JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Variability in the Structure of Rye Flour Alkali-Extractable Arabinoxylans

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The variability in rye flour alkali-extractable arabinoxylan (AE-AX) structures was examined by extensive fractionation and enzymic degradation studies. AX were isolated from destarched rye water-unextractables by sequential extraction with saturated barium hydroxide solution, water, 1.0 M sodium hydroxide, and water. The isolated AE-AX contained ca. 51% AX with an arabinose to xylose (A/X) ratio of 0.71. Fractionation of the isolated AE-AX by ethanol precipitation yielded a range of AE-AX fractions containing AX molecules with different A/X ratios and substitution patterns. Degradation of these structurally different AE-AX fractions by an *Aspergillus aculeatus* endoxylanase (XAA) and a *Bacillus subtilis* endoxylanase (XBS) resulted in AX fragments with various structural features. Further fractionation of the degraded AE-AX fractions by ethanol precipitation showed that a strong correlation exists between the structural features of the AX fragments, that is, average degree of polymerization (DP) of the xylan backbone, A/X ratio, and substitution pattern. Results indicated that the rye flour AE-AX consist of a continuum of structures rather than of two types of AX or two types of regions in the AX molecule.

KEYWORDS: Rye flour; alkali-extractable arabinoxylan; fractionation; enzymic degradation; endoxylanase; structure

INTRODUCTION

Rye arabinoxylans (AX), similar to other cereal AX, consist of a backbone of 1,4-linked β-D-xylopyranose residues, which are unsubstituted or mono- or disubstituted with single α-L-arabinofuranose residues at the O-2 and/or O-3 position(s). To some of these arabinose residues is ester-linked a ferulic acid moiety at position O-5. Two types of AX can be distinguished: water-extractable AX (WE-AX) and water-unextractable AX (WU-AX). WU-AX, representing approximately 75% of the total AX population of the rye kernel, are unextractable because they are retained in the cell walls by covalent and non-covalent interactions among AX and between AX and other cell wall constituents. WE-AX are thought to be loosely bound at the cell wall surface (*1*).

AX can be degraded by endo-β-1,4-xylanases (EC 3.2.1.8), further referred to as endoxylanases. These enzymes hydrolyze internal β-1,4-linkages between xylose residues, generating (arabino-)xylan fragments with lower molecular weight (MW) and (un)substituted xylo-oligosaccharides. Their action can thus lead to (partial) solubilization of WU-AX and to a decrease in MW of WE-AX and/or solubilized AX (*2*). On the basis of their primary sequence and structure, endoxylanases are classified into two main groups: glycoside hydrolase family (GHF) 10 and GHF 11 (*3*). Fungal and bacterial endoxylanases are present in both GHF 10 and 11, in contrast to plant endoxylanases, which to date have been exclusively classified in GHF 10. GHF 10 endoxylanases have lower substrate specificity than GHF 11 endoxylanases, meaning that the former enzymes produce oligosaccharides with a low degree of polymerization (DP), whereas the latter tend to be more specific for xylan and form larger oligosaccharides (*2*, *4*).

Important structural characteristics of AX are the average DP of the xylan backbone, the A/X ratio, and the substitution pattern. Variability in these structural features determines AX heterogeneity. The structure of WU-AX can be studied only by following alkaline solubilization of the AX. This treatment results in the saponification of the ester bonds linking ferulic acid to arabinose, releasing individual AX molecules from the cell wall structure (*5*). On the basis of the studies of Hromadkova et al. (*6*), Ebringerova et al. (*7*), Nilsson et al. (*8*), and Vinkx et al. (*9*), three different groups of AX can be distinguished in rye bran when isolated by sequential alkaline extraction. A first group consists of lowly substituted AX with a low A/X ratio, a second group contains AX with an intermediate A/X ratio and mainly unsubstituted and monosubstituted xylose residues, and a third group contains AX with a high A/X ratio and mainly mono- and disubstituted xylose residues.

In contrast to rye bran alkali-extractable AX (AE-AX), rye flour AE-AX obtained after sequential alkaline extraction all

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10.1021/jf0623790 CCC: $37.00
Published on Web 02/03/2007

show similar xylose substitution levels (*5*, *8*, *10*). However, further fractionation of the AE-AX from rye flour by ammonium sulfate precipitation yields subfractions that differ in structure (*5*), suggesting variability in the structure of rye AE-AX. Whereas for rye WE-AX, two structural models were proposed, that is, one suggesting the occurrence of two types of AX or two types of regions in the AX molecule (*11*) and a second one hypothesizing the existence of a range of structures (*12*), so far, to the best of our knowledge, no structural models have been reported for rye AE-AX.

The aim of this study was therefore to examine the variability in rye flour AE-AX structures by extensive fractionation and enzymic degradation studies. Thus, rye flour AE-AX were fractionated by gradual ethanol precipitation. The obtained fractions were degraded by endoxylanases with different substrate specificity and further fractionated by ethanol precipitation. The structural characteristics (average DP, A/X ratio, and substitution pattern) of the resulting AX degradation products were studied.

MATERIALS AND METHODS

Chemicals. Thermostable α-amylase (Termamyl 120L) was obtained from Novozymes (Bagsvaerd, Denmark), and amyloglucosidase of *Aspergillus niger* was from Megazyme (Bray, Ireland). These enzymes were free from AX-degrading enzyme side activities. Stabifix super silica gel was from Stabifix Brauerei-Technick KG (Graefelfing/Munich, Germany). Standard P-82 pullulans were purchased from Showa Denko K.K. (Tokyo, Japan), and xylohexaose was from Megazyme. Deuterium oxide (D_2O) was obtained from Acros Organics (Geel, Belgium); barium hydroxide, sodium borohydride, and bovine serum albumin (BSA) were from Sigma-Aldrich (Bornem, Belgium). All chemicals and reagents were of at least analytical grade.

Rye Flour. Commercial rye flour (68% extraction) was obtained from Molens Limbourg (Gooik, Belgium). Flour was boiled under reflux in 80% ethanol (1:5 w/v, 60 min) to inactivate endogenous enzymes. The residue was filtered and air-dried at room temperature (RT). Moisture [percent dry matter (% dm), AACC method 44-15A) (*13*), ash (% dm, AACC method 08-01) (*13*), and protein contents (N × 5.7) (% dm, AOAC official Dumas method 990.03) (*14*) of the rye flour after inactivation were 8.89, 0.77, and 4.74%, respectively. Total AX and WE-AX contents (% dm) (sugar content determination following hydrolysis and derivatization as described below) of the inactivated rye flour were 4.36 and 1.63%, respectively.

