Physicochemical and Functional Properties of Rye Nonstarch Polysaccharides. VI. Variability in the Structure of Water-Unextractable Arabinoxylans

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ABSTRACT

A fraction rich in water-unextractable arabinoxylans was isolated from rye wholemeal. Arabinoxylans were solubilized by a sequence of alkaline extractions. Structural features of these arabinoxylans were investigated by methylation analysis and nuclear magnetic resonance spectroscopy. Three groups of water-unextractable arabinoxylans could be recognized, differing in extractability, solubility, and structure. The first group had an intermediate arabinose-to-xylose ratio (A/X) of 0.55–0.79 and was extractable either with saturated $Ba(OH)_2$ containing 1% $NaBH_4$ or with water after saturated $Ba(OH)_2$ extraction. The polymers were soluble in water after neutralization of the alkaline extract, but they precipitated in saturated ammonium sulfate solution. They contained terminal arabinose residues and unsubstituted, 3-mono-, 2-mono-, and disubstituted xylose residues. A second group had an A/X of ~1.1 and was partly extracted with alkali; more could be extracted after delignification. This group was soluble in water and in saturated ammonium sulfate solution. Typical structural features in this arabinoxylan were: substituted arabinose residues (40% of arabinoses), terminal xylose residues (26% of xyloses), and terminal galactose residues. A third group of arabinoxylans was extractable with 1*M* KOH, had low water solubility after neutralization of the alkaline extract, and had an A/X of ~0.2. Besides arabinoxylan, β-glucan was present in large proportions in rye cell-wall material. Glucomannan and xyloglucan were also detected.

In the previous article of this series (Vinkx et al 1993), we described the isolation, fractionation, and structural variation of water-extractable rye arabinoxylans. However, the major part of the arabinoxylans in rye wholemeal is water-unextractable (Delcour et al 1989, Saini and Henry 1989). Water-unextractable arabinoxylans have an important (deleterious) influence on the breadmaking properties of rye flour (Meuser and Suckow 1986, Kühn and Grosch 1989, Weipert 1993). The use of pentosanase improves bread quality (Kühn and Grosch 1988). Because the degradability of arabinoxylans by endoxylanases depends on the presence of side groups (Düsterhöft et al 1993, Kormelink et al 1993, Viëtor et al 1994), insight in the structure of arabinoxylans is clearly relevant.

However, limited information is available on the structure of rye alkali-extracted arabinoxylans. A/X ratios of 0.1– 1.1 were reported for alkali-extracted arabinoxylans from rye (Casier et al 1967, Holas et al 1972, Ali and D'Appolonia 1979, Hromádková and Ebringerová 1987, Saini and Henry 1989). Saini and Henry (1989) extracted the water-insoluble residue from rye wholemeal with 1.25*M* NaOH and subsequently with 3.75*M* NaOH. The authors found that, in percent of rye grain, the former alkaline extract contained 5.85% arabinoxylans (A/X 0.43), the latter alkaline extract contained 1.39% arabinoxylans (A/X 0.55), and the residue contained 1.05% arabinoxylans (A/X 1.02).

Casier et al (1973) reported a ratio of terminal arabinose to unsubstituted xylose of 1.5, as determined by methylation analysis. The structures of alkali-extractable arabinoxylans from rye bran were investigated in more detail by Hromádková and Ebringerová (1987), Hromádková et al (1987), and Ebringerová et al (1990). These authors divided the arabinoxylans into two groups. The first group remained soluble after extraction (0.3*M* NH_3) and neutralization, and represented arabinoxylans with A/X in the range of 0.5–1.1. For an arabinoxylan with A/X 0.78, methylation analysis revealed that 41% of the nonterminal xylose units were unsubstituted (uxyl), 28% were 3-monosubstituted (3mxyl), 5% were 2-monosubstituted (2mxyl), and 26% were disubstituted (dxyl). Also detected were: terminal xylose (16% of the total content of xylose), substituted arabinoses (22% of arabinoses), and a low proportion of terminal galactose. The second group of arabinoxylans was insoluble after neutralization of the alkaline (1.1*M* NaOH) extract and had a low A/X (0.1–0.3). Arabinose residues were linked at *O*-3 of xylose residues. The latter arabinoxylan group represented ~50% of the nonstarch and noncellulosic polysaccharides from rye bran.

It is clear that, to date, the structural features of only two arabinoxylan preparations from rye bran water-unextractables were reported in detail. As arabinoxylans are very heterogenous, more information is required to describe the structural variability of water-unextractable arabinoxylans from rye wholemeal.

Therefore, this article provides structural information on the various arabinoxylans present in that fraction representing the majority of the water-unextractable arabinoxylans from rye wholemeal. The arabinoxylans were extracted by sequential alkaline extraction. The structural information on the various arabinoxylans was obtained both by nuclear magnetic resonance and methylation analysis. The data obtained were compared with data on water-extractable rye arabinoxylans and on wheat arabinoxylans.

MATERIALS AND METHODS

Rye Wholemeal

Rye (cv. Halo, 1992 harvest, grown in Belgium) was milled with a Tecator sample mill to pass a 0.5-mm sieve. The meal was heated for 90 min at 130°C to inactivate enzymes. Defatting was accomplished by extraction with *n*-hexane (Soxhlet, 4 hr).

Enzymes

Bacillus licheniformis α-amylase (type XII-A, A-3403, 17,980 units/ml [one unit will liberate 1 mg of maltose from starch in 3 min at pH 6.9 at 20°C]) was from Sigma Chemical Co. (St Louis, MO). *Streptomyces griseus* pronase E (4,000,000 PU/g, activity measured with casein, pH 7.4, 10 min, Folin-Ciocalteu phenol reagent) was obtained from Merck (Darmstadt, Germany). Englyst Fiberzym Kits for nonstarch polysaccharide (NSP) analysis were obtained from Novo Nordisk Bioindustries (Farnham, Surrey, UK).

Fractionation of Rye Wholemeal (Isolation of WU_I)

A fractionation method for wheat flour published by Gruppen et al (1989) was modified. The fractionation was performed in duplicate. All centrifugations were for 30 min at 10,000 × *g* at 20°C.

