using HPLC. The fragments consisting of less than 7 pentose residues contained relatively low amounts of O-2- and O-2,3-substituted xylose, compared to the original substrate. Xylose residues substituted at O-2 or both O-2 and O-3 appeared to be concentrated in the larger fragments. It was concluded from these results that substituted xylose residues were not randomly distributed in the polysaccharide, but were arranged according to a pattern in which isolated unsubstituted residues are separated by one or two substituted residues. This pattern was interrupted by sequences of contiguous unsubstituted xylose residues. From the available data it was concluded that those sequences could reach a length of at least four residues, but the presence of longer unsubstituted sequences could not be excluded.

5.1 INTRODUCTION

Arabinoxylans are one of the major non-starch polysaccharides (nsp) present in cereal endosperm and aleurone cell walls. They play an important role in the processing of cereals (e.g. baking and brewing)[1]. In brewing, an excess of arabinoxylans in barley malt or adjuncts (maize, wheat, rice) can cause processing problems like poor filtration rates or the formation of beer hazes and precipitates[1,2].
In barley the arabinoxylans comprise 20-25 % of the endosperm cell wall polysaccharides[3,4] and 85 % of the aleurone cell wall polysaccharides[5]. They consist of a range of ß-(1→4)-xylans, substituted to varying extents with single arabinofuranose residues (Ara*f*) at O-2, O-3 or at both O-2 and O-3[5,6].
As arabinoxylans are especially abundant in aleurone cell walls of barley, insufficient degradation of these polysaccharides during malting could impair release of cell wall and starch degrading enzymes into the endosperm. This would result in retarded degradation of the cell walls in germinating barley. Total degradation of the cell wall arabinoxylans requires the presence of several enzymes, e.g. endo- and exo-(1→4)-ß-xylanase and arabinofuranosidase. These enzymes are induced during the malting of barley[7,8]. Degradation of arabinoxylans during malting was found to be only partial[9].
The extent to which the arabinoxylans can be degraded, as well as other features such as solubility and interaction with other polymeric cell wall components, depends on the presence of substituents (mainly Ara*f*) and their distribution over the xylan backbone[5].
Knowledge of this distribution would therefore help in understanding the properties and degradation of the arabinoxylans e.g. during malting. Furthermore, such knowledge would also be helpful in optimization of

the use of exogenous enzymes in processes involving arabinoxylans, e.g. baking.

In previous studies, we determined the chemical composition of water-insoluble barley and malt arabinoxylans extracted from cell wall material[6] and the structure of small oligomers formed during digestion of these arabinoxylans with a purified endoxylanase[10]. The barley and malt arabinoxylans were shown to contain xylose residues substituted at O-2, O-3 or both O-2 and O-3 with Ara*f*[6].

Aim of this study was to elucidate the distribution of arabinose residues over the xylan backbone. We studied the enzymic digestibility of various polymeric arabinoxylan fractions. Also the composition of the less degradable part of the arabinoxylans was studied by ^{1}H-nmr spectroscopy and methylation analysis. From the results of these experiments, in combination with knowledge of the structures of small fragments formed by enzymic degradation of barley arabinoxylans[10] and the results of computer simulations of arabinoxylan structure and degradation, a model for the distribution of arabinose substituents over the xylan chain was proposed.

5.2 EXPERIMENTAL

General

Arabinoxylans used were extracted from water-insoluble cell wall material of barley and malt using a saturated solution of $Ba(OH)_2$ containing $NaBH_4$ (BE fractions)[6]. The barley and malt BE fractions were further fractionated by graded ethanol precipitation (fractions BE-nn, with nn indicating the ethanol concentration in % (v/v) at which the fraction was precipitated)[6]. Purified endoxylanase 1 (E.C. 3.2.1.8) from *Aspergillus awamori*[11] was used for enzymic degradation of arabinoxylan

samples. Conditions of enzymic degradation and methods used for determination of sugar composition and methylation analysis were described before[6,10]. Preparation of the sample for 1D ^{1}H-nmr and recording of the spectrum (at 600 MHz) was performed as described by Hoffmann[12].

HPLC analysis

A Dionex system was used for high performance anion exchange chromatography (HPAEC) as described elsewhere[10]. The column was eluted with a gradient of sodium acetate in 100 mM NaOH (starting at 0 mM sodium acetate, increase to 150 mM acetate in 10 min, further increase to 500 mM sodium acetate in 45 min) at a flow rate of 1 ml/min. For calculation of fragment distributions the responses per mole were assumed to be equal for the known components. Coding and structures of the known fragments are described elsewhere[10].

For high performance size exclusion chromatography (HPSEC), TSK 40-XL, 30-XL and 20-Xl columns (250 x 4 mm) were used in series and eluted with 0.4 M sodium acetate/acetic acid buffer pH 3 (0.8 ml/min). The eluent was monitored with refractive index detection.

Simulation of arabinoxylan chains

Simulations of arabinoxylan structure and degradation were performed using programs written in Turbo Pascal version 5.5 (Borland Corp.), running on IBM-PC compatible microcomputers. Chain lenghts of 2000 xylose units were used, as this was deemed sufficient to obtain representative results in reasonable calculation times. Moreover, a chain length of 2000 xylose residues gave molar weigths for the arabinoxylan chains within the range of experimental values for cereal arabinoxylans[1].

For simulation of the arabinoxylan chains, all substituents of the xylose were assumed to be single α-arabinofuranose groups. Arabinoxylan

chains were simulated by random assignment of one of the four possible substituted xylose residues (no substituents, one substituent at O-2 or O-3 or substituents at both O-2 and O-3) to each of the 2000 xylose positions in the chain. The probabilities for selecting each of these four possible units were set equal to the mole fractions of the corresponding xylopyranose residues in the actual arabinoxylan fraction. These mole fractions were determined by methylation analysis[6] (total xylose = 1, Table 5.II).

For simulation of enzymic degradation of the generated chains a large number of attacks was assumed, each leading to zero or one hydrolysed xylose-xylose linkage.

The specificity of the endoxylanase 1 was determined from the structures found in digests of barley and wheat arabinoxylans[10,13] and is described in detail elsewhere[14]. From these structures, it was concluded that the endoxylanase 1 could hydrolyse a ß-(1→4)-linkage only if the new reducing end consisted of an unsubstituted xylopyranose residue (Xyl*p*). The Xyl*p* attached to O-4 of this unsubstituted residue could be substituted at O-3, but not at O-2. The new non-reducing end Xyl*p* could be unsubstituted or substituted at O-2 and/or O-3. The enzyme was not able to remove a single unsubstituted Xyl*p* from the non-reducing end of an arabinoxylan chain or fragment. Oligomers smaller than xylotriose could not be degraded. Action of the enzyme near O-2-substituted xylopyranose residues was identical to action near O-2,3-disubstituted xylopyranose residues.

Calculations (for both chain simulation and degradation) were repeated 10 times for each set of parameters, the results of these 10 runs were averaged.

5.3 RESULTS

Enzymic degradation of arabinoxylan samples

Fig. 5.1 shows the results from HPSEC of arabinoxylan fractions after digestion with endoxylanase 1. The extent of degradation of the arabinoxylans decreased with increasing ratio of arabinose to xylose (ara:xyl ratio). Arabinoxylans with ara:xyl ratio's of 0.40 were almost totally degraded to small oligomers (retention time longer than 35 min.)

Table 5.I. Relative amounts by weight of the main products of arabinoxylan degradation, measured by HPAEC (total of fragments **1-63** = 100).

Fraction	Fragment[a]										
	1	**2**	**31**	**32**	**41**	**42**	**51**	**52+62**	**53**	**61**	**63**
Barley (experiment)											
BE	7	16	11	7	9	14	11	5	6	11	2
BE-20[b]	10	17	9	8	3	21	8	5	6	12	1
BE-30	11	15	8	8	4	18	11	5	6	15	2
BE-40	10	16	8	7	4	19	11	5	6	16	1
BE-50	10	15	8	7	5	17	13	5	5	16	3
BE-60	10	13	7	7	6	14	20	4	8	19	7
BE-70	9	16	7	9	6	14	21	1	4	20	6
Malt (experiment)											
	7	21	11	5	7	19	10	6	5	9	1
BE-20	12	17	10	8	3	21	6	6	6	9	2
BE-30	11	16	10	8	6	20	8	6	6	10	1
BE-40	9	21	2	8	5	22	9	5	6	10	2
BE-50	12	18	6	8	6	19	10	5	6	9	3
BE-60	11	17	5	7	7	16	13	5	6	9	5
BE-70	11	19	5	7	6	15	15	5	4	8	5
Barley (simulation)[c]											
BE-20	10	20	14	13	4	9	12	3	4	6	3
BE-60	12	16	8	11	7	5	20	4	4	6	8
BE-70	15	10	3	14	10	3	20	5	6	5	8

[a]: Coding of fragments is described in Viëtor *et al.*[10] and Fig. 5.2.
[b]: numbers indicate ethanol concentration at which the fraction precipitated.
[c]: Underlined values differ significantly from experimental values for the corresponding fraction

