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# Cell Wall Fractions Isolated from Outer Layers of Rye Grain by Sequential Treatment with α-Amylase and Proteinase: Structural Investigation of Polymers in Two Ryes with Contrasting Breadmaking Quality

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Recent studies have indicated that some structural features of arabinoxylans, the major cell wall polysaccharides, might be potential quality markers in the selection of rye breeding materials. To specify the most appropriate characteristics, the differences in the structure of cell wall components were studied in two ryes with high and low breadmaking qualities. Two cell wall fractions were isolated from the outer layers of the grain (pooled shorts and bran fractions) by a consecutive water extraction with α-amylase (WE-A) and proteinase K (WE-P). Polysaccharides predominated in the WE-A fraction (∼64%, mainly arabinoxylans). By contrast, the WE-P fraction contained mostly protein (∼63%), and its level of polysaccharides was relatively low (∼18%). The $^1$H NMR and sugar analysis of the ammonium sulfate precipitated subfractions revealed that the WE-A was built of four arabinoxylan populations with marked structural differences (arabinose-to-xylose ratios, Ara/Xyl, of ∼0.3, 0.5, 0.8, and 1.2). Instead, the arabinoxylans present in the WE-P were generally enriched in disubstituted xylopyranosyl residues. The ratio of phenolic components to arabinose residues in the WE-P fraction (indicated by $^1$H NMR) and the proportion of polymers with the highest molecular weights in the WE-A fraction (revealed by HPSEC) distinguished well two ryes with diverse breadmaking qualities. Much less obvious differences between both ryes were observed in the ratio of amide I to amide II band intensities of FTIR spectra for the WE-P and in the level of phenolic acids and ferulic acid dehydrodimers for both cell wall preparations.

**KEYWORDS: Rye outer layers; water extractable cell walls; fractionation; arabinoxylan structure**

## INTRODUCTION

In northern, central, and eastern parts of Europe, rye bread is a staple ingredient of human diet that constitutes an essential source of dietary fiber. Usually, flours of different extraction rates are used for rye bread production. It can be used alone or mixed with wheat flour in varying proportion to produce so-called "light" rye bread. However, in some countries, a whole meal rye flour is commonly used to make various types of bread and other speciality products (*1*).

The level of dietary fiber in rye is much higher (15−21%) than that in wheat (11−13%) (*2*, *3*). This is ascribed to the higher amount of cell wall polysaccharides, being a fundamental part of dietary fiber complex. Rye grain contains 8.0−12.0% arabinoxylans, 1.3−2.2% β-glucans, and 1.0−1.7% cellulose (*2*), whereas in wheat these polysaccharides make up, on average, 6.4, 0.6, and 1.6%, respectively (*4*−6). In rye milling, the outer layers including shorts and bran fractions form a byproduct accounting for 32−56% of the grain (*7*). These two milling fractions together contain a bulk of dietary fiber components: 72% of arabinoxylans, 70% of β-glucans, and 76% of cellulose (*8*, *9*). For this reason, bakery products made from whole grain are enriched in total dietary fiber in comparison to highly refined items. On the other hand, the water extractability of arabinoxylans, the principal dietary fiber components, is almost 2 times higher in flour than in whole meal rye (*10*). Products made from rye flour, therefore, provide fiber fraction with a higher ratio of soluble components. It should be stressed here that the type of fiber consumed determines the physiological response and, consequently, the specific health-related effects (*11*−*13*).

It is well-documented that arabinoxylans, the predominant cell wall polysaccharides with high water-holding and gelling capacities, influence the breadmaking quality of rye flour and whole meal (*14*−*17*). However, it is assumed that water-extractable (WE) and water-unextractable (WU) arabinoxylans affect it differently. The higher proportion of WE fraction had

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a beneficial effect on dough structure and bread characteristics (*18*), whereas the WU fraction negatively influenced the baking functionality of rye flour (*14*). This is also confirmed by the fact that the amount of WE arabinoxylans present in rye flour was positively correlated with flour and bread quality parameters; in contrast, the WU fraction had a negative relationship with these features (*7*). Furthermore, results of the recent investigations in flour and whole meal from rye breeding materials suggest that not only the amount but also the structural features of arabinoxylans, mainly influenced by genotype, might be closely related to the baking performance of rye (*7*, *19*).

The main elements of arabinoxylan backbone, (1→4)-linked *β*-D-xylopyranosyl residues (Xyl*p*), are present as unsubstituted (u-Xyl*p*) and mono- and disubstituted with α-L-arabinofuranosyl residues (Ara*f*) attached through *O*-3 (3-Xyl*p*) and/or *O*-2,3 linkages (2,3-Xyl*p*) (*20*, *21*). The occurrence of minor substituents, such as residues of uronic or acetic acids, directly substituted to Xyl*p* as well as phenolic acids linked via *O*-5 of Ara*f* has been also reported in cereal arabinoxylans *(20*, *22*, *23*). The degree of branching and different arrangement of the substituents over the xylan chain along with a wide range of molecular size illustrate a large heterogeneity in the arabinoxylan structure that controls its functionality (*24*, *25*).

In a previous study in rye flour (*26*, *27*), a fractional precipitation of both WE and alkali extractable (AE) arabinoxylans with ammonium sulfate permitted us to isolate a major arabinoxylan population, containing almost exclusively monosubstituted 3-Xyl*p* as a branched residue (arabinose-to-xylose ratio, Ara/Xyl, of ∼0.5), as well as intermediately (Ara/Xyl ∼ 0.8) and highly substituted (Ara/Xyl ∼ 1.2) arabinoxylan populations, enriched in both singly and doubly substituted Xyl*p*. In addition, a subfraction extremely rich in disubstituted Xyl*p* (54−57% of total Xyl*p*) has been found among WE arabinoxylans with a high degree of substitution. It is evident that in some cases, the Ara/Xyl ratio of the parent population is not a good indicator of arabinoxylans-specific structure, because its similar values might result from different proportions of arabinoxylan subfractions with diverse substitution degrees. It has been shown that arabinoxylans isolated from rye flour of high breadmaking quality had somewhat higher molecular weight and a lower content of 2,3-Xyl*p*, in comparison to those present in the corresponding subfractions from low breadmaking quality flour. Moreover, the high breadmaking quality polymers were characterized by a significantly lower ratio of phenolic components to Ara*f*, observed in their proton nuclear magnetic resonance ($^1$H NMR) spectra (*26*, *27*).

The present work is a part of an investigation undertaken to point out the differences in the structure of cell wall components between two ryes with diverse breadmaking quality which, in the future, could be tested as an index of breadmaking quality in a large number of samples. The objective of this study was to discriminate such structural elements in the WE fractions isolated from rye outer layers by a consecutive treatment with α-amylase and proteinase. It is well-known that granules of starch, the major storage polysaccharide in cereal grain, are embedded in the protein matrix. The use of α-amylase for starch degradation is a prerequisite and, usually sufficient, as the first step in the isolation of cereal cell walls. Because the protein content in the external part of the kernel, especially in aleurone cells, is much higher than in the inner layers (*9*, *10*), a subsequent proteinase treatment seems to be rational to observe its effect on extraction yield and to distinguish WE cell wall material released during amylolysis from that isolated upon proteolysis. Until now, to the best of our knowledge, there is no report in the literature on such an investigation.

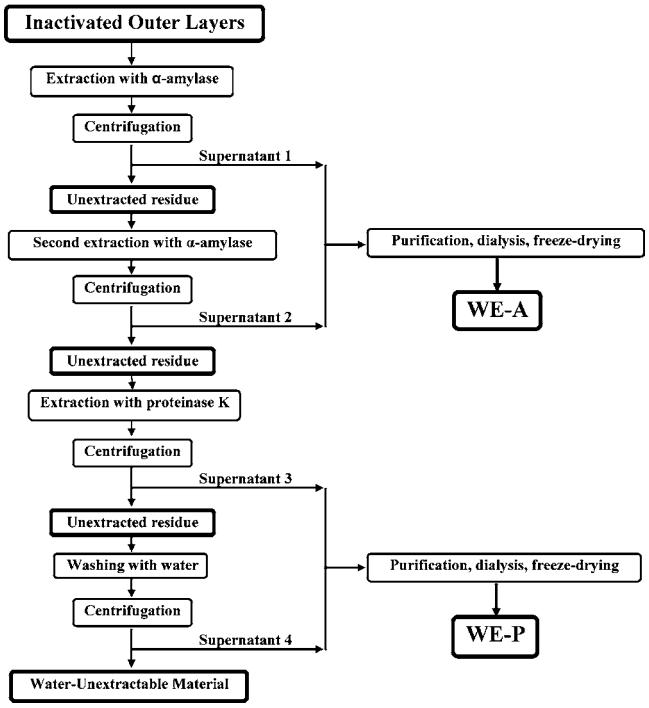


**Figure 1.** Scheme for consecutive extraction and isolation of α-amylase (WE-A) and proteinase (WE-P) extractable fractions from rye outer layers.

