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Immunoblot Quantification of Three Classes of Proteinaceous Xylanase Inhibitors in Different Wheat (*Triticum aestivum*) Cultivars and Milling Fractions

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In wheat (*Triticum aestivum*) grains, TAXI- (*T. aestivum* xylanase inhibitor), XIP- (xylanase inhibiting protein), and TLXI-type (thaumatin-like xylanase inhibitor) xylanase inhibitors (XIs) are expressed in considerable levels and under different forms. As these proteins have a significant impact on microbial xylanases frequently used in cereal-based biotechnological processes, knowledge of their quantitative and qualitative variability in wheat is of great interest. This paper reports the successful use of immunoquantification by Western blotting to determine the intercultivar variation in the three structurally different classes of XIs, as well as their distribution among various industrial milling fractions. TAXI and XIP protein levels in eight wheat cultivars ranged from 81 to 190 ppm and from 156 to 371 ppm, with average values of 133 and 235 ppm, respectively. Using immunoblotting, TLXI protein levels could be measured directly for the first time. They ranged from 51 to 150 ppm and amounted to 112 ppm on average. The three classes of XIs were distributed among different wheat milling fractions in a similar way, with 4 and 10 times higher concentrations in the aleurone-enriched fraction than in white flour and pericarp fractions, respectively. Immunoblot patterns suggested that the observed intercultivar and spatial variabilities within the wheat grain are not due to the presence or absence of specific members of the large polymorphic XI families but to differences in the overall level and/or proportions of the specific members.

KEYWORDS: Immunoblot; wheat (*Triticum aestivum*); xylanase inhibitors

INTRODUCTION

Microbial endo-1,4-β-D-xylanases (EC 3.2.1.8, further referred to as xylanases), key enzymes in the degradation of arabinoxylan, are widely used as technical aids to improve cereal processing and/or product end quality, for example, in breadmaking (*1*) and gluten−starch separation (*2*). They are also added to some animal feeds (*3*). Due to differences in their substrate specificities, action patterns, and optimal working conditions, not all microbial xylanases are equally suited for a given cereal-based application (*1, 4, 5*). Moreover, their sensitivity to cereal proteinaceous inhibitors can highly reduce their efficacy (*4, 6, 7*).

So far, three structurally different cereal xylanase inhibitors (XIs) have been documented, that is, TAXI (*Triticum aestivum* xylanase inhibitor), XIP (xylanase inhibiting protein), and TLXI (thaumatin-like xylanase inhibitor). TAXI-type inhibitors are basic proteins occurring in two molecular forms. The first (form A) is made up of a single polypeptide chain of ∼40 kDa, whereas the second (form B) consists of two polypeptides of ∼30 and ∼10 kDa, held together by a single disulfide bridge (*8*). XIP- (*9*) and TLXI-type (*10*) XIs are single chain, glycosylated, high-p*I* proteins with molecular masses of ∼30 and ∼18 kDa, respectively. Whereas TAXI- and TLXI-type XIs are specifically active on microbial glycoside hydrolase (GH) family 11 xylanases (*10, 11*), XIP-type proteins, in general, can inhibit microbial xylanases of both GH families 10 and 11 (*12*). None of these inhibitors, though, shows activity toward plant xylanases, indicating that these highly abundant proteins (∼2.5% of the albumin/globulin fraction of wheat seed proteins) are probably involved in plant defense, rather than being produced as such for in planta regulatory purposes.

Variation in TAXI and XIP inhibitor levels in wheat has previously been studied using colorimetric activity measurements and conversion of XI activities with dose−response curves. TAXI and XIP levels in whole grains of different wheat cultivars range from 17 to 190 ppm and from 210 to 560 ppm, respectively (*13, 14*). However, activity-based methods suffer from interference of xylanases, inherently present in wheat milling fractions (*15*). In addition, determining inhibitor contents by means of an activity assay may well overlook the effect of

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a heterogeneous population of XI forms. Differences in xylanase inhibition activity and/or specificity within one family of XIs have already been demonstrated for, for example, TAXI-I and TAXI-II (*11*) and for XIP-I and XIP-R1 (*16*). Thus, the applied dose−response curves from a certain cultivar may not be representative for all wheat samples. Apart from that, it is relevant that the three types of XIs share the ability to bind to nonstarch polysaccharides and, in particular, to arabinoxylans (*17*), used as substrate in activity assays. The most stringent drawback of activity-based measurements for TLXI is the lack of an enzyme, which is solely inhibited by TLXI. Thus, so far, TLXI levels could not be assayed.