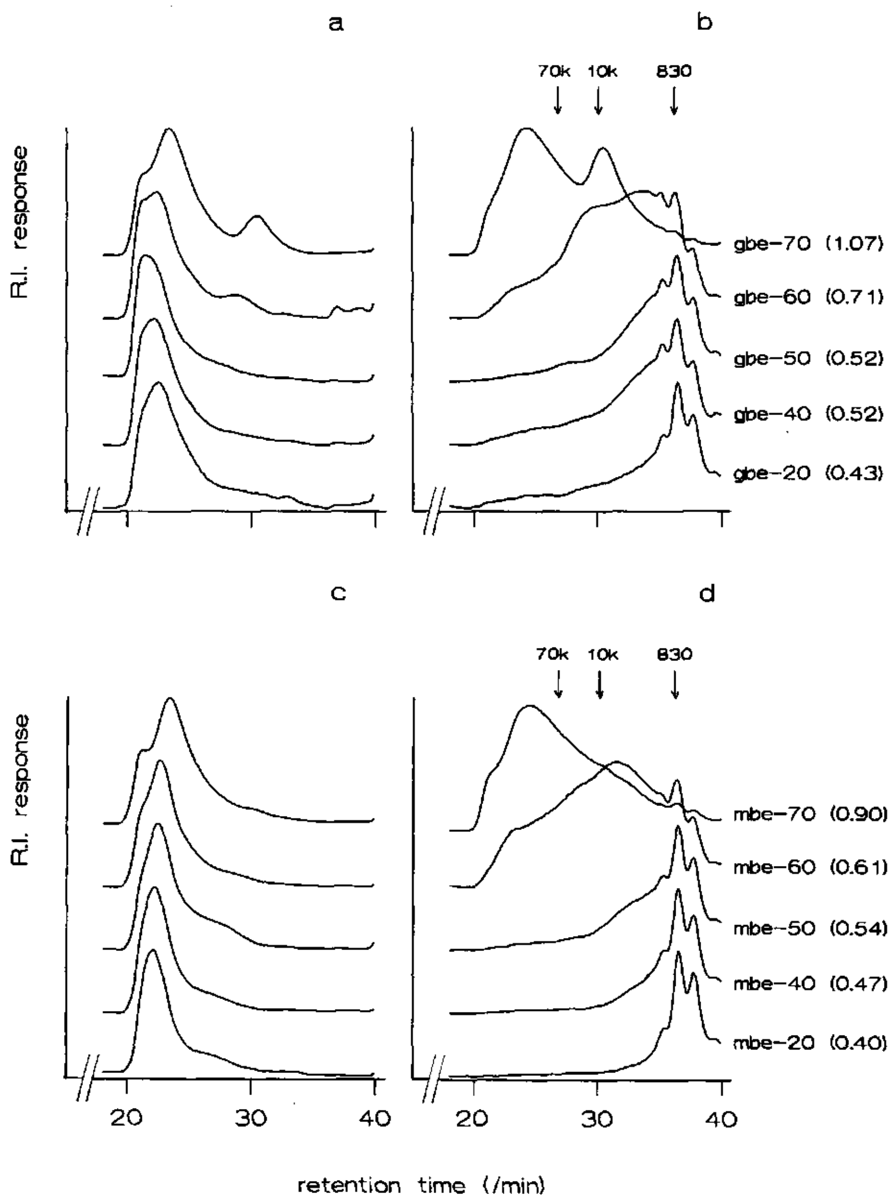

Figure 5.1. HPSEC of digested arabinoxylans from barley and malt (a,b: barley, c,d: malt;, a,c: blanks, b,d: digests; values between parenthesis indicate the ara:xyl ratio of the fraction)

whereas samples with ara:xyl ratio's above 0.9 showed little degradation. In all samples however, some small fragments were formed. Also the patterns in the low molecular weight region of the chromatogram (Fig. 5.1) were quite similar for all samples.

The results of HPAEC (Table 5.I) were also very similar for all fractions. The relative proportions of the small fragments differ little between the various samples.

The main differences between the barley and malt arabinoxylans were found in the fragments **51** and **61**, which were present in the barley digests in larger relative amounts.

Comparison of the arabinoxylan fragments from barley shows that the relative amounts of the fragments **51**, **61** and **63** increase with increasing ara:xyl ratio, whereas relative amounts of fragment **42** decrease.

For the malt arabinoxylans, variations in relative amounts are mainly found for the fragments **31**, **42**, **51** and **63**.

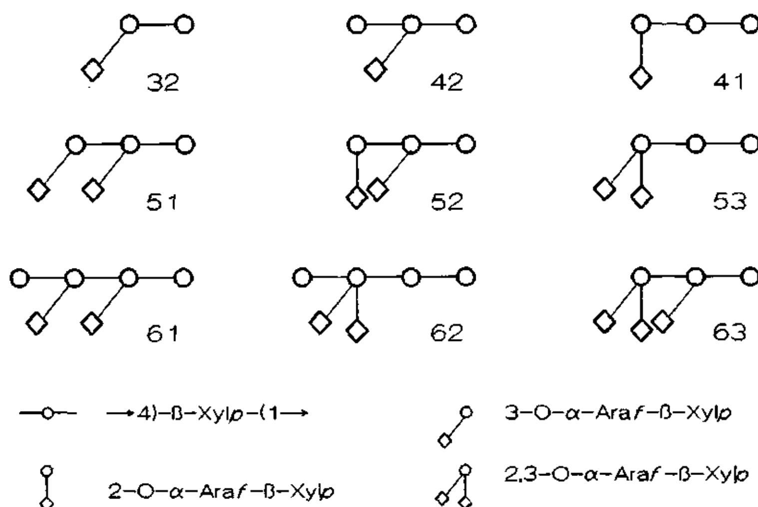

Figure 5.2. Structures of arabinoxylan fragments isolated by HPAEC[10].

For fractions from both barley and malt, the amounts of xylose substituted at O-2 or at both O-2 and O-3 were quite small in the fragments analysed by HPAEC, much smaller than expected from methylation analysis of the undegraded parental polysaccharides (Table 5.II).

Methylation analysis of material collected in the void volume of a Bio-Gel P-2 column after separation of a BE-digest (fraction **P2-void** in table 5.II) showed that 55 % and 65 % of the total xylose in these fractions were substituted for barley and malt respectively. The material was enriched in 2-O- and 2,3-di-O-substituted xylose, compared to the

Table 5.II. Relative linkage composition for xylose residues (in mol% of total xylose) in known fragments in digest and in parental substrates.

Fraction	Substrate[a]				HPAEC fragments[b]			
	4[c]	2,4	3,4	2,3,4	4	2,4	3,4	2,3,4
Barley (experiment)								
BE	56	10	14	19	75	4	18	3
P2-void	45	13	15	27	--	--	--	--
BE-20	71	4	15	9	76	2	20	2
BE-30	65	6	14	15	74	2	21	3
BE-60	55	9	14	22	71	3	21	6
BE-70	33	15	18	33	73	2	19	6
Malt (experiment)								
BE	57	12	14	17	77	4	17	3
P2-void	35	22	13	30	--	--	--	--
BE-20	78	3	15	4	77	2	19	2
BE-30	73	20[d]	8	76	3	19	2	
BE-60	61	10	14	16	74	4	18	4
BE-70	36	18	16	31	76	3	16	5
Barley (simulation)								
BE-20					71	2	22	3
BE-60					67	3	27	4
BE-70					62	4	27	5

[a]: Calculated from Viëtor *et al.*[9].
[b]: Calculated from Table 1 using structures of the fragments as described by Viëtor *et al.*[10].
[c]: Numbers indicate positions at which xylose residues are substituted.
[d]: Sum of 2,4- and 3,4-xylose.

parental polysaccharide. Incubation of the **P2-Void** fractions with fresh endoxylanase 1 did not yield any small fragments (data not shown).

*NMR of barley **P2-Void***

The barley **P2-void** fraction was further analysed by ^{1}H-nmr. The spectrum (Fig. 5.3) shows very broad peaks, due to the presence of material with a high molecular weight. Signals were assigned by comparison of

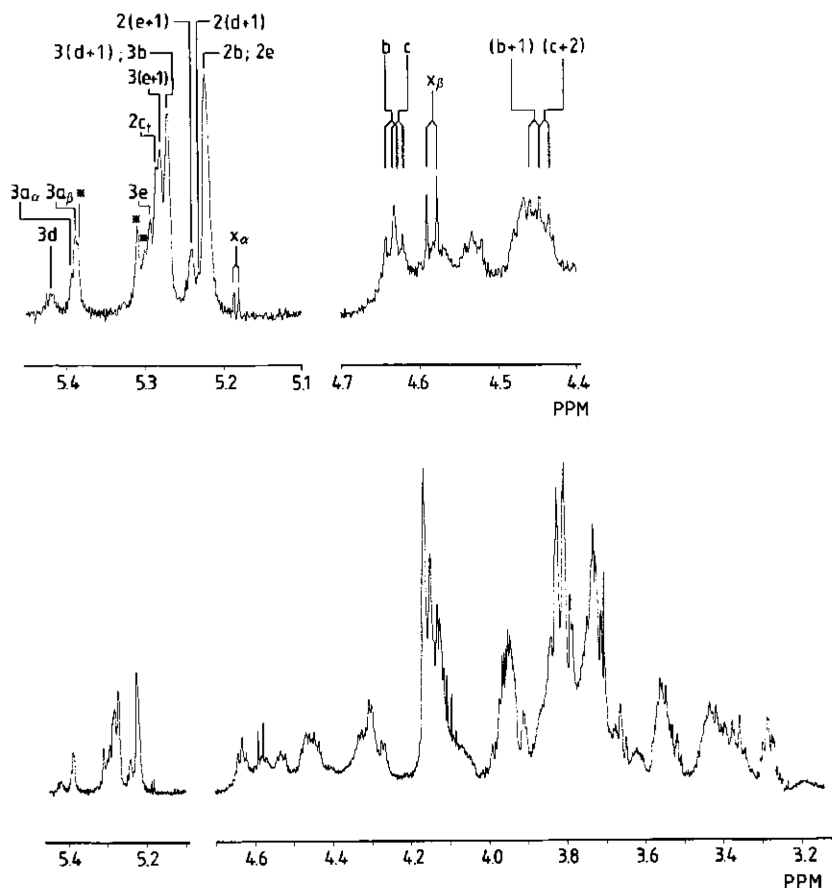

Figure 5.3. 1-Dimensional ^{1}H-nmr spectrum of the barley **P2-void** fraction. Coding of peaks refers to structural elements shown in Fig. 5.4, an asterisk marks unknown signals arising from Ara*f*.

observed chemical shifts (δ) with values previously reported[10,12,13]. Due to large differences in relaxation times, the intensities of the xylopyranose signals (δ between 4.4 and 4.7 ppm) were in general much lower than the intensities of the arabinofuranose signals (δ > 5.2). This made it impossible to assign all xylose signals.

From the arabinose signals several structural elements could easily be identified (Fig. 5.4). The chemical shifts of those signals are summarized in table 5.III. The most prominent arabinose signals were from arabinose residues attached to double substituted xylose residues, present isolated (**b** in Figs. 5.3 and 5.4) or next to another substituted xylose residue (**d** and **e**). Also the signals from arabinose attached to terminal 2-substituted xylose (**c**) were clearly visible. Signals of arabinose

Table 5.III. Chemical shifts of signals from anomeric protons (H-1) marked in Fig. 5.3.