## MATERIALS AND METHODS

**Starting Material.** Two Polish rye cultivars, Amilo, with high breadmaking quality, and Nawid, with low breadmaking quality (harvest year 2000), were selected from breeding materials screened for this characteristic in DANKO, Plant Breeding Co., Laski, Poland. Grain samples, after tempering to 14% moisture, were milled into flour on a Quadrumat Senior laboratory mill (Brabender, Duisburg, Germany). The shorts and bran fractions, produced during this milling, were combined and in the following are referred to as rye outer layers. Their milling yields were 42 and 37% for Amilo and Nawid, respectively. Samples were suspended in 90% ethanol and boiled in a water bath under reflux for 1.5 h. The residues were filtered, washed with ethanol, and dried overnight at 40 °C. Inactivated samples were ground on a Cyclotec 1093 sample mill (Tecator, Höganäs, Sweden) equipped with a 0.5 mm screen, and afterward they constituted a starting material for water extraction.

**Sequential Water Extraction at the Presence of α-Amylase and Proteinase K.** Samples (80−100 g) were suspended in boiling, deionized water (1:10 w/v) using an Ultraturrax (IKA Labortechnik, Staufen, Germany) and incubated with thermostable α-amylase from *Bacillus licheniformis* (1 mL, 3000 units/mL) (EC 3.2.1.1, Megazyme International Ireland Ltd., Bray, Ireland) for 30 min in a boiling water bath (**Figure 1**). The suspension was centrifuged (10000*g*, 20 min, room temperature), and the pellet was again mixed with boiling water and treated in the Ultraturrax. The starch digestion was repeated once at the same conditions, using the same dosage of α-amylase. Both supernatants were combined, purified (as described below), and designated WE-A. The unextracted residue, left after double water extraction in the presence of α-amylase, was resuspended in water (500 mL) using an Ultraturrax and incubated with proteinase K from *Tritirachium album* (1 mL, 600 units/mL) (EC 3.4.21.64, Roche Diagnostics, Mannheim, Germany) at 40 °C for 16 h (pH 7.0, 0.03% $NaN_3$) with continuous stirring. The suspension was centrifuged (10000*g*, 20 min, room temperature) and the pellet washed with water (250 mL). The supernatants were combined, purified (as described below), and designated WE-P. Proteinase K was checked for any endoxylanase activity by monitoring the viscosity of pure arabinoxylans

**Table 1.** Chemical Composition (Grams per 100 g of Dry Matter) of Rye Outer Layers[a]

| | | | | dietary fiber constituents[b] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | starch[c] | protein[c] | ash[c] | Ara | Xyl | Man | Gal | Glc[d] | cellulose | UA | Klason lignin | total | Ara/Xyl |
| Amilo | 38.8 ± 0.8 | 11.89 ± 0.05 | 3.28 ± 0.01 | 5.65 | 10.89 | 0.54 | 0.65 | 7.20 (3.41) | 2.79 | 1.29 | 3.90 | 32.92 | 0.52 |
| Nawid | 37.5 ± 0.6 | 12.68 ± 0.03 | 3.18 ± 0.01 | 5.35 | 10.50 | 0.56 | 0.61 | 6.91 (4.03) | 2.97 | 1.37 | 4.21 | 32.47 | 0.51 |

[a] Abbreviations: Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose, Glc, glucose; UA, uronic acids; Ara/Xyl, arabinose-to-xylose ratio. [b] Results obtained from triplicates; the coefficient of variation was <5%. [c] Results are expressed as mean of duplicate determination ± standard deviation. [d] Noncellulosic glucose, values in parentheses represent β-glucan.

solutions with added enzyme. Two rye flour arabinoxylans have been used for the viscosity test; the first was purchased from Megazyme (purity 95%, Ara/Xyl = 0.96), and the second one (WE.I.60 from rye cultivar Amilo, purity = 92%, Ara/Xyl = 0.49) was isolated by cold water extraction and ammonium sulfate precipitation at 40−60% saturation as described previously (*26*). No change in viscosity was observed up to 20 h.

**Purification and Fractionation of WE-A and WE-P.** The crude WE-A extract was incubated with α-amylase from porcine pancreas (100 μL, 33 units/μL) (EC 3.2.1.1, Sigma-Aldrich) and proteinase K from *T. album* (1 mL, 600 units/mL) (EC 3.4.21.64, Roche Diagnostics) at 40 °C, overnight (pH 6.8, 10 mM $CaCl_2$, 0.03% $NaN_3$) with continuous stirring. The enzymes were inactivated by heat treatment (95 °C, 20 min), and precipitated material was separated by centrifugation (10000*g*, 20 min). The supernatant was dialyzed against deionized water (48 h, 6 °C) using membrane tubing (MW cutoff of 12000; Sigma-Aldrich). Thereafter, the WE-A extract was incubated with amyloglucosidase from *Aspergillus niger* (2 mL) (EC 3.2.1.3, Roche Diagnostics) at 40 °C, overnight (pH 4.8, 0.03% $NaN_3$). After heat treatment (95 °C, 20 min) and centrifugation (10000*g*, 20 min), the supernatant was dialyzed at 6 °C for 72 h.

The crude WE-P extract was incubated with amyloglucosidase from *A. niger* (0.5 mL) (EC3 .2.1.3, Roche Diagnostics) at 40 °C, overnight (pH 4.8, 0.03% $NaN_3$). The enzyme was inactivated by heat treatment (95 °C, 20 min), and the residual solids were separated by centrifugation (10000*g*, 20 min). The supernatant was dialyzed against deionized water (48 h, 6 °C) using membrane tubing (MW cutoff of 12000; Sigma-Aldrich). Aliquots of the purified WE-A and WE-P extracts were freeze-dried. The residual portion of each extract was directly fractionated by graded ammonium sulfate precipitation technique as described previously (*28*). Four freeze-dried subfractions were obtained from WE-A and denoted A.40, A.60, A.80, and A.100 [numbers refer to the saturation level of $(NH_4)_2SO_4$ at which subfractions were collected]. Also, WE-P yielded four subfractions denoted P.40, P.60, P.80, and P.100.

**Chemical Analyses.** Moisture and ash contents were analyzed according to AACC methods 44.15A and 46.11A, respectively (*29*). Protein content (N × 6.25) was determined according to the Kjeldahl method using a Kjeltec Auto 1030 analyzer (Tecator, Höganäs, Sweden). Starch was assayed enzymically according to the colorimetric method of Bach Knudsen et al. (*30*). β-Glucan content was evaluated according to the enzymatic method of McCleary and Glennie-Holmes (*31*) using a β-glucan assay kit (Megazyme International Ireland Ltd.). Klason lignin was measured gravimetrically as the residue left after two-step acid hydrolysis [72% (w/w) $H_2SO_4$, 1 h, 35 °C; 1 M $H_2SO_4$, 3 h, 100 °C] and corrected for ash (*32*). The content of uronic acids was determined by using the method of Scott (*33*). The monosaccharide composition was estimated by gas chromatography of alditol acetates obtained after hydrolysis with 1 M $H_2SO_4$ (2 h, 100 °C) (*34*). Alditol acetates were separated on a wide-bore Rtx capillary column (30 m; 0.53 mm i.d.; Restek, Bellefonte, PA) in a Hewlett-Packard model 5890 Series II Plus gas chromatograph (Waldbronn, Germany) equipped with a flame ionization detector. The column was heated at 190 °C for 2 min, and 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), using β-D-allose (Sigma-Aldrich) as internal standard. Cellulose content was calculated as the difference between amounts of glucose determined by two methods of hydrolysis with and without the pretreatment with 72% (w/w) $H_2SO_4$ (*35*). The constituent sugars were expressed as polysaccharides; for example, experimentally determined values for monosaccharides were converted to polysaccharides using factors of 0.88 for pentoses and 0.90 for hexoses.

Phenolic acids and ferulic acid dehydrodimers and dehydrotrimer were determined in the cell wall fractions after alkaline extraction under argon with 2 M NaOH at 35 °C for 30 min. The solution of 3,4,5-trimethoxy-(*E*)-cinnamic acid (TMCA, Sigma-Aldrich) was added as an internal standard. Maize bran samples, included in each set of samples, were used as a reference material for ferulic acid dehydrodimers and dehydrotrimer analysis (*36*, *37*). The mixture was acidified to pH 2 with 2 M HCl and extracted with diethyl ether. Ether phases were evaporated to dryness, dissolved in MeOH/$H_2O$ (50:50, v/v), and analyzed by HPLC as described previously (*36*).

**$^1$H NMR Spectroscopy.** Cell wall fractions and subfractions were dissolved in $D_2O$ (99.8%) with overnight stirring at room temperature and freeze-dried. Deuterium exchange was repeated once, and finally material was redissolved in $D_2O$ (10−20 mg/mL). $^1$H NMR spectra (400 MHz) were recorded at 60 °C on a Bruker ARX spectrometer (Karlsruhe, Germany). Acetone was used as a standard (δ2.23).

**HPSEC with Triple Detection.** Samples were dissolved in 50 mM $NaNO_3$, containing 0.02% $NaN_3$ (5 mg/mL), with continuous stirring at room temperature, filtered over 0.45 μm membrane, and injected at 25 °C on a high-performance size exclusion chromatography (HPSEC). The system comprised two Shodex OH-pak SB HQ 804 and 805 columns (Showa Denko K.K., Tokyo, Japan) eluted at 0.7 mL/min with 50 mM $NaNO_3$, containing 0.02% $NaN_3$. On-line molar mass analysis was performed using a multiangle laser light scattering (MALLS) detector (mini-Dawn, Wyatt Technology, Santa Barbara, CA, operating at three angles: 41, 90, and 138°), a differential refractometer (ERC 7517 A) ($dn/dc = 0.146$ mL/g), and a UV detector ($\lambda = 280$ nm). The 1.4 Astra software (Wyatt Technology) was used for data collection and calculations.

