JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Indirect Enzyme–Antibody Sandwich Enzyme-Linked Immunosorbent Assay for Quantification of TAXI and XIP Type Xylanase Inhibitors in Wheat and Other Cereals

JOHNNY BEAUGRAND,[†] KURT GEBRUERS,*[,†] CEDRIC VERVERKEN,[‡] ELLEN FIERENS,[†] EMMIE DORNEZ,[†] BRUNO M. GODDEERIS,[‡] JAN A. DELCOUR,[†] AND CHRISTOPHE M. COURTIN[†]

Laboratory of Food Chemistry and Biochemistry and Laboratory of Livestock Physiology, Immunology and Genetics, Katholieke Universiteit Leuven, Kasteelpark Arenberg 30, B-3001 Leuven, Belgium

To quantify *Triticum aestivum* xylanase inhibitor (TAXI) and xylanase inhibiting protein (XIP) type proteins in cereals in general and wheat (*T. aestivum*) in particular, a robust enzyme-linked immunosorbent assay (ELISA) using an uncommon enzyme–antibody sandwich format was developed. *Bacillus subtilis* glycoside hydrolase family (GH) 11 and *Aspergillus oryzae* GH 10 xylanases were selected for coating ELISA plate wells to capture TAXI and XIP, respectively, prior to probing with antibodies. The detection threshold of the developed ELISA was much lower than that of the currently used xylanase inhibitor assay and the recently described Western blot approach. Because of its broad dynamic range (TAXI, 30–600 ng/mL, and XIP, 3–60 ng/mL), one proper standard extract dilution can be used for analyzing different wheat varieties, whereas for the currently used colorimetric assay, often different dilutions need to be analyzed. The TAXI ELISA for wheat was successfully adapted for barley (*Hordeum vulgare*) and could also be used for other cereals.

KEYWORDS: Cereals; wheat; xylanase; xylanase inhibitor; ELISA

INTRODUCTION

Endo-(1→4)-β-xylanases (EC 3.2.1.8), further referred to as xylanases, are key enzymes in the degradation of arabinoxylans, one of the most important groups of polysaccharides in cereal cell walls (*1*), and are frequently used in optimization of cereal-based food biotechnological processes and products (*2, 3*) and feed performance (*4*). Their technological importance mainly stems from the effect that these enzymes have on the water-holding capacity of water unextractable arabinoxylans and/or on the viscous nature of their water extractable counterparts (*2–4*). Xylanases are mainly classified in the glycoside hydrolase (GH) families 10 and 11 based on amino acid sequences and structural similarities (*5*). In addition to structural differences, xylanases belonging to these two families differ in substrate specificity (*6, 7*), substrate selectivity (*8, 9*), and interaction with cereal proteinaceous xylanase inhibitors (*10–13*). Xylanase inhibitors have a strong impact on the activity and thus functionality of xylanases, which are either added (*14–17*) or already present in cereal raw materials (*18*), and are implicated in plant defense (*11, 12, 19*). The quantitatively most important xylanase inhibitors in cereal grains are TAXI [*Triticum aestivum* xylanase inhibitor (*20*)] and XIP [xylanase inhibiting protein (*21*)] type inhibitors. TAXI proteins specifically inhibit microbial GH 11 xylanases and have a molecular mass (MM) of ca. 40 kDa. After reduction, part of the TAXI proteins yields two polypeptides of ca. 30 and 10 kDa. They have been identified and characterized in bread wheat (*T. aestivum*) (*22*), durum wheat (*Triticum durum*), barley (*Hordeum vulgare*), and rye (*Secale cereale*) (*23*). The structurally different XIP proteins have a MM of ca. 30 kDa and inhibit microbial GH 10 as well as GH 11 xylanases (*24, 25*). These proteins have been studied in the above-cited cereals and also rice (*Oryza sativa*) and maize (*Zea mays*) (*10, 26, 27*). Very recently, a third type of xylanase inhibitor belonging to the thaumatin protein family, i.e., TLXI (thaumatin-like xylanase inhibitor), was identified and characterized in wheat (*13, 28*). TLXI inhibits some microbial GH 11 xylanases (*13*). All xylanase inhibitors identified so far are inactive towards plant xylanases, which exclusively belong to GH 10 (*29*).

To date, xylanase inhibitor quantification has mainly been performed by enzyme inhibition assays using standard curves plotted with purified xylanase inhibitor (*30*). A recently developed Western blot analysis using specific polyclonal antibodies (PAbs) also proved to be a powerful tool in the

* To whom correspondence should be addressed. Tel: +32(0)16 32 16 34. Fax: +32(0)16 32 19 97. E-mail: kurt.gebruers@biw.kuleuven.be.
[†] Laboratory of Food Chemistry and Biochemistry.
[‡] Laboratory of Livestock Physiology.

10.1021/jf071087b CCC: $37.00 © 2007 American Chemical Society
Published on Web 08/24/2007

analysis of xylanase inhibitors (*28*). However, although Western blot analysis allows detection and quantification of low levels of the xylanase inhibitors in complex backgrounds, the technique is rather complex and does not allow high-throughput analysis. The present report for the first time describes a robust and sensitive quantitative enzyme-linked immunosorbent assay (ELISA), based on an uncommon indirect enzyme–antibody sandwich format. The reported method allows quantification of TAXI and XIP in wheat or wheat-derived fractions in particular and cereals in general. The assay permits full automation and high-throughput screening of raw materials for specific cereal applications. In plant science, the availability of this ELISA and the earlier described Western blot technique may facilitate the assessment of the physiological function of the xylanase inhibitors *in planta*. For plant breeders, ELISA allows rapid screening of cultivars.

