JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2007,** *55,* 7149−7155 **7149**

Impact of Wheat Flour-Associated Endoxylanases on Arabinoxylan in Dough after Mixing and Resting

EMMIE DORNEZ, KURT GEBRUERS, SVEN CUYVERS, JAN A. DELCOUR, AND CHRISTOPHE M. COURTIN*

Laboratory of Food Chemistry and Biochemistry, Katholieke Universiteit Leuven, Kasteelpark Arenberg 20, B-3001 Leuven, Belgium

The impact of varying levels of endoxylanase activity in wheat flour on arabinoxylan (AX) in mixed and rested dough was studied using eight industrially milled wheat flour fractions with varying endoxylanase activity levels. Analysis of the levels of reducing end xylose (RX) and solubilized AX (S-AX) formed during mixing and resting and their correlation with the endoxylanase activity in the flour milling fractions showed that solubilization of AX during the mixing phase is mainly due to mechanical forces, while solubilization of AX during resting is caused by endoxylanase activity. Moreover, solubilization of AX during the dough resting phase is more outspoken than that during the mixing phase. Besides endoxylanase activity, there were significant xylosidase and arabinofuranosidase activities during the dough resting phase. The results indicate that wheat flour-associated endoxylanases can alter part of the AX in dough, thereby changing their functionality in bread making and potentially affecting dough and end product properties.

KEYWORDS: Wheat; endoxylanase; xylosidase; arabinofuranosidase; arabinoxylan; dough; bread making

INTRODUCTION

Arabinoxylans (AX) are the major nonstarch polysaccharides in wheat flour. They make up 65−70% of the endosperm cell walls (*1*, *2*) and occur in rather small concentrations of approximately 1.5−2.5% in flour (*3*). They are often classified in two groups with different physicochemical properties: 20−30% of the AX are water-extractable (WE-AX), while the remaining 70−80% are water-unextractable (WU-AX). The latter can be solubilized by mechanical work input, chemical, or enzyme treatments. Although AXs are minor constituents of wheat flour, because of their unique physicochemical properties, they largely affect dough and bread characteristics (*3*). It is often stated that WE-AX and solubilized AX (S-AX) are beneficial, while WU-AX are detrimental for bread making, but the mechanisms behind this are not entirely clear (*3*−*6*).

To alter AX functionality in bread making, endoxylanases (EC 3.2.1.8) of microbial origin are often used to change the water extractability and/or the molecular mass (MM) of AX. Numerous reports are available dealing with the effects of such added microbial enzymes on both AX properties and dough and bread quality. The effects of endoxylanase supplementation on bread volume and quality largely depend on the dosage used (*3*, *7*), the substrate selectivity (*8*), the inhibition sensitivity (*7*, *9*), and other biochemical characteristics of the enzyme.

Despite the large number of reports dealing with the effects of added microbial endoxylanases in bread making, little is known about the impact of wheat flour-associated endoxylanases on AX in dough. Rouau et al. (*10*, *11*) and Cleemput et al. (*12*) found that part of the AX was indeed solubilized during dough mixing and fermentation, even in the absence of added microbial endoxylanases. However, they were unable to reveal the underlying mechanisms of this solubilization, which could simply be a physical phenomenon resulting from mechanical work input or the temperature increase or could be due to hydrolysis of AX by wheat-associated endoxylanases. In the latter case, and taking into account the large variability in wheat-associated endoxylanase activity in wheat wholemeal (*13*) and flour (*12*, *14*), it can reasonably be expected that such wheat-associated endoxylanases affect the AX molecules and their functionality in bread making. In this context, it is important to realize that wheat flour not only contains endogenous endoxylanases but can also contain wheat-associated microbial endoxylanases that end up as a contamination in wheat flour after milling (*15*, *16*). Sørensen and Etzerodt (*15*) already reported that flour-associated microbial endoxylanase activity can impact the functional properties of cereal flour and that it can contribute to year to year and batch to batch variations in flour quality. Debyser et al. (*17*) demonstrated the impact of wheat flour-associated endoxylanases in bread making. The addition of TAXI type inhibitors, which inactivate wheat flour-associated microbial endoxylanases, decreased loaf volume by 8%. In addition, the wheat flour-associated endoxylanase level is a major determinant of refrigerated dough syruping (*15*, *18*, *19*).

Furthermore, it is generally accepted that other AX degrading enzymes, such as xylosidases (EC 3.2.1.37) and arabinofura-

* To whom correspondence should be addressed. Tel: + 32 16 32 19 17. Fax: + 32 16 32 19 97. E-mail: christophe.courtin@biw.kuleuven.be.

10.1021/jf071363m CCC: $37.00
Published on Web 07/28/2007

nosidases (EC 3.2.1.55), work synergistically with endoxylanases (*20*−*23*). In contrast to endoxylanases, xylosidases and arabinofuranosidases have an exospecificity, cleaving off single sugar residues from polysaccharides' nonreducing ends.

The purpose of this work was to gain insight into the impact of varying wheat flour-associated endoxylanase activity levels on AX in dough after mixing and resting, to estimate their potential influence on the bread-making process and their contribution to flour variability. In contrast to previously published work dealing with the impact of wheat flour-associated endoxylanases in bread making (*10*−*12*), a more in depth analysis of the changes in AX was performed using a well-characterized set of wheat flour milling fractions.