**FTIR Spectroscopy.** Samples were dissolved in deionized water at room temperature. Aliquots of the solutions (0.5 mL) were placed on a Teflon plate and dried in the oven at 40 °C. Dry films were removed from the plate and mounted in the atmosphere-controlled chamber of a Vector 22 Bruker spectrometer equipped with a DTGS detector. FTIR spectra were recorded in duplicate between 400 and 4000 $cm^{-1}$ at 4 $cm^{-1}$ resolution. Background spectra were taken in the empty chamber.

## RESULTS AND DISCUSSION

**Chemical Characteristics of Starting Materials, WE Cell Wall Fractions, and Subfractions.** The composition of rye outer layers obtained from cultivars Amilo and Nawid is summarized in **Table 1**. Samples were characterized by a similar level of two major components, starch and dietary fiber (38 and 33%, respectively). These values are within the range previously reported for shorts and bran fractions obtained by roller milling of the rye grain (*9*, *38*). Such a high level of starch clearly indicates that, besides the outer coverings and aleurone layer, the starting material contained a significant amount of starchy endosperm. These components originated mainly from an external part of endosperm, that is, the subaleurone (peripheral) and prismatic cells (*39*), because during rye milling the central endosperm cells are recovered in white flour. This is

**Table 2.** Yield and Composition of α-Amylase (WE-A) and Proteinase (WE-P) Extractable Cell Wall Fractions and Subfractions Isolated from Rye Outer Layers[a]

| fraction/ subfraction | yield[c] | arabinoxylans[c] | polysaccharides[d] | nitrogen[d] | molar composition[b] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Ara | Xyl | Man | Gal | Glc | Ara/Xyl |
| | | | | Amilo | | | | | | |
| WE-A | 9.62 | 4.80 | 64.4 | 1.0 | 28.4 | 49.1 | 4.2 | 2.5 | 15.8 | 0.58 |
| A.40 | 1.70 | 0.64 | 67.9 | na | 13.1 | 42.6 | 2.2 | 1.0 | 41.1 | 0.31 |
| A.60 | 3.56 | 2.60 | 83.3 | na | 28.9 | 58.8 | 3.4 | 0.9 | 8.0 | 0.49 |
| A.80 | 1.52 | 1.15 | 81.3 | na | 41.5 | 51.8 | 2.8 | 1.2 | 2.7 | 0.80 |
| A.100 | 1.00 | 0.37 | 41.0 | na | 49.3 | 40.5 | 2.4 | 2.7 | 5.1 | 1.22 |
| WE-P | 2.62 | 0.31 | 17.2 | 9.8 | 30.2 | 37.8 | 5.2 | 5.9 | 20.9 | 0.80 |
| P.40 | 0.71 | 0.04 | 10.7 | na | 16.8 | 31.8 | 8.4 | 2.8 | 40.2 | 0.53 |
| P.60 | 0.38 | 0.07 | 22.3 | na | 30.9 | 52.9 | 3.1 | tr | 13.1 | 0.58 |
| P.80 | 0.60 | 0.18 | 32.9 | na | 39.2 | 52.9 | 2.1 | tr | 5.8 | 0.74 |
| P.100 | 0.38 | 0.03 | 7.9 | na | 49.4 | 40.5 | 10.1 | tr | tr | 1.22 |
| | | | | Nawid | | | | | | |
| WE-A | 9.41 | 4.29 | 63.3 | 1.3 | 25.8 | 46.3 | 4.9 | 2.7 | 20.3 | 0.56 |
| A.40 | 2.16 | 0.75 | 71.9 | na | 11.2 | 37.3 | 2.5 | 1.0 | 48.0 | 0.30 |
| A.60 | 3.36 | 2.43 | 84.1 | na | 27.9 | 58.0 | 4.9 | 1.0 | 8.2 | 0.48 |
| A.80 | 1.15 | 0.77 | 73.1 | na | 40.5 | 50.9 | 3.7 | 1.5 | 3.4 | 0.80 |
| A.100 | 0.60 | 0.28 | 51.5 | na | 49.5 | 40.4 | 2.3 | 3.1 | 4.7 | 1.23 |
| WE-P | 2.87 | 0.34 | 19.2 | 10.5 | 26.6 | 34.9 | 5.2 | 5.7 | 27.6 | 0.76 |
| P.40 | 1.18 | 0.06 | 18.0 | na | 10.6 | 20.0 | 6.1 | 2.8 | 60.5 | 0.53 |
| P.60 | 0.36 | 0.07 | 25.9 | na | 28.6 | 48.6 | 2.7 | tr | 20.1 | 0.59 |
| P.80 | 0.58 | 0.19 | 36.3 | na | 38.9 | 53.7 | 2.2 | tr | 5.2 | 0.72 |
| P.100 | 0.20 | 0.02 | 8.5 | na | 48.2 | 40.0 | 11.8 | tr | tr | 1.21 |

[a] Abbreviations: Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose, Glc, glucose; Ara/Xyl, arabinose-to-xylose ratio; na, not analyzed; tr, traces. [b] Expressed as percentage (mol/100 mol). Results were obtained from triplicates; the coefficient of variation was <2%. [c] Expressed as weight percentage of rye outer layers. Results are presented as means of two extractions; the coefficient of variation was <6%. [d] Expressed as weight percentage of sample. Results were obtained from triplicates; the coefficient of variation was <4%.

also evidenced by relatively low protein and ash contents as well as a much higher level of starch usually observed in white flour when compared to those of bran fractions (*9*, *38*). In our systematic study, the WE cell wall polysaccharides of rye grain are divided into two groups. The first group, composed of the central endosperm polymers, was described earlier (*26*), and the second group of polymers, originating from the outer part of rye, are the subject of the present work. Therefore, the differences in the structure between both groups of polymers can be ascribed to their origin from morphologically different cell walls. It is also consistent with the rye milling streams produced for breadmaking, that is, flour and whole meal. The latter comprises the cell wall polysaccharides of both groups.

*WE Cell Wall Fractions.* The WE-A fractions, isolated by hot water extraction at the presence of α-amylase, constituted 9.6 and 9.4% of the outer layers, respectively, for Amilo and Nawid (**Table 2**). Polysaccharides were the main components of these fractions (64 and 63%). Arabinoxylans and glucose-containing polymers made up, respectively, 72−78 and 16−20% of the polysaccharides. Small amounts of polymers built of mannose and galactose were also found. The WE-A fractions contained only 6 and 8% of protein (N × 6.25). The WE-P fractions, solubilized during proteolysis of the water unextractable residue left after α-amylase treatment, represented 2.6 and 2.9% of the outer layers for Amilo and Nawid, respectively. Quite the opposite, they were characterized by high protein contents (61 and 65%) and comparatively low amounts of polysaccharides (17 and 19%). Such a high protein level was rather unexpected after prolonged proteolysis followed by a thermal denaturation step. However, the specific composition of this fraction, for example, the high proportion of aromatic constituents, as revealed by $^1$H NMR and FTIR spectroscopy (**Figures 2** and **3**), could partly explain the limited action of the enzyme. Due to a single proteinase treatment in our experiment, there is no guarantee that the digestion of storage proteins was completed, but definitely, the fact that the cell wall material was released by proteinase action suggests protein association with material isolated during this extraction. In general, the polysaccharide composition was similar in both WE fractions. Nevertheless, polysaccharides from WE-P had higher levels of glucose-containing polymers (20 and 28%), and consequently, they contained less arabinoxylans (68 and 62%) when compared to those of the WE-A. Arabinoxylans present in the WE-P also had notably higher degrees of substitution with Ara*f*, as pointed out by their Ara/Xyl ratios (0.80 and 0.76), than counterparts in the WE-A (0.58 and 0.56).

Rye outer layers from Amilo had a higher content of WE arabinoxylans, which were recovered in the WE-A fraction (4.8%), than the outer layers from Nawid (4.3%). They represented 61 and 60% of the total WE arabinoxylan population found in rye grain, respectively, for Amilo and Nawid. Both samples of rye outer layers contained practically the same level of arabinoxylans solubilized during digestion with proteinase and recovered in the WE-P (0.31 and 0.34%). The proteinaceous material that remained in the WE-A fractions comprised 5 and 6% of total protein content in rye outer layers, whereas that of the WE-P fractions amounted to 14 and 15% for Amilo and Nawid, respectively.

