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Heterogeneity in the Fine Structure of Alkali-Extractable Arabinoxylans Isolated from Two Rye Flours with High and Low Breadmaking Quality and Their Coexistence with Other Cell Wall Components

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The alkali extractable (AE) arabinoxylans from two rye flours differing in baking quality were studied following sequential extraction of water-unextractable and starch-free rye flour residue with saturated barium hydroxide solution, water and 1 M sodium hydroxide solution (Ba, BaH, and Na, respectively), and further fractionation of isolated fractions by ammonium sulfate precipitation. ^{1}H NMR and sugar analyses of AE subfractions provided evidence for the presence of lowly branched arabinoxylans (average arabinose-to-xylose ratio, Ara/Xyl ~ 0.5), containing mainly un- and monosubstituted xylopyranosyl residues (Xyl*p*) in the chain. The proportion of this subfraction decreased from 50% in the Ba fraction to 35 and 17% in the Na and BaH fractions, respectively. Other subfractions, rich in both mono- and disubstituted Xyl*p*, represented arabinoxylan populations with intermediate (Ara/Xyl ~ 0.8) and high substitution degree (Ara/Xyl ~ 1.1). The Ba and Na fractions contained phenolic compounds, whereas they were absent in the BaH fraction. The higher ratio of such phenolic compounds to arabinose (PhC/Ara) found in AE arabinoxylans from rye flour of inferior baking quality was one of the most pronounced differences between arabinoxylan populations from rye flours with high and low baking quality. The arabinoxylans from rye flour of high baking quality present in Ba and Na fractions had slightly higher apparent molecular weights (MWs) when compared to those from rye flour with low baking quality. The arabinoxylans present in the BaH fractions, characterized by the highest MWs, had similar MWs.

KEYWORDS: Rye flour; alkali-extractable arabinoxylans; cell wall components; fractionation; structure

INTRODUCTION

Arabinoxylans are building blocks of cereal cell walls and a major component of cereal dietary fiber (*1*, *2*). The significance of these cell wall polymers in human nutrition and cereal processing is related to the specific properties of both water-extractable (WE) and water-unextractable (WU) arabinoxylans (*3*−*7*). They are composed of a main chain of unsubstituted and mono- and/or disubstituted (1→4)-linked β-D-xylopyranosyl residues (Xyl*p*) with terminal α-L-arabinofuranosyl residues (Ara*f*). Furthermore, to a lesser extent, the chain can be substituted with residues of ferulic, acetic, or uronic acids (*1*). Variability in both the degree and distribution of substituents as well as in molecular size determines arabinoxylan heterogeneity. It is thought that water-unextractability of part of the arabinoxylan population is to an appreciable extent due to the presence of diferulic acid bridges between individual macromolecules, leading to a well-developed, spatial network in the cell wall. An involvement of other cell wall components in cell wall association has also been considered (*2*, *8*−*10*).

In a preceding article (*11*), we described the considerable differences in the structure of arabinoxylans extracted with water at different temperatures from two rye flours with high and low baking quality. A significant proportion of the remaining polysaccharides, left after water extraction, can be efficiently solubilized by alkaline treatment. It is known that hydroxyl ions cause swelling of cellulose, hydrolysis of ester linkages, and disruption of intermolecular hydrogen bonds between cellulose and hemicellulose, bringing a portion of hemicellulosic material into solution. Higher concentrations of hydroxide result in higher yields of extraction when performed at room temperature, indicating a disruption of stronger linkages (*12*). This may involve also diferulic acid bridges between arabinoxylans and lignin (*13*).

A sequential extraction scheme proposed by Gruppen et al. (*14*) introduced the use of $Ba(OH)_2$ as a primary extractant for arabinoxylans, because Ba^{2+} forms insoluble complexes with

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β-glucans (*15*). A large proportion of β-glucans, remaining in such complexes after $Ba(OH)_2$ extraction, can be solubilized subsequently with distilled water acidified to pH 5.0. This allows an enhanced separation of these two major polysaccharides. Some undesirable changes in the physicochemical properties of solubilized polysaccharides cannot be excluded, as alkaline peeling (β-elimination) might be initiated upon drastic alkaline conditions. The presence of sodium borohydride in alkaline extract prevents this action. So far, the consecutive alkaline extraction is one of the most important tools in the structural characterization of WU cell wall polymers. The application of enzymes of high specificity, such as feruloyl esterases (*16*), might prove to be an alternative. This would require, however, further knowledge on the whole spectrum of existing linkages by which the WU components are held together in the cell walls.

Research efforts published to date on the structure of rye WU nonstarch polysaccharides have focused mostly on those of whole rye (*17*−*19*) or bran (*20*−*23*). Much less attention has been paid to their rye flour counterparts (*18*). It is known that large differences in the structure and distribution of biopolymers exist between different botanical tissues of cereal kernels. In matured grain, the outer layers are rich in lignified cells walls. Consequently, the arabinoxylans in these tissues are partly substituted with residues of uronic and acetic acids. The cell walls of aleurone, starchy endosperm, and scutellum are characteristic of unlignified, primary cell walls (*1*, *24*) containing essentially neutral arabinoxylan. In rye flour, the cell wall polymers originate mainly from the endosperm cell walls of rye grains (*24*). Therefore, the cell wall preparations from such material contain, in general, only one type of tissue, enabling its easier fractionation and characterization.

When compared to starch and protein, WU cell wall polysaccharides only constitute a minor part of rye flour. It is thought, however, that they have a deleterious impact on its baking functionality. In this respect, especially arabinoxylans have received considerable attention, as the major cell wall components with discernible water holding capacity (*7*, *25*−*26*). Recently, the negative relationships between WU arabinoxylan content and baking quality parameters have been found in rye flours from a 3-year breeding experiment, clearly validating this statement (*27*).

The aim of this study was to elucidate the structure of WU arabinoxylan from rye flour with high and low baking quality, isolated by sequential alkaline treatment and fractionated by ammonium sulfate precipitation and to point out the potential structural differences that could help in explaining their different baking quality.

MATERIALS AND METHODS

Rye Flours. Two Polish rye cultivars, Amilo and Nawid, with high and low baking quality, respectively, were selected from breeding material grown in central Poland (DANKO, Plant Breeding Co., Laski) in 2000. Samples were tempered to 14.0% moisture and milled on a Quadrumat Senior laboratory mill (Brabender, Duisburg, Germany) to obtain a straight grade flour. Milling yields (14.0% moisture base), protein, and ash contents (percent dm) were 58 and 63%, 7.2 and 7.6%, and 0.9 and 1.0%, for Amilo and Nawid, respectively. More detailed quality characteristics of both flours were reported earlier (*11*).

Consecutive Alkaline Extraction. The WU materials that were obtained from the above-mentioned rye flours after sequential extraction of the flour with water at 4, 40, and 100 °C in the presence of a thermostable α-amylase (*11*) were used as a starting point for alkaline extraction according to the method by Gruppen et al. (*14*). The samples were thus suspended in saturated $Ba(OH)_2$ solution (500 mL) containing 1% (w/v) $NaBH_4$, stirred continuously for 16 h at room temperature, and centrifuged (10 000*g*, 20 min) (**Figure 1**). The residue was re-extracted with the same extractant (400 mL) for 3 h at room temperature and again centrifuged (10 000*g*, 20 min). The combined supernatants were neutralized with glacial acetic acid and purified as described below. The isolated material is referred to as Ba. The residue left after extraction with saturated $Ba(OH)_2$ was suspended in deionized water (500 mL), acidified to pH 5.0, stirred continuously for 15 h at room temperature, and centrifuged (10 000*g*, 20 min). The residue was re-extracted with deionized water (400 mL) for 3 h at room temperature. Both supernatants were combined and underwent purification as described below. The obtained material is referred to as BaH. The residue was further extracted with 1 M NaOH (500 mL) containing 1% (w/v) $NaBH_4$ for 16 h at room temperature with continuous stirring and centrifuged (10 000*g*, 20 min). The residue was again extracted with the same extractant (400 mL) for 3 h at room temperature. The pooled supernatants were neutralized with glacial acetic acid and purified, as described below. The resulting material is referred to as Na.

