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Enzymic Degradability of Hull-less Barley Flour Alkali-Solubilized Arabinoxylan Fractions by Endoxylanases

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The impacts of the arabinose to xylose (A/X) ratio of arabinoxylans (AX) and the endoxylanase substrate specificity on the enzymic degradability of hull-less barley flour AX by endoxylanases were studied by using alkali-solubilized AX (AS-AX) fractions with different A/X ratio, on the one hand, and glycoside hydrolase family 10 and 11 endoxylanases of *Aspergillus aculeatus* (XAA) and *Bacillus subtilis* (XBS), respectively, on the other hand. AS-AX were obtained by saturated barium hydroxide treatment of hull-less barley flour water-unextractable AX. Fractionation of AS-AX by stepwise ethanol precipitation resulted in structurally different hull-less barley flour AS-AX fractions. Their A/X ratios increased with increasing ethanol concentration, and this increase in A/X ratio was reflected in their xylose substitution levels. For both XAA and XBS, the enzymic degradability of AX and apparent specific endoxylanase activity decreased with increasing A/X ratio of the AS-AX substrates, implying that both endoxylanases were sterically hindered by arabinose substituents. However, for all AS-AX fractions, hydrolysis end products of lower average degree of polymerization were obtained after incubation with XAA than with XBS, indicating that the former enzyme has a lower substrate specificity toward hull-less barley flour AS-AX than the latter. In addition, apparent specific endoxylanase activities indicated that XBS was ∼2 times more sensitive to variations in the A/X ratio of AS-AX fractions than XAA. Furthermore, AS-AX with higher A/X ratio were relatively resistant to degradation by XBS.

KEYWORDS: Arabinoxylan; endoxylanase; degradability; hull-less barley flour

INTRODUCTION

Arabinoxylans (AX) are nonstarch polysaccharides that consist of a backbone of β-(1,4)-linked D-xylopyranosyl residues (xylose) to which α-L-arabinofuranose (arabinose) can be linked at the *O*-2 and/or *O*-3 positions (*1*−*3*). AX are either water-extractable (WE-AX) or water-unextractable (WU-AX) (*4*). Although they are minor constituents in cereals such as wheat and barley, AX are of importance, from both technological and nutritional points of view. In a number of cereal-based processes, WE-AX form highly viscous aqueous solutions, whereas WU-AX have strong water-binding capacity (*5*−*7*). In breadmaking, WU-AX are detrimental, whereas WE-AX and solubilized AX (S-AX) have positive effects on dough and bread characteristics such as bread loaf volume and crumb structure (*8*, *9*). Furthermore, AX are important dietary fiber and especially soluble dietary fiber components. Soluble AX reduce postprandial blood glucose and insulin responses in humans (*10*) and probably can lower blood cholesterol levels because of their highly viscous properties (*11*, *12*). Insoluble dietary fiber components, in general, improve bowel function (*13*). In addition, AX are selectively fermented by intestinal health-promoting bacteria, implying that they are prebiotics (*14*, *15*).

Endo-β-(1,4)-xylanases (EC 3.2.1.8), further referred to as endoxylanases, are used to impact AX functionality in, for example, breadmaking and gluten−starch separation. They cut internal β-(1,4)-linkages in the AX backbone. In general, endoxylanases can hydrolyze WU-AX (insoluble dietary fiber), resulting in the release of S-AX (soluble dietary fiber) and, consequently, an increased viscosity of the aqueous phase. The viscosity decreases when S-AX and native WE-AX are degraded to low molecular weight (MW) AX fragments (*9*, *16*). Endoxylanases mainly belong to glycoside hydrolase families (GHF) 10 and 11 (*17*, *18*). In the literature, differences in substrate specificity (or hydrolysis behavior) toward either non-cereal xylo-polysaccharides or artificial xylo-oligosaccharides were found between several endoxylanases from both families (*Cryptococcus albidus* and *Streptomyces lividans* endoxylanases from GHF 10 and *Trichoderma reesei* and *Streptomyces lividans* endoxylanases from GHF 11) (*19*). Endoxylanases of GHF 10 require less unsubstituted xylose residues adjacent to substituted xyloses and can degrade smaller MW fragments than those of GHF 11. The former enzymes can also hydrolyze glycoside linkages next to branching points and toward the non-reducing end of xylan chains. Thus, endoxylanases of GHF 10 have a lower substrate specificity toward the above-mentioned substrates than those of GHF 11 (*19*, *20*). Earlier, Kormelink et al. (*21*) found differences in the mode of action between two

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endoxylanases from *Aspergillus awamori*, Endo I and Endo III, toward wheat flour alkali-solubilized AX (AS-AX). On the basis of similarities in hydrolysis behavior, Biely et al. (*19*) concluded that Endo I is probably a member of GHF 10, whereas Endo III probably belongs to GHF 11.

Because endoxylanases modify the AX population and thereby AX functionality, it is important to determine which parameters influence the degradability of AX by endoxylanases. In the present study, the impact of the arabinose to xylose (A/X) ratio, which is a measure for the degree of substitution, of the AX chain in combination with substrate specificity of endoxylanases on the enzymic degradability of AX was examined. This was done by using AX fractions with different A/X ratios, on the one hand, and a GHF 10 *Aspergillus aculeatus* endoxylanase (XAA) and a GHF 11 *Bacillus subtilis* endoxylanase (XBS), on the other hand. AS-AX isolated from hull-less barley flour were chosen as AX source because hull-less barley flour is a potential food ingredient and a good source of AX and especially WU-AX, which have been studied to only a very limited extent (*22*). XAA and XBS were used because of their importance in cereal-based processes such as breadmaking and gluten−starch separation (*9*, *23*, *24*). Furthermore, both endoxylanases belong to different families (GHF 10 and 11, respectively), implying that they will probably have a different substrate specificity toward AX.