*Subfractions Obtained by Ammonium Sulfate Precipitation.* Fractionation of the WE cell wall preparations with ammonium sulfate resulted in four subfractions precipitated at 40, 60, 80, and 100% salt saturation. Their yield, polysaccharide content, and composition are given in **Table 2**. Generally, only subfractions isolated at 40% saturation level comprised substantial amounts of glucose-containing polysaccharides. They constituted 41 and 48% of polysaccharides in subfractions precipitated from

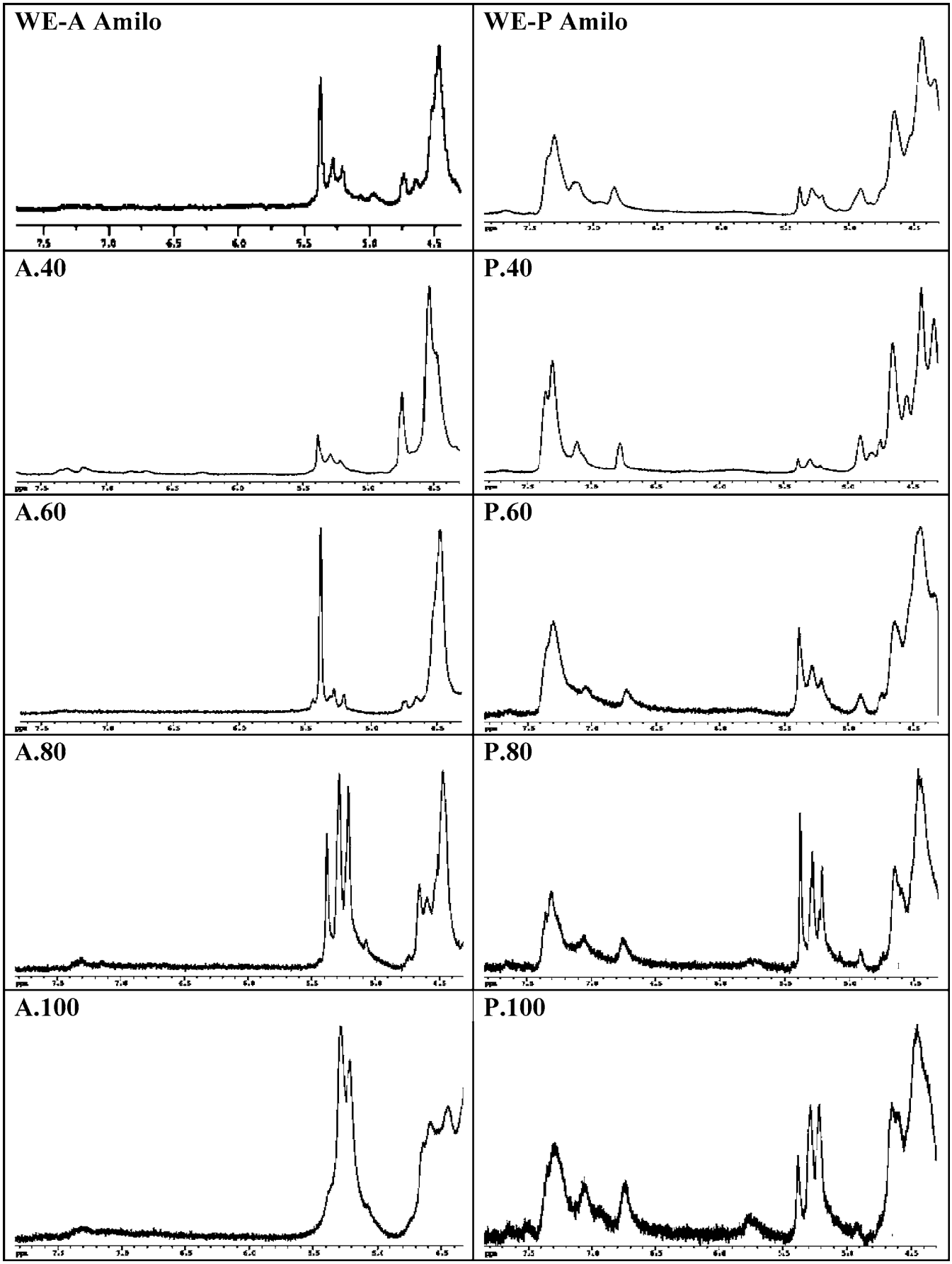


**Figure 2.** $^{1}$H NMR spectra of α-amylase (WE-A) and proteinase (WE-P) extractable fractions and their subfractions obtained from outer layers of Amilo.

WE-A, and 40 and 61% of those obtained from WE-P, respectively, for Amilo and Nawid. In the other subfractions, isolated at higher salt saturation, arabinoxylans made up most of the polysaccharide fraction.

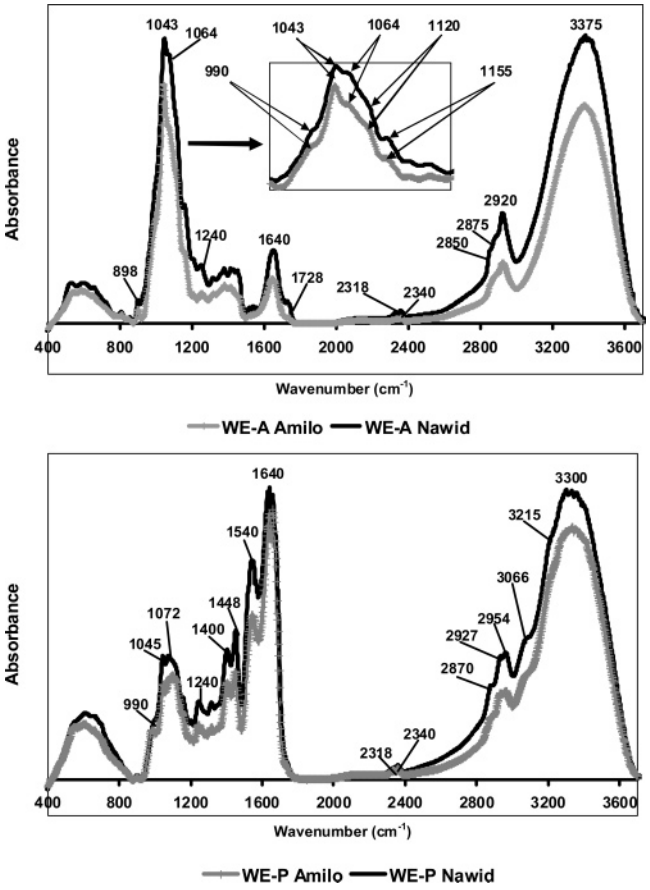


**Figure 3.** FTIR spectra of α-amylase (WE-A) and proteinase (WE-P) extractable fractions isolated from outer layers of Amilo and Nawid.

As could be expected, the arabinoxylan populations precipitated upon increased salt saturation had a progressively higher substitution degree. However, subfractions obtained from WE-A at 40 and 60% ammonium sulfate saturation were characterized by very low (Ara/Xyl ∼ 0.3) and low (Ara/Xyl ∼ 0.5) substitution degrees, whereas the corresponding subfractions produced from WE-P had evidently higher degrees of branching (Ara/Xyl, 0.5 and 0.6, respectively, for 40 and 60% saturation). The arabinoxylans with intermediate (Ara/Xyl = 0.7−0.8) and high substitution degrees (Ara/Xyl ∼ 1.2) were obtained from both WE fractions at 80 and 100% saturation levels, respectively. It is of interest that xylan of very low substitution with Ara*f* (Ara/Xyl ∼ 0.3) was not found among WE arabinoxylans originating from the central part of rye endosperm, which principally comprised three structural classes representing lowly (Ara/Xyl ∼ 0.5), intermediately (Ara/Xyl ∼ 0.8), and highly (Ara/Xyl ∼ 1.2) branched analogues (*26*). This leads to the conclusion that such a structure with a very low Ara/Xyl ratio (0.3) is a characteristic feature of cell wall polymers present in the outer part of the grain. Also, this explains the lower Ara/Xyl ratio of WE arabinoxylans from rye outer layers than that found for counterparts isolated from rye flour (*26*). Xylans with a very low degree of arabinosylation (Ara/Xyl = 0.1−0.3) are a typical structure of water unextractable cell wall polysaccharides. They have been reported for rye whole meal and bran as polymers extracted under strong alkaline conditions (1 and 4 M KOH or NaOH) (*40*, *41*). There is no information, to the best of our knowledge, on the occurrence of their WE analogues in rye. However, recently a similar arabinoxylan population (Ara/Xyl ∼ 0.3) was isolated from WE cell wall material of wheat bran by graded ethanol precipitation (*42*).