Endoxylanases. A GHF 10 endoxylanase of *Aspergillus aculeatus* (XAA) was obtained from Puratos (Groot-Bijgaarden, Belgium). A GHF 11 endoxylanase of *Bacillus subtilis* (XBS) was obtained from Danisco (Brabrand, Denmark). Both enzymes were free from other AX-degrading enzyme side activities. All endoxylanase solutions were made with sodium acetate buffer (25 mM, pH 5.0) containing BSA (0.5 mg/mL).

The endoxylanase activity of both enzymes was determined by a colorimetric method using azurine-cross-linked wheat AX (Xylazyme AX) tablets (Megazyme) as substrate. The endoxylanases were suspended in sodium acetate buffer (25 mM, pH 5.0, 0.5 mg/mL BSA). After shaking (30 min, 6 °C) and centrifugation (5000*g*, 10 min, 6 °C), filtered supernatant was used for the estimation of endoxylanase activity. A substrate tablet was added to preincubated filtrate (1.0 mL, 10 min, 30 °C). The suspension was incubated for 10 min at 30 °C. The reaction was stopped by the addition of 1.0% (w/v) tris-(hydroxymethyl)aminomethane solution (10.0 mL) and vigorous vortex-mixing. After 10 min at RT, the tubes were shaken and the contents were filtered. The absorbance values (590 nm) were measured with an Ultraspec III UV−visible spectrophotometer (Pharmacia Biotech, Uppsala, Sweden) and corrected for the loss of color of the native substrate tablet and for color originating from the filtrate. One unit (U_X) of endoxylanase activity was defined as the amount of enzyme needed to yield a change in absorbance value (at 590 nm) of 1.0 under the conditions of the assay.

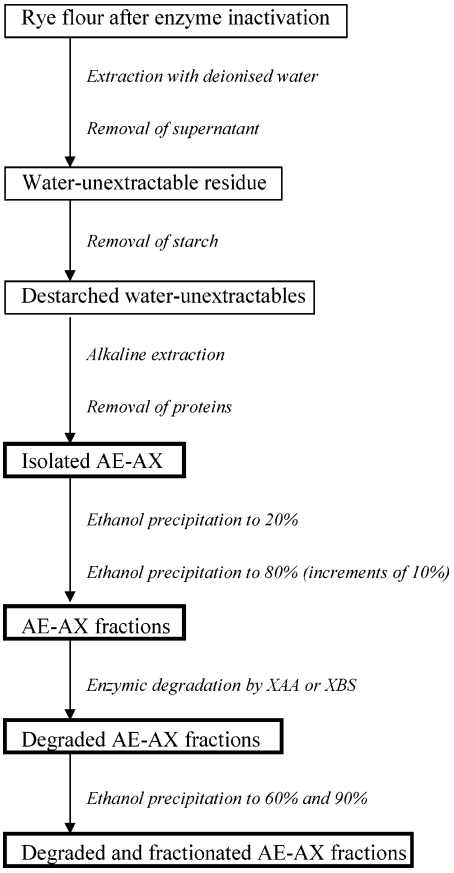

Figure 1. Schematic overview of the main steps in the procedure for the formation, purification, fractionation, and enzymic degradation of AE-AX from rye flour.

Isolation and Fractionation of AE-AX from Rye Flour. *Formation and Purification of AE-AX*. Rye flour (300 g), after inactivation of endogenous enzymes, was extracted with deionized water (1:6 w/v, 90 min, 6 °C) under continuous stirring. After centrifugation (10000*g*, 30 min, 6 °C), the residue was washed with deionized water and centrifuged (10000*g*, 20 min, 6 °C). To remove residual starch, the residue was suspended in deionized water and heated to 75 °C prior to incubation with Termamyl (20.0 mL, 60 min, 90 °C) under continuous stirring. After boiling (20 min) to inactivate the enzyme and cooling to RT, the suspension was centrifuged (10000*g*, 20 min, 18 °C), and the residue was washed as indicated above to remove water-extractable components. The washed residue was suspended in deionized water and incubated with amyloglucosidase (15.0 mL) (16 h, 60 °C, pH 4.5) under continuous stirring. The suspension was then boiled (20 min), cooled to RT, and centrifuged (10000*g*, 20 min, 18 °C). The pellet was washed as above. The obtained residue was suspended in deionized water and dialyzed against deionized water (48 h, 6 °C). The product obtained following lyophilization is further referred to as "destarched rye water-unextractables" (**Figure 1**).

Destarched rye water-unextractables were used for sequential alkaline extraction according to the method by Gruppen et al. (*15*). An aliquot (25 g) was extracted under continuous stirring (16 h, RT) with saturated barium hydroxide solution (1:30 w/v) (750 mL) containing 1% (w/v) sodium borohydride. After centrifugation (10000*g*, 20 min, 18 °C), the residue was re-extracted with the same solvent (375 mL) (2 h, RT) and centrifuged (10000*g*, 20 min, 18 °C). The supernatants, containing the formed AE-AX, were combined and purified as described below. The residue left after extraction with saturated barium hydroxide was suspended in deionized water (750 mL), acidified to pH 5.0 with 1.0 M HCl, stirred continuously for 16 h at RT, and centrifuged (10000*g*, 20 min, 18 °C). The residue was re-extracted with deionized water (375 mL) (2 h, RT) and centrifuged (10000*g*, 20 min, 18 °C). Both supernatants were combined and underwent purification as described below. The residue was further extracted under continuous stirring (16

h, RT) with 1.0 M sodium hydroxide (750 mL) containing 1% (w/v) sodium borohydride and centrifuged (10000*g*, 20 min, 18 °C). The residue was again extracted with the same solvent (375 mL) (2 h, RT) and centrifuged (10000*g*, 20 min, 18 °C). The supernatants were pooled and purified as described below. The residue left after extraction with 1.0 M sodium hydroxide was suspended in deionized water (750 mL), acidified to pH 5.0 with 1.0 M HCl, stirred continuously for 16 h at RT, and centrifuged (10000*g*, 20 min, 18 °C). The residue was re-extracted with deionized water (375 mL) (2 h, RT) and centrifuged (10000*g*, 20 min, 18 °C). Both supernatants were combined and underwent purification as described below.