Structural element[a]	Arabinose residue[b]	Chemical shift (ppm)	Xylose residue[b]	Chemical shift (ppm)
-	--		x_α	5.18
-	--		x_β	4.59
a	$3a_\alpha$	5.39		
	$3a_\beta$	5.39		
b	2b	5.22	b	4.64
	3b	5.27	(b+1)	4.45
c	$2c_t$	5.28	c	4.63
			(c+2)	4.44
d	3d	5.42		
	2(d+1)	5.23		
	3(d+1)	5.27		
e	2e	5.22		
	3e	5.29		
	2(e+1)	5.24		
	3(e+1)	5.28		
-	*	5.31, 5.30, 5.38		

[a]: Letters refer to structural elements shown in Fig. 5.4; -: no element from Fig. 5.4 can be associated with this signal.

[b]: Meaning of subscripts: α,b: reducing end terminus has the α or β conformation respectively; t: arabinose substituent is attached to xylose at non-reducing end of a xylan chain. For explanation of e.g. (d+2) see Fig. 5.4.

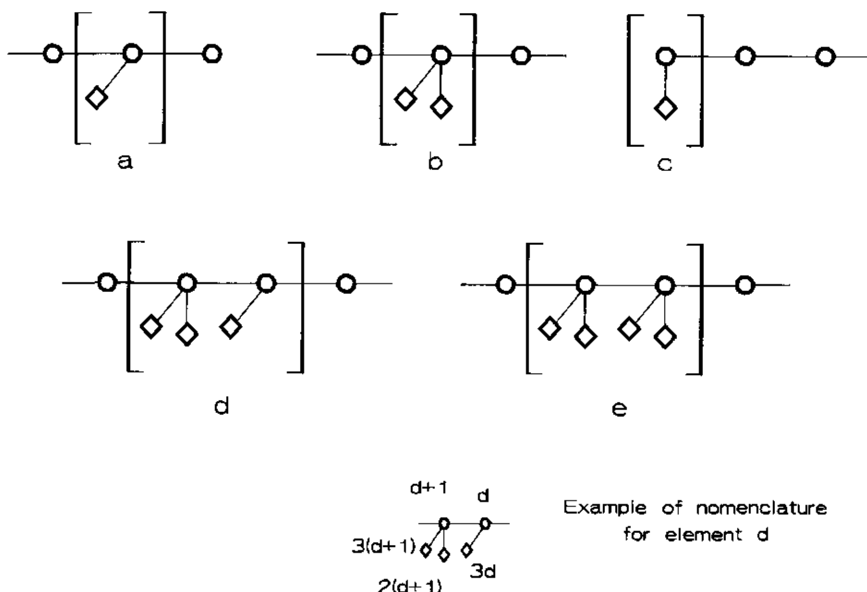

Figure 5.4. Structural elements observed by ^{1}H-nmr in the undegradable part of barley arabinoxylan (fraction **P2-Void**). For an explanantion of the symbols used see Figure 5.2. For all fragments the right hand xylose is the reducing end.

attached to isolated 3-substituted xylose residues were also present (**a**), but of rather low intensity.

Some important signals from anomeric protons of arabinose (marked with an asterisk in Fig. 5.3) could not be assigned. Considering the chemical shifts of these signals, one (at δ = 5.386) probably arises from an arabinose attached to a 3-substituted xylose next to another substituted xylose residue. The others (at δ = 5.300 and δ = 5.308) can arise from arabinoses attached to either a double substituted xylose or a 2-substituted xylose.

Most prominent of the xylose signals were those arising from terminal unsubstituted xylose residues present as reducing end ($\mathbf{x}_\alpha$ and $\mathbf{x}_\beta$). Other signals which could be assigned arose from 2,3-disubstituted xylose residues (**b**, **c**) or xylose residues next to such a residue (**(c+2)**).

Simulation of arabinoxylan structure and degradation

Simulations of arabinoxylan structures and digestion using a random distribution of substituted xylose residues, showed that the relative amounts of the various fragments as predicted from this model differ clearly from the observed amounts (Tables 5.I and 5.II). Also, variations in relative amounts of certain fragments with arabinoxylans show differences between the model arabinoxylans and the experimental data.

For instance, in the simulation the relative amounts of the unsubstituted fragments **2** and **31** (xylobiose and xylotriose respectively) decrease with increasing degree of substitution, whereas the experimental data show these to be more or less constant. Also, the random model predicts the presence of a large amount of fragments with DP > 6 in the digest, while the amounts actually found were relatively low[10]. These results show that a random distribution of substituted xylose residues does not give an adequate description of the arabinoxylans from barley and malt.

5.4 DISCUSSION

The fragments analysed by HPAEC (**1-63**) contained very little 2-O- and 2,3-di-O-substituted xylose. This implies that the larger fragments in the digests were relatively more substituted than the original substrate. This was indeed found for the barley and malt BE fractions (**P2-Void**). The degree of substitution of the **P2-Void** fractions from barley and malt was comparable to the degree of substitution of the corresponding BE-70 fractions.

A structural model for the arabinoxylans

As shown above, a random distribution of substituents over the xylan backbone does not give a good description for arabinoxylans from

barley and malt WIS.

As discussed above, the fractions BE-70 and **P2-Void** were hardly or not degradable by endoxylanase 1. This enzyme is able to hydrolyse the xylan backbone at every position where two or more consecutive unsubstituted xyl*p*-residues are present (Experimental section and ref. 14). Therefore, sequences of two or more consecutive unsubstituted xyl*p* residues have to be absent or very rare in the BE-70 and **P2-Void** fractions. As the degree of substitution of these fractions was not very high (e.g. barley **P2-Void**: 55 % of the xylose is substituted, Table 5.II), the distribution of the substituents had to be quite regular in these fractions.

At the same time, some sequences consisting of at least 4 consecutive unsubstituted xylose residues had to be present in the parent polysaccharides to account for the release of xylose, xylobiose and xylotriose from these substrates by endoxylanase 1.

This lead to a structure containing two types of sequences (Fig. 5.5). The major type consisted of isolated unsubstituted residues separated by one or two substituted residues (a in Fig. 5.5). This gives an average length of 1 xylose residue for the unsubstituted sequences and of 1-2 for the substituted sequences.

Blocks of this type were separated from one another by short sequences consisting of 2 or more unsubstituted xylose residues (b in Fig. 5.5). The presence of blocks of this latter type is required to explain the formation of xylose, xylobiose and xylotriose.

The fact that the relative distributions of the fragments were very similar for all fractions analysed, indicates that the same model is valid for the fractions with a lower degree of substitution. By varying the relative amount of sequences of several unsubstituted xylose residues (type b) variations in ara:xyl ratio can be explained.

The structure proposed above for the barley arabinoxylans closely re-

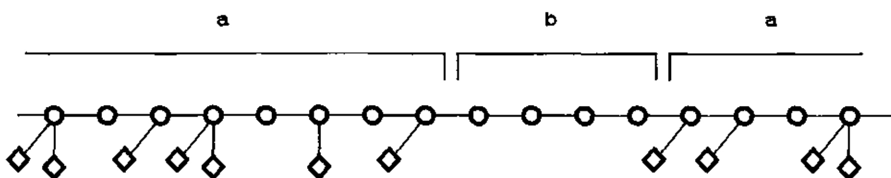

Figure 5.5. Schematic view of a model for the distribution of substituents over an arabinoxylan chain as proposed in the text. Symbols are explained in Fig. 5.2.

sembles the structure proposed by Goldschmid and Perlin[15] for wheat arabinoxylans. In their model, the substituted xylose residues are present isolated or in pairs, separated by a single unsubstituted xylose residue. Blocks with sequences of this type are separated by sequences of 2 or more unsubstituted xylose residues. In their model, these latter sequences were the only site for hydrolysis by endoxylanases.

We showed that another point of attack for the endoxylanase used here is a sequence formed by an unsubstituted Xyl*p* with a 3-O-substituted Xyl*p* attached at O-4. In this sequence, the unsubstituted residue will form the new reducing end after hydrolysis.

ACKNOWLEDGEMENTS

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Chapter 6

Nsp composition of wort and spent grain from brewing trials with a good malting quality barley and a feeding barley

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SUMMARY

Worts and spent grains were obtained from pilot scale brews using malts of two barley varieties differing in malting quality. Triumph was used as an example of a good malting quality barley, Golf as a typical feeding grade barley. From the prepared worts, polysaccharide fractions were isolated by ethanol precipitation and characterized. Results indicated the presence of high molecular weight arabinoxylans and ß-glucans. Arabinoxylan concentrations were similar in worts of both varieties, whereas ß-glucan concentrations were much higher in wort from Golf malt than in wort from Triumph malt. Methylation analysis showed little differences between wort arabinoxylans from both varieties. In comparison to water-insoluble arabinoxylans from barley and malt, the wort arabinoxylans were richer in xylopyranose residues substituted with arabinose residues at both O-2 and O-3.

Viscosities of the hopped worts of both varieties decreased after treatment with endoxylanase 1 from *Aspergillus awamori*. This confirms that arabinoxylans play a role in determining wort viscosity, possibly through interactions with ß-glucans. The endoxylanase was not able to release arabinoxylans from the spent grain.

6.1 INTRODUCTION

In wort a wide range of oligo- and polysaccharides is present. The main oligosaccharides are starch derived maltodextrins, maltotriose, maltose and glucose, but also non-starch polysaccharides (nsp) like arabinoxylans and (1→3),(1→4)-ß-D-glucans (ß-glucans) and their degradation products. Reported levels for polymeric ß-glucan in congress worts[1] and lager beers[2] range from 300 to 500 mg/l, whereas hard data for wort arabinoxylan content or composition were not available. It is known that high concentrations of nsp can adversely influence the brewing process or storage of the beer[3,4]. On the other hand, it has been suggested that the presence of some nsp can improve the sensoric properties of the beer[4].
Several enzymes are important during malting and mashing. These include amylases (convert starch to fermentable sugars), proteinases (provide the brewing yeast with an assimilable nitrogen source) and several ß-glucanases, which can degrade part of the residual ß-glucans from the malt to low molecular weight products during mashing[5]. All of the enzymes mentioned develop during malting. Most of them, however, are inactivated to a certain extent during the kilning process[5,6].
The amounts of amylases and proteinases present after kilning in well modified malts are usually sufficient to allow addition of a certain amount of adjuncts (unmalted barley, maize, rice). However, especially when barley is used as an adjunct, the amounts of ß-glucanases found in malt are not sufficient to degrade all ß-glucans from the adjunct, which can lead to high molecular weight ß-glucans remaining in the wort[5,7]. This in turn can cause high viscosities of wort and beer[8,9] and the formation of gel-like precitates during the final filtration of the beer[10]. The increased viscosity and the precipitates lead to lower filtration rates, especially in the beer filtration at low temperature. In such

cases, addition of a microbial ß-glucanase at mashing or in the fermentation stage can improve brewhouse performance of the malt/adjunct grist[7,11].