**Table 3.** Content of Ferulic, *p*-Coumaric, and Sinapic Acids and Ferulic Acid Dehydrodimers (diFA) in α-Amylase (WE-A) and Proteinase (WE-P) Extractable Cell Wall Fractions and Subfractions Isolated from Rye Outer Layers[a]

| | μg g$^{-1}$ | | | | μg g$^{-1}$ of xylose | |
|---|---|---|---|---|---|---|
| | ferulic acid[b] | *p*-coumaric acid | sinapic acid | diFA[c] | phenolic acids | diFA[c] |
| | | | Amilo | | | |
| WE-A | 2351 ± 21 | 30 ± 2 | 482 ± 4 | 40 ± 5 | 9060 | 127 |
| A.40 | 5984 | 94 | 994 | 62 | 24471 | 214 |
| A.60 | 2387 | 18 | 281 | 30 | 5482 | 61 |
| A.80 | 1090 | 13 | 210 | 34 | 3119 | 81 |
| A.100 | 391 | 8 | 119 | 19 | 3120 | 114 |
| WE-P | 759 ± 12 | 13 ± 1 | 145 ± 2 | 19 ± 3 | 14108 | 292 |
| P.40 | 1249 | 30 | 197 | 38 | 43411 | 1118 |
| P.60 | 825 | 10 | 136 | 20 | 8229 | 169 |
| P.80 | 545 | 6 | 61 | 18 | 3517 | 103 |
| P.100 | 119 | 11 | 15 | 5 | 4531 | 156 |
| | | | Nawid | | | |
| WE-A | 2637 ± 32 | 37 ± 2 | 592 ± 8 | 56 ± 6 | 11146 | 191 |
| A.40 | 6619 | 86 | 515 | 66 | 26940 | 246 |
| A.60 | 2406 | 18 | 173 | 32 | 5322 | 66 |
| A.80 | 1542 | 16 | 148 | 38 | 4586 | 102 |
| A.100 | 874 | 17 | 172 | 43 | 5111 | 207 |
| WE-P | 867 ± 18 | 14 ± 1 | 148 ± 5 | 24 ± 2 | 15358 | 358 |
| P.40 | 1373 | 25 | 210 | 45 | 44667 | 1250 |
| P.60 | 1241 | 17 | 157 | 31 | 11230 | 246 |
| P.80 | 680 | 6 | 73 | 33 | 3892 | 169 |
| P.100 | 80 | 5 | 17 | 6 | 3000 | 176 |

[a] Data represent means ± standard deviation ($n = 2$). [b] Sum of *E* and *Z* isomers. [c] Sum of four ferulic acid dehydrodimers; 8-5′-diFA, 5-5′-diFA, 8-*O*-4′-diFA, and 8-5′-benzofuran-diFA, including traces of ferulic acid dehydrotrimer; 8-*O*-4′, 5-5′-TriFA.

Significant differences in the relative distribution of the arabinoxylan subfractions were observed between both WE fractions (**Table 2**). Clearly, the lowly substituted populations (A.60; Ara/Xyl ∼ 0.5), constituted 55 and 58% of the total amount of arabinoxylans recovered after fractionation from WE-A fractions. In contrast, the structures with an intermediate degree of substitution (P.80; Ara/Xyl ∼ 0.7) dominated among arabinoxylans obtained from WE-P fractions (56%). The highly branched structures were the minor components in all WE fractions; they comprised only 7−8% of total arabinoxylan populations recovered after fractionation. Notable differences in the distribution of arabinoxylan subfractions of both rye samples were found in the relative proportion of polymers isolated at 40% sulfate saturation, which were characterized by very low (Ara/Xyl ∼ 0.3) and low (Ara/Xyl ∼ 0.5) degrees of branching for WE-A and WE-P, respectively. Arabinoxylans isolated from the outer layers of superior breadmaking quality rye Amilo had a lower proportion of such structures (∼13% for both WE-A and WE-P) than those from inferior breadmaking quality rye Nawid (∼18%). This resulted in slightly higher Ara/Xyl ratios of parental arabinoxylan populations derived from Amilo when compared with those from Nawid.

*Phenolic Components in Cell Wall Fractions and Subfractions.* The content of phenolic acids in the WE-A fractions (2863 and 3266 μg g$^{-1}$) was 3 times higher than in the WE-P (917 and 1029 μg g$^{-1}$, respectively, for Amilo and Nawid) (**Table 3**). Similarly, the WE-A fractions had a 2 times higher amount of ferulic acid dehydrodimers (diFA) (40 and 56 μg g$^{-1}$) than WE-P (19 and 24 μg g$^{-1}$). Noticeably, both cell wall fractions isolated from Nawid contained slightly more phenolic components than did their equivalents from Amilo. Ferulic acid made

up 81−84%, sinapic 14−18% and *p*-coumaric only ∼1% of the total phenolic acids in both WE fractions. The level of phenolic acids in the cell wall subfractions, in general, progressively decreased as the salt saturation increased from 40 to 100%.

It is well-known that the ferulic acid residues are esterified to the *O*-5 position of some Ara*f* in heteroxylans (*43*−*45*), as is their dimerization, in the presence of oxidizing agents, which leads to cross-linking of adjacent arabinoxylans chains (*46*). Because of the distinctly low arabinoxylan content in WE-P fractions, the amounts of phenolic acids and diFA were also expressed on a xylose basis, showing that, in contrast, arabinoxylans present in the WE-P were slightly more substituted with phenolic acids. Interestingly, their substitution degrees with diFA (292 and 358 $\mu$g g$^{-1}$ of xylose) were almost 2 times higher when compared to those from WE-A (127 and 191 $\mu$g g$^{-1}$ of xylose for Amilo and Nawid, respectively). The arabinoxylan subfractions obtained from both WE fractions at 40% sulfate saturation exhibited a remarkably high degree of feruloylation.

**$^1$H NMR Analysis of Arabinoxylan Structural Features.** The $^1$H NMR spectra of both WE fractions and their subfractions obtained from Amilo are presented in **Figure 2**. All spectra clearly exemplified differences in the fine structure of arabinoxylan subfractions. The spectra of subfractions obtained from WE-A at 60, 80, and 100% salt saturation (A.60, A.80, and A.100, respectively) showed the distinct arabinoxylan structures characterized by different proportions of mono- and disubstituted Xyl*p* in the backbone. The arabinoxylans in A.60 represented a monosubstituted structure, as indicated by a strong resonance at $\delta$ 5.38, which was assigned to anomeric protons of terminal Ara*f* linked to *O*-3 of Xyl*p*. However, the two signals of relatively low intensity at $\delta$ 5.21 and 5.28, originating from the anomeric protons of terminal Ara*f* linked to *O*-2 and *O*-3 of the same Xyl*p* (*21*, *47*, *48*), were also visible in the spectrum. The arabinoxylans from A.80 contained an appreciable proportion of both mono- and disubstituted branching sites, whereas those in A.100 were almost exclusively composed of disubstituted Xyl*p*, as evidenced by dominating resonances of Ara*f* linked to doubly substituted Xyl*p* ($\delta$ 5.21 and 5.28). The doublet at $\delta$ 4.74 and 4.75 observed in the spectrum of A.40, originating from $\beta$-glucans (*49*), implied that at the lowest saturation level, arabinoxylans and $\beta$-glucans coprecipitated. Nevertheless, unlike the structure present in A.60, which was characterized generally by monosubstituted branches, that present in A.40 was also slightly enriched in disubstituted Xyl*p*.

The presence of arabinoxylans with different, but appreciable, proportions of both mono- and disubstituted Xyl*p* is well-illustrated in the spectra of subfractions obtained from WE-P fraction at 60, 80, and 100% ammonium sulfate saturation (P.60, P.80, and P.100, respectively) (**Figure 2**). There was a considerable increase in the relative intensities of Ara*f* linked to *O*-2 and *O*-3 of the same Xyl*p* and, concurrently, a decline in the intensity of Ara*f* linked to *O*-3 of the singly substituted Xyl*p* ($\delta$ 5.21−5.38) in the spectra of subfractions precipitated with increasing saturation level. The most striking, however, was the presence of some phenolic components in the WE-P fractions and their subfractions, as revealed by very intense resonances in their spectra that appeared in the region of the phenolic moiety ($\delta$ 6.74−6.84, 7.05−7.11, and 7.29−7.35). By contrast, they were practically absent in the spectra of WE-A fraction and subfractions. Because in both WE-A and WE-P fractions, the relative degree of arabinoxylan feruloylation was relatively low (**Table 3**), such intense resonances cannot correspond to phenolic acids. This indicates that other phenolics occur at appreciable levels in the proteinase extractable cell wall material. Considering the high amount of protein in the WE-P fractions as well as their trace level of Klason lignin (data not shown), the presence of aromatic amino acids cannot be excluded. It has been suggested that ferulic acid esterified to arabinoxylans, which participate in gel formation, might also be linked to tyrosine residues (*50*, *51*). On the other hand, tyrosine residues in protein may be cross-linked by oxidative dimerization (*52*). The presence of an oxidatively coupled dimer of tyrosine (isodityrosine) as well as its tetrameric derivative (di-isodityrosine) in plant cell walls has been reported in the literature (*53*, *54*). Notable resonances with similar profiles, but of lower intensity in the region of anomeric protons of phenolic residues ($\delta$ 6.68−7.58), were also observed in the arabinoxylan preparations obtained from rye flour by water extraction without and with α-amylase addition (*26*). This suggests that the similar structures can be distributed throughout the whole endosperm.

