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

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Thaumatin-like xylanase inhibitor from wheat

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Synopsis

Wheat (*Triticum aestivum*) contains a previously unknown type of xylanase (EC 3.2.1.8) inhibitor, which is described here for the first time. Based on its up to 60 % similarity with thaumatin-like proteins (TLPs) and the fact that it contains the Prosite PS00316 thaumatin family signature, it is referred to as thaumatin-like xylanase inhibitor (TLXI). TLXI is a basic ($pI \geq 9.3$ in isoelectric focusing) protein with a molecular mass of approximately 18 kDa (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 successfully expressed in *Pichia pastoris* resulting in a 21 kDa (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 non-competitive 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 approximately 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 hence represents a novel type of inhibitor within this group of proteins.

Introduction

The plant cell is protected from its surrounding environment by the cell wall, which forms a structurally heterogeneous barrier. In 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, E.C. 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 substituted 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) ([3] <http://afmb.cnrs-mrs.fr/CAZY/>). In both families, a pair of glutamate residues catalyzes 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]. Not only micro-organisms, but also plants produce xylanases. 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. During the past decade, studies revealed the presence of two types of proteinaceous xylanase inhibitors in cereals, i.e. the *Triticum aestivum* xylanase inhibitor (TAXI)-type [6, 7] and the xylanase inhibitor protein (XIP)-type inhibitors [8, 9]. These proteins have been purified and biochemically, genetically and structurally characterized.

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 ($pI \geq 8.0$) proteins which specifically inhibit GH11 xylanases [12]. Crystallographic analysis of a complex between a GH11 xylanase of *Aspergillus niger* and TAXI-I showed His³⁷⁴ 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 \geq 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]. In both cases, 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 thaumatin-like xylanase inhibitor (TLXI). 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, AVEVE, Landen, Belgium) whole meal was prepared using a Cyclotec 1093 sample mill (Tecator, Hogänäs, Sweden). All electrophoresis and chromatography media, and molecular mass and pI markers were from GE Healthcare (Uppsala, Sweden), 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 NV Puratos (Ir Filip Arnaut, Groot-Bijgaarden, Belgium). Two GH11 xylanases from *Trichoderma longibrachiatum* (also known as *T. reesei*), i.e. Xyn I and Xyn II, GH11 xylanases from *A. niger* and *T. viride*, and Xylazyme-AX tablets were from Megazyme (Bray, Ireland). GH10

xylanases from *A. oryzae* and *Penicillium purpurogenum* were kindly made available by VTT Biotechnology (Finland, Prof Maija Tenkanen, now Prof at University of Helsinki, Finland) and the ‘Laboratorio de Bioquímica’ (Prof Jaime Eyzaguirre, ‘Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Chile’), respectively. The Institute of Food Research (Dr Nathalie Juge, Norwich, United Kingdom) kindly provided a *Pseudomonas fluorescens* GH10 and a *Pen. funiculosum* GH11 xylanase. Thermophilic *Thermobacillus xylanilyticus* GH10 and GH11 xylanases were made available by INRA (Dr Michael O’Donohue, 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 (Brabrand, Denmark) and Quest International (Naarden, the Netherlands), respectively. The Pro-Q Emerald 300 Glycoprotein Stain Kit was from Invitrogen (Carlsbad, CA, USA). Thaumatin, oat spelt xylan, goat anti-rabbit horseradish peroxidase antibodies, substrate (3,3’,5,5’-tetramethylbenzidine) for the horseradish peroxidase, PNGase F and all other chemicals were from Sigma-Aldrich (Bornem, Belgium). Water soluble oat spelt xylan was prepared as described by He *et al.* [20]. 4-Methylumbelliferyl- β -D-xylobioside (4-MUX₂) was kindly provided by Dr Wim Nerinckx (Laboratory of Glycobiology, University of 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 (7.0 ml and 30.0 ml, respectively) according to Gebruers *et al.* [21].