MATERIALS AND METHODS

Materials. Eight flour milling fractions (A−H) were obtained from an industrial roller mill (Ceres-Soufflet, Brussels, Belgium). These fractions were of different bread-making qualities, as evaluated by bread-making trials, and were selected out of 54 flour milling fractions to cover a large range of endoxylanase activities, that is, 0.01−0.15 enzyme units (EU)/g (*16*). All chemicals and reagents were of at least analytical grade and supplied by Sigma-Aldrich (Bornem, Belgium), unless specified otherwise. Azurine cross-linked AX tablets were purchased from Megazyme (Bray, Ireland), and standard P-82 pullulans were from Showa Denko K.K. (Tokyo, Japan).

Standard Analyses. Moisture and ash contents were measured in duplicate according to the AACC methods 44-15a and 08-21, respectively (*24*). The coefficients of variation for determination of moisture and ash contents were typically 1%. Protein contents were determined in triplicate using the Dumas combustion method, an adaptation of the AOAC Official Method (*25*) to an automated Dumas protein analysis system (EAS, varioMax N/CN, Elt, Gouda, The Netherlands), using 5.7 as a factor for conversion from nitrogen to protein content. The coefficient of variation for the determination of protein contents was typically 3%. Ash and protein contents were expressed on a dry matter basis.

Preparation of Dough Samples. Dough was prepared on a 10.0 g scale according to the straight dough method of Shogren and Finney (*26*). Flour (10.0 g on a 14% moisture base) was mixed for 3.5 min with water (60%) in a 10.0 g pin mixer (National Manufacturing, Lincoln, NE). After mixing, the dough sample was divided in two. One part was immediately frozen with liquid nitrogen and freeze-dried. The other part was allowed to rest in a fermentation cabinet at 30 °C for 3 h, frozen with liquid nitrogen, and freeze-dried. The freeze-dried dough samples were ground with a mortar and pestle and sieved ($\varnothing$ = 250 μm).

Determination of Noncellulosic Carbohydrate Composition and Levels. Noncellulosic carbohydrate compositions and contents of flour milling fractions and aqueous extracts of both flour and ground dough samples were determined by gas−liquid chromatography (GLC) after hydrolysis and conversion of the resulting monosaccharides to alditol acetates as described by Dornez et al. (*16*). The coefficient of variation for the determination of both WE-AX/S-AX and total AX (TOT-AX) was typically 2%. The coefficient of variation for the determination of starch contents was typically 1%.

Determination of Reducing End Sugar Residue Levels and Free Sugar Residues. Reducing end xylose (RX) and arabinose (RA) levels in aqueous extracts were estimated by GLC as described by Courtin et al. (*27*). In this procedure, which strongly resembles that for determination of noncellulosic carbohydrate composition and levels described above, reduction with sodium borohydride was performed prior to hydrolysis and acetylation to alditol acetates. To determine free xylose (FX) and arabinose (FA) residues, the same procedure was performed, but hydrolysis was omitted. For the GLC analysis of both reducing end and free sugar residues, a split ratio of 1:10 instead of 1:20 was used.

Determination of Apparent Endoxylanase Activity Levels. Apparent endoxylanase activities were measured in flour and ground dough sample fractions with the Xylazyme AX method (Megazyme) as described by Dornez et al. (*16*) and expressed in EU/g. One EU is the amount of enzyme needed to increase the extinction at 590 nm with 1.0 per hour incubation, under the conditions of the assay. The endoxylanase activity could not be expressed in nanokatal, as this colorimetric assay does not provide a direct measure for the formation of reducing end sugars. In view of the results by Dornez et al. (*28*), the endoxylanase activity levels measured by this assay must be regarded as apparent activity levels because of the presence of endoxylanase inhibitors in the flour milling fractions and the inhibition of a variable proportion of wheat-associated microbial endoxylanases during aqueous extraction by these proteins. The coefficient of variation for the determination of endoxylanase activity levels was typically 1%.

Determination of Xylosidase and Arabinofuranosidase Activity Levels. Wheat flour extracts were made by suspending 2.0 g of flour in 10.0 mL of sodium acetate buffer (25 mM, pH 5.0) and shaking the suspensions for 30 min (Laboshake). The samples were subsequently centrifuged (10 min, 10000*g*, 7 °C) and filtered (MN 615 Filter). Xylosidase and arabinofuranosidase activity levels in the wheat flour extracts were determined using *p*-nitrophenyl-glycoside substrates. To this end, the procedure of Cleemput et al. (*29*) was adapted to microtiter plates. *p*-Nitrophenyl-β-D-xylopyranoside (PNP-Xyl) and *p*-nitrophenyl-α-L-arabinofuranoside (PNP-Ara) solutions (5.0 mM) were prepared in sodium acetate buffer (25 mM, pH 5.0). Aliquots of these solutions (50 μL) were incubated with an equal level of wheat flour extract for 60 min at 40 °C in a microtiter plate heating block (VWR International). The reaction was stopped by adding 100 μL of TRIS solution (1%). In a control assay, TRIS was added before the wheat flour extract. The release of *p*-nitrophenol (PNP) from the PNP-glycoside was determined colorimetrically at 415 nm with a microtiter plate reader (model 680, Biorad Laboratories, Nazareth, Belgium). Calibration curves, made with PNP (0−0.05 mM), allowed expression of the activity as nanokatal of PNP. All analyses were made in duplicate, and the experimental error, calculated from the difference between the individual values and the mean value, was typically 6%.

