TLXI, a novel type of xylanase inhibitor from wheat (*Triticum aestivum*) belonging to the thaumatin family

Ellen FIERENS*[1], Sigrid ROMBOUTS†[1], Kurt GEBRUERS*[2], Hans GOESAERT*, Kristof BRIJS*, Johnny BEAUGRAND*, Guido VOLCKAERT†, Steven VAN CAMPENHOUT*, Paul PROOST‡, Christophe M. COURTIN* and Jan A. DELCOUR*

*Laboratory of Food Chemistry and Biochemistry, Katholieke Universiteit Leuven, Kasteelpark Arenberg 20, B-3001 Leuven, Belgium, †Laboratory of Gene Technology, Katholieke Universiteit Leuven, Kasteelpark Arenberg 21, B-3001 Leuven, Belgium, and ‡Laboratory of Molecular Immunology, Katholieke Universiteit Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium

Wheat (*Triticum aestivum*) contains a previously unknown type of xylanase (EC 3.2.1.8) inhibitor, which is described in the present paper for the first time. Based on its > 60 % similarity to TLPs (thaumatin-like proteins) and the fact that it contains the Prosite PS00316 thaumatin family signature, it is referred to as TLXI (thaumatin-like xylanase inhibitor). TLXI is a basic (pI ⩾ 9.3 in isoelectric focusing) protein with a molecular mass of approx. 18 kDa (determined by SDS/PAGE) and it occurs in wheat with varying extents of glycosylation. The TLXI gene sequence encodes a 26-amino-acid signal sequence followed by a 151-amino-acid mature protein with a calculated molecular mass of 15.6 kDa and pI of 8.38. The mature TLXI protein was expressed successfully in *Pichia pastoris*, resulting in a 21 kDa (determined by SDS/PAGE) recombinant protein (rTLXI). Polyclonal antibodies raised against TLXI purified from wheat react with epitopes of rTLXI as well as with those of thaumatin, demonstrating high structural similarity between these three proteins. TLXI has a unique inhibition specificity. It is a noncompetitive inhibitor of a number of glycoside hydrolase family 11 xylanases, but it is inactive towards glycoside hydrolase family 10 xylanases. Progress curves show that TLXI is a slow tight-binding inhibitor, with a K_i of approx. 60 nM. Except for zeamatin, an α-amylase/trypsin inhibitor from maize (*Zea mays*), no other enzyme inhibitor is currently known among the TLPs. TLXI thus represents a novel type of inhibitor within this group of proteins.

Key words: heterologous expression, thaumatin-like protein (TLP), thaumatin-like xylanase inhibitor (TLXI), slow tight binding, wheat (*Triticum aestivum*), xylanase inhibitor.

INTRODUCTION

The plant cell is protected from its surrounding environment by the cell wall, which forms a structurally heterogeneous barrier. In the case of plant attack, pathogenic micro-organisms produce a diverse array of enzymes which depolymerize the different polysaccharides in the cell walls [1]. One type of such enzymes are xylanases (also referred to as endo-β-1,4-xylanases or endoxylanases, EC 3.2.1.8). They depolymerize xylan, which, next to cellulose, is one of the most abundant polysaccharides in the cell wall of higher plants. It consists of a main chain of β-1,4-xylopyranosyl residues that, depending on the origin, may be replaced with, e.g., glucuronyl, acetyl or arabinofuranosyl groups to form heteroxylans. Xylanases hydrolyse the β-1,4-xylosidic linkages in the xylan main chain [2]. The majority of the xylanases belong either to glycoside hydrolase family 10 (GH10) or to the structurally unrelated glycoside hydrolase family 11 (GH11) (http://afmb.cnrs-mrs.fr/CAZY/ [3]). In both families, a pair of glutamate residues catalyses the cleavage of the glycosidic bond, one acting as a nucleophile and the other as the acid–base catalyst. Recently, a xylanase was shown to be indispensable in the infection of plants by the pathogen *Botrytis cinerea* [4]. Xylanases are produced not only by micro-organisms, but also by plants. The latter belong to GH10 and play important physiological roles in

several tissues, such as contribution to seed germination and fruit ripening [5].

At the same time, some plants produce proteins which can inhibit xylanases. Over the last decade, studies have revealed the presence of two types of proteinaceous xylanase inhibitors in cereals, i.e. the TAXI (*Triticum aestivum* xylanase inhibitor)-type [6,7] and the XIP (xylanase inhibitor protein)-type inhibitors [8,9]. These proteins have been purified and characterized biochemically, genetically and structurally.

TAXI-type proteins occur in common wheat (*Triticum aestivum*), durum wheat (*Triticum durum*), barley (*Hordeum vulgare*) and rye (*Secale cereale*) [10,11] as monomeric (40 kDa) as well as heterodimeric (30 + 10 kDa) basic (pI ⩾ 8.0) proteins which specifically inhibit GH11 xylanases [12]. Crystallographic analysis of a complex between a GH11 xylanase of *Aspergillus niger* and TAXI-I showed His[374] of TAXI-I to be a key residue in xylanase inhibition. This histidine residue interacts in the active site with the two active glutamate residues of the xylanase, clearly indicating a competitive type of inhibition [13]. XIP-type proteins have been isolated from the above-cited cereals as well as from maize (*Zea mays*) and rice (*Oryza sativa*) [11,14,15]. These monomeric proteins (30 kDa, pI ⩾ 6.7) inhibit GH10 and GH11 xylanases, provided that they are from fungal origin [15]. The crystal structures of XIP-I in complex with GH10 *Aspergillus*

---

nidulans xylanase on the one hand, and with GH11 *Penicillium funiculosum* xylanase on the other hand, reveal that XIP-I possesses an independent enzyme-binding site for each family of xylanases. Like TAXI, XIP is a competitive inhibitor, interacting in the active site of the xylanases [16]. For both families, the inhibition mechanism is based on substrate mimicry.

A regulatory role of TAXI and XIP in plant development is disaffirmed by their lack of effectiveness against endogenous xylanases, their distinct specificity towards xylanases of microbial origin, the ability of TAXI to inhibit two GH11 xylanases of the cereal pathogen *Fusarium graminearum* [17] and the fact that both TAXI and XIP genes are induced by pathogens and wounding [18].

The present study reports on the existence of a third, structurally unrelated, type of xylanase inhibitor in wheat which belongs to the thaumatin family. It is further referred to as TLXI (thaumatin-like xylanase inhibitor). More particularly, the purification of this protein from wheat, the identification, cloning and heterologous expression of its corresponding gene is described. Additionally, the biochemical characteristics and the kinetic parameters of inhibition of both native and recombinant TLXI are discussed.