**Table 4.** Relative Percentage of Un-, Mono-, and Disubstituted Xylose Residues (u-Xyl*p*, 3-Xyl*p*, and 2,3-Xyl*p*) in Arabinoxylans from α-Amylase (WE-A) and Proteinase (WE-P) Extractable Cell Wall Fractions and Subfractions of Rye Outer Layers and Their Ratio of Total Resonance in Phenolic Compounds Region to Total Resonance of Arabinose Anomeric Protons (PhC/Ara)[a]

| fraction/ subfraction | % of total Xyl*p* | | | 2,3-Xyl*p* (% of branched Xyl*p*) | PhC/Ara |
|---|---|---|---|---|---|
| | u-Xyl*p* | 3-Xyl*p* | 2,3-Xyl*p* | | |
| | | | Amilo | | |
| WE-A | 57.7 | 26.5 | 15.8 | 37.4 | 0.18 |
| A.40 | 77.5 | 14.0 | 8.5 | 37.8 | 0.45 |
| A.60 | 57.8 | 38.4 | 3.8 | 9.0 | 0.08 |
| A.80 | 52.7 | 14.9 | 32.4 | 68.5 | 0.06 |
| A.100 | 34.5 | 8.8 | 56.7 | 86.6 | 0.11 |
| WE-P | 50.2 | 19.6 | 30.2 | 60.6 | 4.42 |
| P.40 | 66.2 | 14.7 | 19.1 | 56.5 | 9.92 |
| P.60 | 58.3 | 25.3 | 16.4 | 39.3 | 2.43 |
| P.80 | 52.7 | 20.9 | 26.4 | 55.8 | 1.35 |
| P.100 | 32.2 | 13.3 | 54.5 | 80.4 | 3.83 |
| | | | Nawid | | |
| WE-A | 61.3 | 21.2 | 17.5 | 45.2 | 0.26 |
| A.40 | 78.5 | 12.9 | 8.6 | 40.0 | 0.74 |
| A.60 | 59.9 | 35.3 | 4.8 | 12.0 | 0.13 |
| A.80 | 53.2 | 12.8 | 34.0 | 72.6 | 0.06 |
| A.100 | 35.2 | 6.4 | 58.4 | 90.1 | 0.13 |
| WE-P | 51.7 | 16.7 | 31.6 | 65.4 | 9.16 |
| P.40 | 66.4 | 14.4 | 19.2 | 57.1 | 15.16 |
| P.60 | 59.9 | 21.0 | 19.1 | 47.6 | 3.83 |
| P.80 | 56.4 | 15.0 | 28.6 | 65.6 | 2.00 |
| P.100 | 35.2 | 8.2 | 56.6 | 87.3 | 5.72 |

[a] Abbreviations: u-Xyl*p*, unsubstituted $\beta$-(1→4)-linked D-Xyl*p*; 3-Xyl*p*, $\beta$-(1→4)-linked D-Xyl*p* substituted with α-L-Ara*f* at *O*-3; 2,3-Xyl*p*, $\beta$-(1→4)-linked D-Xyl*p* substituted with α-L-Ara*f* at *O*-2 and *O*-3.

*Substitution Pattern of Arabinoxylans.* On the basis of the signal intensity of Ara*f* linked to mono- and disubstituted Xyl*p* in the $^1$H NMR spectra and the sugar analysis, the relative proportions of un-, mono-, and disubstituted Xyl*p* (u-Xyl*p*, 3-Xyl*p*, and 2,3-Xylp, respectively) were calculated for each arabinoxylan population (**Table 4**). It is obvious that subfractions isolated from WE-A at 60 and 100% salt saturation essentially represented arabinoxylans with one type of branching. The 3-Xyl*p* constituted 88−91% of branched Xyl*p* in arabinoxylans from A.60, whereas the 2,3-Xyl*p* represented 87−90% in those from A.100. In polymers obtained at 80% saturation (A.80), containing both mono- and disubstituted residues, their levels ranged from 27 to 32% for 3-Xyl*p* and from 69 to 73% for 2,3-Xyl*p*. Similar structures, corresponding to these three groups of arabinoxylans, were found in WE cell wall materials originating from flours of the same rye cultivars, Amilo and

**Table 5.** Yield, Relative Proportion, and Structural Characteristics of WE Arabinoxylan Classes Isolated from Rye Grain by Graded Ammonium Sulfate Precipitation Technique

| class | level of salt saturation | Ara/Xyl[a] | degree of substitution | major type of branching | relative proportion (%) flour[b] | relative proportion (%) outer layers | relative proportion (%) whole grain[c] | yield (% of whole grain) |
|---|---|---|---|---|---|---|---|---|
| AX-IV | 40 | 0.31 | very low | 3-Xyl*p*, 2,3-Xyl*p* | nd | 13–18 | 8–11 | 0.27–0.28 |
| AX-I | 60 | 0.47–0.51 | low | 3-Xyl*p* | 69–70 | 55–58 | 60–62 | 1.60–1.94 |
| AX-II | 80 | 0.74–0.91 | intermediate | 3-Xyl*p*, 2,3-Xyl*p* | 23 | 18–24 | 20–24 | 0.52–0.76 |
| AX-III | 100 | 1.00–1.28 | high | 2,3-Xyl*p* | 7–8 | 7–8 | 7–8 | 0.18–0.24 |

[a] Ara/Xyl, arabinose-to-xylose ratio. [b] Cyran et al. (*26*). [c] Data recalculated from the values obtained for flour and for outer layers using the yields of both milling streams; nd, not detected. Data represent minimum and maximum values for two rye cultivars, Amilo and Nawid.

Nawid (*26*). Because in the $^1$H NMR spectra there was not any substantial difference between both resonances of Ara*f* linked to *O*-2 and *O*-3 of the same Xyl*p* ($\delta$ 5.21 and 5.28), suggesting overlapping of one of them with that of Ara*f* attached through *O*-2 to Xyl*p* (2-Xyl*p*) ($\delta$ 5.30), the level of 2-Xyl*p* could not be evaluated by $^1$H NMR analysis. However, in the corresponding subfractions obtained from rye flour and whole meal, in addition to 3-Xyl*p*, the 2-Xyl*p* was observed at significant level, especially in the arabinoxylan subfractions with high degrees of substitution (from 7−10 to 28−37% of total Xyl*p*, respectively, for cold and hot water extractable polymers of flour and ∼14% for polymers extracted from whole meal at room temperature) (*26*, *55*). This indicates that 2-Xyl*p*, being a scarce element of rye arabinoxylan structure, is mainly concentrated in the central endosperm cell walls.

Another totally different group of arabinoxylans has been isolated at 40% saturation (A.40). It was characterized by a very high proportion of u-Xyl*p* in the chain (∼78%) and the presence of both 3-Xyl*p* and 2,3-Xyl*p* in the backbone. However, disubstituted Xyl*p* made up 38−40% of branched Xyl*p*, which also deviated substantially from their levels found in other subfractions. These very lowly substituted arabinoxylans might be partly responsible for non-covalent and covalent types of association in the cell walls. Their unusually low degree of substitution with Ara*f* indicates possible intermolecular alignment via hydrogen bonds between continuous u-Xyl*p* fragments of the individual chains, whereas the high degree of substitution with diFA reflects the arabinoxylans covalent cross-linking via diFA bridges. However, a relatively very low proportion of 3-Xyl*p*, as a main site of feruloylation, did not correspond well with the outstanding level of phenolic acids and diFA observed in A.40 when compared to the remaining subfractions.

**Table 5** summarizes the yield, relative proportion, and principal structural elements of four classes of WE rye arabinoxylans (denoted AX-I, AX-II, AX-III, and AX-IV), which have been isolated from two general millstreams, flour and outer layers, by graded ammonium sulfate precipitation. The structural characteristic of each class is ascribed to polymers present in the inner and/or the outer part of the grain. Their different substitution pattern is reflected by the degree of substitution with Ara*f* and the presence of one or two types of branched Xyl*p* in the chain. Undoubtedly, the AX-I polymers, with almost one type of branching (3-Xyl*p*) and low degree of substitution (Ara/Xyl ∼ 0.5), dominated among WE arabinoxylans of the whole grain (62−65%). The structural features of this group of arabinoxylans have been previously described by Bengtsson and co-workers (*56*), as the polymers eluted with water from a DEAE-cellulose column. Nevertheless, their yield (0.4% of the grain dry matter) was much lower than that recovered in our study (1.60−1.94%). The second class of polymers, of intermediate degree of substitution (Ara/Xyl ∼ 0.8) and both types of branching (3-Xyl*p* and 2,3-Xyl*p*) constituted ∼20% of the parent population, whereas the highly branched polymers (Ara/Xyl ∼ 1.1) of the third class, having virtually one type of branching, but in this case with disubstituted residues (2,3-Xyl*p*), made up only 7%. The fourth class of arabinoxylans, with an extremely low degree of substitution (Ara/Xyl ∼ 0.3) and again both branching types, represented 8−11% of total arabinoxylan populations. The polymers isolated from rye flour, besides the lack of AX-IV class, were enriched in AX-I equivalents when compared to those from the outer layers of the grain.