Purification of xylanase inhibitors from wheat

Wheat whole meal (1.0 kg) was suspended in aqueous 0.1 % (w/v) L-ascorbic acid solution (5.0 l), extracted overnight at 7 °C and centrifuged (10000 g, 30 min, 7 °C). L-Ascorbic acid reduced the oxidation of phenolic compounds during the extraction. Calcium chloride (2.0 g/l) was added to the supernatant and the pH was raised to 8.5 by adding 2.0 M NaOH. The extract was left overnight at 7 °C and the resulting precipitate (containing pectins) was removed by centrifugation (10000 g, 30 min, 7 °C). The pH of the supernatant was adjusted

to 4.5 by adding 2.0 M HCl. Proteins with xylanase inhibition activity in the supernatant were retained by CEC on a SP Sepharose Big Beads column [180 x 130 mm, equilibrated with sodium acetate buffer (25 mM, pH 4.5)]. The bound protein fraction was eluted in one step with NaCl solution (1.0 M, 1000 ml), dialyzed against deionized water (48 h, 7 °C) and freeze-dried, resulting in the CEC fraction (approximately 7.76 g protein). Portions of the CEC fraction (6.0 g) were extracted with sodium acetate buffer (25 mM, pH 5.0, containing 0.2 M NaCl, 100.0 ml), centrifuged (10000 g, 30 min, 7 °C) and filtered through a paper filter. The filtrate was loaded on the *B. subtilis* xylanase affinity column [10 x 70 mm, equilibrated with sodium acetate buffer (25 mM, pH 5.0, containing 0.2 M NaCl), flow rate of 0.33 ml/min]. TAXI was eluted from the column with TRIS solution (250 mM, pH 12.0, 5.0 ml, flow rate of 1.0 ml/min) and immediately neutralized with acetic acid solution (1.0 M). The resulting run-through was used to isolate XIP and TLXI. Portions of the run-through (30 ml) were applied on the *A. niger* xylanase affinity column [16 x 150 mm, equilibrated with sodium acetate buffer (25 mM, pH 5.0, containing 0.2 M NaCl), flow rate of 1.0 ml/min]. This column was then eluted with buffers with increasing pH (250 mM TRIS, pH 10.0 to 12.0, 50.0 ml, flow rate of 3.0 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.

Protein sequencing

To determine the *N*-terminal amino acid sequence, inhibitor proteins (approximately 50 µg) were separated with SDS-PAGE in a 12 % polyacrylamide gel using the Hoeffer Mighty Small unit, electro blotted onto a PVDF membrane with the Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA) (electric potential difference of 10 V, 1 h, room temperature) and subjected to Edman degradation. Sequence analysis was performed on a Procise cLC 491 Sequencer (Applied Biosystems, Foster City, CA, USA). For internal amino acid sequence determination, TLXI (500 µg) was dissolved in 70 % formic acid (0.3 ml) containing a small amount of cyanogen bromide (two crystals, exact amount 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 TRIS-HCl buffer (25 mM, pH 8.0, 1.0 ml)

containing 2-mercaptoethanol (1.0 %) and kept in boiling water for 5 min. The peptides were separated with RP-HPLC on a Microsorb 300 Å C8 reversed phase column (Varian, CA, USA). Solvent A was MilliQ water with 0.1 % trifluoroacetic acid and solvent B was acetonitrile with 0.1 % trifluoroacetic acid. Separation was performed by applying a gradient from 2 to 100 % solvent B in 66 min (flow rate of 1.0 ml/min). The isolated polypeptides were subjected to Edman degradation and analyzed as described above.

Gene isolation and characterisation

Genomic DNA was isolated from young leaflets of wheat cultivar Estica using the DNeasy Plant Mini kit (Qiagen, Hilden, Germany). 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, Berlin-Buch, Germany). mRNA was purified from the total RNA with the Oligotex mRNA Mini kit (Qiagen). Primers were designed based on EST sequences BE399034 and BE427320 corresponding to the N-terminal sequence of native TLXI. RACE 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, respectively, whereas for 5'RACE and 5'nested RACE I5race (5'-TTGGTGGAGCACGAGCGCC-AC-3') and I5nested (5'-CCGGCCACACCGTGAAGTG-3'), respectively, were used for this purpose. For PCR on genomic DNA Iintf (5'-CAAGCGCGGCACCGCTCACCA-TC-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). Assembling of the obtained sequences (see Supplementary Figure S2) 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'-CACAGATCTGCACCGCTCACCATCACGAAC-3') and Ximatrstop (5'-CACAGATCTTCATGGGCAGAAGACGATC-TG-3') using *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA) 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 *Bgl*II fragment in the *Bsm*BI site of expression vector pPICZ α C (Invitrogen). The ligation mixture was used to transform *Escherichia coli* TOP10F' cells [F' {*proAB*, *lacI^q*, *lacZ* Δ M15, Tn10 (TetR)} *mcrA*, Δ (*mrrhsdRMS-mcrBC*), ϕ 80*lacZ* Δ M15, *AlacX74*, *deoR*, *recA1*, λ -*araD139*, Δ (*ara-leu*)7697, *galU*, *galK*, *rpsL*(StrR), *endA1*, *nupG*].

