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Molecular identification of wheat endoxylanase inhibitor TAXI-I, member of a new class of plant proteins

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Abstract ***Triticum aestivum*** **endoxylanase inhibitors (TAXIs) are wheat proteins that inhibit family 11 endoxylanases commonly used in different (bio)technological processes. Here, we report on the identification of the TAXI-I gene which encodes a mature protein of 381 amino acids with a calculated molecular mass of 38.8 kDa. When expressed in *Escherichia coli*, the recombinant protein had the specificity and inhibitory activity of natural TAXI-I, providing conclusive evidence that the isolated gene encodes an endoxylanase inhibitor. Bioinformatical analysis indicated that no conserved domains nor motifs common to other known proteins are present. Sequence analysis revealed similarity with a glycoprotein of carrot and with gene families in *Arabidopsis thaliana* and rice, all with unknown functions. Our data indicate that TAXI-I belongs to a newly identified class of plant proteins for which a molecular function as glycoside hydrolase inhibitor can now be suggested.**

Key words: Wheat; Xylanase inhibitor; Xylanase; Expressed sequence tag

1. Introduction

Endoxylanases (EC 3.2.1.8, further referred to as xylanases) are key plant or microbial enzymes in the degradation of xylan, the most abundant natural polysaccharide after cellulose, as well as of arabinoxylan, an important quality determining non-starch polysaccharide in cereals. Plant xylanases are the subject of recent work [1–3]. Microbial xylanases modify (arabino)xylan functionality in biotechnological processes such as bread-making [4], pasta processing [5], wheat gluten–starch separation [6] and paper and pulp production [7,8] as well as in animal feed preparations [9].

Our group previously discovered and partially characterized proteinaceous xylanase inhibitors in wheat (*Triticum aestivum*) [10,11] and denominated them TAXIs (for *T. aestivum* endoxylanase inhibitors) [12]. Wheat contains TAXI-I and TAXI-II protein types [13], which differ in enzyme inhibition specificity. Both types have similar structures and N-terminal amino acid sequences and occur in two forms, A and B. While form A consists of a 40 kDa single polypeptide chain with at least one intramolecular disulfide bond, form B is made up of two disulfide-linked subunits of 29 and 11 kDa. Glycosylation of TAXI-I and TAXI-II was not detected. TAXI-like xylanase inhibitors also occur in other cereals [14,15]. So far, our experimental results in general indicate that the TAXI-type inhibitors from different cereals [10–16] are active against glycoside hydrolase family 11 but not against family 10 xylanases [17].

Besides TAXI-like inhibitors, a second type of xylanase inhibitors, i.e. that of the XIPs (for xylanase inhibiting proteins), was purified from wheat [18] and rye [19]. XIPs are 29 kDa monomeric glycoproteins that show overall homology with concanavalin B and class III chitinases [20] but they do not exhibit chitinase activity. Their xylanase specificities differ from those of the TAXI-like proteins [18–20] in that they inhibit fungal but not bacterial xylanases irrespective of whether these belong to glycoside hydrolase families 10 or 11.

To date, the interaction mechanism between xylanases and their TAXI-like inhibitors is far from understood because of the novelty of the protein and the lack of TAXI protein structural information. We here report on the molecular identification of TAXI-I. Sequence similarity was found with a dermal glycoprotein of carrot and with multi-copy genes in rice and *Arabidopsis thaliana*, but not with functionally characterized proteins. Recombinant expression of the isolated TAXI-I encoding sequence provides conclusive evidence for the xylanase inhibition activity of this novel protein. Hence, TAXI-I belongs to a new class of plant proteins to which a function can be ascribed now.

2. Materials and methods

2.1. Peptide sequencing

TAXI-I (300 μg) was hydrolyzed with endoproteinase Lys-C according to the instructions of the supplier (Roche, Basel, Switzerland). The resulting peptides were reduced (1 h, 60°C) with 2-mercaptoethanol (1.0% v/v) and separated with high performance liquid chromatography on a Microsorb 300 Å C8 reversed phase column (Var-

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[1] The nucleotide sequence reported in this paper is available at the EMBL/GenBank/DDBJ databases (accession number AJ438880).

