FEBS Letters 519 (2002) 66–70 FEBS 26079

Functional identification of the cDNA coding for a wheat endo-1,4-β-D-xylanase inhibitor[1]

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Received 23 March 2002; accepted 28 March 2002

First published online 24 April 2002

Edited by Marc Van Montagu

Abstract Using expressed sequence tag data, we obtained a full-length cDNA encoding a wheat protein inhibitor of xylanases (XIP-I). The 822 bp open reading frame encoded a protein of 274 amino acids with a molecular mass of 30.2 kDa, in excellent agreement with the native protein. Expression in *Escherichia coli* confirmed that the cDNA encoded a functional endo-1,4-β-D-xylanase inhibitor. Its deduced amino acid sequence exhibited highest similarity to sequences classified as class III chitinases, but the inhibitor did not exhibit chitinase activity. This is the first full-length cDNA sequence that encodes a novel class of protein which inhibits the activity of endo-1,4-β-D-xylanases.

Key words: Xylanase; Protein inhibitor; Wheat; Expressed sequence tag; Heterologous expression; *Triticum aestivum*

1. Introduction

A novel class of protein inhibitors has recently been discovered that inhibited fungal but not bacterial xylanases (endo-1,4-β-D-xylanase, EC 3.2.1.8) [1,2]. This inhibitor protein, named XIP-I, was purified from wheat flour (*Triticum aestivum* var. Soisson) and partially characterised [1]. It is a monomeric, glycosylated protein with a p*I* of 8.7–8.9 and a molecular mass of 29.0 kDa. It inhibited reversibly, and in a competitive manner, family 11 xylanases [3] from *Aspergillus niger* and *Trichoderma viride* [1,2]. A second protein named *Triticum aestivum* xylanase inhibitor (TAXI) that exhibited inhibitory activity towards fungal and bacterial xylanases has also been isolated from wheat flour [4]. It was subsequently shown that two forms of TAXI were present in wheat (denoted TAXI-I and TAXI-II) [5]. TAXI-I and TAXI-II have molecular masses of approx. 40.0 kDa, are not glycosylated and occur in two molecular forms, i.e. a non-proteolytically processed one and a proteolytically processed one. However, TAXI-I and TAXI-II were distinguished by different inhibition activities towards various xylanases and different p*I* values (8.8 and ~9.3 respectively) [4]. The N-terminal amino acid sequences of TAXI-I and TAXI-II showed a high degree of identity, but there was no similarity to XIP-I.

The physiological function of these inhibitors is still unknown but it has been suggested that they play a role in plant defence [1,5]. In addition these inhibitors are of particular importance to industrial applications such as baking [4], wheat processing [6] brewing [7], or animal feed [8] where they hamper the efficacy of xylanases, which are added during the manufacturing process.

In this paper we identify the first full-length cDNA sequence encoding a xylanase inhibitor from wheat. We show that this sequence encodes a soluble and active recombinant protein when expressed heterologously in *Escherichia coli*.

2. Materials and methods

2.1. Strains and plasmids

E. coli DH5α (*supE*44, hsdR17, *recA*1, *endA*1, *gyrA*96, *thi*-1, *relA*1) was from Stratagene and BL21 (DE3) (F^-, *omp*T, *hsd*S_B, (r_B^-, m_B^-), *dcm*, *gal*, λDE3) from Novagen. The *E. coli* vector pGEM-Teasy was from Promega, pBluescript KS from Stratagene, and pET24a from Novagen. Both expressed sequence tags (ESTs) were cloned in the Lambda Uni-ZAP XR vector (Stratagene), provided by Dr. Olin Anderson (U.S. Department of Agriculture, Albany, USA) and excised to give pBluescript phagemids.

2.2. Peptide sequencing of native XIP-I

N-terminal amino acid sequencing was performed using automated Edman degradation at the John Innes Centre, UK. For internal sequencing, native XIP-I was first digested with trypsin [1].

