

cDNA cloning and heterologous expression of a wheat proteinase inhibitor of subtilisin and chymotrypsin (WSCl) that interferes with digestive enzymes of insect pests

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Abstract

A cDNA encoding the proteinase inhibitor WSCI (wheat subtilisin/chymotrypsin inhibitor) was isolated by RT-PCR. Degenerate oligonucleotide primers were designed based on the amino acid sequence of WSCI and on the nucleotide sequence of the two homologous inhibitors (CI-2A and CI-2B) isolated from barley. For large-scale production, *wsci* cDNA was cloned into the *E. coli* vector pGEX-2T. The fusion protein GST-WSCI was efficiently produced in the bacterial expression system and, as the native inhibitor, was capable of inhibiting bacterial subtilisin, mammalian chymotrypsins and chymotrypsin-like activities present in crude extracts of a number of insect larvae (*Helicoverpa armigera*, *Plodia interpunctella* and *Tenebrio molitor*). The recombinant protein produced was also able to interfere with chymotrypsin-like activity isolated from immature wheat caryopses. These findings support a physiological role for this inhibitor during grain maturation.

Keywords: cDNA cloning; *E. coli*; insect proteinases; proteinase inhibitor; recombinant expression; wheat proteinase.

Introduction

Plant protein inhibitors of proteinases have been widely investigated. Their possible endogenous function as storage compounds or as regulators of proteolytic enzymes has been extensively reviewed (Garcia-Olmedo et al., 1987; Richardson, 1991; Birk, 2003).

Since the first report on plant defense response to insect attack (Green and Ryan, 1972), many plant proteinase inhibitors have been shown to act as defensive molecules against phytophagous insects by inhibiting their gut proteinases (Ryan, 1990; Hilder et al., 1992). Evidence of such a defensive role was demonstrated by *in vitro* and *in vivo* assays carried out with native proteins (Shewry and Lucas, 1997) as well as by their recombinant forms expressed in a number of transgenic plants (Hilder and Boulter, 1999; Lawrence and Koundal, 2002).

Recently, we isolated and characterized a small monomeric protein from endosperm of hexaploid wheat (*Triti-*

cum aestivum, c.v. San Pastore) that has been classified as a member of the potato inhibitor I family of the serine proteinase inhibitors (Poerio et al., 2003). This protein, named WSCI (wheat subtilisin chymotrypsin inhibitor) on the basis of its strong interactions with mammalian chymotrypsins and with bacterial subtilisin, has a high content of essential amino acid residues (41 out of a total of 72). The levels of threonine and lysine account for approximately 7 and 8 mol%, respectively. This amino acid composition makes this inhibitor, or an engineered form of it, potentially useful for improving the nutritional quality of this cereal. Since WSCI is also effective in inhibiting chymotrypsin-like activities present in the mid-gut of some insect larvae (*Helicoverpa armigera*, *Plodia interpunctella* and *Tenebrio molitor*) (Poerio et al., 2003), it could also function as a wheat defense mechanism.

The concentration of WSCI in the aqueous protein extract of wheat endosperm is approximately 0.8–0.9% (w/w). This low content makes it difficult to obtain sufficient quantities of homogeneous protein to be used for characterization studies. In order to overcome this difficulty and to investigate WSCI physiological roles, we have expressed the mature protein in a prokaryotic system.

Results

Cloning of *wsci* cDNA

RT-PCR was performed using total wheat RNA isolated from immature caryopses and the two degenerate primers FW1 and RW1. These primers were designed on the basis of the amino acid sequence of the N- and C-terminus traits of purified WSCI, as well as on the nucleotide sequences of the cDNAs coding two highly homologous barley isoinhibitors: CI-2A (accession number X05404) and CI-2B (accession number X05405). The amplification product was then cloned in the pGEM-T easy vector. The resulting recombinant plasmid contained the entire nucleotide region (216 bp) encoding the native inhibitor WSCI (Figure 1). The accession number of the reported nucleotide sequence is AJ422055.

