Contribution of Wheat Endogenous and Wheat Kernel Associated Microbial Endoxylanases to Changes in the Arabinoxylan Population during Breadmaking

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

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

Wheat kernel associated endoxylanases consist of a majority of microbial endoxylanases and a minority of endogenous endoxylanases. At least part of these enzymes can be expected to end up in wheat flour upon milling. In this study, the contribution of both types of these endoxylanases to changes in the arabinoxylan (AX) population during wheat flour breadmaking was assessed. To this end, wheat flour produced from two wheat varieties with different endoxylanase activity levels, both before and after sodium hypochlorite surface treatment of the wheat kernels, was used in a straight dough breadmaking procedure. Monitoring of the AX population during the breadmaking process showed that changes in AX are to a large extent caused by endogenous endoxylanases, whereas the contribution of microbial endoxylanases to these changes was generally very low. The latter points to a limited contamination of wheat flour with microbial enzymes during milling or to an extensive inactivation of these wheat flour associated microbial endoxylanases by endoxylanase inhibitors, present in wheat flour. When all wheat kernel associated microbial endoxylanases were first washed from the kernels and then added to the bread recipe, they drastically affected the AX population, suggesting that they can have a large impact on whole meal breadmaking.

KEYWORDS: Wheat; breadmaking; arabinoxylan; endoxylanase; microbial; endogenous

INTRODUCTION

Although selected endoxylanases of microbial origin are routinely added in breadmaking applications to improve dough and bread characteristics by changing the physicochemical properties of cell wall arabinoxylans (AX), the potential impact of wheat kernel associated endoxylanases in this process has largely been disregarded. As it was recently demonstrated that the levels of wheat kernel associated endoxylanases are of the same order of magnitude as the endoxylanase activity levels commonly added to wheat flour as commercial endoxylanase-containing bread improvers (*1*), it is reasonable to expect that they can affect wheat breadmaking quality.

Wheat kernel associated endoxylanases are mainly microbial and to a lesser extent wheat endogenous (*1*). After wheat roller milling, most microbial endoxylanases end up in bran and shorts fractions. Nevertheless, several studies suggest that part of the microbial endoxylanases end up in flour as contaminant and affect its functional properties. Sörensen and Etzerodt (*2*) reported that a reduction of the level and/or activity of microbial enzymes in flour by pretreatment of wheat kernels improves

both the viscosity and water-holding capacity of refrigerated dough as a function of time. Furthermore, the addition of *Triticum aestivum* xylanase inhibitors (TAXI), which inactivate only microbial endoxylanases and do not affect endogenous endoxylanases, delays syruping in refrigerated doughs (*3*). Debyser et al. (*4*) concluded that microbial endoxylanases, present in wheat flour, were active during breadmaking, as addition of TAXI-type inhibitors to a dough recipe decreased loaf volume. There are hence indications in the literature for the contribution of wheat flour associated microbial endoxylanases to wheat flour quality, despite the presence of an excess of endoxylanase inhibitors in wheat flour.

The level of endoxylanases ending up in wheat flour depends both on the endoxylanase activity level of the wheat sample and its different tissues and, hence, on the milling process. Indeed, both endogenous and microbial endoxylanase activity levels in wheat vary strongly as a function of genetic, climatic, and agronomic factors (*5, 6*), and a strong correlation has been found between apparent endoxylanase activity levels and ash contents in flour milling fractions (*7*). These variations in enzyme levels may very well add to year-to-year and batch-to-batch wheat flour quality variation.

* Corresponding author (telephone + 32 16 32 19 17; fax + 32 16 32 19 97; e-mail christophe.courtin@biw.kuleuven.be).

Arabinoxylan Population during Breadmaking

J. Agric. Food Chem., Vol. 56, No. 6, 2008 **2247**

Earlier work by Rouau et al. (*8, 9*), Cleemput et al. (*10*), and Jimenez et al. (*11*) showed that part of the AX population is solubilized during the breadmaking process, even in the absence of added microbial endoxylanases. However, these researchers were unable to reveal the underlying mechanisms of the AX solubilization, which could simply be due to the mechanical work input or temperature increase or could be due to hydrolysis of AX by wheat flour associated endoxylanases. More recently (*12*), it was demonstrated that wheat flour associated endoxylanases affect the AX population only during the dough resting/fermentation phase and not during the mixing phase. It was, however, impossible to distinguish between the effects of endogenous and flour-contaminating microbial endoxylanases and to establish their effect on the final product. The purpose of this work was therefore to study the contribution of both wheat endogenous and wheat flour/kernel associated microbial endoxylanases to the changes in the AX population during the breadmaking process.

EXPERIMENTAL PROCEDURES

Materials. Wheat varieties Legat and Astuce were from AVEVE (Landen, Belgium) and Clovis Matton (Avelgem-Kerkhove, Belgium), respectively. All chemicals, hemoglobin, and reagents were of at least analytical grade and supplied by Sigma-Aldrich (Bornem, Belgium), unless specified otherwise. Sodium hypochlorite solution (commercial household bleach, 3.0% active chlorine) was from Loda (Westmalle, Belgium) *Bacillus subtilis* glycoside hydrolase family (GHF) 11 endoxylanase (Grindamyl H640, Swissprot accession no. P18429) was from Danisco (Brabrand, Denmark) and *Penicillium purpurogenum* GHF 10 endoxylanase (Swissprot accession no. Q9P8J1) was kindly made available by Prof. Jaime Eyzaguirre (Laboratorio de Bioquimica, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile). Termamyl 120L, an enzyme preparation containing a thermostable α-amylase, was obtained from Novozymes (Bagsvaerd, Denmark). Prior to use, the preparation was incubated for 60 min at 90 °C to inactivate all non-thermostable enzymes potentially present. Azurine-cross-linked AX (AZCL-AX) and amylose (AZCL-amylose) tablets were purchased from Megazyme (Bray, Ireland), and standard P-82 pullulans were from Showa Denko K.K. (Tokyo, Japan).