Much less is known about the role of arabinoxylans and of the endogenous arabinoxylan degrading enzymes in malting and mashing. The addition of endo-ß(1,4)-xylanase has been shown to improve brewhouse yield by lowering the wort viscosity[12]. Arabinoxylans are especially likely to be of importance when unmalted wheat is used in the brew, since wheat endosperm contains much more arabinoxylans than e.g. barley or rice endosperm[13].

The aim of this research was to determine the structural characteristics of wort arabinoxylans and to assess the importance of arabinoxylans in determining wort viscosity. In order to evaluate whether the importance of arabinoxylans in brewing differs between varieties, worts and spent grains obtained from malts of two barley varieties were studied.

Triumph was taken as an example of a good malting quality barley and Golf as a specimen of typically feed grade barley. Both malts were mashed and after lautertun filtration the monosaccharide composition of the nsp in the worts and the spent grains was determined. In addition, the polysaccharide fractions from the worts were isolated by precipitation with ethanol and characterized.

6.2 EXPERIMENTAL

Materials

Triumph and Golf were used from the 1988 harvest and were grown in the Netherlands. Golf was grown at two sites, viz. Overijssel and the Wieringermeer, whereas Triumph was only grown in the Wieringermeer. Samples of Golf grown at each site were analyzed to assess variations

in flour composition as a result of the difference in growth region.

Both barley varieties were malted according to a standard pilot scale malting procedure. The barley was steeped for 41 h at 16 °C. The steeped grain was germinated for 6 days at 14-16 °C. The green malt was kilned using the following program: 3 h at 40 °C, 5 h at 50 °C, in 10 h heating to 80 °C, 2 h at 82 °C and 2 h at 85 °C. For Golf only material grown in Overijssel was used for malting and brewing.

For analysis of the flours, barley kernels were dehusked using 50 % (v/v) sulphuric acid[14,15] and malt grist was dehusked by flotation in chloroform/ethanol[15].

Worts were produced in a 1 hl pilot-scale brewery at TNO from all malt grists of both barley varieties. Both mashes were produced using a standard one-step decoction method with a final mashing temperature of 75 °C. During lautering the filter bed was left intact. Total wort volume after lautering was 120 l for Triumph and 100 l for Golf. Half of the sweet wort was boiled after addition of a standard amount of hop pellets (95 g/hl wort) until the wort extract content had reached 16 °Plato. Samples of the spent grain were taken at three levels: from the top layer (the fines), and from the middle and the bottom parts of the filter cake. The top layer samples of the spent grain comprised 10 % (w/w) and 24 % (w/w) for Golf and Triumph respectively. Samples from the middle and bottom parts of the filter cake each accounted for half of the remaining material (respectively 45 and 38 % (w/w) per fraction for Golf and Triumph), The precipitate formed during boiling of the wort was also collected for analysis.

Isolation of polysaccharides from wort samples

Polysaccharides were isolated from sweet and hopped wort by addition of ethanol to a final concentration of 80 % (v/v). The mixture was

stored overnight at 4 °C. After centrifugation (15 min, 5000 *g*), the supernatant was discarded and the precipitate redissolved in water and precipitated a second time at 80 % (v/v) ethanol. A concentration of 80 % (v/v) ethanol was chosen as this concentration gave the highest yield of polysaccharide material (data not shown). The precipitate formed after the second precipitation step was redissolved in water and made up to a volume equal to that of the starting material. The solution obtained was stored frozen.

Determination of sample composition

For the determination of monosaccharide composition, polysaccharides in the samples were hydrolysed using 2 M trifluoroacetic acid (TFA) for water-soluble samples and using subsequently 12 M and 2 M sulphuric acid for water-insoluble samples[15].

Neutral sugar composition of the hydrolysates was determined by GLC after conversion of the monosaccharides to alditol acetates as described elsewhere[15]. Neutral sugar composition of flour samples from dehusked grain or malt was determined according to Englyst and Cummings[15,16].

Starch (starch derived glucose, including maltodextrins) and fructose were determined enzymatically as described before[15]. ß-Glucan was determined enzymatically using a test kit from Biocon, according to the method of McCleary et al.[17]. Polymeric ß-glucans were precipitated from wort samples by the addition of ammonium sulphate[17]. Precipitated ß-glucan was determined as described above. Protein content was determined by a micro-Kjeldahl method[15], using a conversion factor of 6.25.

For the determination of linkage composition, polysaccharide samples were methylated using dimsyl-anion[18]. The methylated polysaccharides were hydrolyzed with 2 M TFA and converted to alditol acetates[18]. The

obtained mixture of partially methylated alditol acetates was analyzed[18]. The polysaccharide material precipitated from wort with 80 % (v/v) ethanol contained a large excess amount of starch and dextrins. In order to remove most of this starch-like material, these samples were treated with amyloglucosidase (16 h at 40 °C) prior to the determination of their linkage composition. After the treatment, amyloglucosidase activity was destroyed by heating the samples for 10 min in a boiling water bath. To recover the undegraded polymeric material ethanol was added at 0 °C to a concentration of 85 % (v/v). The precipitates were collected and dried in a stream of air prior to methylation of the samples.

Total nsp in the spent grain fractions obtained from the brewing trials was determined from total monosaccharide content minus starch content. For the wort samples and the precipitates from wort, nsp was defined as the sum of arabinose, xylose, mannose and galactose as determined by GLC and glucose present as ß-glucan.

Determination of the influence of endoxylanase treatment on wort viscosity

To degrade the residual polymeric arabinoxylans in wort samples, 1 ml of a solution containing 20 µg of purified endoxylanase 1 from *Aspergillus awamori*[19] in 50 mM sodium acetate buffer (pH 5.0) was added to 50 ml of hopped wort (pH 5.5). To controls, 1 ml of 50 mM sodium acetate buffer (pH 5.0) was added instead of endoxylanase solution. The mixtures were incubated overnight at 40 °C. Enzyme action was stopped by heating the samples in a boiling water bath for 10 min. Relative viscosities of the wort samples were measured against water using an Ubbelohde viscosimeter at 30 °C.

6.3 RESULTS

Comparison of dehusked barley and malt flours from Golf and Triumph

The composition of the flours of dehusked barleys and malts is shown in Table 6.I. Small differences were found in starch and protein contents of the unmalted barley flours from both varieties. For the Golf samples, the nsp content was higher than for the Triumph samples. These results indicate that harvest year (Triumph) in this case has little influence on gross sample composition.

The Golf samples grown in Overijssel had a higher protein content than material grown in the Wieringermeer, possibly due to the difference in growth region.

Differences between varieties in the malt samples were negligible, with all samples showing ß-glucan contents well below 0.5 g/100 g flour.

Protein content of the malt flours appears to be quite low compared to the corresponding barley flours. This is most probably an artifact due to the method used to remove the husk. The loss of protein had no

Table 6.I. Composition of flours from dehusked barley and malt samples prepared from two barley varieties, grown at different locations and in different seasons.

Sample	Site	Year of harvest	Nsp[a]	Starch	Fructose	Protein
			% w/w on dry matter			
Barley						
Golf	Wieringermeer	1988	10 (5)	74	1	10
Golf	Overijssel	1988	10 (6)	68	1	13
Triumph	Wieringermeer	1988	8 (4)	76	1	11
Triumph	Wieringermeer	1984	8 (5)[b]	72[b]	2[b]	11[b]
Malt						
Golf	Overijssel	1988	2 (tr)	83	1	6
Triumph	Wieringermeer	1988	2 (tr)	88	1	6
Triumph	Wieringermeer	1984	3 (tr)[b]	82[b]	2[b]	5[b]

[a]: Values between parenthesis: ß-glucan content in % w/w on dry matter.
[b]: Values taken from Viëtor et al.[15]

influence on the composition of the nsp in the malt flour, although some nsp will have been lost[15]. Due to this loss of protein and nsp the apparent starch content of the malt flours was increased in comparison to the contents in the corresponding unmalted grain samples. Nsp in the flour samples showed some differences in monosaccharide composition (Table 6.II). The main difference was the lower ratio of arabinose to xylose (ara:xyl ratio) for the Golf samples in comparison to the Triumph samples (for both the barley and the malt flours).

Both samples from Golf barley had a comparable ara:xyl ratio; the total amounts of arabinose and xylose found in these flours were also similar (since both the total amount and the composition of the nsp in these fractions were equal, Tables 6.I and 6.II). This indicates that growth region had little influence on the amount or the composition of arabinoxylans in the barley flour.

Both barley and malt from Triumph 1984 showed a higher glucose

Table 6.II. Monosaccharide composition of nsp in flours from dehusked barley and malt, prepared from two barley varieties, grown at different locations and in different seasons. (Values are mol% of total nsp monosaccharides).

Sample	Site	Year of harvest	Ara	Xyl	Man	Gal	Glc[a]	Ara:Xyl Ratio
			mol% of anhydro-sugar in nsp					
Barley								
Golf	Wieringermeer	1988	18	29	3	1	50 (44)	0.62
Golf	Overijsel	1988	17	28	2	1	52 (52)	0.61
Triumph	Wieringermeer	1988	19	27	3	1	50 (48)	0.70
Triumph	Wieringermeer	1984	15[b]	21[b]	3[b]	1[b]	60 (54)[b]	0.71[b]
Malt								
Golf	Overijsel	1988	17	34	1	1	47 (14)	0.50
Triumph	Wieringermeer	1984	26	38	1	1	34 (17)	0.68
Triumph	Wieringermeer	1984	20[b]	25[b]	1[b]	1[b]	53 (3)[b]	0.80[b]

[a]: Values between parenthesis: ß-glucan content in mol% of total nsp monosaccharides.
[b]: Values taken from Vietor et al.[15]

content of the nsp compared to the other samples. Also, the ara:xyl ratio in malt flour of the 1984 harvest Triumph was higher than in 1988 malt flour, although this ratio in the corresponding barley flours was equal. This could indicate a variation due to the malting process.

