JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Wheat-Kernel-Associated Endoxylanases Consist of a Majority of Microbial and a Minority of Wheat Endogenous Endoxylanases

EMMIE DORNEZ, IRIS J. JOYE, KURT GEBRUERS, JAN A. DELCOUR, AND CHRISTOPHE M. COURTIN*

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

The endoxylanases associated with wheat kernels consist of wheat endogenous endoxylanases on one hand and kernel-associated microbial endoxylanases on the other hand. Assessment of their presence, based on analysis of their enzymic activity, can be expected to be hampered by the presence in wheat of high levels of endogenous endoxylanase inhibitors, which are able to inhibit the wheat-kernel-associated microbial endoxylanases. On the basis of preliminary experiments aimed at clarifying the distribution of the wheat-associated endoxylanases, a method to estimate total endoxylanase activities in wheat kernels was developed. Extensive washing of wheat kernels with universal buffer of pH 8.0 provided near-quantitative separation of the microbial endoxylanases located on the surface of wheat kernels from the endogenous endoxylanases and endoxylanase inhibitors located in such kernels. The microbial or endogenous nature of the endoxylanases was confirmed by making use of the inhibition specificity of endoxylanase inhibitors. Determination of the endoxylanase activity in the washing liquid, corresponding to the microbial endoxylanase population, and the washed kernels, corresponding to the endogenous endoxylanase population, allowed estimation of the total endoxylanase activities associated with the wheat kernel. Results showed that microbial endoxylanases can account for over 90% of the total wheat-associated endoxylanase activity and that the latter can be at least 5 times higher than the apparent endoxylanase activity.

KEYWORDS: Wheat; total endoxylanase activity; debranning; washing

INTRODUCTION

Arabinoxylans (AXs) are nonstarch polysaccharides that, due to their unique physicochemical properties, have a great impact on the functionality of wheat in biotechnological processes and applications (*1*, *2*). Endoxylanases are enzymes which hydrolyze the AX backbone internally. They can have a strong impact on AX functionality in breadmaking (*2*), gluten−starch separation (*3*), and animal feeding (*4*). Endoxylanases, typically produced by a large number of bacteria, fungi, and plants, are usually classified in different glycoside hydrolase families (GHF) on the basis of genetic information and sequence similarities (*5*). Whereas fungal and bacterial endoxylanases usually belong to GHF 10 or 11, all plant endogenous endoxylanases identified so far belong to GHF 10 (*6*). It is known that, during germination, endogenous endoxylanases are initially synthesized and accumulated in the aleurone cells as inactive precursors and that they are released from the aleurone cells during programmed cell death. The release of the endoxylanase from the aleurone layer is accompanied by the proteolytic cleavage of amino and carboxyl terminal regions of the precursor molecule, which activates the enzyme. This active endoxylanase then contributes to the breakdown of aleurone and endosperm cell walls (*6*, *7*). However, little if anything is known about the location of the low levels of endogenous endoxylanases in nongerminating wheat kernels.

Endoxylanases associated with wheat kernels originate, however, not only from the wheat plant itself but also from microorganisms populating the outer layers of the wheat kernel (*8*, *9*). This corresponds with the previous observations that endoxylanases in wheat grains are mainly concentrated in the outer kernel layers (*10*, *11*) and that a considerable part of wheat endoxylanase activity can be removed by debranning of wheat kernels (*8*).

Next to endoxylanases, wheat also contains proteins that can inhibit bacterial and/or fungal endoxylanases. Three different types of such inhibitors are known to date: TAXI (*Triticum aestivum* xylanase inhibitor) (*12*), XIP (xylanase-inhibiting protein) (*13*) and TL-XI (thaumatin-like xylanase inhibitor) (*14*). TAXI and TL-XI inhibit most GHF 11 endoxylanases from fungal and bacterial origin, but are unable to inhibit GHF 10 endoxylanases (*14*, *15*). XIP typically inhibits fungal endoxyl-

* To whom correspondence should be addressed. Phone: + 32 16 321917. Fax: + 32 16 321997. E-mail: christophe.courtin@biw.kuleuven.be.

10.1021/jf060129d CCC: $33.50
Published on Web 05/05/2006

anases, but does not show activity against bacterial or plant endoxylanases (*16*, *17*).

Because of the presence of high levels of endoxylanase inhibitors in wheat, the endoxylanase levels measured in wholemeal or flour extracts can be expected to be apparent levels. After all, during the aqueous extraction preceding each activity measurement, complexation of endoxylanases with endoxylanase inhibitors can occur. Endoxylanases in such enzyme−inhibitor complexes do not contribute to endoxylanase activity measurements that are based on the hydrolysis of an (arabino)xylan substrate. Therefore, all endoxylanase activity measurements in wheat, using the current methods, can be expected to underestimate the level of endoxylanases.

The underestimation of endoxylanase levels in wheat may have resulted in an underestimation of the importance of wheat-kernel-associated endoxylanases in cereal processing and in year-to-year wheat quality variation. To assess whether indeed a distinction can be made between total and apparent endoxylanase activities and, if so, to assess its importance, a procedure to measure total endoxylanase activities in wheat kernels, based on a physical separation of microbial and plant endoxylanases and endoxylanase inhibitors in wheat, was developed. This procedure also allows assessment of the relative contributions of wheat-associated microbial as well as endogenous endoxylanases to these total activities. The development of the procedure required preliminary experiments assessing the distribution of the different types of endoxylanases.