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Rye wholemeal (200 g) was extracted with 1,200 ml of distilled water for 90 min at room temperature by end-over-end rotation. The slurry was sieved over a 125-μm sieve, and the residue on the sieve was washed with 600 ml of water. The throughs were centrifuged and three fractions were obtained: a supernatant; an upper, brown tailing fraction; and an underlaying white starch fraction. The starch fraction was resuspended in 100 ml of water and centrifuged again, yielding three fractions that were pooled with the corresponding fractions from the former centrifugation. The starch fraction (ST) and the supernatants (WE) were lyophilized.

The fraction on the sieve and the tailing fraction were purified by amylolysis and proteolysis. Each was suspended in 800 ml of buffer (0.05*M* maleic acid, 0.05*M* tris, 0.001*M* $CaCl_2$, 0.05% NaN_3) and adjusted to pH 7.5 with diluted NaOH. The suspension was incubated with 20 mg of pronase E at 40°C. After 3 hr, a second 20-mg portion of pronase was added, and the pH was adjusted to 7.5 (from 7.1). After 6 hr, the suspension was acidified to pH 6.5 using diluted HCl. For amylolysis, 1 ml of α-amylase solution was added, and the suspension was incubated at 70°C for 90 min. The suspension was tested for residual starch (iodine-iodide solution). Only the tailing fraction was incubated further with 0.5 ml of α-amylase solution for 30 min at 70°C. After cooling and adjusting to pH 7.5 using diluted NaOH, a second proteolysis step was performed on both fractions with 20 mg of pronase E (11 hr at 40°C). The suspensions were neutralized with diluted HCl and centrifuged. The supernatants and corresponding residues were SUP_I and WU_I (originating from the material on the sieve), and SUP_{II} and WU_{II} (originating from the tailings). All fractions were lyophilized.

Alkali Extraction of Arabinoxylans from WU_I

The extraction sequence (according to Gruppen et al 1992) was conducted in duplicate. All dialysis steps were performed in the cold room (6°C) for 48 hr against distilled water.

WU_I from fractionation A (3 g) was extracted for 16 hr at room temperature with 500 ml of saturated $Ba(OH)_2$ containing $NaBH_4$ (1%). After centrifugation, the residue was extracted again for 1 hr with 200 ml of the same solution. After centrifugation, the supernatants were combined, acidified to pH 5.0 with acetic acid, dialyzed, and lyophilized (BE1). To the residue, 100 ml of water was added, and the suspension was acidified to pH 5.0 using acetic acid and then extracted for 1 hr at room temperature. After centrifugation, the residue was extracted twice more with 100 ml of water. The supernatants were combined, dialyzed, and lyophilized (BE2). The residue was further extracted with 200 ml of 1*M* KOH (containing 1% $NaBH_4$) for 16 hr at room temperature. After centrifugation, the residue was extracted again for 1 hr at room temperature with the same extractant and then centrifuged. The supernatants were combined, acidified to pH 5.0 with acetic acid, dialyzed, and lyophilized (1M). The residue was then extracted for 16 hr with 200 ml of 4*M* NaOH (containing 1% $NaBH_4$). After centrifugation, the residue was extracted once more with 100 ml of the same solution for 1 hr. The residue was finally washed three times with 100 ml of water for 30 min and centrifuged. The supernatants obtained were combined, acidified to pH 5.0 with acetic acid, dialyzed, and lyophilized (4M). The residue was lyophilized (RES).

Ammonium Sulfate Fractionation

Solutions of BE1a (200 mg in 40 ml of water) were saturated with ammonium sulfate, kept overnight at room temperature, and centrifuged. The fractions were dialyzed and lyophilized (BE1asup, BE1ares).

Delignification and Further Extraction of the Residue Obtained After Sequential Alkaline Extraction

RESa (300 mg) and RESb (300 mg) were pooled and delignified (Brillouet and Mercier 1981, Düsterthöft et al 1991). To that end, 20 ml of a sodium chlorite solution (1 g of $NaClO_2$ + 0.25 ml of HOAc/50 ml of water) were added to 600 mg of RES. The mixture was incubated for 2 hr at 70°C. After centrifugation (15 min, 10,000 × *g*), the residue was extracted with 20 ml of 1*M* KOH (1% $NaBH_4$) for 120 min and subsequently extracted with 20 ml of 4*M* NaOH (1% $NaBH_4$) for 120 min at room temperature. The final residue was washed with 20 ml of water, and the washings were added to the 4M extract. All extracts were acidified to pH 5.0 using acetic acid, dialyzed and lyophilized (RES1M, RES4M). The relationship between meal fractions, extracts, and fractions of extracts is expressed in Figure 1.

Analysis of Meal and Meal Fractions

Protein (N × 6.25) was measured using a Kjeldahl method (Bremner 1965). Ash and moisture were measured by standard methods (AACC 1983). NSP content and composition were measured by the method of Englyst and Cummings (1984). Total polysaccharides were measured by omitting the isolation step of NSP. For SUP, ST, and wholemeal, the arabinose, xylose, mannose, and galactose residues were measured as NSP, whereas WE, WU_I, and WU_{II} were measured as total polysaccharides. Uronic acids from NSP were measured colorimetrically with the dimethylphenol method according to Scott (1979).

Analysis of Extracts

Protein content was measured according to Lowry et al (1951) with bovine serum albumin as standard. Total monosaccharide composition and total uronic acids were measured as described above.

Methylation analysis was performed as described in Gruppen et al (1992). The fractions were methylated by a modification of the Hakomori method (Sanford and Conrad 1966), dialyzed, and dried in a stream of air. This procedure was repeated once to improve completeness of the reaction. Glycitol acetates were prepared (Englyst and Cummings 1984) after hydrolysis of the samples using 2*M* trifluoroacetic acid (1 hr, 121°C). Samples were analyzed with gas chromatography (GC) with flame ionization detection and identities were confirmed by GC-mass spectrometry as described by Gruppen et al (1992). The 2- and 3-*O*-methylated xylitol acetates coeluted. Their relative amounts were calculated from the relative abundance of the ions at the ratio molecular mass to charge m/z 117 and m/z 129, respectively.