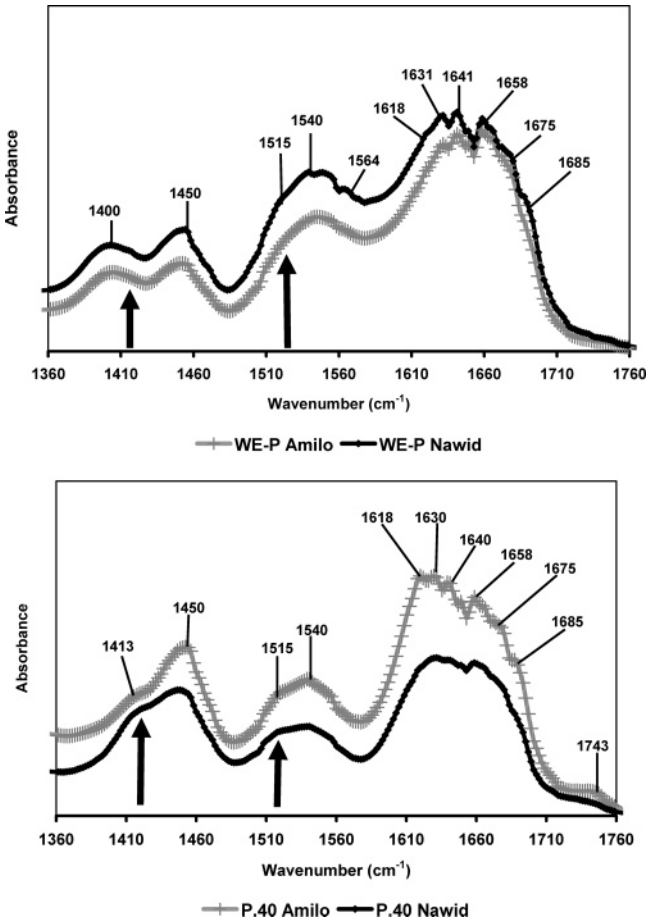


**Figure 4.** FTIR spectra in the 1360–1760 cm$^{-1}$ region of proteinase extractable fractions (WE-P) and their subfractions precipitated at 40% ammonium sulfate saturation (P.40) from outer layers of Amilo and Nawid.

The above findings make clear the existence of a range of polymer structures in the parent population of rye WE arabinoxylans, proposed by Vinkx et al. (*25*). In contrast to previous suggestions of two classes hypothesized by Bengtsson et al. (*56*), Vinkx et al. (*25*) isolated three main arabinoxylan classes from two rye whole meals at 50, 70, and 100% of ammonium sulfate saturation with somewhat higher Ara/Xyl ratios (0.52−0.55, 0.81−1.09, and 1.34−1.42) and the structural features corresponding to AX-I, AX-II, and AX-III, respectively, of this paper.

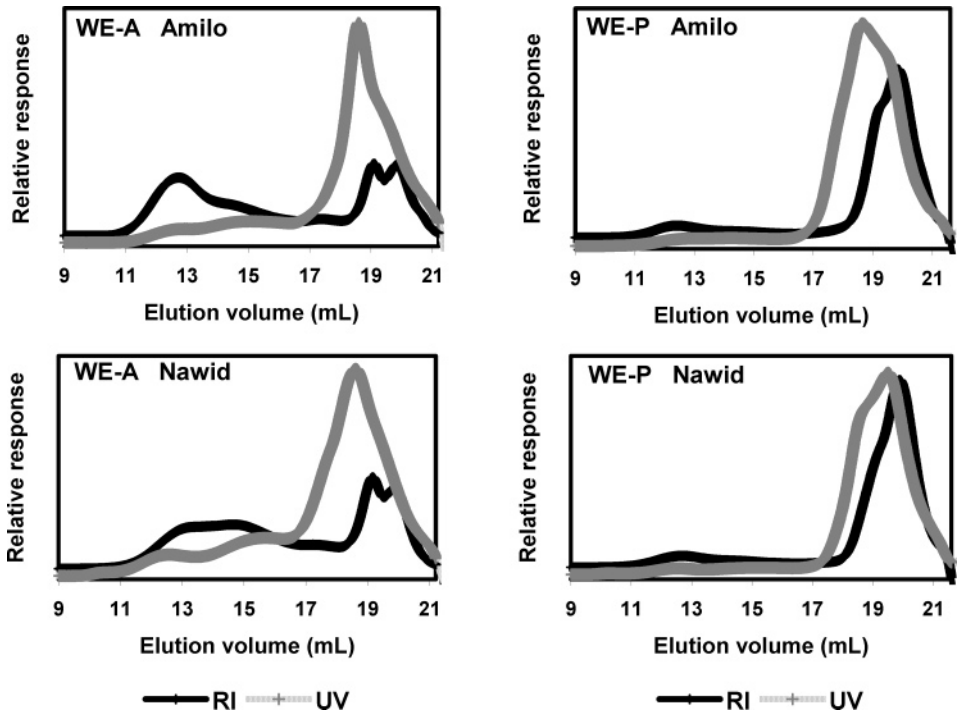


**Figure 5.** HPSEC profiles of polysaccharides and UV-absorbing substances of α-amylase (WE-A) and proteinase (WE-P) extractable fractions from outer layers of Amilo and Nawid.

Their total yields, however, were very low (<1%) in comparison to WE arabinoxylan content reported in rye grain (2.6−3.8%) (*19*).

In this paper we report on the occurrence of four major WE arabinoxylan populations with evident structural differences in the rye grain. Arabinoxylans of both millstreams amounted to 3.21 and 2.58% of the rye grain for Amilo and Nawid, respectively. However, some very small populations isolated from rye flour at 40% ammonium sulfate saturation (Ara/Xyl ∼ 0.6 and 0.7) (*26*) were not taken into consideration. Therefore, it is important to recognize that other structures, differing from the general four classes, also exist among rye WE arabinoxylans.

Unlike the α-amylase extractable cell wall fractions, these solubilized upon proteinase digestion were enriched in 2,3-Xyl*p* (**Table 4**). Also, the subfractions precipitated from WE-P at 40 and 60% salt saturation were rich in 2,3-Xyl*p*, in contrast to their equivalents obtained from WE-A. In the case of Nawid, the subfractions obtained from WE-P at the highest saturation (P.100) resembled in the substitution pattern its equivalent from WE-A (A.100), whereas the P.100 from Amilo had the higher level of 3-Xyl*p*.

*Phenolic Constituents in WE Cell Preparations*. Evidently, the ratio of total resonances in the region of phenolic compounds to those of arabinose anomeric protons (PhC/Ara) ($\delta$ 6.7−7.4 and 5.2−5.4, respectively), estimated from the $^1$H NMR spectra of the WE-P, made a distinction between materials from high and low breadmaking quality rye samples (**Table 4**). Its value was 2 times higher for low breadmaking quality Nawid (9.2) than that obtained for high breadmaking quality Amilo (4.4). A particularly high ratio of PhC/Ara was found for subfractions obtained at 40% saturation (P.40), in contrast to its markedly lower value observed for other subfractions.

**FTIR Spectroscopic Analysis.** As shown in **Figure 3**, the FTIR spectra highlighted well the differences in the chemical nature between α-amylase and proteinase extractable cell wall fractions. However, the spectra of both rye samples were generally similar. Clearly, the spectrum of WE-A showed the signal pattern typical for xylan-type polysaccharides (*57*−*60*). It was characterized by the multiple signals in the carbohydrate fingerprint region (1200−950 cm$^{-1}$), arising predominantly from a combination of C−O stretching and C−OH bending vibrations of the polymer backbone. Among them, an intense ring and side group band (C−C and C−O) at 1043 cm$^{-1}$ attributable to Xyl*p* (*60*) is indicative of $\beta$-(1→4)-linked structures. The WE-A spectra also show the amide I band (1640 cm$^{-1}$), which is mainly assigned to polypeptide carbonyl stretching (*61*), whereas a small absorption band at 1728 cm$^{-1}$ represents carbonyl stretching vibrations of esters (*57*). A relatively broad band centered at 3375 cm$^{-1}$ is assigned to the OH stretching modes of the polysaccharide chains. The signals, seen as a triplet in the region 2954−2850 cm$^{-1}$, indicate the methyl and methylene stretching in the cell wall preparations.

In the FTIR spectra of the proteinase extractable fractions, quite the opposite of those extracted in the presence of α-amylase, the amide I and II bands dominated (1640 and 1540 cm$^{-1}$, respectively). The latter results largely from N−H bending and C−N stretching vibrations of protein amide bonds (*61*). The presence of both amide bands in the WE-P spectra and only amide I in those of WE-A suggests that the cell wall preparations are composed of different kinds of proteins. In the case of the WE-P spectra, the broad absorption band with a maximum at 3300 cm$^{-1}$ is mainly due to the N−H stretching of the protein backbone. It is overlapped, to some extent, with that of the OH stretching modes of polysaccharides present in the samples. This was observed in the spectra of the Nawid subfractions, where such an absorbance appeared as a shoulder at 3400 cm$^{-1}$ (results not shown). Interestingly, there were notable differences in the ratio of the amide I to amide II band intensities between both rye samples in the WE-P spectra. The proteinase extractable fraction from Amilo was found to have a higher band intensity ratio (1.62) than that of Nawid (1.32). Apart from bands of fundamental aliphatic CH and $CH_2$ stretching modes in the region 2954−2850 cm$^{-1}$, the WE-P spectra displayed the distinct absorption band at 3066 cm$^{-1}$, which is assigned to an aromatic CH stretching (*62*). It seemed to be the only evidence for the presence of phenolic components

in the WE-P fractions provided by FTIR spectroscopy, because their diagnostic bands overlap with those of proteins in the 1700−1400 $cm^{-1}$ region. However, a closer examination of the spectra of subfractions obtained at 40% saturation level (P.40) in this region revealed two other bands, the former at 1515 $cm^{-1}$, corresponding to phenolic ring absorbance (*63*), and the latter at 1413 $cm^{-1}$, which can be assigned to symmetric stretching vibrations of carboxylate anions (*64*) (**Figure 4**). Both bands were discernible in the spectra of subfractions obtained from the WE-P, but they could not be observed in these of unfractionated materials. It was also evident that the amide I band is a composite of overlapping component bands, which can be related to different protein secondary structures such as α-helices, β-strands, turns, and nonordered polypeptide fragments (*61*).