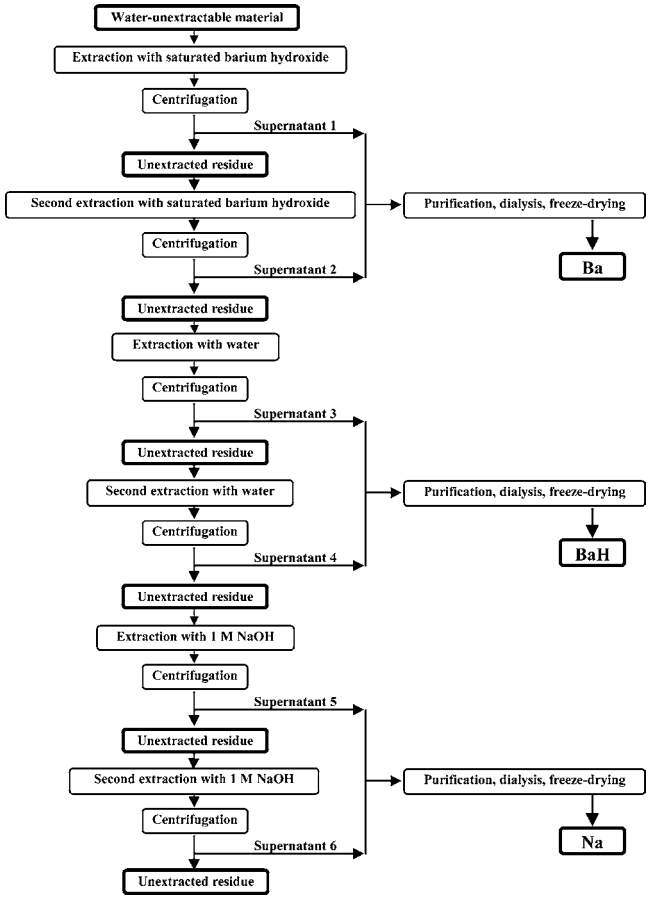

Figure 1. Scheme for sequential extraction and isolation of AE fractions from rye flour.

Purification and Fractionation of AE Materials. The crude alkaline extracts were incubated with α-amylase from porcine pancreas (100 μL, 33 units/μL) (EC 3.2.1.1, Sigma-Aldrich) and proteinase K from *Tritirachium album* (1.25 mL, 600 units/mL) (EC 3.4.21.64, Roche Diagnostics GmbH, Mannheim, Germany) at 40 °C, overnight (pH 6.8, 10 mM $CaCl_2$, 0.03% NaN_3) with occasional stirring. Heat treatment was used to inactivate enzymes (20 min, 95 °C), and precipitated material was separated by centrifugation (10 000*g*, 20 min). The supernatants were dialyzed against deionized water (48 h, 6 °C) using membrane tubing (MW cutoff of 12 000; Sigma-Aldrich). The resulting extracts were incubated with amyloglucosidase from *Aspergillus niger* (1.0 mL) (EC 3.2.1.3, Roche Diagnostics GmbH, Mannheim, Germany) at 40 °C, overnight (pH 4.8, 0.03% NaN_3), heat treated (20 min, 95 °C), and centrifuged (10 000*g*, 20 min). The low molecular weight components were eliminated by dialysis against deionized water at 6 °C. Aliquots of the purified extracts (200−400 mL) were freeze-dried.

Table 1. Yield and Composition of Three Alkaline Fractions (Ba, BaH, and Na) Obtained by Consecutive Extraction with Barium Hydroxide, Water, and Sodium Hydroxide from Two Experimental Rye Flours[a]

						molar composition[d]						
fraction	yield[b]	arabinoxylans[b]	total sugars[c]	protein[c]	ash[c]	Ara	Xyl	Man	Gal	Glc	total Ara + Xyl	Ara/Xyl
					Amilo							
Ba	1.66	0.69	50.1	27.1	2.8	34.3	49.2	0.8	0.9	14.8	83.5	0.70
BaH	0.52	0.14	84.5	5.8	na	15.8	20.1	1.1	1.0	62.0	35.9	0.79
Na	2.54	0.51	51.2	37.6	6.3	17.3	24.6	2.6	1.6	53.9	41.9	0.70
					Nawid							
Ba	1.54	0.61	45.1	28.4	9.2	41.0	59.0	nd	nd	nd	100.0	0.69
BaH	1.37	0.45	78.2	2.8	na	21.9	26.0	2.4	2.5	47.2	47.9	0.84
Na	1.87	0.33	43.8	36.3	9.1	20.1	26.0	6.4	2.5	45.0	46.1	0.77

[a] Abbreviations: arabinoxylans = 0.88 × (Ara + Xyl); Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose; Ara/Xyl, arabinose-to-xylose ratio; na, not analyzed; nd, not detected. [b] Expressed as weight percentage of flour. Results are presented as means of two extractions; the coefficient of variation was less than 4%. [c] Expressed as weight percentage of Ba, BaH, and Na, respectively. Results obtained from triplicates; the coefficient of variation was less than 2%. [d] Expressed as percentage (mol/100 mol). Results obtained from triplicates; the coefficient of variation was less than 2%.

The remaining extracts were directly fractionated by graded ammonium sulfate precipitation, as described earlier (*28*). The AE subfractions were thus precipitated from each extract by stepwise addition of $(NH_4)_2SO_4$, left overnight at 4 °C, and separated by centrifugation (10 000*g*, 20 min). The precipitated material was redissolved in deionized water, dialyzed at 6 °C until free of $(NH_4)_2SO_4$ (judged by conductivity measurement), and freeze-dried. Collection was accomplished at 40, 60, 80, and 100% saturation level. Generally, four subfractions were obtained from each fraction. Ba yielded Ba.40, Ba.60, Ba.80 and Ba.100 (numbers refer to the saturation level of $(NH_4)_2SO_4$ at which subfractions were collected). In the case of rye flour Nawid, no precipitate could be collected at 40% saturation. BaH produced BaH.40, BaH.60, BaH.80 and BaH.100 and, accordingly, four subfractions (Na.40, Na.60, Na.80, and Na.100) were collected from Na from both flours.

Chemical Analyses. Protein content in purified cell wall materials was determined according to Lowry et al. (*29*) using bovine serum albumin as a standard. Ash was determined by AACC method, 46.11A (*30*). Sugar analysis was performed after hydrolysis with 1 M sulfuric acid (2 h, 100 °C) followed by conversion of the monosaccharides to their alditol acetates (*31*). Samples were quantified on a Hewlett-Packard model 5890 Series II Plus gas chromatograph (Waldbronn, Germany) equipped with a 30-m × 0.53-mm i.d. wide bore Rtx 225 capillary column (Restek, Bellefonte, PA) and flame ionization detector. The injector and detector were maintained at 230 and 250 °C, respectively. The column was heated at 190 °C for 2 min, then the temperature program was 190−220 °C at 5 °C/min and 220 °C for 5 min. Output signals were collected and integrated by ChemStation software (Hewlett-Packard). β-D-allose (Sigma-Aldrich) was used as internal standard. The arabinoxylan content was estimated as 0.88 times the sum of arabinose and xylose.