2.3. Polymerase chain reaction (PCR)

Oligonucleotides were purchased from Sigma-Genosys. Nested-PCR using degenerate primers, based on the peptidic sequence data, was used to produce a nucleotide fragment corresponding to part of the sequence encoding XIP-I. First round amplification reactions were performed using the primers 5′-GBAACACBGGBCARGTSAC-3′ (F_n5) and 5′-ACVGTBGCVGTNAGRTG-3′ ($R_{ii}5$); second round reactions were performed using the primers 5′-GBCARGTSACBGTSTTCTG-3′ (F_n3) and 5′-TGNAGSGGGTTVCCSGG-3′ ($R_{ii}3$). Reaction mixtures (50 μl) for both first and second round reactions were in 10 mM Tris–HCl buffer, pH 9.0 containing 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% (v/v) Triton X-100, 5 μM tetramethylammonium chloride (Sigma), 0.2 mM each of dATP, dTTP, dGTP and dCTP and 5 U Taq DNA polymerase (Promega). Amplifications were carried out on a Model 2400 thermal cycler (Perkin Elmer). First round reactions contained 25 pmol F_n5 and $R_{ii}5$ and genomic DNA (250 ng) purified from shoots of 7 day old *T. aestivum* var. Soisson wheat using the DNEasy Plant Mini kit (Qiagen). PCR conditions were: 94°C for 2 min; 94°C for 1 min, 50°C for 1 min,

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[1] The XIP-I sequence has been submitted to the EMBL database under accession number AJ422119

Abbreviations: EST, expressed sequence tag; ORF, open reading frame; PCR, polymerase chain reaction; xylanase, endo-1,4-β-D-xylanase; XIP-I, xylanase inhibitor protein I; *xip*-I, cDNA encoding XIP-I; reXIP-I, recombinant XIP-I

PII: S0014-5793(02)02710-2

72°C for 3 min (30 cycles); 72°C for 10 min. Second round reactions contained 25 pmol of F_n3 and $R_{ii}3$ and an aliquot (2 μl) of the first round reaction as template. PCR conditions were: 95°C for 5 min; 95°C for 1 min, 52°C for 1 min, 72°C for 2 min (30 cycles); 72°C for 7 min. A 450 bp product (called XI47) was obtained, purified using a QIAquick gel extraction kit (Qiagen), subcloned into the pGEM-T Easy vector, and subjected to DNA sequencing.

The overlap extension PCR was performed to amplify the complete open reading frame (ORF) encoding XIP-I [9]. The 5′-end of XIP-I from the EST WHE1701-1704_I04_I04ZS (kindly donated by Dr Olin Anderson) was amplified by PCR with the primers 5′-ACCTGTCACTCATATGGGCGGGGGGAAAGAC-3′ (named *Nde*I 5′) and 5′-AGGGGCGGGGGACGCTCGGCTTG-3′ (named *Nde*I 3′). Reaction mixtures (100 μl) were in 20 mM Tris–HCl buffer, pH 8.8, containing 1.5 mM $MgCl_2$, 110 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% (v/v) Triton X-100, 0.1 mM each of dATP, dTTP, dGTP and dCTP, 1 mg/ml nuclease free BSA, 2.5 U *Pfu* DNA polymerase (Stratagene), 0.4 μg plasmid and 500 pmol of each primer. PCR conditions were: 94°C for 5 min; 94°C for 1 min, 42°C for 2 min, 72°C for 3 min (30 cycles); 72°C for 7 min. A PCR product of the expected size (360 bp) was obtained. The 3′-end of XIP-I from the EST WHE1205_C08_E15ZS (kindly donated by Dr Olin Anderson) was amplified by PCR with the primers 5′-ACTCCTACTTCGGCGGGTCCAAGCCGAG-3′ (named *Bam*HI 5′) and 5′-TCACTGTCCAGGATCCTTAGGCGTAGTAGTACTTGATC-3′ (named *Bam*HI 3′). The PCR conditions were as described above to yield a 540 bp product. Both PCR products, obtained from the first round of reactions, were gel-purified and subjected to a second round of PCR. Reaction mixtures (100 μl) were in 20 mM Tris–HCl buffer, pH 8.8, containing 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% (v/v) Triton X-100, 0.1 mM each of dATP, dTTP, dGTP and dCTP, 2 U Vent DNA polymerase (New England Biolabs), and 0.4 μg of each of the first round PCR products. PCR conditions were: 94°C for 1 min, 42°C for 2 min, 72°C for 3 min (five cycles), after which 500 pmol of the primers *Nde*I 5′ and *Bam*HI 3′ were added, and a further 25 cycles (under the same PCR conditions) were used to generate an 850 bp product. The PCR product was digested with *Nde*I/*Bam*HI, gel-purified, and cloned into pET24a. The final construct was referred to as pETXIP-I and transformed into BL21(DE3) for protein expression and sequencing.