EXPERIMENTAL

Materials

Wheat (cultivar Soissons) (from Aveve) wholemeal was prepared using a Cyclotec 1093 sample mill. All electrophoresis and chromatography media, and molecular mass and pI markers were from GE Healthcare, unless specified otherwise. The producers of the kits and enzymes used in cloning and heterologous expression of TLXI are mentioned below. *Bacillus subtilis* GH11 xylanase and an *Aspergillus aculeatus* GH10 xylanase were supplied by Puratos (by Ir Filip Arnaut). Two GH11 xylanases from *Trichoderma longibrachiatum* (also known as *Trichoderma reesei*), i.e. Xyn I and Xyn II, GH11 xylanases from *A. niger* and *Trichoderma viride*, and Xylazyme-AX tablets were from Megazyme. GH10 xylanases from *Aspergillus oryzae* and *Penicillium purpurogenum* were kindly made available by VTT Biotechnology (from Professor Maija Tenkanen, now at Department of Applied Chemistry and Microbiology, University of Helsinki, Helsinki, Finland) and the Laboratorio de Bioquímica (Professor Jaime Eyzaguirre, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile) respectively. Dr Nathalie Juge (Institute of Food Research, Norwich, U.K.) kindly provided a *Pseudomonas fluorescens* GH10 and a *Penicillium funiculosum* GH11 xylanase. Thermophilic *Thermobacillus xylanilyticus* GH10 and GH11 xylanases were made available by Dr Michael O'Donohue (INRA, Reims, France). *A. niger* GH10 xylanase was purified from an *A. niger* CBS 110.42 culture filtrate [19]. Grindamyl H 640 bakery enzyme, containing the wild-type *B. subtilis* GH11 xylanase, and Biobake 710, containing the above-cited *A. niger* GH11 xylanase, were from Danisco and Quest International respectively. The Pro-Q Emerald 300 Glycoprotein Stain kit was from Invitrogen. Thaumatin, oat spelt xylan, horseradish-peroxidase-conjugated goat anti-rabbit antibodies, substrate (3,3′,5,5′-tetramethylbenzidine) for the horseradish peroxidase, PNGase F (peptide N-glycosidase F) and all other chemicals were from Sigma–Aldrich. Water-soluble oat spelt xylan was prepared as described by He et al. [20]. 4-MUX$_2$ (4-methylumbelliferyl-β-D-xylobioside) was kindly provided by Dr Wim Nerinckx (Laboratory of Glycobiology, University of Ghent, Ghent, Belgium).

Affinity matrix preparation

B. subtilis and *A. niger* GH11 xylanases were purified from Grindamyl H 640 and Biobake 710 enzyme preparations respectively, and N-hydroxysuccinimide-activated Sepharose 4 Fast Flow matrix was used for the preparation of affinity matrices with these two enzymes (7 and 30 ml respectively) according to Gebruers et al. [21].

Purification of xylanase inhibitors from wheat

Wheat wholemeal (1 kg) was suspended in 5 litres of aqueous 0.1 % (w/v) L-ascorbic acid solution, extracted overnight at 7 °C and centrifuged at 10 000 g for 30 min at 7 °C. L-Ascorbic acid reduced the oxidation of phenolic compounds during the extraction. Calcium chloride (2 g/l) was added to the supernatant, and the pH was raised to 8.5 by adding 2 M NaOH. The extract was left overnight at 7 °C, and the resulting precipitate (containing pectins) was removed by centrifugation at 10 000 g for 30 min at 7 °C. The pH of the supernatant was adjusted to 4.5 by adding 2 M HCl. Proteins with xylanase-inhibition activity in the supernatant were retained by CEC (cation-exchange chromatography) on a SP Sepharose Big Beads column (180 mm × 130 mm, equilibrated with 25 mM sodium acetate buffer, pH 4.5). The bound protein fraction was eluted in one step with 1 litre of 1 M NaCl solution, dialysed against deionized water for 48 h at 7 °C and freeze-dried, resulting in the CEC fraction (approx. 7.76 g of protein). Portions (6 g) of the CEC fraction were extracted with 25 mM sodium acetate buffer, pH 5.0, containing 100 ml of 0.2 M NaCl, centrifuged at 10 000 g for 30 min at 7 °C and filtered through a paper filter. The filtrate was loaded on the *B. subtilis* xylanase-affinity column (10 mm × 70 mm, equilibrated with 25 mM sodium acetate buffer, pH 5.0, containing 0.2 M NaCl) at a flow rate of 0.33 ml/min. TAXI was eluted from the column with 5 ml of 250 mM Tris solution, pH 12.0, at a flow rate of 1 ml/min, and immediately neutralized with 1 M ethanoic (acetic) acid solution. The resulting run-through was used to isolate XIP and TLXI. Portions (30 ml) of the run-through were applied on the *A. niger* xylanase-affinity column (16 mm × 150 mm, equilibrated with 25 mM sodium acetate buffer, pH 5.0, containing 0.2 M NaCl) at a flow rate of 1 ml/min. Bound proteins were then eluted from the column with buffers of increasing pH (50 ml of 250 mM Tris, pH 10.0–12.0), at a flow rate of 3 ml/min. XIP was removed from the column in the elution step with pH 10.0. TLXI eluted when the pH was raised to 12.0. The flow scheme is shown in Supplementary Figure S1 (http://www.BiochemJ.org/bj/403/bj4030583add.htm).

Protein sequencing

To determine the N-terminal amino acid sequence, inhibitor proteins (approx. 50 μg) were separated by SDS/PAGE in a 12 % polyacrylamide gel using the Hoeffer Mighty Small unit, electroblotted on to a PVDF membrane with the Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) (electric potential difference of 10 V) for 1 h at room temperature (20 °C) and subjected to Edman degradation. Sequence analysis was performed on a Procise cLC 491 Sequencer (Applied Biosystems).

For internal amino acid sequence determination, TLXI (500 μg) was dissolved in 0.3 ml of 70 % methanoic (formic) acid containing a small amount of CNBr (two crystals; the exact amount was not critical). Protein cleavage was performed by incubating the solution in the dark for 27 h at room temperature, after which all volatiles were evaporated under a nitrogen gas stream. The resulting peptides were dissolved in 1 ml of 25 mM Tris/HCl buffer, pH 8.0, containing 1 % (v/v) 2-mercaptoethanol

and kept in boiling water for 5 min. The peptides were separated using reverse-phase HPLC on a Microsorb 300 Å (1 Å = 0.1 nm) pore-size C_8 reverse-phase column (Varian). Solvent A was MilliQ water with 0.1 % trifluoroacetic acid, and solvent B was aceto-nitrile with 0.1 % trifluoroacetic acid. Separation was performed by applying a gradient from 2 to 100 % solvent B in 66 min at a flow rate of 1 ml/min. The isolated polypeptides were subjected to Edman degradation and analysed as described above.

Gene isolation and characterization

Genomic DNA was isolated from young leaves of wheat cultivar Estica using the DNeasy Plant Mini kit (Qiagen). Total RNA was extracted from young embryos (3 weeks post-anthesis) of the same cultivar by means of the Invisorb Spin Plant-RNA Mini kit (Invitek). mRNA was purified from the total RNA with the Oligotex mRNA Mini kit (Qiagen). Primers were designed based on EST (expressed sequence tag) sequences BE399034 and BE427320 corresponding to the N-terminal sequence of native TLXI. RACE (rapid amplification of cDNA ends) reactions were performed using the GeneRacer kit (Invitrogen) according to the manufacturer's instructions with HotStarTaq polymerase as enzyme. 3′-RACE and 3′-nested RACE were performed with I3race (5′-GTGCCAGACCGGCGACTG-3′) and I3nested (5′-GTGGCAGCTCGCTGACTTG-3′) as gene-specific primers res-pectively, whereas, for 5′-RACE and 5′-nested RACE, I5race (5′-TTGGTGGAGCACGAGCGCCAC-3′) and I5nested (5′-CCGGCCACACCGTGAAGTG-3′) respectively were used for this purpose. For PCR on genomic DNA, Iintf (5′-CAAG-CGCGGCACCGCTCACCATC-3′) was used in combination with XI2 (5′-AATACCTGACACACGTGTACGG-3′). PCR products were purified using the PCRapid kit (Invitek) and subsequently sequenced on a 377 DNA Sequencer using ABI PRISM Big Dye Terminator chemistry (Applied Biosystems). Assembly of the obtained sequences (see Supplementary Figure S2 at http://www.BiochemJ.org/bj/403/bj4030583add.htm) resulted in a contiguous sequence.