Expression and purification of the recombinant product

In order to express the proteinase inhibitor in *E. coli*, *wsci* cDNA was cloned in the pGEX-2T vector at BamHI and EcoRI sites. Bacterial cells, transformed with the recombinant plasmid pGEX-2T-*wsci*, expressed a fused protein product [glutathione S-transferase (GST)-WSCl] containing a cleavage site (Arg-Gly) specific for the proteolytic enzyme thrombin. SDS-PAGE analysis performed on cell

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ACGGACACGGGCGACCATCACAACCAGAAGACGGAGTGGCCAGAGTTGGTGGGGAAGTCG 60
T D T G D H H N Q K T E W P E L V G K S 18

GTGGAGGAGGCCAAGAAGGTGATTTGCAGGACAAGTCAGAGGCACAGATCGTAGTTCTA 120
V E E A K K V I L Q D K S E A Q I V V L 38

CCGGTGGGGACAATTGTGACCATGGAATATCGAATCGACCGTCCGCCTCTTTGTGAC 180
P V G T I V T M E Y R I D R V R L F V D 58

AGTCTCGACAAAATTGCCAGGTCCCCAGGGTCGGCTGA 219
S L D K I A Q V P R V G - 72

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Figure 1 Nucleotide sequence of the *wsci* cDNA.

The nucleotide sequences of the FW1 and RW1 primers are reported in bold italics. The deduced amino acid sequence of the wheat inhibitor is also reported (letters on gray background).

extracts obtained from uninduced and IPTG-induced bacterial cultures indicated (in the latter culture) the presence of an abundant protein band with electrophoretic mobility corresponding to a molecular mass of approximately 34 kDa (Figure 2, lane b). This mass value is consistent with that of the expected GST-WSCI fused product. The active recombinant protein was isolated from the cell extract and purified to homogeneity (Figure 2, lane c) by carrying out affinity chromatography on glutathione-Sepharose. The affinity step ensured a four-fold purification of the fusion protein and recovery of the inhibitory activity at close to 90%. In conclusion, 12 mg of pure GST-WSCI was obtained from 1 l of transformed induced *E. coli* culture.

Enzymatic cleavage of the recombinant GST-WSCI

Purified GST-WSCI was subjected to enzymatic hydrolysis with thrombin as described in the materials and methods section; under these conditions, approximately 20% of the fused protein remained uncleaved in the digestion mixture (Figure 2, lane d). The product of interest (the protein band with the highest electrophoretic mobility) was purified by submitting the digestion mixture to affinity chromatography on a glutathione-Sepharose column. The overall yield of the digestion and purification steps was approximately 50%. We named the recombinant inhibitor WSCI*; it has a molecular mass of 8270.39 ± 0.5 Da as determined by electrospray mass

spectrometry (ES-MS) analysis. Our recombinantly produced WSCI* contains two additional residues (Gly-Ser) at its N-terminus with respect to the native inhibitor. The presence of this extra dipeptide is due to the cloning site chosen, BamHI, as well as to the thrombin cleavage site specificity.

Isolation and partial characterization of chymotrypsin-like activities from insect and from wheat caryopses

Fourth-instar larvae of *T. molitor*, *H. armigera* and *P. interpunctella* were separately extracted as described under materials and methods. These extraction procedures yielded 23.5, 2.8 and 2.45 U of chymotrypsin-like activity, respectively. Immature wheat caryopses (10 g) yielded, under the extraction conditions described, 350 U of chymotrypsin-like activity, as defined in the materials and methods section.

The serine proteinase activities isolated from insect and from immature wheat caryopses were identified as chymotrypsin-like activities using *N*-benzoyl-L-tyrosine ethyl ester (BTEE) (as a synthetic substrate) and by testing their susceptibility to *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and *N*-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) reagents. The crude extracts of insect larvae contained both chymotrypsin-like and trypsin-like activities, whereas the extracts of immature caryopses did not contain TLCK-susceptible proteinases. The chymotrypsin-like activity found in both insect and wheat samples was fully inactivated by TPCK.

Inhibitory properties of the recombinant protein

The native WSCI, the fusion protein GST-WSCI and WSCI* exhibited similar behavior when tested against bovine pancreatic chymotrypsin or *B. subtilis* subtilisin (Table 1). As already reported for the native inhibitor (Poeiro et al., 2003), GST-WSCI and WSCI* were both very effective in inhibiting the bacterial subtilisin; the percentage of inhibition was a linear function of the amount of the recombinant inhibitors over the full activity range (0–100%). The extent of inhibition of pancreatic chymotrypsin by native WSCI, GST-WSCI and WSCI* was comparable: full inhibition of the bovine enzyme, in all three cases, was achieved at very high values of the I/E molar ratio. Appropriate controls excluded the influence of the GST portion on the inhibitory activity of the fused protein (data not shown). All the inhibition assays of the chymotrypsin-like activities (from insect and wheat extracts)