Purification of the different AE-AX containing supernatants was done by removing the proteins with silica gel as described by Trogh et al. (*16*).

The lyophilized fractions obtained after extraction with the different alkaline solvents were mixed, suspended in deionized water, and lyophilized to obtain a homogeneous fraction, which was indicated as the "isolated AE-AX" (**Figure 1**). The yield of AX in the isolated AE-AX is expressed as a percentage of the total WU-AX content of the rye flour.

Fractionation of AE-AX. The isolated AE-AX were solubilized in deionized water (1.0% w/v). Fractionation was carried out at RT by stepwise increase in ethanol concentration. Ethanol (95%) was slowly added under continuous stirring at RT to a concentration of 20%. After ethanol addition, the mixture was stirred for 30 min at RT and kept overnight at 6 °C. The precipitate was then recovered by centrifugation (10000*g*, 30 min, 6 °C), solubilization in deionized water, and lyophilization. The obtained fraction was designated AE-$AX_{0-20\%}$. Then, the supernatant was brought to an ethanol concentration of 30%. After ethanol addition, the mixture was stirred (30 min, RT), kept overnight at 6 °C, and centrifuged (10000*g*, 30 min, 6 °C). The precipitate was collected as described above and designated AE-$AX_{20-30\%}$. This procedure was repeated with stepwise increase in ethanol concentration (increments of 10%) until a final ethanol concentration of 80% was reached. Following removal of the aqueous ethanol solvent by rotary evaporation (40 °C), the remaining fraction (AE-$AX_{80+\%}$) was dissolved in deionized water and lyophilized. The resulting fractions (AE-$AX_{0-20\%}$, AE-$AX_{20-30\%}$, AE-$AX_{30-40\%}$, AE-$AX_{40-50\%}$, AE-$AX_{50-60\%}$, AE-$AX_{60-70\%}$, AE-$AX_{70-80\%}$ and AE-$AX_{80+\%}$) are further referred to as the "AE-AX fractions" (**Figure 1**). Yields of the AX recovered in the AE-AX fractions after gradual ethanol precipitation are expressed as a percentage of the isolated AE-AX.

Enzymic Degradation and Fractionation of AE-AX Fractions. The AE-AX fractions (AE-$AX_{20-30\%}$, AE-$AX_{30-40\%}$, AE-$AX_{40-50\%}$, AE-$AX_{50-60\%}$, AE-$AX_{60-70\%}$, and AE-$AX_{70-80\%}$) (250 mg) were dissolved in sodium acetate buffer (25 mM, pH 5.0) (124 mL) and incubated (18 h, 30 °C) with 1.0 mL of a solution containing 0.0003 U_X XAA or 0.05 U_X XBS in the same buffer. Under these incubation conditions, hydrolysis end products were obtained (results not shown). After incubation, the solutions were heat treated (30 min, 100 °C), cooled, and lyophilized. The obtained digests are further designated the "degraded AE-AX fractions" (**Figure 1**).

The degraded AE-AX fractions, in their turn, were further fractionated by ethanol precipitation. The fractions were solubilized in deionized water (1% w/v). Ethanol (95%) was slowly added under continuous stirring at RT to a concentration of 60%. After ethanol addition, the mixture was stirred for 30 min at RT and kept overnight at 6 °C. The precipitate was recovered by centrifugation (10000*g*, 30 min, 6 °C), solubilization in deionized water, and lyophilization. The obtained fraction was designated $F_{0-60\%}$. Then, the supernatant was brought to an ethanol concentration of 90%. After ethanol addition, the mixture was stirred (30 min, RT), kept overnight at 6 °C, and centrifuged (10000*g*, 30 min, 6 °C). The precipitate was collected as described above and designated $F_{60-90\%}$. Following removal of the aqueous ethanol solvent from the supernatant by rotary evaporation (40 °C), the remaining fraction ($F_{90+\%}$) was dissolved in deionized water and lyophilized. The resulting fractions are further referred to as the "degraded and fractionated AE-AX fractions" (**Figure 1**). Yields of the AX recovered in the degraded and fractionated AE-AX fractions were expressed as a percentage of the AX present in the degraded AE-AX fractions.

Analytical Methods. *Protein and β-Glucan Contents.* Protein contents were estimated colorimetrically with the Folin−Ciocalteu reagent as described by Lowry et al. (*17*), using BSA as a standard. Mixed linkage (1,3), (1,4)-β-D-glucan contents were determined enzymically [lichenase (60 min, 50 °C) and β-glucosidase (10 min, 50 °C)] with a (1,3), (1,4)-β-D-glucan test kit from Megazyme following the method described in the technical sheet provided with it.

Monosaccharide and Reducing End Sugar Content and Composition. Monosaccharide content and composition of samples was determined by gas−liquid chromatography of alditol acetates obtained after acid hydrolysis (60 min at 110 °C) (*18*) and reduction and acetylation (*19*) of carbohydrate-containing samples. The derivatives were separated on a Supelco SP-2380 polar column (Supelco, Bellefonte, PA) in an Agilent chromatograph (Agilent 6890 series, Wilmington, DE) equipped with a flame ionization detector. The inlet and detector temperatures were 270 °C, whereas separation took place at 225 °C. The carrier gas was He. The internal standard was β-D-allose (Sigma, Bornem, Belgium).

Reducing end sugar contents in the AX-containing fractions were estimated by gas−liquid chromatography of alditol acetates as described by Courtin et al. (*20*), thus performing an analysis similar to that above but with reduction prior to hydrolysis and acetylation.

High-Performance Size Exclusion Chromatography (HPSEC). Apparent peak molecular weights of the AX were estimated by HPSEC on a Shodex SB-806 HQ column (300 mm × 8 mm i.d.) with a Shodex SB-G guard column (50 mm × 6 mm i.d.) from Showa Denko K.K. The MW separation range of the SB-806 HQ column is between 1.0×10^3 and 20.0×10^6. Samples (16.0 mg) were solubilized in sodium acetate buffer (25 mM, pH 5.0) (8.0 mL) and filtered (pore size = 0.45 μm). Elution of the samples (20 μL) was with sodium acetate buffer (25 mM, pH 5.0) (0.5 mL/min at 30 °C) on a Kontron 325 pump system (Kontron, Milan, Italy) with autoinjection. The separation was monitored with a refractive index (RI) detector (VDS Optilab, Berlin, Germany). MW markers (1.5 mg/mL) were Shodex standard P-82 pullulans with molecular weights of 160.0×10^4, 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, and 0.59×10^4 and xylohexaose (MW = 810), glucose (MW = 180), and xylose (MW = 150).