Cloning, mutagenesis and protein expression

Construction of expression plasmids

The DNA sequence encoding mature TLXI was amplified with primer combination Ximatf (5′-CACAGATCTGCACCGCTCA-CCATCACGAAC-3′) and Ximatrstop (5′-CACAGATCTTCA-TGGGCAGAAGACGATCTG-3′) using Pfu DNA polymerase (Stratagene) and PCR product Iintf/XI2 as template. The resulting PCR product was cloned in a pCR®4-TOPO® vector (Invitrogen), verified by DNA sequencing, and subsequently subcloned as a BglII fragment in the BsmBI site of expression vector pPICZαC (Invitrogen). The ligation mixture was used to transform *Escherichia coli* TOP10F′ cells [F′ {*proAB*, *lacI�q*, *lacZΔM15*, Tn*10* (TetR)} *mcrA*, Δ(*mrrhsdRMS-mcrBC*), φ80*lacZΔM15*, Δ*lacX74*, *deoR*, *recA1*, λ-*araD139*, Δ(*ara-leu*)7697, *galU*, *galK*, *rpsL*(StrR), *endA1*, *nupG*].

Expression and purification of rTLXI (recombinant TLXI)

A sequence-verified pPICZαC-*tlxi* construct, linearized with PmeI, was used to transform competent *Pichia pastoris* KM71H cells (*arg4 aox1::ARG4*) using the EasySelect *Pichia* Expression kit (Invitrogen). For large-scale expression, a single colony was grown in 10 ml of buffered minimal glycerol-complex medium (pH 6.0), supplemented with 0.35 M NaCl, for 24 h at 30 °C, with shaking at 250 rev./min. The volume was increased to 500 ml in a 2 litre flask and incubated overnight under the same conditions. A pre-induction transition phase was included in which 10 %

glycerol/13.4 % yeast nitrogen base was added to the primary culture at a ratio of 1:10 (v/v). When the cells reached an attenuance at 600 nm (D_{600}) of 0.20–0.24 in a 1:50 dilution, the cell culture was harvested by centrifugation at 2500 *g* for 10 min. To induce protein expression, the cells were suspended in 100 ml of buffered minimal methanol-complex medium (pH 6.0) and cultured in baffled flasks for another 50 h at 20 °C in the presence of 1.25 % (v/v) methanol. To harvest culture supernatants, yeast cells were removed by centrifugation at 2500 *g* for 10 min. Before purification, the medium was dialysed overnight against 25 mM sodium acetate buffer, pH 5.0. rTLXI was purified by CEC using a SP-Sepharose Fast Flow column (10 mm × 200 mm) equilibrated with the same buffer. Bound proteins were eluted using a linear salt gradient of 0–1 M NaCl in 100 min (flow rate of 1 ml/min). Inhibition activity and purity of the resulting fractions were assessed using the Xylazyme-AX method (see below) and SDS/PAGE respectively.

Protein content determination

Protein concentrations were determined by the Bradford Coomassie Brilliant Blue method with BSA as standard [22]. For pure TLXI samples, protein concentrations were determined spectrophotometrically at 280 nm using a specific absorbance value of 1.457 absorbance units for 1 mg/ml TLXI (1 cm pathlength UV cell).

Xylanase inhibition assay (Xylazyme-AX method)

Inhibition activities were determined with the colorimetric Xylazyme-AX method [23]. All xylanase solutions were prepared in 25 mM sodium acetate buffer, pH 5.0, with 0.5 mg/ml BSA, and contained 2 units of xylanase per 1 ml. Xylanase units were defined as described by Gebruers et al. [23], and, under the conditions of the assay, the xylanase concentration corresponding to 1 unit was approximately 5.1 nM for the *A. niger*, 8.9 nM for the *T. longibrachiatum* (Xyn I), 2.1 nM for the *T. longibrachiatum* (Xyn II), 11.6 nM for the *T. viride*, 5.8 nM for the *B. subtilis*, 0.1 nM for the *Thermobacillus xylanilyticus* and 2.1 nM for the *Penicillium funiculosum* GH11 xylanases. The corresponding concentrations for the GH10 xylanases were 53.7 nM for the *A. aculeatus*, 36.6 nM for the *A. niger*, 30.9 nM for the *Ps. fluor-escens*, 14.2 nM for the *Thermobacillus xylanilyticus* and 2.9 nM for the *A. oryzae* enzyme. Different inhibitor concentrations up to 8000 nM and up to 3000 nM were used for TLXI and rTLXI respectively. All measurements were performed in triplicate.

Electrophoresis

SDS/PAGE under non-reducing and reducing conditions was performed on 20 % polyacrylamide gels with a PhastSystem unit, as described in GE Healthcare Separation Technique File 110. 2-Mercaptoethanol (5 %) was used as reducing agent. The low-molecular-mass markers were from GE Healthcare. The pI value of the inhibitor protein was determined by isoelectric focusing as described in GE Healthcare Separation Technique File 100, using the PhastSystem unit with polyacrylamide gels containing ampholytes (pH 3–9). Broad-range pI markers (3.5–9.3) were used. All gels were silver-stained as described in GE Healthcare Development Technique File 210.

Glycan detection

The proteins (0.1 mg) were separated by SDS/PAGE in a 12 % polyacrylamide gel (see above). Glycan detection was performed in-gel, using the Pro-Q Emerald 300 Glycoprotein Stain kit, according to the instructions of the manufacturer. To gain insight

into the extent of glycosylation, TLXI was analysed by electrospray-ionization MS on an Esquire-LC/MS system (Bruker).

Deglycosylation

Enzymic N-deglycosylation

The N-linked glycans of the purified TLXI and rTLXI were removed with PNGase F (Enzymic In-solution N-Deglycosylation kit) under reducing and denaturing conditions according to the instructions of the manufacturer (Sigma–Aldrich). The different deglycosylated and control samples were analysed by SDS/PAGE followed by silver staining.

Chemical deglycosylation

TLXI and rTLXI were treated with anhydrous TFMSA (trifluoromethanesulfonic acid), which effectively cleaves N- and O-linked glycans from glycoproteins, leaving the primary structure of the protein intact [24]. Anisole was used as a scavenger to neutralize reactive groups formed during the deglycosylation reaction. Excess TFMSA was neutralized by reaction with pyridine.

Briefly, the purified inhibitors (100 μg) were freeze-dried in glass tubes for 24 h to ensure complete dryness. A 10 % anisole solution in TFMSA (150 μl) was cooled to 0 °C, and then added to the inhibitor samples. The reaction was performed at 0 °C. After 3 h, the reaction mixture was placed in a methanol/solid CO_2 bath and neutralized with an equal volume of ice-cold aqueous pyridine (60 %). The excess pyridine was removed by gel-filtration on a PD-10 column and, after freeze-drying, the different deglycosylated samples were analysed by SDS/PAGE followed by silver staining.

Western blot and immunoprobing

Rabbit polyclonal antibodies against native TLXI were prepared, and Western blot analysis was performed on samples of TLXI (0.50 μg), rTLXI (0.56 μg), wheat CEC fraction (18 μg) and commercial thaumatin (4 μg), as described by Beaugrand et al. [25].