For nuclear magnetic resonance (NMR) spectroscopy, ^{1}H-NMR spectra were recorded with a Bruker AM-300 (300 MHz) apparatus at 85°C. Samples were dissolved in D_2O (99.8% D), freeze-dried, and dissolved again in D_2O. Pulse repetition time was 2 sec. Number of scans varied from 4,000 to 16,000. Acetone was used as standard (δ2.23 ppm). Peak assignments were made on the basis of the data by Joseleau et al (1977) for arabinans from *Rosa glauca*, and by Hoffmann et al (1992) for wheat flour water-extractable arabinoxylans.

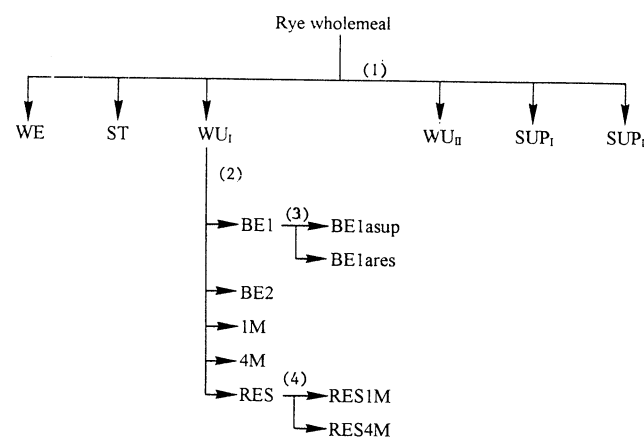

Fig. 1. Fractionation of rye wholemeal: (1) physical enzymatic separation; (2) sequential alkaline extraction; (3) ammonium sulfate fractionation; and (4) delignification followed by sequential alkaline extraction. WE, ST, WU_x and SUP_x = water-extractable, starch, water-unextractable arabinoxylans, and the supernatants of amylolysis and proteolysis treatment of water-unextractable arabinoxylans.

Commercial barley β-glucan (Novo Industri, Copenhagen, Denmark) produced anomeric resonance peaks at δ4.78, δ4.75, δ4.58, δ4.57, δ4.55, and δ4.54 ppm. For α-dextrin at 60°C in D_2O, anomeric resonance peaks at δ5.37 and δ5.38 ppm were reported for internal unbranched residues (McIntyre and Vogel 1990).

A ^1H-decoupled ^{13}C-NMR spectrum was recorded with a Bruker AM-300 apparatus (75 MHz) at 85°C. The sample was dissolved (~30 mg/ml) in D_2O (99.8% D). Pulse repetition time was 2 sec, and the number of scans was 10,000. Dioxane was used as standard (δ67.4 ppm). Peak assignments were made on the basis of the data by Kovac et al (1980) for xylo-oligosaccharides, by Brillouet and Joseleau (1987) for alkali-extractable heteroxylans from wheat bran, by Ebringerová et al (1990) for alkali-extractable arabinoxylans from rye bran, and by Hoffmann et al (1992) for water-extractable arabinoxylans from wheat flour.

Size-exclusion chromatography of dissolved fractions (500 μl, 1 mg/ml) was performed on a Sephacryl S500 HR column (30 × 1 cm, Pharmacia, dextran fractionation range 40–20,000 kDa). The eluent was 0.3% NaCl (0.4 ml/min). Fractions (150 sec) were collected and analyzed (0.8 ml) for total carbohydrates with the phenol-sulfuric acid method according to Dubois et al (1956). Dextrans (10, 40, 70, and 500 kDa) were used as standards.

RESULTS AND DISCUSSION

Fractionation of Rye Wholemeal (Isolation of WU_I)

The yield and composition of the various fractions obtained with fractionations A and B are shown in Table I. The results show that the fractionation method was reproducible. Recovery of arabinoxylans (measured as the sum of arabinose and xylose) was 100%.

Proteolysis did not produce WU fractions totally free of protein, which is in accordance with other reports (Brillouet et al 1988, Düsterhöft et al 1991). Because amylolysis was used to remove adhering starch in the WU fractions, starch degradation products were present in the SUP fractions. No arabinoxylans were detected in the starch fraction.

Arabinoxylans were concentrated in the WU fractions: WU_I (yield 9%) contained on average (of A and B) 48% of all recovered arabinoxylans and WU_{II} (yield 6%) contained 14%. Of all recovered arabinoxylans, 31% was cold water-soluble (WE) and 7% was hot water-soluble (SUP). The choice of a 125-μm sieve resulted in two fractions of a considerable yield, containing a different population of arabinoxylans (A/X of 0.52 and 0.73 for WU_I and WU_{II}, respectively).

NSP glucose was recovered predominantly in the WU fractions (48 and 17% in WU_I and WU_{II}, respectively) and in the SUP fractions (22%). A low amount was extracted with cold water (13%). The NSP glucose content of wholemeal (3.61%) probably included β-glucan, cellulose, glucomannan, and xyloglucan. The content of soluble NSP glucose, predominantly β-glucans, was 0.48% in rye wholemeal (dmb) in accordance with the 0.3–1.2% range reported in literature data (Bengtsson et al 1992).

Mannose was present in low amounts in WU and WE fractions. The mannose detected in the WE fraction may have resulted from sucrose and fructans. Indeed, rye wholemeal contains sucrose and fructans (Henry 1985, Bengtsson and Åman 1990). Fructose was detected as a mixture of glucose and mannose. Glucomannans probably were present in the WU fractions (Gruppen et al 1989, 1992).

Water-extractable arabinogalactans have been reported before for rye (Meuser et al 1986) and could explain the presence of galactose in the WE fractions.

Uronic acids were only detected in low proportions (0.32% in meal). They were concentrated by the fractionation in WU_I (1.17%, or 38% of total uronic acids) and in WU_{II} (0.89%, or 17% of total recovered uronic acids). Uronic acids and galactose in the WU fractions may be structural moieties of rye bran heteroxylans (Ebringerová et al 1990).

Extraction of WU_I

In Table II, the yield and composition of extracts from WU_I are reported. The data show that the major part (96%) of the arabinoxylans were recovered. Total mass recovery was only 78% of WU_I, probably because part of WU_I was dialyzable. The presence of starch amylolysis products (Table I) may have contributed to the low recovery rate of glucose (71%).