EXPERIMENTAL PROCEDURES

Materials. Wheat cultivars Koch, Decan, and Corvus (harvest 2004) were obtained from AVEVE (Landen, Belgium). Chemicals, bovine serum albumin (BSA), hemoglobin, and reagents were purchased from Sigma-Aldrich (Bornem, Belgium) and were of at least analytical grade. Sodium hypochlorite (commercial household bleach) was from Loda (Westmalle, Belgium). *Bacillus subtilis* 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, Chile). Azurine-cross-linked arabinoxylan (AZCL-AX) tablets were purchased from Megazyme (Bray, Ireland). TAXI, XIP, and TL-XI were isolated from wheat by an affinity-based purification procedure (*14*, *18*).

Debranning of Wheat Kernels. Intact wheat kernels (Koch, 100 g) were debranned using a Satake batch debranner, type TM05C (Satake, Bredbury, U.K.) for 5, 10, 15, 20, 30, 60, and 90 s. The material that was removed from the kernels, further referred to as "Satake bran", and the debranned kernels were recovered and weighed. The percentage of debranning was calculated from the weight difference between the kernels before and after debranning.

Surface Sterilization of Wheat Kernels. Surface sterilization of wheat kernels was based on the method described by Rabie et al. (*19*) for barley kernels. Thus, intact wheat kernels (Koch, 100 g) were shaken for 1, 5, 10, 20, and 30 min (Laboshake, VWR International, Leuven, Belgium) with sodium hypochlorite (200 mL, 3.0% active chlorine). After removal of the sodium hypochlorite, the kernels were rinsed twice with deionized water (800 mL, 10 min) and freeze-dried.

Washing of Wheat Kernels. Liquids used for washing of wheat kernels were deionized water, 250 mM NaCl, 20% ethanol, two detergent solutions, i.e., 0.01% Triton and 0.1% CHAPS, universal buffer (UB) at pH 4.0, 6.0, and 8.0, and a mixture (UB pH 8.0, 250 mM NaCl, and 0.1% CHAPS). UB was made by dissolving citric acid (6.0 g), monopotassium phosphate (3.9 g), boric acid (1.8 g), diethyl barbituric acid (5.3 g), and sodium azide (0.2 g) in deionized water (1.0 L) and adjusting the pH with a NaOH solution (2.0 M).

Intact wheat kernels (Koch, 100 g) were shaken (Laboshake) with 200 mL of each of the above-mentioned washing liquids for 30 min at room temperature or at 7 °C. After washing, the liquid was recovered by sieving over a 2.0 mm sieve and used for further analysis. After this washing step, two rinsing steps were executed. In each one, deionized water (800 mL) was added to the kernels. After 10 min, the rinsing liquids were recovered by sieving and used for further analysis. The rinsed wheat kernels were frozen with liquid nitrogen and freeze-dried.

To adequately compare the different samples, all washing and rinsing liquid samples were desalted and transferred in the same buffer (25 mM sodium acetate, pH 5.0) using a PD10 buffer exchange column with a Sephadex G-25 matrix (Amersham Biosciences, Uppsala, Sweden).

A similar washing procedure, using UB pH 8.0, was performed with washing times of 30 min and 1, 3, 6, 9, 12, 15, 17, 24, and 48 h. Finally, the washing procedure, using UB pH 8.0 and a washing time of 17 h, was performed on wheat cultivars Koch, Decan, and Corvus.

Grinding of Wheat Kernels. Untreated, debranned, and washed wheat kernels (100 g) were ground with a Cyclotec 1093 sample mill (Tecator, Hogänäs, Sweden).

Extraction of Wheat Material. Extraction of ground untreated, debranned, and washed wheat kernels (2.0 g) or Satake bran (0.5 g) was done by suspending the sample in sodium acetate buffer (10.0 mL, 25 mM, pH 5.0) and shaking (Laboshake) for 30 min at room temperature. After centrifugation for 10 min at 10000*g* in a Beckman J2-21 centrifuge (Beckman, Fullerton, CA), the suspensions were filtered through an MN 615 filter (Macherey-Nagel, Düren, Germany).

Determination of Endoxylanase Activities. Endoxylanase activities in the extracts and washing and rinsing liquids were determined with the Xylazyme AX method (Megazyme). The samples (1.0 mL) were equilibrated for 10 min at 40 °C before addition of an AZCL-AX tablet. After appropriate incubation times, the reaction was stopped by adding 10.0 mL of 1.0% (w/v) Tris solution and by vigorous vortex stirring. After 10 min at room temperature, the solutions were filtered, and the extinction values at 590 nm (E_{590}) [Ultraspec III UV/vis spectrophotometer (Pharmacia Biotech, Uppsala, Sweden)] were measured against a control, prepared by incubating the extracts without the AZCL-AX tablet. Correction was made for nonenzymic color release by the AZCL-AX tablets. Activities were expressed in enzyme units (EU). One EU is the amount of enzyme needed to yield an E_{590} of 1.0 per hour of incubation, under the conditions of the assay. The endoxylanase activities from the washing and rinsing liquids were expressed as enzyme units per gram of originally treated wheat rather than per milliliter of liquid. With this method, endoxylanase activities as low as 0.02 EU/g of kernel can be determined accurately. The coefficient of variation for the determination of endoxylanase activities is typically 1%.

Determination of Endoxylanase Inhibitor Activities. Endoxylanase inhibitor activities in extracts and washing and rinsing liquids were measured with a modified Xylazyme AX method as described by Gebruers et al. (*20*). TAXI inhibitor activities were measured by using GHF 11 *B. subtilis* endoxylanase. This bacterial GHF 11 endoxylanase is inhibited by TAXI (*15*) and not by XIP or TL-XI (*14*, *17*). XIP inhibitor activities were measured using a GHF 10 *P. purpurogenum* endoxylanase solution as fungal GHF 10 endoxylanases are only inhibited by XIP and not by TAXI or TL-XI (*14*, *15*, *17*). As to date no endoxylanase is known which is only inhibited by TL-XI and not by TAXI or XIP, this third type of inhibitor could not be measured separately. The *B. subtilis* and *P. purpurogenum* enzyme solutions (2.0 EU/mL) were prepared in sodium acetate buffer (25 mM, pH 5.0) containing BSA (0.5 g/L). The extracts were diluted to ensure a linear response between the concentration of inhibitor and the inhibition activity measured with the assay below.