Expression and purification of rTLXI - A sequence verified pPICZ α C-*tlxi* construct, linearised with *Pme*I, 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 buffered minimal glycerol-complex medium (pH 6.0) supplemented with 0.35 M sodium chloride for 24 h (30 °C, shaking at 250 rpm). The volume was increased to 500 ml in a 2 l flask and incubated overnight under the same conditions. A preinduction transition phase was included in which 1:10 (v/v) of 10 % glycerol/13.4 % yeast nitrogen base was added to the primary culture. When the cells reached an optical density at 600 nm of 10-12, the cell culture was harvested by centrifugation at 2500 *g*. To induce protein expression, the cells were suspended in buffered minimal methanol-complex medium (pH 6.0) to an optical density (600 nm) of 50 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*. Prior to purification, the medium was dialysed overnight against sodium acetate buffer (25 mM, pH 5.0). rTLXI was purified with CEC using a SP-Sepharose Fast Flow column (10 x 200 mm) equilibrated with the same buffer. Bound proteins were eluted using a linear salt gradient of 0-1.0 M NaCl in 100 min (flow rate of 1.0 ml/min). Inhibition activity and purity of the resulting fractions were assessed with the Xylazyme-AX method 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 AU for 1 mg/ml TLXI (1.000 cm UV-cell path length).

Xylanase inhibition assay (Xylazyme-AX method)

Inhibition activities were determined with the colorimetric Xylazyme-AX method [23]. All xylanase solutions were prepared in sodium acetate buffer (25 mM, pH 5.0) with BSA (0.5 mg/ml) and contained 2.0 xylanase units per 1.0 ml. Xylanase units were defined as described by Gebruers *et al.* [23] and under the conditions of the assay, the xylanase concentration corresponding to 1.0 unit were 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 *Th. xylanilyticus* and 2.1 nM for the *Pen. 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. fluorescens*, 14.2 nM for the *Th. 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 the 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 the GE Healthcare separation technique file 100, using the PhastSystem unit with polyacrylamide gels containing ampholytes (pH 3-9). The 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 with SDS-PAGE in a 12 % polyacrylamide gel (cfr. supra). The glycan detection was performed in gel, using the Pro-Q Emerald 300 Glycoprotein Stain Kit, according to the instructions of the manufacturer (Invitrogen). To gain insight in the extent of glycosylation, TLXI was analyzed by electrospray ionization mass spectrometry on an Esquire-LC/MS system (Bruker, Bremen, Germany).

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 analyzed by SDS-PAGE followed by silver staining.

Chemical deglycosylation - TLXI and rTLXI were treated with anhydrous trifluoromethane sulfonic acid (TFMSA), 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 neutralised 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 in TFMSA solution (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-dry ice 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 analyzed by SDS-PAGE followed by silver staining.

Western blot and immuno-probing

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-MUX₂ was used as substrate. TLXI and 4-MUX₂ 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 Na₂HPO₄/0.1 M citric acid), pH 5.0]. The mixtures were incubated for different periods of time up to 90 min at 40 °C. The reaction was stopped by adding 15 µl of glycine-NaOH buffer (500 mM, pH 13.0) and putting the samples on ice. The hydrolysis products formed were quantified on a fluorimeter

(Horiba Jobin Yvon Inc, Edison, Florida, USA) based on a standard curve of 4-methylumbelliferone (0-6 μM). Excitation was at 360 nm and the emission was recorded at 446 nm, during 0.1 sec (5 trials, standard error < 1.0 %). Both monochromator slit widths were 1 nm. All measurements were performed in triplicate in 75 μl capillaries.

Binding to substrate

TLXI (9 μM in 25 mM sodium acetate buffer, pH 5.0, 1.0 ml) 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 (10 min, 15000 g) and the pellet was washed twice with buffer (25 mM sodium acetate, pH 5.0, 1.0 ml). The 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 (cfr. supra) and compared to the inhibition activity of the original TLXI sample. For insoluble xylan, supernatant and pellet were also analyzed by SDS-PAGE.