2.4. Sequence analysis

DNA sequencing was carried out with an Applied Biosystems Model 373A automated DNA sequencer using dye termination chemistry at the University of Durham. Homology searches for nucleotide sequences against the EST database dbEST [10] were performed using BLAST [11] at the National Centre for Biotechnology Information (NCBI). Homology searches for protein sequences against the swall database was carried out using FASTA3 [12] at the European Bioinformatics Institute. Sequence data was analysed using the Genetics Computer Group Wisconsin Package version 10 (Accelrys).

2.5. Heterologous expression of XIP-I in E. coli

Luria–Bertani (LB) medium (50 ml) was inoculated with an overnight culture (0.5 ml) derived from a single colony of BL21(DE3)[pETXIP-I] or BL21(DE3)[pET24a]. The cultures were grown at 37°C (200 rpm) until A_{600nm} 1.0 and then transferred to 25°C. After addition of isopropyl-thio-β-D-galactopyranoside (Sigma; 1 mM final concentration) and incubation for 18 h at 200 rpm, cells were harvested by centrifugation (3000×*g*, 15 min, 4°C), resuspended in McIlvaine's buffer (0.1 M citric acid/0.2 M Na_2HPO_4, pH 5.5), sonicated (4×15 s on ice), and centrifuged (13 000×*g*, 15 min, 4°C). The soluble protein extracts (0.5 ml) were desalted on Sephadex G-25 columns (NAP-5; Amersham Pharmacia Biotech.) and tested for xylanase inhibitor activity.

2.6. Activity assays

Xylanase activity was determined using the 3,5-dinitrosalicylic acid (Sigma) method [13] with 1% w/v birchwood (1,4)-β-xylan (Fluka) at 30°C in McIlvaine's buffer. One unit of xylanase activity was defined as the amount of protein that released 1 μmol xylose per min at 30°C and pH 5.5.

Xylanase inhibitor activity was determined by measuring the activity of a family 11 xylanase from *Aspergillus awamori* var. *awamori* [14] in the presence and absence of XIP-I [1]. Reactions (in triplicate) containing *Aspergillus* xylanase (6 μl, 277 ng) alone, or pre-incubated (5 min, 30°C) with soluble protein extracts (94 μl) from transformed *E. coli*, were assayed for xylanase activity. One unit of xylanase inhibitor activity was defined as the amount of protein that caused a reduction in absorbance at 550 nm of 0.1 absorbance units at 30°C and pH 5.5.

Recombinant XIP-I (reXIP-I) was assayed for chitinase activity by measuring the release of Remazol Brilliant Violet 5R from dyed chitin at 35°C in McIlvaine's buffer [1,15].

2.7. Gel electrophoresis and immunoblotting

SDS–PAGE gel electrophoresis was carried out on 10% bis–Tris pre-cast NuPAGE gels (Invitrogen) with biotinylated marker proteins (New England Biolabs). Proteins were transferred to nitrocellulose membranes by semi-dry blotting (Bio-Rad) [16]. The blots were probed with a 5000-fold dilution of polyclonal antiserum raised in rabbits against XIP-I purified from wheat. Immunoreactive proteins were visualised using a horseradish peroxidase anti-rabbit secondary antibody (Sigma; 1:2000) together with the chemiluminescent detection reagents (ECL Plus Detection Kit, Amersham Pharmacia Biotech.). Isoelectric focusing gels were run using the Phast system (Amersham Pharmacia Biotech.).