^{1}H Nuclear Magnetic Resonance Spectroscopy. Samples were dissolved in D_2O (99.8% D) with overnight stirring at room temperature and freeze-dried. This step was repeated once, and finally the deuterium-exchanged material was redissolved in D_2O (5 mg/mL) and centrifuged (10 000*g*, 10 min) before analysis. ^{1}H NMR spectra were recorded on a Bruker 500-MHz spectrometer (Bruker, Karlsruhe, Germany) at 85 °C. Acetone was used as standard (δ 2.23 ppm).

GPC. Samples (6.0−12.0 mg) were solubilized overnight at room temperature in 0.3% NaCl (3 mL). The solution was filtered (Profill 0.45 μm disposable filter, Alltech Associates Inc., Illinois, USA) and the polysaccharide materials were fractionated with a Kontron Instruments 325 System HPLC (Milan, Italy) equipped with a Kontron Instruments 465 autosampler and on line monitoring with a refractive index detector (VDS Optilab, Berlin, Germany) and a Kontron Instruments 332 UV detector (280 nm). Samples were eluted isocratically with 0.3% NaCl (0.5 mL/min) at 30 °C on a 300-mm × 8-mm i.d. Shodex SB-806 HQ GPC column (Showa Denko K. K., Tokyo, Japan) connected to a 50-mm × 6-mm i.d. SB-800P guard column. The column was calibrated with Shodex standard P-82 pullulan standards (Showa Denko K. K.) with MWs of 78.8×10^4, 40.4×10^4, 21.2×10^4, 11.2×10^4, 4.73×10^4, 2.28×10^4, 1.18×10^4, 0.59×10^4.

Lichenase Treatment. Samples containing substantial levels of β-glucan, as revealed by ^{1}H NMR spectroscopy, were solubilized in 0.3% NaCl and incubated with lichenase, (1→3), (1→4)-β-D-glucan-4-glucanohydrolase from *Bacillus subtilis* (EC 3.2.1.73, 50 μL, 50 units/mL) (Megazyme International Ireland Ltd., Bray, Ireland) overnight at room temperature. Following heat inactivation (20 min, 95 °C) and centrifugation (10 000*g*, 10 min), the supernatants were analyzed using GPC as described above. As a control, the same sample without enzyme addition was run.

RESULTS AND DISCUSSION

Extraction of Hemicellulosic Material. The yield and chemical composition of three AE fractions, isolated from two rye flours with saturated barium hydroxide solution, water and 1 M sodium hydroxide solution (Ba, BaH and Na, respectively), are shown in **Table 1**. The total yields of arabinoxylans solubilized by a sequential alkaline treatment constituted 1.34 and 1.39% of rye flour for Amilo and Nawid, respectively. This represented 36.8 and 43.4% of the overall arabinoxylan in the flour, while the WE arabinoxylans comprised 61.0 and 52.5%. Only 2.2 and 4.1% of arabinoxylans were left in the final residues (data not shown).

With saturated $Ba(OH)_2$ solution, 52 and 44% of total AE arabinoxylans in Amilo and Nawid, respectively, were extracted. For the BaH fractions, this was 10% for Amilo and 32% for Nawid. The latter fractions were enriched in sugar residues (85 and 78%), while in Ba and Na fractions, the total content of sugars was only up to 51%. Similar trends were previously observed in AE material from sieved rye flour by Nilsson et al. (*18*). Despite intensive proteolysis during purification of the solubilized hemicellulosic materials, a large amount of protein was found in the Ba and Na fractions (27.1 and 37.6% for Amilo, and 28.4 and 36.3% for Nawid, respectively). The BaH fractions were characterized by a substantially lower protein content, 5.8 and 2.8%, respectively (**Table 1**).

Saturated $Ba(OH)_2$ solution preferentially extracted arabinoxylans: they constituted 84 and 100% of polysaccharides in Ba fractions from Amilo and Nawid. Subsequent water extraction solubilized a majority of the β-glucans from Amilo (as shown by sugar analysis and ^{1}H NMR, **Figure 2b**). This tendency was not pronounced in the case of Nawid, where similar proportions of arabinoxylans and β-glucans were found in BaH and Na fractions. It is pertinent to point out here that

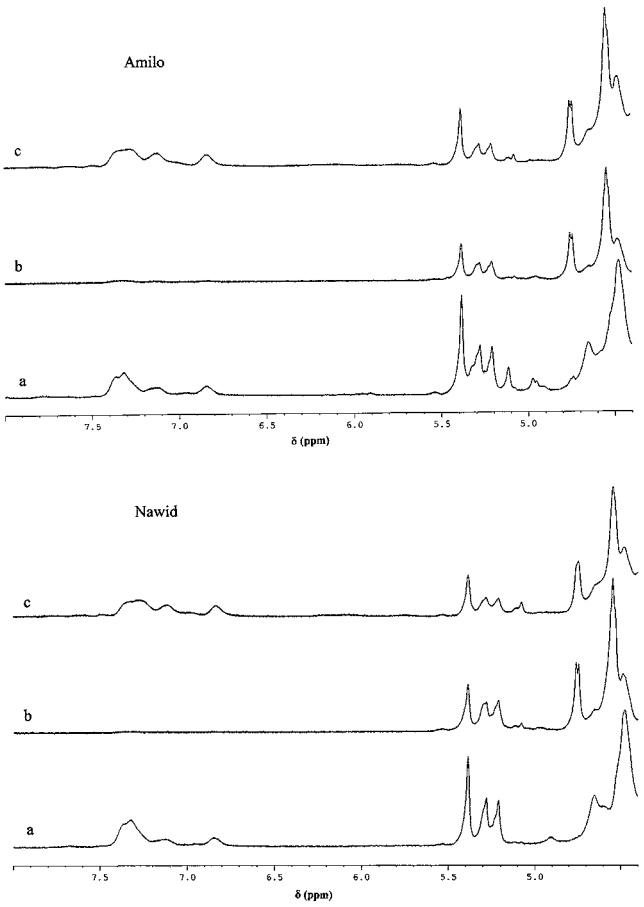

Figure 2. ^{1}H NMR spectra of the AE fractions (Ba, BaH, and Na) obtained from two rye flours Amilo and Nawid by successive extraction with: (a) $Ba(OH)_2$, (b) water, and (c) NaOH.

the Ara/Xyl of arabinoxylans in the BaH fraction was notably higher in comparison to those present in the Ba and Na fractions, indicating a higher substitution degree of arabinoxylans coextracted with β-glucans during a second step of sequential extraction.