MATERIALS AND METHODS

Chemicals and Enzymes. Stabifix super silica gel was from Stabifix Brauerei-Technik KG (Gräfelfing, Germany). Standard P-82 pullulans were purchased from Showa Denko K.K. (Tokyo, Japan), and xylohexaose was from Megazyme (Bray, Ireland). Deuterium oxide was obtained from Acros Organics (Geel, Belgium), whereas barium hydroxide and sodium borohydride were from Sigma-Aldrich (Bornem, Belgium). All chemicals and reagents were of at least analytical grade. Thermostable α-amylase, Termamyl 120L, was obtained from Novozymes (Bagsvaerd, Denmark), whereas *A. niger* amyloglucosidase and *B. subtilis* lichenase were from Megazyme. These enzymes were free from AX-degrading enzyme side-activities.

XAA (NCBI accession no. AAE69552), a GHF 10 endoxylanase, was supplied by Puratos (Groot-Bijgaarden, Belgium), whereas the GHF 11 XBS (Swiss-Prot accession no. P18429, XynA_BACSU) was from Danisco (Brabrand, Denmark). Both endoxylanases were free from side-activities. All endoxylanase solutions were made with sodium acetate buffer (25 mM, pH 5.0) containing bovine serum albumin (BSA) (0.5 mg/mL).

The endoxylanase activity of both enzymes was determined with Xylazyme AX tablets (Megazyme) according to the method of Trogh et al. (*25*), with the exception that endoxylanase solutions were incubated for 10 min instead of 14 h. One unit (U) of endoxylanase activity is defined as the amount of enzyme (6.47 μg for XAA and 0.58 μg for XBS) needed to yield a change in absorbance value (at 590 nm) of 1.0 under the conditions of the assay.

Hull-less Barley Flour. A sifted hull-less barley flour from the variety SW 1290 H (Svalöf Weibulls AB, Svalöf, Sweden) obtained after industrial-scale milling was provided by Cerealia (Malmö, Sweden). Ash and protein contents (percent dry matter) were 1.3 and 13.2%, respectively, whereas total AX and WU-AX contents (percent dry matter) of the hull-less barley flour were 2.9 and 2.4%, respectively (analysis methods described further).

Isolation and Purification of AS-AX and Different AS-AX Fractions from Hull-less Barley Flour. *Isolation and Purification of AS-AX.* The isolation procedure used was a modification of that described by Gruppen et al. (*26*, *27*) and Viëtor et al. (*2*). Hull-less barley flour was refluxed in 80% ethanol (1:5 w/v, 120 min) to inactivate endogenous enzymes. The residue was filtered, washed with ethanol (80%), and air-dried at room temperature (RT). The inactivated hull-less barley flour (200 g) was extracted with deionized water (1:5 w/v, 60 min, 4 °C) under continuous stirring. After centrifugation (10000*g*, 30 min, 4 °C), the residue was washed twice with deionized water and centrifuged (10000*g*, 20 min, 4 °C). To remove residual starch, the residue was suspended in deionized water and heated to 75 °C prior to incubation with Termamyl (5.0 mL, 60 min, 90 °C) under continuous stirring. After boiling (20 min) of the suspension to inactivate the enzyme and cooling to RT, it was centrifuged (10000*g*, 20 min, 18 °C), and the residue obtained was washed twice as outlined above to remove water-extractable components. The residue was further suspended in deionized water and incubated (5 h, 40 °C) with lichenase (400 μL) to remove residual β-glucan (*28*). After boiling (20 min), cooling to RT, and centrifugation (10000*g*, 20 min, 18 °C), the residue was washed again. It was suspended in deionized water and incubated (15 h, 60 °C, pH 4.5) with amyloglucosidase (1.0 mL) under continuous stirring. Afterward, it was boiled (20 min), cooled to RT, centrifuged (10000*g*, 20 min, 18 °C), and washed. The suspension of partially purified WU-AX was dialyzed against deionized water (48 h, 4 °C) and lyophilized.

Partly purified WU-AX was extracted under continuous stirring (16 h, RT) with saturated barium hydroxide solution (1:30 w/v) containing 1% (w/v) sodium borohydride. After centrifugation (10000*g*, 20 min, 18 °C), the residue was re-extracted with the same solvent (2 h, RT) and centrifuged (10000*g*, 20 min, 18 °C). The combined supernatants, containing the formed AS-AX, were neutralized to pH 7.0, dialyzed against deionized water (48 h, 4 °C), and lyophilized. Proteins were removed with silica gel as described by Trogh et al. (*25*). The composition of the lyophilized fraction obtained, further referred to as AS-AX, was analyzed.

Fractionation of AS-AX. Fractionation of AS-AX material by graded ethanol precipitation was performed as follows. AS-AX material was solubilized in deionized water, and ethanol (95%) was added stepwise under continuous stirring at RT to a concentration of 20%. The mixture was stirred for 30 min at RT, kept during 8 h at 4 °C, and centrifuged (10000*g*, 30 min, 4 °C). The precipitate was recovered by centrifugation (10000*g*, 30 min, 4 °C), solubilization in deionized water, and lyophilization. The obtained fraction was denoted $F_{0-20\%}$. Then, the supernatant was brought to an ethanol concentration of 30%. The mixture was stirred (30 min, RT), kept during 8 h at 4 °C, and centrifuged (10000*g*, 30 min, 4 °C). The precipitate was collected as described before and is further referred to as $F_{20-30\%}$. This procedure was repeated with stepwise increases in ethanol concentration (per 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 ($F_{80+\%}$) was dissolved in deionized water and lyophilized. The coefficient of variation calculated for the yield of AS-AX recovered with this isolation procedure was <3.9%.

The composition of each of the AS-AX fractions was analyzed. Fractions $F_{20-30\%}$ to $F_{60-70\%}$ were used to study the enzymic degradability of hull-less barley AX.