Much as in the case of the extraction of wheat water-unextractables (Gruppen et al 1991), saturated $Ba(OH)_2$ (containing 1% $NaBH_4$) was a selective extractant for rye arabinoxylan. The BE1 fraction contained only 2% glucose and 2% galactose (Table II). However, BE1 had a high protein content (23%), because WU_I had a relatively high protein content (9.13%). BE1 contained only 25% of the sum of the arabinoxylans recovered from WU_I. Gruppen et al (1991, 1992) reported an extraction of 80% of the arabinoxylans of wheat flour water-unextractables and of 29% of the arabinoxylans of wheat bran, under comparable experimental conditions.

The variability of structures within the BE1 fraction was demonstrated by monosaccharide analysis of fractions obtained by ammonium sulfate fractionation. Saturated ammonium sulfate yielded a supernatant fraction with A/X 1.06 and a residue with

TABLE I
Composition of Wholemeal Fractions[a]

Fraction	Yield	Moisture	Ash	Protein	Ara	Xyl	Man	Gal	Glc	NSP-Glc	UA	A + X	A/X
Fractionation A													
WE	14.7	8.85	5.65	20.53	6.0	9.0	2.21	1.07	25.3	(2.94)	0.24	15.0	0.667
ST	24.0	7.21	0.19	0.38	...[b]	...	...	...	92	(TR)	0.08	...	...
WU_I	9.3	5.06	3.40	9.13	13.1	24.9	1.51	1.97	21.1	(17.2)	1.17	38.0	0.526
WU_{II}	5.5	10.26	3.45	12.67	7.1	9.8	1.55	1.22	38.7	(9.5)	0.87	16.9	0.731
SUP_I	18.6	ND[c]	ND	ND	0.62	1.00	TR[d]	TR	40.1	(1.96)	0.15	1.62	0.622
SUP_{II}	34.5	ND	ND	ND	0.33	0.47	...	TR	63	(1.11)	0.14	0.80	0.708
Fractionation B													
WE	16.3	13.39	5.59	19.16	5.7	8.6	2.07	1.02	23.4	(2.69)	0.33	14.3	0.662
ST	25.4	11.34	0.18	0.37	...	...	...	...	90	(...)	0.11	...	...
WU_I	9.4	12.54	2.71	8.04	12.5	24.4	1.17	1.94	17.9	(16.0)	1.40	36.9	0.513
WU_{II}	6.2	8.54	3.37	11.89	7.9	10.9	1.58	1.24	40.5	(9.5)	0.90	18.8	0.727
SUP_I	15.1	ND	ND	ND	0.57	0.87	TR	TR	35.9	(1.76)	0.17	1.44	0.653
SUP_{II}	36.2	ND	ND	ND	0.31	0.43	...	TR	64	(1.17)	0.14	0.74	0.727
Wholemeal		8.11	ND	9.85	2.72	4.6	0.39	0.45	65	(3.61)	0.32	7.32	0.590

[a]Expressed as weight percent of fraction (as is basis); yield as weight percent of rye wholemeal. Ara = arabinose, Xyl = xylose, Man = mannose, Gal = galactose, Glc = glucose, NSP-Glc = nonstarch polysaccharide (in parentheses), UA = uronic acids, A + X = arabinose and xylose, A/X = arabinose to xylose ratio. WE, ST, WU_x and SUP_x = water-extractable, starch, water-unextractable arabinoxylans, and the supernatants of amylolysis and proteolysis treatment of water-unextractable arabinoxylans, respectively, from rye wholemeal.
[b]Not detected.
[c]Not determined.
[d]Trace.

A/X 0.55 (Table III).

Water extraction after $Ba(OH)_2/NaBH_4$ extraction yielded the BE2 extract. The BE2 fraction contained β-glucan (as shown by ^{1}H-NMR) (Fig. 2b), 62% arabinoxylan, and only 3% protein. The A/X (0.56) was lower than that of BE1 (0.65). BE2 contained 17% of all arabinoxylans recovered from WU_I.

The 1M fraction contained arabinoxylans with a very low A/X (0.21), representing a considerable proportion (32%) of the arabinoxylans of WU_I. The uronic acid content of 1M (1.2%) was slightly higher than that of BE1 and BE2 (0.5 and 0.9%, respectively). A high content of glucose (24%) was measured (β-glucan as shown by ^{1}H-NMR). As for wheat flour (Gruppen et al 1992), most of the β-glucan was found in the 1M fraction (and BE2).

The 4M extract contained almost all the extracted mannose and only a minor proportion of the extracted arabinoxylans, as was the case for wheat flour (Gruppen et al 1992), wheat bran (Brillouet and Mercier 1981), and rye bran (Hromádková and Ebringerová 1987). The A/X (0.32) was comparable to the 0.20–0.38 figures from Hromádková and Ebringerová (1987) but lower than the 0.55 reported by Saini and Henry (1989).

The residue of WU_I after alkaline extraction (RES) had a high A/X (1.10). For rye wholemeal, it was reported that 13% of the water-unextractable arabinoxylans remained in the residue after alkaline (up to 3.75*M* NaOH) extraction, and this fraction had an A/X of 1.02 (Saini and Henry 1989). In this study, 18% of the arabinoxylans of WU_I were not extracted. Therefore, we delignified RES and extracted the obtained residue with alkali. After delignification, only part (33%) of the thus far alkali-unextractable arabinoxylans were recovered in alkaline extracts. All fractions had high A/X (Table III). The results were in agreement with data reported for wheat bran. Indeed, Brillouet and Mercier (1981) reported that a total delignification was not obtained, and that the residue contained a high level of hemicellulosic material firmly bound to the lignin-cellulose complex (representing 19.8% of original arabinoxylans). However, Hromádková and Egringerová (1987) reported that the arabinoxylan that remained in the residue after delignification and alkaline extraction of rye bran had an A/X of 0.35.