Kinetic analysis

Kinetic parameters were derived from reaction progress curves of TLXI and rTLXI with *T. longibrachiatum* GH11 xylanase (Xyn I). 4-MUX$_2$ was used as substrate. TLXI and 4-MUX$_2$ solutions were mixed, and the reaction was started by adding the enzyme solution, yielding a final volume of 150 μl in McIlvaine buffer (0.2 M Na_2HPO_4/0.1 M citric acid), pH 5.0. The mixtures were incubated for various lengths of time up to 90 min at 40 °C. The reaction was stopped by adding 15 μl of 500 mM glycine/NaOH buffer, pH 13.0, and putting the samples on ice. The hydrolysis products formed were quantified on a fluorimeter (Horiba Jobin Yvon) based on a standard curve of 4-methylumbelliferone (0–6 μM). Excitation was at 360 nm and the emission was recorded at 446 nm for 0.1 s (five trials; S.E.M. < 1 %). Both monochromator slit widths were 1 nm. All measurements were performed in triplicate in 75 μl capillaries.

Substrate binding

TLXI (9 μM in 1 ml of 25 mM sodium acetate buffer, pH 5.0) was mixed with soluble or insoluble oat spelt xylan (50 mg). These mixtures were shaken for 30 min at room temperature. In the case of insoluble xylan, the supernatant was removed after centrifugation at 15 000 g for 10 min, and the pellet was washed twice with 1 ml of 25 mM sodium acetate buffer, pH 5.0. The
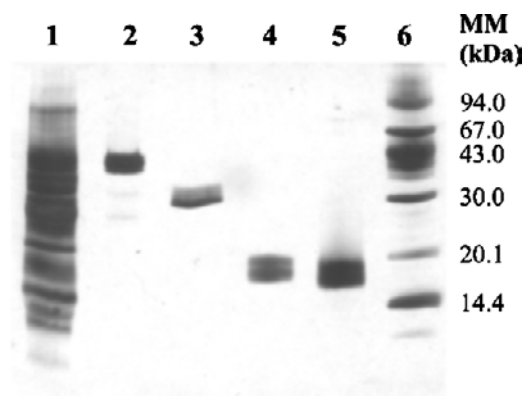
Figure 1 SDS/PAGE profiles of a wheat CEC fraction and the isolated xylanase inhibitors

A CEC fraction (lane 1) was obtained by fractionation of wheat wholemeal extract by CEC. Pure TAXI (lane 2), XIP (lane 3) and TLXI (lanes 4 and 5) were obtained in this order from the CEC fraction by affinity chromatography on *B. subtilis* and *A. niger* GH11 xylanase-affinity columns. TLXI was analysed by SDS/PAGE under non-reducing (lane 4) and reducing conditions (lane 5). The size of the molecular mass (MM) markers (lane 6) is indicated on the right-hand side in kDa. The gel was silver-stained.

inhibition activity against the *T. longibrachiatum* xylanase (Xyn I) in the mixture (for soluble xylan) and in the supernatant (for insoluble xylan) (50 μl) was determined with the Xylazyme-AX method (see above) and compared with the inhibition activity of the original TLXI sample. For insoluble xylan, supernatant and pellet were also analysed by SDS/PAGE.

RESULTS AND DISCUSSION

Xylanase inhibitor purification

Starting from wholemeal, the two already known xylanase inhibitors, TAXI and XIP, and the novel xylanase inhibitor, TLXI, were purified using affinity chromatography with immobilized xylanases (Figure 1). The purification yields of TAXI and XIP were similar to those described earlier (14–15 mg/kg of wholemeal [9,26]). The yield of TLXI was approx. 2.5 mg/kg of wholemeal. The successful use of affinity chromatography with immobilized xylanases indicates that the complexation between TLXI and xylanase is reversible.

Molecular characterization

Primers, designed on Triticeae EST sequences, allowed us to amplify and subsequently sequence the complete *tlxi* gene coding sequence, including the 5′- and 3′-untranslated regions. At present, several wheat ESTs can be found in the database that show 99–100 % identity with the *tlxi* sequence and correspond to proteins expressed in root (CK199869), grain (CD914550 and CD914871) and ovary (CD937830). In addition, EST sequences from barley (BQ760132) and durum wheat (AJ611730) are 65 % and 47 % identical (69 % and 59 % similar) respectively with the protein sequence of TLXI.

The *tlxi* gene contains no introns and encodes a mature protein of 151 amino acids preceded by a signal sequence of 26 amino acids (PSORT [27]). Approx. 50 % of the mature protein sequence was confirmed by sequencing peptides of native TLXI obtained after CNBr cleavage. The sequence includes a potential glycosylation site at Asn[95] as well as ten cysteine residues (Figure 2) that are probably involved in intramolecular disulfide

```
 -26   M  A  S  P  A  R  S  A  S  A  S  P  V  L  L  L  L  V  V  L
 -78   ATGGCGTCTCCAGCCAGAAGCGCGAGCGGCCTCTCCAGTCCTCCTCTTGCTCGTCGTCCTC
  -6   A  A  G  A  S  A  A  P  L  T  I  T  N  R  C  H  F  T  V  W
 -18   GCCGCCGGGGCAAGCGCGGCACCGCTCACCATCACGAACCGTTGCCACTTCACGGTGTGG
  15   P  A  V  A  L  V  L  H  Q  G  G  G  G  T  E  L  H  P  G  A
  42   CCGGCCGGTGGCGCTCGTGCTCCACCAAGGGGCGGCGGCACCGAGCTCCACCCAGGGGCC
  35   S  W  S  L  D  T  P  V  I  G  S  Q  Y  I  W  G  R  T  G  C
 102   AGCTGGAGCCTCGACACGCCCGTGATCGGCTCCCAGTACATATGGGGGCGCACGGGCTGC
  55   S  F  D  R  A  G  K  G  R  C  Q  T  G  D  C  G  G  S  S  L
 162   TCCTTCGACCGGGCCGGCAAGGGGCGGTGCCAGACCGGCGACTGCGGTGGCAGCTCGCTG
  75   T  C  G  G  N  P  A  V  P  V  T  M  A  E  V  S  V  L  Q  G
 222   ACTTGCGGGGGCAACCCGGCGGTGCCGGTGACCATGGCCGAGGTGTCCGTGCTCCAGGGC
  95   N  Y  T  Y  G  V  T  S  T  L  K  G  F  N  L  P  M  D  L  K
 282   AACTATACCTACGGCGTCACGTCGACGCTCAAGGGGTTCAACCTGCCCATGGACCTGAAG
 115   C  S  S  G  D  A  L  P  C  R  K  A  G  C  D  V  V  Q  P  Y
 342   TGCAGCTCCGGCGACGCGCTCCCGTGCCGGAAGGCTGGGTGCGACGTTGTCCAGCCGTAC
 135   A  K  S  C  S  A  A  G  S  R  L  Q  I  V  F  C  P  *
 402   GCGAAGAGCTGCAGCGCGGCTGGAAGCCGGCTCCAGATCGTCTTCTGCCCATGA
```

Figure 2   Complete coding sequence of TLXI

The first methionine residue of the signal sequence (italic) is at position −26. The mature protein starts at position 1, stops at position 151 and contains ten cysteine residues (bold) and one possible N-glycosylation site (italic bold) at position 95. Parts of the amino acid sequence were confirmed by sequencing peptides obtained after CNBr cleavage of native TLXI (underlined). The Prosite thaumatin family signature PS00316 is shaded. * indicates a stop codon.

Table 1   MS data and corresponding glycosyl moieties

The Table shows the different masses obtained with MS with their corresponding glycosyl entities as determined with GlycoMod [41]. HexNAc, N-acetylhexosamine (e.g. N-acetylglucosamine or N-acetylgalactosamine); deoxyhexose, e.g. fucose or rhamnose; hexose, e.g. galactose, mannose or glucose; NeuAc, N-acetylneuraminic acid (sialic acid).