Standard Analyses. Moisture and ash contents (percent dry matter) were estimated according to AACC methods 44-15A and 08-01, respectively (*29*). Protein content (N × 6.25) (percent dry matter) was determined using the Dumas method, an adaptation of AOAC method 990.03 (*30*) to an automated Dumas protein analysis system (EAS variomax N/CN, Elt, Gouda, The Netherlands). The coefficients of variation of the results were less than 0.9, 1.6, and 1.1% for moisture, ash, and protein content determinations, respectively.

Carbohydrate Composition. Carbohydrate compositions of hull-less barley flour, purified flour AS-AX material, and ethanol-precipitated flour AS-AX fractions were estimated by gas−liquid chromatography (GLC) of alditol acetates obtained after acid hydrolysis, reduction, and acetylation of the samples as described by Trogh et al. (*25*). Flour (0.100 g) and AX samples (0.010 g) were hydrolyzed in 2.0 M trifluoroacetic acid (5.0 mL) at 110 °C for 120 and 60 min, respectively. Alditol acetates (1.0 μL) were separated on a Supelco SP-2380 polar column (30 m × 0.32 mm i.d.; 0.2 μm film thickness) (Supelco, Bellefonte, PA) in an Agilent chromatograph (Agilent 6890 series, Wilmington, DE) equipped with autosampler, splitter injection port (split ratio 1:20), and flame ionization detector. The carrier gas was helium. Separation was at 225 °C, and injection and detection were at 270 °C. The coefficient of variation of the results of this analysis

was <5.0%. The AX content of the samples (percent dry matter) was defined as 0.88 times the sum of arabinose and xylose.

Proton Nuclear Magnetic Resonance Spectroscopy. Xylose substitution levels of purified AS-AX samples were studied by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy. Fractions $F_{0-20\%}$ to $F_{60-70\%}$ (4.0 mg) were dissolved in deuterium oxide (1.0 mL), stirred (8 h, RT), and lyophilized. This step was repeated three times. The lyophilized samples were then dissolved in deuterium oxide (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 by the High-Resolution NMR Centre (VUB, Brussel, Belgium). Pulse repetition time was 2.0 s, and the number of scans was 128. The proportions (percent) of un-, *O*-2 mono-, *O*-3 mono-, and *O*-2,*O*-3 disubstituted xylose residues were calculated by combining the GLC results with the ^{1}H NMR spectral data according to the method of Roels et al. (*31*), which itself relies on the approach by Westerlund et al. (*32*). The level of *O*-2 monosubstituted xylose was estimated using the difference in peak surface between the two peaks of anomeric protons of arabinose residues linked to *O*-2,*O*-3 disubstituted xylose (*33*). The coefficient of variation of the results of this analysis was <4.5%.

Enzymic Degradability of AX Studied by High-Performance Size Exclusion Chromatography (HPSEC). In a preliminary experiment, aiming to find a suitable endoxylanase dosage for enzymic degradation of AX, hull-less barley flour AS-AX substrates ($F_{20-30\%}$, $F_{30-40\%}$, $F_{40-50\%}$, $F_{50-60\%}$, and $F_{60-70\%}$) (8.0 mg) were dissolved in sodium acetate buffer (25 mM, pH 5.0) (4.0 mL). Endoxylanase preparations were diluted in the same sodium acetate buffer with BSA (0.5 mg/mL). Enzyme dosages ranging from 0.001 to 5 U for XAA and from 0.01 to 10 U for XBS were added (0.1 mL) to the AS-AX fractions (1.4 mL). The mixtures were then incubated at 30 °C for different incubation times (0−48 h). The apparent MW distribution of the resulting mixtures was studied 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. (Tokyo, Japan). Elution of the samples (20 μL) was with 0.3% NaCl (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 MW of 78.8×10^4, 40.4×10^4, 21.2×10^4, 11.2×10^4, 4.73×10^4, 2.28×10^4, 1.18×10^4, 0.59×10^4, xylohexaose, and glucose.

On the basis of preliminary experiments, dosages of 0.004 U of XAA and 0.03 U of XBS were used to monitor the gradual enzymic degradation of AX fractions as a function of incubation time.

Enzymic Degradability of AX Studied by Reducing End Sugar Residue Content. AS-AX fractions ($F_{20-30\%}$, $F_{30-40\%}$, $F_{40-50\%}$, $F_{50-60\%}$, and $F_{60-70\%}$) (10.0 mg) were dissolved in sodium acetate buffer (25 mM, pH 5.0) (4.5 mL). To the control samples was added 0.5 mL of the same sodium acetate buffer with BSA (0.5 mg/mL), whereas 0.5 mL of endoxylanase solution was added to the other samples. The same endoxylanase dosages as for HPSEC were used (0.004 U for XAA and 0.03 U for XBS). All the mixtures of AX and endoxylanase were incubated at 30 °C for 15 h. After incubation, the solutions were inactivated (30 min, 120 °C), cooled, and filtered. Reducing end sugar residue contents of the filtrates (2.5 mL) were estimated by GLC of alditol acetates as described by Courtin et al. (*34*). The coefficient of variation of the results of this analysis did not exceed 5.0%.

The apparent specific activity of the endoxylanases (nanokatals per milligram of protein) of the different AS-AX fractions was expressed as the amount of terminal reducing xylose (RX) (nanomoles) formed per second and per milligram of endoxylanase under the experimental conditions used. The coefficient of variation of the results of this analysis was <7.0%.