Methods. *Sodium Hypochlorite Surface Treatment of Wheat Kernels.* Wheat kernels were surface treated by shaking for 10 min in sodium hypochlorite solution, followed by rinsing with deionized water as described earlier (*1*). Kernels were subsequently air-dried.

Isolation of Wheat Kernel Associated Microbial Endoxylanases. Intact wheat kernels (100 g) were shaken at 150 strokes/min (Laboshake, VWR International, Leuven, Belgium) with 200 mL of sodium acetate buffer (25 mM, pH 5.0, 0.02% sodium azide) for 17 h to remove the wheat kernel associated microbial endoxylanases. After washing, the liquid phase was removed from the kernels by sieving over a 2.0 mm sieve. It was then dialyzed against sodium acetate buffer (10 mM, pH 5.0) at 7 °C for 24 h. The dialysate was then concentrated (factor 20) by freeze-drying and resuspending in a small volume of water. In this way, 1.0 mL of washing liquid (WL) contained a level of microbial endoxylanases which, by approximation, is equal to that in 10.0 g of wheat kernels. Freeze-drying and concentration of WLs did not change the activity of the endoxylanases.

Milling of Wheat. Both untreated and surface-treated wheat kernels were milled with a Bühler MLU-202 laboratory mill (Uzwil, Switzerland) into six flour fractions, bran, and shorts. The extraction rate is the weight of the flour fractions over the combined weight of the flour, bran, and shorts fractions. After milling, the three break flour and three reduction flour fractions were mixed thoroughly to obtain straight run flour.

Standard Analyses. Moisture and ash contents were measured according to AACC methods 44-15a and 08-12, respectively (*13*). Protein contents were determined using the Dumas combustion method, an adaptation of the AOAC Official Method (*14*) to an automated

Table 1. Schematic Overview of the Three Bread Recipes

		RWF bread[a]	STWF bread[a]	RWF+WL bread[a]
flour from untreated kernels	10.0 g	x		x
flour from surface-treated kernels	10.0 g		x	
NaOAc buffer (200 mM, pH 5.0)	1.0 mL	x	x	
microbial endoxylanases in NaOAc buffer (200 mM, pH 5.0)	1.0 mL			x
fresh yeast	2.35%	x	x	x
water	3.4/4.4 mL[b]	x	x	x
sucrose	6.0%	x	x	x
NaCl	1.5%	x	x	x

[a] RWF bread, bread from regular wheat flour; STWF bread, bread from wheat flour from surface-treated kernels; RWF+WL bread, bread from regular wheat flour supplemented with washing liquid containing the isolated microbial endoxylanases. [b] Amounts of water added to Legat and Astuce wheat flours, respectively.

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.

Breadmaking. Bread was prepared in triplicate on a 10.0 g scale based on the straight dough method of Shogren and Finney (*15*). Flour (10.0 g on a 14% moisture base) was mixed in a 10.0 g pin mixer (National Manufacturing, Lincoln, NE) with fresh yeast (2.35%), sugar (6.0%), salt (1.5%), water, and sodium acetate buffer (1.0 mL, 200 mM, pH 5.0). Baking absorptions and mixing times were estimated with farinograph and mixograph analyses, respectively, and further optimized in several preliminary breadmaking trials. They were 5.06 mL and 144 s for Astuce flour and 6.06 mL and 252 s for Legat flour, respectively.

Table 1 outlines the three slightly different bread recipes. The first recipe made use of flour derived from untreated wheat kernels and yielded regular wheat flour (RWF) breads. The second recipe made use of wheat flour derived from surface-treated wheat kernels and yielded surface-treated wheat flour (STWF) breads (**Table 1**). STWF and RWF breads were produced with the same baking absorption and mixing time. The third bread recipe made use of RWF, but differed from the RWF bread recipe by the presence of 1.0 mL of WL, containing isolated microbial endoxylanases in sodium acetate buffer (200 mM, pH 5.0), that was added instead of the same sodium acetate buffer. These breads are referred to as RWF+WL breads.

After mixing, dough was fermented for 180 min in a fermentation cabinet at 30 °C with punching at 105, 155, and 180 min. Final proof was 57 min, and baking was performed for 13 min at 232 °C in a rotary oven (National Manufacturing). Bread was weighed immediately after baking. Loaf volume was measured 60 min after baking by glass bead displacement as described by Vanhamel et al. (*16*). Bread height was determined with a graduated ruler. Dough samples were withdrawn after mixing and after 180 min of fermentation. Bread and dough samples were frozen in liquid nitrogen, freeze-dried, ground with a mortar and pestle, and sieved over a 250 μm sieve.

Analysis of Noncellulosic Carbohydrate Composition and Content. Total AX (TOT-AX) and starch contents of the flours were determined by gas chromatography (GC) of alditol acetates after acid hydrolysis, monosaccharide reduction, and acetylation. Flour (10–15 mg) was hydrolyzed with 2.0 M trifluoroacetic acid (TFA) (5.0 mL) for 60 min at 110 °C. Reduction with sodium borohydride and acetylation with acetic acid anhydride of the obtained monosaccharides were executed according to the procedure of Englyst and Cummings (*17*), and analysis of the alditol acetates with GC was as described before (*7*). For soluble AX contents of flour, dough, and bread samples, the procedure was preceded by an aqueous extraction. Flour and dough extraction was as described before (*7*). For bread extraction, 1.0 g was suspended in deionized water (10.0 mL) with heat-treated Termamyl 120L (60 μL). The suspension was shaken for 30 min at 37 °C and subsequently centrifuged for 15 min at 10000*g* and filtered (MN 615 Filter, Macherey-Nagel, Düren, Germany). The aqueous extracts (2.5 mL)

were hydrolyzed with 4.0 M TFA (2.5 mL) for 60 min at 110 °C, and the obtained monosaccharides were subsequently converted to alditol acetates and analyzed with GC as described before (*7, 17*).