We, for the first time, embarked on a study determining TAXI, XIP, and TLXI levels in different wheat cultivars and revealing their spatial distribution in various milling fractions by means of immunoblotting and densitometric analysis. This method allows quantification of all types of XIs through the use of polyclonal antibodies (PAbs), specifically interacting with TAXI, XIP, or TLXI proteins (*18*, *19*). Furthermore, the abovementioned difficulties, inherent to activity assays, are mostly overcome. Interactions with xylanases or other interfering substances are ruled out under denaturing conditions, so that TAXI, XIP, and TLXI levels can be determined in a broad range of wheat samples. We here report on the outcome of this study.

MATERIALS AND METHODS

Materials. Wheat cultivars Glenlea, Lona, Klein-Estrella, Red-River, Martonvasari-17, Kirkpinar-79, Hereward, and Bilancia (harvest 2005), grown on the same field and under identical climatological and agronomical conditions, were obtained from Dr. Zoltan Bedo (Agricultural Research Institute of the Hungarian Academy of Sciences, Martonvasar, Hungary) and ground into wholemeal using a Perten SKCS 3100 laboratory mill (Huddinge, Sweden). Milling fractions of wheat cultivar Tiger (harvest 2005) were provided by Dr. Walter von Reding (Bühler AG, Uzwil, Switzerland). The following fractions, originating from different parts of the wheat caryopsis (*20*), were selected for analysis: peeling fraction, which represents the outermost part of the wheat kernel (consisting of 53.2% outer pericarp) and makes up 3.5% of the wheat kernel weight; pearling fraction, which is obtained by further abrading the wheat kernel after peeling (3.0% of wheat kernel weight) and is for the largest part made up of intermediate layers (21.4%, consisting of inner pericarp, testa, and nucellar tissue), and aleurone tissue (32.1%); and the remaining part of the grain, which is further divided into the residual bran fraction (22.4% of wheat kernel weight) and the flour fraction (71.1% of wheat kernel weight). The residual bran largely consists of aleurone (38.5%) and starchy endosperm (22.7%), whereas the flour fraction almost exclusively contains starchy endosperm cells. In addition, a fraction enriched in aleurone tissue consisting of about 80% aleurone cells was included.

Polyclonal antibodies, specifically interacting with TAXI-, XIP-, or TLXI-type XIs, were those described by Beaugrand and co-workers (*18*). Grindamyl H640 bakery enzyme, containing a *Bacillus subtilis* GH family 11 xylanase, and Biobake 710, containing an *Aspergillus niger* GH family 11 xylanase, were provided by Danisco (Brabrand, Denmark) and Quest International (Naarden, The Netherlands), respectively. *Penicillium purpurogenum* GH family 10 xylanase was kindly made available by Prof. Jaime Eyzaguirre (Laboratorio de Bioquimica, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Chile). Azurine cross-linked wheat Xylazyme AX tablets were from Megazyme (Bray, Ireland). One-dimensional electrophoretic equipment and the Trans-blot semidry transfer cell were from Bio-Rad (Hercules, CA), whereas 2D electrophoretic media were obtained from GE Healthcare (Uppsala, Sweden). Horseradish peroxidase-conjugated goat anti-rabbit IgG PAbs and reagents were purchased from Sigma-Aldrich (Bornem, Belgium) and were of analytical grade, unless specified otherwise.

Extraction of Wheat Seed Xylanase Inhibitors. Extracts of wheat kernel wholemeal and wheat milling fractions were prepared by suspending 2.0 g of material in 10.0 mL of sodium acetate buffer (25.0 mM, pH 5.0) and shaking on a horizontal shaker [150 strokes/min, 30 min, room temperature (20 ± 2 °C)]. The supernatant was recovered after centrifugation (10000*g*, 15 min, 7 °C).

Wheat extracts for 2D gel electrophoresis were prepared as described previously (*21*). Briefly, samples were crushed in liquid nitrogen, and 250 mg was suspended in 1.0 mL of Tris-HCl buffer (50.0 mM, pH 7.8). After centrifugation (14000*g*, 15 min, 4 °C), proteins (albumins/globulins) were precipitated from the supernatant with 4 volumes of 10% trichloroacetic acid (TCA) in acetone (overnight, −20 °C), and the resultant pellets were air-dried.

Purification of Three Types of XIs from Wheat. TAXI-, XIP-, and TLXI-type proteins were purified from wheat wholemeal (cv. Claire) as described earlier (*22*). In brief, a combination of cation-exchange chromatography, affinity chromatography with an immobilized *B. subtilis* xylanase to selectively bind TAXI-type proteins, and *A. niger* xylanase affinity chromatography to bind the two other classes of XIs, was used. The latter column was eluted consecutively at pH 10.0 and 12.0 to separate XIP- and TLXI-type proteins, respectively (*10*).