Endoxylanase solutions (0.5 mL) were preincubated for 30 min at room temperature with an equal volume of buffer (reference) or (diluted) sample to allow formation of enzyme−inhibitor complexes in the latter case. After preincubation, the mixtures were equilibrated for 10 min at 30 °C. An AZCL-AX tablet was then added. The reaction was stopped after 1 h of incubation by adding 1.0% (w/v) Tris solution (10.0 mL) and vortex-mixing. After 10 min at room temperature, the tubes were shaken vigorously, and the contents were filtered. The E_{590} values of the reference and the sample were measured against those of controls,

Table 1. Endoxylanase (EX) and Endoxylanase Inhibitor (TAXI and XIP) Levels of Debranned Kernels and Satake Bran Obtained after Debranning of Wheat for 0, 5, 10, 15, 20, 30, 60, and 90 s

		debranned kernels			Satake bran			removal from the kernel		
time of debranning (s)	degree of debranning (%)	EX (EU/g)	TAXI (ppm)	XIP (ppm)	EX (EU/g)	TAXI (ppm)	XIP (ppm)	EX (%)	TAXI (%)	XIP (%)
0	0.0	1.70	64	317	0.0	0	0	0	0	0
5	3.4	0.48	80	341	40.9	0	97	75	0	1
10	5.2	0.34	87	351	27.0	16	182	81	1	3
15	5.8	0.34	82	343	33.8	21	225	86	2	4
20	7.3	0.28	76	338	25.2	23	268	88	2	6
30	10.8	0.35	92	343	14.7	32	344	83	4	11
60	20.7	0.20	88	328	5.8	36	356	88	10	22
90	28.6	0.21	79	298	3.0	37	366	85	16	33

prepared by incubation with buffer instead of enzyme solution. While both controls allowed correction for nonenzymic color release, the second control allowed correction for endoxylanase activity present in the samples. The percentage inhibition was calculated from the difference between the E_{590} values of samples and references.

For conversion of endoxylanase inhibitor activities into inhibitor levels, dose−response curves with pure inhibitors were determined, allowing expression of the endoxylanase inhibitor levels in wheat in micrograms per gram or parts per million (ppm) as described by Bonnin et al. (*21*). The coefficient of variation for the determination of endoxylanase inhibitor levels is typically 6%.

Determination of Proteolytic Activities. Hemoglobin [1.0% (w/v) in 200 mM sodium acetate buffer, pH 4.0] was used as the substrate to measure both endo- and exoproteolytic activity in washing liquids. Equal amounts of hemoglobin solution and sample (0.25 mL) were incubated for 24 h at 40 °C. The reaction was stopped by addition of cold trichloroacetic acid [0.4 mL, 10.0% (w/v)], and the precipitated proteins were removed by centrifugation (10000*g*, 10 min). The free α-amino nitrogen levels of the supernatant were assayed with trinitrobenzenesulfonic acid (TNBS) reagent [0.3% (v/v) in 200 mM sodium phosphate buffer, pH 8.0]. To this end, the supernatant (0.025 mL) and TNBS reagent (0.225 mL) were incubated for 20 min at 50 °C. The reaction was stopped with 200 mM HCl (0.75 mL). The extinction of the solution at 340 nm was a measure for the proteolytic activity in the sample. The coefficient of variation for the determination of proteolytic activities is typically 7%.

Microbial Analyses. Serial decimal dilutions from the washing liquids were made by dilution in the corresponding buffers. A 1 mL sample of each dilution was pour-plated in duplicate on plate count agar (PCA) for mesophilic aerobic plate counts and incubated at 30 °C for 3 days. For moulds and yeasts, spread-plating of 0.1 mL of each dilution was carried out in duplicate on prepoured oxytetracycline glucose yeast extract (OGYE) agar plates. The plates were incubated at 20 °C for 3 days. All colonies on PCA and OGYE plates were counted, regardless of their color or size, and expressed as colony-forming units (cfu) per gram of wheat sample.

RESULTS AND DISCUSSION

Distribution of Endoxylanases over the Wheat Kernel. As development of a procedure for measuring total plant and microbial endoxylanase activities in wheat requires a more detailed knowledge of the distribution of these different types of endoxylanases in the wheat kernel, two preliminary experiments were carried out which could provide insight into the distribution of microbial and endogenous endoxylanases over the wheat kernel, i.e., debranning and surface sterilization of wheat kernels.

Debranning of Wheat Kernels. Debranning of Koch wheat kernels for 0, 5, 10, 15, 20, 30, 60, and 90 s resulted in removal of 0%, 3.4%, 5.2%, 5.8%, 7.3%, 10.8%, 20.7%, and 28.6% of the kernel. This linear relationship between time and degree of debranning ($R^2 > 0.99$, $P < 0.01$) was also observed by Gys et al. (*8*) and Laca et al. (*22*). Debranning decreased the apparent endoxylanase activity in the kernels drastically, while the endoxylanase inhibitor levels remained relatively constant (**Table 1**). More than 80% of the apparent endoxylanase activity was located in the outer 6% of the wheat kernel. These results are in agreement with those of Gys et al. (*8*). The apparent endoxylanase activity in the Satake bran also decreased with increasing degree of debranning. There are two explanations for this decrease: the dilution of the bran material with material that contains less endoxylanase activity and the gradual increase in the removed bran of plant endoxylanase inhibitors that inactivate the microbial endoxylanases. This last explanation was confirmed by the increase of TAXI and XIP levels in the bran material with increasing degree of debranning. The endoxylanases in Satake bran were predominantly from microbial origin as they could be inhibited by 90% on average by addition of an excess of endoxylanase inhibitors. The endoxylanases in the debranned kernels, however, were not all from plant origin as still 30% on average could be inhibited by addition of an excess of endoxylanase inhibitors. Laca et al. (*22*) and Gys et al. (*8*) also noticed that it was impossible to remove all wheat-kernel-associated microorganisms and microbial endoxylanases, respectively, as part of them remained in the crease of the debranned kernels. In conclusion, debranning of wheat kernels showed that the largest part of the endoxylanases is situated on and in the outer 6% of the wheat kernel. However, this technique was not able to homogeneously remove the outer layers of the kernels as the outer layers in the crease of the kernels could not be abraded.