Fractionation of AE Materials. Ammonium sulfate precipitation permitted the isolation of 4 polysaccharide populations from each AE fraction. Their yields and composition are presented in **Tables 2** and **3** for Amilo and Nawid, respectively. Polysaccharide material that precipitated at 40% saturation was almost exclusively composed of β-glucans (as revealed by sugar analysis and ^{1}H NMR, **Figures 3a** and **4a**). Relatively small amounts of arabinoxylans coprecipitated with β-glucans in these subfractions. The subfractions obtained at 60, 80, and 100% salt saturation contained mainly arabinoxylan with a progressively higher substitution degree, as pointed out by their Ara/Xyl ratios for both flours (**Tables 2** and **3**). The 60% saturation produced lowly branched arabinoxylan structures (Ara/Xyl, 0.48−0.54) from all AE fractions. The arabinoxylans with an intermediate substitution degree (Ara/Xyl, 0.74−0.85) precipitated by increasing the saturation level up to 80%, while 100% ammonium sulfate saturation yielded highly branched arabinoxylan subfractions (Ara/Xyl, 0.95−1.23). Lowly branched structures composed 48 and 52% of total amount of arabinoxylans recovered in Ba and 33 and 36% in Na, whereas they made up only 18 and 17% in BaH fractions for Amilo and Nawid, respectively. For the highly branched structures, the opposite trend was observed; they made up only 5 and 4% of the Ba fraction, 12 and 9% of the Na fraction, and 27 and 14% of the BaH fraction for Amilo and Nawid, respectively. The higher Ara/Xyl ratios of unfractionated arabinoxylan populations present in BaH, when compared with those from Ba and Na fractions (**Table 1**), is due to a low proportion of arabinoxylans with low substitution degrees combined with a high proportion of those with intermediate and high substitution degrees. It is of note that the very lowly branched structures (Ara/Xyl ~0.2) observed by Vinkx and co-workers in rye whole meal (*17*) could not be found here.

Clearly, the BaH subfractions had a low protein content (1−3%). Substantially higher protein concentrations were found in the Ba subfractions (9−17%), while the highest level was observed in subfractions obtained from Na fractions at 80 and 100% ammonium sulfate saturation (35−47%), suggesting a strong association between arabinoxylans and proteinaceous material in the cell wall structure.

Structural Features of Arabinoxylan Populations by ^{1}H NMR. *Unfractionated Alkali-Extractable Cell Wall Materials.* The ^{1}H NMR spectra of the three AE fractions obtained from rye flours Amilo and Nawid can be seen in **Figure 2**. All spectra showed a well-resolved resonance at δ 5.38 ppm, which was assigned to anomeric protons of terminal Ara*f* linked to *O*-3 of singly substituted Xyl*p*. The signals at δ 5.21 and δ 5.28 ppm, representing anomeric protons of terminal Ara*f* linked to *O*-2 and *O*-3 of doubly substituted Xyl*p* (*32*−*35*), were broad. A small signal at δ 5.25 ppm, between the signals of Ara*f* from doubly substituted Xyl*p* and partly overlapping with them, was visible, especially in the Ba fraction (**Figure 2a**). This may indicate the presence of arabinogalactans (*36*, *37*) in the cell wall preparations isolated by alkaline treatment. The doublet at δ 4.74 and δ 4.75 ppm, assigned to the β-anomer in 3-linked glucopyranosyl residues (Glc*p*) in β-glucan (*38*), was prominent in the spectra of BaH and Na fractions (**Figure 2**, parts **b** and **c**), confirming a substantial proportion of this polymer in these samples.

The cell wall material present in BaH was practically free of phenolic compounds, whereas those from Ba and Na contained a substantial amount of phenolics, as indicated by strong resonances in the region of phenolic moieties (δ 6−8 ppm) of their spectra (*39*−*41*). Because alkaline treatment cleaves covalent and noncovalent bonds between arabinoxylans and other cell wall polymers, in general, and feruloyl ester linkages, releasing ferulates and diferulates, in particular, the strong resonances observed at δ 6−8 ppm clearly indicate that also other types of phenolic components or linkages, which are still present in the solubilized hemicellulosic material, might be responsible for the association of polymers in the cell walls. These may be phenolic compounds ether-linked to sugar units of the wall polysaccharides, because is known that such linkages are likely to be resistant to alkaline hydrolysis (*42*). However, it is also possible that a small quantity of ferulates and diferulates, being physically enclosed in a densely branched structures, could survive an alkaline treatment, as noted earlier for maize bran heteroxylans (*43*). In addition, the presence of some lignin structures in arabinoxylan preparations, possibly at very low concentration, cannot be excluded (*43*).

The AE arabinoxylans were further characterized by the relative proportions of un- (u-Xyl*p*), mono- (3-Xyl*p*) and disubstituted (2,3-Xyl*p*) xylopyranosyl residues in the chain, on the basis of results obtained by ^{1}H NMR spectroscopy and sugar analysis. In general, small differences were observed in the level of differently linked Xyl*p* in the arabinoxylan populations from Ba, BaH, and Na (**Table 4**). From this viewpoint, there were no considerable differences between arabinoxylans from the high and low baking quality rye flours.

Table 2. Yield and Composition of Subfractions Isolated by Ammonium Sulfate Precipitation from Three Alkaline Extracts (Ba, BaH and Na) of Rye Flour Amilo[a]

					molar composition[d]						
subfraction	yield[b]	arabinoxylans[b]	total sugars[c]	protein[c]	Ara	Xyl	Man	Gal	Glc	total Ara + Xyl	Ara/Xyl
Ba.40	0.07	0.01	46.3	12.0	7.9	11.2	1.2	nd	79.7	19.1	0.70
Ba.60	0.45	0.29	73.2	10.7	31.4	64.9	nd	nd	3.7	96.3	0.48
Ba.80	0.45	0.27	64.5	12.1	44.3	55.7	nd	nd	nd	100.0	0.79
Ba.100	0.11	0.03	34.0	17.2	51.5	48.5	nd	nd	nd	100.0	1.06
BaH.40	0.24	0.01	56.6	1.3	3.1	3.1	1.9	nd	91.9	6.2	1.00
BaH.60	0.09	0.02	74.6	3.0	13.4	25.3	nd	nd	61.3	38.7	0.53
BaH.80	0.13	0.05	47.0	3.3	35.4	47.7	nd	nd	16.9	83.1	0.74
BaH.100	0.08	0.03	36.5	3.1	45.9	48.5	nd	0.7	4.9	94.4	0.95
Na.40	0.57	0.05	95.6	2.9	3.0	5.5	1.7	nd	89.8	8.5	0.55
Na.60	0.44	0.14	62.7	15.3	17.0	34.6	1.5	nd	46.9	51.6	0.49
Na.80	0.58	0.18	34.5	35.4	39.9	54.0	0.7	nd	5.4	93.9	0.74
Na.100	0.27	0.05	20.8	41.3	51.8	48.2	nd	nd	nd	100.0	1.08

[a] Abbreviations: arabinoxylans = 0.88 × (Ara + Xyl); Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose; Ara/Xyl, arabinose-to-xylose ratio; nd, not detected. [b] Expressed as weight percentage of flour. Results are presented as means of two extractions; the coefficient of variation was less than 4%. [c] Expressed as weight percentage of corresponding subfractions. Results obtained from triplicates; the coefficient of variation was less than 2%. [d] Expressed as percentage (mol/100 mol). Results obtained from triplicates; the coefficient of variation was less than 2%.

