Structural Characteristics of Water-Extractable Nonstarch Polysaccharides from Barley Malt[1]

M. Cyran,[2] M. S. Izydorczyk,[3,4] and A. W. MacGregor[3]

Cereal Chem. 79(3):359–366

ABSTRACT

Water-extractable (WE) material was isolated from a Canadian barley malt (cv. Harrington). The purified WE material contained mainly arabinoxylans, β-glucans, proteins, and small amounts of arabinogalactans and mannose-containing polymers. WE material was treated with specific enzymes to obtain two fractions: one enriched in arabinoxylan (AX) and another enriched in β-glucan (BG). The AX fraction was further fractionated by stepwise precipitation in $(NH_4)_2SO_4$ into five arabinoxylan subfractions. ^{1}H-NMR spectroscopy and sugar analyses revealed a relatively high content of unsubstituted xylose residues (48–58%) as well as a relatively high content of doubly substituted xylose residues (28–33%) in the structure of the arabinoxylans. β-Glucans constituted a minor portion of water-extractable malt polysaccharides and were characterized by high levels of tri- and tetrasaccharide residues (93.4%) with a molar ratio of 2.19 for cellotriosyl to cellotetraosyl units. Size-exclusion chromatography revealed that the WE material contained several polymer populations. One population had a very high molecular weight that appeared to be the result of aggregation. The AX fraction contained higher molecular weight polymers than the BG fraction.

β-Glucans and arabinoxylans are the most important nonstarch polysaccharides of cereal grains. Sound barley grain contains 3–8% β-glucan (Oscarsson et al 1996; Izydorczyk et al 2000) and 4–10% arabinoxylan (Henry 1986). The level of these polymers is affected by genotype and environment (Henry 1986; Pérez-Vendrell et al 1996). β-Glucans are predominant (75% of total polysaccharides) in endosperm cell walls (Fincher 1975), while arabinoxylans constitute the majority (70%) of aleurone cell walls (Bacic and Stone 1981). General structures of β-glucan and arabinoxylan are well known. However, both polymers are highly heterogeneous in chemical structure and molecular weight. Barley arabinoxylan consists of a backbone of β-(1→4)-D-xylopyranosyl residues, partly substituted with single α-L-arabinofuranosyl residues at O-2, and O-3, or at both O-2 and O-3 positions of the xylose residues (McNeil et al 1975; Viëtor et al 1992, 1994). The presence of arabinosyl substituents and their distribution over the xylan backbone affect such arabinoxylan properties as solubility and interaction with other polymeric cell wall components (McNeil et al 1975; Andrewartha et al 1979) as well as restrict the enzymic degradation by endoxylanase (Viëtor et al 1994). Some arabinose residues are covalently linked through ester linkages to ferulic acid (4-hydroxy-3-methoxycinnamic acid) (Smith and Hartley 1983). Feruloylated arabinoxylans may form gels through interchain cross-linking and dimerization of their esterified ferulic acid (Geissmann and Neukom 1973; Hoseney and Faubion 1981; Izydorczyk et al 1990; Figueroa-Espinoza and Rouau 1998).

Unlike arabinoxylan, β-glucan is an unbranched polysaccharide composed mainly of cellotriosyl and cellotetraosyl units linked by single β-(1→3)-linkages and thus contains ≈70% β-(1→4) and 30% β-(1→3) linkages (Woodward et al 1983). Cellotriosyl and cellotetraosyl units constitute ≈90% of the polysaccharide, with the remaining structure containing longer consecutive β-(1→4)-linked D-glucopyranosyl units (Woodward et al 1988; Wood et al 1996; Izydorczyk et al 1998b). The presence of β(1→3) linkages gives an irregular shape to the molecule, preventing intermolecular association and making β-glucan partially water-soluble (Buliga et al 1986). On the other hand, long blocks of up to 14 adjacent β-(1→4) linkages have the potential to aggregate through hydrogen bonds and precipitate from solution during enzymatic hydrolysis of β-glucan (Woodward et al 1983).

Intact cell walls constitute an effective barrier to the movement of hydrolytic enzymes in the endosperm of barley during malting, causing low extract yields (Brown and Morris 1890; Bamforth 1982). Due to their specific properties, arabinoxylans and β-glucans can provoke filtration problems during brewing and may participate in the formation of hazes during beer storage (Coote and Kirsop 1976; Wainwright 1990). These problems have been mainly associated with β-glucans. However, despite having different chemical structures, both β-glucans and arabinoxylans possess the ability to form viscous solutions or gels (Medcalf et al 1968; Bamforth 1982). Therefore, extensive degradation of β-glucan is a prerequisite for successful beer production, but the role played by arabinoxylans during beer production and storage is not clear. Arabinoxylan is more resistant to degradation during malting (Viëtor et al 1991); hence, its content in commercial beers (514–4,211 mg/L) is several times higher than that of β-glucan (0.3–248 mg/L) (Schwarz and Han 1995). β-Glucans, even at quite low polymer concentration, may form gelatinous precipitates. Thermal and mechanical stresses as well as changes in constitution of wort and beer during production and storage may be responsible for formation of β-glucan gels (Linemann and Krüger 1997). It is speculated that maltose prevents association of β-glucan chains and, therefore, β-glucan gels cannot usually be detected in wort. On the other hand, the increased alcohol content in wort after fermentation may enhance the gelation process (Bamforth 1994). Very little information is available on the possible contribution of arabinoxylans to filtration problems or haze formation in beer. Therefore, it seems evident that knowledge about the structure and functionality of both polysaccharides and their hydrolytic products arising during brewing is of fundamental importance to gaining a better understanding of the formation of hazes, gels, and precipitates during brewing and beer storage.