2.8. Purification of native and reXIP-I

XIP-I was purified from wheat (*T. aestivum* var. Soisson) flour as previously described [1]. ReXIP-I was purified from an induced culture (500 ml) of BL21(DE3)[pETXIP-I]. Crude soluble protein extract in McIlvaine's buffer (10 ml) was exchanged into 20 mM Tris–HCl, pH 7.0 buffer on PD-10 columns (Amersham Pharmacia Biotech.) and loaded onto a Mono-Q HR 10/10 column equilibrated in the same buffer using an ÄKTA fast protein liquid chromatography system (Amersham Pharmacia Biotech.). Proteins were eluted at 1 ml/min with a linear gradient of 0–1 M NaCl in the same buffer. Fractions containing inhibitory activity were pooled (8 ml), dialysed extensively against McIlvaine's buffer, and concentrated to 0.5 ml using a Centricon-3 (Millipore). The sample was applied to a Superdex 75 10/30 column equilibrated in the same buffer, eluted at 0.5 ml/min, and fractions (1 ml) were assayed for inhibitory activity and analysed by Western blotting.

3. Results and discussion

3.1. Cloning of XIP-I full-length cDNA and sequence analysis

The overall strategy for obtaining a full-length cDNA corresponding to *T. aestivum* var. Soisson XIP-I involved (1) generation of a partial *xip*-I cDNA using PCR with degenerate primers (designed from peptide sequences obtained with highly purified XIP-I) and genomic DNA, (2) generation of a consensus sequence by alignment of EST sequences that were highly similar to the partial *xip*-I, and (3) PCR cloning of full-length *xip*-I from two overlapping ESTs that showed 100% identity with the partial *xip*-I cDNA and peptide sequences for native XIP-I.

We have previously reported the N-terminal sequence of the first 15 amino acids of native XIP-I purified from *T. aestivum* var. Soisson. From the purified protein we have now sequenced the first 50 amino acids (AGGKTGQVTVFWGRNKAEGSLREACDSGMYTMVTMSFLDVFGAN(C/G)KYHLD) as well as three internal peptides (1. NVYYGVAPVAQK, 2. GGPGKPLHLTATVR, 3. FYVGLTADDKSHQWVHPK). A 450 bp putative *xip*-I PCR product (XI47) was obtained using degenerate primers based on the N-terminal and internal amino acid sequences of the purified XIP-I. The XI47 DNA sequence was used as a probe to screen the EST databases at the NCBI. A total of 19 ESTs were obtained, which when aligned, produced a consensus sequence which showed high similarity with the deduced amino acid sequence of the XI47 cDNA fragment (Fig. 1). The N-terminal peptide and three internal peptides could be aligned