RESULTS

Purification and Characterization of AS-AX Material. Analysis of WU-AX structure is mostly indirectly performed on its constituent AX fragments, released from cereal cell walls by alkaline extraction (AS-AX) (*2*, *26*, *27*), because the analysis techniques require soluble samples. Prior to the formation of AS-AX, WU-AX were partially purified from hull-less barley flour. Starch was removed with Termamyl and amyloglucosidase, whereas lichenase was used for the removal of β-glucans. The partially purified WU-AX material contained 20.3% AX and ~84% of the original hull-less barley flour WU-AX (**Table 1**). WU-AX were then partially solubilized by alkaline treatment with saturated barium hydroxide solution containing 1.0% sodium borohydride. Approximately 55% of the WU-AX present in the partially purified WU-AX fraction were recovered as AS-AX. After protein removal, the purified AS-AX material contained 85.8% AX and had an A/X ratio of 0.71 (**Table 1**). The latter value was higher than that obtained for purified WE-AX from the same hull-less barley variety (0.62; *25*), which was in accordance with data from Izydorczyk et al. (*22*) for hull-less Canadian barley flour AS-AX and WE-AX. Furthermore, the AX content and A/X ratio of hull-less barley flour AS-AX were similar to the values (80.1% and 0.72, respectively) reported by Viëtor et al. (*2*) for AS-AX of a hulled European barley flour. For European wheat flour AS-AX, these values were 87.4% and 0.51, respectively (*35*).

Table 1. Monosaccharide and Arabinoxylan (AX) Contents (Percent Dry Matter), Arabinose to Xylose (A/X) Ratio, $Yield_{WU-AX}$ [Percent of Water-Unextractable AX (WU-AX) in the Flour], and Protein Content (Percent Dry Matter) of Hull-less Barley Flour, Partially Purified WU-AX, and Alkali-Solubilized AX (AS-AX) Fractions

sample	Ara[a]	Xyl	Gal	Glc	AX[b]	A/X	$yield_{WU-AX}$	protein
barley flour	1.4	1.9	0.4	76.4	2.9	0.74	100.0	13.2
WU-AX fraction	9.8	13.3	1.0	2.9	20.3	0.74	83.8	67.7
AS-AX fraction	40.6	56.9	1.6	1.0	85.8	0.71	39.0	7.9

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

The levels of un-, *O*-2 mono-, *O*-3 mono-, and *O*-2,*O*-3 disubstituted xylose residues were 51.5, 13.4, 12.2, and 22.9%, respectively, for AS-AX compared to 61.1, 8.3, 7.5, and 23.1%, respectively, for WE-AX of the same variety (*25*). These results indicated that the AX chains of AS-AX from hull-less barley flour are generally more substituted with arabinose than those of WE-AX. For hulled barley flour AS-AX and wheat flour AS-AX, the un-, *O*-2 mono-, *O*-3 mono-, and *O*-2,*O*-3 disubstituted xylose levels were 57.4, 9.6, 14.4, and 18.5 and 65.4, 2.1, 18.1, and 14.5%, respectively (*2*, *35*).

Fractionation and Characterization of AS-AX Fractions. The purified AS-AX of hull-less barley flour were fractionated by stepwise ethanol precipitation into different AS-AX fractions ($F_{0-20\%}$ to $F_{70-80\%}$ and a remaining fraction $F_{80+\%}$). After ethanol precipitation, 55.6% of the originally formed AS-AX were recovered in the different AS-AX fractions, mainly in $F_{60-70\%}$ (**Table 2**). The relatively low yield was due to general losses of AS-AX during fractionation, rather than selective AX removal. The combined yields of $F_{50-60\%}$ and $F_{60-70\%}$ amounted to 50.2% of the totally recovered AS-AX obtained after ethanol precipitation.

Apart from the fraction $F_{80+\%}$, the AX content of the AS-AX fractions was >80%. The A/X ratios increased with increasing ethanol concentrations (0−80%) from 0.42 to 1.25 (**Table 2**). Similar trends were found for AS-AX fractions of hulled European barley flour (*2*).

Figure 1 shows the proportions of un-, mono- (*O*-2 or *O*-3), and *O*-2,*O*-3 disubstituted xylose residues in the $F_{0-20\%}$ to $F_{60-70\%}$ fractions. The levels of un- and *O*-3 monosubstituted xyloses decreased, whereas those of *O*-2 mono- and *O*-2,*O*-3 disubstituted xylose residues increased with increasing ethanol

Table 2. $Yield_{AX}$ [Percent of Arabinoxylan (AX) in the Starting Material], AX Content (Percent Dry Matter), and Arabinose to Xylose (A/X) Ratio of Different Hull-less Barley Flour Alkali-Solubilized AX (AS-AX) Fractions

AS-AX fraction	$yield_{AX}$	AX[a]	A/X
$F_{0-20\%}$	4.0	80.6	0.42
$F_{20-30\%}$	6.8	90.3	0.44
$F_{30-40\%}$	7.6	90.3	0.48
$F_{40-50\%}$	5.3	91.9	0.56
$F_{50-60\%}$	9.2	93.7	0.71
$F_{60-70\%}$	18.7	95.4	0.96
$F_{70-80\%}$	2.8	82.5	1.25
$F_{80+\%}$	1.1	23.8	1.26

[a] AX = (arabinose + xylose) × 0.88; A/X = arabinose/xylose.

concentration or increasing A/X ratio. Only $F_{60-70\%}$ did not follow the trend for the level of *O*-3 monosubstituted xylose (16.0%). Viëtor et al. (*2*) observed no changes in *O*-3 monosubstituted xylose levels for hulled barley flour AS-AX fractions, whereas Gruppen et al. (*35*) also described a decrease in *O*-3 monosubstituted xylose residues with increasing A/X ratio for $F_{0-20\%}$ to $F_{50-60\%}$ fractions of AS-AX from wheat flour. As outlined in **Figure 1**, the levels of un-, *O*-2 mono-, and *O*-2,*O*-3 disubstituted xylose residues were linearly correlated with the A/X ratio (R^2 = 0.99, 0.97, and 0.94, respectively). Furthermore, the ^{1}H NMR spectra (not shown) indicated that both *O*-3 mono- and *O*-2,*O*-3 disubstituted xyloses occurred isolated and/or next to *O*-2,*O*-3 disubstituted xylose residues in the AX chain of the different AS-AX fractions and that the level of paired *O*-2,*O*-3 disubstituted xylose residues increased with increasing A/X ratio.