The present work is part of an investigation undertaken to study the details of the structure of arabinoxylans and β-glucans in barley and malt. The objectives of this work were to provide new insights into the fate of these polysaccharides during malting and mashing as well as to highlight structural features that have the potential to cause processing problems. Such information could be used to alleviate processing problems either by changing the appropriate characteristics of these polysaccharides in barley through breeding programs or by changing processing conditions. The results of an investigation into the structural features of water-extractable arabinoxylans and β-glucans from malt are presented here. A stepwise ammonium sulfate precipitation technique was used to obtain more structurally homogenous fractions for more detailed molecular characterization of polysaccharide populations.

[1] Contribution No. 824 of the Grain Research Laboratory, Canadian Grain Commission, 1404-303 Main Street, Winnipeg, Manitoba R3C 3G8, Canada.

[2] Department of Nutritional Evaluation of Plant Materials, Institute of Plant Breeding, Radzikow, 05-870 Blonie, Poland.

[3] Grain Research Laboratory, 1404-303 Main Street, Winnipeg, Manitoba, R3C 3G8 Canada.

[4] Corresponding author. E-mail: mizydorczyk@cgc.ca Phone: 204-983-1300. Fax: 204-983-0724.

Publication no. C-2002-0401-07R.

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MATERIALS AND METHODS

Preparation of Barley Malt

A commercial sample of barley malt (Harrington) was ground in a Wiley mill (0.5 mm screen). The grist was autoclaved (5 min, 100°C) and boiled in 80% ethanol for 1.5 hr to inactivate the endogenous enzymes, washed with fresh ethanol, and oven-dried (40°C).

Malt Analysis

Total proteins, fine and coarse grind extracts, viscosity, and the Ps/Pt (soluble to total protein) ratio were analyzed as described by Edney and Tipples (1997).

Extraction and Purification of Water-Extractable (WE) Nonstarch Polysaccharides

Enzyme-inactivated malt grist (500 g) was mixed with water (1 L) containing thermostable α-amylase (*B. licheniformis*, 4,500 units/L, Megazyme). The suspension was extracted at 65°C for 90 min with stirring and centrifuged (10,000 × *g*, 30 min). The extraction was repeated once. Combined extracts were stirred with Vega clay (Pembina Mountain Clay, Winnipeg, MB) for 20 min (10 g/L) to remove proteins by adsorption on clay, then centrifuged (10,000 × *g*, 30 min). The extract was incubated with α-amylase (porcine pancreas, Sigma) for 3 hr (pH 6.5, 10 m*M* $CaCl_2$, 0.03% NaN_3 at 35°C) and with amyloglucosidase (Boehringer Mannheim) for 24 hr (pH 5.0, 35°C), then dialyzed against distilled water (1,000 molecular weight cutoff [MWCO], 4°C) for two days. The incubation with starch-degrading enzymes was repeated once for 6 hr, then the extract was incubated with proteinase K (Boehringer Mannheim, 0.5 mL/L) for 24 hr at 35°C and dialyzed until the dialyzate was free of sugars. The enzymes were inactivated by heat (95°C, 20 min) and removed by centrifugation (10,000 × *g*, 30 min). The supernatant was treated by adsorption filtration (Sartobind Membrane Adsorbers, Sartorius AG, Germany) in an attempt to remove residual proteins. Finally, the extract was freeze-dried and designated water-extractable (WE).

Isolation of β-Glucan (BG)

WE (4.0 g) was dissolved in acetate buffer (0.05*M*, pH 4.5), incubated with xylanase (Megazyme, 5,000 units) and arabinofuranosidase (Megazyme, 150 units) at 40°C for 2.5 hr with constant stirring. Enzymes were inactivated and removed in the manner described above. The solution was dialyzed, using dialysis membranes of 1,000 MWCO. Incubation with xylanase and arabinofuranosidase was repeated once after two days of dialysis and, after a further dialysis, the material was freeze-dried. Xylanase and arabinofuranosidase were checked for any lichenase activity by monitoring viscosity of pure β-glucan solutions with added enzymes. No change in the viscosity was observed up to 6 hr.

Isolation and Fractionation of Arabinoxylan (AX)

WE (3.0 g) was dissolved in phosphate buffer (0.02*M*, pH 6.5) and incubated with lichenase (Megazyme, 200 units/g) for 2 hr at 40°C and with β-glucosidase (Megazyme, pH 4.0, 30 min). The solution was dialyzed against distilled water (6,000–8,000 MWCO, 4°C), heated (95°C, 20 min), centrifuged (10,000 × *g*, 30 min) and freeze-dried. The purity of the enzymes was checked as described above using pure arabinoxylan solutions.

TABLE I
Composition and Properties of Barley Malt[a]

P_T (%)	FE (%)	CE (%)	ED (%)	V (Cps)	P_S/P_T	AX (%)	BG (%)	FA (%)
11.8	81.6	80.4	1.2	1.48	41.5	6.97	0.50	0.104

[a] P_T, total protein content; FE, fine extract content; CE, coarse extract content; ED, extract difference; V, viscosity; P_S/P_T, soluble to total protein ratio; AX, arabinoxylan; BG, β-glucan; FA, ferulic acid.