Methylation Analysis and NMR Investigation of Arabinoxylan Structures

Intermediate A/X arabinoxylans. The spectrum of BE1ares (Fig. 2a) revealed that the structural features of water-extractable arabinoxylans were also present in alkali-extractable rye arabinoxylans. Methylation analysis revealed that this arabinoxylan, in molar percent of total xylose residues, consisted of 58% uxyl, 25% 3mxyl, 2% 2mxyl, and 9% dxyl. A proportion of the xylose residues (5%) occurred as nonreducing terminal units (Tables IV and V).

The spectrum of BE2 (Fig. 2b) was similar to that of BE1ares. However, β-glucan and a minor amount of α-dextrin were also present, as well as minor peaks similar to those in BE1asup. The presence of β-glucan in BE2 was confirmed by methylation analysis that detected both 3-linked and 4-linked glucose (Table IV). ^{1}H-NMR spectroscopy and methylation analysis showed that BE2 contained less 3-monosubstituted xyloses than BE1ares. Substituted arabinoses and terminal xyloses were measured in higher proportions. The content of 2-monosubstituted xylose residues was low for all samples (Tables IV and V), which is in accord with results obtained by Ebringerová et al (1990) for rye alkali-extractable arabinoxylans.

The majority of the arabinoxylans in BE1 and BE2 extracts from WU_I can be considered to belong to a group of arabinoxylans with intermediate A/X, which are soluble in water after alkaline

TABLE II
Composition of Extracts from WU_I[a]

	Yield	Protein	Ara	Xyl	Man	Gal	Glc	UA	A + X	A/X
Extraction a										
BE1	13.7	22.5	25.7	39.6	...[b]	2.37	.88	0.51	65	0.648
BE2	9.5	3.1	22.4	39.6	TR[c]	3.07	21.8	0.87	62	0.565
1M	22.4	11.0	9.3	42.7	0.8	1.18	24.7	1.21	52	0.219
4M	14.2	8.0	5.4	15.8	6.6	1.13	16.6	1.34	21.1	0.340
RES	17.0	ND[d]	19.3	17.5	TR	2.65	29.5	2.26	36.8	1.103
Extraction b										
BE1	14.0	23.2	26.1	39.8	...	2.45	2.43	0.58	66	0.655
BE2	9.8	3.0	22.3	40.5	TR	2.82	21.2	0.89	63	0.551
1M	22.8	11.1	9.2	42.8	TR	1.19	22.9	1.24	52	0.214
4M	13.7	7.7	5.1	17.3	7.5	1.12	11.5	1.28	22.3	0.294
RES	18.9	ND	19.0	17.3	TR	2.62	30.0	2.23	36.3	1.097

[a] WU_I = Water-unextractable arabinoxylans from rye wholemeal. Expressed as weight percent of fraction (as is basis); yield as weight percent of WU_I. Ara = arabinose, Xyl = xylose, Man = mannose, Gal = galactose, Glc = glucose, UA = uronic acids, A + X = arabinose and xylose, A/X = arabinose to xylose ratio. BE1, BE2, 1M, and 4M = extracts obtained by sequential alkaline extraction from WU_I. RES = nonalkaline-extractable residue from WU_I.
[b] Not detected.
[c] Trace.
[d] Not determined.

TABLE III
Composition of WU_I[a] Subfractions Obtained from Ammonium Sulfate Fractionation and Delignification[b]

	Yield	Protein	Ara	Xyl	Man	Gal	Glc	UA	A + X	A/X
BE1asup	23	6.2	27.1	25.5	...[c]	5.2	TR[d]	1.00	53	1.064
BE1ares	68	21.0	22.7	41.2	...	TR	TR	0.43	64	0.550
RES1M	9.1	1.4	37.3	33.9	...	5.1	4.7	1.91	71	1.102
RES4M	9.1	1.2	32.4	30.7	TR	4.4	13.7	1.55	63	1.056

[a] WU_I = Water-unextractable arabinoxylans from rye wholemeal.
[b] Expressed as weight percent of fraction (as is basis); yield as weight percent of unfractionated sample. Ara = arabinose, Xyl = xylose, Man = mannose, Gal = galactose, Glc = glucose, UA = uronic acids, A + X = arabinose and xylose, A/X = arabinose to xylose ratio. BE1asup and BE1ares = ammonium sulfate fractionation fractions from the WU_I $Ba(OH)_2$ extract. RES1M, RES4M = extracts obtained after delignification of the nonalkaline-extractable residue from WU_I.
[c] Not detected.
[d] Trace.

extraction and precipitate in a saturated ammonium sulfate solution.

High A/X arabinoxylans. Apart from the known arabinose peaks, the spectrum of BE1asup (Fig. 3a) contained a variety of unidentified peaks, which revealed the complexity of the material. Some resonances in the range of δ5.0–5.4 ppm possibly originated from substituted arabinoses (Joseleau et al 1977). The ^{1}H-NMR spectra of the fractions released after delignification of the alkali-unextractable residue (RES1M and RES4M) much resembled the spectra of the ammonium sulfate-soluble fraction of the BE1 extract (BE1asup), as shown in Figure 3. This suggested that saturated barium hydroxide (containing 1% $NaBH_4$) can release a proportion of arabinoxylans that seems to be associated with lignin. Peak broadening occurred and peaks of paired disubstituted xylose units could not be recognized (Fig. 3).