Inactivation of Enzymes in Flour and Dough Samples. Flour samples (15 g) were boiled under reflux in 80% aqueous ethanol (200 mL) to inactivate enzymes. After 2 h, the solution was filtered over a Buchner filter and rinsed with 95% aqueous ethanol. The samples were subsequently dried by exposure to air for 2 days.

Determination of MM by High-Performance Size Exclusion Chromatography (HPSEC). The flour and dough samples (100 mg) were extracted in water (1.0 mL) by shaking (Laboshake) the suspensions for 30 min at 7 °C. They were subsequently centrifuged (10 min, 10000*g*, 7 °C), and the supernatants were heated for 30 min at 100 °C (Eppendorf heating block, VWR International) to inactivate the enzymes. Subsequently, the samples were filtered through a 0.45 μm membrane (Regenerated cellulose, Grace Davison Discovery Sciences, Deerfield, MA).

The apparent MM distribution of the flour and dough extracts 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 ammonium acetate (25 mM, pH 5.8, 0.5 mL/min at 30 °C) on a Kontron 325 pump system (Kontron, Milan, Italy) equipped with autoinjection. The separation was monitored with an evaporative light scattering detector (Alltech 3300 ELSD, Grace Davison Discovery Sciences). MM markers (1.5 mg/mL) were Shodex standard P-82 pullulans with MM of 788, 404, 212, 112, 47.3, 22.8, 11.8, and 5.9 kDa.

RESULTS AND DISCUSSION

Selection and Characterization of Flour Samples. Out of the 54 industrial wheat roller mill flour milling fractions (*16*), eight fractions (A−H) were selected to cover a broad range of apparent endoxylanase activity levels. **Table 1** lists the bread-making quality, ash, starch, protein, TOT-AX, and WU-AX contents, and apparent endoxylanase activity levels of these eight selected flour samples. Endoxylanase activity levels ranged from 0.01 to 0.15 EU/g and were positively correlated with ash

Table 1. Bread-Making Quality, Ash, Starch, and Protein Contents, TOT-AX and WU-AX Levels, and Endoxylanase, Arabinofuranosidase, and Xylosidase Activity Levels of the Eight Selected Industrial Wheat Milling Fractions and Straight Run Flour[a]

flour samples	bread-making quality	ash (% dm)	starch (% dm)	protein (% dm)	TOT-AX (% dm)	WU-AX (% dm)	endoxylanase (EU/g)	arabinofuranosidase (nkat/g)	xylosidase (nkat/g)
A. CR1F-1 F2	first	0.34	81.4	9.7	2.32	1.86	0.01	0.01	0.46
B. CR3-2 F2	second	0.60	79.8	11.2	2.11	1.65	0.04	0.11	0.48
C. B1-2-1 F1	first	0.50	80.6	11.1	1.85	1.46	0.05	0.06	0.48
D. SG2-2 F1	second	0.62	77.3	12.1	2.32	1.92	0.06	0.11	0.50
E. B4F-1 F1	third	0.88	73.7	16.3	2.37	2.02	0.07	0.16	0.46
F. CR4 F1	third	0.84	77.5	11.0	2.35	1.96	0.09	0.22	0.61
G. SG BF1/BF2 F1	third	0.95	74.3	14.4	2.83	2.42	0.12	0.23	0.55
H. BF2 F1	third	1.22	71.5	13.8	2.82	2.44	0.15	0.31	0.54
straight run flour		0.51	80.0	11.0	2.13	1.69	0.04	ND	ND

[a] Legend: B, break; BF, bran finisher; CR, coarse reduction; FR, fine reduction; SG, semolina grader; Fx, fraction number; and ND, not determined.

Table 2. Soluble AX Levels (%) in Flour, Dough after Mixing, and Dough after Resting

	soluble AX (%)		
flour samples	flour	dough after mixing[a]	dough after resting[b]
A	0.46	0.61	0.72
B	0.46	0.52	0.69
C	0.39	0.47	0.65
D	0.40	0.51	0.72
E	0.35	0.43	0.64
F	0.39	0.47	0.67
G	0.41	0.47	0.75
H	0.38	0.44	0.80

[a] Incubation time, 3.5 min. [b] Incubation time, 180 min.

contents and negatively with starch contents, suggesting that the endoxylanase activity in flour is strongly influenced by the level of bran contamination as previously discussed (*16*). In addition, arabinofuranosidase and xylosidase activity levels were determined in the eight selected flour milling fractions (**Table 1**). Arabinofuranosidase activity levels ranged from 0.01 to 0.31 nkat/g and were strongly positively correlated with endoxylanase activity levels ($R^2 = 0.92$, P value < 0.01) and hence also positively with ash content ($R^2 = 0.96$, P value < 0.01) and negatively with starch content ($R^2 = 0.77$, P value < 0.01). Under the experimental conditions, xylosidase activity levels consistently exceeded arabinofuranosidase levels as already reported by Cleemput et al. (*29*), but they did not differ to a large extent between the different flour milling fractions (0.46−0.61 nkat/g). In contrast to arabinofuranosidase and endoxylanase activity levels, they were not significantly correlated with any of the above-mentioned parameters.

Analysis of Soluble AX Levels in Dough. Soluble AX levels, that is, the sum of WE-AX and S-AX levels, were determined in the eight flour samples and in the respective dough samples after mixing and after 3 h of resting. It was clear that soluble AX levels increased during processing: They were the lowest in flour milling fractions and the highest in doughs frozen after 3 h of resting at 30 °C (**Table 2**). The levels of S-AX formed during the mixing phase were calculated as the difference between the soluble AX levels in dough after mixing and in flour, while the levels of S-AX formed during the resting phase were calculated as the difference between the soluble AX levels in rested dough and in dough after mixing. The percentage of solubilization of WU-AX was further calculated as a percentage of the WU-AX content of the straight run flour (1.69%). As the WU-AX levels of the milling fractions differed largely, the WU-AX of straight run flour was chosen as reference to avoid bias and to make comparison with literature possible.