Analysis of Reducing End Sugar and Free Sugar Contents. Reducing end xylose (RX) contents were determined by GC as described by Courtin et al. (*18*). In this procedure, which strongly resembles that for the determination of noncellulosic carbohydrate composition and contents described above, reduction with sodium borohydride is performed prior to acid hydrolysis and acetylation to alditol acetates. To determine free xylose (FX) and arabinose (FA) contents in the samples, the same procedure was performed, but hydrolysis was omitted.

Analysis of Enzyme Activity Levels. Endoxylanase activity levels in flour and whole meal samples were determined with AZCL-AX tablets as substrate (*7*). Because of the presence of endoxylanase inhibitors in the samples and the inhibition by these proteins of a variable proportion of wheat-associated microbial endoxylanases during aqueous extraction, and, in keeping with our earlier work (*1*), endoxylanase activity levels obtained for such samples are further referred to as apparent endoxylanase activity levels.

Microbial and endogenous endoxylanase activity levels of wheat were also determined. Microbial endoxylanases were first separated from the endogenous endoxylanases and endoxylanase inhibitors by washing wheat kernels (*5*). The activity levels in the washing liquids and washed kernels were subsequently determined using AZCL-AX tablets (*7*). They correspond to the microbial and endogenous endoxylanase activity levels, respectively. Total endoxylanase activity levels were then calculated as the sum of microbial and endogenous activity levels.

All endoxylanase activity levels are expressed in endoxylanase units (EU) per gram, with 1 EU corresponding to the amount of enzyme needed to increase the extinction value at 590 nm (E_{590}) by 1.0 per hour of incubation under the conditions of the assay.

α-Amylase activity levels were determined using AZCL-amylose tablets as substrate as described before (*7*) and are expressed in amylase units (AU) per gram. One AU is the amount of enzyme needed to increase the E_{590} by 1.0 per hour of incubation under the conditions of the assay.

Protease activity levels were determined by measuring the free α-amino nitrogen levels formed with trinitrobenzene−sulfonic acid reagent (*7*). Hemoglobin was used as substrate. Protease activity levels were expressed in protease units (PU) per gram, with 1 PU corresponding to the amount of enzyme needed to increase the extinction value at 340 nm (E_{340}) by 1.0 per hour of incubation under the conditions of the assay.

Endoxylanase, α-amylase, and protease activity levels could not be expressed in nanokatal or international units, as (i) the colorimetric assays used to measure them do not provide a direct measure for the formation of reducing end sugars or free α-amino nitrogen levels in mole and (ii) conversion of the absorption units to nanokatal or international units using calibration curves is meaningless for complex enzyme mixtures.

Arabinofuranosidase and xylosidase activity levels were determined by measuring the release of *p*-nitrophenol (PNP) from the corresponding PNP glycosides at 415 nm (E_{415}) (*12*). Calibration curves made with PNP (0–0.05 mM) allowed expression of the activity levels as nanokatals per gram.

Analysis of Endoxylanase Inhibitor Contents. TAXI and xylanase-inhibiting protein (XIP) endoxylanase inhibitor contents were determined with a modified Xylazyme-AX method, using *B. subtilis* and *P. purpurogenum* endoxylanases, respectively (*7*).

Determination of Molecular Mass by High-Performance Size Exclusion Chromatography (HPSEC). The apparent molecular mass (MM) distribution of AX in the flour, dough, and bread extracts was 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. as described before (*12*). The separation was monitored with an evaporative light scattering detector (Alltech 3300 ELSD, Grace Davison Discovery Sciences, Deerfield, MA). MM markers (1.5 mg/mL) were Shodex standard P-82 pullulans with MMs of 788, 404, 212, 112, 47.3, 22.8, 11.8, and 5.9 kDa.

Table 2. Apparent, Microbial, Endogenous, and Total Endoxylanase Activity Levels of Wheat Varieties Astuce and Legat

	Astuce	Legat
apparent endoxylanase (EU/g)	0.18 ± 0.01	1.29 ± 0.10
microbial endoxylanase (EU/g)	1.29 ± 0.30	3.42 ± 0.90
endogenous endoxylanase (EU/g)	0.05 ± 0.01	0.49 ± 0.15
total endoxylanase (EU/g)	1.34 ± 0.31	3.91 ± 0.91

Table 3. Yields of Flour, Bran, and Shorts Obtained after Bühler Milling of Untreated and Surface-Treated Wheat Kernels of Astuce and Legat

	Astuce			Legat		
	flour (%)	bran (%)	shorts (%)	flour (%)	bran (%)	shorts (%)
untreated	71.3	16.6	12.1	75.5	11.1	13.4
surface treated	70.0	20.2	9.8	75.8	10.8	13.4

Table 4. Starch, Ash, Protein, TOT-AX, WE-AX, TAXI, and XIP Contents and Endoxylanase, α-Amylase, Protease, Arabinofuranosidase, and Xylosidase Activity Levels of Flour Derived from Untreated and Surface-Treated Wheat Kernels of Astuce and Legat[a]

	Astuce		Legat	
	RWF	STWF	RWF	STWF
ash (%)	0.51a	0.53a	0.44a	0.44a
starch (%)	79.6a	82.6a	82.8a	81.0a
protein (%)	12.5a	12.5a	11.7a	11.9a
TOT-AX (%)	2.15a	2.19a	2.77a	2.69a
WE-AX (%)	0.50a	0.44a	0.51a	0.55a
TAXI (ppm)	64a	71a	57a	52a
XIP (ppm)	272a	299a	226a	261a
endoxylanase (EU/g)	0.02a	0.01b	0.20a	0.22a
α-amylase (AU/g)	1.26a	0.86b	1.68a	1.79a
protease (PU/g)	nd	nd	nd	nd
arabinofuranosidase (nkat/g)	0.11a	0.10b	0.10a	0.09b
xylosidase (nkat/g)	0.50a	0.37b	0.44a	0.44a

[a] Per variety, values with the same letter in one row are not significantly different from each other. nd, not detectable; RWF, regular wheat flour; STWF, wheat flour from surface-treated kernels.