Abbreviations: TAXI, *Triticum aestivum* xylanase inhibitor; EST, expressed sequence tag; XIP, xylanase inhibiting protein; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; IPTG, isopropyl-1-thio-β-D-galactopyranoside; UTR, untranslated region

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ian, CA, USA). Solvent A was 0.1% (v/v) trifluoroacetic acid in water and solvent B 0.1% (v/v) trifluoroacetic acid in acetonitrile. The peptides were separated in a gradient of 0–100% solvent B in solvent A (66 min, 1.0 ml/min) and sequenced by automated Edman degradation with an Applied Biosystems 477 A Protein Sequencer, connected on-line with a 120 A phenylthiohydantoin-amino acid analyzer (Applied Biosystems, Foster City, CA, USA).

2.2. Molecular cloning

Genomic DNA was isolated from young leaves 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). 5′-Rapid amplification of cDNA ends (RACE) was performed on the isolated mRNA using the GeneRacer kit (Invitrogen, Carlsbad, CA, USA), with gene-specific primer TAXIN3 (CCCAGCGTCTTCGTGTCGTAG). HotStarTaq DNA polymerase (Qiagen) was used for the amplification of the 5′ cDNA ends with the GeneRacer 5′ primer and the gene-specific primer, TAXRACE (TGTACGGGTACGCCGTGCA). Polymerase chain reaction (PCR) on genomic DNA was conducted with primers TAXI5 (CAAGAAAGATGCCACCAGTG) and TAXI3 (GTAGTGGACGAATCCACCTGTC) for amplification of the complete TAXI-I coding sequence. PCR products were purified using the QIAquick PCR purification kit (Qiagen) prior to direct sequencing on a 377 DNA Sequencer using ABI PRISM Big Dye Terminator chemistry (Applied Biosystems).

2.3. Construction of expression plasmids

The DNA sequence encoding mature TAXI-I was amplified with primer combination XIF (CCAAGATCTCTTCCGGTGCTCGCTCCG)/XIR (CCTAGATCTTTACAGGCCGCCGCAACCCGTAAAG) and *Pfu* DNA Polymerase (Stratagene, La Jolla, CA, USA) using a TAXI5/TAXI3 PCR product as template. Both primers contain *Bgl*II restriction sites for cloning. The resulting PCR product was cloned in the pBAD/TOPO Thiofusion expression vector (Invitrogen), verified by DNA sequencing and subsequently subcloned as *Bgl*II fragment in expression vector pQE16-SSPelB. This vector is derived from the pQE16 vector (Qiagen) in which the mouse dehydrofolate reductase gene was replaced by the *Erwinia carotovora* PelB signal sequence [21] using the *Eco*RI/*Bgl*II restriction sites. The ligation mixture was used to transform electrocompetent *Escherichia coli* XL1-Blue MRF′ (Stratagene) cells. Sequence-verified pBAD/Thio TAXI-I and pQE16-SSPelB TAXI-I constructs were retained for expression experiments.

2.4. Expression of recombinant TAXI-I

E. coli TOP 10 (Invitrogen) and WK6 cells (genotype: Δ(*lac-pro*AB) *gal*E *str*A *nal*^{r} [F′ *tra*D36 *lac*I^q *lacZ*ΔM15 *pro*AB$^+$]) were respectively transformed with pBAD/Thio TAXI-I and pQE16-SSPelB TAXI-I. Small-scale expressions were carried out according to standard procedures [22]. For large-scale production of *E. coli* WK6 [pQE16-SSPelB TAXI-I], a batch fermentation (5 l) was carried out in a 6.6 l bioreactor (BioFlo 3000 Benchtop fermentor, New Brunswick Scientific, Edison, NJ, USA) inoculated with 50 ml of cells grown overnight at 37°C in Luria–Bertani medium with 100 μg/ml ampicillin (airflow: 1.5 volumes per volume of medium and per minute, and at 500 rpm agitation rate). To avoid excessive foaming, the culture was supplemented with 10.0 μl/l antifoam 289 (Sigma-Aldrich, Steinheim, Germany). The culture was kept at pH 7.0 by adding 3.0 M NaOH or 3.0 M HCl. The cell culture was grown at 37°C until an optical density of 0.6 at 600 nm was reached and subsequently induced by addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG, Promega, Madison, WI, USA) to a final concentration of 0.1 mM. Cells were further grown for 24 h at 25°C. Small samples were taken to measure the xylanase inhibition activities in both cell culture medium and periplasmic fraction. The latter was obtained by cold osmotic shock [22]. Xylanase inhibition activities were measured as described in Section 2.5. As a negative control, *E. coli* WK6 [pQE16-SSPelB] cells were identically processed.