During the mixing phase, 3−9% of the flour WU-AX was solubilized. This is partially in the range of the 7−14% previously reported by Rouau et al. (*11*) and the 7−12% reported by Cleemput et al. (*12*). During the resting phase, more AX became water extractable, and at the end of the resting phase, 14−25% of the flour WU-AX was solubilized. The rather high levels of AX solubilized during the resting phase (7−21%) are in contrast with the very low levels reported for straight run flour samples by Cleemput et al. (*12*) (0−5%) but in agreement with results of Rouau et al. (*10*, *11*). The differences in AX solubilization between the different studies can be due to differences in dough recipe, mixing equipment, and levels of wheat flour-associated endoxylanase activity.

AX in dough can be solubilized by hydrolysis of AX by wheat flour-associated endoxylanases or by disaggregation of AX chains, weakly bound in endosperm cell walls, by a temperature increase or mechanical work input (*10*−*12*). To elucidate the mechanism of AX solubilization, we tried to correlate the solubilization of WU-AX with the endoxylanase activity level of the flour. No significant relation was found between the percentage of WU-AX solubilized during the mixing phase and the apparent endoxylanase activity of the flour. However, the percentage of WU-AX solubilized during the resting phase was significantly correlated with the endoxylanase activity of the flour ($R^2 = 0.92$, P value < 0.01) (**Figure 1A**). This seems to indicate that solubilization of WU-AX during the short mixing phase is caused by mechanical forces, rather than by endoxylanase hydrolysis, while that during the long resting phase occurs by enzymic hydrolysis. Cleemput et al. (*12*) found much more solubilization during mixing than during fermentation for flours of three wheat varieties. It is hence not surprising that they could not find a relation between the degree of AX solubilization and endogenous AX hydrolyzing enzyme activities. Possibly, the levels of wheat flour-associated endoxylanase activity levels were very low in the wheat flour samples of the 1994 harvest year studied (*12*). It has indeed been shown that wheat-associated endoxylanase activity levels strongly vary with harvest year (*13*).

Analysis of Reducing End and Free Sugar Levels in Dough. To confirm the above hypotheses about the mechanisms that determine AX solubilization in dough, RX levels were also measured in the different flour and dough samples (**Table 3**). There was little or no difference between the RX levels in flour and dough after mixing (**Table 3** and **Figure 1B**), and the RX levels formed were not correlated with the endoxylanase activity levels of the flour samples. This indicates that there is little if any endoxylanase activity during the mixing phase, and it can hence be concluded that mainly physical phenomena are important for AX solubilization during mixing. Enzymic solu-

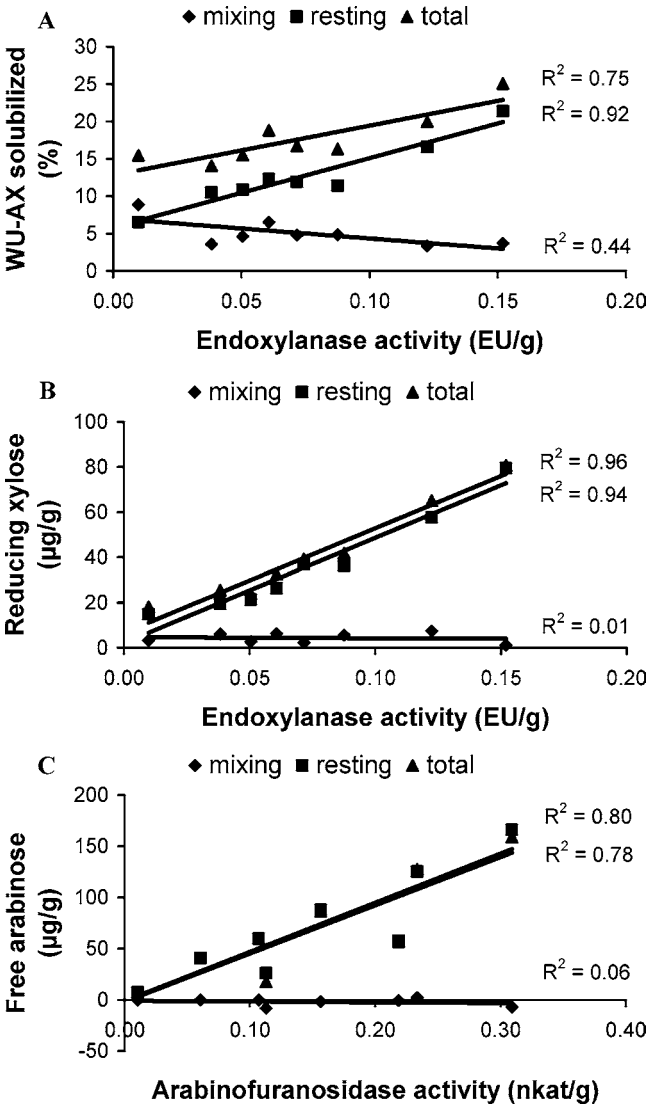

Figure 1. WU-AX solubilized (%) (**A**), RX formed (μg/g) (**B**), and FA formed (μg/g) (**C**) during processing in the function of the endoxylanase activity level (EU/g) (**A**,**B**) and arabinofuranosidase activity level (nkat/g) (**C**) of the flour.