Statistical Analysis. Tukey's tests (*P* value < 0.05) were performed with the Statistical Analysis System software 8.1 (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Milling of Surface-Treated and Untreated Wheat Kernels. Two wheat varieties, Legat and Astuce, with different endoxylanase activity levels (**Table 2**) were selected for breadmaking experiments. Although both microbial and endogenous endoxylanase activity levels were higher for Legat than for Astuce wheat (**Table 2**), the ratio of microbial to endogenous endoxylanase activity levels was much higher for the former, making the contamination of flour with microbial endoxylanases more easily perceivable in this case.

During the milling process, part of the microbial endoxylanases associated with the outer wheat kernel layers end up in the flour fractions as contamination (*7*). To produce wheat flour that is to a very large extent free of microbial enzymes, wheat kernels were surface treated with sodium hypochlorite solution prior to milling. The treatment had virtually no impact on the flour, bran, and shorts yields, and the extraction rates of untreated and surface-treated wheat kernels were hence very similar for both wheat varieties (**Table 3**).

Table 4 lists the composition of flours derived from untreated and surface-treated wheat kernels. No significant differences

Arabinoxylan Population during Breadmaking

J. Agric. Food Chem., Vol. 56, No. 6, 2008 **2249**

Table 5. Endoxylanase, α-Amylase, Protease, Arabinofuranosidase, and Xylosidase Activity Levels in Unconcentrated Washing Liquids of Astuce and Legat Wheat Kernels

	Astuce	Legat
endoxylanase (EU/mL)	0.65 ± 0.15	1.71 ± 0.45
α-amylase (AU/mL)	0.17 ± 0.02	1.15 ± 0.13
protease (PU/mL)	1.44 ± 0.05	2.72 ± 0.10
arabinofuranosidase (nkat/mL)	0.07 ± 0.01	0.16 ± 0.02
xylosidase (nkat/mL)	0.09 ± 0.01	0.10 ± 0.01

were found in the contents of ash, starch, protein, TOT-AX, water extractable AX (WE-AX), TAXI, and XIP for flours derived from untreated and surface-treated wheat kernels (*P* value > 0.05). For enzyme activity levels of the flours, the two wheat varieties showed different responses to the surface treatment (**Table 4**). For Legat, preliminary surface treatment did not significantly affect apparent endoxylanase, α-amylase, and xylosidase activity levels of the resultant flour. Arabinofuranosidase activity levels, in contrast, were significantly lower in flour derived from pretreated wheat (*P* value < 0.05). For Astuce, all enzyme activity levels were significantly lower (*P* value < 0.05) in flour derived from surface-treated wheat than in that derived from untreated wheat. For both Astuce and Legat, protease activity levels were below the detection limit (**Table 4**).

Isolation of Wheat Kernel Associated Microbial Endoxylanases. Microbial endoxylanases and other enzymes were removed from the wheat kernels by washing. All measured enzyme activity levels in the WLs were higher for Legat than for Astuce wheat (**Table 5**). After isolation, WLs were dialyzed and concentrated (factor 20) to reduce the volume needed in breadmaking experiments.

Changes in the AX Population during Breadmaking. The effects of xylanolytic enzymes on the AX population during the breadmaking process were examined by analysis of the contents of soluble AX, RX, FX, and FA for the different bread recipes at the different stages of the breadmaking process (**Figure 1**). Soluble AX comprise both WE-AX, which are already present in wheat flour, and solubilized AX, which become soluble during the breadmaking process.

Impact of Endogenous Endoxylanases on AX during Breadmaking. Monitoring of the AX population during the different stages of the breadmaking process for the STWF breads allowed evaluation of the impact of endogenous endoxylanases. For both Astuce and Legat wheats, AX solubilization is observed during mixing, fermentation, and baking. To find out whether the observed solubilization is due to mechanical or enzymic mechanisms, the RX contents were studied as they are expected to rise much more as a result of enzymic than of mechanical action (**Figure 1**). RX contents did not significantly increase during mixing, indicating mechanical solubilization in this case. During fermentation, RX contents increased, but the increase was stronger for Legat than for Astuce, reflecting the higher endogenous endoxylanase activity in Legat than in Astuce wheat flour. Solubilization during the fermentation phase occurs, hence, by enzymic hydrolysis. These results confirm earlier findings and hypotheses maintaining that endogenous endoxylanases affect the AX population only during the fermentation phase and not during the mixing phase of breadmaking (*12*). During baking, RX contents increased as well.

To investigate the role of xylosidases and arabinofuranosidase activities during the breadmaking process, FX and FA contents were determined (**Figure 1**). Xylosidases were apparently not active during the mixing phase. During the fermentation phase,

the contents of FX increased, indicating such action. As a result of baking, FX contents decreased again. FA contents also increased during the dough fermentation stage of the breadmaking process, but decreased as a result of baking.

Impact of Wheat Flour Associated Microbial Endoxylanases on AX during Breadmaking. Comparison of the AX population in RWF and STWF bread recipes allowed assessment of the functionality of microbial enzymes that actually end up as a contamination in wheat flour. Slightly different results were obtained for the two wheat varieties. For Legat wheat, no significant differences in soluble AX, RX, and FX were observed between the two recipes, indicating either that only limited levels of wheat-associated microbial endoxylanases and/or xylosidases end up in wheat flour after milling or that the majority of microbial enzymes present in wheat flour are hardly active due to rapid inhibition by enzyme inhibitors present in wheat flour.