MATERIALS AND METHODS

Materials. The cereals used in this study were from Aveve (Landen, Belgium), and the corresponding whole meals were obtained on a Cyclotec 1093 sample mill (Tecator, Hogänäs, Sweden). All reagents were purchased from Sigma-Aldrich (Bornem, Belgium) and were of analytical grade, unless specified otherwise. Chromatographic and electrophoresis media and markers were purchased from GE Healthcare (Uppsala, Sweden). *Trichoderma viride* GH 11 xylanase (NCBI accession number AJ012718), *Trichoderma longibrachiatum* GH 11 xylanase (pI 5.5) (CAA49294), *Aspergillus niger* GH 11 xylanase (DQ147775), and a xylanase from rumen microorganisms (no accession number available) were from Megazyme (Bray, Ireland). *Bacillus subtilis* GH 11 xylanase (ABH92520) was purified from Grindamyl H640 bakery enzyme preparation (Danisco, Brabrand, Denmark) as described by Gebruers et al. (*31*). *Aspergillus oryzae* GH 10 xylanase (BAA75475) was a kind gift from Prof. M. Tenkanen (University of Helsinki, Finland) and was purified on a Sephacryl S-100 gel permeation column (26 mm × 670 mm) with sodium acetate buffer (250 mM NaOAc, pH 5.0) as the eluent.

Sample Preparation. Whole meals (100 g) from wheat (cv. Ordéal), barley (cv. Majestic), rye (cv. Halor), oats (*Avena sativa* cv. Evita), and maize were extracted on a horizontal shaker (150 strokes/min) for 30 min at room temperature in sodium acetate buffer (25 mM, pH 5.0, 500 mL). The resulting extract was subsequently centrifuged (10000*g*, 10 min, 7 °C), and the supernatant was used for further analysis.

Purification of Xylanase Inhibitors. Wheat (cv. Soissons) TAXI and XIP and barley (cv. Majestic) *H. vulgare* xylanase inhibitor (HVXI) were purified from whole meal using cation exchange chromatography and *A. niger* and *B. subtilis* xylanase affinity chromatography as described earlier (*23, 31*). Affinity chromatography run-through material was sampled and stored at −20 °C for use as a negative control. In the purified xylanase inhibitor samples, protein concentrations were determined (cfr. infra) after buffer exchange on PD-10 columns in sodium acetate buffer (25 mM, pH 5.0). The solutions were then aliquoted and stored at −20 °C prior to the ELISA and Western blot experiments.

Protein Quantification. Protein quantification was performed colorimetrically in triplicate with the microscale Bradford (*32*) reagent from Sigma-Aldrich according to the manufacturer's instructions. Bovine serum albumin was used as a standard. Pure xylanase inhibitor protein concentrations were determined by measuring UV absorbance at 280 nm (UV cell path length of 1.0 cm) and using calculated specific absorbance values of 0.775 and 2.471 for 1.0 mg/mL TAXI and XIP, respectively.

Xylanase Inhibitor Polyclonal Antibody Production and Purification. Antixylanase inhibitor sera were produced in New Zealand white rabbits using the immunization protocol described by Beaugrand et al. (*28*). The specificity and titer of the final bleed antisera were analyzed by Western blotting and indirect ELISA, respectively. After polishing on a set of antigen affinity columns, PAbs with improved specificity towards TAXI and XIP were obtained (*28*). The polished PAbs were concentrated up to ca. 1.0 mg/mL with 5 kDa cutoff Vivaspin concentrators (Vivascience, Hannover, Germany). Aliquots thereof were stored at –20 °C.

ELISA Procedure. For the quantification of TAXI and XIP in cereal extracts, an indirect enzyme–antibody sandwich ELISA format was used. To specifically capture TAXI or XIP in the microtitre plate wells, the surface of multiwell Polystyrene Maxisorp Microtiter Plates (Nunc, Roskilde, Denmark) was coated with appropriate xylanases, which are specifically inhibited by one of the inhibitors, or with uninhibited xylanases (cfr. infra) by pipetting xylanase solutions (15 μg/mL in 30 mM sodium bicarbonate buffer of pH 9.6, 100 μL) in the wells and incubating the plates overnight at 4 °C. The plates were then washed three times with sodium phosphate buffer (10 mM, pH 7.4) containing 0.05% Tween 20, 100 mM sodium chloride, and 2 mM potassium chloride [further referred to as phosphate buffer saline (PBS)–Tween buffer] using a SkanWasher 300 (Skatron/Instrument AS, Norway). The remaining protein-binding sites in the wells were blocked with casein solution (1.0% in PBS, 300 μL) for 120 min at room temperature. The unbound proteins were washed away with PBS–Tween (cfr. supra). Cereal extracts were appropriately diluted in PBS–Tween (final volume of 100 μL) and then incubated in the wells for 60 min at room temperature allowing TAXI or XIP to bind on the xylanase coated on the well surface. The proteins not caught by the xylanase were washed away with PBS–Tween (cfr. supra). Rabbit anti-TAXI or -XIP PAbs (100 μL) were added and incubated for 60 min at 37 °C. After three washings with PBS–Tween, a solution of horseradish peroxidase (HRP)-conjugated goat antirabbit IgG antibodies (100 μL of 1/2000 diluted in PBS–Tween, Dako, Glostrup, Denmark) was added and incubated for 60 min at 37 °C. After the plates were washed again, ABTS [2,2′-azino-di-(3-ethylbenzthiazoline-6-sulfonate)] peroxidase substrate solution (100 μL, Kirkegaard-Perry, Gaithersburg, MD) was added. After 40 min of development time, the optical density (O.D.) at 405 nm was measured with a Multiscan ELISA Plate Reader (Titertek, Huntsville, AL). Standard curves were constructed by using pure inhibitor solutions instead of extract.

To assess the effectiveness of the TAXI and XIP capture step in the above-described ELISA, diluted wheat (cv. Ordéal) extracts were incubated in xylanase-coated wells (one complete ELISA plate for each xylanase inhibitor) and control wells (reference wells, completely blocked with casein, one complete plate) as described above. To check the binding of TAXI, the extract was diluted 50 times, while for XIP an 800-fold dilution was used. After incubation, the solutions were recovered from the wells, pooled, and concentrated with 5 kDa cutoff Vivaspin concentrators to ca. 0.5 mL and 50 μL, respectively. The concentrates were analysed by Western blot (cfr. infra).