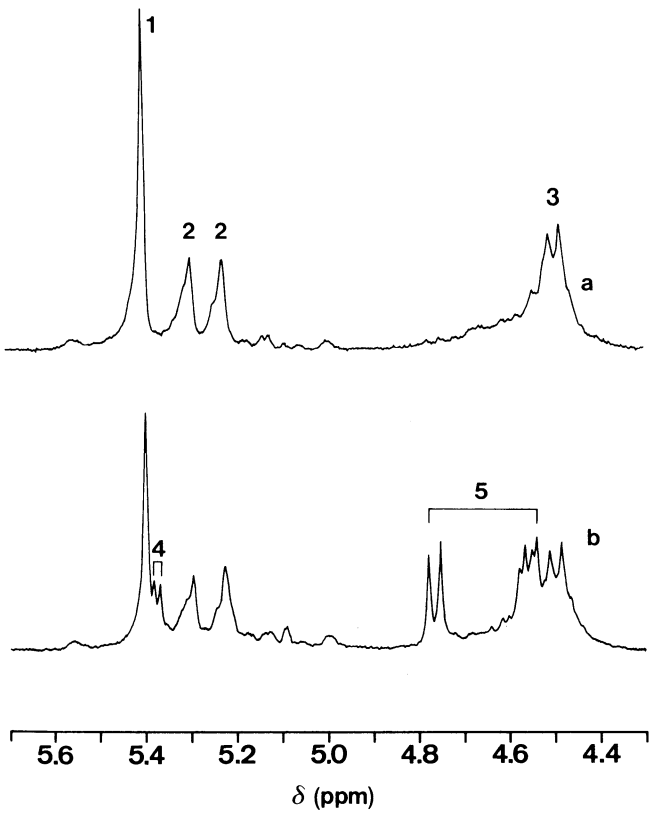

Fig. 2. Diagnostic regions of the ^{1}H-nuclear magnetic resonance spectra (D_2O, 85°C, 300 MHz) of rye alkali-extracted arabinoxylan fractions: BE1ares (a); BE2 (b); arabinose from 3-monosubstituted xylose (1); arabinose from disubstituted xylose (2); xylose (3); α-dextrin(4); β-glucan (5).

The ^{13}C-NMR spectrum (Fig. 4) revealed more information about the structure of RES1M. About the same proportion of xylose residues containing one or two arabinose residues were present, but the structure was more complex; there were additional peaks in the anomeric region and in the remaining part of the spectrum. The peaks at δ69.7 ppm and δ65.8 ppm were well resolved and intense. They can be attributed to terminal xylose units (Kovac et al 1980, Brillouet and Joseleau 1987, Ebringerová et al 1990, Ebringerová et al 1992). The presence of 2-linked or 5-linked arabinose, which can be recognized by resonances at δ89.8 ppm and δ67.1 ppm, respectively (Ebringerová et al 1992), could not be deduced from the spectrum (Fig. 4). Unassigned resonances could originate from other substituted arabinose residues or from galactose and glucose residues, which were also present in low proportions in the sample (Table IV).

The resemblance between BE1asup and RES1M observed using ^{1}H-NMR (Fig. 3) was confirmed by methylation analysis (Table IV). While ^{1}H-NMR revealed only the complexity of the polymer, methylation analysis showed substituted arabinose residues (2-, 3-, 5-, and 2,3-linked arabinose) up to 40% of total arabinose. Terminal xylose, already detected by ^{13}C-NMR (Fig. 4), made up 26% of the xyloses. Terminal galactose was also detected. All these observations indicated the presence of oligomer side chains. Indeed, terminal xylose and terminal galactose units can be linked through arabinose residues to the xylan chain (Wilkie 1979). The ratio of terminal galactose to substituted arabinose was ~0.23, and the ratio of terminal xylose to substituted arabinose was ~0.59 (samples from Table IV; a correction for xyloglucan had to be made in 4M). Substituted arabinoses were consistent with the observation of unknown peaks in the α-anomeric region of the ^{1}H-NMR spectra. According to Ebringerová et al (1990), 20% of the arabinose moieties were linked 2-, 3-, and 5- in some rye bran arabinoxylans. Substituted arabinoses, terminal xylose, and terminal galactose have been reported as features in arabinoxylans from rye bran (Ebringerová et al 1990); from wheat bran (Brillouet et al 1982, Brillouet and Joseleau 1987, DuPont and Selvendran 1987, Shiiba et al 1993); from rice bran (Shibuya and Iwasaki, 1985); from maize kernels

TABLE IV
Methylation Analysis of Water-Unextractable Arabinoxylan Fractions[a]

Methylated Compound	Linkage Mode	Fraction (Mol %)					
		BE1asup	BE1ares	BE2a	1Ma	4Ma	RES1M
2,3,5-Me_3-Ara	t-Ara*f*	29.3	32.6	22.9	10.9	9.0	29.2
3,5-Me_2-Ara	2-Ara*f*	4.9	1.4	2.0	0.5	0.8	5.0
2,5-Me_2-Ara	3-Ara*f*	7.7	2.1	2.1	0.9	1.6	8.4
2,3-Me_2-Ara	5-Ara*f*	3.3	0.9	1.2	0.6	1.0	2.9
5-Me-Ara	2,3-Ara*f*	3.2	0.5	0.7	...[b]	1.0	2.9
2,3,4-Me_3-Xyl	t-Xyl*p*	11.5	2.9	3.7	2.1	5.1	10.9
2,3-Me_2-Xyl	4-Xyl*p*	7.4	35.4	29.5	65.7	39.5	6.5
2-Me-Xyl	3,4-Xyl*p*	13.5	16.8	11.3	6.1	6.2	14.7
3-Me-Xyl	2,4-Xyl*p*	(7.6)[c]	(10.4)	(9.2)	(9.3)	(6.6)	(6.2)
Xyl	2,3,4-Xyl*p*	11.6	5.7	5.5	1.9	3.7	10.5
2,3,4,6-Me_4-Gal	t-Gal*p*	4.3	1.2	1.3	...	1.2	4.3
2,4-Me_2-Gal	3,6-Gal*p*	1.1	...	TR[d]	...	...	...
2,4,6-Me_3-Glc	3-Glc*p*	1.0	...	3.5	2.1	1.3	1.1
2,3,6-Me_3-Glc	4-Glc*p*	0.9	0.8	16.7	9.6	20.1	3.7
2,3,-Me_2-Glc	4,6-Glc*p*	...	...	TR	...	2.7	...
2,3,6-Me_3-Man	4-Man*p*	TR	...	TR	...	8.3	TR

[a]BE1asup, BE1ares = ammonium sulfate fractionation fractions from the WU_I $Ba(OH)_2$ extract (BE1). BE2a, 1Ma, 4Ma = extracts obtained by sequential alkaline extraction from WU_I. RES1M = extract obtained after delignification of the alkaline-unextractable residue from WU_I. WU_I = water unextractable arabinoxylans from rye wholemeal.
[b]Not detected.
[c]2-Me-Xyl and 3-Me-Xyl coeluted, the ratio of 2-Me-Xyl to 3-Me-Xyl was measured using MS and presented within parentheses.
[d]Trace.