Quantification of Purified XIs. Concentrations of affinity-purified XIs were determined by high-performance anion exchange chromatography with integrated pulsed amperometric detection after hydrolysis of the protein samples for 24 h at 110 °C using hydrogen chloride (6.0 M). A Dionex BioLC system (Sunnyvale, CA) consisting of a GS50 gradient pump with online degasser, an AS50 autosampler with a thermal compartment, and an ED50 electrochemical detector equipped with a gold working electrode and a pH reference electrode was used (*23*). Separation was performed at 30 °C on an AminoPac PA10 guard column (50 × 2 mm) and analytical column (250 × 2 mm) at a flow rate of 0.25 mL/min. Eluents and gradient conditions were described by Lamberts et al. (*24*). An amino acid standard mix (Standard Reference Material 2389, National Institute for Standards and Technology, Gaithersburg, MD) was run in parallel. The chromatographic system control, data acquisition, and data analysis were performed using Chromeleon version 6.70 software (Dionex).

Immunoblotting. *Gel Electrophoresis.* Regular SDS-PAGE was performed on equal volumes of wheat extracts or defined quantities of affinity-purified XIs in 12.5% polyacrylamide gels (160 V; 65 min) using the Mini-PROTEAN-3 device (Bio-Rad) according to the method of Laemmli (*25*).

Two-dimensional gel electrophoresis for the separation of wheat grain albumins/globulins, extracted as described above, was performed as described previously (*21*). In brief, protein pellets (see above) were fully solubilized in 2D lysis buffer (2.0 M thiourea, 7.0 M urea, 4.0% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 20 mM dithiothreitol, 0.5% Immobiline pH gradient buffer pH 6−11, traces of bromophenol blue). Isoelectric focusing (∼30 kVh, 20 °C) was performed on Immobiline pH gradient strips in a p*I* range of 6−11 using the Ettan IPGphor II IEF unit (GE Healthcare) and was followed by SDS-PAGE on 15% homogeneous polyacrylamide gels (molecular mass range of 10−60 kDa) using the Ettan Daltsix vertical electrophoresis system (GE Healthcare) in conjunction with a Tris−glycine buffer system at 20 °C (*25*).

Transfer, Immunodetection, and Densitometric Analysis. One- or two-dimensional separated proteins were electroblotted (16 V, 40 min) onto an activated Protran nitrocellulose membrane (0.45 μm pore size, Schleicher and Schuell, Dassel, Germany). Overnight blocking of free binding sites was performed at 4 °C in a 1.0% casein solution prepared in phosphate-buffered saline (1.76 mM KH_2PO_4, 0.01 M Na_2HPO_4, 2.68 mM KCl, 0.137 M NaCl) at pH 7.4 containing 0.01% Tween (PBS−Tween). Membranes were further incubated with a 1:2000 dilution of the anti-TAXI, anti-XIP, or anti-TLXI primary PAbs in PBS−Tween (60 min, room temperature) (*18*). After six washes (5 min each) with PBS−Tween, membranes were incubated (60 min, room temperature) with secondary horseradish peroxidase-conjugated goat anti-rabbit PAbs (1:30000 dilution). Blots were washed again in PBS−Tween before they were developed with 3,3′,5,5′-tetramethylbenzidine substrate solution (30 min, room temperature).

The membranes were scanned using an ImageScanner and accompanying MagicScan 4.6 UMAX software (GE Healthcare). Den-