Western Blot Analysis. For Western blot analysis, samples were handled as described earlier (*28*). Briefly, they were first separated by electrophoresis in 12% sodium dodecyl sulfate–polyacrylamide gels together with GE Healthcare MM standards. The separated proteins were electrotransferred onto activated nitrocellulose membranes (Protran Schleicher & Schuell, Dassel, Germany) using a Trans-Blot semidry electrophoretic transfer cell (Bio-Rad, Nazareth, Belgium). After an overnight blocking step of unoccupied binding sites on the blot with 1.0% casein solution in PBS, the membranes were incubated for 60 min with anti-TAXI PAbs (0.36 μg/cm^2) or anti-XIP PAbs (0.16 μg/cm^2) in PBS–Tween. After the membranes were washed in PBS–Tween, they were incubated with HRP-conjugated goat antirabbit IgG antibodies (Sigma-Aldrich). The membranes were washed in PBS–Tween, developed in TMB (3,3′,5,5′-tetramethylbenzidine, Sigma-Aldrich) substrate solution for the peroxidase, washed again, dried, and scanned.

RESULTS

Specificity of TAXI and XIP Antibodies towards Wheat Protein Samples. The specificity of the anti-TAXI and -XIP PAbs was evaluated by Western blot analysis. If the PAbs have absolute specificity towards their antigen, they can be used in a direct ELISA format or an indirect double antibody sandwich ELISA format for quantification of TAXI and XIP, respectively. However, when some cross-reacting proteins are observed, ELISA has to be performed with an alternative antigen-capture

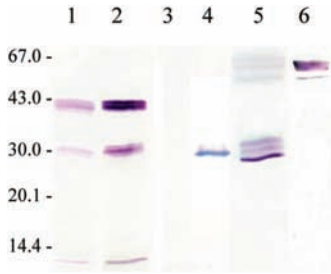

Figure 1. Western blot analysis of proteins extracted from wheat whole meal (cv. Ordéal) and the run-through fraction obtained after the xylanase affinity purification of TAXI and XIP. Lanes 1 and 2, wheat extract (5 and 35 μg of protein loaded, respectively) probed with anti-TAXI PAbs; lane 3, the run-through fraction (35 μg loaded) probed with anti-TAXI PAbs; lanes 4 and 5, wheat extract (5 and 35 μg loaded, respectively) probed with anti-XIP PAbs; and lane 6, the run-through fraction (35 μg loaded) probed with anti-XIP PAbs. The MM values based on the migration of GE Healthcare low molecular weight markers are indicated on the left side.

step, which, in the case of xylanase inhibitors, is possible through the use of specific xylanases.

Hence, wheat whole meal extract proteins (cv. Ordéal) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto activated nitrocellulose for immunodetection using the anti-TAXI (**Figure 1**, lanes 1 and 2) and -XIP PAbs (**Figure 1**, lanes 4 and 5). With the anti-TAXI PAbs, only the three TAXI polypeptides of ca. 40, 30, and 10 kDa were detected, even when as much as 35 μg of protein was loaded on gel (**Figure 1**, lanes 1 and 2, respectively). No signal could be detected at all when 35 μg of wheat protein from the run-through fraction obtained after the xylanase affinity purification of TAXI and XIP (cfr. supra) was analyzed (**Figure 1**, lane 3). The anti-XIP PAbs only revealed their target (band at ca. 30 kDa) with a 5 μg load of wheat whole meal extract protein (**Figure 1**, lane 4). When 35 μg was applied, three different XIP isoforms could easily be distinguished, probably due to different degrees of glycosylation. In addition to these XIP bands, bands appeared at ca. 60 kDa (**Figure 1**, lane 5). The XIP bands were absent in the affinity chromatography run-through fraction, whereas a high level of the cross-reacting proteins was present (**Figure 1**, lane 6). Higher quantities of wheat extract proteins were similarly analyzed on Western blot and probed with PAbs against TAXI or XIP. Similar results were obtained (data not shown).

In the case of XIP, the cross-reacting proteins did not allow the use of the direct ELISA approach or the indirect double antibody sandwich ELISA format, as these methods would overestimate the XIP levels. To overcome this problem, an indirect enzyme–antibody sandwich ELISA format was optimized. In this format, the xylanase inhibitors are selectively captured by xylanases coated on the surface of ELISA plate wells. In spite of the good specificity of the TAXI antibodies, this approach was also used for TAXI, because the enzyme–antibody format increases the robustness of the assay. After all, from earlier studies (*22*, *31*), one can derive that only active xylanase inhibitor proteins are captured by the enzymes and, hence, will be quantified by the optimized ELISA. Inactive homologues that might interfere in the direct ELISA approach or the indirect double antibody sandwich ELISA format may be present in cereals. Indeed, a number of genes coding for TAXI or XIP type proteins for which no inhibition activity has yet been demonstrated [unpublished data and (*29*)] have already been identified.

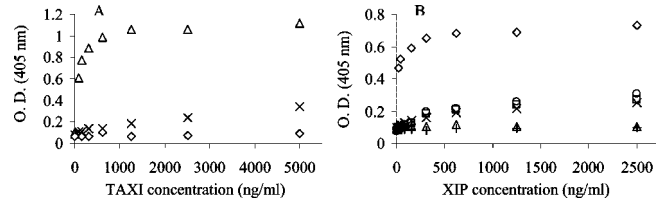

Figure 2. (**A**) TAXI and (**B**) XIP ELISA signals (O.D.) in the function of xylanase inhibitor concentration using xylanase–antibody sandwich formats. Different xylanases were coated on the ELISA plate well surface to capture the xylanase inhibitors: *B. subtilis* GH 11 xylanase (Δ); *T. longibrachiatum* GH 11 xylanase (□); *A. oryzae* GH 10 xylanase (◊); *T. viride* GH 11 xylanase (○); *A. niger* GH 11 xylanase (×); and xylanase from rumen microorganisms (+).