TABLE II
Yield (%) and Composition of Water-Extractable (WE) Material and Arabinoxylan (AX) and β-Glucan (BG) Fractions[a]

				Monosaccharides (mol %)						
Sample	Yield[b]	Protein[c]	Ferulic Acid[d]	Ara	Xyl	Man	Gal	Glc	Total Ara+Xyl	Xyl/Ara Ratio[e]
WE	1.39	15.27 ± 0.06	0.43 ± 0.01	28.1	36.9	2.7	5.4	26.9	65.0	1.31 (1.51)
AX	1.11	16.89 ± 0.28	0.59 ± 0.02	36.8	47.6	1.9	6.3	7.4	84.4	1.29 (1.46)
BG	0.62	12.03 ± 0.05	nd	26.6	28.8	2.4	8.5	33.7	55.4	1.08 (1.37)

[a] AX, arabinoxylan-enriched fraction obtained from WE; BG, β-glucan-enriched fraction obtained from WE; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose, Glc, glucose; Total Ara+Xyl, sum of arabinose and xylose obtained from monosaccharide analysis; Xyl/Ara, xylose to arabinose ratio; nd, not determined.
[b] Yield based on amount of malt used for extraction.
[c] Lowry method; $n = 2 \pm$ standard deviation.
[d] Sum of *trans* and *cis* isomers; $n = 3 \pm$ standard deviation.
[e] Values in parentheses indicate Xyl/Ara ratio after correction for the presence of arabinogalactans.

TABLE III
Yield (%) and Composition of Arabinoxylan (AX) Subfractions[a]

				Monosaccharides (mol %)						
Sample	Yield[b]	Protein[c]	Ferulic Acid[d]	Ara	Xyl	Man	Gal	Glc	Total Ara+Xyl	Xyl/Ara Ratio[e]
AX-50	21.5	29.0 ± 0.1	1.23 ± 0.01	38.0	47.2	1.2	5.5	8.1	85.2	1.24 (1.37)
AX-60	12.4	12.3 ± 0.2	0.80 ± 0.01	39.6	57.1	· · ·	1.4	2.0	96.7	1.44 (1.47)
AX-80	21.5	12.2 ± 0.6	0.67 ± 0.01	42.2	54.2	0.1	1.0	2.5	96.4	1.28 (1.30)
AX-100	4.3	13.0 ± 0.2	0.54 ± 0.03	41.2	54.1	0.3	1.8	2.6	95.3	1.31 (1.31)
AX-100S[f]	40.3	5.7 ± 0.2	0.11 ± 0.02	32.6	38.6	4.6	17.8	6.4	71.2	1.18 (1.92)

[a] Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose, Glc, glucose; Total Ara+Xyl, sum of arabinose and xylose as a percentage of total carbohydrate material; Xyl/Ara, xylose to arabinose ratio.
[b] Yield of subfractions based on the amount of material recovered after fractionation.
[c] Lowry method; $n = 2 \pm$ standard deviation.
[d] Sum of *trans* and *cis* isomers.
[e] Values in parentheses indicate Xyl/Ara ratio after correction for the presence of arabinogalactans.
[f] AX-100S subfraction soluble in $(NH_4)_2SO_4$ at 100% saturation.

Freeze-dried AX (2.3 g/L) was dissolved in phosphate buffer (0.1*M*, pH 7) and fractionated by a graded ammonium sulfate precipitation technique (Izydorczyk et al 1998a) at $(NH_4)_2SO_4$ saturation levels of 50, 60, 80, and 100%, then it was dialyzed and freeze-dried. The corresponding subfractions were designated AX-50, AX-60, AX-80, AX-100, respectively; the subfraction soluble in $(NH_4)_2SO_4$ at 100% saturation was designated AX-100S.

Analytical Methods

Protein contents of fractions and subfractions were determined by the method of Lowry et al (1951) with bovine serum albumin (Sigma-Aldrich Canada, Ltd.) as a standard. The monosaccharide composition of polysaccharide fractions was determined (in triplicate) by gas chromatography after hydrolysis with 1.0*M* H_2SO_4 for 2 hr at 100°C.

Alditol acetates, prepared according to the method of Englyst and Cummings (1984), were separated on a Supelco SP-2380 column (30 m, 0.32 mm i.d., 0.2 µm film thickness) using a Hewlett Packard gas chromatograph equipped with a flame-ionization detector. High purity hydrogen was used as a carrier gas. The injector port and detector were heated at 230 and 250°C, respectively. The column oven program started at 190°C for 2 min, then was programmed to rise at 5°C/min to 220°C. The final temperature was maintained for 4 min. Erythritol was used as an internal standard.

β-Glucan content was assayed by the enzymatic method of McCleary and Glennie-Holmes (1985) with a hexokinase kit (Boehringer and Manheim) for determination of glucose.

Phenolic acids were isolated according to the method of Izydorczyk et al (1991) and analyzed by HPLC using an LC-18 column (33 × 4.6 mm, particles 3 µm in diameter, 37°C; Supelco Canada, Ltd., Oakville, ON) and an absorbance detector (model 490, Waters Associates, Milford, MA) operated at 280 nm. Gradient elution was performed using two solvents: A, 10% (v/v) aqueous methanol plus 1 m*M* trifluoroacetic acid (TFA); and B, 80% (v/v) aqueous methanol plus 1 m*M* TFA acid (Parr et al 1996). The run was programmed isocratically for 5 min with 100% solvent A at 1 mL/min followed by a 10-min linear gradient to 70% solvent A and 30% solvent B and finally maintained at this level for the next 4 min.

Polysaccharide fractions and subfractions (4 mg/mL) were dissolved in phosphate buffer (0.01*M*, pH 6.5) and digested with lichenase (20 µL, Megazyme) for 20 hr at 40°C. Oligosaccharides released by lichenase from the β-glucan fractions were analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection as described previously (Izydorczyk et al 1998b).