Surface Sterilization of Wheat Kernels. To inactivate the microbial endoxylanases on the outer layers of the wheat kernels, they were surface treated by washing with sodium hypochlorite for variable times. The apparent endoxylanase activity decreased drastically from 1.7 to 0.2 EU/g of kernel in the first 5 min of washing and subsequently remained relatively constant (**Figure 1**). The TAXI- and XIP-related endoxylanase inhibitor activities, in contrast, did not change appreciably, confirming that the inactivation procedure used only affected the surface of the kernel. As the residual endoxylanase activity could not be inhibited by addition of an excess of endoxylanase inhibitors, it can be concluded that all microbial endoxylanases were inactivated. The activity remaining in the wholemeal of kernels after surface sterilization (0.2 EU/g of kernel) was hence considered to be caused merely by endogenous endoxylanases.

These preliminary experiments indicated that the microbial endoxylanases are only located at the surface of the wheat kernels, while the endogenous endoxylanases and endoxylanase inhibitors are located in the kernels. On the basis of these results, it is obvious that a method to estimate total endoxylanase activities in wheat kernels can be obtained by physically separating the microbial endoxylanases from the endogenous

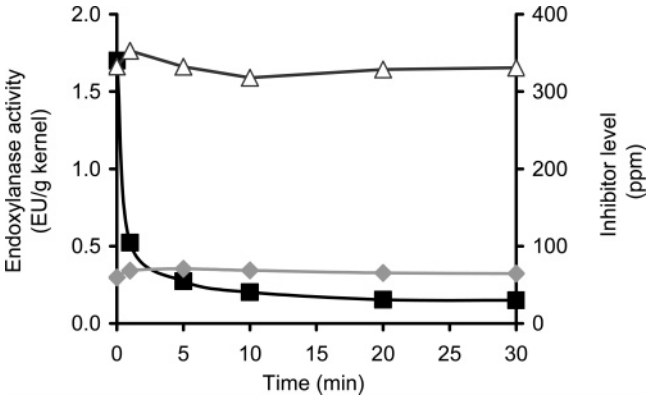

Figure 1. Apparent endoxylanase activities (EU/g of kernel) (black squares) and TAXI (gray tilted squares) and XIP (white triangles) levels (ppm) in Koch wheat wholemeal as a function of the sodium hypochlorite treatment time (min).

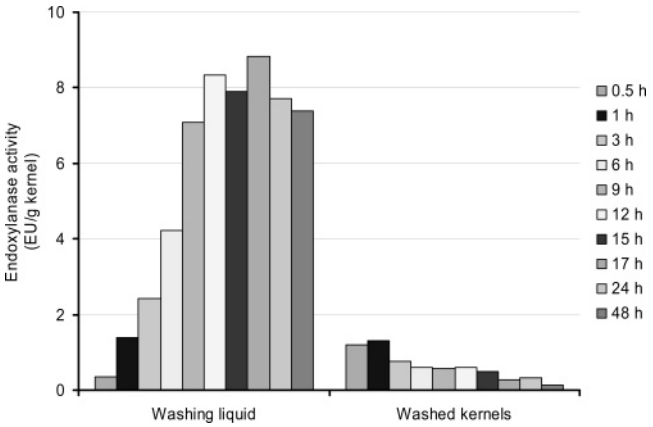

Figure 2. Endoxylanase activities (EU/g of kernel) in the washing liquids and washed kernels obtained after washing of Koch with UB pH 8.0 for different times (h).

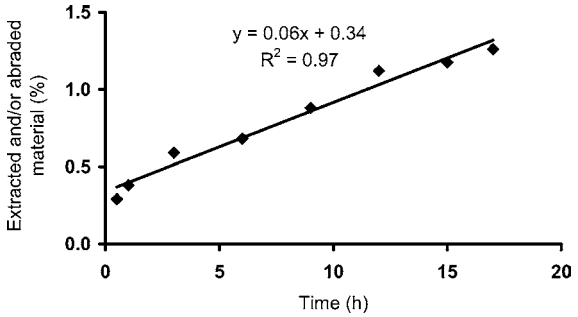

Figure 3. Material abraded from the Koch wheat kernels (%) during washing with deionized water as a function of the washing time (h).

endoxylanases and endoxylanase inhibitors. To that end, a washing procedure in which microbial endoxylanases are removed from the wheat kernels was developed as described below.

Development of a Washing Procedure. *Different Washing Liquids.* Different noninactivating aqueous washing liquids were tested for their ability to remove microbial endoxylanases quantitatively from the wheat kernels. Endoxylanase activities were measured in the extracts of the washed wheat kernels and in the washing and rinsing liquids after PD10 buffer exchange (**Table 2**). Endoxylanase activities in the washed kernels were lower than in the untreated kernels, but the differences were rather small. None of the washing liquids seemed capable of removing all microbial endoxylanases and reaching an endogenous endoxylanase activity in the washed kernels of 0.2 EU/g of kernel as obtained after surface sterilization of the wheat kernels. This implied that endoxylanase activities in the washing liquids were rather low. UB pH 8.0 was considered to be the best washing liquid because it extracted ca. 20% of the apparent endoxylanase activity in the wheat wholemeal. Addition of 0.1% CHAPS and 250 mM NaCl to this buffer did not result in extra removal of endoxylanases from the wheat kernels. Endoxylanase activities recovered in each of the rinsing liquids were negligible.