Optimization of the ELISA for Wheat TAXI and XIP Type Xylanase Inhibitors. *Screening of Different Xylanases.* On the basis of the available knowledge on TAXI and XIP enzyme specificity (*10–13*, *24*), several xylanases were screened for their ability to efficiently capture TAXI and XIP proteins. For TAXI ELISA, GH 11 xylanases of *B. substilis* and *A. niger* were tested. For XIP ELISA, a GH 10 xylanase from *A. oryzae*, GH 11 xylanases of *A. niger*, *T. viride*, and *T. longibrachiatum*, and a xylanase from rumen microorganisms were evaluated for their ability to bind the xylanase inhibitors. The GH 10 xylanase from *A. oryzae* and the GH 11 *B. subtilis* xylanase were screened as negative controls for TAXI and XIP capture, respectively. **Figure 2** shows TAXI and XIP ELISA signals using these enzymes and increasing dosages of the purified xylanase inhibitors. For TAXI, as expected, no significant signal was observed with the *A. oryzae* GH 10 xylanase, while the *A. niger* xylanase gave slowly increasing O.D. readings. With the *B. subtilis* xylanase, under the experimental conditions, a plateau was already reached at 800 ng/mL, at which the signal was ca. 3.5 times higher than that with the *A. niger* xylanase at 5000 ng/mL.

The xylanases used for capturing XIP proteins could be classified in three distinct categories based on the signals obtained in ELISA. A first category, comprised of the *B. subtilis* and rumen microorganism xylanases, gave no signal at all. A second one, comprised of the *T. longibrachiatum*, *A. niger*, and *T. viride* xylanases, gave a moderate signal. The curves were superimposable and gradually increased with increasing amounts of XIP protein. The *A. oryzae* xylanase representing the third category yielded very high signals and already reached a plateau at 250 ng/mL. The above results are in agreement with the xylanase specificities of both xylanase inhibitors (cfr. supra). These results demonstrate the feasibility of an enzyme–antibody sandwich ELISA setup and show that the *B. subtilis* and *A. oryzae* xylanases are the best enzymes to specifically capture TAXI and XIP, respectively.

Optimization of the Assay Conditions. After optimization of the enzyme–antibody sandwich ELISA with the *B. subtilis* and *A. oryzae* xylanases as xylanase inhibitor catchers, the best results were obtained when (i) the coating of the xylanases to the well surface was performed overnight in 30 mM sodium bicarbonate buffer, pH 9.6, at 4 °C, (ii) the concentration of the xylanase solution used for coating was ca. 15 μg/mL and 100 μL of this solution was applied per well, (iii) an equal volume of the xylanase inhibitors/extracts was incubated for 60 min at room temperature in the

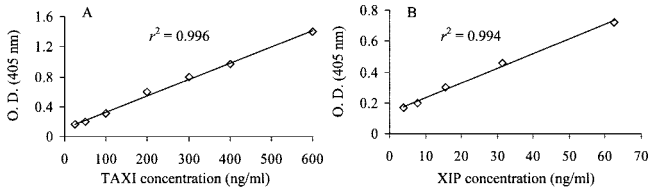

Figure 3. ELISA standard curves for (**A**) TAXI and (**B**) XIP. The presented regions correspond to the linear parts of the response curves obtained with *B. subtilis* GH 11 xylanase and *A. oryzae* GH 10 xylanase, respectively, which are presented in Figure 2.

Table 1. Performance Characteristics of the TAXI and XIP ELISA

	TAXI	XIP
typical linear range (ng/mL)	~30–600	~3–60
detection threshold (O.D.)[a]	0.096	0.131
maximal O.D. after 40 min of development	0.7–1.4	0.6–1.2
average correlation coefficient (r^2) of 10 standard curves	0.99	0.99
intra-assay CV (%, $n = 33$)	6.6	7.2
interassay CV (%, $n = 142$, 5 different days)	11.8	12.6
linear dilution range of wheat whole meal extract	10–100	200–2000

[a] The detection threshold is calculated as the average O.D. measured with the run-through fraction (obtained after the xylanase affinity purification of TAXI and XIP) plus three standard deviations when essayed 43 times in three independent tests.

xylanase-coated wells, and (iv) the incubation of the xylanase inhibitors/extracts was done in PBS–Tween buffer. The washing steps, the blocking step with casein, the binding of primary and secondary antibodies, and the incubation with peroxidase substrate were performed under standard conditions. In contrast to what could be expected based on the optimal inhibition conditions of TAXI and XIP (*20, 24*), the use of 25 mM acetate buffer, pH 5.0, instead of PBS–Tween buffer, pH 7.4, for incubation of the xylanase inhibitors/extracts in the wells did not yield significantly higher signals in the ELISA (data not shown).

Under the above-described experimental conditions, the ELISA responses to pure TAXI and XIP proteins increased linearly in a range of 30–600 ng/mL and 3–60 ng/mL, respectively (**Figure 3** and **Table 1**). The lower limits of these ranges correspond to the minimum xylanase inhibitor concentrations needed to exceed the detection threshold. This threshold is equal to the average O.D. reading obtained when ELISA is performed on "wheat extracts", which do not contain TAXI and XIP [run-through fraction material obtained after xylanase affinity purification of xylanase inhibitors (cfr. supra): 800 μg/mL for TAXI ELISA and 100 μg/mL for XIP ELISA] plus three times the standard deviation that was obtained when the assay was done 43 times divided over three independent runs. The upper limits of the above linear ranges correspond to the xylanase inhibitor concentrations at the end of the linear part of the curve, thus before the plateau O.D. (**Table 1**). The TAXI and XIP standard curves obtained in the ELISA are of good quality as demonstrated by the high average correlation coefficients (**Table 1**).

To assess the right dilution factor for quantifying TAXI and XIP proteins in crude wheat extracts, the ELISA was performed on various extract dilutions. The sigmoidal curves obtained for the wheat cultivar Ordéal each contained a linear part, that is, between 10- and 100-fold dilutions for TAXI and between 200- and 2000-fold dilutions for XIP (**Figure 4** and **Table 1**). For proper quantification, the extract dilution factors should be

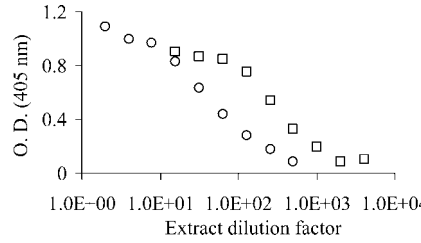

Figure 4. TAXI (○) and XIP (□) ELISA signals (O.D.) in the function of the logarithmic value of the wheat whole meal extract dilution factor. TAXI and XIP were captured on the well surface by *B. subtilis* GH 11 xylanase and *A. oryzae* GH 10 xylanase, respectively.