Composition of wort and spent grain obtained from pilot-scale brewing trials

The amounts of apparent starch (including maltodextrins), ß-glucan, total nsp (for definition see Experimental) and protein were determined for the fractions obtained from brewing trials with malts from Triumph (1988 harvest, Wieringermeer) and Golf (1988 harvest, Overijssel). The results are summarized in Table 6.III. The wort fractions from Golf contained more nsp (including ß-glucan) and less protein than the wort fractions from Triumph. The difference in nsp content was in part due to the higher ß-glucan content of Golf wort, but other nsp had also increased. In the ethanol precipitates about 58 % of the nsp was recovered for the sweet wort samples of both Triumph and Golf (calculated from Table 6.III). In the precipitates from the hopped worts, recovery was 62 % for Triumph and 67 % for Golf. The differences in recoveries between the sweet and hopped wort fractions of Golf were mainly due to differences in the recoveries of ß-glucan.

The concentration of 'starch' (glucose derived from starch and starch fragments) was comparable in both the hopped worts. This was to be expected since the worts were concentrated by boiling to 16 °P.

The main difference in the composition of the spent grain was found in the 'starch' content, which was much higher for the Triumph spent grain than for the spent grain from Golf. For both Triumph and Golf, the top layer contained less nsp and more protein than the other layers. The difference was most pronounced for the spent grain from Triumph.

Table 6.III. Composition of brewing fractions from pilot-scale trials with Triumph and Golf malt[a].

Fraction	Component		
	Nsp[b]	'Starch'	Protein
Triumph			
sweet wort	2.4 (0.30)	104	9
hopped wort	2.9 (0.36)	125	11
hot break	------------- 69.8[c]	--------------	17
spent grains:			
top	24 (0.67)	26	24
middle	38 (0.74)	13	16
bottom	38 (0.89)	14	16
ethanol precipitates[d]:			
sweet wort	1.4 (0.20)	22	ND[e]
hopped wort	1.8 (0.27)	29	ND
Golf			
sweet wort	3.3 (1.07)	86	7
hopped wort	4.5 (1.46)	127	9
hot break	-------------- 64.0[c]	------------	24
spent grains:			
top	37 (0.61)	5	27
middle	44 (0.60)	6	24
bottom	47 (1.38)	7	20
ethanol precipitates[d]:			
sweet wort	1.9 (0.45)	22	ND
hopped wort	3.0 (1.13)	30	ND

[a]: Values are expressed in g/l for the sweet and hopped worts and the ethanol precipitates from these worts, and in % w/w on dry matter for the other fractions.
[b]: Values between parenthesis are ß-glucan contents in % (w/w) on dry matter.
[c]: Hot breaks: values given are for total neutral monosaccharides, including starch.
[d]: Ethanol precipitates obtained as described in Experimental section.
[e]: ND: not determined.

Monosaccharide compositions of the nsp were determined for the wort and spent grain fractions (Table 6.IV). Main sugars present in nsp of all fractions were arabinose, xylose and glucose. Small amounts of mannose and galactose were also found.

Arabinoxylans (sum of arabinose and xylose contents) were the predominant nsp in wort fractions of both varieties, with the arabinoxylans in

Golf showing a slightly higher arabinose to xylose ratio (ara:xyl ratio) (0.54 and 0.50 for Golf and Triumph wort respectively). In the material precipitated from the worts by 80 % ethanol, arabinose and xylose still were the main sugars. The ara:xyl ratio in the precipitates was considerably higher than in total wort. For Golf, the ara:xyl ratio in the wort precipitate was 0.72 vs. 0.54 in the total wort, for Triumph these values were 0.80 vs. 0.50 respectively (Table 6.IV). This implies that the ethanol soluble arabinoxylan fragments contained much less arabinose than the precipitated arabinoxylans.

Table 6.IV. Monosaccharide composition of nsp in brewing fractions from pilot-scale brewing trials with Triumph and Golf malt.

Fraction	Ara	Xyl	Man	Gal	Glc[a]	Ara:Xyl Ratio
	mol% of anhydro-sugar in total nsp					
Triumph						
sweet wort	25	51	9	5	11 (11)	0.49
hopped wort	25	50	10	4	11 (11)	0.50
hot break[b]	1	3	1	1	95 (0)	0.33
spent grains:						
top	25	35	3	5	34 (3)	0.71
middle	22	47	1	2	27 (2)	0.47
lower	22	46	1	3	29 (2)	0.48
ethanol precipitate:						
sweet wort	36	45	2	5	12 (12)	0.80
hopped wort	36	45	2	5	13 (13)	0.80
Golf						
sweet wort	22	41	5	4	29 (29)	0.54
hopped wort	23	43	6	0	29 (29)	0.53
hot break[b]	2	3	1	1	94 (0)	0.60
spent grain:						
top	21	44	2	3	31 (2)	0.48
middle	19	44	1	2	34 (1)	0.43
lower	19	45	1	2	34 (3)	0.42
ethanol precipitate:						
sweet wort	31	43	2	4	20 (20)	0.72
hopped wort	26	36	1	4	33 (33)	0.72

[a]: Values between parenthesis: ß-glucan as a fraction of total nsp monosaccharides (in mol%).
[b]: Values for hot break include starch and maltodextrins.

From the data in tables 6.III and 6.IV it can be seen that the precipitates from the hopped worts consist predominantly of arabinoxylans. From these data, calculations show that the hopped worts contain about 1.7 g/l and 1.5 g/l of polymeric arabinoxylan for Golf and Triumph respectively, assuming arabinose and xylose are present only as arabinoxylan. This means that the worts from both varieties contain more polymeric arabinoxylan than ß-glucan.
Glucose was the major remaining sugar residue in the ethanol precipitates. It was present mainly as a building block of ß-glucan. The relative amount of ß-glucan was considerably higher in the ethanol precipitates from Golf worts than in those from Triumph worts, which is in accordance with the higher ß-glucan level found in the Golf worts. Worts from both Golf and Triumph contained a rather large amount of mannose (6 and 5 mol% for Golf and Triumph respectively). In the precipitated material, the mannose levels were much lower (2 mol% for both varieties) indicating that most of the mannose was present in the form of oligosaccharides[20] or possibly ethanol soluble glycoproteins.
For both Triumph and Golf, the monosaccharide composition of the total worts did not change during boiling. For the Golf fractions, the ethanol precipitates obtained from hopped wort (i.e. after boiling of the wort) contained relatively more glucose (as ß-glucan), while the ara:xyl ratio remained constant. This is reflected in the larger amounts of ß-glucan precipitated from the hopped wort from Golf relative to the sweet wort (Table 6.III). For the Triumph fractions, the sugar composition of the 80 % (v/v) ethanol precipitates obtained from the wort samples did not change with boiling of the wort.
As in the worts, xylose and arabinose were also the main sugars present in the nsp of the spent grain. The ara:xyl ratio was somewhat lower than the ratio in the wort fractions, except for the Triumph top layer.

Of the glucose present in the spent grain nsp, only a small proportion was ß-glucan. The remainder was most likely derived from cellulose in the husks.

Linkage composition of wort polysaccharides

The linkage composition of polysaccharides precipitated from sweet wort with 80 % (v/v) ethanol was determined after treatment with amyloglucosidase to remove starch and dextrins (see Experimental). The results are summarized in Table 6.V. The major sugars in the samples were arabinose and xylose. Glucose was recovered mainly as (1,3)- and (1,4)-linked glucopyranose, consistent with the presence of

Table 6.V. Methylation analysis of ethanol precipitates from sweet worts prepared from Golf and Triumph malts after treatment of the samples with amyloglucosidase (results presented as mol% of total anhydro-sugars).

Sugar[a]	Linkages	Samples	
		Golf	Triumph
Ara	1	35	32
	1,2	--	1
Xyl	1	2	2
	1,4	28	30
	1,2,4/1,3,4	7	6
	1,2,3,4	11	11
Glc	1,3	5	5
	1,4	14	14
Total ara		35	33
Total xyl		48	49
Total glc		19	19
% of xyl residues substituted		38	34
Substituents per xyl residue		0.61	0.56

[a]: Ara = arabinose, xyl = xylose, glc = glucose.

ß-glucans. Terminal and (1,4,6)-linked glucopyranose could not be detected. This indicates that starchy saccharides were almost completely removed by the treatment with amyloglucosidase.
Arabinose was recovered as terminal furanose groups. Xylose was mainly found as (1,4)-linked xylopyranose (58 % and 61 % of total xylose for Golf and Triumph respectively); lesser amounts of (1,2,3,4)-linked xylopyranose (respectively 23 % and 22 %) and (1,2,4)- or (1,3,4)-linked xylopyranose (together respectively 15 % and 12 %) were also found. Terminal xylopyranose was found in very small amounts. Differences between samples from Golf and Triumph were negligible.

Influence of endoxylanase treatment on wort viscosity
The relative viscosity of untreated hopped wort was higher for Golf than for Triumph (1.60 mPa.s and 1.48 mPa.s respectively). For both varieties the relative viscosities of the wort could be lowered to 1.42 mPa.s after 16 h incubation with endoxylanase. The endoxylanase used was not able to release any arabinoxylans from the spent grain (data not shown).

6.4 DISCUSSION

The composition of nsp in wort and spent grain of two barley varieties showed small differences, except in ß-glucan content.
Earlier reports[21-23] on the composition of spent grain give widely differing values for protein (21-36 % w/w) and neutral sugar content (40-56 % w/w). Our results for the total neutral sugars in the various spent grain fractions all fall within the range of reported values, as does the protein content of the Golf fractions (Table 6.III). For the

Triumph spent grain, the middle and bottom layers have a protein content below the reported values, whereas the top layer falls within this range.