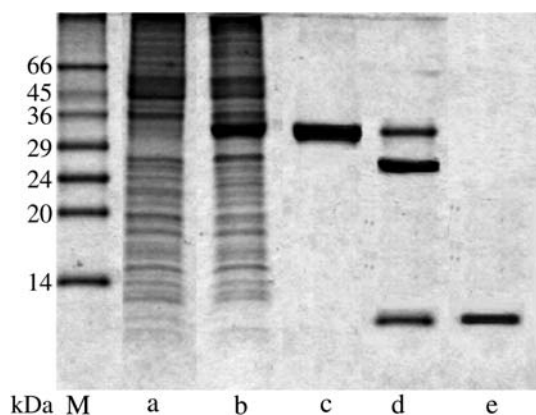


Figure 2 SDS-PAGE analysis of the recombinant product, GST-WSCI.

Lane M, markers; lane a, extract from uninduced cells; lane b, extract from IPTG-induced cells; lane c, purified GST-WSCI; lane d, thrombin digestion mixture of GST-WSCI; lane e, native WSCI (as control).

Table 1 Inhibitory effect of the recombinant GST-WSCI, native WSCI and WSCI* on the activity of bacteria subtilisin and pancreatic chymotrypsin.

	Inhibitor concentration (nM)	Subtilisin residual activity (%)	Inhibitor concentration (nM)	Chymotrypsin residual activity (%)
GST-WSCI	25	50.3±2.0	45	49.7±2.2
	50	0.89±0.6	2×10 ³	0.8±0.4
WSCI	24	51.0±1.6	47	51.5±1.8
	46.7	0.8±0.3	1.9×10 ³	0.3±0.1
WSCI*	26.7	50.7±1.8	48.3	50.7±2.3
	51.7	0.7±0.4	2.1×10 ³	0.5±0.2

Inhibition assays were carried out with BTEE as substrate (as described in materials and methods). In the assay mixtures the final concentrations of subtilisin and chymotrypsin were 53.3 and 18.7 nM, respectively. The residual activity values for the two proteolytic enzymes were obtained from three independent experiments.

were performed with samples of uncleaved recombinant GST-WSCI. This was based on the observation that although native WSCI and recombinant inhibitor WSCI* exhibited almost identical inhibitor activity, both were less effective (2.5-fold lower than GST-WSCI) when tested in an assay mixture containing crude extracts of insect larvae or of wheat caryopses. The chymotrypsin-like activities isolated from larvae of three insects (*H. armigera*, *P. interpunctella* and *T. molitor*) were all susceptible to the recombinant protein and exhibited similar inhibition patterns (Figure 3). Inhibition assays were carried out by incubating aliquots of insect extracts, each containing 1 U of chymotrypsin-like activity, with increasing amounts of the recombinant GST-WSCI. The inhibition of the insect proteolytic activities was not complete: the lowest residual activity values (31%, 29% and 37%, respectively) were detected with a GST-WSCI concentration of approximately 140 nM; these values remained substantially unchanged even using GST-WSCI at a concentration as high as 1000 nM (Figure 3).

GST-WSCI was also effective in inhibiting chymotrypsin-like activity isolated from developing wheat caryopses: approximately 30% inhibition was obtained by preincubating 1 U of wheat enzyme with GST-WSCI at a

concentration of 12 nM. The residual activity was a linear function of the amount of recombinant inhibitor in the range 100–30%. Inhibition of the wheat enzyme was not complete and residual activity of approximately 30% was obtained using GST-WSCI concentration values ranging from 30 up to 1000 nM (Figure 3).

Discussion

We have described here the molecular cloning and heterologous expression of a cDNA encoding a wheat subtilisin/chymotrypsin inhibitor classified as a member of the potato inhibitor I family (Poerio et al., 2003). Up until now, no nucleotide sequences encoding proteinase inhibitors belonging to this protein family have been identified in *Triticum aestivum*. In *Hordeum vulgare*, two different cDNAs coding for the inhibitors CI-2A and CI-2B, highly homologous (89%) to the wheat protein WSCI, have been isolated and investigated with regard to their expression in different tissues of the barley plant (Williamson et al., 1987). cDNAs coding for a number of proteins classified under the same inhibitor family have been isolated from a few more plant species, including *Zea mays* (Cordero et al., 1994), *Solanum tuberosum* (Cleveland et al., 1987), *Nicotiana glauca* (Criqui et al., 1992), *Lycopersicon esculentum* (Margossian et al., 1988) and *Lycopersicon peruvianum* (Wingate et al., 1989). However, these cDNAs, with the exception of that encoding the *Zea mays* inhibitor MPI, do not exhibit significant nucleotide sequence similarity with the *wsci* cDNA.