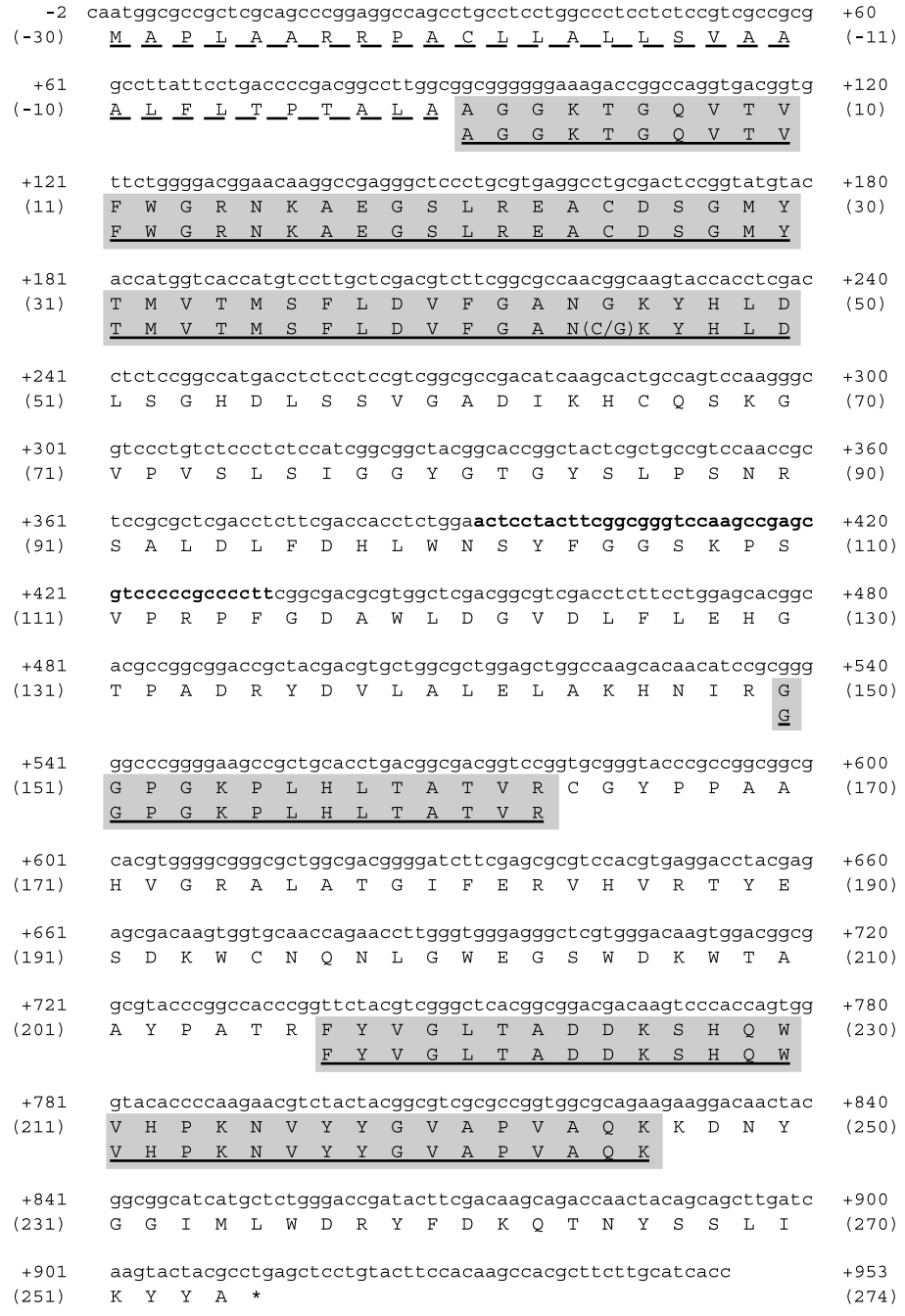

Fig. 1. Nucleotide and amino acid sequence of XIP-I, and alignment with XIP-I peptides. XIP-I peptides are underlined, identical residues are shaded, the signal peptide is indicated by a dashed line and the stop codon is denoted by an asterisk. The sequence in bold corresponds to the overlapping nt region of the two wheat ESTs (accession numbers: BE605137 and BE404812).

with the translated ORF of the consensus sequence with an overall identity of 99% over 94 amino acids (Fig. 1). Therefore this sequence was a very good candidate to represent the cDNA clone encoding XIP-I. This consensus sequence differed from the peptide data derived from native XIP-I by one amino acid (L37F). The complete nucleotide sequence of this cDNA consisted of 955 bp. It contained an ORF of 822 bp encoding a protein of 274 amino acids (Fig. 1). The predicted signal peptide was 30 amino acids long, in good agreement with the N-terminal sequence of the mature protein [1]. The calculated molecular mass of 30 222 Da was in excellent agreement with that of 30 657 Da obtained by matrix-assisted laser desorption time-of-flight mass spectrometry on purified XIP-I (Flatman et al., unpublished results). Native XIP-I has been reported to be weakly glycosylated [1], and the slight difference in molecular mass could be accounted to three sugar residues.