Ara:xyl ratios in the spent grain were comparable to or slightly lower than ratios published earlier (0.49-0.58)[21,22], except for the top layer of Triumph, which gave an ara:xyl ratio close to values published earlier for barley and malt flour[15]. The ara:xyl ratios in all spent grain samples except the top layer of Triumph were much lower than ratios published for barley and malt flour (0.71 and 0.80 respectively)[15]. This might be attributed to the fact that spent grain contains large amounts of husk. Arabinoxylans in barley husk are known to have a very low ara:xyl ratio (0.20)[24]. One fraction, the spent grain of the top layer of Triumph, was exceptional in this respect, having an ara:xyl ratio of 0.71.

For both Triumph and Golf, arabinoxylans prevailed in the wort, although in the Golf worts, the concentration of ß-glucan is much higher than in Triumph worts.

The ara:xyl ratio in total wort was considerably lower than the ratio of the arabinoxylans precipitated from the wort with 80 % (v/v) ethanol. This indicates that the arabinoxylan fragments of low molecular weight contain much less arabinose than the polymeric fraction in the precipitates. A similar observation was made for the digests of enzyme-degraded arabinoxylans extracted from barley and malt cell wall material[18]: the large fragments and undegraded fractions had a much higher ara:xyl ratio than the small fragments[25].

The results of methylation analysis of the arabinoxylans precipitated from wort with ethanol can be compared to the results of methylation analysis of arabinoxylans extracted from water-insoluble cell wall material (WIS) of barley and malt[18]. This comparison shows that the precipitated wort arabinoxylans contain much less single substituted

xylose (12 % and 15 % for Golf and Triumph respectively, Table 6.V) than the barley and malt WIS arabinoxylans (24 % and 25 % for barley and malt, respectively[18]). On the other hand the amounts of double substituted xylose present in arabinoxylans from wort precipitates (23 %) are higher than the amounts found in barley or malt WIS arabinoxylans (20 and 16 % respectively[18]).

We have shown before that substituents on the xylan backbone limit degradation of arabinoxylan by endoxylanase[25]. The action of endo-xylanase 1 of *A. awamori* was particularly hampered by xylose residues substituted at O-2 or at both O-2 and O-3[25,26]. The results presented here indicate that these substituents are also important for practical applications in limiting the degradation of arabinoxylans. Treatment of hopped worts with endo-ß(1,4)-xylanase lead to a lower wort viscosity. This confirms that arabinoxylans do contribute to wort viscosity and indicates that the use of endoxylanase might help in reducing viscosity problems in the brewhouse. It also confirms the results of Ducroo and Frelon[12], who used a commercial preparation containing endo-ß(1,4)-xylanase and ß-glucanase and measured a reduction in viscosity of an all-malt wort of 24 %. When using a preparation devoid of endoxylanase activity, the wort viscosity was reduced by 11 %. We obtained for the Golf wort a reduction of 11 % in wort viscosity by treatment of the wort with endoxylanase. For the Triumph wort which had a similar arabinoxylan content but a much lower ß-glucan content the reduction in viscosity due to enzyme treatment was only 5 %. This seems to indicate that interactions between arabinoxylan and ß-glucan can be an important factor in determining wort viscosity.

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Chapter 7
Concluding remarks

The aims of the research presented here were (i) elucidation of the structural features of water-insoluble arabinoxylans from barley and malt flour, (ii) analysis of the factors influencing the enzymic degradability of these arabinoxylans and (iii) determination of the changes in arabinoxylan structures occurring during malting and brewing.

7.1 METHODS USED

Research started with the isolation of water-insoluble cell wall material (WIS) from dehusked barley and malt (Chapter 2). First, the husk had to be removed from the barley and the malt. A good method to remove husk from raw barley, based on soaking the grain in 50 % (v/v) sulphuric acid, was already available[1,2]. However, this method could not be used to dehusk malted barley properly (Chapter 2). For this material we devised another method which made use of the difference in density between malt husk and malt endosperm when suspended in an ethanol-chloroform mixture. A flour with a low protein content (compared to published values for whole malt) was obtained (Chapter 2).
For the next step, arabinoxylans had to be extracted from barley and malt WIS. A novel method developed for the extraction of arabinoxylans from wheat WIS[3] was also applied successfully for barley WIS. In this method a saturated solution of $Ba(OH)_2$ containing $NaBH_4$ was used as extractant. The extracts obtained consisted for over 90 mol% (based on neutral sugars) of arabinose and xylose. The remainder was mainly glucose. For the isolation of arabinoxylans, extraction with

$Ba(OH)_2$ solution was far superior to extraction with NaOH solutions. Using this latter extractant with barley WIS resulted in extracts typically consisting for up to 75 % of (1→3),(1→4)-ß-D-glucans (ß-glucans)[4,5] next to arabinoxylans.

7.2 STRUCTURES OF ARABINOXYLANS FROM BARLEY, MALT AND WORT

The results presented in the previous chapters confirmed that barley arabinoxylans consist of (1→4)-linked ß-D-xylopyranose (Xyl*p*) chains, substituted with single unit α-L-arabinofuranose (Ara*f*) groups. Three types of substituted Xyl*p* residues were found. The presence of two of these types (3-O- and 2,3-di-O-Ara*f*-Xyl*p*) in barley arabinoxylans is generally acknowledged.

We were able to prove the presence of a third type of substituted xylose residue, 2-O-Ara*f*-Xyl*p*, by both methylation analysis of intact arabinoxylans and by ^{1}H-nmr studies of isolated arabinoxylan fragments (Chapters 3 and 4). This unit was an important structural element in the arabinoxylans and appears to be unique for barley and malt.

It was confirmed that the arabinoxylans in barley and malt consist of a population of xylan polymers varying in arabinose content. We were able to quantify the nature of this variation by detailed methylation studies of arabinoxylan fractions obtained by graded precipitation with ethanol, which varied in their arabinose to xylose ratios (ara:xyl ratios). There was a strong correlation between the ara:xyl ratio of a fraction and the relative amounts of 2-O- and 2,3-di-O-Ara*f*-Xyl*p* in that fraction (Chapter 3). The amount of 3-O-Ara*f*-Xyl*p* was found to be independent from the ara:xyl ratio of the fraction.

Also arabinoxylans isolated from wort samples were studied by methyla-

tion analysis. In comparison to arabinoxylans isolated from barley and malt WIS, the arabinoxylans from wort contained less substituted Xyl*p* residues. Of these substituted Xyl*p* residues, a larger proportion was double substituted than in the barley and malt arabinoxylans (Chapter 6).

7.3 ENZYMIC DEGRADATION AND SUBSTITUENT DISTRIBUTION OF ARABINOXYLANS

A number of arabinoxylans fragments from barley was isolated after digestion with endoxylanase 1 from *Aspergillus awamori*[6] (Chapter 4). From the structure of these fragments, it was confirmed that Ara*f* substituents block the enzymic hydrolysis of certain (1→4)-links in the xylan backbone. For the enzyme we used, Kormelink et al.[7] showed that the presence of 2,3-di-O-Ara*f*-Xyl*p* blocked the adjacent two ß-(1→4)-links towards the reducing end in the xylan backbone from hydrolysis, whereas a substituent at O-3 (in 3-O-Ara*f*-Xyl*p*) only blocked a single adjacent ß-(1→4)-link in the same direction. Neither of the substitutions blocked linkages at the non-reducing side of the substituted Xyl*p* residue. On the basis of our results (Chapters 4 and 5) we concluded that the presence of 2-O-Ara*f*-Xyl*p* also blocked the two next (1,4)-links towards the reducing end, as described for 2,3-di-O-Ara*f*-Xyl*p*. From these results and the results from methylation studies of intact arabinoxylans and undegradable material left after enzymic degradation, a qualitative model was derived for the distribution of Ara*f* residues over the xylan backbone (Chapter 5). To enable prediction of the properties of the arabinoxylan (e.g. interactions with other polymer chains) a quantitative model would be required. For such a model, the arabinoxylan can be seen as a linear chain, constructed from four basic units (Xyl*p* and 2-O-, 3-O- and 2,3-di-O-Ara*f*-Xyl*p*). However, the formulation

of such a model is complicated by this relatively large number of basic units required. Published models for galactomannans[8] and ß-glucans[9] needed only two basic units, which lead to only 4 or 8 parameters in the model. Using the same class of model for arabinoxylans would require 16 or 64 parameters.

7.4 MODIFICATION OF ARABINOXYLANS DURING MALTING AND BREWING

The information gathered in this research in combination with previous reports allows an assessment of the changes in arabinoxylans occurring during malting and brewing.

From the mass balances, it became clear that both ß-glucan and arabinoxylan were degraded during malting (Chapter 2). The ß-glucan content of the malt flour was very low (0.1 % (w/w) versus 4.2 % (w/w) in the barley flour) confirming the almost complete degradation of ß-glucans during malting (Chapter 2). Arabinoxylan content in the flour was still quite high after malting (3.0 and 1.4 % w/w in barley and malt flour, respectively), but a considerable proportion of the arabinoxylan material was recovered in the ethanol-soluble fraction of the malt flour indicating substantial degradation of the arabinoxylans.

Very little water-soluble (ethanol-insoluble) polymeric arabinoxylan is recovered after malting (Chapter 2). No differences in composition were found between the water-insoluble arabinoxylans from barley and malt. However, some differences were observed in the distribution of arabinoxylans over the various fractions isolated by ethanol precipitation (Chapter 3).

During mashing, a proportion of the malt arabinoxylans appears as polymeric arabinoxylan in the wort. This fraction of arabinoxylan is rela-

tively rich in 2,3-di-O-substituted Xyl*p* and poor in single substituted Xyl*p* compared to arabinoxylans from barley and malt WIS (Chapter 6).

7.5 THE ROLE OF ARABINOXYLANS IN BREWING

We showed that arabinoxylans can increase wort (and beer) viscosity (Chapter 6). In a wort from good malting barley, the concentration of polymeric arabinoxylan is as high as that of ß-glucan. Degradation of the arabinoxylans reduced the wort viscosity significantly.
In our studies on viscosity reduction by treatment of two wort samples with endoxylanase 1, similar viscosities were found after this treatment. This final viscosity was independent of the ß-glucan concentration.