The total arabinoxylan content in malt was estimated by gas chromatography. Ground malt samples were hydrolyzed in 1.0*M* H_2SO_4 for 2 hr at 100°C after starch digestion and precipitation of soluble nonstarch polysaccharides with 80% ethanol (Englyst and Cummings 1984). Arabinoxylan was calculated as 0.88 × [xylose + (arabinose – 0.7 Gal)], taking into account that some of the arabinose was derived from arabinogalactan. The arabinose-galactose ratio of 0.7 was used as reported for wheat arabinogalactan (Fincher and Stone 1974; Izydorczyk et al 1991).

NMR Spectroscopy

^{1}H-NMR spectra of arabinoxylan subfractions were recorded on a Bruker AM 300 FT spectrometer at 85°C. Samples were deuterium exchanged by dissolving in D_2O (0.5%, w/v). Approximately 256 pulses were collected; pulse repetition time was 7.05 sec and radio frequency pulse angle 68.6°.

Molecular Weight Determination

Freeze-dried fractions and subfractions (3 mg) were dissolved in distilled water (10 mL) and filtered at 50°C through a 3-µm cellulose acetate filter. The high-performance size exclusion chromatography (HPSEC) system consisted of a pump (Waters 510), an injection valve (model 7010, Rheodyne) with a 200-µL sample loop, a guard column (TSK PWH, Tosoh Corp., Tokyo, Japan), and a TSK G5000 PW column (7.8 × 600 mm, TSK PW). The columns were maintained at room temperature. The samples were eluted with 0.1*M* $NaNO_3$ containing 0.02% NaN_3 at a rate of 0.5 mL/min. The mobile phase was filtered through 0.1-µm cellulose acetate membranes. Three detectors were connected online to HPSEC: multiple-angle laser light scattering (MALLS, Dawn DSP-F, Wyatt Technology, Santa Barbara, CA), refractive index (RI, Waters 410) (dn/dc = 0.146 mL/g) and a UV detector (Waters

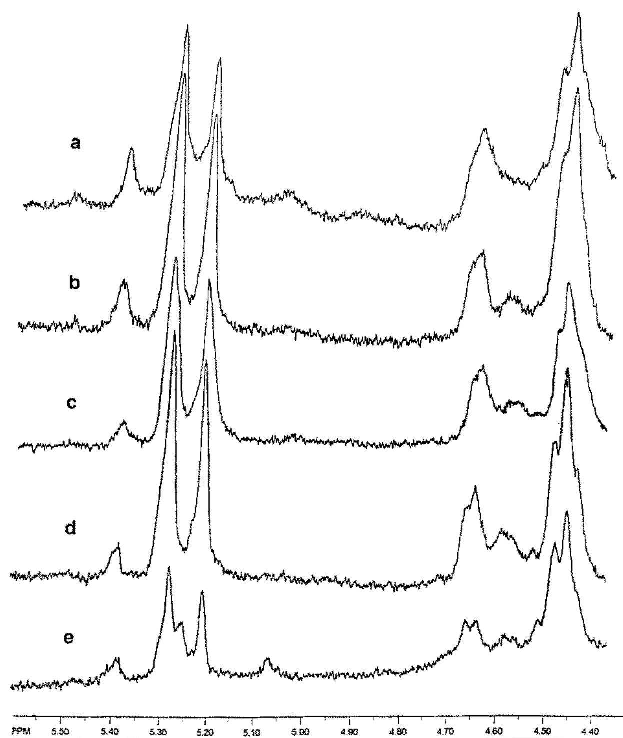

Fig. 1. ^{1}H NMR spectra of water-extractable arabinoxylan subfractions from barley malt obtained by ammonium sulphate precipitation: (a–e, respectively) AX-50, AX-60, AX-80, AX-100, AX-100S.

TABLE IV
Proportion (mol %) of Un-, Mono-, and Disubstituted Xylose in Arabinoxylan (AX) Subfractions[a]

Sample	u-Xyl*p*	2-Xyl*p*	3-Xyl*p*	2,3-Xyl*p*	Unsubstituted/ Substituted Xyl*p*	Doubly/Singly Xyl*p*
AX-50	48.0	12.8	10.7	28.5	0.92	1.21
AX-60	58.1	8.1	6.0	27.8	1.37	1.97
AX-80	54.4	8.0	4.8	32.6	1.19	2.55
AX-100	56.6	6.8	3.4	33.2	1.30	3.22
AX-100S[b]	44.7	15.3	10.4	29.6	0.80	1.15
AX-100S[b,c]	65.8	9.4	6.5	18.3	1.92	1.15

[a] u-Xyl*p*, [→4(Xyl*p*)1→]; 2-Xyl*p*, [→2,4(Xyl*p*)1→]; 3-Xyl*p*, [→3,4(Xyl*p*)1→]; 2,3-Xyl*p*, [→2,3,4(Xyl*p*)1→].
[b] AX-100S subfraction soluble in $(NH_4)_2SO_4$ at 100% saturation.
[c] Corrected for the presence of arabinogalactan.

490). The Astra 4.72 software (Wyatt Technology) was used for analysis.

RESULTS AND DISCUSSION

Results of malt analyses are summarized in Table I. Based on extract difference, viscosity of the malt extract, and the P_S/P_T ratio, the malt can be considered to be well modified. Substantially more arabinoxylans than β-glucans were found in the malt sample; these results were in accordance with literature data (Manzanares and Sendra 1996; Debyser et al 1997b). The level of total ferulic acid in malt was much higher than that reported by Viëtor et al (1991) for dehusked malt, but it was within the range found in barley grain (Nordkvist et al 1984; Zupfer et al 1998).