Analysis of the ESTs used in the construction of the consensus sequence identified two sequences (WHE1701-1704_I04_I04ZS, accession BE605137 and WHE1205_-C08_E15ZS, accession BE404812) that covered the entire consensus sequence and which overlapped by 30 bp (nt 392–434, Fig. 1). The 436 bp sequence of WHE1701-1704_I04_I04ZS was detected in a cDNA library from the whole spikes of heat

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XIP-I MAPLAARRPACLLALLSVAAALFLTPTALAAGGKTGQVTVFWGRNKAEGSLREACD  56
rice  MVALGRRSWLVPLAMV-LAVSSCLAGPAMAA-GKTGQMTVFWGRNKNEGTLKETCD  54
hevA  ~~~MAKRTQAILLLLLAISLI--MSSSHVDGGG----IAIYWGQNGNEGTLTQTCS  47
conB  ~~MGCERKALILMVVIWIMSFWTLSLADISSTE----IAVYWGQR-EDGLLRDTCK  49

XIP-I SGMYTMVTMSLLDVFGANGKY-HLDLSGH-------DLSSVGADIKHCQSKGVPVS 104
rice  TGLYTTVVISFYSVFG-HGRY-WGDLSGH-------DLRVIGADIKHCQSKNIFVF 101
hevA  TRKYSYVNIAFLNKFG-NGQTPQINLAGHCNPAAGG-CTIVSNGIRSCQIQGIKVM 101
conB  TNNYKIVFISFLDKFGCEIRKPELELEGVCGPSVGNPCSFLESQIKECQRMGVKVF 105

XIP-I LSIGGYGTGYSLPSNRSALDLFDHLWNSYFGGSKPSVPRPFGDAWLDGVDLFLEHG 160
rice  LSIGGAGKDYSLPTSKSAADVADNIWNAHMDGRRPGVFRPFGDAAVDGIDFFIDQG 157
hevA  LSLGGGIGSYTLASQADAKNVADYLWNNFLGGKSSS--RPLGDAVLDGIDFDIEHG 155
conB  LALGGPKGTYSACSADYAKDLAEYLHTYFLSERREG---PLGKVALDGIHFDIQKP 158

XIP-I TPADRYDVLALELAKHNIRGGPGKPLHLTATVRCGYPPAAHVGRALATGIFERAHV 216
rice  AP-DHYDDLARNLYAYNKMYRARTPVRLTATVRCAFPD-PRMKKALDTKLFERIHV 211
hevA  -STLYWDDLARYLSAYSKQG--KKVY-LTAAPQCPFPDR-YLGTALNTGLFDYVWV 206
conB  VDELNWDNLLEELYQIKDVY--QSTFLLSAAPGCLSPDE-YLDNAIQTRHFDYIFV 211

XIP-I RTYESDKWCNQN----LGWEGSWDKWTAA-YP-ATRFYVGLTADD--KSHQWVHPK 264
rice  RFYD-DATCSYNHAGLAGVMAQWNKWTAR-YP-GSHVYLGLAAANVPGKNDNVFIK 264
hevA  QFY-NNPPCQYSSGNINNIINSWNRWTTSI--NAGKIFLGLPAAPEAA-GSGYVPP 258
conB  RFY-NDRSCQYSTGNIQRIRNAWLSWTKSVYPRDKNLFLELPASQATAPGGGYIPP 266

XIP-I NVYYG-VAPVAQKKD-NYGGIMLWDRYFDKQTNYSSLIKYYA~~~~~~~~~~~~~~ 304
rice  QLYYD-LLPNVQKAK-NYGGIMLWDRFYDKQTGYGKTVKYWA~~~~~~~~~~~~~~ 304
hevA  DVLISRILPEIKK-SPKYGGVMLWSKFYDDKNGYSSSILDSVLFLHSEECMTVL~~ 311
conB  SALIGQVLPYLPDLQTRYAGIALWNRQADKETGYSTNIIRYLNATAMPFTSNLLKY 322

XIP-I ~~   -
rice  ~~   -
hevA  ~~   -
conB  PS 324
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Fig. 2. Multiple sequence alignments of XIP-I and related proteins. The EMBL accession numbers of rice class III chitinase, hevamine A, and concanavalin B are 049828, AJ007701, and X83426, respectively. Residues which are identical in all sequences are shaded.