Results and discussion

Xylanase inhibitor purification

Starting from whole meal, 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 whole meal [9, 26]). The yield of TLXI was approximately 2.5 mg/kg whole meal. 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 EST 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, 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]). Approximately half of the mature protein sequence was confirmed by sequencing peptides of native TLXI obtained after cyanogen bromide cleavage. The sequence includes a potential glycosylation site at Asn⁹⁵ as well as 10 cysteine residues (Figure 2), which are probably involved in intramolecular disulfide bonds. The calculated molecular mass and pI of the mature protein were determined to be 15632.8 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 thaumatin-like proteins (TLPs) 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]. Up 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 [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 mainly found in cereals. The proteins in this group all contain 10 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 permeabilisation. 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). Subsequent purification yielded 4 mg pure rTLXI per litre culture medium.

Biochemical characterization

Native TLXI appears in SDS-PAGE under reducing as well as non-reducing conditions as a broad band at approximately 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 (result 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). 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 (result not shown). For native TLXI this was confirmed by mass spectrometry. Next to a peak at 15632.5 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 moiety are listed in Table 1. The peak with the highest mass corresponded to 16638.0 Da, a shift in molecular mass which could be accounted for by five sugar residues: two *N*-acetyl hexosamines (e.g. GluNAc, GalNAc), one 6-deoxy-hexose (e.g. fucose, rhamnose), one hexose (e.g. mannose, 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 appeared no longer 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 little 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), nor the GH10 xylanases were affected 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 the table. Since TLXI binds to xylan, present in the Xylazyme-AX tablets (cfr. infra), it is clear that the results have an indicative value only, implying that one should be careful when comparing these results to those of other inhibitors. However, they give a very good indication of the xylanase specificity of TLXI and rTLXI.

Its xylanase specificity further supports the assumption that TLXI plays a role in plant defence. Indeed, like TAXI, TLXI is only active 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].

Binding to substrate

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 to that in the original TLXI solution. Little if any inhibition 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 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. Xylan could hence 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, is 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-MUX₂ 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 is reached instantaneously. The establishment of the equilibrium between enzyme, inhibitor and enzyme-inhibitor complex occurs over a period of approximately 30 minutes. For rTLXI, similar progress curves were obtained, but the establishment of equilibrium took even longer than for TLXI, approximately 45 minutes (Figure 4 B). Therefore the time course for rTLXI was followed up to 90 minutes 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 :

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

Where v_s and v_o are the steady-state and initial velocities respectively and t is time [42].

Integration of eqn. 1 gives :

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

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 presented 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 slow [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.

$$k = k_{-I} + k_{+I} [I] \quad (3)$$

The slope is the association rate constant for inhibition (k_{+I}) and the intercept is the dissociation rate constant (k_{-I}). From these two values an inhibition constant ($K_i = k_{-I}/k_{+I}$) can be calculated. All 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 to 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 to the two known cereal xylanase inhibitor proteins, TAXI and XIP, the association rate of TLXI is lower, while the dissociation occurs at more similar rate. The Surface Plasmon Resonance (SPR) 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 preincubation 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 fulfill 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 an even tighter complexes [12, 49].

Mode of interaction – To classify the type of inhibition of a time-dependent inhibitor, it is convenient to analyze 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 $E + S \rightleftharpoons ES$ equilibration is rapid relative to other rates) [50].

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

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

$$k = k_{-1} + k_{+1} [I] \quad (6)$$

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 Scheme 1. 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 GH10 as well as the GH11 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 :

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

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

For TLXI and rTLXI respectively, values of about 57.2 ± 5.2 nM 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 's (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 Xylazym-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 inhibitor

concentration necessary to obtain 50 % inhibition, IC_{50} , can be derived from the Henderson equation [42].

$$IC_{50} = \frac{1}{2}[E] + K_i \quad (8)$$

where $[E]$ is the total enzyme concentration.

The IC_{50} -value measured with 4-MUX₂ 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 times 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, this work presents a novel type of xylanase inhibitor from wheat. TLXI is the first thaumatin-like protein for which xylanase inhibition activity is described. We demonstrated that both native TLXI, and recombinant TLXI, expressed in *P. pastoris*, are slow-tight binding inhibitors. In contrast to 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 thaumatin-like proteins, TLXI is believed to play a role in plant defense.