Thus, fractionation of AS-AX by gradual ethanol precipitation yielded structurally different AS-AX fractions.

Enzymic Degradability of Hull-less Barley Flour AS-AX Fractions Studied by HPSEC. To investigate the impact of the A/X ratio on the enzymic degradability of hull-less barley flour AX, the AS-AX fractions recovered after graded ethanol precipitation ($F_{20-30\%}$, $F_{30-40\%}$, $F_{40-50\%}$, $F_{50-60\%}$, and $F_{60-70\%}$) of hull-less barley flour AS-AX were incubated with XAA or XBS. On the basis of preliminary trials (results not shown), in which wide ranges of enzyme dosages of XAA (0.001−5 U) and XBS (0.01−10 U) were tested for each AS-AX fraction, XAA and XBS dosages of 0.004 and 0.03 U, respectively, were used to obtain a gradual enzymic degradation of AX as a function of incubation time. For both endoxylanases, the maximum degradation of the AS-AX fractions was reached after 15 h of incubation (hydrolysis end products) or earlier. Incubation for >15 h and/or addition of fresh endoxylanase after 15 h did not change the MW profiles of the hydrolysis end products (results not shown).

Figure 2 shows the HPSEC apparent MW profiles of the $F_{20-30\%}$ and $F_{60-70\%}$ fractions incubated (0−15 h) with 0.004 U of XAA or 0.03 U of XBS. The profiles of the other AS-AX fractions (not shown) ranged between these two extremes. For both XAA and XBS, the apparent peak MW shifted to lower MW with increasing incubation times for all AS-AX fractions. During the first 4 h, the shift in apparent peak MW toward lower MW was high. Thereafter, degradation of AX slowed, indicating that the level of easily accessible hydrolysis sites in the AX chain decreased, irrespective of the endoxylanase used. An additional element may be that endoxylanase activity decreases because of inhibition by hydrolysis products.

Figure 3 combines the HPSEC profiles of control AS-AX fractions (without endoxylanase) and the hydrolysis end products

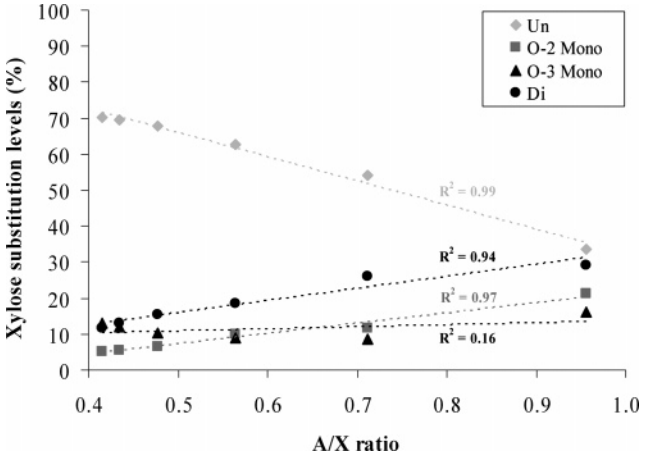

Figure 1. Percentages of total xylose occurring as un- (Un), *O*-2 mono- (*O*-2 Mono), *O*-3 mono- (*O*-3 Mono), and *O*-2,*O*-3 disubstituted (Di) xylose residues as a function of the arabinose to xylose (A/X) ratio of different alkali-solubilized arabinoxylan fractions ($F_{0-20\%}$ to $F_{60-70\%}$) from hull-less barley flour.

(after 15 h) thereof for each endoxylanase separately. The apparent peak MW of the fragments formed after hydrolysis of $F_{20-30\%}$ and $F_{60-70\%}$ was the lowest and highest, respectively, whereas that of the other AS-AX fractions varied between these two. For XAA, the estimated apparent peak MW of the hydrolysis end products ranged from 810 to 5600, whereas for XBS it varied between 2100 and 230000 from $F_{20-30\%}$ to $F_{60-70\%}$ (**Table 3**). The hydrolysis end product MW increased with increasing A/X ratio, implying that the enzymic degradability of hull-less barley flour AX decreased with increasing A/X ratio of the AX substrate, irrespective of the endoxylanase used. Both enzymes were thus sterically hindered by the presence of arabinose substituents.

The data in **Figure 3** and **Table 3** confirm the hypothesis that XAA and XBS indeed differ in substrate specificity, most likely because they belong to GHF 10 and 11, respectively. For each AS-AX fraction, incubation with GHF 10 XAA resulted in hydrolysis end products with a lower apparent peak MW than those obtained after incubation with XBS of GHF 11. The differences in hydrolysis end product MW were larger between AS-AX fractions with higher A/X ratios ($F_{50-60\%}$ and $F_{60-70\%}$) and were 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.

Enzymic Degradability of Hull-less Barley Flour AS-AX Fractions Studied by Reducing End Sugar Residue Content. **Table 3** lists the levels of RX formed after incubation of the structurally different AS-AX fractions of hull-less barley flour with XAA or XBS for 15 h. Under the experimental conditions used, these RX levels are a measure of the level of hydrolysis sites for the endoxylanase in the AX chain. They decreased with increasing A/X ratio of the AS-AX substrate and were much lower for XBS than for XAA, implying that, particularly for XBS, fewer β-(1,4)-bonds in the xylan backbone were hydrolyzed, which resulted in higher MW fragments (**Figure 3** and **Table 3**). The enzymic degradability of hull-less barley flour AS-AX fractions decreased with increasing A/X ratio of the AX substrate, in accordance with the results from the HPSEC analyses.