Proton Nuclear Magnetic Resonance (^{1}H NMR) Spectroscopy. Substitution profiles of AX were studied by ^{1}H NMR spectroscopy. Samples (4.0 mg) were dissolved in D_2O (1.0 mL), stirred (8 h), and lyophilized. This step was repeated three times. The lyophilized samples were then dissolved in D_2O (0.5 mL) and analyzed. The ^{1}H NMR spectra were recorded with a Bruker AMX 500 MHz NMR spectrometer (Karlsruhe, Germany) at 85 °C. Pulse repetition time was 2.0 s, and the number of scans was 64. The proportions (percent) of un-, mono-, and disubstituted xylose residues were calculated by combining the gas−liquid chromatography results with the ^{1}H NMR spectral data according to the method of Roels et al. (*21*), which itself relies on the approach by Westerlund et al. (*22*).

RESULTS AND DISCUSSION

Yield and Chemical Composition of Isolated AE-AX and AE-AX Fractions. AX were isolated from destarched rye water-unextractables by sequential extraction with saturated barium hydroxide solution, water, 1.0 M sodium hydroxide, and again water. The resulting extracts were mixed to obtain a homogeneous fraction, which was designated the "isolated AE-AX". About 42% of the WU-AX originally present in the rye flour could be solubilized by the alkaline solvents. When calculated on the level of total AX present in the rye flour, 30% of the AX were recovered as AE-AX. This yield was lower than those reported by Cyran et al. (*5*), who solubilized 37 and 43% of the overall AX content in rye flour with high and low bread-making qualities, respectively. The relatively low yield obtained in this study can be explained on the basis of two observations. On the one hand, the residue after alkaline extraction still contained ca. 15% of the WU-AX originally present in the rye flour. These AX could be part of bran particles present in the

Table 1. Yield (Percent of Isolated AE-AX), Monosaccharide, AX, and Protein Contents (Percent Dry Matter), and A/X Ratio of Isolated AE-AX and AE-AX Fractions

fraction	yield	Ara[a]	Xyl[a]	Man[a]	Gal[a]	Glc[a]	AX[b]	A/X	protein
AE-AX	100.0	24.0	33.8	2.7	2.0	40.9	50.8	0.71	11.2
AE-AX$_{0-20\%}$	12.7	4.1	9.5	3.6	0.4	71.8	12.0	0.44	5.4
AE-AX$_{20-30\%}$	12.5	25.9	64.7	0.7	0.4	27.0	79.7	0.40	4.2
AE-AX$_{30-40\%}$	5.4	28.6	62.7	1.1	0.6	23.6	80.4	0.46	4.1
AE-AX$_{40-50\%}$	10.9	35.2	65.8	2.4	0.9	17.8	88.8	0.53	3.2
AE-AX$_{50-60\%}$	11.5	42.5	62.8	2.7	1.6	11.1	92.7	0.68	1.8
AE-AX$_{60-70\%}$	16.9	50.7	57.4	3.4	3.9	7.0	95.2	0.88	3.4
AE-AX$_{70-80\%}$	25.8	46.6	44.9	2.4	6.0	6.0	80.5	1.04	6.0
AE-AX$_{80+\%}$	4.4	8.8	5.2	2.2	0.9	16.9	12.3	1.71	46.6

[a] Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose. [b] AX = (Ara + Xyl) × 0.88.

rye flour, which had an ash content of 0.77%. Such AX are significantly less extractable than endosperm AX (*8*, *16*). On the other hand, AX were lost during the Termamyl and amyloglucosidase treatments (ca. 17 and 19% of the WU-AX originally present in the rye flour, respectively). These treatments were performed at elevated temperature, and Cyran et al. (*23*) showed that, with increasing extraction temperature, more AX could be extracted from rye flour.

The isolated AE-AX contained ca. 51% AX with an A/X ratio of 0.71 (**Table 1**). The fraction was highly contaminated with glucose (ca. 41%). Despite the amylolysis in the isolation procedure, the isolated AE-AX still contained a large level of starch. As part of the glucose (ca. 11%) originated from β-glucan, the remaining ca. 30% is considered to originate from starch. The protein content was ca. 11%.

The isolated AE-AX were fractionated by graded ethanol precipitation into different AE-AX fractions (AE-AX$_{0-20\%}$, AE-AX$_{20-30\%}$, AE-AX$_{30-40\%}$, AE-AX$_{40-50\%}$, AE-AX$_{50-60\%}$, AE-AX$_{60-70\%}$, AE-AX$_{70-80\%}$, and AE-AX$_{80+\%}$). Apart from the fractions AE-AX$_{0-20\%}$ and AE-AX$_{80+\%}$, which were more contaminated with glucose and protein, respectively, the AX contents of the other fractions were very high (**Table 1**). Due to the low level of AX in AE-AX$_{0-20\%}$ and AE-AX$_{80+\%}$, the characteristics of these fractions are not further reported.

With the exception of the fraction AE-AX$_{20-30\%}$, the level of AX recovered in the AE-AX fractions increased with ethanol concentration (**Table 1**). Fraction AE-AX$_{70-80\%}$ contained the highest level of AX. With increasing ethanol concentration, AX of increasing A/X ratio were precipitated (**Table 1**). A similar trend was found for AE-AX fractions of rye flour precipitated by ammonium sulfate (*5*). An increase in A/X ratio with increasing ethanol concentration was also observed for AE-AX fractions of wheat flour (*24*) and barley flour (*25*, *26*).

Structural Characteristics of Isolated AE-AX and AE-AX Fractions. Table 2 shows the A/X ratio, the average DP of the xylan backbone, the estimated apparent peak MW, and the substitution pattern of the AX present in the isolated AE-AX and AE-AX fractions. The average DP of the xylan backbone of AX was estimated on the basis of the total xylose and reducing end xylose (RX) contents of the AE-AX fractions. The AX of the isolated AE-AX had an average DP of ca. 255 and an estimated apparent peak MW of 157000, with the former value mainly being determined by the smaller fragments in the AE-AX$_{80+\%}$ fraction (results not shown). With increasing ethanol concentration, the AX of the AE-AX fractions showed variability in average DP as well as in apparent peak MW. **Figure 2** illustrates that the AE-AX populations precipitating at increasing ethanol concentration had broad MW distributions.