2.5. Purification and characterization of recombinant TAXI-I

The 5 l fermentation broth was centrifuged (10 000 × *g*, 30 min, 4°C). The culture supernatant containing recombinant TAXI-I was dialyzed overnight at 4°C. Following centrifugation (10 000 × *g*, 30 min, 4°C), the supernatant was adjusted to pH 5.0 with 1.0 M HCl and concentrated on a SP-Sepharose Fast Flow column (Amersham Biosciences, Uppsala, Sweden), equilibrated with sodium acetate buffer (25 mM, pH 5.0). Bound proteins were eluted with 0.5 M NaCl and applied on a *Bacillus subtilis* xylanase affinity column as described earlier [23]. Pure recombinant TAXI-I was collected by elution with sodium phosphate buffer (200 mM, pH 11.8). The recovered fraction was instantly adjusted to pH 5.0 with 1.0 M acetic acid.

The inhibition activities against xylanases from *Aspergillus niger* (SWISS-PROT P55329, xylanase M4, Megazyme, Bray, Ireland) and *B. subtilis* (Puratos, Groot-Bijgaarden, Belgium) were determined with a variant of the colorimetric Xylazyme-AX method of Megazyme as described by Gebruers et al. [13]. One enzyme unit corresponds to an increase in absorbance at 590 nm of 1.0 under the conditions of the assay. One inhibition unit (IU) is the amount of inhibitor that, under the conditions of the assay, reduces the $A_{590\,nm}$ of one enzyme unit by 50% (to 0.5). SDS–PAGE and isoelectric focusing analysis of the recombinant inhibitor were performed as described earlier [13].

3. Results and discussion

3.1. Gene isolation and characterization

Enzymatic digestion, peptide purification and Edman degradation of purified natural TAXI-I protein yielded, besides the previously identified N-terminal amino acid sequences [13], novel amino acid sequences of three internal peptides. All sequences were used to screen *Triticeae* expressed sequence tag (EST) sequence databases with tBLASTn [24] yielding five matching wheat nucleotide sequences (Table 1). Assembly and manual editing resulted in a contiguous draft sequence of 1182 bp, corresponding to 95% of the mature TAXI-I protein as well as the 3′ untranslated region (UTR), but lacking the N-terminal coding part and 5′ UTR. Based on this draft sequence, 5′-RACE was performed on mRNA isolated from young wheat embryos (3 weeks post anthesis) with internal primers TAXIN3 and TAXRACE (Fig. 1). A PCR fragment of 413 bp allowed for the determination of the missing 5′ end of the TAXI-I cDNA. Another set of primers, TAXI5 and TAXI3, was designed for PCR amplification of the complete

Table 1
Wheat ESTs[a] matching identified TAXI-I peptides

GenBank Id.	Size (nt)	Tissue	Developmental stage	Similarity[b] (%)
BE430407[c]	780	n.s.	n.s.	99
BM140380	460	*F. graminearum* infected spike	adult plant	99
BE420158[c]	649	scutellum callus	n.s.	94
BE402285[c]	551	endosperm	8–12 days post anthesis	93
BE419727[c]	288	scutellum callus	n.s.	93
BQ171519	399	drought stressed leaf	full tillering	92
BF428535[c]	298	leaf	full tillering	89

[a]ESTs from GenBank release 128.
[b]FASTA scores [31] of encoding protein similarities.
[c]ESTs from GenBank release 121 used to build the TAXI-I draft sequence. n.s., not specified.

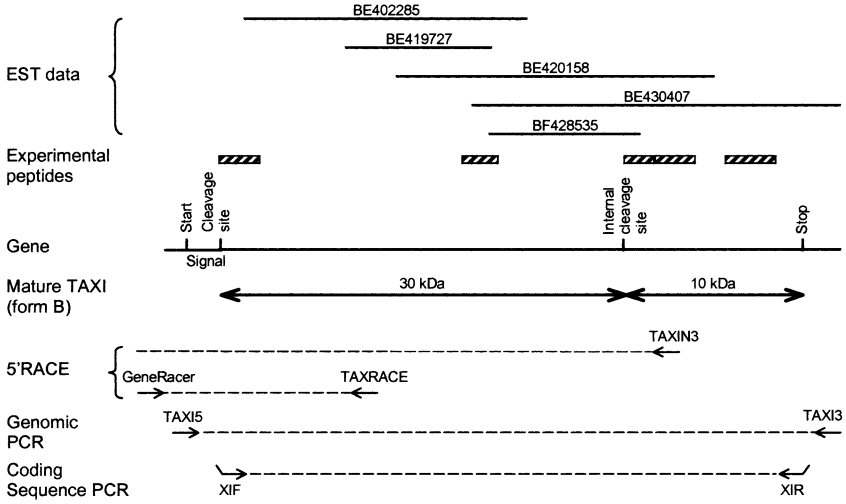

Fig. 1. Schematic overview of TAXI-I sequence data and relative positions of primers used for characterization and cloning. We refer to the text for explanation of the details.