On the basis of the arabinose and xylose contents of the different AS-AX fractions and the RX levels obtained after incubation of these fractions with XAA or XBS, the average degree of polymerization of AX fragments (DP_{AX}) formed after

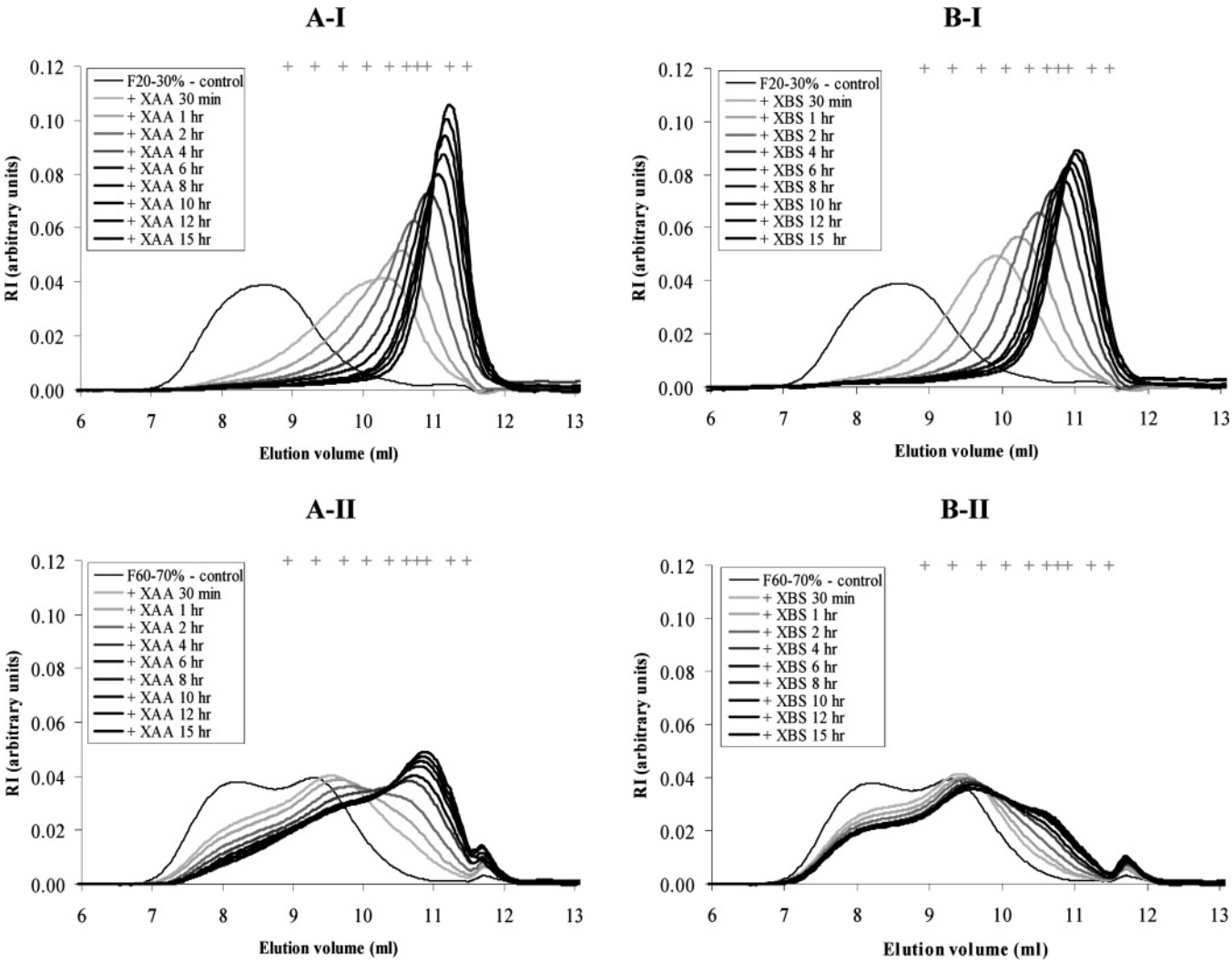

Figure 2. HPSEC apparent MW profiles of the hydrolysis products obtained after incubation of the $F_{20-30\%}$ (**A-I** and **B-I**) and $F_{60-70\%}$ (**A-II** and **B-II**) alkali-solubilized arabinoxylan fractions from hull-less barley flour with *A. aculeatus* endoxylanase (XAA) (**A-I** and **A-II**) or *B. subtilis* endoxylanase (XBS) (**B-I** and **B-II**) for different times (0–15 h). MW markers (+) from left to right are 78.8×10^4, 40.4×10^4, 21.2×10^4, 11.2×10^4, 4.73×10^4, 2.28×10^4, 1.18×10^4, 0.59×10^4, xylohexaose, and glucose.

enzymic degradation was estimated (**Table 3**). For both XAA and XBS, the average DP_{AX} increased with increasing A/X ratio, and large differences in average DP_{AX} values were obtained between $F_{50-60\%}$ and $F_{60-70\%}$. Furthermore, the fragments formed by incubation with XBS were larger than those formed by XAA, which was in accordance with the HPSEC data (**Figure 3**).