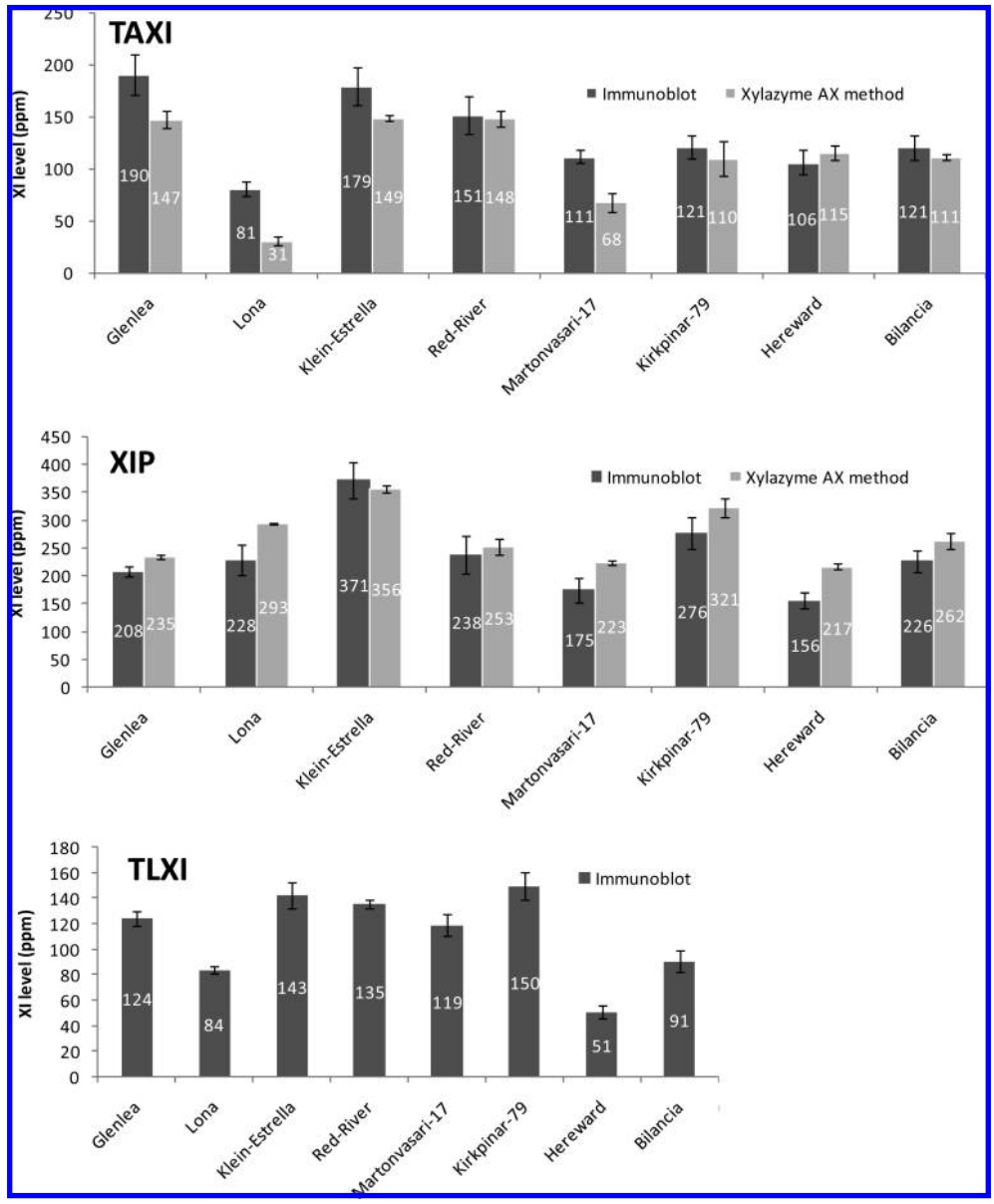

Figure 1. TAXI, XIP, and TLXI levels (ppm) in wholemeal of eight different wheat cultivars as determined by immunoblotting (dark gray bars) and comparison with TAXI and XIP levels (ppm), measured with the Xylazyme AX method (light gray bars). Error bars represent the standard deviations of XI levels measured in triplicate in wheat samples of independent extractions performed on separate days.

sitometric analysis of protein bands was performed using UN-SCAN-IT 5.1 software (Silk Scientific, Orem, UT). To convert densities of XI bands into inhibitor levels (ppm equivalents), standard curves of purified XIs (cv. Claire, see above) were analyzed in parallel with the samples. The coefficient of variation for the determination of XI levels by immunoblotting is typically 9%.

Determination of "Apparent" XI Activity. The XI activities of wheat extracts were determined colorimetrically with the Xylazyme AX method (Megazyme) described by Gebruers et al. (*11*). A GH family 11 *B. subtilis* xylanase and a GH family 10 *P. purpurogenum* xylanase were used to measure the levels of TAXI- and XIP-type proteins, respectively.

For conversion of XI activities (%) to inhibitor levels, dose−response curves with affinity-purified XIs (cv. Claire, see above) were created, allowing expression of the apparent XI levels in wheat in parts per million equivalents as described by Dornez et al. (*15*).