Table 2. A/X Ratio, Average Degree of Polymerization (DP) of the Xylan Backbone, Estimated Apparent Peak Molecular Weight (MW), and Relative Proportions (Percent of Total Xylose) of Un-, Mono-, and Disubstituted Xylose Residues of AX from Isolated AE-AX and AE-AX Fractions

fraction	A/X	DP[a]	MW	un-[b]	mono-[b]	di-[b]
AE-AX	0.71	255	157000	49.2	30.6	20.2
AE-AX$_{20-30\%}$	0.40	400	713000	65.0	30.0	5.0
AE-AX$_{30-40\%}$	0.46	355	713000	62.4	29.2	8.4
AE-AX$_{40-50\%}$	0.53	395	1263000	59.1	28.8	12.1
AE-AX$_{50-60\%}$	0.68	450	1306100	50.5	31.0	18.5
AE-AX$_{60-70\%}$	0.88	345	235000	40.2	31.6	28.2
AE-AX$_{70-80\%}$	1.04	240	137200	29.9	36.1	34.0

[a] DP = Xyl/RX with Xyl = monosaccharide content of xylose and RX = reducing xylose content. [b] Un-, mono-, and di- represent percentages of total xylose occurring as un-, mono-, and disubstituted xylose residues.

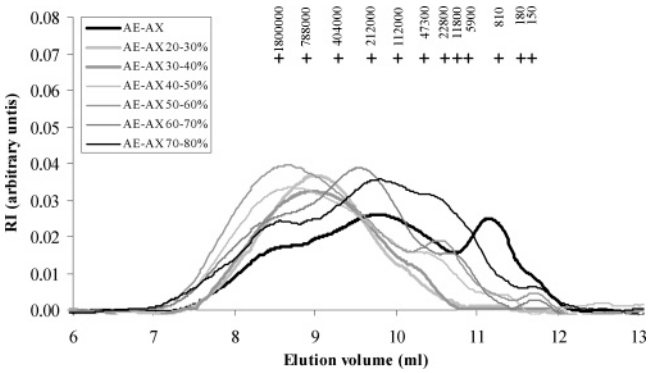

Figure 2. HPSEC apparent MW profiles of the AX from the isolated AE-AX and the AE-AX fractions. MW markers (+) from left to right are 160.0×10^4, 78.8×10^4, 40.4×10^4, 21.2×10^4, 11.2×10^4, 4.73×10^4, 2.28×10^4, 1.18×10^4, and 0.59×10^4, xylohexaose, glucose, and xylose.

Taken together, these data indicate that the precipitation behavior of the isolated AX is to a large extent determined by A/X ratio and substitution pattern and only to lesser extent by DP and apparent peak MW.

The AX of the isolated AE-AX contained ca. 49% un-, 31% mono-, and 20% disubstituted xylose residues (**Table 2**). These substitution levels were comparable to those reported by Cyran et al. (*5*) for AX extracted with saturated barium hydroxide solution from rye flour (51% un-, 29% mono-, and 20% disubstituted xylose residues).

The AX that precipitated between 20 and 30% ethanol were built up predominantly of un- and monosubstituted xylose residues (ca. 65 and 30%, respectively) with a relatively low level of disubstituted xylose residues (ca. 5%) (**Table 2**). The fractions obtained at higher ethanol concentrations contained progressively more disubstituted xylose residues and, at the same time, fewer unsubstituted xylose residues. The highly substituted AX population isolated between 70 and 80% ethanol was enriched in both mono- and disubstituted xylose residues (ca. 36 and 34%, respectively). **Figure 3** shows the relative proportions of the differently substituted xylose residues present in each AE-AX fraction as a function of the A/X ratio. It shows that the levels of unsubstituted xylose residues decreased linearly, whereas those of disubstituted xylose residues increased linearly with increasing A/X ratio. The level of monosubstitution remained almost constant with increasing A/X ratio. The relationships observed between A/X ratio and substitution pattern are in agreement with those reported earlier for rye flour AE-AX fractionated by ammonium sulfate precipitation (*5*). The same trends were also observed for wheat flour (*24*) and barley

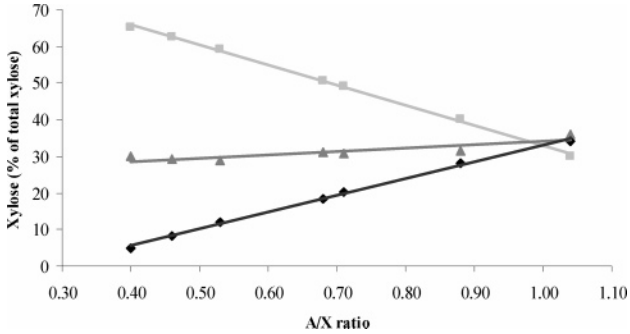

Figure 3. Levels of un- (squares), mono- (triangles), and disubstituted (diamonds) xylose residues (percent of total xylose) as a function of the A/X ratio of the AX from the AE-AX fractions.

Table 3. A/X Ratio, Estimated Apparent Peak Molecular Weight (MW), and Average Degree of Polymerization (DP) of the Xylan Backbone of the AX from the Degraded AE-AX Fractions after Incubation with XAA or XBS

		MW		DP[a]	
fraction	A/X	XAA	XBS	XAA	XBS
$AE\text{-}AX_{20-30\%}$	0.40	950	1500	5	8
$AE\text{-}AX_{30-40\%}$	0.46	950	1700	5	9
$AE\text{-}AX_{40-50\%}$	0.53	1400	2500	6	11
$AE\text{-}AX_{50-60\%}$	0.68	3400	4400	9	18
$AE\text{-}AX_{60-70\%}$	0.88	6500	40600	16	34
$AE\text{-}AX_{70-80\%}$	1.04	57800	79400	48	69

[a] DP = Xyl/RX with Xyl = monosaccharide content of xylose and RX = reducing xylose content.

flour (*25*, *26*) AE-AX fractionated by ethanol precipitation, except for the level of monosubstitution. Gruppen et al. (*24*) observed a decrease in monosubstituted xylose residues with increasing A/X ratio for wheat flour AE-AX, whereas Viëtor et al. (*26*) found an increase in monosubstituted xylose residues with increasing A/X ratio for barley flour AE-AX.