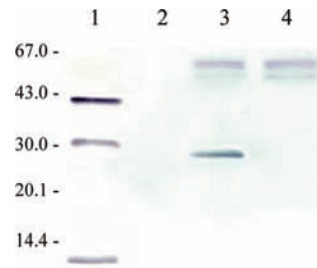

Figure 5. Western blot analysis of diluted wheat whole meal extracts (25 μg of protein) after incubation in xylanase-coated and -noncoated ELISA plate wells. Lane 1, extract incubated in noncoated wells and probed with anti-TAXI PAbs; lane 2, extract incubated in *B. subtilis* xylanase-coated wells and probed with anti-TAXI PAbs; lane 3, extract incubated in noncoated wells and probed with anti-XIP PAbs; and lane 4, extract incubated in *A. oryzae* xylanase-coated wells and probed with anti-XIP PAbs. The MM values based on the migration of GE Healthcare low molecular weight markers are indicated on the left side.

within these ranges as the calculated amounts of TAXI and XIP are then independent of the dilution used.

To determine whether all of the xylanase inhibitors present in the diluted samples are captured by the xylanases coated on the well surface, samples were recovered from the wells after incubation, concentrated, and analyzed by Western blot (30 μg of protein loaded) (**Figure 5**, lanes 2 and 4), which was previously shown to have a detection limit of ca. 20 ng of xylanase inhibitor (*28*). As a control, the same samples were incubated in wells without xylanases (surface completely casein blocked), recovered, concentrated, and analyzed (**Figure 5**, lanes 1 and 3). The results clearly showed that TAXI and XIP are quantitatively captured by the *B. substilis* and *A. oryzae* xylanases, respectively, as no xylanase inhibitors could be detected in the recovered samples. The control samples still contained the TAXI and XIP proteins. The cross-reacting proteins detected with the anti-XIP PAbs were present in comparable levels in the control and xylanase-incubated samples. This demonstrates that the XIP cross-reacting protein does not interfere in the present ELISA format.

Using the above-described optimized ELISA, the concentrations of TAXI and XIP were determined in wheat whole meal sample (cv. Ordéal). The measurements were performed on two different days and each time in triplicate and yielded 110 ± 13 (2.75 ± 0.33 nmol/g) and 224 ± 26 ppm (7.47 ± 0.87 nmol/g) for TAXI and XIP, respectively.

Statistical Evaluation of the Optimized Assay. The intra-assay coefficient of variation (CV) of the ELISA was ca. 7% for TAXI as well as XIP, based on 33 replicates. The interassay CV, calculated from five independent ELISA plates, was ca. 12% (**Table 1**). Both CVs are very acceptable (*33*)

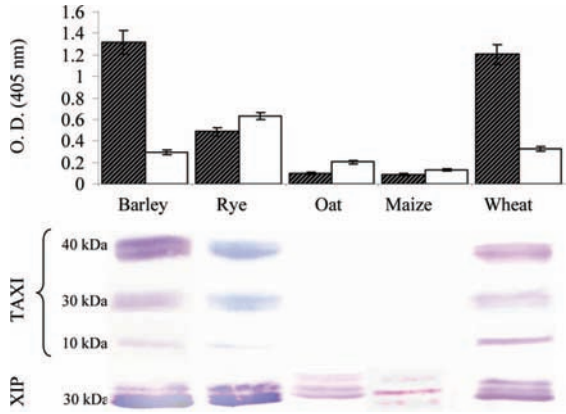

Figure 6. Western blot of whole meal extracts from barley (90 μg of protein loaded), rye (90 μg of protein), oat (40 μg of protein), maize (65 μg of protein), and wheat (30 μg of protein) after probing with anti-TAXI and -XIP PABS. The identities and MM of the bands are presented on the left side. The histogram presents the TAXI and XIP ELISA signals (O.D.) using *B. subtilis* and *A. oryzae* xylanases, respectively, as xylanase inhibitor catchers. Wheat extract was diluted 20 times, and all other cereal extracts were diluted 10 times for TAXI measurements (dark bars). For XIP measurements (light bars), a dilution factor of 800 was used for barley, rye, and wheat extracts; one of 150 was used for oat, and one of 50 times was used for maize. The error bars represent the standard deviation from three replicates.

and comparable to those of some commercialized ELISA kits (e.g., the IkBα ELISA kit, Sigma-Aldrich, St. Louis, MO) and other quantification techniques (cfr. infra).

Application of the ELISA for Other Cereals than Wheat. The Western blot results presented in **Figure 6** show that the anti-TAXI and anti-XIP PAbs recognize their target homologues in other cereals. In line with earlier studies, TAXI homologues were detected in barley (referred to as HVXI) and rye [referred to as *S. cereale* xylanase inhibitor (SCXI)] but not in oat and maize (*10, 23*). XIP homologues appeared on blot for all four cereals as three bands at ca. 30 kDa, but for oat and maize, the overall intensities were low, in line with their very low XIP contents mentioned elsewhere (*10, 26*). Because the specificities of the TAXI and XIP homologues from the nonwheat cereals towards different xylanases are very similar to the specificities of the wheat xylanase inhibitors (*10*), also for these cereals, the above-used enzyme–antibody ELISA setup should give a good signal over noise ratio.

As for TAXI in wheat (20-fold diluted whole meal extract), the ELISA on barley and rye (10-fold diluted whole meal extracts) gave very clear HVXI and SCXI signals, respectively, whereas the signals for oat and maize (10-fold diluted whole meal extracts) did not exceed the threshold O.D. of the method (**Figure 6**). Much as for wheat, pronounced XIP ELISA signals were observed for barley and rye with 800-fold diluted extracts. Also significant but very low signals were measured for oat (150-fold diluted extract) and maize (50-fold diluted extract) (**Figure 6**). The latter signal was very close to the ELISA threshold O.D. (**Table 1**). These ELISA results are in line with the observations on Western blot (cfr. supra).