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  1 ATCCACTTCCACACCCACAAACAATTCCACGCTCCATCTGTGCAAGAAAGatgccaccag 60
-21                                                M  P  P  V -18

 61 tgctcctcctcgtcctggccgcctcgctcgtggcgctgccgtcgtgccaaagccttccgg 120
-17  L  L  L  V  L  A  A  S  L  V  A  L  P  S  C  Q  S  L  P  V  3

121 tgctcgctccggtcaccaaggaccccgccacctccctctacacaatccccttccacgacg 180
  4  L  A  P  V  T  K  D  P  A  T  S  L  Y  T  I  P  F  H  D  G 23

181 gcgccagcctcgtcctcgacgtcgccggccctctcgtctggtccacgtgcgatggcggcc 240
 24  A  S  L  V  L  D  V  A  G  P  L  V  W  S  T  C  D  G  G  Q 43

241 agccgcccgcggagatcccgtgcagcagccccacctgcctcctcgccaacgcctaccccg 300
 44  P  P  A  E  I  P  C  S  S  P  T  C  L  L  A  N  A  Y  P  A 63

301 ccccgggctgccccgctcccagctgcggcagcgataagcacgacaaaccgtgcacggcgt 360
 64  P  G  C  P  A  P  S  C  G  S  D  K  H  D  K  P  C  T  A  Y 83

361 acccgtacaacccggtcagcggcgcgtgcgccgcagggagcctctcccacacgagattcg 420
 84  P  Y  N  P  V  S  G  A  C  A  A  G  S  L  S  H  T  R  F  V 103

421 tggccaacaccaccgacgggagcaagccggtgagcaaggtgaacgtcggggtcctggcgg 480
104  A  N  T  T  D  G  S  K  P  V  S  K  V  N  V  G  V  L  A  A 123

481 cgtgcgcgccgagcaagctcctggcgtcgctgccccggggctccacgggcgtggccgggc 540
124  C  A  P  S  K  L  L  A  S  L  P  R  G  S  T  G  V  A  G  L 143

541 tcgcgaactccggcttggcgctgccggcgcaggtggcatccgcgcagaaggtcgccaaca 600
144  A  N  S  G  L  A  L  P  A  Q  V  A  S  A  Q  K  V  A  N  R 163

601 ggttcctcctctgcctccccaccggcggccctggcgtggccatatttggcggcggcccgg 660
164  F  L  L  C  L  P  T  G  G  P  G  V  A  I  F  G  G  G  P  V 183

661 tcccgtggccgcaattcacgcagtcgatgccttacacgccgctcgtcaccaagggcggca 720
184  P  W  P  Q  F  T  Q  S  M  P  Y  T  P  L  V  T  K  G  G  S 203

721 gccccgcgcactacatctcggccaggtccattgtagtgggggacacccgcgtccccgtac 780
204  P  A  H  Y  I  S  A  R  S  I  V  V  G  D  T  R  V  P  V  P 223

781 cggagggcgcgctcgccaccggcggcgtgatgctcagcacgaggctaccctacgtcttgc 840
224  E  G  A  L  A  T  G  G  V  M  L  S  T  R  L  P  Y  V  L  L 243

841 tccgccccgacgtgtaccgcccgttgatggacgcgttcaccaaggccctggcggcgcagc 900
244  R  P  D  V  Y  R  P  L  M  D  A  F  T  K  A  L  A  A  Q  H 263

901 atgccaacggagcgcccgtggcgcgcgcagtggaggctgtggcgccgttcggggtgtgct 960
264  A  N//G  A  P  V  A  R  A  V  E  A  V  A  P  F  G  V  C  Y 283

961 acgacacgaagacgctgggcaacaacctcggcgggtacgcggtgcccaacgtccagctgg 1020
284  D  T  K  T  L  G  N  N  L  G  G  Y  A  V  P  N  V  Q  L  G 303

1021 ggctcgatggcggcagtgactggacgatgaccgggaagaactcgatggtggacgtcaagc 1080
304  L  D  G  G  S  D  W  T  M  T  G  K  N  S  M  V  D  V  K  Q 323