Structural Characteristics of Degraded AE-AX Fractions. The structurally different AE-AX fractions obtained after gradual ethanol precipitation were degraded by two endoxylanases differing in substrate specificity, that is, XAA or XBS, to gain further insight into the structural features of the rye flour AE-AX. In preliminary experiments with HPSEC (results not shown), incubation conditions were tested for each AE-AX fraction to obtain hydrolysis end products. Maximum degradation of the AE-AX fractions was reached after 18 h of incubation at 30 °C with 0.0003 U_X XAA or 0.05 U_X XBS.

Table 3 summarizes the estimated apparent peak MW of the hydrolysis end products obtained after incubation of the AE-AX fractions with XAA or XBS. For XAA as well as for XBS, the apparent peak MW of the degraded AX increased with increasing A/X ratio of the AE-AX fractions. These results show that highly substituted substrates were more difficult to degrade, implying that the endoxylanases were sterically hindered by the presence of arabinose substituents. With increasing A/X ratio of the AE-AX fractions, the increase in hydrolysis end product apparent peak MW became larger and was more pronounced after incubation of these fractions with XBS than with XAA. The latter indicates that XBS was more sterically hindered by arabinose substituents than XAA. XAA formed AX fragments with lower apparent peak MW than XBS. The two latter observations are in agreement with the lower substrate specificity of XAA compared with that of XBS (*2*, *4*).

Table 3 further lists the average DP of the xylan backbone of the AX fragments formed after enzymic degradation of the

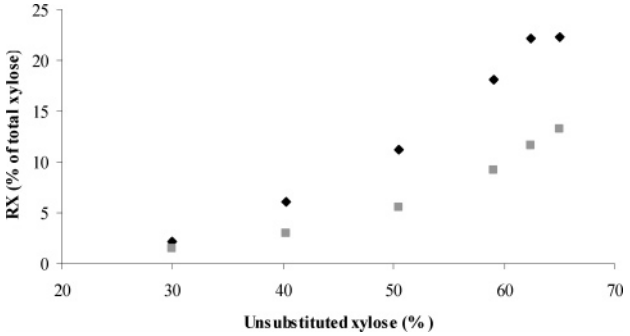

Figure 4. Reducing xylose (RX) levels (percent of total xylose) obtained after incubation of the AE-AX fractions with XAA (diamonds) or XBS (squares) as a function of the levels of unsubstituted xylose residues of the AX from the AE-AX fractions.

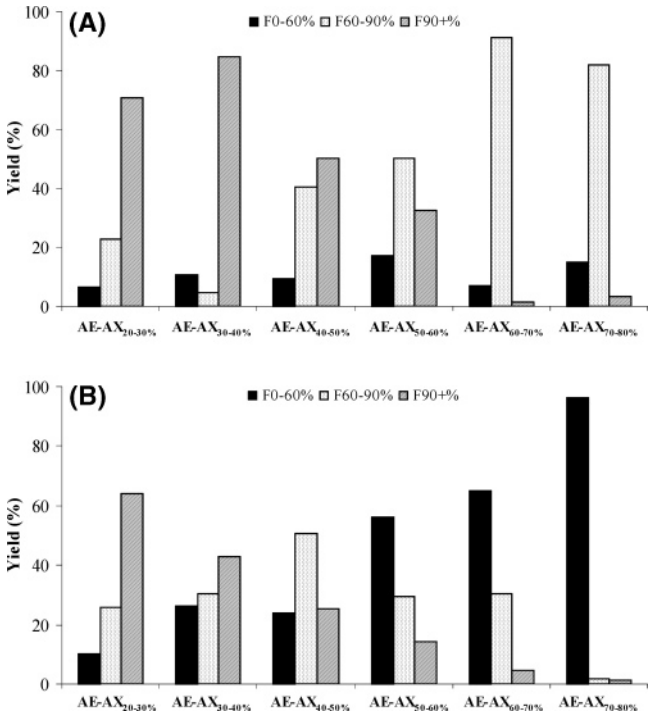

Figure 5. Yield (percent of AX in degraded AE-AX fractions) of AX in the degraded and fractionated AE-AX fractions, with degradation by XAA (**A**) and degradation by XBS (**B**).

different AE-AX fractions with XAA or XBS. For both endoxylanases, the average DP of the AX fragments increased with increasing A/X ratio of the AE-AX fractions, and the fragments formed by XAA had a lower average DP than those formed by XBS. These results are in agreement with the apparent peak MW data, indicating decreasing enzymic degradability of the rye flour AE-AX with increasing A/X ratio and confirming the lower substrate specificity of XAA compared with that of XBS.

A plot of the levels of RX formed after incubation of the AE-AX fractions with XAA or XBS as a function of the levels of unsubstituted xylose residues of these AE-AX fractions (**Figure 4**) shows that the enzymic degradability of the rye flour AE-AX is also determined by the substitution pattern of the xylan backbone. The levels of RX formed by XAA and XBS increased with increasing levels of unsubstituted xylose residues (corresponding with decreasing levels of disubstituted xylose residues, **Figure 3**). At similar levels of unsubstituted xylose residues, the AX fragments formed by XAA contained more RX than those formed by XBS. This difference in action