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Abbreviations

4-MUX₂: 4-methylumbelliferyl-beta-D-xylobioside; AX: arabinoxylan; CEC: cation exchange chromatography; EST: expressed sequence tag; GH: glycoside hydrolase family; IC₅₀: inhibitor concentration necessary to obtain 50 % inhibition; [I]/[E]₅₀: ratio of inhibitor concentration to enzyme concentration necessary to obtain 50 % inhibition; RACE: rapid amplification of cDNA ends; S.E.M.: standard error of mean; (r)TLXI: (recombinant) thaumatin-like xylanase inhibitor; TAXI: *Triticum aestivum* xylanase inhibitor; TFMSA: trifluoromethane sulfonic acid; TLP: thaumatin-like protein; XIP: xylanase inhibitor protein

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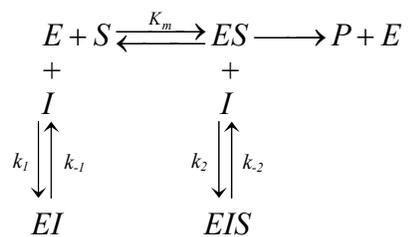
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Accession number

The nucleotide sequence data reported in this paper have been deposited in the GenBank2/EMBL/DDBJ Nucleotide Sequence Databases under the accession number AJ786602.

Schemes

Scheme 1



TABLES

Table 1 Mass spectrometry data and corresponding glycosyl moiety

The different masses obtained with mass spectrometry with their corresponding glycosyl entities as determined with GlycoMod [41].

Molecular mass	Glycosyl moiety
15632.5	-
15833.9	HexNAc
16040.5	HexNAc ₂
16185.5	HexNAc ₂ – Deoxyhexose
16347.0	HexNAc ₂ – Deoxyhexose – Hexose
16638.0	HexNAc ₂ – Deoxyhexose – Hexose – NeuAc

HexNAc = e.g. *N*-acetylglucosamine, *N*-acetylgalactosamine

Deoxyhexose = e.g. fucose, rhamnose

Hexose = e.g. galactose, mannose, glucose

NeuAc = *N*-acetyl neuramic acid (sialic acid)

Table 2 Inhibitor sensitivity of different GH10 and GH11 xylanases

[I]/[E]₅₀ values (ratio of inhibitor concentration to enzyme concentration necessary to measure 50 % inhibition) were determined with the Xylazyme-AX method, as described in the experimental section and are presented between brackets. Results are expressed means ± S.E.M. for 3 measurements.

	NCBI Accession	[I]/[E] ₅₀	
		TLXI	rTLXI
GH11 xylanases:			
<i>Trichoderma longibrachiatum</i> (Xyn I)	CAA49294	+++ (4.2 ± 0.2)	+++ (7.8 ± 1.2)
<i>Aspergillus niger</i>	CAA01470	++ (135.0 ± 2.5)	+ (1063 ± 19)
<i>Trichoderma viride</i>	CAB60757	++ (170.4 ± 1.1)	n.d.
<i>Thermobacillus xylanilyticus</i>	CAJ87325	+ (234.7 ± 50.1)	n.d.
<i>Penicillium funiculosum</i>	CAC15487	+ (289.6 ± 4.4)	n.d.
<i>Bacillus subtilis</i>	AAA22897	–	n.d.
<i>Trichoderma longibrachiatum</i> (Xyn II)	CAA49293	–	n.d.
GH10 xylanases:			
<i>Aspergillus aculeatus</i>	AAE69552	–	n.d.
<i>Aspergillus niger</i>	CAA03655	–	n.d.
<i>Aspergillus oryzae</i>	BAA75475	–	n.d.
<i>Penicillium purpurogenum</i>	AAF71268	–	n.d.
<i>Pseudomonas fluorescens</i>	CAA33469	–	n.d.
<i>Thermobacillus xylanilyticus</i>	CAA76420	–	n.d.