The inhibitor WSCI is present in the aqueous extract of wheat endosperm at a concentration of 0.8–0.9% (w/w). The purification procedure for this protein, although already reported, is time consuming and gives very low overall yield (approx. 6%). In order to produce pure active inhibitor for characterization studies, we decided to express *wsci* cDNA in *E. coli*. Attempts to express the recombinant unfused WSCI protein were unsuccessful. In fact, when *E. coli* strain BL21-DE3 was transformed with the recombinant vector pET-3a-*wsci*, we were unable to detect either the protein inhibitor or antichymotrypsin activity in both the soluble and insoluble fractions of bacterial cells. When the same bacterial strain was transformed with the recombinant vector pGEX-2T-*wsci*, we obtained the expression of a recom-

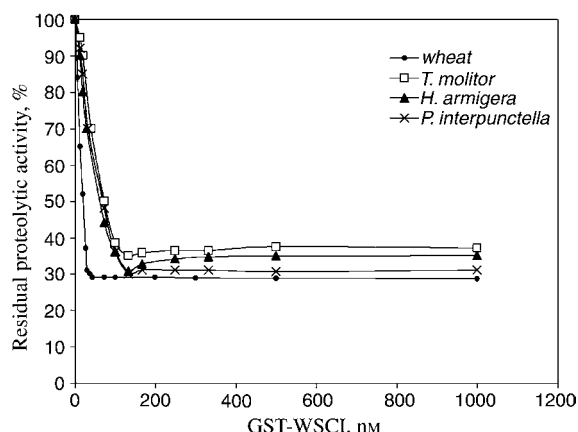


Figure 3 Effect of GST-WSCI concentration on chymotrypsin-like activities isolated from insect larvae extracts (*H. armigera*, *P. interpunctella* and *T. molitor*) and from immature wheat caryopses.

The inhibition assays were performed with 1 U (as defined in the materials and methods section) of chymotrypsin-like activity of each extract.

binant fused protein (GST-WSCI) that was fully active. The overall yield of this expression, including the purification step on the glutathione-Sepharose column, was 12 mg of recombinant fused product per liter of culture broth. Theoretically, when submitted to enzymatic hydrolysis with thrombin, this amount of recombinant product would yield approximately 3 mg of pure recombinant inhibitor WSCI*. However, in our hands and under the experimental conditions chosen, the thrombin digestion of GST-WSCI was not complete. In addition, chromatographic purification of the digestion mixture yielded a poor amount of pure active WSCI*.

Currently, we are working to improve the digestive yield of the recombinant GST-WSCI, as well as the purification of WSCI*. However, our data clearly show that the expression in *E. coli* of the recombinant GST-WSCI is a valid and efficient system to produce sufficient quantities of recombinant inhibitor to use in characterization studies.

The inhibitory activity of GST-WSCI, tested against pure commercial enzymes, was identical to that of the native WSCI and recombinant inhibitor WSCI* (Table 1). When tested with crude preparations of *H. armigera*, *P. interpunctella* and *T. molitor* larvae, GST-WSCI exhibited enhanced activity with respect to WSCI and WSCI*; such behavior could be due to improved stability of the WSCI portion of the fused product with respect to the action of a number of endo- and exo-peptidases, the presence of which has been detected in the midguts of the insects under study (Terra et al., 1996). Since GST is unable to inhibit chymotrypsin-like activities, we suggest that the GST portion of the fused GST-WSCI protein acts as a shield of the WSCI domain and minimizes its proteolytic degradation. The insect enzyme activities were not fully inhibited, even in the presence of a molar excess of the inhibitor GST-WSCI. This behavior could be due to the presence of proteolytic activities in the insect extracts that do not interact with GST-WSCI, but are capable of hydrolysis of the synthetic substrate BTEE. Such a conclusion is supported by several reports that underline the variety of proteinase activities identified in the midguts of a number of lepidopteran and coleopteran larvae (Terra et al., 1996; Bown et al., 1998; Patankar et al., 2001), as well as by data on incomplete inhibition of insect proteolytic enzymes by class-specific inhibitors (Purcell et al., 1992).