stressed wheat (*T. aestivum* var. Chinese Spring) at 5, 10, 15 and 20 days after anthesis. The 562 bp sequence of WHE1205_C08_E15ZS was detected in a cDNA library from the roots of 5 day old aetiolated seedlings (*T. aestivum* var. Chinese Spring). Fusion of these two wheat EST sequences by PCR led to a full-length cDNA encoding XIP-I (EMBL accession number AJ422119). The signal sequence was not incorporated into the final product and the TGA stop codon was changed to TAA for protein expression purposes since the former can be mistranslated to tryptophan when expressed in *E. coli* [17]. Direct sequencing of the EST fusion cDNA (*xip*-I) revealed that the amino acid change (L37F) observed in the consensus sequence is not present in the actual cDNA, and that this change was in fact a sequencing error reported in the EST WHE1701-1704_I04_I04ZS. This also confirms that the cDNA is 100% identical to the peptide sequences derived from native XIP-I (Fig. 1).

3.2. Sequence alignment and structural homology

XIP-I showed overall homology with a number of class III chitinases (family 18 glycosidases [3]) from rice, pokeweed, apple, strawberry, grape, tobacco, maize, cowpea, the rubber plant (*Hevea brasiliensis*), and concanavalin B (a lectin from jackbean) [3] (Fig. 2). Among these sequences, only *H. brasiliensis* chitinase cDNA is known to encode a protein (hevamine A) exhibiting chitinase activity [18]. XIP-I shared 63, 47, and 42% similarity and 55, 36, and 33% identity with the rice class III chitinase [19], hevamine A [20], and concanavalin B [21] sequences, respectively.

Molecular modelling of XIP-I based on the known crystal structures of hevamine A [22] and concanavalin B [23], showed that all three proteins presented a typical 'TIM-barrel' [24] $(\beta/\alpha)_8$-fold (data not shown).

3.3. Functional expression in E. coli

Conclusive demonstration that the ORF included in the consensus sequence encoded XIP-I was obtained by heterologous expression in *E. coli*. Full-length *xip*-I cDNA was cloned into pET24a to give the expression construct pETXIP-I. The expressed protein lacked the signal peptide and was a non-fusion protein with no additional amino acids at the N- and C-termini. The soluble protein fraction obtained from a culture of *E. coli* transformed with pETXIP-I contained an immunoreactive polypeptide of M_r 29 kDa that co-migrated on SDS–PAGE gels with native XIP-I (Fig. 3) and also contained

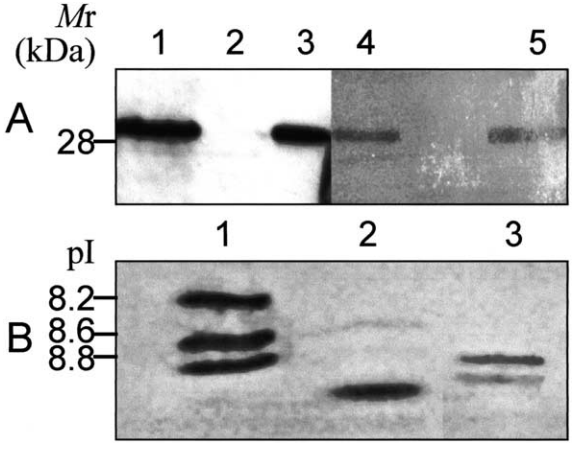

Fig. 3. Gel electrophoresis of reXIP-I. A: Western blot analysis of (lane 1) crude soluble protein extracts of *E. coli* BL21(DE3)[pET-XIP-I], (lane 2) crude soluble protein extracts of *E. coli* BL21(DE3)[pET24a], (lane 3) native XIP-I (20 ng) (lane 4) reXIP-I after purification by gel-filtration, (lane 5) native XIP-I (20 ng). B: Isoelectric focusing of (lane 1) p*I* markers (from lentil lectin; Sigma) (lane 2) partially purified reXIP-I, (lane 3) native XIP-I (25 ng).