1081 aagggacggcgtgcgttgcgttcgtggagatgaagggagtggcggccggcgacggcaggg 1140
324  G  T  A  C  V  A  F  V  E  M  K  G  V  A  A  G  D  G  R  A 343

1141 cgccggcggtgatcctcggaggggcccagatggaggacttcgtgctcgacttcgacatgg 1200
344  P  A  V  I  L  G  G  A  Q  M  E  D  F  V  L  D  F  D  M  E 363

1201 agaagaagcggctcgggtttagcaggctgccgcactttacgggttgcggcggcctgTAAT 1260
364  K  K  R  L  G  F  S  R  L  P  H  F  T  G  C  G  G  L  *    381

1261 AATAAATCTGTTTAACGACAGGTGGATTCGTCCACTAC                      1298
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Fig. 2. Nucleotide and amino acid sequences of TAXI-I, including transcription start, signal sequence and 3′ UTR. Shaded sequences denote positions of identified TAXI-I peptides. The signal sequence is indicated in bold and the stop codon is marked by an asterisk. The internal cleavage site discerning forms A and B is indicated by a double slash.

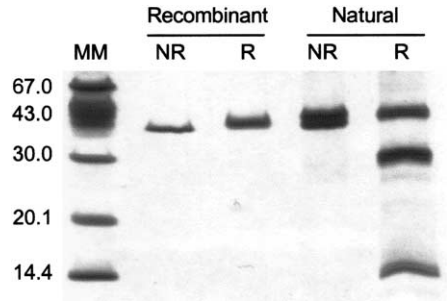

Fig. 3. SDS–PAGE of purified natural and recombinant TAXI-I. R: reduced; NR: non-reduced; MM: molecular mass markers (kDa).

gene coding sequence from wheat (var. Estica) genomic DNA (Fig. 1). Sequencing of PCR-amplified as well as cloned products revealed that the TAXI-I coding sequence is 1206 bp long and G/C-rich (68%). Aligning of the matching ESTs and the obtained cDNA and genomic sequences showed that the gene does not contain introns. The open reading frame encodes a protein of 402 amino acids (Fig. 2). Using the PSORT program [25] for protein localization prediction, the highest score was given to the possibility that TAXI-I is secreted outside the plant cell. The predicted cleavage site is situated after Ser-(−1) whereby Leu-1 corresponds to the experimentally determined N-terminus of mature TAXI-I. Hence, the open reading frame encodes a signal peptide of 21 amino acids followed by a mature protein of 381 amino acids (calculated molecular mass of 38.8 kDa) containing all experimentally determined peptides (Fig. 2). The calculated p*I* of the mature protein is 8.24 and therefore slightly less basic than the 8.8 value determined for the natural TAXI-I protein [13]. ScanProsite [26] indicates the presence of a potential *N*-glycosylation site at Asn-105 although no clear evidence for glycosylation was found for natural TAXI-I [13].

3.2. Occurrence of TAXI-like xylanase inhibitors

As outlined above, TAXI-like xylanase inhibiting proteins were found in barley and rye [14,15]. Screening of the available *Triticeae* EST sequences confirmed the occurrence of expressed TAXI orthologues in both cereals (GenBank BQ471743, BQ472163 and BE586568, release 129). So far, nothing is known about the regulation of TAXI gene expression. However, the wheat EST libraries suggest that the gene is expressed in different plant tissues at different stages of plant development (Table 1). TAXI also is expressed under plant stress conditions such as those induced by *Fusarium graminearum* infection and drought.

3.3. A new class of plant proteins

TAXI-I shows protein sequence similarity (45%) with an extracellular dermal glycoprotein (EDGP) [27] from carrot (GenBank BAA03413). The molecular function of EDGP remains to be elucidated but it is clear that the protein is specific to the epidermis and dermal tissues. The EDGP mRNA level increases rapidly in response to wounding which may indicate that EDGP is a plant defense protein [27]. In addition, the rice genome appears to have a cluster of eight uncharacterized genes on chromosome 1 (GenBank AP003269) with protein sequence similarities ranging from 59 to 72%, while two uncharacterized genes from *A. thaliana* chromosome 1 (GenBank AC005278) show protein sequence similarities of 53 and 56%. The products of the cited genes are annotated as putative EDGPs due to their similarity with EDGP. It may be speculated that the presence of duplicated TAXI-like genes in wheat, *A. thaliana* and rice broadens the inhibitory spectra of the translated proteins. Conserved domain searches [28] failed to find any motif or domain in the TAXI-I protein common to other known proteins. Hence, it is clear that TAXI-I belongs to a novel class of proteins commonly present in cereals and other plants.