Isolation and Purification of WE Material and AX and BG Fractions

The isolation and purification procedures developed in this study for WE material were based partially on a method that worked well for barley and wheat nonstarch polysaccharides (Izydorczyk et al 1991, 1998b). To ensure maximum purity of material isolated from malt, a double treatment with α-amylase and amyloglucosidase was used to remove starch and starch dextrins. Protein contaminants were adsorbed on clay, digested with proteinase K, and retained on membrane ion exchangers. Dialysis tubing of low (1,000) MWCO has been used to avoid losses of small molecular weight polysaccharides. The WE material constituted 1.39% of the malt grist and contained 15.27% protein, despite the application of three techniques specifically to remove the protein (Table II). Further purification attempts using ion-exchange chromatography were not successful because substantial losses of polysaccharide material were observed. Debyser et al (1997b) reported slightly higher levels of protein (18.1–24.4%) in water-extractable arabinoxylan fractions from barley malts prepared by ethanol precipitation. The WE material contained 0.43% ferulic acid, which represented only ≈2.5% of that present in malt. Ferulic acid may be responsible for covalent cross-linking of arabinoxylans and, consequently, for their poor solubility (Izydorczyk et al 1990). Only a small proportion of the ferulic acid present in wheat and rye has been found in the water-extractable arabinoxylans from these cereals (Izydorczyk et al 1991; Vinkx et al 1993; Dervilly et al 2000). Xylose, arabinose, and glucose were the major monosaccharide residues detected after acid hydrolysis, pointing to the presence of arabinoxylan and β-glucan polymers in the WE material (Table II). A considerable amount of galactose and mannose residues found in WE indicates the presence of other polymeric species, possibly arabinogalactan or mannose-containing polymers. Wheat arabinogalactans have been studied extensively (Izydorczyk et al 1991; Loosveld et al 1997), but not much information is available on arabinogalactans in barley. When the Xyl/Ara ratio was calculated from the total amounts of Ara and Xyl residues found in the WE material, a value of 1.31 was obtained. The ratio increased to 1.51 when a correction for the presence of arabinogalactan was applied (assuming a value of 0.67 for the ratio of Ara/Gal in arabinogalactans) (Loosveld et al 1997).

A portion of the WE material was further treated with lichenase and β-glucosidase in an attempt to remove the β-glucan component and generate an AX-enriched fraction. This resulted in the fraction designated AX. Another portion of WE was treated with xylanase and arabinofuranosidase to remove arabinoxylans; this treatment resulted in the fraction designated BG. Analyses of these two fractions are also shown in Table II. The AX fraction contained appreciably more ferulic acid and arabinoxylans than did the parent WE

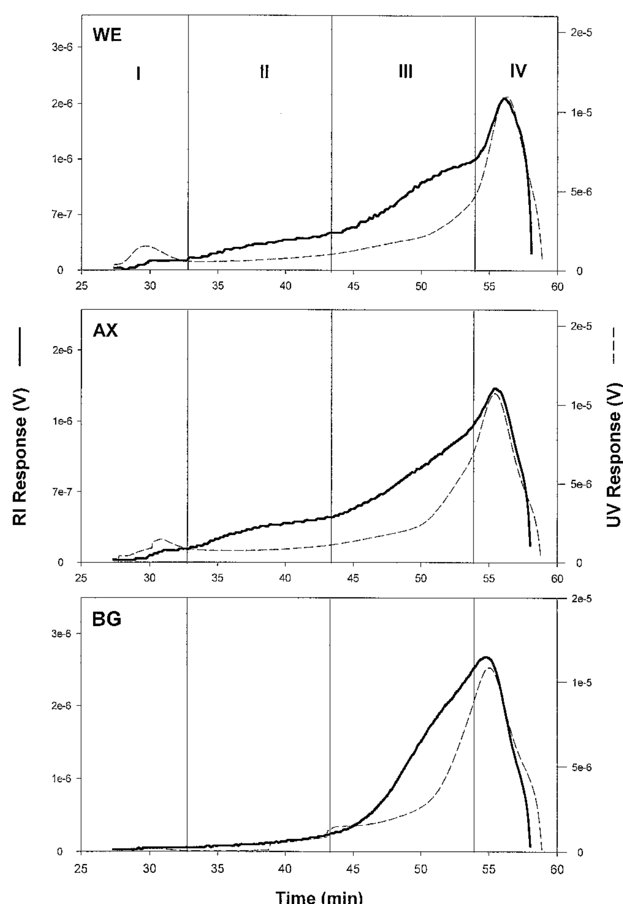

Fig. 2. Elution profiles of water-extractable (WE) material, arabinoxylan (AX), and β-glucan (BG) fractions from barley malt obtained by high-performance size-exclusion chromatography.

TABLE VI
Average Molecular Weight Values of Various Polymeric Populations in Water-Extractable (WE) Material and Arabinoxylan (AX) and β-Glucan (BG) Fractions

	M_w (g/mol) and Relative Amount[a]			
Sample	**Region I**	**Region II**	**Region III**	**Region IV**
WE	541,300,000 (2.5%)	10,047,000 (16.5%)	1,587,000 (46.1%)	647,000 (34.9%)
AX	216,400,000 (2.5%)	3,409,000 (19.2%)	482,800 (47.8%)	222,100 (30.5%)
BG	80,650,000 (1.0%)	1,910,000 (6.2%)	80,190 (57.1%)	36,970 (35.7%)

[a] Average of at least three chromatographic analyses. Relative amounts (in parentheses) were calculated by integration of the area under the size-exclusion chromatography profile for each region as indicated in Fig. 2.