RESULTS

Intercultivar Variability of Three Types of XIs in Wheat Wholemeal. *Quantification of XIs Using Immunoblotting and Densitometric Analysis.* TAXI, XIP, and TLXI levels in wholemeal from eight wheat cultivars, grown at the same location and under similar climatological and agronomical conditions, were determined using densitometric analysis of Western blots, probed with anti-TAXI, anti-XIP, or anti-TLXI specific PAbs. TAXI, XIP, and TLXI levels in the eight wheat cultivars ranged from 81 to 190 ppm, from 156 to 371 ppm, and from 51 to 150 ppm and were, on average, 133, 235, and 112 ppm, respectively (**Figure 1**). Consistent with previous findings (*14*), no correlation was found between TAXI and XIP levels. Likewise, on the basis of these novel results, neither TAXI nor XIP levels are correlated with TLXI contents.

Overall, the immunoreactive bands were built up of several smaller bands with slightly differing molecular masses, representing different (iso)forms of each type of inhibitor. For XIP and TLXI, this effect was more obvious than for TAXI. The intensity of the separate bands on immunoblot varied among the eight cultivars as exemplified for four cultivars in **Figure 2**.

As SDS-PAGE was performed under reducing conditions, the cleaved form of TAXI-type XIs migrated as two bands of

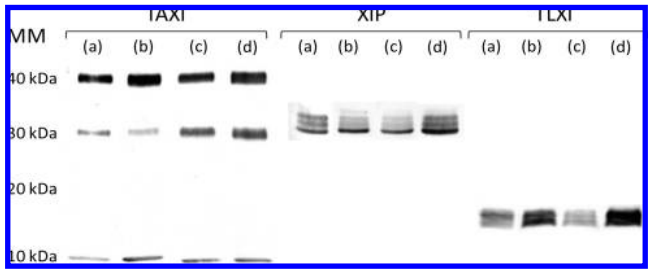

Figure 2. Western blots showing quantitative as well as qualitative differences in immunoreactive bands of TAXI- (40, 30, and 10 kDa bands), XIP-, and TLXI-type XIs in wheat cultivars: (lane a) Lona, (lane b) Martonvasari-17, (lane c) Hereward, and (lane d) Klein-Estrella. Molecular masses (MM) are indicated on the left side.

Table 1. Ratios of Immunoreactive Band Densities of the Nonprocessed Form of TAXI-Type XIs (40 kDa Polypeptides) over the Cleaved Form (30 + 10 kDa Polypeptides) for Wholemeal of Eight Different Wheat Cultivars

cultivar	TAXI 40 kDa form/30 + 10 kDa form
Glenlea	0.9
Lona	2.5
Klein-Estrella	0.9
Red-River	1.1
Martonvasari-17	2.1
Kirkpinar-79	1.1
Hereward	0.7
Bilancia	0.9
av	1.3

about 30 and 10 kDa and, hence, was separated from the unprocessed form (about 40 kDa). Unlike with the Xylazyme AX method, immunoblotting provides the opportunity to determine the extent to which the TAXI proteins are processed. Apparently, the ratio of the 40 kDa form of TAXI over the cleaved (30 + 10 kDa) form is cultivar-dependent and ranged from 0.7 to 2.5 (**Table 1**).

Quantification of XIs Using the Xylazyme AX Method. By way of comparison, the intercultivar variability of TAXI and XIP levels in the wheat samples was also determined using the Xylazyme AX method, which is based on the specific activity of the inhibitors against a *B. subtilis* and a *P. purpurogenum* xylanase, respectively (**Figure 1**). On average, TAXI levels were slightly higher, that is, 133 ppm, using immunoblotting, compared to 110 ppm, determined by inhibition activity measurements. In contrast, for XIP-type XIs, immunoblotting resulted in slightly lower levels with an average of 235 ppm for the eight cultivars tested, compared to 270 ppm using the Xylazyme AX method. Overall, a significant correlation ($R^2 = 0.73$; $P < 0.01$ for TAXI levels; $R^2 = 0.88$, $P < 0.01$ for XIP levels) was found between the methods, indicating that a strong relationship exists between the physical occurrence of XIs and their activity against the microbial xylanases used.

The cultivar-dependent variation in XIP levels (factor of 2) was comparable for the two applied methods. TAXI levels, however, varied by a factor of 5 based on the Xylazyme AX method, whereas only a 2-fold variation could be observed by immunoblotting. The observed discrepancy can largely be explained by the results for the cultivars Lona and Martonvasari-17. Conversion of XI activities for these two cultivars resulted in lower levels of TAXI-type XIs, that is, 31 and 68 ppm, respectively, than those obtained based on immunoblotting, that is, 81 and 111 ppm, respectively. These two cultivars showed a much higher ratio (2.1−2.5) of the unprocessed (40 kDa) over the processed (30 + 10 kDa) form (**Table 1**).