Table 3. RX and FX Levels (μg/g) in Flour, Dough after Mixing, and Dough after Resting

	RX (μg/g)			FX (μg/g)		
flour samples	flour	dough after mixing[a]	dough after resting[b]	flour	dough after mixing[a]	dough after resting[b]
A	32	35	50	14	14	18
B	34	40	59	21	9	22
C	38	41	62	17	17	32
D	39	46	72	16	14	31
E	34	37	74	18	17	42
F	43	48	85	18	11	29
G	52	59	117	18	14	50
H	77	78	158	24	23	66

[a] Incubation time, 3.5 min. [b] Incubation time, 180 min.

bilization hardly takes place, most likely because of the relatively short mixing time and perhaps also because of the relatively low temperature for enzymic hydrolysis.

The RX levels obtained after the resting phase were much higher, indicating significant enzymic hydrolysis of AX. This was confirmed by the strong correlation between the endoxylanase activity and the RX levels formed during the resting phase ($R^2 = 0.94$, P value < 0.01) (**Figure 1B**). As there is no mechanical work input or temperature change during resting, hydrolysis of AX by wheat flour-associated endoxylanases seems the only mechanism for AX solubilization.

Table 4. FA Levels (μg/g) in Flour, Dough after Mixing, and Dough after Resting

	FA (μg/g)		
flour samples	flour	dough after mixing[a]	dough after resting[b]
A	20	19	27
B	36	27	53
C	53	52	93
D	47	47	106
E	99	98	186
F	42	42	99
G	63	65	190
H	110	103	269

[a] Incubation time, 3.5 min. [b] Incubation time, 180 min.

However, RX is not only formed by endoxylanase activity but also can be produced by xylosidase activity in dough. To assess the impact of xylosidase activity on the RX levels, FX levels were also determined. In contrast to what was expected, FX levels could not be neglected. Therefore, not the level of RX but the difference between RX and FX levels is a more correct prediction of the number of β-1,4-bonds in the AX chain cleaved by endoxylanase activity. After this correction for FX, the level of RX formed during the resting phase was still significantly correlated with the endoxylanase activity of the flour ($R^2 = 0.74$, P value < 0.01), while during the mixing phase, again no correlation could be found. The level of FX formed during the mixing or during the resting phase could surprisingly not be correlated with the xylosidase activity of the flour. Remarkably, a significant relation was found between the level of FX formed during resting and endoxylanase activity of the flour ($R^2 = 0.93$, P value < 0.01). At first sight, this seems to indicate that wheat flour-associated endoxylanases are able to produce FX residues. This is, however, unlikely as endoxylanases normally only produce FX when the polymeric substrate gets depleted. It is more likely that this correlation is caused by a synergistic working of the two enzymes (*20*, *21*). Xylosidases work much more efficiently on smaller AX fragments than on a large AX chain, and xylosidase activity hence depends on endoxylanase activity for the supply of substrate. Most likely, smaller AX fragments need to be produced first by endoxylanase hydrolysis of AX, and these smaller fragments can then further be degraded by xylosidase hydrolysis. It is tempting to speculate that the breakdown of AX in smaller fragments by endoxylanase activity occurs much more slowly than the breakdown of the smaller oligosaccharides by xylosidase activity. As endoxylanase activity is the rate-determining factor, the FX formed would be correlated with endoxylanase and not with xylosidase activity levels, which is indeed what was observed.

FA levels (**Table 4**) were similar to RA levels (results not shown). FA was not formed during the mixing phase, most likely because the time frame was again too short for enzymic hydrolysis. After the resting phase, FA levels were much higher. The level of FA formed during resting correlated well with the arabinofuranosidase activity level of the flour ($R^2 = 0.80$, P value < 0.01) (**Figure 1C**).

Analysis of Arabinose over Xylose (A/X) Ratio of Soluble AX in Dough. The A/X ratio is often used as a measure for the

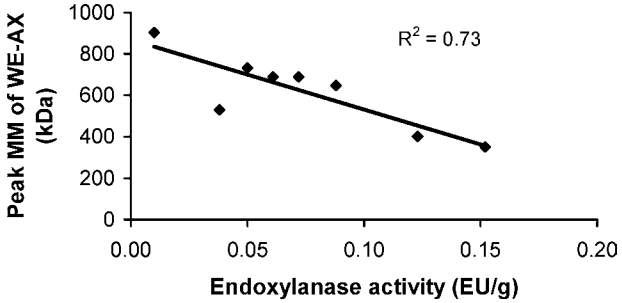

Figure 2. Peak MM of WE-AX (*k*) in the function of the arabinofuranosidase activity level (EU/g) of the flour.