TABLE V
Composition of Oligosaccharides (mol %) Released by Lichenase from Water-Extractable (WE) Material and β-Glucan (BG) Fraction

	Degree of Polymerization									
Sample	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10+11**	**3+4 Total**	**3:4 Ratio**
WE	64.1	29.3	3.4	1.2	0.4	0.4	0.7	0.5	93.4	2.19
BG	63.9	30.6	3.6	1.2	0.1	0.1	0.2	0.2	94.6	2.09

material. Enzyme treatment had indeed lowered significantly the glucose containing material, but the combined mannose and galactose level had not changed although the proportion of the two sugars had changed slightly. There was only a very small difference in the Xyl/Ara ratio (corrected and uncorrected) between WE and AX, indicating that the degree of branching had not been changed much during the enzyme treatment.

Attempts to remove arabinoxylans from the WE material and generate a fraction enriched in β-glucan for further detailed studies were only partially successful. The enzyme treatment increased the level of glucose-containing material from 26.9% in WE to 33.7% in BG, but arabinoxylans still constituted a major portion of this fraction. Many endoxylanases preferentially hydrolyze the xylan backbone at unsubstituted domains and, therefore, are less active on highly substituted xylans (Coughlan and Hazlewood 1993; Cleemput et al 1997). The arabinoxylans remaining in the BG fraction were more highly branched than the arabinoxylans present in the original WE fraction as indicated by the substantially lower Xyl/Ara ratio in BG (Table II). Some types of arabinofuranosidase appear to be able to cleave arabinofuranose from xylo-oligosaccharides but not from arabinoxylans (Wood and McCrae 1996). The relatively high proportion of arabinoxylans remaining in the BG fraction may be due to the high degree of substitution of the arabinoxylans. There was a relatively high level of galactose residues in this fraction, suggesting that it may contain appreciable amounts of arabinogalactan. Assuming a Ara/Gal ratio of 0.67, arabinogalactan in the BG fraction amounted to 14.5%. However, if the BG fraction also contains some galactomannans, as indicated by the presence of mannose residues, then the calculated amount of arabinogalactan in this fraction is likely overestimated.

Fractionation of the AX Fraction

Fractionation of the AX fraction using the graded ammonium sulfate fractionation technique resulted in five arabinoxylan subfractions (Table III). Interestingly, a large proportion of the material did not precipitate even at 100% saturation with $(NH_4)_2SO_4$. Protein contents had a range of 5.7–29.0%. Significant differences in the concentration of ferulic acid were observed among the arabinoxylan subfractions. The least soluble subfraction (i.e., precipitated most readily) contained the highest amount of ferulic acid. A similar trend was reported for wheat water-soluble arabinoxylan fractions obtained by $(NH_4)_2SO_4$ and by ethanol precipitation (Izydorczyk and Biliaderis 1992; Dervilly et al 2000). Monosaccharide analysis of subfractions revealed that the carbohydrate content of AX-60, AX-80, and AX-100 consisted of >95% arabinose and xylose, and only very small amounts of mannose, galactose, and glucose. The subfraction AX-100S, especially, contained a high amount of galactose and mannose residues. Again, assuming Ara/Gal ratio of 0.67 in arabinogalactans, the amount of this polymer in the subfraction AX-100S amounted to >29%. It appears, therefore, that arabinogalactans in barley, like their counterparts in wheat (Izydorczyk et al 1991), are more soluble than arabinoxylans and remain in solution despite the high concentration of $(NH_4)_2SO_4$. Surprisingly, however, the AX-50 subfraction also contained some galactose, indicating that a different population of arabinogalactans may be present in this subfraction. All subfractions contained some glucose. Glucose was almost equally distributed among the AX-60, AX-80 subfractions and AX-100 whereas a greater proportion of glucose was found in AX-50 and AX-100S. The Xyl/Ara ratio decreased from 1.44 to 1.18 as the saturation level increased from 60 to 100%, including the subfraction soluble at 100% saturation. This indicates a higher degree of branching in subfractions precipitated at higher saturation levels of $(NH_4)_2SO_4$, with the exception of AX-50. However, after correction for the presence of arabinogalactan in AX-100S, it appears that arabinoxylan in this subfraction had the lowest degree of branching (the highest Xyl/Ara ratio).

^{1}H-NMR Analysis of AX Subfractions

Figure 1 shows the ^{1}H-NMR spectra of the AX subfractions obtained by $(NH_4)_2SO_4$ precipitation. Signals were assigned based on previously published spectral data (Bengtsson and Åman 1990; Hoffman et al 1992; Viëtor et al 1994). The ^{1}H-NMR spectra revealed signals for anomeric protons of terminal α-L-arabinofuranosyl residues at δ 5.23, δ 5.28, and δ 5.38 ppm and of α-D-xylopyranosyl residues at δ 4.4 to 4.7 ppm. The signal at δ 5.38 ppm was assigned to the protons of arabinofuranosyl residues linked to singly substituted xylose residue at O-3, and the signals at δ 5.23 and δ 5.28 ppm to the protons of arabinofuranosyl residues linked to doubly substituted xylose at O-3 and O-2, respectively. The relative intensity of the resonances clearly showed that the majority of xylose residues carried two Ara*f* substituents linked at O-2 and O-3. The resonance at δ 5.26 ppm, especially visible for the AX-100S, originated from α-linked arabinofuranosyl residues linked to galactose residues (Loosveld et al 1997) and confirmed the substantial presence of arabinogalactan in this subfraction. A small resonance at δ 5.1 also may originate from α-arabinofuranose residues in arabinogalactan