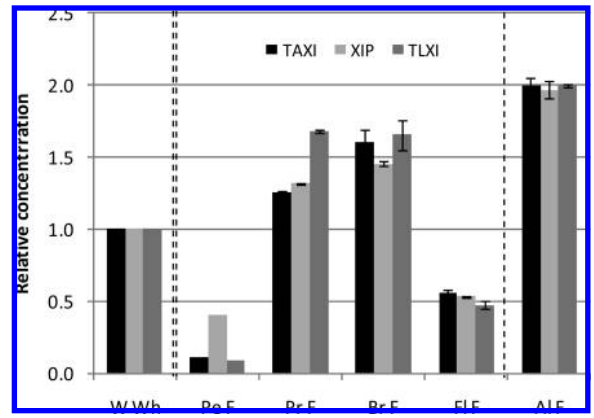

Figure 3. Concentrations of TAXI- (black bars), XIP- (light gray bars), and TLXI- (dark gray bars) type XIs in different wheat milling fractions (cv. Tiger), determined by immunoblotting and normalized on the basis of their concentrations in wheat wholemeal (W Wh; relative concentration = 1.0). The peeling fraction (Pe F), the pearling fraction (Pr F), and the residual bran (Br F) and flour (Fl F) fractions after pearling, together, make up the wholemeal sample. The aleurone-enriched fraction (Al F) is an individual fraction containing up to 80% aleurone cells.

Distribution of Three Classes of XIs over Different Wheat Grain Milling Fractions. Milling fractions (cv. Tiger) were examined for the presence of the three types of XIs. **Figure 3** represents the relative intensities of the inhibitor bands from the three classes of XIs in each milling fraction normalized to the signal of the wholemeal extract (cv. Tiger), obtained after probing with anti-TAXI-, anti-XIP-, or anti-TLXI-specific PAbs. These results provide a good indication of the tissue-specific concentration of each class of inhibitors.

Only weak immunoreactive signals were observed for extracts of either the peeling or the flour fractions. In contrast, in extracts of the residual bran and the pearling fractions, the concentrations of the three classes of XIs were 3 times higher than in the flour fraction. The highest XI concentrations were found for the aleurone-enriched fraction. On average, the relative intensities of the bands were about 4 times higher in this fraction than in the flour fraction, which consists almost exclusively of starchy endosperm. The ratios of the levels of the three types of XIs were very similar in the different milling fractions, indicating a similar distribution of these XIs in the wheat grain.

As observed above for the different cultivars, also for the wholemeal of cv. Tiger, immunoreactive bands extending over a small range of molecular masses were observed for each of the XIs, thus, representing different (iso)forms (**Figure 2**). Moreover, a similar variability in (iso)forms was discovered for all milling fractions derived from this cultivar (results not shown). With regard to the extent of processing of TAXI-type proteins, approximately equal amounts of both molecular forms were present in the different milling fractions (ratios of form A to form B varying from 1.0 to 1.2).

Taking into account the milling yields, the contribution of each fraction to the total population of TAXI-, XIP-, and TLXI-type XIs present in the wheat kernel (cv. Tiger) was evaluated (**Table 2**). The levels of XIs in the peeling fraction, representing the outer pericarp tissue, were almost negligible. Because of the high yield of the flour fraction, the inner part of the kernel, despite its low inhibitor concentrations, contained a large part of the total pool of XIs (49.4, 49.9, and 44.1% of TAXI, XIP, and TLXI, respectively). Next, comparable proportions of the TAXI (45.4%), XIP (43.1%), and TLXI (48.8%) populations were present in the residual bran fraction after pearling, which

Table 2. Proportions of TAXI-, XIP-, and TLXI-Type XIs on the Total Population of Each Type of Inhibitor in the Entire Wheat Kernel in Different Milling Fractions (Cv. Tiger), Determined by Immunoblotting

milling fraction	TAXI (%)	XIP (%)	TLXI (%)
peeling fraction	0.5	1.9	0.4
pearling fraction	4.7	5.2	6.6
bran fraction after pearling	45.4	43.1	48.8
flour fraction after pearling	49.4	49.9	44.1
aleurone-enriched fraction	17.6	18.2	18.4