The standard curve determined for barley with purified HVXI was linear between 5 and 80 ng/mL (**Figure 7**) and was unexpectedly much steeper than the TAXI curve from wheat (**Figure 3**). This indicated that to use the ELISA for the determination of xylanase inhibitor levels in a nonwheat cereal, standard curves have to be constructed using the xylanase inhibitor homologues of the cereal under consideration. After

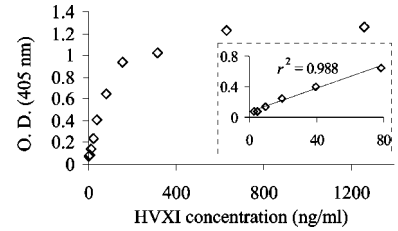

Figure 7. ELISA standard curve for HVXI from barley. The frame inside the chart zooms in on the linear part of the curve.

all, the affinity of these homologues towards the xylanase coated on the surface of the ELISA plate wells and the affinity of the PAbs towards these homologues can be different. ELISA HVXI quantification performed with four replicates on 50-fold diluted barley whole meal extract (cv. Majestic) gave 21 ± 2 ppm.

DISCUSSION

This report for the first time describes the use of an indirect enzyme–antibody sandwich format for the ELISA quantification of TAXI and XIP type xylanase inhibitors in cereals. Because of the cross-reactivity of the anti-XIP PAbs, this format was chosen over the classical and frequently used direct and indirect double antibody sandwich approaches to avoid overestimation of XIP levels. To ensure robustness of the assay, a similar setup was used in the TAXI ELISA, although, based on the specificity of anti-TAXI PAbs in Western blot, here the classical direct and indirect approaches could be used. In this context, it is important to note that, for TAXI and XIP, several homologues have been identified in cereals at the genetic level, for which to date no inhibition activity has been demonstrated (unpublished data and *29*). For both TAXI and XIP ELISA, a xylanase with very high specificity towards the xylanase inhibitors was coated on the ELISA plate well surface to capture the inhibitors. Analysis of the uncaptured proteins by Western blot validated the effectiveness of this step under optimized conditions, in line with the tight binding properties of the inhibitors in the presence of the enzymes used (*24, 34, 35*). In the case of XIP, the high MM cross-reacting noninhibitor proteins were recovered in the unbound fraction, demonstrating the specificity of the ELISA. Because of the high molecular mass of these proteins, they cannot be precursors or degradation products of XIP. After all, XIP is expressed as a 30.2 kDa protein with a 30 amino acid signal sequence (ca. 3 kDa). Because the cross-reacting proteins are present in the final run-through fraction obtained during the affinity purification of XIP and the anti-XIP PAbs were purified by XIP-affinity chromatography, the cross-reaction cannot be explained by impurity of the sample used for immunization. Therefore, these proteins must have at least one epitope similar to one in XIP.

The ELISA method described here is more sensitive than the currently used Xylazyme-AX colorimetric xylanase inhibitor assay based on the measurement of residual xylanase activity in the presence of xylanase inhibitor (*18, 30*). Standard curves constructed for the latter assay with *B. subtilis* xylanase and TAXI and with *Penicillium purpurogenum* xylanase and XIP show that ca. 540 ng/mL TAXI and ca. 200 ng/mL XIP, respectively, are needed to yield 20% inhibition, which is the lower limit of degree of inhibition to ascertain accurate quantification. Below this percentage of inhibition, relatively high experimental errors are observed. Because the standard curves are typically linear up to 80%, the upper limit of the dynamic range is reached at 2160 and 800 ng/mL, respectively. Keeping in mind the 12- and 2.7-fold variation in TAXI and

XIP levels, respectively, observed in wheat grains (*30*, *36*), this range is relatively narrow and sometimes necessitates the analysis of a number of extract dilutions when a batch of wheat samples is analyzed. In contrast, for the present ELISA, there is a 20-fold difference between the lower and the upper limit of the TAXI and XIP dynamic ranges, allowing one to use one standard dilution. In the case of wheat whole meal extracts, respectively, 100- and 2000-fold dilutions could be used. Much as the colorimetric assay (*18*, *30*), the new ELISA yields apparent xylanase inhibitor levels, because, during extraction of wheat flour or whole meal, part of the xylanase inhibitors may be lost by complexation with xylanases synthesized by the microbial flora on the grain surface. However, recent studies demonstrated that the total xylanase activity in wheat grains is negligible when compared to the total xylanase inhibitor activity, and thereby, the xylanases do not have a significant impact on the level of xylanase inhibitor that can be measured (*18*). In line with this, no xylanase inhibitors were detected in the unbound protein fraction after incubation in the xylanase-coated ELISA plate wells. In contrast, for xylanases in wheat grains, large differences have been observed between apparent and real activities due to inhibition.

The inter- and intra-assay variation of the TAXI and XIP ELISA is in the range of magnitude of results of the cited colorimetric assay (*18*, *30*), some quantitative sandwich ELISAs of plant antigens (*37*–*39*), and commercialized ELISA kits. For the colorimetric assay, an intra-assay CV of ca. 6% has been described by Dornez et al. (*18*). The inter-assay CV is ca. 12% (unpublished data). The TAXI and XIP contents measured in the Ordéal whole meal sample using ELISA, that is, 110 and 224 ppm, respectively (cfr. supra), are in the range of values obtained colorimetrically by others (*30*, *36*), reporting TAXI and XIP levels of 17–200 and 210–560 ppm, respectively.

The ELISA method was much more sensitive than the quantification method based on Western blot. With the Western blot technique, dynamique ranges of 375–3125 and of 775–6250 ng/mL for TAXI and XIP, respectively, were determined, each with ca. 10-fold difference between the extremes. This difference is less than with ELISA but still more than with the colorimetric assay. Calculated from data described by Beaugrand et al. (*28*), the intra-assay CV of the immunoblot method is ca. 8%, which again is in the same order of magnitude as for the ELISA.

The ELISA can be applied for the quantification of TAXI and XIP homologues in other cereals than wheat, provided that standard curves are constructed with the corresponding xylanase inhibitor homologues from those cereals. For these xylanase inhibitors, affinity-based purification methods are available (*23*, *26*). The ELISA-based HVXI level in a barley whole meal sample (cv. Majestic) was 21 ppm, which is 5.2 times lower than the TAXI level in the Ordéal whole meal. This is in line with earlier reports on HVXI levels in barley, in which a factor 10 difference is described between one wheat and one barley variety (*10*, *23*).