Table 3. Yield and Composition of Subfractions Isolated by Ammonium Sulfate Precipitation from Three Alkaline Extracts (Ba, BaH, and Na) of Rye Flour Nawid[a]

					molar composition[d]						
subfraction	yield[b]	arabinoxylans[b]	total sugars[c]	protein[c]	Ara	Xyl	Man	Gal	Glc	total Ara + Xyl	Ara/Xyl
Ba.60	0.46	0.25	59.3	11.6	33.5	66.5	nd	nd	nd	100.0	0.50
Ba.80	0.47	0.21	50.0	8.6	45.7	54.3	nd	nd	nd	100.0	0.84
Ba.100	0.06	0.02	26.0	17.1	51.0	49.0	nd	nd	nd	100.0	1.04
BaH.40	0.60	0.03	74.2	1.5	3.2	3.9	1.4	nd	91.5	7.1	0.82
BaH.60	0.15	0.06	83.3	1.6	18.5	35.6	nd	nd	45.9	54.1	0.52
BaH.80	0.27	0.21	93.0	2.2	38.7	49.5	0.8	1.1	9.9	88.2	0.78
BaH.100	0.10	0.05	59.1	2.9	51.1	45.3	nd	1.2	2.4	96.4	1.13
Na.40	0.30	0.02	68.9	4.4	3.7	6.9	4.8	0.8	83.8	10.6	0.53
Na.60	0.28	0.08	66.6	9.4	15.4	28.4	4.2	1.0	51.0	43.8	0.54
Na.80	0.31	0.10	42.3	43.9	37.9	44.8	4.3	1.4	11.6	82.7	0.85
Na.100	0.12	0.02	19.5	46.8	55.2	44.8	nd	nd	nd	100.0	1.23

[a] Abbreviations: arabinoxylans = 0.88 × (Ara + Xyl); Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose; Ara/Xyl, arabinose-to-xylose ratio; nd, not detected. [b] Expressed as weight percentage of flour. Results are presented as means of two extractions; the coefficient of variation was less than 4%. [c] Expressed as weight percentage of corresponding subfractions. Results obtained from triplicates; the coefficient of variation was less than 2%. [d] Expressed as percentage (mol/100 mol). Results obtained from triplicates; the coefficient of variation was less than 2%.

Table 4. Relative Percentage of Un-, Mono-, and Disubstituted Xylose Residues (u-Xyl, 3-Xyl, and 2,3-Xyl) of Arabinoxylans Isolated from AE Fractions of Rye Flours Amilo and Nawid[a]

	Amilo						Nawid					
fraction	u-Xyl	3-Xyl	2,3-Xyl	sub/un	di/mono	PhC/Ara	u-Xyl	3-Xyl	2,3-Xyl	sub/un	di/mono	PhC/Ara
Ba	51.0	27.9	21.1	0.96	0.76	0.67	50.8	29.6	19.6	0.97	0.66	0.86
BaH	43.5	34.2	22.3	1.30	0.65	tr	41.8	32.0	26.2	1.39	0.82	tr
Na	48.3	33.2	18.5	1.07	0.56	1.30	45.2	32.7	22.1	1.21	0.68	1.53

[a] Abbreviations: u-Xyl, unsubstituted β-(1→4)-linked D-xylopyranosyl residue; 3-Xyl, β-(1→4)-linked D-xylopyranosyl residue substituted with α-L-arabinofuranosyl residue at *O*-3; 2,3-Xyl, β-(1→4)-linked D-xylopyranosyl residue substituted with α-L-arabinofuranosyl residue at *O*-2 and *O*-3; sub/un, ratio of substituted to unsubstituted xylopyranosyl residues; di/mono, ratio of 2,3-Xyl to 3-Xyl; PhC/Ara, the ratio of total resonance in phenolic compounds region to total resonance from arabinose anomeric protons; tr, traces.

The arabinoxylans from the BaH fractions displayed somewhat higher proportion of 2,3-Xyl*p* (22.3 and 26.2%, for Amilo and Nawid, respectively), in comparison to the level of such residues in the Ba and Na fractions, ranging from 18.5 to 22.1% for both flours. Consequently, the level of u-Xyl*p* in arabinoxylans from BaH was lower (43.5 and 41.8%) than that in Na (48.3 and 45.2%) and Ba fractions (51.0 and 50.8%, for Amilo and Nawid, respectively), whereas the levels of 3-Xyl*p* were slightly higher for arabinoxylans from the BaH and Na fractions (34.2 and 33.2% for Amilo and 32.0 and 32.7% for Nawid) when compared to those from Ba fraction (27.9 and 29.6%). A difference in the ratio of phenolic compounds to arabinose residues (PhC/Ara) was noticed in the Ba and Na fractions; higher values of these attributes were observed for rye flour Nawid of inferior baking quality.

Subfractions Obtained by Ammonium Sulfate Precipitation. The anomeric region of Ara*f* in the ^{1}H NMR spectra of fractionated AE materials confirmed, on one hand, a large

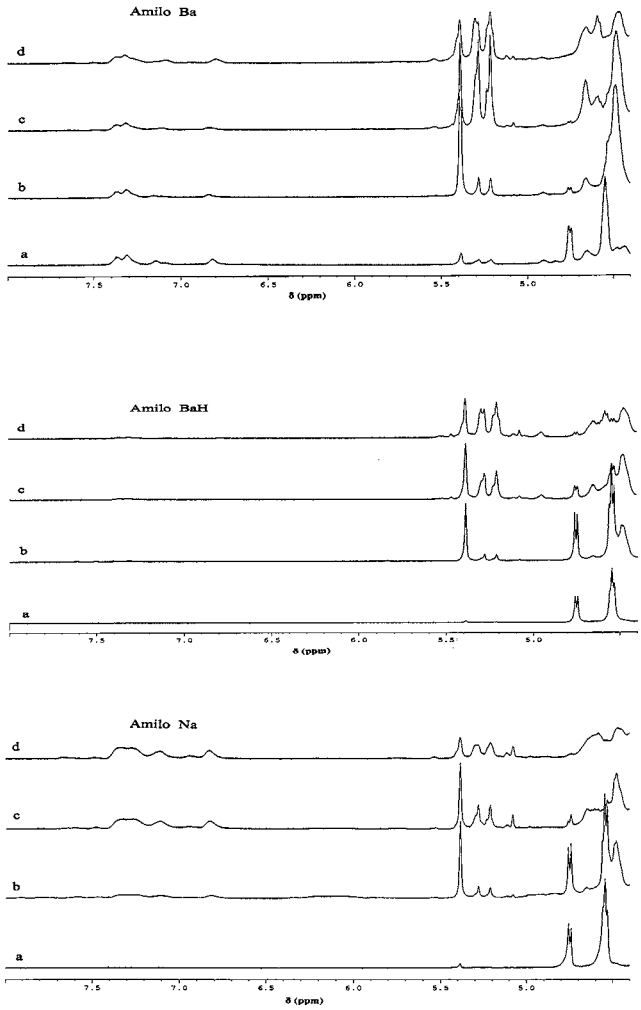

Figure 3. [1]H NMR spectra of the of the subfractions obtained from three main AE fractions (Ba, BaH, and Na) of rye flour Amilo by ammonium sulfate precipitation at different saturation levels: (a) 40, (b) 60, (c) 80, and (d) 100%, respectively.

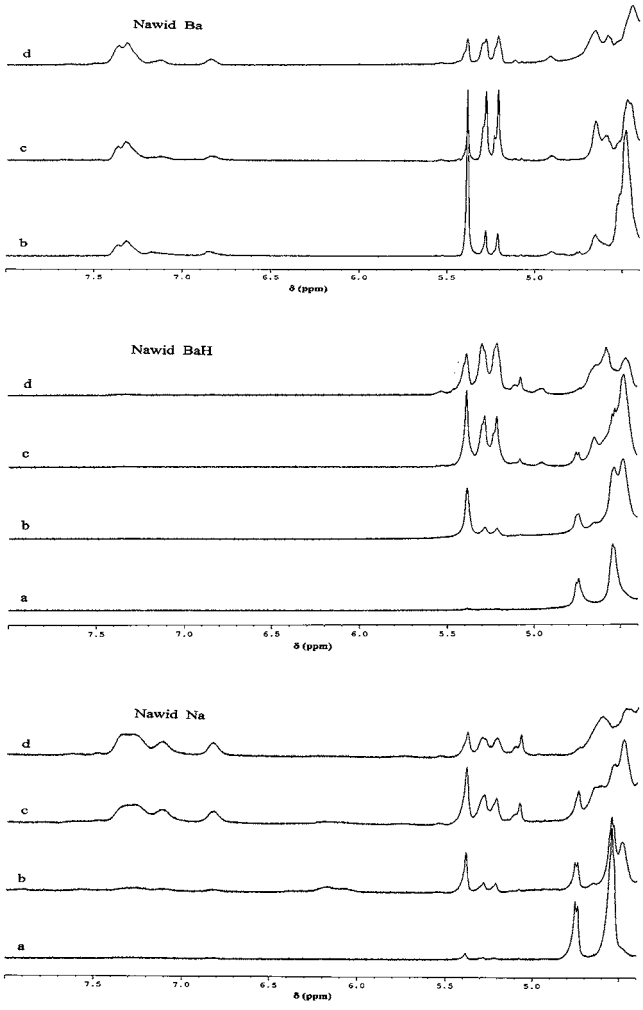

Figure 4. [1]H NMR spectra of the of the subfractions obtained from three main AE fractions (Ba, BaH, and Na) of rye flour Nawid by ammonium sulfate precipitation at different saturation levels: (a) 40, (b) 60, (c) 80, and (d) 100%, respectively.