Different Washing Times. Because washing for 30 min clearly did not remove all microbial endoxylanases from the wheat kernels to reach an endoxylanase level of 0.2 EU/g of kernel as obtained after surface sterilization of wheat, longer washing times (1, 3, 6, 9, 12, 15, 17, 24, and 48 h) with UB pH 8.0 were tested (**Figure 2**). Endoxylanase activities in the washing liquids increased linearly with time when the kernels were washed longer ($R^2 > 0.99$, $P < 0.01$). After 12 h, the activities remained relatively constant. For practical reasons, the final washing time was set at 17 h (overnight). Proteolytic activity in the washing liquids was very low [<0.05 (abs/g)/h]. This indicates that the proteolytic cleavage of endoxylanases during washing is negligible. The remaining endoxylanase activity in the washed kernels was 0.27 EU/g of kernel, which corresponds rather well to the endoxylanase activity in the kernels that were treated with sodium hypochlorite for 30 min.

To investigate whether the increase in endoxylanase activity with increasing washing time was caused by extraction of the enzymes or by a combination of extraction and a soft abrasion potentially taking place during the long washing period, the washing procedure was also executed with deionized water instead of UB. The washing water was freeze-dried to determine the amount of kernel material that was removed during washing. The amount of extracted and/or abraded material increased linearly with increasing washing time ($R^2 = 0.97$, $P < 0.01$) (**Figure 3**). After 17 h of washing, 1.3% of dry material was removed from the wheat kernels.

Total Endoxylanase Activities. The total endoxylanase activity associated with the wheat kernel prior to possible complexation of endoxylanases with endoxylanase inhibitors, further referred to as total endoxylanase activity, can be calculated as the sum of the endoxylanase activities in the washing liquid (microbial endoxylanases) and in the washed kernels (endogenous endoxylanases) (**Figure 4**). For Koch, the enzyme activity due to microbial endoxylanase amounted to 8.83 EU/g of kernel while the endogenous activity amounted to 0.27 EU/g of kernel, yielding a total endoxylanase activity of 9.1 EU/g of kernel. The contribution to this total endoxylanase activity by the microbial endoxylanase is thus 33 times higher that that of the endogenous enzymes. In addition, the microbial endoxylanase activity is more than 5 times higher than the apparent activity measured for Koch wholemeal, i.e., 1.7 EU/g of kernel.

Validation of the Washing Procedure. Next to the assumption that the washing procedure yields high levels of endoxylanase activity due to the separation of inhibition-sensitive endoxylanases and endoxylanase inhibitors, other possible explanations for these very high total endoxylanase activities could be in situ production of endoxylanases due to microbial growth and germination of the kernels during the washing procedure. To investigate these possibilities, a number of experiments were set up.

Assessment of Potential Microbial Growth. Although it was considered unlikely that microorganisms can grow and produce endoxylanases during washing of the kernels because of the presence of sodium azide (0.02%) in the UB medium, chloram-

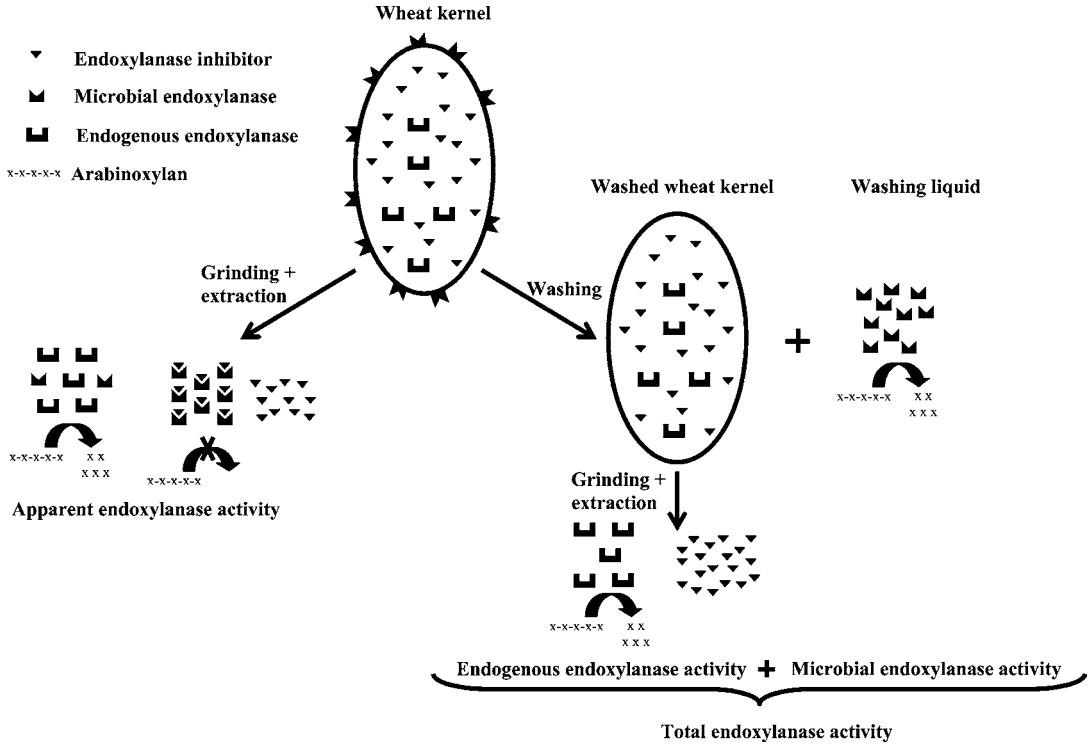

Figure 4. Schematic representation of the measurement of apparent, endogenous, microbial, and total endoxylanase activities in wheat.