3.4. Expression, purification and characterization of recombinant TAXI-I

To confirm that the identified gene encodes a xylanase inhibitor, the coding sequence was used for recombinant expression in *E. coli*. Some characteristics of the recombinant protein were then compared to those of the natural inhibitor protein.

The mature TAXI-I encoding sequence, 1143 bp long, was amplified by PCR from wheat genomic DNA using primers XIF and XIR (Fig. 1). The sequence was cloned into expression plasmid pQE16 that was modified for periplasmic protein secretion by insertion of the *E. carotovora* pectate lyase signal sequence [21]. Protein expression in *E. coli* is then controlled by the phage T5 promoter and induced by addition of IPTG. A 39.5 kDa protein was detected by SDS–PAGE, consistent with the size of the recombinant TAXI-I protein containing seven additional N-terminal linker-derived amino acids (ANTPGRS). Inhibition activity against xylanases from *A. niger* and *B. subtilis* confirmed the presence of active xylanase inhibitor proteins in the periplasmic fraction and the culture medium. Although most of the inhibition activity was found in the culture medium probably due to membrane leakage and cell lysis during expression, the recombinant protein expression level in the culture medium was unexpectedly low (an estimated 1.9 mg/l, based on specific inhibition activity measurements (see below)). It could not significantly be increased by using other periplasmic expression systems such as pHOS31 [29], pMALp2x (New England Biolabs) and pBADgIII (Invitrogen) (unpublished results). In contrast, cytoplasmic expression with the pBAD/TOPO Thiofusion expression vector resulted in high level expression and aggregation of inactive Thioredoxin–TAXI-I fusion proteins into inclusion bodies. Apparently, only the oxidizing conditions in the periplasm yielded soluble and properly folded active recombinant TAXI-I. This probably has to do with the presence of intramolecular disulfide bridges in the protein [13].

TAXI-I was concentrated from the culture supernatant by cation exchange chromatography and after a *B. subtilis* xylanase affinity purification step [23], pure recombinant TAXI-I was obtained. Both under reducing and non-reducing conditions, only one protein band was visible by SDS–PAGE (Fig. 3). This indicates that only the non-processed form A of TAXI-I was expressed. Form B of the natural inhibitor is therefore probably formed by a specific plant processing mechanism, the function of which remains unsolved. The experimentally determined p*I* of recombinant TAXI-I (∼9.0) is close to the ∼8.8 value of native TAXI-I [13]. The specific inhibition activity of recombinant TAXI-I against *A. niger* and *B. subtilis* xylanase was respectively 5260 IU/mg protein and 2500 IU/mg protein (as compared to 10 000 IU/mg protein and 5000 IU/mg protein for the natural inhibitor [13]).

During purification of the recombinant xylanase inhibitor, little or no loss of inhibition activity was detected. Furthermore, recombinant TAXI-I appeared stable for at least 2 h at room temperature in a pH 3.0–11.0 range. Xylanase inhibition activity was only partly reduced after 40 min at 70°C similarly to observations with natural TAXI-I.

3.5. Conclusions

The first TAXI-like xylanase inhibitor has been cloned, recombinantly expressed and biochemically characterized. Corresponding EST data from cereals show that TAXI genes are expressed in different tissues at different stages of plant development and under plant stress conditions. Furthermore, TAXI-like proteins occur in many plant species. Especially TAXI-I shows high similarity with EDGP, a suggested plant defense protein in carrot. We therefore can conclude that TAXIs belong to a novel class of plant proteins to which a function as plant protective microbial glycoside hydrolase inhibitor can now be ascribed. Knowledge of the primary protein structure and the availability of experimental amounts of the recombinant protein will help to resolve the three-dimensional structure of TAXI-I and that of its complexes with xylanases. The latter holds promise for the elucidation of the inhibitor–enzyme interactions essential for inhibition. Getting deeper insight in the inhibition mechanism is especially relevant for biotechnological applications where xylanases are used and where TAXI proteins have shown to have a considerable impact [12,30]. The availability of (mutant) TAXI-I proteins and xylanases with well defined inhibition characteristics in the future may eventually lead to a new generation of bio-active proteins with improved performance in feed and food applications (such as refrigerated doughs and breadmaking) and in (bio)technological processes (such as gluten–starch separation and paper/pulp processing).

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