As described elsewhere (*22*, *40*, *41*), wheat contains TAXI-I type and TAXI-II type proteins based on their specificity towards xylanases. The *B. subtilis* GH 11 xylanase is inhibited by both types and in ELISA will yield the sum of the TAXI-I and TAXI-II protein levels. The *A. niger* GH 11 xylanase is inhibited by TAXI-I type and XIP proteins and could be used in combination with anti-TAXI PAbs to specifically quantify TAXI-I proteins. However, the ELISA signal obtained with the *A. niger* enzyme was rather low.

ABBREVIATIONS USED

ELISA, enzyme-linked immunosorbent assay; GH, glycoside hydrolase family; HVXI, *Hordeum vulgare* xylanase inhibitor; O.D., optical density; PAbs, polyclonal antibodies; PBS, phosphate buffer saline; SCXI, *Secale cereale* xylanase inhibitor; TAXI, *Triticum aestivum* xylanase inhibitor; TLXI, thaumatin-like xylanase inhibitor; XIP, xylanase inhibiting protein; xylanase, endo-(1→4)-β-xylanase.

ACKNOWLEDGMENT

We thank Dr. H. Goesaert of our group for providing pure HVXI.

LITERATURE CITED

(1) Biely, P. Microbial xylanolytic systems. *Trends Biotechnol.* **1985**, *3*, 286–290.
(2) Courtin, C. M.; Delcour, J. A. Arabinoxylans and ndoxylanases in wheat flour bread-making. *J. Cereal Sci.* **2002**, *35*, 225–243.
(3) Christophersen, C.; Andersen, E.; Jacobsen, T. S.; Wagner, P. Xylanases in wheat separation. *Starch/Stärke* **1997**, *1*, 5–12.
(4) Bedford, M. R.; Schulze, H. Exogenous enzymes for pigs and poultry. *Nutr. Res. Rev.* **1998**, *11*, 91–114.
(5) Bourne, Y.; Henrissat, B. Glycoside hydrolases and glycosyltransferases: families and functional modules. *Curr. Opin. Struct. Biol.* **2001**, *11*, 593–600.
(6) Beaugrand, J.; Chambat, G.; Wong, V. W. K.; Goubet, F.; Rémond, C.; Paës, G.; Benamrouche, S.; Debeire, P.; O'Donohue, M.; Chabbert, B. Impact and efficiency of GH 10 and GH 11 thermostable endoxylanases on wheat bran and alkali-extractable arabinoxylans. *Carbohydr. Res.* **2004**, *339*, 2529–2540.
(7) Jeffries, T. W. Biochemistry and genetics of microbial xylanases. *Curr. Opin. Biotechnol.* **1996**, *7*, 337–342.
(8) Frederix, S. A.; Courtin, C. M.; Delcour, J. A. Impact of xylanases with different substrate selectivity on gluten-starch separation of wheat flour. *J. Agric. Food Chem.* **2003**, *51*, 7338–7345.
(9) Moers, K.; Celus, I.; Brijs, K.; Courtin, C. M.; Delcour, J. A. Endoxylanase substrate selectivity determines degradation of wheat water-extractable and water-unextractable arabinoxylan. *Carbohydr. Res.* **2005**, *340*, 1319–1327.
(10) Goesaert, H.; Elliott, G.; Kroon, P. A.; Gebruers, K.; Courtin, C. M.; Robben, J.; Delcour, J. A.; Juge, N. Occurrence of proteinaceous endoxylanase inhibitors in cereals. *Biochim. Biophys. Acta* **2004**, *1696*, 193–202.
(11) Juge, N. Plant protein inhibitors of cell wall degrading enzymes. *Trends Plant Sci.* **2006**, *11*, 359–367.
(12) Beliën, T.; Van Campenhout, S.; Robben, J.; Volckaert, G. Microbial endoxylanases: effective weapons to breach the plant cell-wall barrier or, rather, triggers of plant defense systems? *Mol. Plant–Microbe Interact.* **2006**, *19*, 1072–1081.
(13) Fierens, E.; Rombouts, S.; Gebruers, K.; Goesaert, H.; Brijs, K.; Beaugrand, J.; Volckaert, G.; Van Campenhout, S.; Proost, P.; Courtin, C. M.; Delcour, J. A. TLXI, a novel type of xylanase inhibitor from wheat (*Triticum aestivum*) belonging to the thaumatin family. *Biochem. J.* **2007**, *403*, 583–591.
(14) 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.
(15) Trogh, I.; Courtin, C. M.; Andersson, A. A. M.; Åman, P.; Sørensen, J. F.; Delcour, J. A. The combined use of hull-less barley flour and xylanase as a strategy for wheat/hull-less barley flour breads with increased arabinoxylan and (1→3,1→4)-β-glucan levels. *J. Cereal Sci.* **2004**, *40*, 257–267.
(16) Sørensen, J. F.; Kragh, K. M.; Sibbesen, O.; Delcourv, J.; Goesaert, H.; Svensson, B.; Tahir, T. A.; Brufau, J.; Perez-Vendrell, A. M.; Bellincampi, D.; D'Ovidio, R.; Camardella, L.; Giovane, A.; Bonnin, E.; Juge, N. Potential role of glycosidase inhibitors in industrial biotechnological applications. *Biochim. Biophys. Acta* **2004**, *1696*, 275–287.
(17) 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*, 1604–1609.

(18) 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 endogeneous endoxylanases. *J. Agric. Food Chem* **2006**, *54*, 4028–4034.

(19) Bellincampi, D.; Camardella, L.; Delcour, J. A.; Desseaux, V.; D'Ovidio, R.; Durand, A.; Elliot, G.; Gebruers, K.; Giovane, A.; Juge, N.; Sørensen, J. F.; Svensson, B.; Vairo, D. Potential physiological role of plant glycosidase inhibitors. *Biochim. Biophys. Acta* **2004**, *1696*, 265–274.