For Astuce, in contrast, there were some small, although statistically significant, differences in the contents of soluble AX and RX after fermentation, indicating that the microbial endoxylanases that ended up in Astuce wheat flour had a measurable impact on the AX population during the breadmaking process.

As both TAXI and XIP-type inhibitor contents were higher in Astuce than in Legat flour (**Table 4**), the difference in endoxylanase functionality of the two flour samples cannot be due to this factor. A possible explanation is the ca. 20 times lower apparent endoxylanase activity level in Astuce than in Legat flour, which represents a much reduced background activity compared to what is observed with Legat. Also, Astuce flour may have been contaminated with microbial endoxylanases that are not sensitive to endoxylanase inhibitors, such as bacterial GHF 10 endoxylanases. This would also explain the significant difference found in apparent endoxylanase activity levels between flour derived from untreated and treated wheat kernels of Astuce, whereas for Legat, no such difference was observed (**Table 4**). For both wheat samples, at all stages of the breadmaking process, FA contents were consistently lower for STWF bread recipes than for RWF bread recipes. This indicates that the arabinofuranosidases in wheat flour are at least partially from microbial origin and that they can be active during the breadmaking process.

Impact of Wheat Kernel Associated Microbial Endoxylanases on AX during Breadmaking. Evaluation of the impact of the WL fractions allowed assessment of the maximum possible impact of wheat kernel associated microbial enzymes during the breadmaking process. The contents of soluble AX, RX, FX, and FA were consistently significantly higher for the RWF+WL bread recipe, indicating a strong potential impact of the microbial endoxylanases associated with wheat kernels. The differences were higher for Legat than for Astuce, as Legat contains higher enzyme activity levels (**Table 2**). During RWF+WL breadmaking, enzymes were already active during the relatively short mixing phase. Indeed, not only soluble AX but also RX contents increased significantly during mixing, indicating that, in the presence of the isolated microbial enzymes, solubilization is due to both mechanical work input and endoxylanase activity. Furthermore, FX and FA contents increased during mixing, demonstrating that also xylosidases and arabinofuranosidases showed significant activity during the mixing phase. As a result of baking, RX, FX, and FA contents decreased for samples supplemented with WL.

MM Distribution of Soluble AX. **Figure 2** shows the MM distribution of the water extractables for the different bread recipes. Because of the ungelatinized state of the starch and,
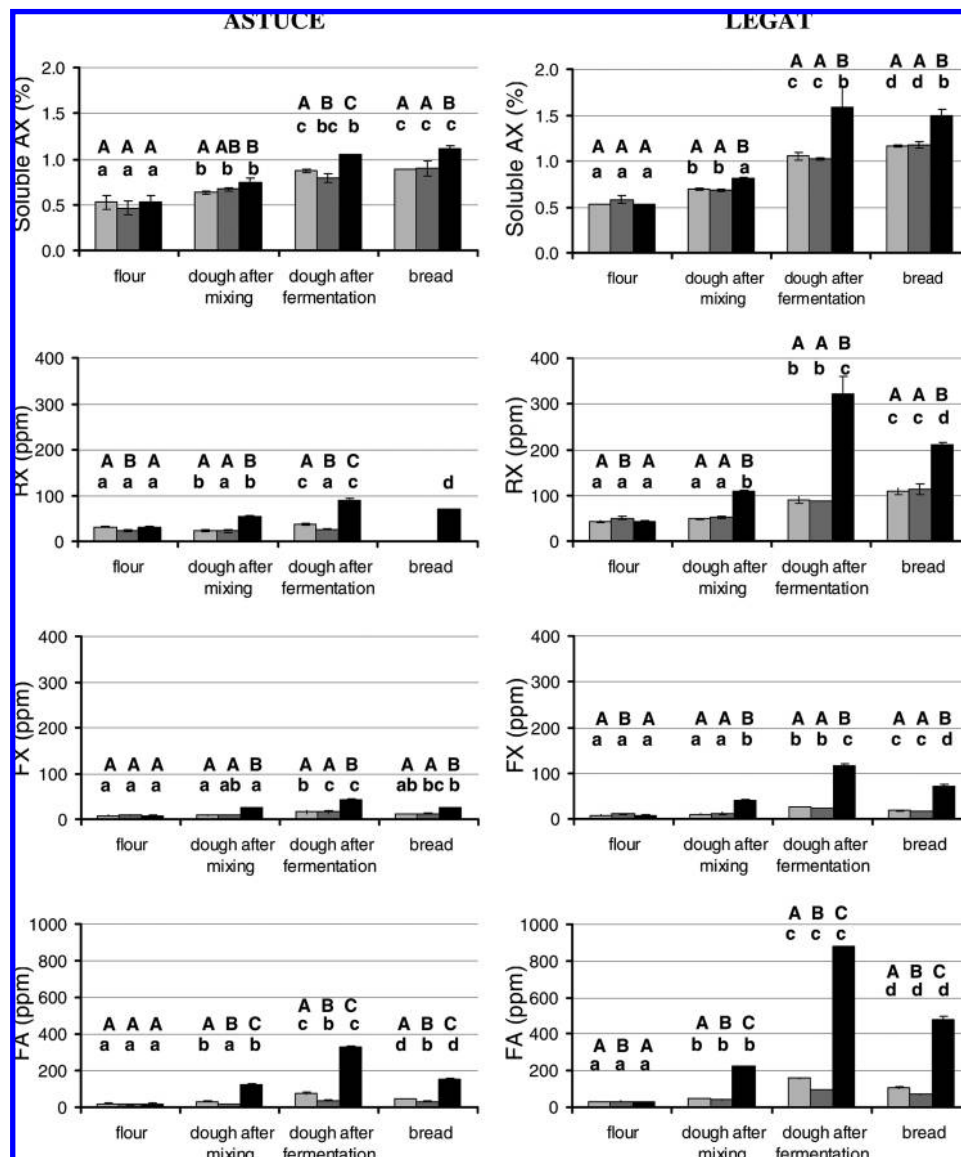
Figure 1. Contents of soluble AX, RX, FX, and FA for Astuce and Legat in the different stages of the breadmaking process for RWF breads (light gray bars), STWF breads (dark gray bars), and RWF+WL breads (black bars). A, AB, B, C: Tukey groups with *P* value < 0.05 for comparison of different bread recipes within one stage of the breadmaking process. a, ab, b, bc, c, d: Tukey groups with *P* value < 0.05 for comparison of different stages of the breadmaking process within one bread recipe.