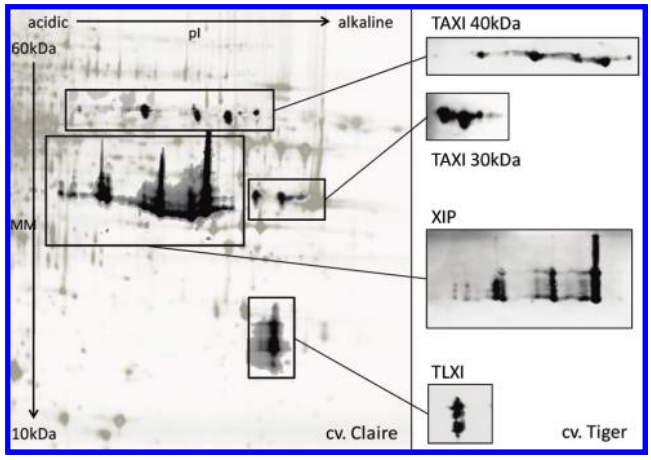

Figure 4. 2D immunoblot patterns (p*I* 6−11; 15% polyacrylamide gels) of TAXI- (40 and 30 kDa polypeptides), XIP-, and TLXI-type XIs in wholemeal of cv. Claire in comparison to those in the aleurone-enriched milling fraction of cv. Tiger.

includes a large part of the aleurone layer. Although the XI concentrations in the pearling fraction, largely consisting of intermediate layers and aleurone, were relatively high, this part represented only 4.7−6.6% of the entire XI population. Finally, the aleurone-enriched fraction, which represented only about 7% of the total weight of the caryopsis, contained a considerable portion of the total level of TAXI (17.6%), XIP (18.2%), and TLXI (18.4%).

Isoform Heterogeneity of Three Classes of XIs in Wheat Grain. To reveal whether the intercultivar or spatial variation in XI levels and activities could be (partially) ascribed to the presence or absence of specific (iso)forms, the use of anti-TAXI-, anti-XIP-, and anti-TLXI-specific PAbs was extended to probe 2D blots. The 2D pattern of wholemeal proteins from cv. Claire was compared with the 2D immunoblot of proteins extracted from the aleurone-enriched fraction from cv. Tiger, in which the three types of XIs are most highly abundant (**Figure 4**).

Overall, a high similarity of the 2D gel patterns was observed for TAXI-, XIP-, and TLXI-type XIs. The high multiplicity of (iso)forms was visible in both samples, and the spots were positioned at similar molecular masses and p*I* values. Nevertheless, differences in abundance of the various forms could be observed for the two cultivars/fractions.

DISCUSSION

The acquired results underscore the effectiveness of immunoblotting in combination with densitometric analysis in estimating XI levels and demonstrate the added value of this method. First, immunoblotting provides a means to measure TAXI, XIP, and TLXI protein levels in complex protein mixtures simultaneously, whereas the colorimetric method could only be used for TAXI and XIP quantification, due to the xylanase specificity of TLXI. Second, immunoblotting offers the opportunity to obtain information on the variability of the multiple XI (iso)forms. Indeed, wheat contains a heterogeneous population of XIs and previous studies revealed that different genetic variants as well as post-translational modifications (e.g., glycosylation) are responsible for this polymorphism (*21*). As the PAbs used in the immunoblot assay were raised against the native mixture of TAXI, XIP, or TLXI forms, which were affinity-purified from wheat wholemeal, it can be expected that all inhibitor forms are immunoreactive and, hence, included in the measurement. In fact, this was supported by the broad immunoreactive bands, visible after the blots had been probed with the PAbs. Third, xylanases or other substances in aqueous wheat extracts, affecting the Xylazyme AX method, cannot interfere in immunoblot quantification as non-covalent complex formation with XIs is ruled out in SDS-PAGE.

The observed intercultivar variability in TAXI and XIP levels (both 2-fold) by immunoblotting was in the range previously documented by Bonnin et al. (*13*) (70−200 and 210−560 ppm for TAXI and XIP, respectively). Whereas Dornez and coworkers (*14*) found a comparable variation in XIP levels (factor of 1.5), based on activity measurements, they reported an 8-fold difference in TAXI levels. However, this could be ascribed to one cultivar having an extremely low level of TAXI-type XIs. TAXI levels of the remaining cultivars differed by only a factor of 2. Dornez and colleagues (*26*, *27*) also demonstrated that the largest part of the variability in TAXI and XIP levels can be attributed to genotype, rather than to harvest year or growth environment. The TLXI contents in the eight cultivars analyzed here were on average 112 ppm and varied by a factor of 3. The levels of the three classes of XIs were not interrelated, which may point toward a possible individual role within the plant.