As shown in **Figure 4**, the apparent specific activity of an endoxylanase, calculated on the basis of the formation of RX residues, depends strongly on the A/X ratio of AS-AX substrates. With increasing A/X ratio, it decreased from 1175 to 620 nkat/mg of protein for XAA and from 1390 to 280 nkat/mg of protein for XBS, indicating that both endoxylanases were less active toward highly substituted AX substrates. The activities of XAA and XBS toward $F_{60-70\%}$ (A/X ratio of 0.96) were 47 and 80%, respectively, lower than those toward $F_{20-30\%}$ (A/X ratio of 0.44). As indicated by the slopes of the equations of the linear relations for XAA ($R^2 = 0.91$) and XBS ($R^2 = 0.99$) (**Figure 4**), GHF 11 XBS was ~2 times (2228 compared to 1018) more sensitive to variations in the A/X ratio of hull-less barley flour AS-AX than XAA of GHF 10. Thus, not only the A/X ratio of the AX substrate, but, at a given A/X ratio, also the substrate specificity of the enzyme strongly affected the apparent specific activity of endoxylanases.

DISCUSSION

AS-AX from hull-less barley flour were obtained after saturated barium hydroxide treatment of partially purified WU-AX. In general, hull-less barley flour AS-AX were more substituted with arabinose, particularly monosubstituted, than WE-AX from the same flour. Fractionation of the purified AS-AX by stepwise ethanol precipitation resulted in structurally different hull-less barley flour AS-AX fractions. The A/X ratio of these fractions increased with increasing ethanol concentration. This increase in A/X ratio was reflected in the levels of un- and *O*-3 monosubstituted xyloses, which decreased, and of *O*-2 mono- and *O*-2,*O*-3 disubstituted xyloses, which increased.

Furthermore, the present research clearly demonstrated that the enzymic degradability of AS-AX fractions from hull-less barley flour is strongly affected by the A/X ratio of the AX substrate, on the one hand, and by the substrate specificity of the endoxylanase used, on the other hand. For both XAA and XBS, the enzymic degradability of AS-AX and the apparent specific endoxylanase activity decreased with increasing A/X ratio. Low A/X AS-AX fractions were degraded to lower MW fragments than those with higher A/X ratio. Because >50% of the AS-AX recovered after ethanol precipitation had an A/X ratio ≥0.71, it can be concluded that hull-less barley flour

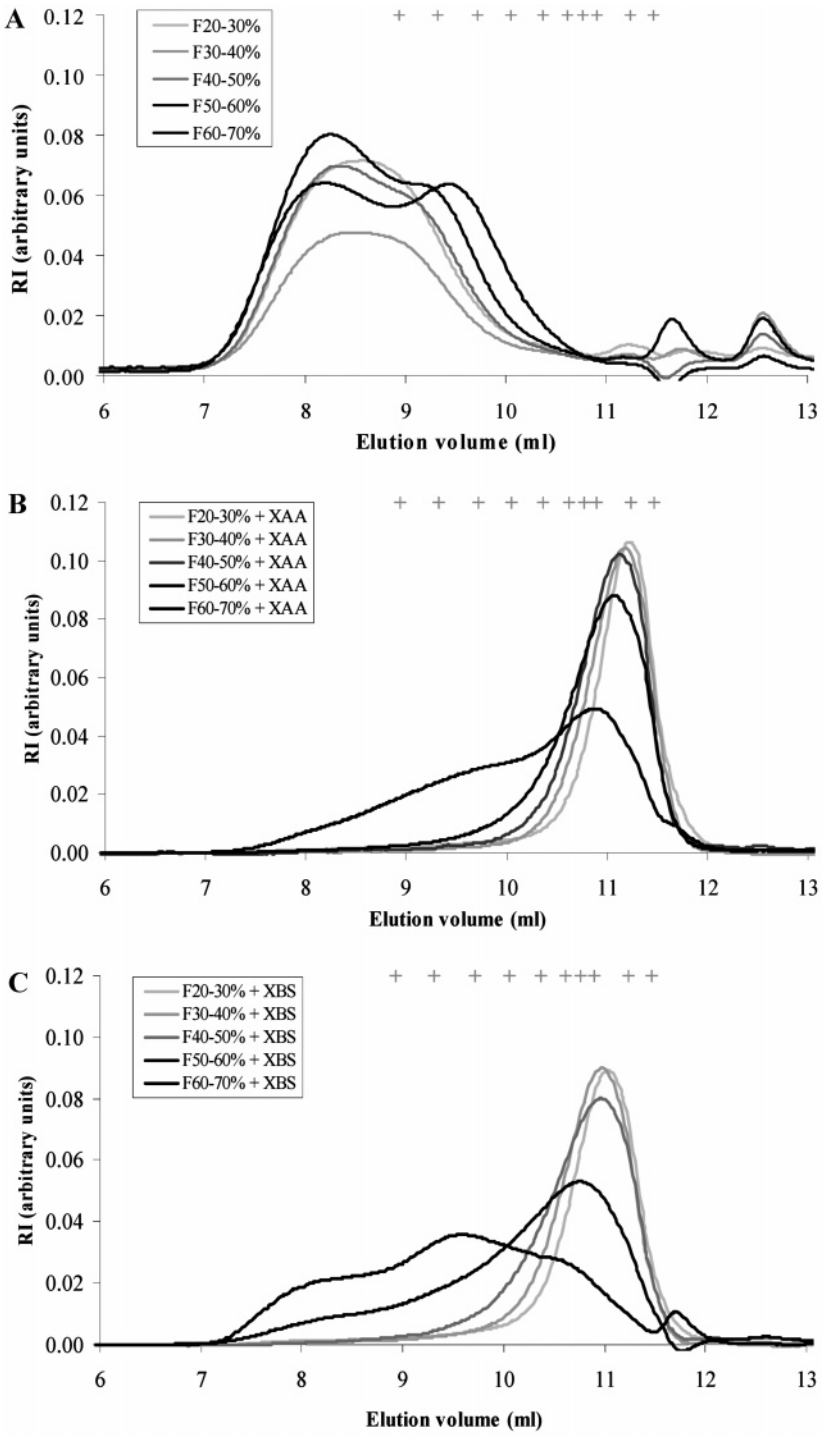

Figure 3. HPSEC apparent MW profiles of different alkali-solubilized arabinoxylan (AS-AX) fractions ($F_{20-30\%}$ to $F_{60-70\%}$) from hull-less barley flour without endoxylanase (**A**) and profiles of the hydrolysis end products obtained after incubation of these AS-AX fractions with *A. aculeatus* endoxylanase (XAA) (**B**) or *B. subtilis* endoxylanase (XBS) (**C**) for 15 h. MW markers (+) from left to right are 78.8×10^4, 40.4×10^4, 21.2×10^4, 11.2×10^4, 4.73×10^4, 2.28×10^4, 1.18×10^4, 0.59×10^4, xylohexaose, and glucose.