hence, its poor enzymic degradability in the case of the dough samples, and because of the extraction in presence of Termamyl for the bread samples, the profiles in a MM range above 100 kDa most likely did not contain significant levels of starchy material. In what follows, we hence consider the profiles in this MM range as merely reflecting AX populations.

For the Astuce STWF recipe, AX with high MMs were solubilized during the mixing phase. During fermentation, there was some breakdown of AX with MMs exceeding 800 kDa, and a clear increase in the contents of AX with MMs of approximately 200–400 kDa was observed. After baking, the HPSEC profile of soluble AX was lower, which can be caused by either enzymic breakdown of AX to MMs below 200 kDa or by a lower extractability of AX due to physical entrapment or chemical cross-linking or a combination of both. When STWF and RWF bread recipes are compared, little, if any, difference in the HPSEC profiles was observed for the two varieties. The addition of WL in the breadmaking process (RWF+WL bread recipes), however, significantly affected the HPSEC profiles of the Astuce wheat solubles. During mixing, there was already

breakdown of AX with MMs above 800 kDa. During fermentation, more drastic degradation was observed, and AX fragments with MMs between 200 and 400 kDa were degraded to lower MM fragments. Similar to what was observed for STWF and RWF breads, the ELSD signal of the bread samples was lower as a result of baking. It is not clear whether this is due to lower extractability or to AX degradation during the baking phase.

For Legat, similar trends were observed, although it was remarkable that the peak MM of soluble AX in flour was found at much lower MMs. This confirms the earlier observation that peak MM of soluble AX is negatively correlated with the apparent endoxylanase activity level of the flour (*12*).

Breadmaking Results. Table 6 lists data on bread volume, weight, and height of the breads made with Astuce and Legat flours. For both Astuce and Legat, no significant differences in bread volume, weight, and height could be found between RWF and STWF breads (*P* value > 0.05). These findings are in agreement with those of Rouau et al. (*19*), who did not observe changes in dough, bread, or AX characteristics when XIP-type inhibitors, inhibiting only fungal endoxylanases, were added to
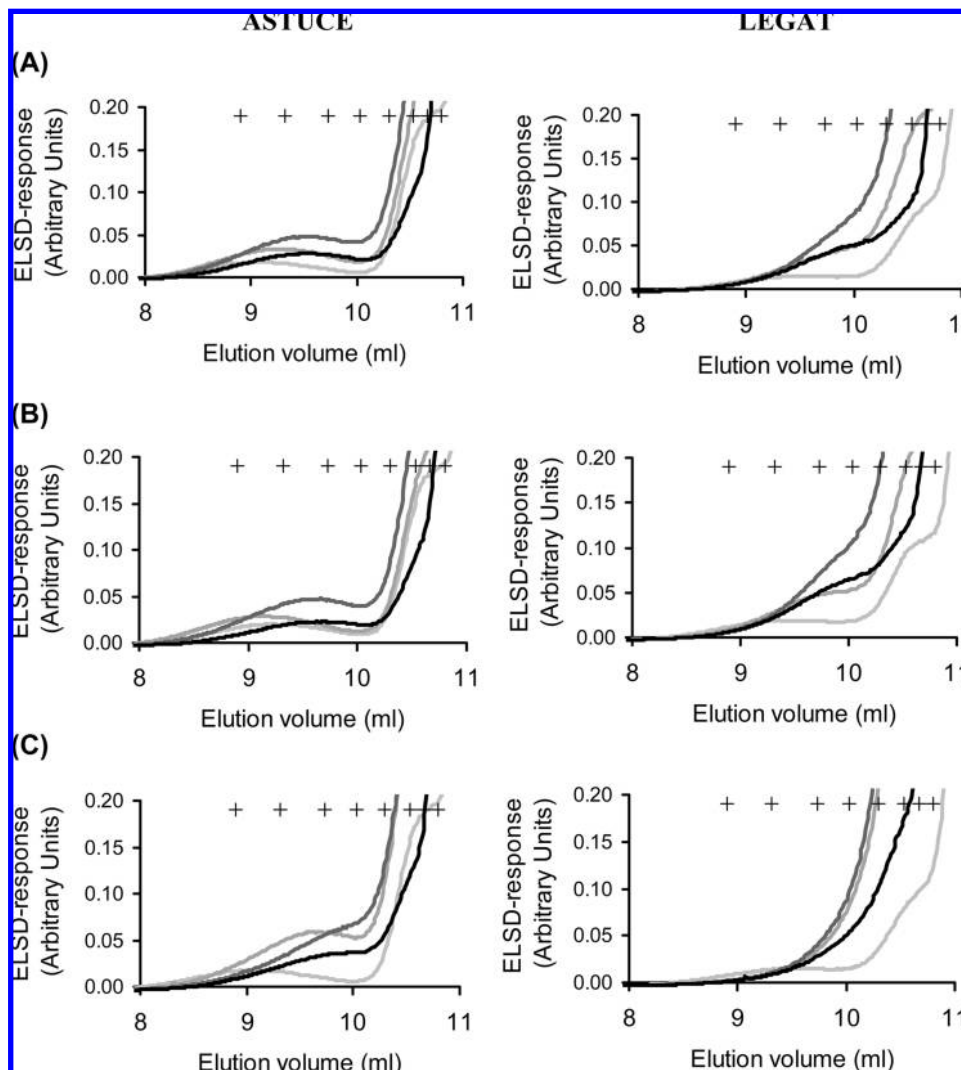
Arabinoxylan Population during Breadmaking