variation in the arabinoxylan structures precipitated from both rye flours at 60, 80, and 100% ammonium sulfate saturation (**Figures 3** and **4** for Amilo and Nawid, respectively). On the other hand, the fact that comparable profiles of Ara*f* resonance were obtained for arabinoxylans precipitated at the same saturation level from all AE fractions illustrates that the entire arabinoxylan population present in Ba, BaH, and Na fractions consists of structural analogues. The changes in the profiles of Ara*f* signals in the spectra of subfractions precipitated at 60, 80, and 100% saturation suggest different patterns of branching of the arabinoxylan backbone (*32*). The subfractions isolated at 60% salt saturation (**Figures 3b** and **4b**) were characterized by relatively low level of resonances at δ 5.21 and δ 5.28 ppm, originating from terminal Ara*f* linked to *O*-2 and *O*-3 of doubly substituted Xyl*p* (*32*−*35*). Generally, an increase in such resonances was observed in the spectra of subfractions precipitated at 80 and 100% saturation with concomitant decrease of resonances at δ5.38 ppm, arising from terminal Ara*f* linked to *O*-3 of singly substituted Xyl*p*. Resonance signals originating from phenolic compounds (δ 6.5−7.5 ppm) were clearly visible in the spectra of the Ba and Na subfractions isolated from both rye flours. The spectra of materials precipitated at 40% saturation from BaH and Na mainly showed signals, corresponding to β-glucan structural features (e.g., a doublet at δ 4.74 and δ 4.75 ppm), assigned to (1→4)-β-Glc*p*(1→3) residues, and triplet at δ 4.55 ppm, assigned to (1→3)-β-Glc*p*(1→4) and (1→4)-β-Glc*p*(1→4) residues (*38*). These signals were distinguishable in the spectra of BaH.60 and Na.60, confirming, as mentioned previously, a significant proportion of β-glucans in such preparations. The spectrum of Ba.40 displayed resonance attributed to β-glucans as well. However, this material, being specific only for rye flour Amilo, also contained resonances from Ara*f* and phenolic compounds.

The relative proportion of differently substituted Xyl*p* present in the arabinoxylan chain was calculated and are presented in **Table 5**. The arabinoxylans that precipitated at 60% saturation were built up predominantly of u-Xyl*p* and 3-Xyl*p* residues (55−59% and 32−37%, respectively), with a relatively low level of 2,3-Xyl*p* (6−11%). The AE subfractions obtained at higher saturation levels contained progressively more 2,3-Xyl*p* residues and simultaneously less u-Xyl*p*. The highly substituted arabinoxylan populations isolated at 100% saturation from all AE fractions were enriched in both 3- and 2,3-Xyl*p*. From the results in **Table 5**, it is clear that the subfractions that progressively precipitated from Ba, excluding Amilo Ba.40 and Ba.80, and especially from Na fractions, had increasing PhC/Ara ratios and that values were distinctly higher for Nawid (low baking quality) than for Amilo (good baking quality). Whether this illustrates an essential differences in the architecture of cell walls from high and low baking quality rye flours, connected to an

Table 5. Relative Percentage of Un-, Mono-, and Disubstituted Xylose Residues (u-Xyl, 3-Xyl, and 2,3-Xyl) of Arabinoxylans Isolated from AE Subfractions of Rye Flours Amilo and Nawid[a]

	Amilo						Nawid					
subfraction	u-Xyl	3-Xyl	2,3-Xyl	sub/un	di/mono	PhC/Ara	u-Xyl	3-Xyl	2,3-Xyl	sub/un	di/mono	PhC/Ara
Ba.40	49.9	30.2	19.9	1.00	0.66	3.15						
Ba.60	58.5	35.2	6.3	0.71	0.18	0.43	56.8	36.4	6.8	0.76	0.19	0.66
Ba.80	49.8	21.4	28.8	1.00	1.35	0.21	50.0	16.0	34.0	1.00	2.13	0.71
Ba.100	32.4	29.1	38.5	2.09	1.32	1.03	33.9	28.1	38.0	1.95	1.35	1.13
BaH.40	27.5	42.9	29.6	2.64	0.69	nd	43.4	31.3	25.3	1.30	0.81	nd
BaH.60	55.3	36.5	8.2	0.81	0.22	nd	56.7	34.7	8.6	0.76	0.25	nd
BaH.80	48.2	29.6	22.2	1.07	0.75	nd	46.9	28.2	24.9	1.13	0.88	nd
BaH.100	38.6	27.7	33.7	1.59	1.22	nd	28.5	30.0	41.5	2.51	1.38	nd
Na.40	60.8	23.5	15.7	0.64	0.67	nd	60.4	26.2	13.4	0.66	0.51	nd
Na.60	59.2	32.6	8.2	0.69	0.25	0.64	57.0	32.0	11.0	0.75	0.34	1.07
Na.80	46.6	32.8	20.6	1.15	0.63	1.12	39.4	36.2	24.4	1.54	0.67	1.22
Na.100	29.0	34.0	37.0	2.45	1.09	1.54	19.8	37.2	43.0	4.05	1.16	1.92

[a] Abbreviations: u-Xyl, unsubstituted β-(1→4)-linked D-xylopyranosyl residue; 3-Xyl, β-(1→4)-linked D-xylopyranosyl residue substituted with α-L-arabinofuranosyl residue at *O*-3; 2,3-Xyl, β-(1→4)-linked D-xylopyranosyl residue substituted with α-L-arabinofuranosyl residue at *O*-2 and *O*-3; sub/un, ratio of substituted to unsubstituted xylopyranosyl residues; di/mono, ratio of 2,3-Xyl to 3-Xyl; PhC/Ara, the ratio of total resonance in phenolic compounds region to total resonance from arabinose anomeric protons; nd, not detected.