Table 2. Endoxylanase Activities in the Different Fractions Obtained after Washing and Rinsing of Wheat Kernels for 30 min with Different Washing Liquids

	endoxylanase activity (EU/g of kernel)					endoxylanase activity (% of total)		
treatment	kernels	washing liquid	rinsing water 1	rinsing water 2	sum	kernels	washing liquid	rinsing waters
untreated	1.70	0.00	0.00	0.00	1.70	100	0	0
UB pH 4.0	1.32	0.04	0.00	0.01	1.37	96	3	1
ethanol, 20%	1.31	0.06	0.01	0.01	1.39	94	4	2
water	1.39	0.07	0.01	0.01	1.48	94	5	1
CHAPS, 0.1%	1.43	0.18	0.02	0.02	1.65	86	11	3
Triton, 0.01%	1.54	0.21	0.03	0.01	1.80	86	12	2
NaCl, 250 mM	1.33	0.21	0.00	0.00	1.55	86	14	0
UB pH 6.0	1.25	0.25	0.02	0.00	1.53	82	17	1
UB pH 8.0	1.21	0.36	0.03	0.01	1.61	75	22	3
mixture	1.16	0.37	0.06	0.02	1.61	72	23	5

phenicol, an antibiotic that inhibits microbial protein synthesis, was used to further substantiate this. No difference in endoxylanase activities in the washing liquids and washed kernels could be detected when sodium azide was omitted or when chloramphenicol (100 ppm) was added in the washing procedure.

When the washing procedure was carried out at 7 °C, the endoxylanase activity in the washing liquid was lower than in the liquid obtained at room temperature, but the remaining endoxylanase activity in the kernels was higher. It can be assumed that the difference in endoxylanase activity between room temperature and 7 °C was caused by a lower washing efficiency at lower temperature. Omitting sodium azide or adding chloramphenicol at this temperature did not change the endoxylanase activities.

Finally, washing liquids obtained after washing of wheat kernels at room temperature with UB pH 8.0 with and without sodium azide were plated on PCA and OGYE agars. Microbial counts were 10^7 cfu/g on PCA and 10^3 cfu/g on OGYE agar both in the presence and in the absence of sodium azide, which excludes microbial growth during washing.

Assessment of Potential Germination. Germination of kernels may also explain the increase in endoxylanase activity as germinating kernels produce different enzymes to mobilize the endosperm reserve components for plant development (*23*). Endogenous endoxylanases produced during germination identified to date are GHF 10 endoxylanases which are not inhibited by TAXI and XIP inhibitors. Addition of an excess of endoxylanase inhibitors, however, inhibited the largest part of the endoxylanase activity present in the washing liquid. The fact that these endoxylanases can be inhibited shows that they are from microbial origin and not from plant origin (**Figure 5**). The remaining endoxylanase activity is most likely due to bacterial GHF 10 endoxylanases as these endoxylanases are not inactivated by the known inhibitors. Addition of an excess of endoxylanase inhibitors to an extract of the wholemeal of washed kernels caused a minimal (if any) decrease in the endoxylanase activity, which shows that the endoxylanases were mainly from plant origin. In comparison, addition of an excess of endoxylanase inhibitors to a wholemeal extract of untreated kernels reduced the endoxylanase activity by approximately 50%. The latter was rather unexpected because of the large concentration of inhibitors already present in wheat.

Reconstitution of Washed Kernels and Washing Liquid. To further substantiate the absence of in situ production of

Table 3. Apparent, Total, Endogenous, and Microbial Endoxylanase Activities for Three Different Wheat Varieties

wheat variety	endoxylanase activity (EU/g of kernel)				contribution to apparent endoxylanase activity (%)		contribution to total endoxylanase activity (%)	
	apparent	total	endogenous	microbial	endogenous	microbial	endogenous	microbial
Koch	1.70 ± 0.03	9.10 ± 0.59	0.267 ± 0.028	8.83 ± 0.56	16	84	3	97
Corvus	0.26 ± 0.01	1.13 ± 0.05	0.054 ± 0.002	1.08 ± 0.05	21	79	4	96
Decan	0.16 ± 0.02	0.46 ± 0.03	0.035 ± 0.001	0.42 ± 0.03	22	78	8	92

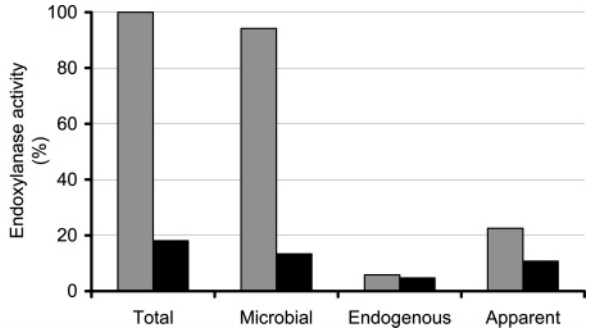

Figure 5. Total, microbial, endogenous, and apparent endoxylanase activities in extracts and washing water of Koch wheat in the absence (gray bars) and presence (black bars) of excess endoxylanase inhibitors expressed as a percent of the total endoxylanase activity.

endogenous or microbial endoxylanases during the washing procedure, reconstitution of the meal of washed kernels and the corresponding washing liquid was executed. This was done by making an extract of 2.0 g of the meal of washed kernels with 5.6 mL of the desalted washing liquid and 4.4 mL of sodium acetate buffer (25 mM, pH 5.0). These amounts give the same ratio as in the washing procedure and take into account the dilution of the washing liquid due to buffer exchange. The endoxylanase activity measured in the reconstituted sample was 1.8 EU/g of kernel, which is similar to the endoxylanase activity in an untreated wholemeal sample (1.7 EU/g of kernel). This again indicates that the high endoxylanase activity in the washing liquid was caused by microbial endoxylanases.