J. Agric. Food Chem., Vol. 56, No. 6, 2008 **2251**

Figure 2. HPSEC profiles of aqueous extracts of flours (light gray), doughs after mixing (middle gray), doughs after fermentation (dark gray), and breads (black), of RWF breads (**A**), STWF breads (**B**), and RWF+WL breads (**C**) for Astuce (left) and Legat (right). MM markers (+) from the left to the right are 788, 404, 212, 112, 47.3, 22.8, 11.8, and 5.9 kDa.

Table 6. Bread Volume, Weight, and Height of RWF, STWF, and RWF+WL Breads Made with Astuce and Legat Wheat Flours[a]

	Astuce			Legat		
bread	vol (cm³)	wt (g)	height (cm)	vol (cm³)	wt (g)	height (cm)
RWF	53.2a	11.8a	4.0ab	56.9a	12.6b	4.2ab
STWF	52.4a	11.9a	3.9a	55.6a	12.6b	4.1a
RWF+WL	56.0b	11.8a	4.2b	60.9b	12.3a	4.4b

[a] Per variety, values with the same letter in one row are not significantly different from each other. RWF bread, bread from regular wheat flour; STWF bread, bread from wheat flour from surface-treated kernels; RWF+WL bread, bread from regular wheat flour supplemented with washing liquid containing the isolated microbial endoxylanases.

control flour. However, they are in contrast with those of Debyser et al. (*4*), who found a decrease in bread volume when TAXI-type inhibitors, inhibiting only microbial endoxylanases, were added to the dough recipe.

RWF+WL breads had bread volumes that were approximately 5 and 7% higher than those of the RWF breads for Astuce and Legat, respectively. For reasons not understood at present, significantly lower bread weights were found for Legat, but not for Astuce RWF+WL breads. No significant differences were found for bread height. RWF+WL breadmaking gave rise to stickier doughs and darker crust color. Effects were more

apparent for Legat than for Astuce, which can be explained by the higher enzyme activity levels in the Legat WL.

The observed effects on bread characteristics can be caused by the impact of endoxylanases present in the WL on the AX population during breadmaking. Endoxylanases can, on the one hand, indeed positively affect bread volume by solubilization of water unextractable AX (*20*), but they can, on the other hand, also lead to unwanted stickiness. However, the observed effects cannot be ascribed unequivocally to differences in endoxylanase activity levels. Other enzymes, present in WL, may also affect bread characteristics. High protease activity levels, for example, can negatively affect bread volume when they destroy the gluten network. However, with wheat varieties that form a strong gluten network, low dosages of proteases can increase the extensibility of gluten films, thereby improving gas retention and bread volume (*21, 22*).

Furthermore, the darker crust color of the RWF+WL breads may be partially ascribed to endoxylanase activity, but other enzymic activities might contribute, too. Glycoside hydrolases, in general, and proteases increase the contents of reducing sugars and free amino groups, respectively, and hence more precursors are available for Maillard browning (*21, 22*).

Relevance of the Present Findings. The experimental approach of this study for the first time allows distinguishing

2252 *J. Agric. Food Chem.,* Vol. 56, No. 6, 2008

Dornez et al.

between the effects of endogenous and flour associated microbial endoxylanases on the AX population during breadmaking. This distinction is important as it is much easier to reduce the level of microbial endoxylanases in wheat flour, for example, by soft debranning of wheat kernels prior to milling (*23, 24*), than to reduce the level of endogenous endoxylanases in wheat flour. By comparing flour derived from untreated and flour derived from surface-treated wheat kernels, it became clear that the changes in AX population during white wheat flour control breadmaking are mainly caused by endogenous endoxylanases, making the selection of an appropriate wheat variety (with low enzymic activity) the only safe choice to avoid unwanted variability problems. The rather limited contribution of microbial endoxylanases can be explained both by a limited contamination of wheat flour with microbial enzymes and by inactivation of these endoxylanases by endoxylanase inhibitors. It can reasonably be assumed that, generally, microbial endoxylanases that end up in wheat flour are not able to affect relatively short-time processes, such as breadmaking, but they may be of importance in much longer processes, such as storage of refrigerated dough (*3*).

When all endoxylanases present on the outer wheat kernel layers were added integrally to the bread recipe, larger effects on the AX population and on dough and bread characteristics were observed. This demonstrates that the amount of bran contamination in wheat flour should be low and that very high extraction rates and bran shattering during milling hence need to be avoided if one wishes to keep the microbial endoxylanase activity level of flour as low as possible. However, we need to emphasize that the experimental setup used implied that the enzymes used were already in solution when added to the system and that, hence, they did not need to be set free from the bran during the breadmaking process itself. Although the results of this study, obtained on white breads, cannot be fully extrapolated to whole meal breadmaking applications, it can reasonably be expected that the large variability in wheat-associated endoxylanases (*5, 6*) can affect wheat functionality in whole meal breadmaking applications. Whereas the addition of external microbial endoxylanases in whole meal breadmaking can increase bread volume (*25, 26*), high levels of wheat kernel associated endoxylanases should, most likely, be avoided as this can lead to unwanted variability problems. Indeed, the functionality of added microbial endoxylanases not only depends on the presence of endoxylanase inhibitors in wheat flour (*27, 28*) and on endoxylanase characteristics (*20*) but may also be affected by the levels of wheat flour associated endoxylanase activities (*29*). Furthermore, high endoxylanase activity levels in wheat are usually accompanied by high levels of other enzymes, which can also complicate processing and negatively affect end-product quality.