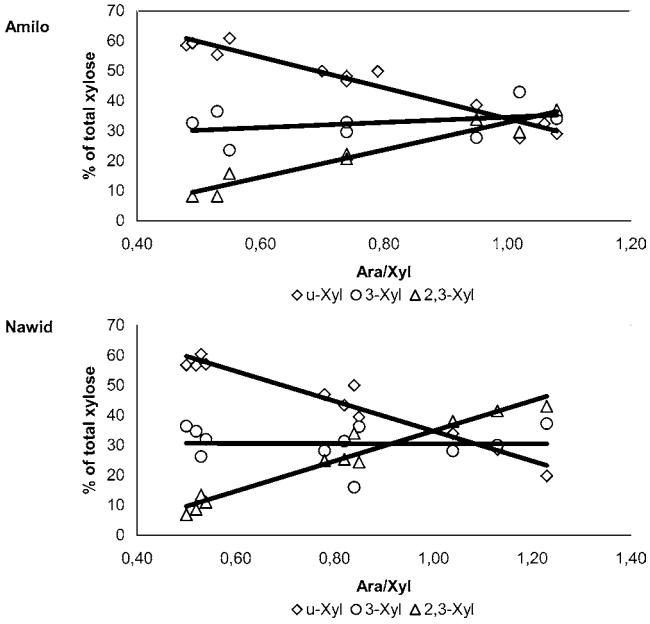

Figure 5. Linear relationships between the relative proportion of differently substituted xylose residues and the ratio of Ara/Xyl of AE arabinoxylans from two rye flours.

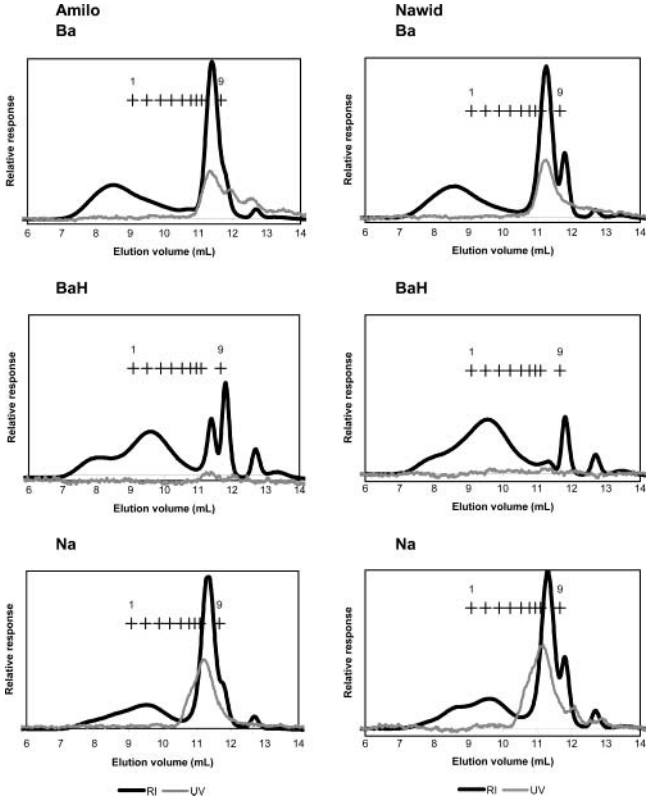

Figure 6. MW profiles of polysaccharides and UV-absorbing materials of three AE fractions (Ba, BaH, and Na) from rye flours Amilo and Nawid. Pullulan calibration standards are (1) 78.8×10^4; (2) 40.4×10^4; (3) 21.2×10^4; (4) 11.2×10^4; (5) 4.73×10^4; (6) 2.28×10^4; (7) 1.18×10^4; (8) 0.59×10^4; (9) glucose.

involvement of phenolic compounds into a more complex structural organization of the cell wall, is not clear.

A plot of the differently substituted Xyl*p* residues as a function of Ara/Xyl ratios using three sets of subfractions (Ba, BaH, and Na), shows that Ara/Xyl was correlated with both levels of un- and 2,3-Xyl*p*, although in opposite way, while the level of monosubstitution remained almost constant (**Figure 5**). The same trend was earlier observed for wheat flour WE arabinoxylans fractionated by graded ethanol precipitation (*44*).

Molecular Weight Distribution. The GPC profiles of the AE fractions from both flours are shown in **Figure 6**. The apparent peak MWs of the polymers that eluted in HMW region were much higher than that of the highest MW pullulan (78.8×10^4) used as standard. The elution volumes of HMW polymers from Ba fraction were 8.55 and 8.64 mL for Amilo and Nawid, respectively, indicating the presence of slightly higher MW polymers in high baking quality rye flour. The cell wall polysaccharides from BaH fractions had the highest apparent MWs and were almost the same for both flours (elution volumes 8.14 and 8.16 mL). As evidenced by lichenase digestion of β-glucans (**Figure 8**), the HMW populations represented arabinoxylans, while the β-glucans showed up in the region of intermediate apparent MW. This was especially visible in the BaH and much less pronounced in the Na profiles. Similar to what was found for the Ba fraction, differences were observed between HMW arabinoxylans from Amilo and Nawid in the elution profiles of the Na samples (elution volumes 8.53 and 8.62 mL, respectively). However, the HMW arabinoxylans present in the Na from Amilo were completely visible only after enzymatic digestion of coexisting β-glucans (**Figure 8**). The

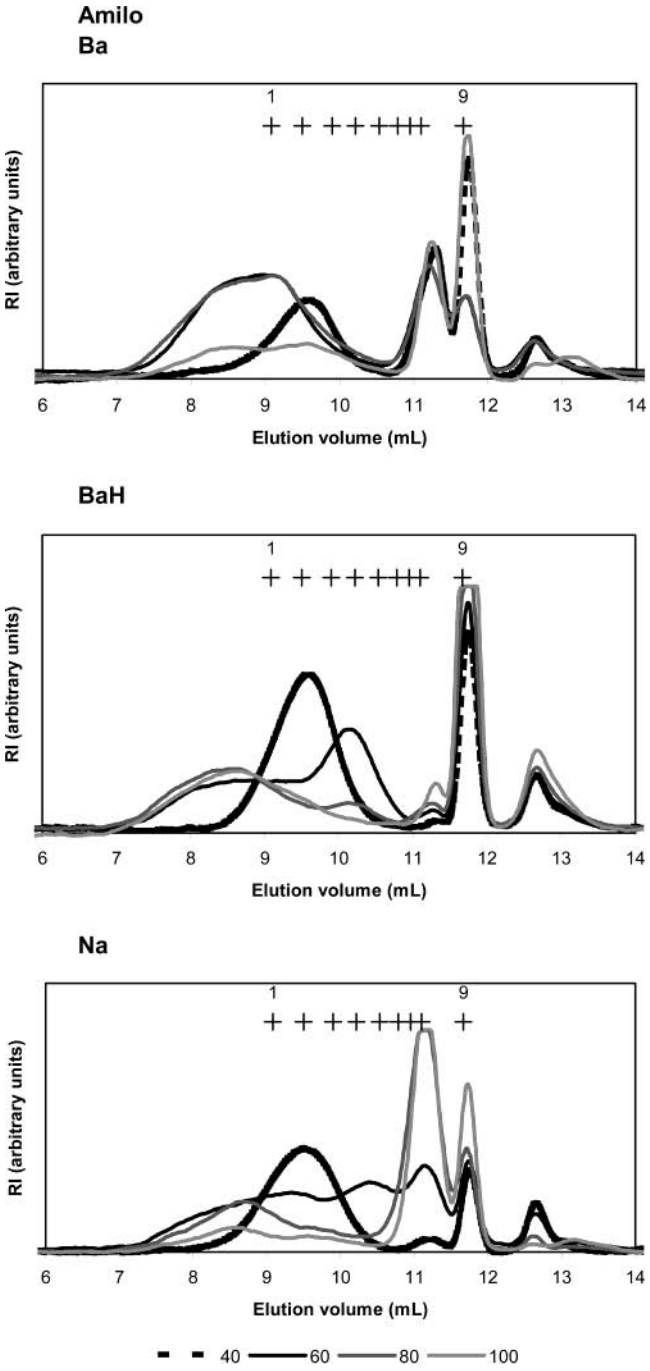

Figure 7. MW distribution of subfractions precipitated at 40, 60, 80, and 100% saturation of ammonium sulfate from three AE fractions (Ba, BaH, and Na) of rye flour Amilo. Elution volumes of pullulan standards are as in **Figure 6**.

strong signals of UV-absorbing materials, which can be ascribed to proteins and/or phenolic components, appeared in the LMW region, indicating a possible association of these components with LMW polysaccharides in both the Ba and Na fractions. Interestingly, three populations of UV-absorbing substances, with different apparent peak MWs, could be recognized in the elution profiles of the Ba fraction from Amilo and Na fraction from Nawid.