Molecular mass	Glycosyl moiety
15 632.5	–
15 833.9	HexNAc
16 040.5	$HexNAc_2$
16 185.5	$HexNAc_2$–Deoxyhexose
16 347.0	$HexNAc_2$–Deoxyhexose–Hexose
16 638.0	$HexNAc_2$–Deoxyhexose–Hexose–NeuAc
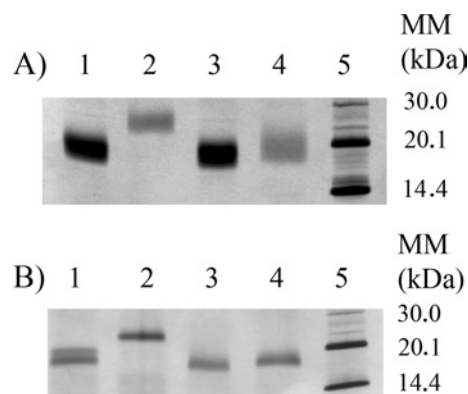
Figure 3   Deglycosylation of TLXI and rTLXI

TLXI and rTLXI were deglycosylated enzymically with PNGase F (**A**) and chemically with TFMSA (**B**). The original TLXI and rTLXI are shown in lanes 1 and 2 respectively. Deglycosylated TLXI and rTLXI are shown in lanes 3 and 4 respectively. The sizes of the molecular-mass (MM) markers (lane 5) are indicated on the right-hand side (in kDa). The gels were silver-stained.

bonds. The calculated molecular mass and pI of the mature protein were determined to be 15 642.8 Da and 8.38 respectively.

BLASTp [28] results show that the xylanase inhibitor belongs to the thaumatin family (Pfam00314) and contains the Prosite PS00316 thaumatin family signature (Figure 2). Similarity up to 60 % was found with several TLPs (thaumatin-like proteins) from various origins. TLPs are a heterogeneous family of proteins which exhibit sequence similarity with thaumatin, a sweet-tasting protein found in the arils of fruits of the African shrub *Thaumatococcus daniellii* [29]. Until now, thaumatin is the only protein of the family for which sweetness has been described. Members of this family have already been identified in wheat (pWIR2 [30]), barley (antifungal proteins R and S [31], TLPs 1–8 [32,33]), rye {AFP (antifreeze protein) [34]}, oats (PR-5 [35]), maize (zeamatin [36]), tobacco (osmotin [37]), many kinds of fruit (cherry [38], apple [39]) and other plants.

TLXI belongs to a group of smaller TLPs, which are found mainly in cereals. The proteins in this group all contain ten cysteine residues, which are involved in five intramolecular disulfide bridges and form the basis for high stability under extreme thermal and pH conditions [33]. Only one TLP, i.e. zeamatin, showing 43 % identity with TLXI, is known to inhibit enzymes, namely α-amylases and trypsin [36]. Most of the TLPs have been shown to possess antifungal activity and therefore have been assigned to class 5 of the pathogenesis-related proteins. Their activity is thought to rely on an interaction of the acidic cleft of TLPs with 1,3-β-D-glucans of fungal membranes [40], causing membrane permeabilization. Based on its homology with TLP, it can be assumed that TLXI also plays a role in plant defence.

The *tlxi* gene was recombinantly expressed in *P. pastoris*, resulting in a 21 kDa protein with xylanase-inhibiting activity against *T. longibrachiatum* xylanase (XynI) (Supplementary Figure S3 at http://www.BiochemJ.org/bj/403/bj4030583add.htm). Subsequent purification yielded 4 mg of pure rTLXI per litre of culture medium.

Biochemical characterization

Native TLXI appears in SDS/PAGE under reducing as well as non-reducing conditions as a broad band at approx. 18 kDa (Figure 1). rTLXI appears in SDS/PAGE as one 21 kDa protein band. Based on isoelectric focusing, TLXI and rTLXI have a pI of 9.3 or higher (results not shown).

The reaction of the antibodies, raised against native TLXI from wheat, with the commercial thaumatin sample on a Western

blot verified that TLXI is a member of the thaumatin family (Supplementary Figure S4 at http://www.BiochemJ.org/bj/403/bj4030583add.htm). On this Western blot, it also became clear that the broad band for native TLXI, seen on SDS/PAGE, consists of three to four finer protein bands, suggesting the presence of different forms with varying extents of glycosylation. The Pro-Q Emerald 300 Glycoprotein Stain kit indeed showed considerable glycosylation, not only for TLXI but also for rTLXI (results not shown). For native TLXI, this was confirmed by MS. Next to a peak at 15 632.5 Da (molecular mass similar to that calculated from the amino acid sequence), peaks corresponding to TLXI proteins with different degrees of glycosylation were observed. The different molecular masses and their corresponding glycosyl moieties are listed in Table 1. The peak with the highest mass corresponded to 16 638 Da, a shift in molecular mass which could be accounted for by five sugar residues: two N-acetylhexosamines (e.g. N-acetylglucosamine or N-acetylgalactosamine), one 6-deoxyhexose (e.g. fucose or rhamnose), one hexose (e.g. galactose, mannose or glucose) and one sialic acid, as determined with GlycoMod [41].

To determine the type of glycosylation, the proteins were deglycosylated, on the one hand enzymically, with PNGase F, which can only cleave N-bound sugars, and on the other hand chemically, with TFMSA, which can remove both N- and O-bound sugars (Figure 3). The enzymic deglycosylation with PNGase F had little or no effect on TLXI, unlike for rTLXI, where the SDS/PAGE molecular mass clearly decreased. TFMSA affected the molecular mass of both proteins. Indeed, TLXI no

Table 2   Inhibitor sensitivity of different GH10 and GH11 xylanases

[I]/[E]$_{50}$ values were determined using the Xylazyme-AX method, as described in the Experimental section, and are given in parentheses. Results are means $\pm$ S.E.M. for three measurements. –, no inhibition at the highest inhibitor concentration; n.d., not determined.

		[I]/[E]$_{50}$	
Xylanase	NCBI accession number	TLXI	rTLXI
GH11			
Trichoderma longibrachiatum (Xyn I)	CAA49294	+++ (4.2 $\pm$ 0.2)	+++ (7.8 $\pm$ 1.2)
Aspergillus niger	CAA01470	++ (135.0 $\pm$ 2.5)	+ (1063 $\pm$ 19)
Trichoderma viride	CAB60757	++ (170.4 $\pm$ 1.1)	n.d.
Thermobacillus xylanilyticus	CAJ87325	+ (234.7 $\pm$ 50.1)	n.d.
Penicillium funiculosum	CAC15487	+ (289.6 $\pm$ 4.4)	n.d.
Bacillus subtilis	AAA22897	–	n.d.
Trichoderma longibrachiatum (Xyn II)	CAA49293	–	n.d
GH10			
Aspergillus aculeatus	AAE69552	–	n.d.
Aspergillus niger	CAA03655	–	n.d.
Aspergillus oryzae	BAA75475	–	n.d.
Penicillium purpurogenum	AAF71268	–	n.d.
Pseudomonas fluorescens	CAA33469	–	n.d.
Thermobacillus xylanilyticus	CAA76420	–	n.d.

longer appeared as a broad band, and both TLXI and rTLXI clearly had a lower apparent molecular mass. From these deglycosylation experiments, we can conclude that native TLXI is O-glycosylated and few or no N-bound sugars are present. rTLXI, on the other hand, is clearly both N- and O-glycosylated. TLXI is the first member of the thaumatin family for which glycosylation is reported.