– = no inhibition at the highest inhibitor concentration

n.d. = not determined

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

The inhibition kinetic assays were conducted with 4-MUX₂ as a substrate in McIlvaine buffer (pH 5.0) at 40 °C. The K_m for this substrate for *T. longibrachiatum* xylanase (XynI) is $577 \pm 102 \mu\text{M}$, the substrate concentration used was $22.7 \mu\text{M}$ and the enzyme concentration was 54.2 and 27.1 nM respectively for TLXI and rTLXI. Results are expressed means \pm S.E.M. for measurements in triplicate. K_i is the inhibition constant, k_{+I} and k_{-I} are the rate constants for association and dissociation, respectively.

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

Figures

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 whole meal 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 analyzed in SDS-PAGE under non-reducing (lane 4) and reducing conditions (lane 5). The size of the molecular mass markers (lane 6) is indicated on the right side. The gel was silver stained.

Figure 2 Complete coding sequence of TLXI

The first methionine of the signal sequence (*italic*) is at position -26. The mature protein starts at position 1, stops at position 151 and contains 10 cysteine residues (**bold**) and one possible *N*-glycosylation site (**bold, italic**) at position 95. Parts of the amino acid sequence were confirmed by sequencing peptides obtained after cyanogen bromide cleavage of native TLXI (underlined). The Prosite thaumatin family signature PS00316 is shaded.

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 markers (lane 5) are indicated on the right side. The gels were silver stained.

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-MUX₂ was used as substrate (22.7 μM). The concentrations of enzyme were 54.2 nM and 27.1 nM respectively, for TLXI and rTLXI. The different concentrations of TLXI were 283 nM (▲), 212 nM (■), 177 nM (▼), 141 nM (◆), 82 nM (●) and 0 nM (□) and of rTLXI 200 nM (▲), 175 nM (■), 150 nM (▼), 125 nM (◆), 100 nM (●) and 0 nM (□).

The apparent first order rate constants k for establishment of equilibrium were calculated from the progress curves and plotted versus the inhibitor concentration $[I]$ (respective insets). The solid line indicates a linear correlation between $[I]$ and k .

Figure 5 Time course of the inhibition with different substrate concentrations

For different quantities of 4-MUX₂ 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 μM (▲), 386 μM (■), 148 μM (▼), 90 μM (◆), 30 μM (●) and 15 μM (□). The apparent first order rate constants k for establishment of equilibrium were calculated from the progress curves and plotted versus the substrate concentration $[S]$ (inset).

Figure 1

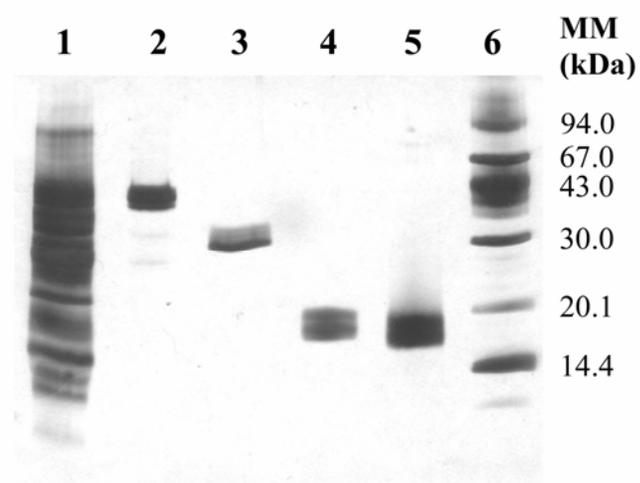


Figure 2

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-26 M A S P A R S A S A S P V L L L L V V L
-78 ATGGCGTCTCCAGCCAGAAGCGCGAGCGCCTCTCCAGTCCTCCTCTTGCTCGTTCCTC
-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 CCGGCGGTGGCGCTCGTGCTCCACCAAGGGGGCGGCGGCACCGAGCTCCACCCAGGGGCC
35 S W S L D T P V I G S Q Y I W G R T G C
102 AGCTGGAGCCTCGACACGCCGGTGATCGGCTCCCAGTACATATGGGGGCGCACGGGCTGC
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 ACTTGCGGGGGCAACCCGGCGGTGCCGGTGACCATGGCCGAGGTGTCCGTGCTCCAAGGC
95 N Y T Y G V T S T L K G F N L P M D L K
282 AACTATACCTACGGCGTCACGTTCGACGCTCAAGGGGTTCAACCTGCCCATGGACCTGAAG
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
    