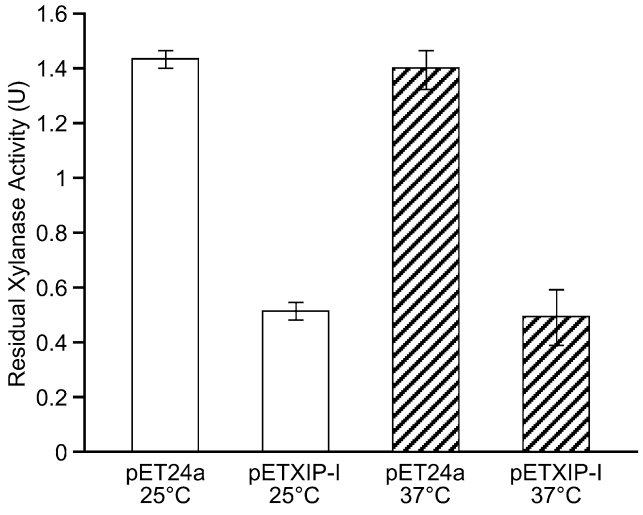

Fig. 4. Xylanase inhibitor activity of reXIP-I. Crude soluble protein extracts were prepared from the soluble fraction of *E. coli* BL21(DE3)[pET24a] and BL21(DE3)[pETXIP-I] cultures grown at 25°C for 18 h, or at 37°C for 3 h (see Section 2) and assayed for xylanase inhibitor activity using birchwood (1,4)-β-xylan as substrate and *A. awamori* xylanase as the target enzyme. The residual xylanase activity is shown for each fraction.

xylanase inhibitor activity (Fig. 4). No similar protein or activity could be detected from the corresponding fraction from a culture transformed with pET24a. The specific inhibitor activity of reXIP-I in the crude extract was 19 200 U/mg, very similar to that of 11 700 ± 1600 U/mg obtained with the native enzyme under the same conditions. This indicated that soluble, active, reXIP-I was expressed in *E. coli*. Isoelectric focusing showed that reXIP-I had an apparent p*I* value of 9.0, which was in excellent accordance with that of the native protein (Fig. 3). Two isoforms of p*I* 8.7 and 8.9 were detected for native XIP-I; the lower value was due to modifications upon storage or during the purification process (Flatman et al., unpublished results).

Analysis of reXIP-I in crude soluble protein extracts and after purification by gel-filtration, showed that the protein rapidly lost its biological activity, in contrast to native XIP-I, which was stable under the same conditions (no loss of inhibitor activity was detected after 48 h at 4°C). The loss in biological activity of reXIP-I could not be attributed to proteolysis of the protein during expression and/or purification since no lower M_r immunoreactive polypeptides were detected on immunoblots (Fig. 3). Moreover, reXIP-I expressed at 37°C for a shorter period of time (3 h) was also soluble, but its activity was similarly unstable (Fig. 4). The solubility of reXIP-I also suggested that the incorrect formation of disulphide bonds in the protein when expressed in the *E. coli* cytoplasm was not a reason for the instability. Moreover, the protein behaved similarly on gels in reducing and non-reducing conditions, as described for native XIP-I [1]. The presence of some degree of glycosylation (approx. 2% w/w) on native XIP-I was previously reported [1] and, taken together, the data suggest that glycosylation is involved in stabilising the activity of native XIP-I.

As previously found for native XIP-I [1], we could not detect any chitinase activity towards the insoluble substrate chitin azure for reXIP-I. These results suggest that XIP-I is a novel class of protein that may have evolved to inhibit xylanases rather than to function as a chitinase. This is particularly relevant to its physiological role in the wheat plant since the activity of plant chitinases are regulated in response to fungal elicitors [25], and they exhibit rapid evolution by acting as prime targets for the co-evolution of plant–pathogen interactions [26].

Acknowledgements: We would like to thank Dr Olin Anderson (U.S. Department of Agriculture, Albany, USA) for the wheat EST clones. This work was funded by the Biotechnology and Biological Sciences Research Council (UK) and Brewing Research International through a CASE studentship to G.O.E. (97/B2/D/03626) and the European Commission (GEMINI QLK1-2000-00811).

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