Assessment of the Presence of Endoxylanase Inhibitors in the Washing Liquid. To assess whether the endoxylanase activity defined above as total is not in itself an underestimation of the actual endoxylanase activity due to the presence of endoxylanase inhibitors in the washing liquids, apparent inhibition activities were measured in the washing liquids. TAXI was absent, and XIP was only present in very small amounts (3.6 ppm).

Application of the Washing Procedure. The developed washing procedure was also executed on two other wheat varieties (Corvus and Decan) with lower apparent endoxylanase activities. As can be seen in **Table 3**, the washing method was repeatable and showed a typical coefficient of variation of 6%. The total endoxylanase activities were always higher than the apparent activities, but the factor difference between apparent and total endoxylanase activities was larger when the endoxylanase activity in the sample was high. The contribution of the microbial endoxylanases to both the apparent and total endoxylanase activities was much higher than the contribution of endogenous endoxylanases. These results indicate that the current methods for the determination of endoxylanase activities can seriously underestimate the activity present in wheat as most microbial endoxylanases become inhibited by wheat endoxylanase inhibitors during extraction and hence escape the measurements.

Relevance of the Present Findings. Extensive washing of wheat kernels with UB pH 8.0 gave a nearly quantitative separation of the microbial endoxylanases on one hand from the endogenous endoxylanases and endoxylanase inhibitors on the other hand and hence allowed separate measurement of wheat endogenous and microbial endoxylanase activities (**Figure 4**). The potential endoxylanase activity in the whole kernel was very high and of the same order of magnitude as the endoxylanase activities commonly added to wheat flour in breadmaking. Indeed, in-house endoxylanase activity measurements on nine commercial endoxylanases containing bread improvers showed that the recommended dosages varied from 0.30 to 17.4 EU/g of flour. The microbial endoxylanase activity of wheat kernels was much higher than the endogenous endoxylanase activity. As current endoxylanase activity measurements do not take the majority of these microbial endoxylanases into account—only the bacterial GHF 10 endoxylanases remain largely uninhibited—it is obvious that this may have resulted in an underestimation of their importance in cereal processing. This is especially the case for wholemeal applications such as wholemeal breadmaking and animal feeds. Indeed, as it can, for example, be expected that no immediate association between endoxylanases and endoxylanase inhibitors occurs in relatively low moisture content processes such as breadmaking because of the limited mobility of molecules, wheat-kernel-associated endoxylanases could exert significant action prior to inactivation through complex formation with an inhibitor. Also in animal feed applications, complexation of endoxylanases with endoxylanase inhibitors probably does not occur immediately due to lack of mobility and the wheat-kernel-associated endoxylanases hence could have a large impact on the feed conversion. In these processes, total endoxylanase activities could therefore turn out to be more effective in helping to predict wheat functionality than apparent activities.

Besides these wholemeal applications, microbial endoxylanases can also be present in significant amounts in wheat flour due to contamination of flour with bran material during milling. Indeed, the results of Gys et al. (*8*) showed that the apparent endoxylanase activity in wheat flour could be reduced by more than 60% when wheat kernels were debranned before milling. This reduction in endoxylanase activity seriously affected the syruping behavior of the refrigerated doughs (*24*). Relatively small amounts of microbial endoxylanases in flour can hence have a large impact on the functionality of wheat flour in certain applications. Addition of TAXI-type endoxylanase inhibitors, which only inhibit GHF 11 endoxylanases and hence not endogenous endoxylanases, was shown to reduce syruping in refrigerated doughs (*25*).

In gluten−starch separation, high-extraction-rate wheat flours are often used. As they are likely to be strongly contaminated with microbial endoxylanases, it can be assumed that even in this high moisture content process, where the increased mobility of the components can be expected to accelerate the complexation of microbial endoxylanases and endoxylanase inhibitors, variations in initial endoxylanase activity could cause significant variations in the processability of wheats with seemingly similar characteristics, a problem which is as yet not accounted for. Indeed, it was recently shown that limited amounts of endoxyl-

anases can have a significant effect, for better or for worse, on the performance of wheat in gluten−starch separation (*3*).

In conclusion, a discrepancy exists between apparent endoxylanase activities and total-wheat-associated endoxylanase activities due to the presence of a majority of inhibition-sensitive microbial endoxylanases in wheat. In biotechnological processes these endoxylanases can be expected to be functional for a certain time before they become inactivated through complexation with endoxylanase inhibitors, thereby affecting the process.

ABBREVIATIONS USED

AX, arabinoxylan; AZCL-AX, azurine-cross-linked arabinoxylan; BSA, bovine serum albumin; cfu, colony forming unit; E_{590}, extinction at 590 nm; EU, enzyme unit; EX, endoxylanase; GHF, glycoside hydrolase family; OGYE, oxytetracycline glucose yeast extract; PCA, plate count agar; ppm, parts per million; TAXI, *Triticum aestivum* xylanase inhibitor; TL-XI, thaumatin-like xylanase inhibitor; TNBS, trinitrobenzenesulfonic acid; XIP, xylanase-inhibiting protein; UB, universal buffer.

ACKNOWLEDGMENT

We thank Prof. C. W. Michiels (Laboratory of Food Microbiology, Katholieke Universiteit Leuven) and his staff for their advice and technical assistance in the microbial experiments.

LITERATURE CITED

(1) Izydorczyk, M. S.; Biliaderis, C. G. Cereal arabinoxylans: advances in structure and physicochemical properties. *Carbohydr. Polym.* **1995**, *28*, 33−48.