Inhibition specificity

To determine the xylanase specificity of TLXI, its inhibition activity towards several microbial xylanases of GH10 and GH11 was determined using the Xylazyme-AX method. TLXI was active towards most of the GH11 xylanases, but the high-pI GH11 xylanases from *T. longibrachiatum* (Xyn II, pI 9.0) and *B. subtilis* (pI 9.3), and the GH10 xylanases were unaffected by TLXI (Table 2). To be able to order the inhibited xylanases according to their sensitivity for TLXI, the [I]/[E]$_{50}$ values (ratio of inhibitor concentration to enzyme concentration necessary to obtain 50 % inhibition) are included in Table 2. Since TLXI binds to xylan, present in the Xylazyme-AX tablets (see below), it is clear that the results have an indicative value only, implying that one should be careful when comparing these results with those of other inhibitors. However, they give a very good indication of the xylanase specificity of TLXI and rTLXI.

Its xylanase specificity supports further the assumption that TLXI plays a role in plant defence. Indeed, like TAXI, TLXI is active only against GH11 xylanases from both fungal and bacterial origin and is inactive towards GH10 xylanases [12]. Since plant xylanases are structurally similar to the microbial GH10 xylanases [5], TLXI probably does not have a regulatory role as such *in planta*. Its specificity clearly differs from that of XIP, which inhibits GH10 and GH11 xylanases [15].

Substrate binding

Preliminary experiments suggest interaction between TLXI and (arabino-) xylan. To confirm this assumption, a binding experiment was performed. TLXI was mixed with insoluble xylan, and, after centrifugation, the inhibition activity in the supernatant was compared with that in the original TLXI solution. Little, if any, inhibitory activity was measured in the supernatant, while the original sample clearly showed inhibition activity, and,

as no SDS/PAGE inhibitor protein band was observed in the supernatant, while such a band was clearly visible when the pellet was examined, binding between TLXI and insoluble xylan was proven. For soluble xylan, the inhibition activity of the mixture of soluble xylan and TLXI was remarkably lower than that of the original TLXI sample. This demonstrates an interaction between TLXI and xylan, preventing the interaction between TLXI and xylanase. Hence, xylan could not be used to study the interaction between enzyme and inhibitor by means of classical kinetic analysis.

Kinetics and mechanism of xylanase inhibition

From the first sensitivity screen of different xylanases, the most sensitive one, i.e. *T. longibrachiatum* (Xyn I) xylanase, was chosen for in-depth characterization of the kinetic parameters. In view of the binding of TLXI to polymeric substrate, all analyses were performed with 4-MUX$_2$ as substrate, to which TLXI nor rTLXI bind.

Time-dependent inhibition

As shown in Figure 4(A), xylanase inhibition by TLXI shows a clearly time-dependent approach to steady state, whereas the steady-state rate of substrate hydrolysis in the absence of inhibitor was reached instantaneously. The establishment of the equilibrium between enzyme, inhibitor and enzyme–inhibitor complex occurred over a period of approx. 30 minutes. For rTLXI, similar progress curves were obtained, but the establishment of equilibrium took even longer than for TLXI, approx. 45 min (Figure 4B). Therefore the time course for rTLXI was followed for up to 90 min, in which the reaction reached the steady-state conditions.

The concentrations of inhibitor which had to be used to obtain these progress curves were of similar order of magnitude as the concentration of enzyme, indicating that TLXI and rTLXI are not only slow-binding, but also tight-binding, inhibitors.

For slow tight-binding inhibitors, the velocity at any time is:

$$v = v_s + (v_o - v_s)e^{-kt} \qquad (1)$$

where v_s and v_o are the steady-state and initial velocities respectively, and t is time [42].
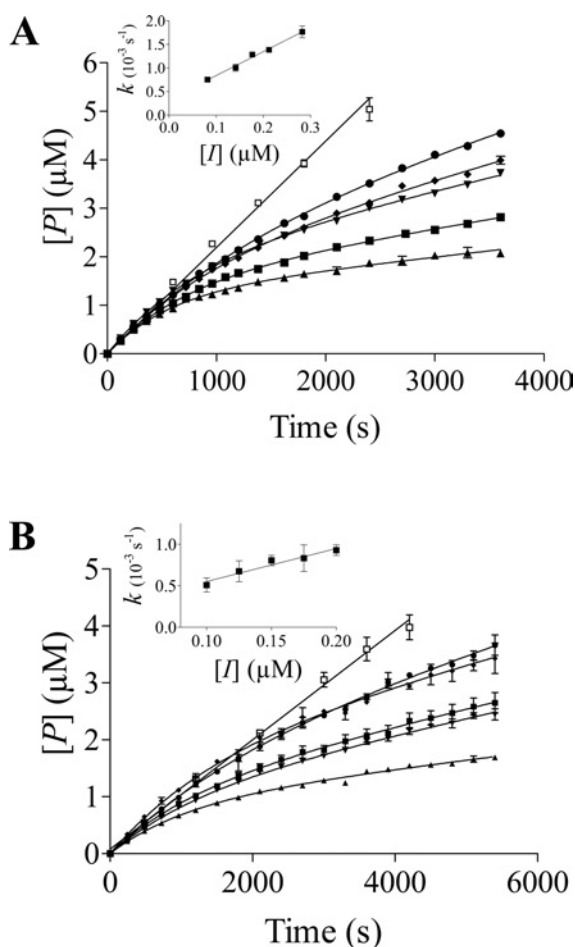
Figure 4   Time course of the inhibition with different inhibitor concentrations

For different quantities of TLXI (**A**) and rTLXI (**B**), progress curves were plotted with *T. longibrachiatum* (Xyn I) xylanase. 4-MUX$_2$ was used as substrate (22.7 μM). The concentrations of enzyme were 54.2 and 27.1 nM for TLXI and rTLXI respectively. The different concentrations were 283 (▲), 212 (■), 177 (▼), 141 (◆), 82 (●) and 0 (□) nM for TLXI and 200 (▲), 175 (■), 150 (▼), 125 (◆), 100 (●) and 0 (□) nM for rTLXI. The apparent first-order rate constants k for establishment of equilibrium were calculated from the progress curves and plotted against the inhibitor concentration, [I] (respective insets), and a linear correlation between [I] and k was found.

Integration of eqn (1) gives:

$$[P] = v_s t + \frac{v_s - v_o}{k}(1 - e^{-kt}) \tag{2}$$

where [P] is the product concentration at any time and k is the apparent first-order rate constant for the interconversion between v_o and v_s.

The progress curves shown in Figure 4 can be described by eqn (2). Since the increase of k with [I] is linear, as shown in the insets of Figure 4, the slow-binding inhibition arises from a simple single-step interaction between E and I in which the rate of complex formation is low [43]. The fact that the initial velocity of hydrolysis is independent of the concentration of inhibitor confirms this single-step mechanism [44].

For this mechanism, k as function of [I] is given by the following equation, provided that [S] $\ll K_m$, under which these experiments were performed:

$$k = k_{-1} + k_{+1}[I] \tag{3}$$

Table 3   Kinetic parameters for the inhibition of *T. longibrachiatum* xylanase (XynI)

The inhibition kinetic assays were conducted with 4-MUX$_2$ as a substrate in McIlvaine buffer (pH 5.0) at 40 °C. The K_m for this substrate for *T. longibrachiatum* xylanase (XynI) is 577 $\pm$ 102 μM, the substrate concentration used was 22.7 μM and the enzyme concentration was 54.2 and 27.1 nM respectively for TLXI and rTLXI. Results are means $\pm$ S.E.M. for measurements in triplicate.