(Saulnier et al 1993); and from sorghum husk (Woolard et al 1977). Quantitatively, the results of methylation analysis of BE1asup and RES1M corresponded quite well with results described for wheat beeswing bran (Brillouet and Joseleau 1987, DuPont and Selvendran 1987).

In rye water-extractables, arabinoxylan fractions with high A/X (1.09, 1.34, and 1.42) were isolated (Vinkx et al 1993, 1995). However, they contained only trace levels of terminal xylose (up to 2% of xyloses) and substituted arabinoses (up to 2% of arabinoses). Therefore, they were not comparable to high A/X alkali-extractable arabinoxylans. In the rye water-extractables, trace amounts of substituted arabinose and terminal xylose are ascribed to contamination with bran polymers of the class of high A/X arabinoxylans. In wheat flour water-extractables, fractions in which up to 10% of arabinoses were substituted were reported by Izydorczyk et al (1993).

Low A/X arabinoxylans. The presence of a third group consisting of low branched xylans, was deduced from the data for 1M (Table IV). Hromádková et al (1987) extracted from rye bran an arabinoxylan with A/X 0.14 with 1.1 *M* NaOH and precipitated it by acidification of the alkaline extract. This arabinoxylan was water-insoluble and was substituted with single arabinose units at position 3 of every sixth or seventh xylosyl residue. It represented ~50% of the nonstarch and noncellulosic polysaccharides in rye bran (Hromádková et al 1987). In 1M, apart from terminal arabinose and unsubstituted and monosubstituted xylose residues, minor amounts of disubstituted xyloses were measured. Because Hromádková et al (1987) detected no disubstituted xyloses in rye arabinoxylans with a low degree of branching and 3% dxyl was measured in 1M, possibly 1M was not a pure arabinoxylan of this class (the A/X of 1M was 0.21). The isolation procedure included no separation between the water-soluble and water-insoluble fraction of the 1M extract. It was observed, however, that 1M had a low solubility.

The presence of both 4-linked glucose and 4-linked mannose in 4M (Table IV) was an indication of glucomannans (Fincher and Stone 1986, Gruppen et al 1992). The detection of 4,6-linked glucose also in 4M was an indication of xyloglucans, β-(1→4)-glucan with terminal α-xylose units linked at *O*-6 of glucose residues, which are known to solubilize in 4.3*M* KOH but not in 0.7*M* KOH (Hayashi 1989).

TABLE V
Relative Proportions of the Partially Methylated Xylose Residues Calculated from Methylation Analysis Data[a]

	BE1asup	BE1ares	BE2a	1Ma	4Ma	RES1M
% uxyl	17	58	59	87	72	15
% 3mxyl	27	25	20	7	10	30
% 2mxyl	4	2	2	1	1	5
% dxyl	26	9	11	3	7	25
% txyl	26	5	7	3	10	26

[a] BE1asup, BE1ares = ammonium sulfate fractionation fractions from the WU_I $Ba(OH)_2$ extract (BE1). BE2a, 1Ma, 4Ma = extracts obtained by sequential alkaline extraction from WU_I. RES1M = extract obtained after delignification of the alkaline-unextractable residue from WU_I. WU_I = water unextractable arabinoxylans from rye wholemeal. uxyl = xylose residue unsubstituted with arabinose, 3mxyl = 3-monosubstituted xylose, 2mxyl = 2-monosubstituted xylose, dxyl = disubstituted xylose, txyl = terminal xylose.

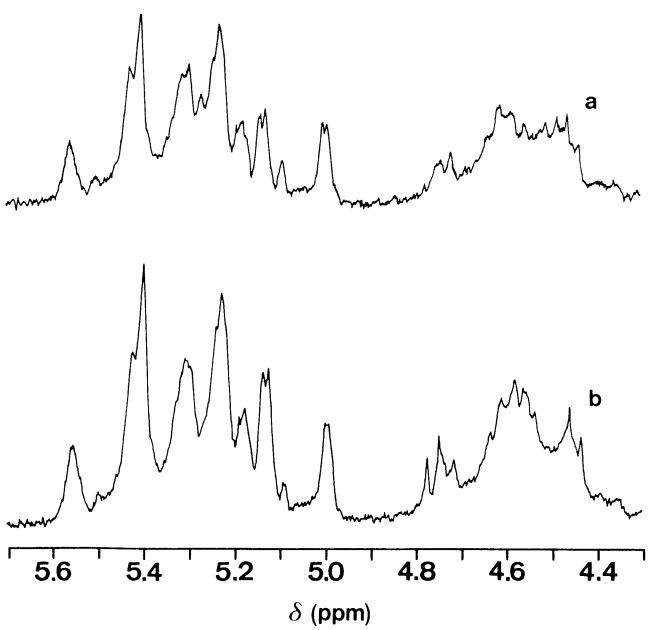

Fig. 3. Diagnostic regions of the ^{1}H-nuclear magnetic resonance spectra (D_2O, 85°C, 300 MHz) of rye alkali-extracted arabinoxylan fractions BE1asup (a) and RES1M (b).

Arabinoxylan Classes and Literature Data

A strong similarity between the alkali-extractable arabinoxylans from rye and wheat was found when examining literature data. Three different groups of alkali-extractable arabinoxylans were described for wheat bran. The first group precipitated upon neutralization of the alkaline extract (hemicellulose A). This group represented about 28% of the arabinoxylan in industrial wheat bran (Brillouet and Mercier 1981). It had an A/X of 0.18–0.25 (Brillouet and Mercier 1981, DuPont and Selvendran 1987) and was probably located in the aleurone (Brillouet and Joseleau 1987). The water-soluble alkali-extracted arabinoxylans were divided into two groups. The intermediate branched arabinoxylans contained the usual structural elements (terminal arabinose, 4-, 3, 4-, and 2,3,4-linked xylose). The main bran arabinoxylan was highly branched (A/X 0.9–1.23) and apart from the usual elements, also contained substantial amounts of substituted arabinoses (2-, 3-, 5-, or 2,3-linked), terminal xylose, terminal galactose, and terminal (4-*O*-methyl-)glucuronic acids (Brillouet et al 1982, Brillouet and Joseleau 1987, DuPont and Selvendran 1987, Shiiba et al 1993).