Table 5. A/X Ratio of Soluble AX in Flour, Dough after Mixing, and Dough after Resting

	A/X of soluble AX		
flour samples	flour	dough after mixing[a]	dough after resting[b]
A	0.46	0.51	0.51
B	0.46	0.48	0.53
C	0.45	0.48	0.50
D	0.42	0.45	0.49
E	0.36	0.38	0.43
F	0.45	0.46	0.51
G	0.42	0.43	0.48
H	0.39	0.39	0.45

[a] Incubation time, 3.5 min. [b] Incubation time, 180 min.

substitution degree of AX. We corrected soluble arabinose levels for both arabinose originating from arabinogalactan peptide (AGP) and FA levels and soluble xylose levels for FX levels. As different AX degrading enzymes, such as endoxylanases, xylosidases, and arabinofuranosidases, work synergistically to degrade AX, it is interesting to analyze the changes occurring in the A/X ratio of soluble AX. From **Table 5**, it is clear that the A/X ratio of soluble AX increased during processing, although the differences were rather small. This indicates that more substituted fragments became solubilized. Another possible explanation is that the soluble AX molecules were to a larger extent degraded by xylosidase activity than by arabinofuranosidase activity. In literature, Cleemput et al. (*12*) found little if any differences in A/X ratio, while Rouau et al. (*11*) found a lower A/X ratio in dough after fermentation than in dough after mixing and flour. However, in these studies, A/X ratios were not corrected for arabinose originating from AGP or for FA and FX levels. When in our own work these corrections are not made, we also observe a decrease in A/X.

Analysis of MM Distribution of Soluble AX. HPSEC profiles of the different flour milling fractions indicated that the peak MM of soluble AX was inversely related to the endoxylanase activity of the flour (**Figure 2**). As this suggests enzymic breakdown of AX fragments during sample preparation or analysis by endoxylanase activity, the experiments were also executed with wheat flours preliminary boiled under reflux in 80% ethanol to inactivate the flour-associated enzyme activities. Endoxylanase activity measurements confirmed that this treatment inactivated the flour endoxylanases. However, no differences were observed in HPSEC profiles between samples in which enzymes were inactivated by heating after extraction and samples in which enzymes were inactivated by boiling of flour under reflux in 80% ethanol before extraction. This indicates that both treatments were effective to avoid enzymic breakdown of AX during sample preparation and analysis.

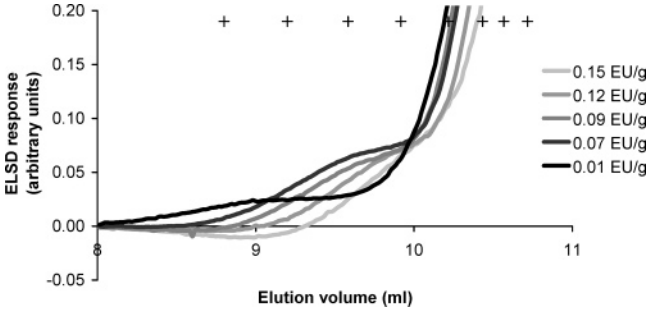

Figure 3. HPSEC profiles obtained after subtracting the profile of aqueous extracts of doughs after mixing from that of extracts of doughs after resting for flour milling fractions with diverse endoxylanase activity levels. MM markers (+) from the left to the right are 788, 404, 212, 112, 47.3, 22.8, 11.8, and 5.9 kDa.

Figure 3 shows the HPSEC profiles obtained after subtracting the HPSEC profile of aqueous extracts of doughs after mixing from that of extracts of doughs after resting. This way of processing allows one to specifically focus on the changes during the resting phase. For the sample with the lowest endoxylanase activity, high MM fragments were formed by solubilization of WU-AX, while for the samples with the higher endoxylanase activity, the high MM material was most likely also formed but then further degraded to smaller MM fragments. Levels of soluble AX with high MM (approximately 800 k) decreased when the endoxylanase activity of the flour increased. For the sample with the highest endoxylanase activity levels, the curve even showed negative values in the high MM region, demonstrating that wheat flour-associated endoxylanases not only solubilize WU-AX but also seriously degrade WE-AX and S-AX to lower MM fragments during the resting phase.

Relevance of the Present Findings for Bread Making. Analysis of the impact of the wheat-associated endoxylanases on bread quality was not possible in the present set up, as the milling fractions used differed largely in overall composition, which makes the analysis of, for example, bread volume meaningless. However, by comparing the changes in AX caused by wheat flour-associated endoxylanases with those found in literature caused by added microbial endoxylanases, an estimation of possible effects on bread volume can be obtained.

Our results showed 6−21% solubilization of WU-AX after resting, and a difference of approximately 15% S-AX between flours with high and low wheat flour-associated endoxylanase activities. In view of the results of Rouau et al. (*11*), this is not insignificant, as additional WU-AX solubilization of 14% after fermentation/resting by added microbial endoxylanases was associated with loaf volume increases of approximately 14%. The results of Trogh et al. (*7*), in contrast, showed a volume increase of not more than 5% when an additional 22% of the WU-AX was solubilized after fermentation/resting by added microbial endoxylanases. Courtin et al. (*8*) found an increase and a decrease in bread volume with approximately 5% when an additional 15% flour WU-AX was solubilized during fermentation/resting with *Bacillus subtilis* and *Aspergillus aculeatus* endoxylanases, respectively. This indicates that, depending on the substrate selectivity of the wheat flour-associated endoxylanases, positive or negative effects on bread volume can be expected.

In contrast to added microbial endoxylanases, the presented results show that wheat flour-associated endoxylanases are not active during mixing but exert their main effect during the fermentation phase of bread making. This suggests that at least part of the wheat flour-associated endoxylanases is not inhibited

by endoxylanase inhibitors during processing. In view of the known specificity of endoxylanase inhibitors, this implies that the active enzymes are either endogenous or bacterial glycoside hydrolase family 10 endoxylanases.