The analysis of different milling fractions showed that TAXI, XIP, and TLXI are distributed in a similar way throughout the wheat kernel. The three types of XIs were highly abundant in the envelope of the wheat caryopsis, with the exception of the outer pericarp. In particular, the highest concentrations of XIs were found in the aleurone layer. These findings are in agreement with Gebruers et al. (*28*), who found 2- and 3-fold higher XI activity levels in wheat bran and shorts, respectively, than in flour. Likewise, on the basis of the analysis of 59 industrial wheat roller mill streams, Dornez et al. (*29*) concluded that TAXI, and to a lesser extent XIP levels, are strongly correlated with bran-related parameters, such as ash and AX contents. High concentrations of the three classes of XIs in the outer wheat kernel layers are consistent with their presumed role in plant defense. Indeed, the timely inhibition of xylanases from phytopathogenic species through a barrier of well-located XIs may interfere with the invasion of these micro-organisms.

For TAXI-type proteins, the ratio of the unprocessed (40 kDa) over the processed form (30 + 10 kDa) was found to be cultivar-dependent. Approximately equal levels of both forms were detected for all cultivars, with the exception of two, for which 2.1 and 2.5 times more of the unprocessed 40 kDa polypeptides could be observed. It is remarkable that the immunoblot-determined TAXI levels were significantly higher for these two cultivars than the levels calculated from their activity against the *B. subtilis* xylanase. This observation suggests that proteolytic cleavage of the 40 kDa form of TAXI-type proteins may serve to enhance its activity against xylanases. Such a link between post-translational processing and activation of plant proteins has been previously reported (*30*, *31*). However, at this point, it is rather unlikely that processing indeed alters the activity or selectivity of the inhibitors in the wheat grain, because

both processed and unprocessed TAXI-I, recombinantly expressed in *Pichia pastoris*, inhibit GH family 11 *B. subtilis* and *A. niger* xylanases to the same extent (*32*). Alternatively, a lower activity of the two cultivars could be due to each cultivar having its own proportion of the different members of the TAXI population, as it has been shown that TAXI-I and TAXI-II proteins have different specificities toward xylanases (*11*).

A comparable qualitative variability in XI (iso)forms was observed for the different cultivars as well as for the various milling fractions. However, the intensity of the separate bands differed among the different samples. As a result, it can be assumed that quantitative intercultivar and spatial differences in XIs in wheat grains probably are not so much a result of the presence or absence of certain XI (iso)forms, but rather caused by variable expression levels of the XI family members. Further evidence for this came from 2D immunoblotting. The large heterogeneity of XI (iso)forms was comparable for the two samples tested (**Figure 4**). As XI levels are to a large degree affected by genotype (*26*), it can be assumed that expression of the various XI (iso)forms, too, is (in part) genetically determined.

From a wheat-processing point of view, a more profound insight in the intercultivar and spatial variability of the three types of XIs and their corresponding (iso)forms is of great interest, because the performance and efficiency of commercial microbial xylanases are highly influenced by the levels and the selectivity of the XIs. Moreover, fractions containing high concentrations of XIs with broad xylanase specificities can enhance the shelf life of refrigerated doughs, as a key quality determining factor called dough syruping is largely a result of arabinoxylan hydrolysis by microbial xylanases naturally occurring on wheat grains (*33*, *34*).

A recent development in cereal processing is the partial replacement of white flour by an isolated aleurone fraction in the formulas of breads, pasta, or other food products (*35*). As a result, the health benefits of whole wheat products, rich in dietary fiber and vitamins (*36*−*38*), are combined with properties such as a soft texture, a mild taste, and a light color, which are more appealing to consumers. Consequently, as this study indicated that 4 times higher XI concentrations are present in aleurone-enriched than in white flour fractions, it must be concluded that the use of aleurone-rich fractions for applications, in which inhibited xylanases are added, can significantly reduce their efficiency. Hence, higher xylanase dosages or inhibitor-insensitive xylanases are required to overcome this issue.

In summary, 1D and 2D immunoblotting enabled us to quantify the three types of XIs for the first time and to draw a comprehensive picture on the total XI population present in different wheat samples. As for TAXI and XIP levels, the level of TLXI-type XIs determined in wheat grains was substantial. The three types of XIs are similarly distributed throughout the wheat caryopsis. Moreover, TAXI, XIP, and TLXI proteins are highly concentrated in the aleurone layer, showing 4 times higher values compared to white flour. These findings are of relevance for cereal applications, in which (inhibitor-sensitive) xylanases, being added or naturally present, have an impact on wheat functionality.

ABBREVIATIONS USED

GH, glycoside hydrolase; PAbs, polyclonal antibodies; PBS, phosphate-buffered saline; TAXI, *Triticum aestivum* xylanase inhibitor; TLXI, thaumatin-like xylanase inhibitor; XI, xylanase inhibitor; XIP, xylanase inhibiting protein.

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