**HPSEC.** Size exclusion profiles of the WE fractions obtained from both rye samples are shown in **Figure 5**. There were notable differences in the elution profiles of the α-amylase extractable polymers from high and low breadmaking quality rye samples, as pointed out by RI detector response in the high molecular weight (HMW) region (11−17 mL). The polymers in the WE-A fraction from Amilo had a higher proportion of counterparts with the highest average molecular weight [$M_w \sim 9.34 \times 10^5$ g/mol, polydispersity index ($I$) ∼ 1.23]. The second, unresolved, population was characterized by lower $M_w$ (∼$5.49 \times 10^5$ g/mol, $I \sim 1.08$). Instead, the WE-A polymers from Nawid had the same proportion of both populations eluted in the HMW ($M_w \sim 9.73 \times 10^5$ g/mol, $I \sim 1.12$, and $M_w \sim 5.56 \times 10^5$ g/mol, $I \sim 1.00$) as well as a slightly higher proportion of two overlapped populations that appeared in the low molecular weight (LMW) region at 18−21 mL ($M_w \sim 3.16$ and $2.92 \times 10^5$ g/mol for Amilo and 2.15 and $1.63 \times 10^5$ g/mol for Nawid, respectively).

The size exclusion chromatograms of proteinase extractable polymers exhibited, at least, two dominating, not-resolved peaks in the LMW region ($M_w \sim 5.71$ and $3.47 \times 10^4$ g/mol, $I \sim 1.65$ and 1.34 for Amilo, and $M_w \sim 6.53$ and $3.88 \times 10^4$ g/mol, $I \sim 1.71$ and 1.19 for Nawid). The small peaks in the HMW region were also visible in the WE-P chromatograms. However, their average $M_w$ values were notably higher (1.55 and $2.02 \times 10^6$ g/mol with $I \sim 1.06$, for Amilo and Nawid, respectively) than those of HMW polymer from the WE-A fractions, having the same hydrodynamic volumes.

Much like the polymers detected by RI, the profiles of UV-absorbing components in the WE-P fractions showed relatively small peaks in the HMW region in comparison to those from LMW region. Again, two superimposed peaks represented the bulk of the UV-absorbing materials, more likely proteins. Clearly, the populations recorded by both detectors coeluted in the region of the lowest molecular weight. Apparently, the UV-absorbing material from Nawid had a higher level of polymers with the lowest hydrodynamic volume, whereas that of Amilo was enriched in polymers, which did not coincide with the peak shown by the RI detector in the LMW region.

**Figure 6** shows the HPSEC profiles of the WE-A subfraction isolated from Amilo. It was obvious that a major subfraction with a low degree of substitution (A.60) had a much higher proportion of the HMW polymers, in comparison with the other subfractions. Interestingly, the A.60 from Nawid had a comparatively lower proportion of HMW polymers than that of Amilo, whereas in the remaining subfractions from both ryes, their proportion was similar (results not shown). This indicates a significant contribution of polymers present in the A.60 (AX-I) to the proportion of the HMW population in the parent fraction.

Taking into account a relatively low protein content in the WE-A fractions, it can be assumed that the HPSEC profiles recorded by the RI detector mainly correspond to polysaccharides. Thus, the profiles of their subfractions from both detectors may suggest partly association between polysaccharides and UV-absorbing components in the LMW region (**Figure 6**). There were two polysaccharide populations with the lowest hydrodynamic volumes, which could be involved in the interactions with UV-absorbing material in the cell wall. Such interactions, to a certain extent, may occur in the HMW region as well, especially in a subfraction obtained at the lowest saturation level (A.40), where both UV and RI signals were almost totally overlapped in this region.

The results presented have shown that the WE arabinoxylans from the outer layers of rye grain consisted of four subfractions

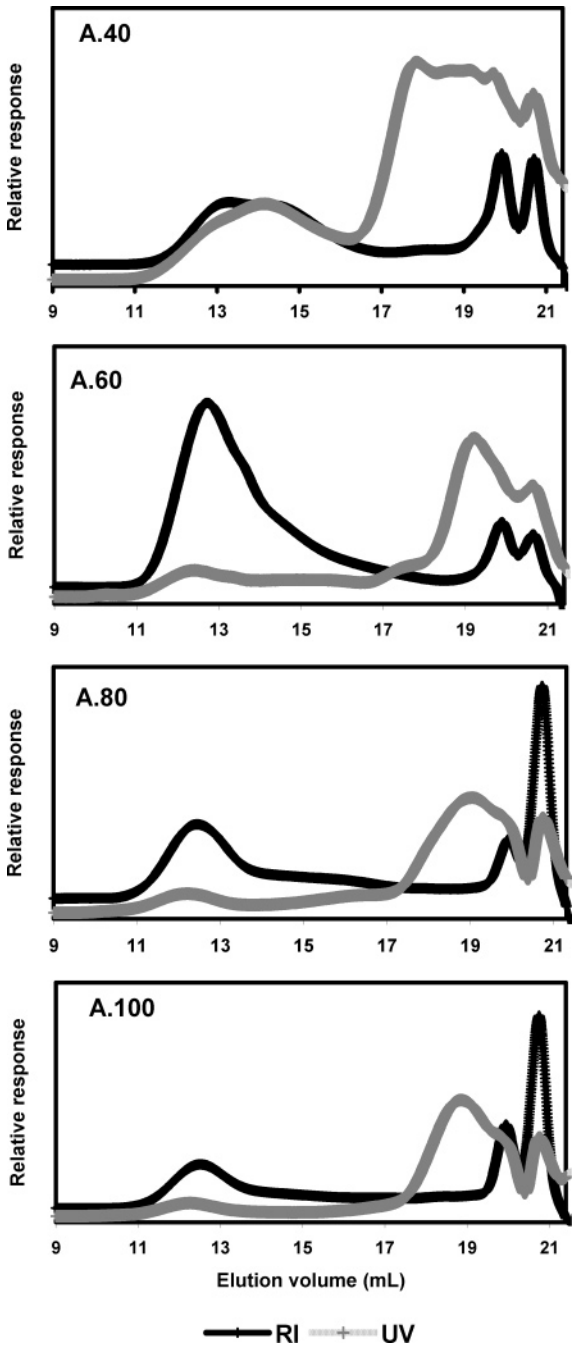


**Figure 6.** HPSEC-RI-UV profiles of α-amylase extractable subfractions obtained from outer layers of Amilo by ammonium sulfate precipitation at 40, 60, 80, and 100% saturation (A.40, A.60, A.80, and A.100, respectively).

with distinct structural differences, in comparison to WE arabinoxylans from rye flour, which contained only three of them (*26*). The obvious and much less clear differences in the structural features of arabinoxylans were observed between two ryes with opposite breadmaking qualities. Arabinoxylans isolated from the outer layers of high breadmaking quality rye were characterized by a higher proportion of HMW polymers as well as their lower proportion of subfractions with the least substituted backbone by Ara*f* (AX-IV) and the lower level of substitution with feruloyl residues, compared with those isolated from low breadmaking quality rye.

Proteolysis following amylolysis did not change much in the yield of arabinoxylans. Nevertheless, it also provided the evidence for structural diversity of proteinase extractable cell walls between two ryes with high and low breadmaking qualities. Additionally, it was possible to demonstrate their different chemical natures, in relation to those extracted in the presence of α-amylase, particularly exemplified by $^1$H NMR, HPSEC, and FTIR analyses. Next to the phenolic acids and ferulic acid dehydrodimers, the proteinase extractable cell walls contained an appreciable amount of other phenolics. Their concentration was higher in the fraction that derived from low breadmaking quality rye when compared with that of high breadmaking quality.

As a preliminary step, in this study we pointed out some elements in the structure of WE cell walls from the outer layers of the grain that may affect rye baking performance. However, their relationship with the breadmaking quality of rye and their usefulness in screening of breeding populations still need to be verified.

## ABBREVIATIONS USED

WE-A, α-amylase extractable cell wall fraction; WE-P, proteinase extractable cell wall fraction; $^1$H NMR, proton nuclear magnetic resonance; Ara/Xyl, arabinose-to-xylose ratio; diFA, ferulic acid dehydrodimers; HPSEC, high-performance size exclusion chromatography; FTIR, Fourier transform infrared spectroscopy; WE, water extractable fraction; WU, water unextractable fraction; Xyl*p*, xylopyranosyl residues; Ara*f*, arabinofuranosyl residues; 2-Xyl*p*, *β*-(1→4)-linked D-Xyl*p* substituted with α-L-Ara*f* at *O*-2; 3-Xyl*p*, *β*-(1→4)-linked D-Xyl*p* substituted with α-L-Ara*f* at *O*-3; 2,3-Xyl*p*, *β*-(1→4)-linked D-Xyl*p* substituted with α-L-Ara*f* at *O*-2 and *O*-3; HPLC, high-performance liquid chromatography; PhC/Ara, phenolic compounds-to-arabinose ratio; HMW, high molecular weight; LMW, low molecular weight.

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