As wheat flour-associated endoxylanases change AX in a later phase than the added microbial ones, they can either enlarge or nullify the impact of added microbial endoxylanases. Added microbial endoxylanases, suitable for bread making, preferentially hydrolyze WU-AX, giving rise to high MM S-AX (*3*). Depending on the biochemical properties of these endoxylanases, levels of 0.30−17.4 EU/g flour are added under the form commercial bread improvers (*16*). After mixing, such microbial endoxylanases are typically largely inactivated by wheat endoxylanase inhibitors, preventing them from further degrading S-AX and WE-AX during fermentation/resting. Wheat flour-associated endoxylanases, in contrast, remain active during the relatively long fermentation/resting phase and can hence further degrade soluble AX to lower MM fragments, which are less effective in increasing loaf volume (*4*, *30*). From **Figure 3**, it was indeed clear that wheat flour-associated endoxylanases not only solubilize WU-AX but also seriously degrade soluble AX to lower MM fragments during the resting phase. As low MM soluble AX are less effective in bread making than their high MM counterparts, it is possible that, in flours with high wheat flour-associated endoxylanase activity levels, the beneficial effect of added microbial endoxylanases can be nullified. Variability in wheat flour-associated endoxylanase activity may hence impact not only AX in dough but also bread volume.

In the above assessment, it is important to know that the maximum levels and the variability in endoxylanase activities in the studied milling fractions are both three-fold lower than the maximum levels and variability found in flours of different wheat varieties and harvest years. Indeed, endoxylanase activity levels in straight run flours of a very large set of different sound wheat varieties ranged from 0.01 to 0.44 EU/g (*14*, unpublished results). This suggests that the effects observed here for the milling fractions with the highest endoxylanase levels will even be larger when dealing with high endoxylanase level straight run flour.

To avoid unwanted variability problems in bread making or, by inference, other wheat-based processes in which endoxylanases play a role, selection of wheat flours with low endoxylanase activity seems the safest choice.

ABBREVIATIONS USED

AGP, arabinogalactan peptide; AX, arabinoxylan; A/X, arabinose over xylose; ELSD, evaporative light scattering detector; EU, enzyme unit; FA, free arabinose; FX, free xylose; GLC, gas−liquid chromatography; HPSEC, high-performance size exclusion chromatography; MM, molecular mass; PNP, *para*-nitrophenol; PNP-Ara, *p*-nitrophenyl-α-L-arabinofuranoside; PNP-Xyl, *p*-nitrophenyl-β-D-xylopyranoside; RA, reducing arabinose; RX, reducing end xylose; S-AX, solubilized arabinoxylan; TOT-AX, total arabinoxylan; WE-AX, water-extractable arabinoxylan; WU-AX, water-unextractable arabinoxylan.

ACKNOWLEDGMENT

We thank Nele Schoonens for technical assistance in preliminary experiments and Ceres-Soufflet (Brussels, Belgium) for supply of wheat flour milling fractions.

LITERATURE CITED

(1) Mares, D. J.; Stone, B. A. Studies on wheat endosperm. I. Chemical composition and ultrastructure of cell walls. *Aust. J. Biol. Sci.* **1973**, *26*, 793−812.

(2) Fincher, G. B.; Stone, B. A. Cell walls and their components in cereal grain technology. In *Advances in Cereal Science and Technology*; Pomeranz, Y., Ed.; American Association of Cereal Chemists Inc.: St. Paul, MN, 1986; Vol. VIII, pp 207−295.

(3) Courtin, C. M.; Delcour, J. A. Arabinoxylans and endoxylanases in wheat flour bread-making. *J. Cereal Sci.* **2002**, *35*, 225−243.

(4) Courtin, C. M.; Roelants, A.; Delcour, J. A. Fractionation-reconstitution experiments provide insight into the role of endoxylanases in bread-making. *J. Agric. Food Chem.* **1999**, *47*, 1870−1877.

(5) Gan, Z.; Ellis, P. R.; Schofield, J. D. Gas cell stabilization and gas retention in wheat bread dough. *J. Cereal Sci.* **1995**, *21*, 215−230.

(6) Jelaca, S. L.; Hlynka, I. Effect of wheat-flour pentosans in dough, gluten, and bread. *Cereal Chem.* **1972**, *49*, 489−495.

(7) Trogh, I.; Sørensen, J. F.; Courtin, C. M.; Delcour, J. A. Impact of inhibition sensitivity on endoxylanase functionality in wheat flour breadmaking. *J. Agric. Food Chem.* **2004**, *52*, 4296−4302.

(8) Courtin, C. M.; Gelders, G. G.; Delcour J. A. Use of two endoxylanases with different substrate selectivity for understanding arabinoxylan functionality in wheat flour breadmaking. *Cereal Chem.* **2001**, *78*, 564−571.

(9) Gebruers, K.; Courtin, C. M.; Moers, K.; Noots, I.; Trogh, I.; Delcour, J. A. The bread-making functionalities of two *Aspergillus niger* endoxylanases are strongly dictated by their inhibitor sensitivities. *Enzyme Microb. Technol.* **2005**, *36*, 417−425.

(10) Rouau, X.; Moreau, D. Modification of some physicochemical properties of wheat-flour pentosans by an enzyme complex recommended for baking. *Cereal Chem.* **1993**, *70*, 626−632.

(11) Rouau, X.; El-Hayek, M.-L.; Moreau, D. Effect of an enzyme preparation containing pentosanases on the bread-making quality of flours in relation to changes in pentosan properties. *J. Cereal Sci.* **1994**, *19*, 259−272.