WU-AX are enzymically degradable to only a limited extent. Furthermore, the RX levels formed after incubation of the AS-AX fractions with XAA or XBS decreased and, thereby, the average DP_{AX} values of the hydrolysis end products increased with increasing A/X ratio. These findings imply that both endoxylanases were sterically hindered by arabinose substituents.

Although similar trends were obtained for the general action of XAA and XBS on AX fractions with different A/X ratio, significant differences were found in substrate specificity or hydrolysis behavior of these enzymes. For all hull-less barley flour AS-AX fractions, the apparent peak MW of the hydrolysis end products obtained after incubation with XAA was lower than that of fragments recovered with XBS and the RX contents were higher for XAA than for XBS, the latter implying that more β-(1,4)-bonds in the xylan backbone were hydrolyzed with XAA. Taken together, the results showed that XAA (of GHF 10) had a lower substrate specificity toward hull-less barley flour AS-AX substrates than XBS (of GHF 11). Thereby, XAA formed smaller MW fragments and was less sterically

Table 3. Estimated Apparent Peak Molecular Weight (MW), Reducing Xylose (RX) Formed (Percent of Total Xylose), and Average Degree of Polymerization (DP_{AX}) of the Hydrolysis End Products Obtained after Incubation (15 h) of Different Hull-less Barley Flour Alkali-Solubilized Arabinoxylan (AS-AX) Fractions with *A. aculeatus* Endoxylanase (XAA) or *B. subtilis* Endoxylanase (XBS)

	apparent peak MW		RX		DP_{AX}[a]	
AS-AX fraction	XAA	XBS	XAA	XBS	XAA	XBS
$F_{20-30\%}$	810	2100	14.5	7.1	10	20
$F_{30-40\%}$	1100	3100	13.9	5.8	11	25
$F_{40-50\%}$	1400	3500	13.4	5.4	12	29
$F_{50-60\%}$	2000	12000	10.9	4.9	16	35
$F_{60-70\%}$	5600	230000	4.7	1.2	42	170

[a] DP_{AX} = (arabinose + xylose)/reducing xylose.

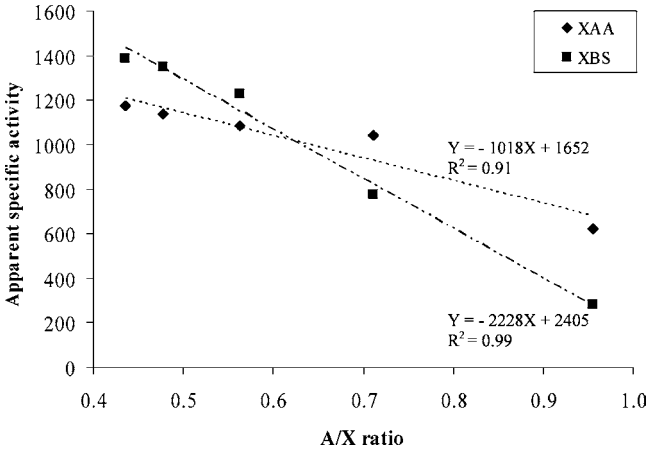

Figure 4. Apparent specific activities (nanokatals per milligram of protein) of the *A. aculeatus* endoxylanase (XAA) and *B. subtilis* endoxylanase (XBS) as a function of the arabinose to xylose (A/X) ratio of different alkali-solubilized arabinoxylan fractions ($F_{20-30\%}$ to $F_{60-70\%}$) from hull-less barley flour.

hindered by arabinose substituents than XBS. Apparent specific activities of both enzymes indicated that XBS was ~2 times more sensitive to variations in A/X ratio of hull-less barley flour AS-AX substrates than XAA.

In addition, incubation of higher A/X AS-AX fractions with XBS resulted in higher MW fragments, indicating that such fractions were relatively resistant to degradation by XBS. This can be of significance for the production of high MW soluble AX levels (important for viscosity-related health benefits) in cereal-based processes such as breadmaking (*36*). Furthermore, the DP_{AX} values of the hydrolysis end products obtained after incubation with XAA were relatively low, irrespective of the A/X ratio of the AS-AX substrates, implying that these fragments (arabinoxylo-oligosaccharides) probably have prebiotic properties, as already shown before for xylo-oligosaccharides (*15*, *37*). Thus, knowledge of parameters influencing the enzymic degradability of AX by endoxylanases will most likely be of importance for the production of health-promoting components.

ABBREVIATIONS USED

AS-AX, alkali-solubilized arabinoxylan; AX, arabinoxylan; A/X ratio, arabinose to xylose ratio; BSA, bovine serum albumin; DP_{AX}, degree of polymerization of arabinoxylan fragments; GHF, glycoside hydrolase family; GLC, gas−liquid chromatography; ^{1}H NMR, proton nuclear magnetic resonance; HPSEC, high-performance size-exclusion chromatography; MW, molecular weight; RI, refractive index; RT, room temperature; RX, reducing xylose; S-AX, solubilized arabinoxylan; U, units; WE-AX, water-extractable arabinoxylan; WU-AX, water-unextractable arabinoxylan; XAA, *Aspergillus aculeatus* endoxylanase; XBS, *Bacillus subtilis* endoxylanase.

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