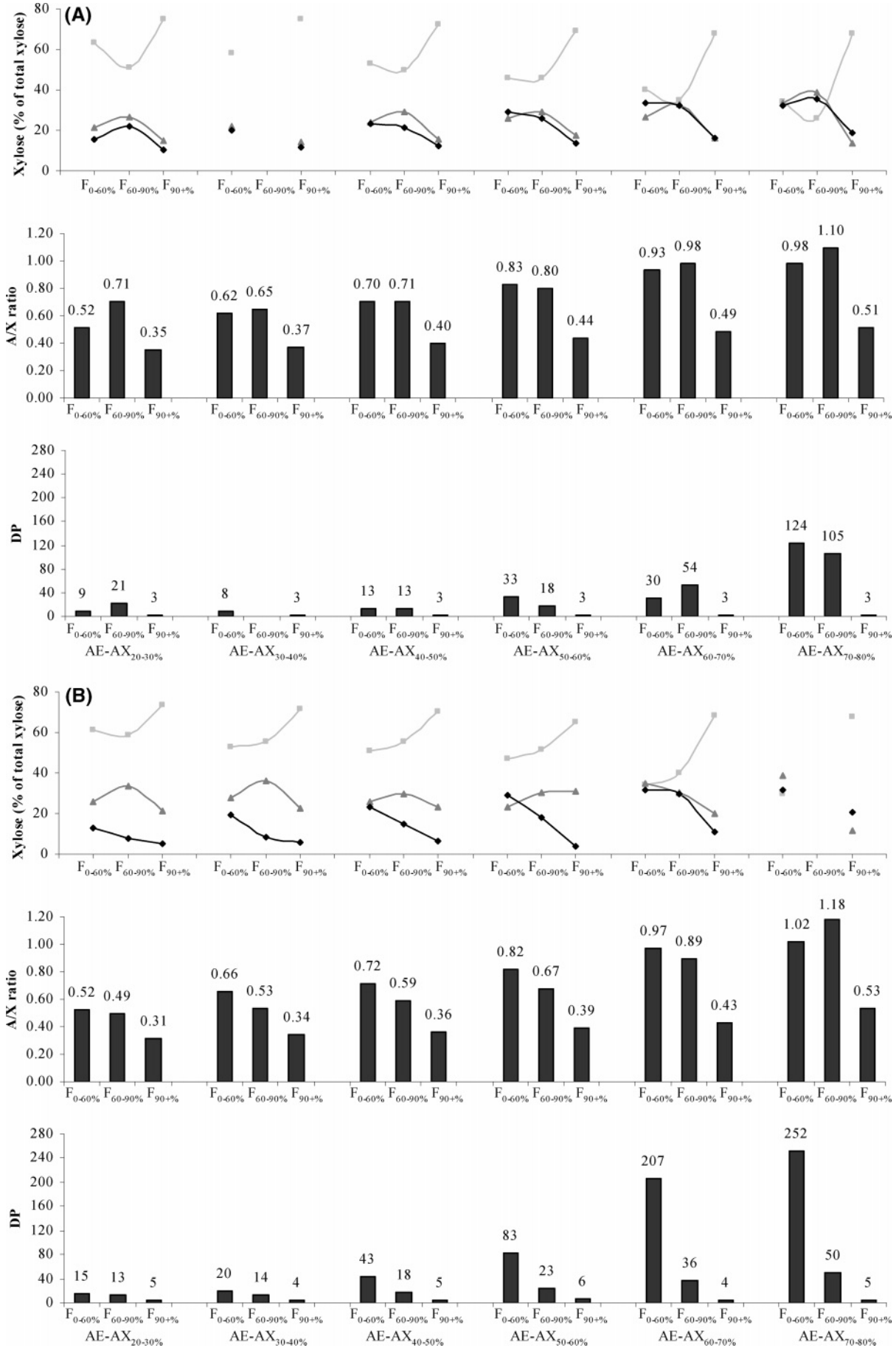

Figure 6. Average degree of polymerization (DP) of the xylan backbone, A/X ratio, and relative proportions (percent) of un- (squares), mono- (triangles), and disubstituted (diamonds) xylose residues of AX from XAA (**A**) and XBS (**B**) degraded and fractionated AE-AX fractions.

between XAA and XBS was more pronounced at higher levels of unsubstituted xylose residues.

Yields and Structural Characteristics of Degraded and Fractionated AE-AX Fractions. To further elucidate the structural features of the rye flour AE-AX, the AX fragments formed after incubation of the AE-AX fractions with XAA or XBS were fractionated by ethanol precipitation to 60 and 90% ethanol.

Figure 5A represents the yields of the AX recovered in the $F_{0-60\%}$, $F_{60-90\%}$, and $F_{90+\%}$ fractions after ethanol precipitation of the fragments formed by XAA. After degradation of the substrates with low A/X ratio (AE-$AX_{20-30\%}$ and AE-$AX_{30-40\%}$), most of the AX were recovered in the $F_{90+\%}$ fraction. With increasing A/X ratio of the AE-AX substrates, the levels of AX recovered in the $F_{90+\%}$ fraction decreased, whereas those of AX recovered in the $F_{60-90\%}$ fraction increased. The yield of AX in the $F_{0-60\%}$ fractions was low and nearly constant for all AE-AX fractions.

Figure 5B represents the yields of the AX recovered in the $F_{0-60\%}$, $F_{60-90\%}$, and $F_{90+\%}$ fractions after ethanol precipitation of the fragments formed by XBS. With increasing A/X ratio of the AE-AX substrates, the levels of AX that precipitated in the $F_{0-60\%}$ and $F_{90+\%}$ fractions increased and decreased, respectively, whereas the yield of AX recovered in the $F_{60-90\%}$ fraction first increased and then decreased. Comparison of the precipitation behavior of the AX fragments formed by XAA or XBS reveals that, for XAA, most of the material was present in the fractions $F_{60-90\%}$ and $F_{90+\%}$, whereas, for XBS, most of the material was recovered in the fractions $F_{0-60\%}$, and $F_{60-90\%}$. This precipitation behavior is in line with the structural characteristics observed for the AX of the degraded AE-AX fractions (**Table 3**).

Figure 6 represents the structural characteristics of the AX that precipitated in the fractions $F_{0-60\%}$, $F_{60-90\%}$, and $F_{90+\%}$ after degradation of the AE-AX fractions by XAA and XBS. For the fractionated AX fragments formed by XAA (**Figure 6A**) it is clear that, with increasing A/X ratio of the AE-AX substrates (fractions AE-$AX_{20-30\%}$−AE-$AX_{70-80\%}$), the average DP of the AX fragments in the fractions $F_{0-60\%}$ and $F_{60-90\%}$ also increased. The AX fragments in fraction $F_{90+\%}$ all had a comparable and low average DP. The A/X ratio of the AX fragments in the fractions $F_{0-60\%}$, $F_{60-90\%}$, and $F_{90+\%}$ increased with increasing A/X ratio of the original AE-AX fractions. The levels of un-, mono-, and disubstituted xylose residues showed a decreasing, constant, and increasing trend, respectively, with increasing A/X ratio of the AE-AX fractions. The hydrolysis end products of XBS present in the fractions $F_{0-60\%}$, $F_{60-90\%}$, and $F_{90+\%}$ showed similar trends for average DP, A/X ratio, and substitution pattern with increasing A/X ratio of the AE-AX substrates (**Figure 6B**). The tendencies observed for A/X ratio and substitution pattern of the degraded and fractionated AE-AX as a function of the A/X ratio of the original AE-AX fractions are analogous to those observed for the AE-AX fractions before enzymic degradation (**Table 2** and **Figure 3**), indicating that the structural differences in the AE-AX fractions manifest themselves in the degraded and fractionated AE-AX fractions.