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

(3) Frederix, S. A.; Courtin, C. M.; Delcour, J. A. Substrate selectivity and inhibitor sensitivity affect xylanase functionality in wheat flour gluten-starch separation. *J. Cereal Sci.* **2004**, *40*, 41−49.

(4) Bedford, M. R.; Schulze, H. Exogenous enzymes for pigs and poultry. *Nutr. Res. Rev.* **1998**, *11*, 91−114.

(5) Henrissat, B. A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.* **1991**, *280*, 309−316.

(6) Simpson, D. J.; Fincher, G. B.; Huang, A. H. C.; Cameron-Mills, V. Structure and function of cereal and related higher plant (1−4)-β-xylan endohydrolases. *J. Cereal Sci.* **2003**, *37*, 111−127.

(7) Caspers, M. P. M.; Lok, F.; Sinjorgo, K. M. C.; van Zeijl, M. J.; Nielsen, K. A. and Cameron-Mills, V. Synthesis, processing and export of cytoplasmic endo-β-1,4-xylanase from barley aleurone during germination. *Plant J.* **2001**, *26*, 191−204.

(8) Gys, W.; Gebruers, K.; Sorensen, J. F.; Courtin, C. M.; Delcour, J. A. Debranning of wheat prior to milling reduces xylanase but not xylanase inhibitor activities in wholemeal and flour. *J. Cereal Sci.* **2004**, *39*, 363−369.

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

(10) Schmitz, J. F.; McDonald, C. E.; Gilles, K. A.; Medcalf, D. G. Arabinoxylanases and cellulases of wheat. *Cereal Chem.* **1974**, *51*, 809−821.

(11) Bonnin, E.; Le Goff, A.; Saulnier, L.; Chauraud, M.; Thibault, J.-F. Preliminary characterisation of endogenous wheat arabinoxylan-degrading enzymic extracts. *J. Cereal Sci.* **1998**, *28*, 53−62.

(12) Debyser, W.; Delcour, J. A. Inhibitors of xylanolytic and β-glucanolytic enzymes. Patent Application WO 98/49278, 1998.

(13) McLauchlan, W. R.; Garcia-Conesa, M. T.; Williamson, G.; Roza, M.; Ravestein, P.; Maat, J. A novel class of protein from wheat which inhibits xylanases. *Biochem. J.* **1999**, *338*, 441−446.

(14) Fierens, E.; Gebruers, K.; Goesaert, H.; Brijs, K.; Courtin, C. M.; Delcour, J. A. TL-XI, a thaumatin-like xylanase inhibitor from wheat (Triticum aestivum). *4th European symposium on enzymes in grain processing (ESEGP-4)*; INRA: Nantes, France, 2005.

(15) Gebruers, K.; Brijs, K.; Courtin, C. M.; Fierens, K.; Goesaert, H.; Rabijns, A.; Raedschelders, G.; Robben, J.; Sansen, S.; Sorensen, J. F.; Van Campenhout, S.; Delcour, J. A. Properties of TAXI-type endoxylanase inhibitors. *BBA−Proteins Proteom.* **2004**, *1696*, 213−221.

(16) Flatman, R.; McLauchlan, W. R.; Juge, N.; Furniss, C.; Berrin, J.-G.; Hughes, R.; Manzanares, P.; Ladbury, J. E.; O'Brien, R.; Williamson, G. Interactions defining the specificity between fungal xylanases and the xylanase-inhibiting protein XIP-I from wheat. *Biochem. J.* **2002**, *365*, 773−781.

(17) Juge, N.; Payan, F.; Williamson, G. XIP-I, a xylanase inhibitor protein from wheat: a novel protein function. *BBA−Proteins Proteom.* **2004**, *1696*, 203−211.

(18) Gebruers, K.; Brijs, K.; Courtin, C. M.; Goesaert, H.; Proost, P.; Van Damme, J.; Delcour, J. A. Affinity chromatography with immobilised endoxylanases separates TAXI- and XIP-type endoxylanase inhibitors from wheat (Triticum aestivum L.). *J. Cereal Sci.* **2002**, *36*, 367−375.

(19) Rabie, C. J.; Lübben, A.; Marais, G. J.; Jansen van Vuuren, H. Enumeration of fungi in barley. *Int. J. Food Microbiol.* **1997**, *35*, 117−127.

(20) Gebruers, K.; Debyser, W.; Goesaert, H.; Proost, P.; Van Damme, J.; Delcour, J. A. Triticum aestivum L endoxylanase inhibitor (TAXI) consists of two inhibitors, TAXI I and TAXI II, with different specificities. *Biochem. J.* **2001**, *353*, 239−244.

(21) Bonnin, E.; Daviet, S.; Gebruers, K.; Delcour, J. A.; Goldson, A.; Juge, N.; Saulnier, L. Variation in the levels of the different xylanase inhibitors in grain and flour of 20 French wheat cultivars. *J. Cereal Sci.* **2005**, *41*, 375−379.

(22) Laca, A.; Mousia, Z.; Diaz, M.; Webb, C.; Pandiella, S. S. Distribution of microbial contamination within cereal grains. *J. Food Eng.* **2006**, *72*, 332−338.

(23) Corder, A. M.; Henry, R. J. Carbohydrate-degrading enzymes in germinating wheat. *Cereal Chem.* **1989**, *66*, 435−439.

(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*, 371−377.

(25) 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.

Received for review January 17, 2006. Revised manuscript received April 3, 2006. Accepted April 4, 2006. Financial support by the Instituut voor de aanmoediging van Innovatie door Wetenschap en Technologie in Vlaanderen (IWT; Brussels, Belgium) is gratefully acknowledged. 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 Katholieke Universiteit Leuven.

JF060129D