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Figure 3

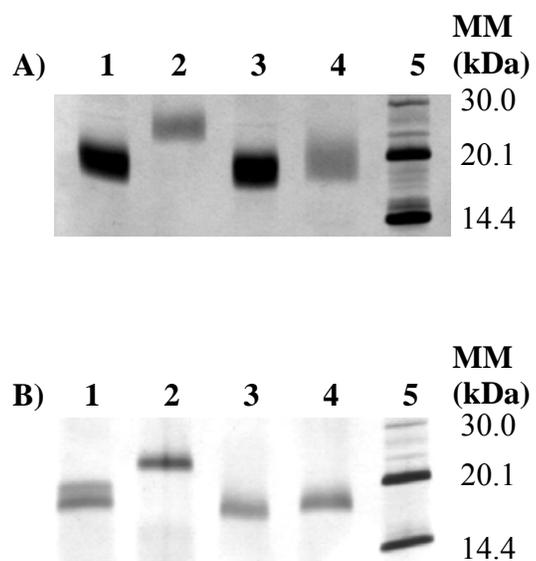
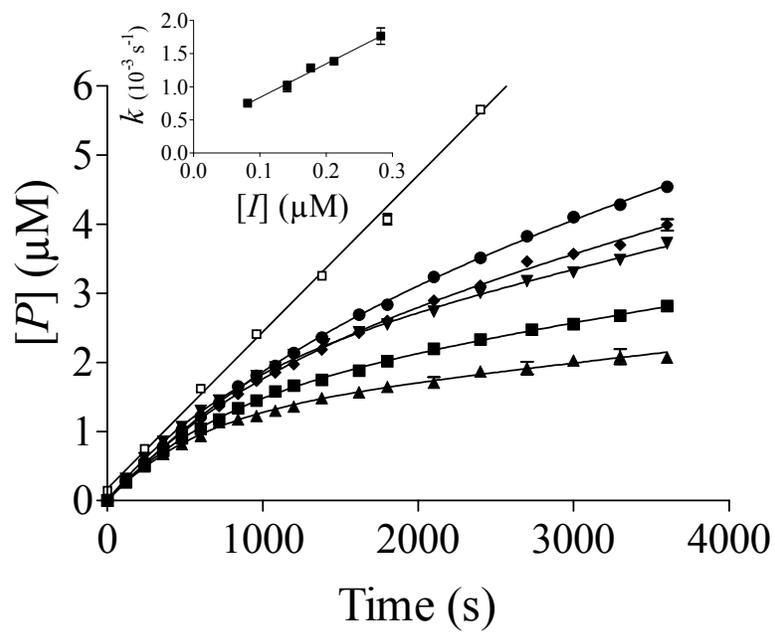


Figure 4

A



B

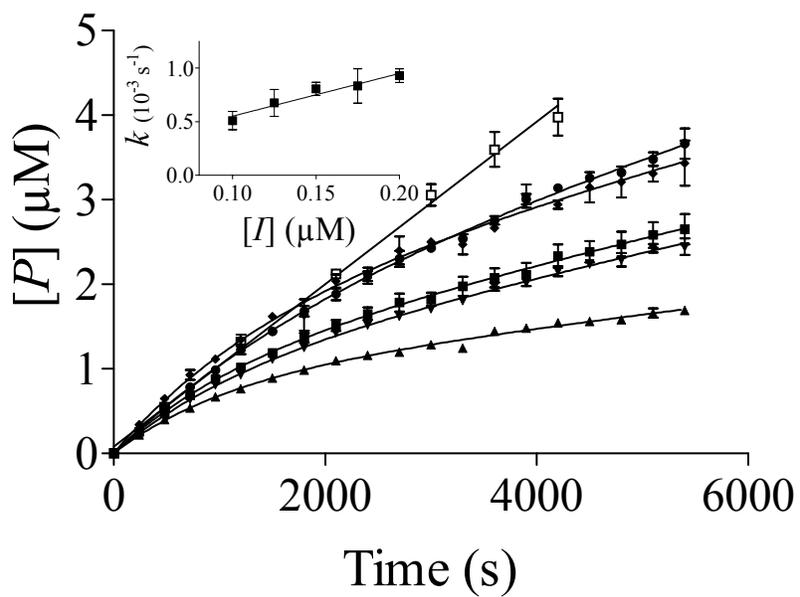


Figure 5