Comparison of the fractions $F_{0-60\%}$, $F_{60-90\%}$, and $F_{90+\%}$ for each AE-AX fraction after degradation by XAA (**Figure 6A**) shows that the AX fragments in the $F_{0-60\%}$ fractions had the highest average DP, whereas the AX fragments in the $F_{90+\%}$ fractions had the lowest average DP, except for the fractions AE-$AX_{20-30\%}$ and AE-$AX_{60-70\%}$. For these AE-AX fractions, the fragments with the highest average DP precipitated in the fraction $F_{60-90\%}$. The AX in the $F_{60-90\%}$ fractions had the highest

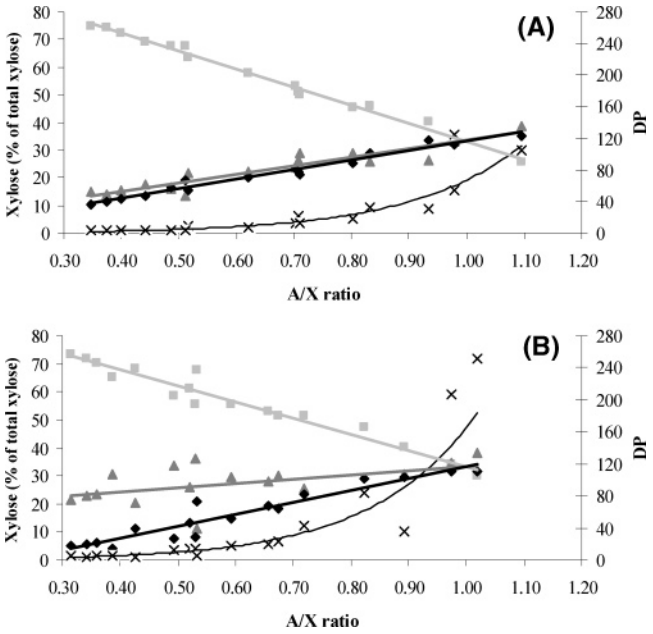

Figure 7. Percentages of un- (squares), mono- (triangles), and disubstituted (diamonds) xylose residues and average degree of polymerization (DP) (times signs) of the xylan backbone as a function of the A/X ratio of the AX from degraded and fractionated AE-AX fractions, with degradation by XAA (**A**) and XBS (**B**).

A/X ratio and contained the lowest level of unsubstituted xylose residues and the highest level of substituted xylose residues. The AX in the $F_{90+\%}$ fractions had the lowest A/X ratio and contained the highest level of unsubstituted xylose residues and the lowest level of substituted xylose residues. The AX of fractions $F_{0-60\%}$ showed intermediate degrees and patterns of substitution. For each AE-AX fraction degraded by XBS and subsequently precipitated by ethanol (**Figure 6B**), comparison of the fractions $F_{0-60\%}$, $F_{60-90\%}$, and $F_{90+\%}$ shows that the AX fragments that precipitated in the fraction $F_{0-60\%}$ had the highest average DP and A/X ratio and contained the highest level of disubstituted xylose residues. The AX in fraction $F_{90+\%}$ had the lowest average DP and A/X ratio and contained the lowest level of disubstituted xylose residues. Enzymic degradation of the rye flour AE-AX fractions by XAA or XBS and subsequent fractionation by ethanol precipitation thus yielded a variety of AX fragments differing in average DP, A/X ratio, and substitution pattern. However, when all of the results were summarized in a plot of substitution pattern and average DP as a function of the A/X ratio (**Figure 7**), clear relationships were found. With increasing A/X ratio of the AX in the AE-AX fractions, the levels of unsubstituted xylose residues linearly decreased, whereas the levels of mono- and disubstituted xylose residues linearly increased. The average DP of the AX fragments showed a marked increase with increasing A/X ratio.

In conclusion, gradual ethanol precipitation of isolated rye flour AE-AX and enzymic degradation of the resulting structurally different AE-AX fractions by endoxylanases with different substrate specificity yielded structurally different AX hydrolysis products. Further fractionation of the degraded AE-AX fractions by ethanol precipitation resulted in a broad set of AX fragments with different structural features. Overall, these results suggest a random distribution of the arabinose molecules on the xylan backbone rather than the occurrence of two types of AX or two types of regions in the AX, as no bimodal precipitation behavior was observed but a large variety of products with different structural features was obtained.

Structure of Rye Flour AE-AX. As outlined above, at present, in the case of rye WE-AX, two views exist on their structure. The model by Bengtsson et al. (*11*) maintains that rye WE-AX contain two types of AX or two types of regions in the AX molecule, one containing mainly un- and disubstituted xylose residues and the other consisting mainly of un- and O-3 monosubstituted xylose residues. In contrast, Vinkx et al. (*12*) hypothesized the existence of a range of structures in the WE-AX of rye grain. Gruppen et al. (*27*) and Viëtor (*28*) proposed structural models for wheat flour AE-AX and barley flour AE-AX, respectively. However, our results for rye flour AE-AX suggest the existence of a range of structures, casting doubt on the validity and usefulness of building hypothetical structural models. Taken together, it is hypothesized that the rye flour AE-AX consist of a continuum of structures with a minority of lowly substituted AX and a majority of more highly substituted AX. Between the two extreme populations, increasing levels of AX containing increasing levels of substitution were present.

ABBREVIATIONS USED

AE-AX, alkali-extractable arabinoxylan; AX, arabinoxylan; A/X ratio, arabinose to xylose ratio; BSA, bovine serum albumin; DP, degree of polymerization; D_2O, deuterium oxide; GHF, glycoside hydrolase family; ^{1}H NMR, proton nuclear magnetic resonance; HPSEC, high-performance size exclusion chromatography; MW, molecular weight; RT, room temperature; RX, reducing xylose; U_X, unit of endoxylanase activity; WE-AX, water-extractable arabinoxylan; WU-AX, water-unextractable arabinoxylan; XAA, *Aspergillus aculeatus* endoxylanase; XBS, *Bacillus subtilis* endoxylanase.

ACKNOWLEDGMENT

We thank Prof. R. Willem (VUB, Brussels, Belgium) for the ^{1}H NMR analyses. We are grateful for technical assistance provided by I. Verbruggen and L. Van den Ende.

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Received for review August 18, 2006. Revised manuscript received November 10, 2006. Accepted December 8, 2006.

JF0623790