7.6 COMPARISON OF ARABINOXYLANS FROM BARLEY AND OTHER CEREALS.

The basic chemical structure of all cereal arabinoxylans is very similar: a backbone of (1→4)-linked Xyl*p* residues, in which part of the xylose residues are substituted with terminal Ara*f* groups. The arabinoxylans isolated from various cereals differ in details of the substitution of the xylan backbone[10] (Table 7.I).
These differences are found in the ratio of arabinose to xylose, in the relative amounts of the various linkages between arabinose and xylose and in the presence of other substituents such as (4-O-methyl-)glucuronic acid and xylopyranosyl-arabinose.
From the data in Table 7.I it appears that the arabinoxylans from different cereals can be divided into two groups, based on their composition: (i) arabinoxylans from barley, wheat and rye and (ii) those from rice and sorghum. Arabinoxylans in group (ii) differ from those in group

(i) in the presence of glucuronic acid substituents on the xylan chain and in the much higher ratio of arabinose to xylose.

Arabinoxylans from the endosperm of barley and malt differ from those from wheat and rye endosperm mainly in that they contain significant amounts of xylose substituted at O-2 with a single Ara*f* group (Chapter 3). This substitution pattern is absent or very rare in arabinoxylans from wheat[12] and rye[14,15]. Differences between barley and malt arabinoxylans and those from sorghum and rice are even bigger: arabinoxylans from the latter cereals contain glucuronic acid residues (partly 4-O-methylated), and are substituted to a higher degree.

It is interesting to note that barley, wheat and rye grow in colder climates than sorghum and rice. It is unknown whether the observed differences in arabinoxylan composition are related to this difference in growing conditions. The exact influence of the various substituents on the properties of the arabinoxylan also remains unknown. These basic questions could therefore be interesting for future study.

Table 7.I. Composition of arabinoxylans from cell wall fractions of various cereal grains, excluding husk.

cereal	AX content[a]	ara:xyl ratio	positions of substituents[b]	other sugars	reference
barley		0.67	2, 3, 2&3	---	11
	27	0.68	2, 3, 2&3	---	Ch. 2, 3
wheat		0.56	(2), 3, 2&3	---	12
	83	0.56		GlcA (?)	12
rye		0.48	3, 2&3	---	13
		0.61	3, (2&3)	---	14
rice	41	0.78	(2), 3, 2&3	Gal GlcA 4Me-GlcA	15
sorghum	49	0.87	---	GlcA	16

[a]: AX = arabinoxylan; arabinoxylan content is given as % (w/w) of arabinose and xylose on total nsp in the endosperm cell walls.

[b]: Substituted positions of Xyl*p* residues of the backbone (values between parenthesis: present in small quantities or absent, 2&3 : substituents on O-2 and O-3 of one Xyl*p* residue).

7.7 PROCESSING OF ARABINOXYLAN-CONTAINING BIOMASS

The results presented in Chapters 4 and 5 showed that the use of an endoxylanase alone leaves large amounts of undegraded high molecular weight fragments, especially when the substrate arabinoxylans have a high degree of substitution. Tests have shown that the endoxylanase 1 from *Aspergillus awamori* (used in our experiments) was the least sensitive to the presence of substituents on the xylan backbone. Other endoxylanases studied resulted in larger amounts of high molecular weight fragments[7]. Even for the endoxylanase 1, total degradation of arabinoxylans to small oligomers would require the addition of an arabinofuranosidase to remove Ara*f* substituents from the xylan backbone. Such a complete degradation of arabinoxylans to small carbohydrates is especially important when the degraded arabinoxylan material is to be used as raw material for fermentations. This area requires much more research e.g. to isolate arabinofuranosidases which are able to remove Ara*f* residues from O-2 of Xyl*p* as found in barley arabinoxylans. To our knowledge, such an arabinofuranosidase has not yet been described.

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Summary

This thesis describes research on the water-insoluble arabinoxylans from barley and malt. In addition, some information is presented about arabinoxylans from wort and spent grain. The information obtained was used to determine the structural features of these arabinoxylans and the changes in arabinoxylan structure during both the malting and the brewing process.

In the first chapter, the structure and composition of the barley kernel and its constituents are described with emphasis on the non-starch polysaccharides (nsp). The malting and brewing process are outlined and the role of and changes in nsp during these processes are discussed, with reference to the relevant literature.

In chapter 2, the fractionation of flours from dehusked barley and malt as well as the characterisation of the obtained fractions are described. Mass balances are given for the fractionation of barley and malt flour, together with the composition of the various fractions. For both barley and malt, more than 95 % (w/w) of the flour dry matter was recovered after fractionation. Main components in both flours were starch, protein and nsp. Arabinose, xylose and glucose were the major monosaccharides present in nsp, with mannose and galactose only present in small amounts. In barley, glucose in the nsp was mainly (1→3),(1→4)-ß-D-glucan (ß-glucan). After fractionation, ß-glucan was recovered in the water-soluble fractions and in the water-insoluble cell wall fraction (WIS). Arabinose and xylose were mainly recovered in the WIS and were assumed to be present as arabinoxylan. In addition, arabinose was found in a fraction soluble in 70 % (v/v) ethanol together with most of the galactose, presumably as arabinogalactan.

After malting, ß-glucan had almost disappeared, due to degradation of the ß-glucan present in the barley flour. Also part of the arabinoxylan had been degraded, as was shown by the appearance of large amounts of xylose and arabinose in the 70 % (v/v) ethanol soluble fraction. The amount of galactose in this fraction was diminished, in comparison to the corresponding fraction from barley flour.

The WIS obtained from barley and malt was subsequently extracted with saturated $Ba(OH)_2$ solution, 1 M NaOH and 4 M NaOH, all containing 0.13 M $NaBH_4$. The material extracted with saturated $Ba(OH)_2$ solution was fractionated by graded ethanol precipitation. Mass balances of these extractions and analysis of the fractions obtained, are described in Chapter 3.

The polysaccharides extracted from WIS with $Ba(OH)_2$ solution (BE-extracts) consisted for over 90 % of arabinoxylans, the remainder was predominantly glucan (starch or ß-glucan). The extracted arabinoxylans comprised more than 60 % of the arabinoxylan present in the original WIS. Further fractionation of the BE-extracts by graded ethanol precipitation yielded a range of polysaccharides, in which the ratio of arabinose to xylose (ara:xyl ratio) increased with the ethanol concentration. Methylation analysis of a number of the arabinoxylan-rich fractions obtained from barley and malt WIS showed that the main sugar residues found in all these fractions were terminal arabinofuranose (Ara*f*) and (1→4)-linked xylopyranose (Xyl*p*). Part of the xylose was substituted at O-2, O-3 or both O-2 and O-3 with Ara*f*. The presence of 2-O-Ara*f*-Xyl*p* was a novel feature for endosperm arabinoxylans from cereals and appears to be unique for barley and malt flours containing endosperm and aleurone. The total amount of substituted xylose present in the arabinoxylan fractions studied ranged from 28-62 % and from 22-56 % on total xylose for barley and malt arabinoxylans respectively. A strong

correlation was found between the ara:xyl ratio of an arabinoxylan fraction and the relative amounts of O-2- and O-2,3-substituted xylose found in that fraction. The level of O-3-substituted Xyl*p* was constant at 15±1 % of total xylose for all fractions studied. Differences in composition between barley and malt arabinoxylans were small. The main difference was that the malt BE-fraction appeared somewhat richer in low substituted arabinoxylans.

In chapter 4 the structures of 12 arabinoxylan fragments consisting of 1 to 6 pentose residues, are described. The fragments were isolated after degradation of barley WIS arabinoxylan (BE fraction) with endoxylanase 1 from *Aspergillus awamori*. After incubation with the enzyme, the mixture of arabinoxylan fragments obtained, was fractionated by a combination of size exclusion chromatography and high performance anion-exchange chromatography. This combination allowed the isolation of 12 pure components, which could be studied by ^{1}H-nmr spectroscopy. Among the fragments isolated, three contained only xylose and were determined to be xylose, xylobiose and xylotriose. The other fragments all contained arabinose and xylose. Arabinose was present only as terminal α-Ara*f*, xylose was found as (1→4)-linked ß-Xyl*p* units, some of which were substituted with α-Ara*f* groups at O-2 or O-3 (or both). Two of the structures contained a 2-O-α-Ara*f*-Xyl*p* unit, confirming the presence of this structural element in barley arabinoxylan, as described in Chapter 3. Structures of this type have not been reported before. Data on the specificity of the endoxylanase 1 were available and could be updated with the structures of the isolated arabinoxylan fragments.

In Chapter 5 the distribution of various fragments in digests of a range of arabinoxylans was studied. These data, together with the structures of isolated fragments and the derived specificity of the endoxylanase 1,

are used to derive a model for the distribution of arabinose substituents over the xylan backbone in barley and malt arabinoxylans. Here again, differences between barley and malt arabinoxylans appeared to be very small. For both materials, the results of degradation experiments indicated that the distribution of substituents over the backbone could not be described by a random model, but had to be more regular. For the larger part of the arabinoxylan, unsubstituted Xyl*p* residues were separated by one or two substituted xylose residues. This pattern was interrupted at a number of places by sequences of several adjacent unsubstituted Xyl*p* residues. These unsubstituted sequences could reach lengths of at least 4 or 5 residues. A model as proposed here indicates that the distribution of Ara*f* residues over the xylan backbone has an important influence on the enzymic degradability of the arabinoxylan.
To gain insight in the role of arabinoxylans in practical applications, viz. brewing, the composition of worts and spent grains produced from malt of two barley varieties was studied. Arabinoxylans appeared to be present in hopped worts of both varieties in concentrations up to 3 g/l. However, only part of these arabinoxylans is present as polysaccharide, the remainder is soluble in 80 % (v/v) ethanol, and therefore present in oligomeric form. The polymeric arabinoxylans were very rich in O-2,3-disubstituted Xyl*p* compared to arabinoxylans from barley and malt WIS. Incubation of wort samples with endoxylanase 1 lead to a reduction in viscosity of worts from both varieties. This indicates that arabinoxylans really do contribute to wort viscosity.
In the final chapter, the arabinoxylans from dehusked barley and malt are compared with arabinoxylans isolated from husk-free kernel material of other cereals. Two groups of cereals could be distinguished. These groups differed in the substituents present in the arabinoxylans and in their arabinose to xylose ratio.