The isolation procedure of Saini and Henry (1989) did not permit a distinction between different arabinoxylan classes in the alkaline extracts from rye grain. Ebringerová et al (1990) probably described a mixture of rye bran high and intermediate A/X arabinoxylans. The authors reported A/X and proportions of substituted arabinoses that were in between the results for the intermediate and the high A/X arabinoxylans described in this work. Only two different groups of arabinoxylans were recognized in rye bran (Hromadková et al 1987, Ebringerová et al 1990), while in this work three groups are described, much as what has been reported before for wheat bran.

Molecular Weight Distributions

Apparent molecular weights of solubilized rye water-unextractable arabinoxylans were in the same range (Fig. 5) as those

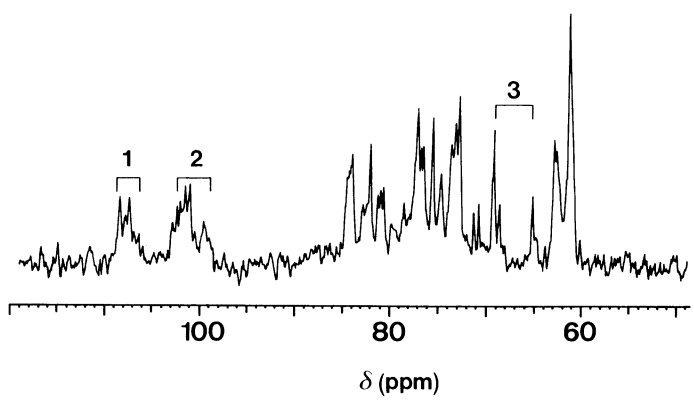

Fig. 4. ^{13}C-nuclear magnetic resonance spectrum (D_2O, 85°C, 75 MHz) of the rye arabinoxylan fraction with high arabinose to xylose ratio (RES1M): arabinose anomeric region (1); xylose anomeric region (2); terminal xylose (3).

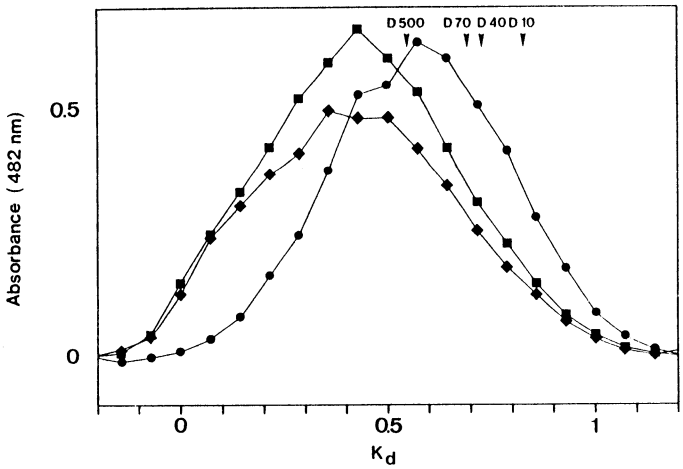

Fig. 5. Size-exclusion chromatography of rye water-soluble alkali-extracted fractions: BE1 (■); BE2 (◆); and BE1sup (●). Dextrans (10, 40, 70, and 500 kDa) were used as standards.

reported for water-extractable rye arabinoxylans (Vinkx et al 1993). No molecular weight distribution was obtained for 1M and 4M fractions because of the low solubility of these preparations.

Extractability of Arabinoxylans

The low A/X of the arabinoxylans in 1M possibly explains why this fraction was neither water-extractable nor water-soluble after alkali-extraction. Andrewartha et al (1979) found that wheat arabinoxylans with A/X <0.43 were water-insoluble because of noncovalent bonds between unsubstituted xyloses. In vitro noncovalent bonds between arabinoxylans (A/X 0.44) and cellulose and among arabinoxylans have been reported for barley aleurone (McNeil et al 1975). The authors found that arabinoxylans with a high A/X (1.38) did not bind to cellulose in vitro. However, the observation that arabinoxylans that could not be solubilized from wheat flour water-unextractables by endoxylanase had a high A/X was interpreted as an indication that in vivo, a high degree of substitution does not hinder binding to cellulose (Gruppen et al 1993).

The other two arabinoxylan classes were water-soluble after alkaline extraction. Extraction involves cleavage of the native bonds in the cell wall matrix. The extent to which these bonds occur causes differences in the extractability of the different arabinoxylans (Hromádková and Ebringerová 1992). Also, Gruppen et al (1992) concluded that the difference in extractability between water-extractable and $Ba(OH)_2$-extractable wheat flour arabinoxylans was not caused by structural differences, but by differences in covalent or noncovalent bonds, while structural differences might have influenced the differences in extractability in different alkaline media. Markwalder and Neukom (1976) found evidence that alkali-labile diferulic acid cross-links reduced the water-extractability of wheat flour arabinoxylans. A comparable mechanism may contribute to the water-unextractability of rye arabinoxylans (Gibeaut and Carpita 1992). In addition, there is ample evidence that different types of covalent bonds maintain lignin and arabinoxylan associated in the secondary cell wall, and that most of these are, to some extent, susceptible to alkaline cleavage (Iiyama et al 1990, Joseleau et al 1992, Iiyama et al 1994). The results of the present work suggest that lignin was an embedding matrix for, or was linked to, arabinoxylans with not only a high A/X, but also specific structural features, such as terminal xylose and substituted arabinoses. Part of these arabinoxylans could be released by $Ba(OH)_2$; a part could be released by $NaClO_2$ treatment, followed by alkaline extraction.

It was not clear why some arabinoxylans were extracted in BE2 and not in BE1. Saturated $Ba(OH)_2$ containing 260 m*M* $NaBH_4$ kept the major part of the water-soluble β-glucans insoluble while it solubilized arabinoxylans (Gruppen et al 1991). Possibly, physical entanglements with β-glucan played a role in the extractability of the intermediate A/X arabinoxylans. A release of the arabinoxylans then occurred when the β-glucans solubilized.

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