The GPC profiles of AE subfractions precipitated at 60, 80, and 100% ammonium sulfate saturation from Amilo overlapped in the HMW region, indicating their similar apparent MWs (**Figure 7**). Such material, representing HMW arabinoxylans (as revealed by lichenase treatment, **Figure 8**) was not present

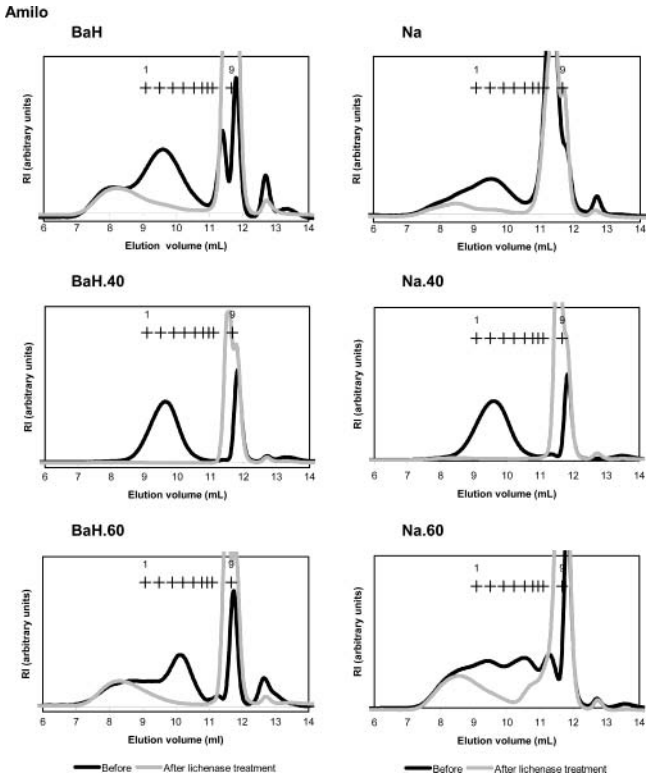

Figure 8. MW distribution of the AE fractions and subfractions of rye flour Amilo before and after lichenase treatment. Elution volumes of pullulan standards are as in **Figure 6**.

in subfractions precipitated at 40% saturation, containing mostly β-glucans with an intermediate apparent peak MW, and small amounts of LMW arabinoxylans (supported by sugar analysis, **Tables 2** and **3**). Analogous trends were observed for subfractions obtained from Nawid (results not shown).

Lichenase treatment of the subfractions enriched in β-glucans (as indicated by ^{1}H NMR, **Figures 3** and **4**) provided evidence that different β-glucan populations, having different peak MWs, occurred in AE fractions (**Figure 8**). Subfractions obtained at 40% saturation were characterized by single, symmetric populations with apparent peak MW of 30×10^4 and 34×10^4 for the BaH and Na samples, respectively. The range of MW for β-glucans precipitated at 60% saturation was much broader. A single β-glucan peak of 12×10^4 was distinguished in the BaH fraction, whereas two β-glucan peaks, MW 45×10^4 and 4.7×10^4 were found in the Na fraction. In this case, the MW of β-glucan populations were not related to the level of ammonium sulfate saturation, suggesting differences in their fine structure.

Comparison Between WE and AE Arabinoxylans of Rye Flour. In our previous work (*11*), the WE arabinoxylan populations of the two rye flours used in this paper were studied after extraction with water at different temperatures and subfractionation with graded ammonium sulfate precipitation. In this concluding section, we try to find out whether, despite of different extraction conditions, similar trends can be observed for WE and AE fractions. ^{1}H NMR spectra and the relative proportion of the differently substituted Xyl*p* showed that similar, lowly branched arabinoxylan structures (Ara/Xyl $\sim$ 0.5), were obtained at 60% ammonium sulfate saturation from both WE and AE fractions. They constituted $\sim$76% of arabinoxylan populations extracted with cold water (*11*), whereas their proportion in the populations extracted with hot water and saturated $Ba(OH)_2$ was lower ($\sim$50% for both fractions). The clear differences in their PhC/Ara ratios (0.12, 0.29, and 0.55%,

on average for both flours, for arabinoxylans extracted with cold and hot water and saturated $Ba(OH)_2$ solution, respectively) as well as in protein concentration (2.0, 8.1, and 11.2%, respectively), reflected the progressively growing association between these components in the cell wall observed from cold water extractable polymers to those extracted with saturated $Ba(OH)_2$ solution. The proportion of lowly substituted structures dropped to ~35% of the overall arabinoxylan population extracted with NaOH, while their PhC/Ara and protein concentration increased to 0.86 and 12.4%, respectively, on average for both flours. The lowest proportion of lowly branched analogues (~17%) was found in the BaH fractions, which were virtually free of both phenolic and proteinaceous materials.

The lack of high-intensity resonances at δ 5.30 ppm, attributed to anomeric protons of Ara*f* linked to *O*-2 of singly substituted Xyl*p* (*45*), was striking in the spectra of AE arabinoxylans obtained at 100% saturation, when compared to those of WE counterparts, precipitated at the same saturation level (*11*). This is in accordance with the low content of 2-Xyl*p* reported for AE arabinoxylans from whole rye grain (*17*) and bran (*22*). Despite a notably higher proportion of 2-Xyl*p* in the highly substituted WE arabinoxylans, both highly branched populations had comparable substitution degrees (Ara/Xyl ~ 1.2 and, ~1.1, for WE and AE, respectively, on average for both flours). However, the AE arabinoxylans, virtually free of 2-Xyl*p*, were characterized by much higher level of 3-Xyl*p*.

There were no significant differences in the range of the peak WMs for WE and AE arabinoxylan populations that appeared in the region of HMW. The highest peak MWs were found for arabinoxylans from BaH fractions of both flours, which were comparable to that of hot WE polymers from Amilo. The lowest peak MWs in the region of HMW were observed for cold WE arabinoxylans that corresponded well to those present in the Ba and Na fractions derived from each rye flour.

On the basis of the above results, it can be concluded that the general agreement on the structural similarities between WE and WU arabinoxylans can be explained by the presence of the same "core" structures in both polymer populations. However, differences in their fine structures exist and result in greater association of AE arabinoxylans with other cell wall components, such as β-glucans, phenolic compounds and proteins, than that of WE counterparts. This may exert a major influence on their functionality in the native systems of rye dough.

ABBREVIATIONS USED

AE, alkali-extractable fraction(s); WE, water-extractable fraction(s); Ara/Xyl, arabinose-to-xylose ratio; ^{1}H NMR, proton nuclear magnetic resonance; Xyl*p*, xylopyranosyl residues; PhC/Ara, phenolic compounds-to-arabinose ratio; Glc*p*, glucopyranosyl residues; WU, water-unextractable fraction(s); HPLC, high-performance liquid chromatography; GPC, gel permeation chromatography; MW, molecular weight; Ara*f*, arabinofuranosyl residues; HMW, high molecular weight; LMW; low molecular weight.

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