	K_i (nM)	k_{+1} (10^4 M$^{-1}\cdot$s^{-1})	k_{-1} (10^{-4} s^{-1})
TLXI	65.1 $\pm$ 7.3	0.51 $\pm$ 0.03	3.31 $\pm$ 0.56
rTLXI	38.7 $\pm$ 16.6	0.40 $\pm$ 0.06	1.54 $\pm$ 0.88

The slope is the association rate constant for inhibition (k_{+1}) and the intercept is the dissociation rate constant (k_{-1}). From these two values, an inhibition constant ($K_i = k_{-1}/k_{+1}$) can be calculated. All of these kinetic parameters are listed in Table 3.

Slow binding, or slow onset of inhibition, is a widespread phenomenon among non-protein glycosidase inhibitors [45]. For two non-cereal xylanase-inhibitor proteins, slow tight binding with xylanases has been described. In contrast with TLXI, they follow the two-step inhibition mechanism, with a rapidly formed initial collision complex, which isomerizes slowly to form the final tight complex [46,47]. The two-step inhibition mechanism is characterized by a hyperbolic increase of k with [I], instead of a linear increase as seen for TLXI.

Compared with the two known cereal xylanase-inhibitor proteins, TAXI and XIP, the association rate of TLXI is lower, while the dissociation occurs at a more similar rate. The SPR (surface plasmon resonance) data of TAXI-I, on the one hand, suggest that this is also a rather slow-binding inhibitor [48]. XIP, on the other hand, has been described as not slow binding, because an increase in pre-incubation time did not affect the inhibition activity and the SPR sensorgram did not exhibit the typical shape characteristic of the slow two-step interaction [49].

TLXI and rTLXI fulfil the requirements for tight-binding inhibition stated by Szedlacsek and Duggleby [44], i.e. that the enzyme concentration used in the study of the kinetic parameters and the K_i value are of the same order of magnitude (Table 3). However, the [I]/[E]$_{50}$ values for TAXI and XIP are smaller (i.e. 0.6 for both) than the one obtained for TLXI, indicating that TAXI and XIP form even tighter complexes [12,49].

Mode of interaction

To classify the type of inhibition of a time-dependent inhibitor, it is convenient to analyse the effect of various substrate concentrations on k at a fixed inhibitor concentration (Figure 5). When [S] approaches K_m, eqn (3) expands to eqn (4), (5) or (6), depending on whether the inhibition is competitive, uncompetitive or non-competitive respectively (these equations are derived with the assumption that E + S $\leftrightarrows$ ES equilibration is rapid relative to other rates) [50]:

$$k = k_{-1} + \frac{k_{+1}[I]}{1 + [S]/K_m} \tag{4}$$

$$k = k_{-1} + \frac{k_{+1}[I]}{1 + K_m/[S]} \tag{5}$$

$$k = k_{-1} + k_{+1}[I] \tag{6}$$
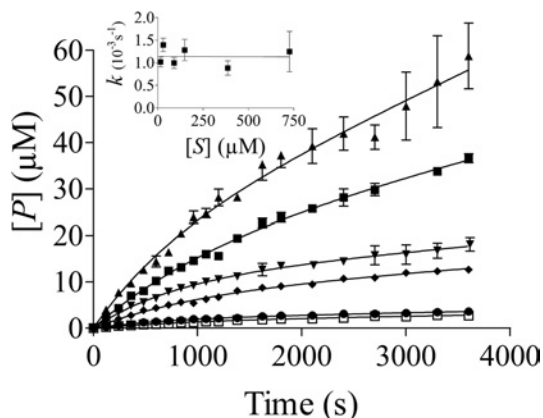
Figure 5   Time course of the inhibition with different substrate concentrations

For different quantities of 4-MUX$_2$ progress curves were plotted with *T. longibrachiatum* (Xyn I) xylanase (54.2 nM). The concentration of TLXI was 113 nM in all progress curves. The different concentrations were 727 (▲), 386 (■), 148 (▼), 90 (◆), 30 (●) and 15 (□) μM. The apparent first-order rate constants k for establishment of equilibrium were calculated from the progress curves and plotted against the substrate concentration, [S] (inset).

The relationship between [S] and k is shown in the inset of Figure 5. Since k is independent of [S], the inhibition of TLXI is non-competitive. The corresponding mechanism is shown in the following scheme:

$$E + S \underset{}{\overset{K_m}{\rightleftharpoons}} ES \longrightarrow P + E$$
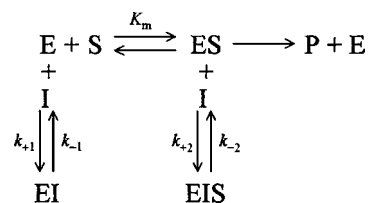
In this mechanism, K_{i1} ($= k_{-1}/k_{+1}$) equals K_{i2} ($= k_{-2}/k_{+2}$), and both will be replaced by K_i.

Thus the inhibition mechanism of TLXI is completely different from the mechanism of the other cereal xylanase inhibitors, as both TAXI and XIP are competitive inhibitors. The crystal structures of the complexes between xylanases and these inhibitors clearly show interactions in the active sites of the xylanases, for both inhibitors [13,16].

Inhibition constant

According to Cha [42], the steady-state velocity of a slow tight-binding inhibitor, following the non-competitive type of inhibition, is described by:

$$v_s = \frac{V_{max}[S]/K_m}{(1 + [I]/K_i)(1 + [S]/K_m)} \tag{7}$$

where V_{max} is the maximal velocity of the enzymatic reaction.

For TLXI and rTLXI respectively, K_i values of about 57.2 ± 5.2 and 25.7 ± 1.6 nM obtained from the progress curves with a fixed substrate concentration using eqn (7) agree well with the above-obtained K_i values (65.1 and 38.3 respectively). Again, the K_i of rTLXI is lower than the K_i of TLXI, although the [I]/[E]$_{50}$ values listed in Table 2 suggest the opposite. This indicates that the overestimation of the [I]/[E]$_{50}$ value made by measurements with the Xylazyme-AX method is bigger for rTLXI than for TLXI.

Another approach to determine the K_i for slow tight-binding inhibitors is the use of the Henderson equation, which accounts

for the depletion of both free inhibitor and free enzyme by binding. For non-competitive inhibition, the following equation for the IC$_{50}$ can be derived from the Henderson equation [42]:

$$IC_{50} = {}^1\!/_2[E] + K_i \tag{8}$$

where [E] is the total enzyme concentration.

The IC$_{50}$ value measured with 4-MUX$_2$ as substrate was 88.5 ± 0.4 nM. Using this equation, an inhibition constant for TLXI of 61.4 ± 4.0 nM was calculated, which is again in good agreement with the above-obtained results for the K_i of TLXI (65.1 and 57.2 nM).

From this IC$_{50}$ value, a [I]/[E]$_{50}$ value 1.6 ± 0.1 was calculated, which is more than 2.5-fold lower than the one determined with the Xylazyme-AX method, indicating that the substrate binding of TLXI is a significant factor in the determination of the inhibition activity, when a (arabino-) xylan-containing substrate is used.

In conclusion, the present paper describes a novel type of xylanase inhibitor from wheat. TLXI is the first TLP for which xylanase inhibition activity is described. We have demonstrated that both native TLXI and rTLXI, expressed in *P. pastoris*, are slow tight-binding inhibitors. In contrast with the two other cereal xylanase inhibitors, i.e. the competitive inhibitors TAXI and XIP, TLXI is a non-competitive inhibitor. Based on its xylanase specificity and its homology with TLPs, TLXI is believed to play a role in plant defence.