(12) Cleemput, G.; Booij, C.; Hessing, M.; Gruppen, H.; Delcour, J. A. Solubilisation and changes in molecular weight distribution of arabinoxylans and protein in wheat flours during bread-making, and the effects of endogenous arabinoxylan hydrolysing enzymes. *J. Cereal Sci.* **1997**, *26*, 55−66.

(13) Dornez, E.; Joye, I. J.; Gebruers, K.; Lenartz, J.; Massaux, C.; Bodson, B.; Delcour, J. A.; Courtin, C. M. Insight into variability of apparent endoxylanase and endoxylanase inhibitor levels in wheat kernels. *J. Sci. Food Agric.* **2006**, *86*, 1610−1617.

(14) Gebruers, K.; Bedo, Z.; Delcour, J. A.; Courtin, C. M. Xylanases and xylanase inhibitors of wheat, barley and rye in the HEALTHGRAIN diversity screen. Manuscript in preparation.

(15) Sørensen, J. F.; Etzerodt, H. Removing enzymes on grain. UK Patent Application GB 2 402 600 A, 2004.

(16) Dornez, E.; Gebruers, K.; Wiame, S.; Delcour, J. A.; Courtin, C. M. Insight into the distribution of arabinoxylans, endoxylanases, and endoxylanase inhibitors in industrial wheat roller mill streams. *J. Agric. Food Chem.* **2006**, *54*, 8521−8529.

(17) Debyser, W.; Peumans, W. J.; Van Damme, E. J. M.; Delcour, J. A. *Triticum aestivum* xylanase inhibitor (TAXI), a new class of enzyme inhibitor affecting breadmaking performance. *J. Cereal Sci.* **1999**, *30*, 39−43.

(18) Gys, W.; Courtin, C. M.; Delcour, J. A. Reduction of xylanase activity in flour by debranning retards syruping in refrigerated doughs. *J. Cereal Sci.* **2004**, *39*, 371−377.

(19) Courtin, C. M.; Gys, W.; Gebruers, K.; Delcour, J. A. Evidence for the involvement of arabinoxylan and xylanases in refrigerated dough syruping. *J. Agric. Food Chem.* **2005**, *53*, 7623−7629.

(20) de Vries, R. P.; Kester, H. C. M.; Poulsen, C. H.; Benen, J. A. E.; Visser, J. Synergy between enzymes from Aspergillus involved in the degradation of plant cell wall polysaccharides. *Carbohydr. Res.* **2000**, *327*, 401−410.

(21) Sørensen, H. R.; Meyer, A. S.; Pedersen, S. Enzymatic hydrolysis of water-soluble wheat arabinoxylan. 1. Synergy between alpha-L-arabinofuranosidases, endo-1,4-beta-xylanases, and beta-xylosidase activities. *Biotechnol. Bioeng.* **2003**, *81*, 726−731.

(22) Suh, J. H.; Cho, S. G.; Choi, Y. J. Synergic effects among endo-xylanase, beta-xylosidase, and alpha-L-arabinofuranosidase from *Bacillus stearothermophilus*. *J. Microbiol. Biotechnol.* **1996**, *6*, 179−183.

(23) Biely, P.; Vrsanska, M.; Kucar, S. Identification and mode of action of endo-(1-4)-β-xylanases. In *Xylans and Xylanases*; Visser, J., Beldman, G., Kusters-Van Someren, M. A., Voragen, A. G. J., Eds.; Elsevier Science Publishers B. V.: Amsterdam, The Netherlands, 1992; pp 81−95.

(24) AACC. *Approved Methods of the American Association of Cereal Chemists*; American Association of Cereal Chemists Inc.: St. Paul, MN, 2000.

(25) AOAC. *Official Methods of Analysis*, 16th ed.; Method 990.03; Association of Official Analytical Chemists: WA, 1995.

(26) Shogren, M. D.; Finney, K. F. Bread-making test for 10 grams of flour. *Cereal Chem.* **1984**, *61*, 418−423.

(27) Courtin, C. M.; Van den Broeck, H.; Delcour, J. A. Determination of reducing end sugar residues in oligo- and polysaccharides by gas−liquid chromatography. *J. Chromatogr. A* **2000**, *866*, 97−104.

(28) Dornez, E.; Joye, I. J.; Gebruers, K.; Delcour, J. A.; Courtin, C. M. Wheat-kernel-associated endoxylanases consist of a majority of microbial and a minority of wheat endogenous endoxylanases. *J. Agric. Food Chem.* **2006**, *54*, 4028−4034.

(29) Cleemput, G.; Bleukx, W.; van Oort, M.; Hessing, M.; Delcour, J. A. Evidence for the presence of arabinoxylan hydrolyzing enzymes in European wheat flours. *J. Cereal Sci.* **1995**, *22*, 139−145.

(30) Biliaderis, C. G.; Izydorczyk, M. S.; Rattan, O. Effect of arabinoxylans on bread-making quality of wheat flours. *Food Chem.* **1995**, *53*, 165−171.

Received for review May 9, 2007. Revised manuscript received June 27, 2007. Accepted June 27, 2007. The "Instituut voor de aanmoediging van Innovatie door Wetenschap en Technologie in Vlaanderen" (I.W.T., Brussels, Belgium) is gratefully acknowledged for financial support. K.G. is a postdoctoral fellow of the "Fonds voor Wetenschappelijk Onderzoek-Vlaanderen" (F.W.O.-Vlaanderen, Brussels, Belgium). The study was in part carried out in the framework of research project GOA/03/10 financed by the Research Fund K.U. Leuven.

JF071363M