ABBREVIATIONS USED

AU, amylase unit; AX, arabinoxylan; AZCL-amylose, azurin-cross-linked amylose; AZCL-AX, azurine-cross-linked arabinoxylan; E_{xxx}, extinction at *xxx* nm; ELSD, evaporative light scattering detector; EU, endoxylanase unit; FA, free arabinose; FX, free xylose; GC, gas chromatography; GHF, glycoside hydrolase family; HPSEC, high-performance size exclusion chromatography; MM, molecular mass; PNP, *p*-nitrophenol; PU, protease unit; RWF, regular wheat flour; RX, reducing end xylose; STWF, wheat flour from surface-treated kernels; TAXI, *Triticum aestivum* xylanase inhibitor; TOT-AX, total arabinoxylan; WE-AX, water extractable arabinoxylan; WL, washing liquid.

LITERATURE CITED

(1) 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.

(2) Sörensen, J. F.; Etzerodt, H. Removing enzymes on grain. GB 2402600A, 2004.

(3) 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.

(4) 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* (1), 39–43.

(5) Dornez, E.; Gebruers, K.; Joye, I. J.; De Ketelaere, B.; Lenartz, J.; Massaux, C.; Bodson, B.; Delcour, J. A.; Courtin, C. M. Effects of genotype, harvest year and genotype-by-harvest year interaction on arabinoxylan, endoxylanase activity and endoxylanase inhibitor levels in wheat kernels. *J. Cereal Sci.* **2008**, doi: 10.1016/j.jcs.2007.03.008.

(6) Dornez, E.; Gebruers, K.; Joye, I. J.; De Ketelaere, B.; Lenartz, J.; Massaux, C.; Bodson, B.; Delcour, J. A.; Courtin, C. M. Effects of fungicide treatment, N-fertilisation and harvest date on arabinoxylan, endoxylanase activity and endoxylanase inhibitor levels in wheat kernels. *J. Cereal Sci.* **2008**, doi: 10.1016/j.jcs.2007.03.009.

(7) 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.

(8) 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* (3), 259–272.

(9) Rouau, X.; Moreau, D. Modification of some physicochemical properties of wheat-flour pentosans by an enzyme complex recommended for baking. *Cereal Chem.* **1993**, *70* (6), 626–632.

(10) 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* (1), 55–66.

(11) Jimenez, T.; Martinez-Anaya, M. A. Amylases and hemicellulases in breadmaking. Degradation by-products and potential relationship with functionality. *Food Sci. Technol. Int.* **2001**, *7* (1), 5–14.

(12) Dornez, E.; Gebruers, K.; Cuyvers, S.; Delcour, J. A.; Courtin, C. M. Impact of wheat flour-associated endoxylanases on arabinoxylan in dough after mixing and resting. *J. Agric. Food Chem.* **2007**, *55*, 7149–7155.

(13) AACC. The definition of dietary fiber. *Cereal Foods World* **2001**, *46* (3), 112−126.

(14) *Official Methods of Analysis*, 16th ed.; Association of Official Analytical Chemists: Washington, DC, 1995.

(15) Shogren, M. D.; Finney, K. F. Bread-making test for 10 grams of flour. *Cereal Chem.* **1984**, *61* (5), 418–423.

(16) Vanhamel, S.; Vandenende, L.; Darius, P. L.; Delcour, J. A. A volumeter for breads prepared from 10-grams of flour. *Cereal Chem.* **1991**, *68* (2), 170–172.

(17) Englyst, H. N.; Cummings, J. H. Simplified method for the measurement of total non-starch polysaccharides by gas−liquid-chromatography of constituent sugars as alditol acetates. *Analyst* **1984**, *109* (7), 937–942.

(18) 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* (1), 97–104.

(19) Rouau, X.; Daviet, S.; Tahir, T.; Cherel, B.; Saulnier, L. Effect of the proteinaceous wheat xylanase inhibitor XIP-I on the performances of an *Aspergillus niger* xylanase in breadmaking. *J. Sci. Food Agric.* **2006**, *86* (11), 1604–1609.

Arabinoxylan Population during Breadmaking

J. Agric. Food Chem., Vol. 56, No. 6, 2008 **2253**

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

(21) Kulp, K. Enzymes as dough improvers. In *Advances in Baking Technology*; Kamel, B. S., Stauffer, C. E., Eds.; Blackie Academic and Professional: London, U.K., 1993; pp 152–178.

(22) Mathewson, P. R. Enzymatic activity during bread baking. *Cereal Foods World* **2000**, *45* (3), 98–101.

(23) Dexter, J. E.; Wood, P. J. Recent applications of debranning of wheat before milling. *Trends Food Sci. Technol.* **1996**, *7* (2), 35–41.

(24) 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* (3), 371–377.

(25) Ter Haseborg, E.; Himmelstein, A. Quality problems with high-fiber breads solved by use of hemicellulase enzymes. *Cereal Foods World* **1988**, *33* (5), 419–422.

(26) Shah, A. R.; Shah, R. K.; Madamwar, D. Improvement of the quality of whole wheat bread by supplementation of xylanase from *Aspergillus foetidus*. *Bioresour. Technol.* **2006**, *97* (16), 2047–2053.

(27) 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* (4), 417–425.

(28) Trogh, I.; Sörensen, 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.

(29) Beldman, G.; Osuga, D.; Whitaker, J. R. Some characteristics of β-D-xylopyranosidases, α-L-arabinofuranosidases and an arabinoxylan α-L-arabinofuranohydrolase from wheat bran and germinated wheat. *J. Cereal Sci.* **1996**, *23* (2), 169–180.