REFERENCES

1   Beliën, T., Van Campenhout, S., Robben, J. and Volckaert, G. (2006) Microbial endoxylanases: effective weapons to breach the plant cell-wall barrier or, rather, triggers of plant defense systems? Mol. Plant Microbe Interact. **19**, 1072–1081

2   Biely, P. (1985) Microbial xylanolytic systems. Trends Biotechnol. **3**, 286–290

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

4   Brito, N., Espino, J. J. and Gonzalez, C. (2006) The endo-β-1,4-xylanase Xyn11A is required for virulence in *Botrytis cinerea*. Mol. Plant Microbe Interact. **19**, 25–32

5   Simpson, D. J., Fincher, G. B., Huang, A. H. C. and Cameron-Mills, V. (2002) Structure and function of cereal and related higher plant (1 → 4)-β-xylan endohydrolases. J. Cereal Sci. **37**, 111–127

6   Debyser, W. and Delcour, J. A. (1997) Inhibitors of xylanolytic and β-glucanolytic enzymes. Eur. Pat. WO 98/49278

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

8   Hessing, M. and Happe, R. P. (2000) A novel class of xylanase inhibitors. Eur. Pat. EP0979830

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

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

11 Goesaert, H., Elliott, G., Kroon, P. A., Gebruers, K., Courtin, C. M., Robben, J., Delcour, J. A. and Juge, N. (2004) Occurrence of proteinaceous endoxylanase inhibitors in cereals. Biochim. Biophys. Acta **1696**, 193–202

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

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

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

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

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

17 Beliën, T., Van Campenhout, S., Van Acker, M. and Volckaert, G. (2005) Cloning and characterization of two endoxylanases from the cereal phytopathogen *Fusarium graminearum* and their inhibition profile against endoxylanase inhibitors from wheat. Biochem. Biophys. Res. Commun. **327**, 407–414

18 Igawa, T., Tokia, T., Kudo, T., Yamaguchi, I. and Kimura, M. (2005) A wheat xylanase inhibitor gene, XIP-I, but not TAXI-1 is significantly induced by biotic and abiotic signals that trigger plant defense. Biosci. Biotechnol. Biochem. **69**, 1058–1063

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

20 He, L., Bickerstaff, G. F., Paterson, A. and Buswell, J. A. (1993) Purification and partial characterisation of two xylanases that differ in hydrolysis of soluble and insoluble xylan fractions. Enzyme Microb. Technol. **15**, 13–18

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

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

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

24 Edge, A. S. B. (2003) Deglycosylation of glycoproteins with trifluoromethanesulphonic acid: elucidation of molecular structure and function. Biochem. J. **376**, 339–350

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

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

27 Nakai, K. and Horton, P. (1999) PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. Trends Biochem. Sci. **24**, 34–35

28 Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25**, 3389–3402

29 Selitrennikoff, C. P. (2001) Antifungal proteins. Appl. Environ. Microbiol. **67**, 2883–2894

30 Rebmann, G., Mauch, F. and Dudler, R. (1991) Sequence of a wheat cDNA encoding a pathogen-induced thaumatin-like protein. Plant Mol. Biol. **17**, 283–285

31 Hejgaard, J., Jacobsen, S. and Svendsen, I. (1991) Two antifungal thaumatin-like proteins from barley grain. FEBS Lett. **291**, 127–131

32 Reiss, E. and Horstmann, C. (2001) *Drechslera teres*-infected barley (*Hordeum vulgare* L.) leaves accumulate eight isoforms of thaumatin-like proteins. Physiol. Mol. Plant Pathol. **58**, 183–188

33 Reiss, E., Schlesier, B. and Brandt, W. (2006) cDNA sequences, MALDI–TOF analyses and molecular modelling of barley PR-5 proteins. Phytochemistry **67**, 1856–1864

34 Yu, X. M. and Griffith, M. (1999) Antifreeze proteins in winter rye leaves form oligomeric complexes. Plant Physiol. **119**, 1361–1369

35 Skadsen, R. W., Sathish, P. and Kaeppler, H. F. (2000) Expression of thaumatin-like permatin PR-5 genes switches from the ovary wall to the aleurone in developing barley and oat seeds. Plant Sci. **156**, 11–22

36 Schimoler-O'Rourke, R., Richardson, M. and Selitrennikoff, C. P. (2001) Zeamatin inhibits trypsin and α-amylase activities. Appl. Environ. Microbiol. **67**, 2365–2366

37 Koiwa, H., Kato, H., Nakatsu, T., Oda, J., Yamada, Y. and Sato, F. (1997) Purification and characterization of tobacco pathogenesis-related protein PR-5d, an antifungal thaumatin-like protein. Plant Cell Physiol. **38**, 783–791

38 Fils-Lycaon, B. R., Wiersma, P. A., Eastwell, K. C. and Sautière, P. (1996) A cherry protein and its gene, abundantly expressed in ripening fruit, have been identified as thaumatin-like. Plant Physiol. **111**, 269–273

39 Krebitz, M., Wagner, B., Ferreira, F., Peterbauer, C., Campillo, N., Witty, M., Kolarich, D., Steinkellner, H., Scheiner, O. and Breiteneder, H. (2003) Plant-based heterologous expression of Mal d 2, a thaumatin-like protein and allergen of apple (*Malus domestica*), and its characterization as an antifungal protein. J. Mol. Biol. **329**, 721–730

40 Trudel, J., Grenier, J., Potvin, C. and Asselin, A. (1998) Several thaumatin-like proteins bind to β-1,3-glucans. Plant Physiol. **118**, 1431–1438

41 Cooper, C. A., Gasteiger, E. and Packer, N. H. (2001) GlycoMod: a software tool for determining glycosylation compositions from mass spectrometric data. Proteomics **1**, 340–349

42 Cha, S. (1975) Tight-binding inhibitors-I: kinetic behavior. Biochem. Pharmacol. **24**, 2177–2185

43 Morrison, J. F. and Walsh, C. T. (1988) The behavior and significance of slow binding enzyme inhibitors. Adv. Enzymol. Relat. Areas Mol. Biol. **61**, 201–301

44 Szedlacsek, S. E. and Duggleby, R. G. (1995) Kinetics of slow and tight-binding inhibitors. Methods Enzymol. **249**, 144–180

45 Legler, G. (1990) Glycoside hydrolases: mechanistic information from studies with reversible and irreversible inhibitors. Adv. Carbohyd. Chem. Biochem. **48**, 319–384

46 Dash, C., Vathipadiekal, V., George, S. P. and Rao, M. (2002) Slow-tight binding inhibition of xylanase by an aspartic protease inhibitor. J. Biol. Chem. **277**, 17978–17986

47 Vathipadiekal, V. and Rao, M. (2004) Inhibition of 1,4-β-D-xylan xylanohydrolase by the specific aspartic protease inhibitor pepstatin. J. Biol. Chem. **279**, 47024–47033

48 Fierens, K., Gils, A., Sansen, S., Brijs, K., Courtin, C. M., Declerck, P. J., De Ranter, C. J., Gebruers, K., Rabijns, A., Robben, J. et al. (2005) His[374] of wheat endoxylanase inhibitor TAXI-I stabilizes complex formation with glycoside hydrolase family 11 endoxylanases. FEBS J. **272**, 5872–5882

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

50 Bakker, A. V., Jung, S., Spencer, R. W., Vinick, F. J. and Faraci, W. S. (1990) Slow tight-binding inhibition of prolyl endopeptidase by benzoyloxycarbonyl-prolyl-prolinal. Biochem. J. **271**, 559–562