Samenvatting

Dit proefschrift beschrijft onderzoek aan de water-onoplosbare arabinoxylanen uit gerst en mout. Verder worden enige gegevens gepresenteerd over arabinoxylanen uit wort en bostel. De verkregen informatie werd gebruikt voor bepaling van de structuur van de arabinoxylanen en van veranderingen in samenstelling van deze arabinoxylanen tijdens het mout- en brouwproces.

In het eerste hoofdstuk worden de structuur en samenstelling van de gerstekorrel en de voornaamste componenten van het graan beschreven, met de nadruk op de niet-zetmeel polysachariden (nsp). Verder wordt globaal ingegaan op de processen van mouten en brouwen met de rol van nsp hierin. Hierbij wordt een overzicht gegeven van de relevante literatuur.

In hoofdstuk 2 wordt de fractionering van melen van ontkafte gerst en mout beschreven. Massabalansen van de fractioneringen en analyses van de verkregen fracties worden gegeven. Voor zowel gerst als mout werd meer dan 95 % van het meel teruggevonden na fractionering. Hoofdcomponenten van beide melen waren zetmeel, eiwit en nsp. Arabinose, xylose en glucose waren de voornaamste suikerbouwstenen van het nsp. Daarnaast waren kleine hoeveelheden mannose en galactose aanwezig. In de nsp uit gerstemeel was glucose voornamelijk aanwezig als $(1\rightarrow3),(1\rightarrow4)$-ß-D-glucaan (ß-glucaan). Het ß-glucaan werd na fractionering van het meel voornamelijk teruggevonden in de water-oplosbare fracties en in het water-onoplosbare celwandmateriaal (WIS). Arabinose en xylose werden vooral teruggevonden in de WIS-fractie. Er werd aangenomen dat deze twee monosachariden aanwezig waren als bouwsteen van arabinoxylaan. Ook werd een deel van het arabinose teruggevonden

in de fractie oplosbaar in 70 % (v/v) ethanol, samen met het grootste deel van het galactose; waarschijnlijk waren arabinose en galactose in deze fractie aanwezig in arabinogalactaan-eiwit complexen.

Na mouten was het ß-glucaan vrijwel verdwenen als gevolg van afbraak tijdens de vermouting. Ook een deel van het arabinoxylaan was afgebroken, gezien de aanwezigheid van grote hoeveelheden arabinose en xylose in de fractie oplosbaar in 70 % (v/v) ethanol. De hoeveelheid galactose in deze fractie was verminderd vergeleken met de vergelijkbare fractie uit gerstemeel.

De WIS verkregen uit gerstemeel en moutmeel werd geëxtraheerd met achtereenvolgens een verzadigde oplossing van $Ba(OH)_2$, 1 M NaOH en 4 M NaOH. Al deze extractiemiddelen bevatten 0,13 M $NaBH_4$. Het materiaal geëxtraheerd met $Ba(OH)_2$ werd verder gefractioneerd door middel van stapsgewijze precipitatie met ethanol. Massabalansen van de extracties en fractioneringen worden beschreven in hoofdstuk 3.

De polysachariden geëxtraheerd met $Ba(OH)_2$ uit WIS (BE-fractie) bestonden voor meer dan 90 % uit arabinoxylanen, de rest was hoofdzakelijk glucaan (zetmeel of ß-glucaan). Meer dan 60 % van de in de WIS aanwezige arabinoxylanen werden geëxtraheerd met $Ba(OH)_2$.

Verdere fractionering van de BE-fracties met ethanol leverde een reeks polysacharidefracties op. In deze fracties nam de verhouding van arabinose tot xylose (ara:xyl-verhouding) toe met de ethanolconcentratie waarbij de fractie precipiteerde.

Uit methyleringsanalyse van een aantal van de verkregen fracties werd afgeleid dat eindstanding arabinofuranose (Ara*f*) en (1→4)-gekoppeld xylopyranose (Xyl*p*) de voornaamste suikerresiduen in al deze fracties waren. Een deel van het Xyl*p* was gesubstitueerd met Ara*f* op O-2, op O-3 of op zowel O-2 als O-3. De aanwezigheid van 2-O-Ara*f*-Xyl*p* is een nieuw kenmerk van arabinoxylanen uit granen en voor zover nu

bekend uniek voor arabinoxylanen uit gerst- en moutmeel. De totale hoeveelheid gesubstitueerd xylose in de bestudeerde arabinoxylaan fracties varieerde tussen 28-62 % en 22-56 % van het totale xylose voor arabinoxylanen uit respectievelijk gerst- en mout-WIS. Er werd een sterke correlatie waargenomen tussen de ara:xyl-verhouding van een fractie en de relatieve hoeveelheden O-2- en O-2,3-gesubstitueerd Xyl*p* in die fractie. Het gehalte aan O-3-gesubstitueerd xylose was 15±1 % in alle onderzochte fracties.

Verschillen tussen arabinoxylanen uit gerst- en mout-WIS waren klein. Het voornaamste verschil was dat de moutfracties wat rijker waren aan laag gesubstitueerde arabinoxylanen in vergelijking met de gerstfracties.

In hoofdstuk 4 wordt de structuur beschreven van 12 arabinoxylaanfragmenten, bestaande uit 1 tot 6 pentose residuen. De fragmenten werden geïsoleerd na degradatie van arabinoxylaan uit gerst-WIS (BE-fractie, Hoofdstuk 3) met endoxylanase 1 van *Aspergillus awamori*. Het verkregen mengsel van arabinoxylaanfragmenten werd gefractioneerd door middel van een combinatie van gelpermeatie-chromatografie en hogedruk-anion-uitwisselingschromatografie. Deze combinatie maakte de isolatie van twaalf zuivere componenten mogelijk. Deze componenten werden vervolgens onderzocht met ^{1}H-nmr spectroscopie.

Drie van de verkregen fragmenten bevatten alleen xylose en bleken overeen te komen met xylose, xylobiose en xylotriose. De andere componenten bevatten alle arabinose naast xylose. Het arabinose was alleen aanwezig als terminaal α-Ara*f*, xylose was aanwezig als (1→4)-gekoppeld ß-Xyl*p*, voor een deel gesubstitueerd met α-Ara*f* op O-2, op O-3 of op zowel O-2 als O-3.

Twee van de geïsoleerde fragmenten bevatten een 2-O-α-Ara*f*-Xyl*p* eenheid, hetgeen de aanwezigheid van dit structuurelement in arabinoxylanen uit gerst- en mout-WIS, als beschreven in hoofdstuk 3,

bevestigd. Structuren met deze eenheid zijn niet eerder gerapporteerd.
Gegevens over de verdeling en de structuur van de gevonden fragmenten in afbraakmengsels van diverse arabinoxylaan fracties en over de specificiteit en het werkingsmechanisme van het endoxylanase 1 zijn in hoofdstuk 5 gebruikt om een model voor de verdeling van arabinose over de xylaanketen in het originele arabinoxylaan af te leiden. Ook hier bleken de verschillen tussen arabinoxylanen uit gerst en uit mout klein te zijn.
Voor beide polysachariden gaven de resultaten van afbraakexperimenten aan dat de verdeling van substituenten over de xylaanketen niet beschreven kon worden met een random-model, maar regelmatiger moest zijn. Uit de resultaten werd afgeleid dat het grootste deel van de xylaanketen moest bestaan uit geïsoleerde ongesubstitueerde Xyl*p*-residuen, van elkaar gescheiden door één of twee gesubstitueerde Xyl*p*-residuen. Om het vrijmaken van xylose, xylobiose en xylotriose door endoxylanase 1 te kunnen verklaren, moet dit patroon op een aantal plaatsen onderbroken worden door een reeks opeenvolgende ongesubstitueerde Xyl*p*-residuen. De lengte van dergelijke reeksen kan varieren van 2 tot tenminste 4 à 5 residuen (gezien de vorming van xylotriose), langere reeksen zijn niet uit te sluiten. Een vergelijking van modellen zoals hier is uitgevoerd, bevestigt dat de verdeling van arabinose substituenten een belangrijke invloed heeft op de enzymatische afbreekbaarheid van het arabinoxylaan.
Om meer inzicht te krijgen in de rol van arabinoxylanen bij het brouwen van bier, werd de samenstelling van wort- en bostelmonsters geproduceerd uit mout van twee gerstrassen bestudeerd.
In wortmonsters van beide rassen bleken arabinoxylanen aanwezig in concentraties tot 3 g/l. Een deel van deze arabinoxylanen is aanwezig als polymeer materiaal, de rest is aanwezig als oligomeren. De polymere

arabinoxylanen waren opvallend rijk aan O-2,3-digesubstitueerd Xyl*p* in vergelijking met oorspronkelijke arabinoxylanen uit gerst- of moutWIS. Incubatie van wortmonsters met endoxylanase 1 leidde tot een reductie van de viscositeit van de monsters. Dit geeft aan dat arabinoxylanen bijdragen aan de viscositeit van de wort.
In het laatste hoofdstuk worden de arabinoxylanen uit gerst en mout vergeleken met arabinoxylanen uit het endosperm van andere granen. Hierbij konden de granen worden verdeeld in twee groepen. Deze groepen verschilden in de aanwezige monosachariden en in het gehalte aan arabinose.

Curriculum Vitae

Remco Jurgen Viëtor werd op 23 augustus 1962 te Apeldoorn geboren. Het VWO-diploma werd behaald in 1980. In hetzelfde jaar werd begonnen met de studie Scheikunde aan de Rijksuniversiteit Utrecht. Het kandidaatsexamen werd afgelegd in juli 1983. Het doctoraal examen met als hoofdvak Bio-Organische Chemie (begeleiders: dr. J. Verhagen en prof. dr. J.F.G. Vliegenthart) en bijvak Biochemie van de Metabole Stoornissen (begeleiders: dr. L. Dorland en prof. dr. S.K. Wadman) werd behaald in 1986. Daarnaast werd de bevoegdheid Stralingsbescherming niveau 3 behaald in december 1985 bij het Interuniversitair Reactor Instituut te Delft. In de periode 1986-1990 was de auteur als wetenschappelijk assistent verbonden aan de sectie Levensmiddelenchemie en -microbiologie van de Landbouwuniversiteit Wageningen, alwaar dit proefschrift werd bewerkt onder leiding van prof. dr. ir. A.G.J. Voragen in samenwerking met dr. ir. S.A.G.F. Angelino (TNO, Zeist).