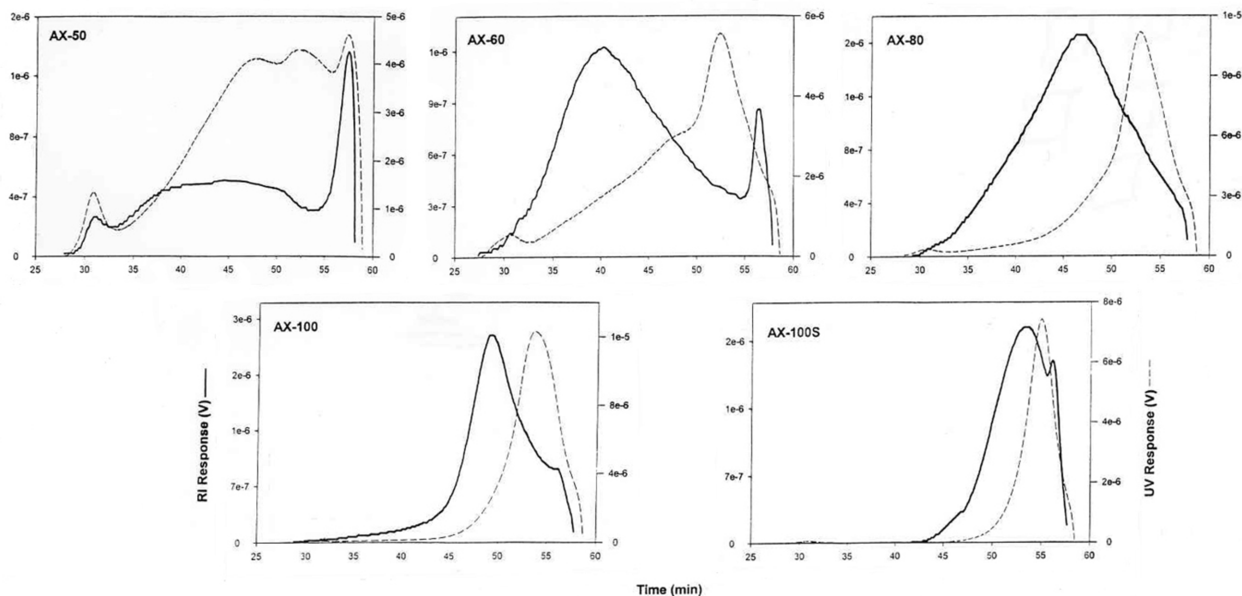

Fig. 3. Elution profiles of water-extractable arabinoxylan subfractions from barley malt obtained by high-performance size-exclusion chromatography.

(Saulnier et al 1992). Although the resonance at δ 5.26 was not clearly visible in the AX-50 subfraction, the signal at δ 5.28 was broad and may have overlapped with the signal from Ara*f* associated with galactose residues. A small signal at δ 5.1 also confirms the presence of arabinogalactans in this subfraction.

The relative proportions un- (u-Xyl*p*), mono- (2-Xyl*p* or 3-Xyl*p*) and di-substituted xylose residues (2,3-Xyl*p*) were calculated from the sugar analysis and from ^{1}H-NMR spectra by quantitative integration of anomeric protons of the individual arabinofuranosyl residues (Westerlund et al 1990; Oscarsson et al 1996; Debyser et al 1997a; Roels et al 1999). The ^{1}H-NMR data clearly showed variability in the arabinoxylan structures (Table IV). The arabinoxylan precipitated at higher saturation levels of $(NH_4)_2SO_4$ had increasing proportions of 2,3-Xyl*p* (28–33%) with decreased levels of both monosubstituted xylose residues (12.8–6.8% and 10.7–3.4%, for 2-Xyl*p* and 3-Xyl*p*, respectively). The amounts of u-Xyl*p* varied from 48–58% and were comparable with those found by Oscarsson et al (1996) for the WE arabinoxylans from barley (47–62%), where a lower level of 2,3-Xyl*p* was observed (18–24%). The increase in $(NH_4)_2SO_4$ saturation from AX-50 to AX-100 produced arabinoxylan with progressively higher ratios of doubly to singly substituted xylose residues (1.21–3.22). Arabinoxylan remaining in solution at 100% saturation of ammonium sulfate (AX-100S) had the lowest ratio of doubly to singly substituted xyloses (1.15). After correction for the presence of arabinogalactans in the AX-100S, this subfraction had the highest level of u-Xyl*p* and the lowest level of 2,3-Xyl*p* residues. In general, the malt arabinoxylans contained relatively high amounts of unsubstituted xylose residues, making the ratios of unsubstituted to substituted Xyl*p* residues higher than those reported for water-extractable barley arabinoxylans (Izydorczyk et al 1998b). Also, the majority of substituted xylose residues carried two Ara*f* rather than one, resulting in higher ratios of doubly to singly substituted Xyl*p* than those reported for water-extractable barley arabinoxylans. It is possible to envisage two types of structural domains in arabinoxylans which may have resisted the enzymic degradation during malting. On one hand, there are domains densely substituted with arabinose residues and thereby protected from enzymic attack; and on the other hand, there are smooth domains with no Ara*f* substitutions. Although it is generally accepted that exposed xylan backbones are most susceptible to enzymic degradation by xylanases, it is also possible that such domains participate in formation of stable aggregates (among arabinoxylans or other polymers) and, in consequence, resist enzyme hydrolysis.