(20) Gebruers, K.; Brijs, K.; Courtin, C. M.; Fierens, K.; Goesaert, H.; Rabijns, A.; Raedschelders, G.; Robben, J.; Sansen, S.; Sørensen, J. F.; Van Campenhout, S.; Delcour, J. A. Properties of TAXI-type endoxylanase inhibitors. *Biochim. Biophys. Acta* **2004**, *1696*, 213–221.

(21) Juge, N.; Payan, F.; Williamson, G. XIP-I, a xylanase inhibitor protein from wheat: a novel function. *Biochim. Biophys. Acta* **2004**, *1696*, 203–211.

(22) Gebruers, K.; Goesaert, H.; Brijs, K.; Courtin, C. M.; Delcour, J. A. Purification of TAXI-like endoxylanase inhibitors from wheat (*Triticum aestivum* L.) whole meal reveals a family of isoforms. *J. Enzyme Inhib. Med. Chem.* **2002**, *17*, 61–68.

(23) Goesaert, H.; Gebruers, K.; Brijs, K.; Courtin, C. M.; Delcour, J. A. "TAXI"-type endoxylanase inhibitors in different cereals. *J. Agric. Food Chem.* **2003**, *51*, 3770–3775.

(24) Flatman, R.; McLauchlan, W. R.; Juge, N.; Furniss, C. S.; Berrin, J. G.; Hughes, R. K.; Manzanares, P.; Ladbury, J. E.; O'Brien, R.; Williamson, G. Interactions defining the specificity between fungal xylanases and the wheat proteinaceaous inhibitor, XIP-I. *Biochem. J.* **2002**, *365*, 773–781.

(25) Payan, F.; Leone, P.; Porciero, S.; Furniss, C.; Tahir, T.; Williamson, G.; Durand, A.; Manzanares, P.; Gilbert, H. J.; Juge, N.; Roussel, A. The dual nature of the wheat xylanase protein inhibitor XIP-I: Structural basis for the inhibition of Family 10 and 11 xylanases. *J. Biol. Chem.* **2004**, *279*, 36029–36037.

(26) Goesaert, H.; Gebruers, K.; Brijs, K.; Courtin, C. M.; Delcour, J. A. "XIP"-type endoxylanase inhibitors in different cereals. *J. Cereal Sci.* **2003**, *38*, 317–324.

(27) Durand, A.; Hughes, R.; Roussel, A.; Flatman, R.; Henrissat, B.; Juge, N. Emergence of a subfamily of xylanase inhibitors within glycoside hydrolase family 18. *FEBS J.* **2005**, *272*, 3227–3227.

(28) Beaugrand, J.; Gebruers, K.; Ververken, C.; Fierens, E.; Croes, E.; Goddeeris, B.; Courtin, C. M.; Delcour, J. A. Antibodies against wheat xylanase inhibitors as tools for the selective identification of their homologues in other cereals. *J. Cereal Sci.* **2006**, *44*, 59–67.

(29) Elliott, G. O.; McLauchlan, W. R.; Williamson, G.; Kroon, P. A. A wheat xylanase inhibitor protein (XIP-I) accumulates in the grain and has homologues in other cereals. *J. Cereal Sci.* **2003**, *37*, 187–194.

(30) 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.* **2006**, *86*, 1610–1617.

(31) 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.

(32) Bradford, M. M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.

(33) Crowther, J. R. The ELISA guidebook. In *Methods in Molecular Biology*; Walker, J. M., Ed.; Humana Press: Totowa, NJ, 2001; Vol. *149*.

(34) Sansen, S.; De Ranter, C. J.; Gebruers, K.; Brijs, K.; Courtin, C. M.; Delcour, J. A.; Rabijns, A. Structural basis for the inhibition of *Aspergillus niger* xylanase by Triticum aestivum xylanase inhibitor-I. *J. Biol. Chem.* **2004**, *279*, 36022–36028.

(35) Fierens, K.; Gils, A.; Sansen, S.; Brijs, K.; Courtin, C. M.; Declerck, P. J.; De Ranter, C. J.; Gebruers, K.; Rabijns, A.; Robben, J.; Van Campenhout, S.; Volckaert, G.; Delcour, J. A. His374 of wheat endoxylanase inhibitor TAXI-I stabilizes complex formation with glycoside hydrolase family 11 endoxylanases. *FEBS J.* **2005**, *335*, 5872–5882.

(36) 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.

(37) Skerritt, J. H. Immunochemistry of cereal grain endosperm proteins. In *Advances in Cereal Sciences and Technology*; Pomeranz, Y., Ed.; AACC: St. Paul, MN, 1988; Vol. *9*, pp 263–238.

(38) Gale, K. R.; Blundell, M. J.; Hill., A. S. Development of a simple, antibody-based test for granule-bound starch synthase Wx-B1b (Null-4A) wheat varieties. *J. Cereal Sci.* **2004**, *40*, 85–92.

(39) Xu, T.; Jacobsen, C. M.; Kyu Cho, I.; Hara, A. H.; Li, Q. X. Application of an enzyme-linked immunosorbent assay for the analysis of imidacloprid in Wiliwili tree, *Erythrina sandwicensis* O. Deg, for control of the wasp *Quadrastichus erythrinae*. *J. Agric. Food Chem.* **2006**, *54*, 8444–8449.

(40) 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*, 23–244.

(41) Raedschelders, G.; Fierens, K.; Sansen, S.; Rombouts, S.; Gebruers, K.; Robben, J.; Rabijns, A.; Courtin, C. M.; Delcour, J. A.; Van Campenhout, S.; Volckaert, G. Molecular identification of wheat endoxylanase inhibitor TAXI-II and the determinants of its inhibition specificity. *Biochem. Biophys. Res. Commun.* **2005**, *335*, 512–522.

Received for review April 13, 2007. Revised manuscript received June 27, 2007. Accepted July 13, 2007. The Research Fund of the K.U. Leuven is acknowledged for the postdoctoral fellowship to J.B. The "Fonds voor Wetenschappelijk Onderzoek Vlaanderen" is thanked for the postdoctoral fellowship to K.G., and the "Instituut voor de Aanmoediging van Innovatie door Wetenschap en Technologie in Vlaanderen" is thanked for the scholarships to E.F. The study was in part carried out in the framework of research project GOA/03/10 financed by the K.U. Leuven Research Fund.

JF071087B