Characterization of β-Glucans in WE Material and the BG Fraction

The proportions of soluble oligomers released by lichenase from WE material and its BG fraction are presented in Table V. The products from both materials were mainly composed of tri- and tetrasaccharides (93.4 and 94.6%), originating from 3-*O*-β-D-cellobiosyl-D-glucose and 3-*O*-β-D-cellotriosyl-D-glucose units in the original β-glucan (Woodward et al 1988). Larger oligomers, up to DP11, were also observed, but DP5 and DP6 oligomers were the most abundant. The total amount of tri- and tetrasaccharides in the malt β-glucan found in this study appears to be higher than that reported for barley β-glucan (87.0 and 89.4%) by Woodward et al (1988) and Izydorczyk et al (1998b), respectively. The molar ratio of tri- to tetrasaccharide residues (DP3/DP4) in β-glucan from WE (2.19) was slightly higher than that reported by Woodward et al (1988) and Izydorczyk et al (1998b) for water soluble barley β-glucan (2.0 and 2.13, respectively). The higher DP3/DP4 ratios have been associated with lower solubility of β-glucans (Izydorczyk et al 1998b). Therefore, it is not surprising that β-glucans remaining in barley after malting have these characteristics.

Molecular Weight Determination

Size-exclusion chromatograms of WE material and of fractions AX and BG are shown in Fig. 2. The elution profile for WE shows several polymer populations, as indicated by the response from the RI detector. The chromatogram was divided into four regions and the amounts and average molecular weights of the different polymer populations are given in Table VI. Although most of the material was eluted in the lower molecular weight end of the chromatogram (regions III and IV), WE material contained considerable amounts of high molecular weight populations (regions I and II) (Table VI). The small peak eluting in the high molecular weight region (region I), which constituted ≈2.5% of WE, may be the result of polymer aggregation. The molecular weight of this material was extremely high. It contained UV-absorbing components, most likely proteins or phenolic acids. It is possible that a small portion of arabinoxylans is cross-linked through diferulic acid bridges and therefore exhibits large molecular weight values as well as UV-absorbing properties. Fractions eluting in regions II and III were of considerably higher molecular weights than that in region IV. The presence of UV-absorbing material throughout the entire elution profile of the WE material may indicate that some of the proteins remaining in the WE preparation are, in fact, covalently bound to the carbohydrate material.

The AX fraction contained mainly arabinoxylans and, therefore, the elution profile of this material shown in Fig. 2 reflects the heterogeneous nature of this polymer in malt. Again, four families of molecules could be differentiated, and their molecular weights are shown in Table VI. Again, the highest molecular weight population (region I) represented 2.5% of the AX fractions and may well be the result of molecular aggregation. A considerable amount of material eluted in region II with an average molecular weight above 3 million. The average molecular weight of the major fraction in region III was about twice as high as of that in region IV. Over 90% of the material in BG, the fraction enriched in β-glucan, eluted in regions III and IV and had a significantly lower molecular weight than the correspondingly eluting material in AX, the arabinoxylan enriched fraction. It appears, therefore, that arabinoxylans were hydrolyzed to a lesser extent than β-glucans during malting. Interestingly, the average molecular weight of materials eluting in all four regions of the WE material was substantially higher than in the corresponding regions of either AX or BG fractions. This may indicate possible interactions between the two polymers, leading to formation of high molecular weight aggregates. It should also be noted that the elution time of material eluting in region IV was longer for WE than for the AX and BG subfractions, although the molecular weight of this material appears to be higher than the corresponding material in the AX and BG subfractions. This may indicate differences in the composition or conformation of the eluting species.

Elution profiles of the AX subfractions are shown in Fig. 3, and molecular weight characteristics of individual peaks are given in Table VII. The small peak of very high molecular weight detected

TABLE VII
Average Molecular Weight Values of Various Populations in Arabinoxylan (AX) Subfractions

Subfractions	M_w (g/mol)[a]
AX-50	11,655,000
	1,299,000
	222,250
AX-60	397,550
	137,500
AX-80	192,250
AX-100	47,246
AX-100S	72,160
	53,300

[a] Average of at least three chromatographic analyses. Molecular weights for individual subfractions correspond to chromatogram peaks in Fig. 3.

in WE and AX fractions can be seen in the AX-50 subfraction profile. It was greatly reduced in the AX-60 subfraction and was not detected in the other subfractions. This peak may contain aggregated material and therefore might be expected to be poorly soluble and precipitated by relatively low concentrations of ammonium sulfate. The large, broad peak of UV absorbing material in AX-50 reflects the high protein content of this subfraction. Interestingly, however, the UV-absorbing material did not always follow the carbohydrate eluting material, which may indicate the possibility that some of the proteins are not covalently linked to the arabinoxylans. There was a clear shift in elution time of the main arabinoxylan population from subfraction AX-60 to AX-100, with a concomitant decrease of the molecular weight.

CONCLUSIONS

Arabinoxylans constituted the majority of water-soluble nonstarch polysaccharides remaining in barley after malting. Some β-glucans and small amounts of arabinogalactans as well as mannose containing polymers were also found. Some proteins (≈15%) appeared to be associated with one or more of the carbohydrate polymers in the water-extracted material from malt; covalent linkages between the proteins and polysaccharides cannot be excluded. Size-exclusion chromatography analysis indicated that the polymers in the WE material have a propensity for interaction and aggregation. The arabinoxylans in the WE material had higher molecular weights than β-glucans in this material. The major portion of Xyl*p* in the arabinoxylans were either unsubstituted or doubly substituted with Ara*f*. Both types of structural features may render arabinoxylans resistant to further enzymatic hydrolysis during mashing and eventually be responsible for wort filtration problems. β-Glucans remaining in barley malt also exhibited some characteristics which potentially may enhance formation of aggregates and be responsible for filtration problems.

ACKNOWLEDGMENTS

We are grateful to Dominion Malt for providing the malt samples, to Michael Edney for providing the malt analysis, to SangGuan You and Sharon Bazin for assistance with the HPLC analysis, and to the Brewing and Malting Barley Research Institute and Agri-Food Research and Development Initiative for financial support.

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[Received May 17, 2001. Accepted December 5, 2001.]