During this study we isolated a chymotrypsin-like activity from immature wheat caryopses that was susceptible to the recombinant GST-WSCI inhibitor, as well as to the native WSCI and WSCI*. Although the recombinant GST-WSCI was a more effective inhibitor than the other two molecules, inhibition of the wheat enzymes was not complete. This result could be explained by the presence of a number of proteinases in the wheat extracts, the complexity and specificity of which have been only partially clarified (Domínguez and Cejudo, 1996). Characterization of the wheat chymotrypsin-like activity is still under study; however, our observations already suggest that the WSCI inhibitor acts as a modulator of the chymotrypsin-like activity during seed maturation.

The study of enzyme/inhibitor systems (i.e., determination of E/I molar ratios and of the inhibition constant,

identification of the inhibitor reactive site, isolation and characterization of the E/I complex) requires discrete amounts of both protein partners. Thus, as already proposed by several authors (Di Giaimo et al., 2002; Ren et al., 2003), the GST-tagged WSCI could be used with affinity-based systems for the isolation and rapid purification of both target and related enzymes. *In vitro* screening with GST-WSCI, or with engineered forms thereof, may then be useful to identify (i) digestive proteinases present in the midguts of insect species attacking plants of agronomic interest and (ii) extracellular proteinases from phytopathogenic microorganisms that are inhibited by the recombinant wheat inhibitor. This investigation could represent a useful approach in plans to adopt insect and/or microorganism control strategies by transforming plants of interest with proteinase inhibitor genes such as *wsci* cDNA. The wheat inhibitor WSCI, with its capability of interacting with animal chymotrypsin-like activities and of suppressing proteinase activities of microorganisms such as *B. subtilis*, may represent a good candidate in designing plants exhibiting resistance to the attack of insects and microorganisms.

A number of genes encoding plant proteinase inhibitors have already been used to obtain transgenic plants with enhanced insect tolerance (Hilder and Boulter, 1999; Lawrence and Koundal, 2002). The retarded growth and increased mortality of insects fed with these transgenic plants have been attributed to a depletion of essential amino acids, which occurs as consequence of stimulated hypersecretion of susceptible digestive enzymes (Broadway and Duffey, 1986; Gatehouse et al., 1992). However, it must be said that transgenic plants expressing exogenous proteinase inhibitors do not guarantee the same levels of insect control as transgenic crops expressing *Bacillus thuringiensis* (Bt) δ -endotoxins. This poor insecticidal activity is due to their low stability (Schuler et al., 1998), as well as to the acquired resistance of some species of phytophagous insects that are able to change the composition of their digestive proteolytic enzymes (Jongsma et al., 1995). The cDNA encoding the wheat inhibitor WSCI might also be used in combination with other insect control-genes (i.e., Bt δ -endotoxin, lectins, α -amylase inhibitors, etc.) to obtain insect-resistant transgenic plants with enhanced defensive properties against pest attack. This strategy is based on the observation that exogenous proteinase inhibitors exert protective action towards other insect control-proteins by interfering with degradation processes activated by plant proteinases and insect digestive enzymes (MacIntosh et al., 1990; Newell et al., 1995; Zhang et al., 2000; Amirhusin et al., 2004).

Materials and methods

Plant material

Wheat seeds (*Triticum aestivum*, c.v. San Pastore) were originally supplied by Istituto Nazionale per la Cerealicoltura (S. Angelo Lodigiano, Milano, Italy) and reproduced both in the open field and in a greenhouse at the University of Tuscia (Viterbo, Italy).

RNA isolation

Developing caryopses (collected 20 days post-anthesis) were used as starting material for RNA extraction. Due to the high starch content in wheat grains, total RNA was purified by several steps of phenol/chloroform extraction (Prescott and Martin, 1987). RNA samples were analyzed by electrophoresis on formaldehyde-agarose gel and by monitoring $A_{260/280 \text{ nm}}$.

cDNA cloning

Degenerate oligonucleotide primers, FW1 (5'-ACN GAY ACN GGN GAC CAT CAC-3') and RW1 (5'-GCC GAC CCT GGG NAC YTG NGC-3'), were designed on the basis of the N- and C-terminal regions of the WSCI amino acid sequence (Poerio et al., 2003) according to the CODEHOP program (Rose et al., 1998). RT-PCR experiments were carried out with 50 pmol of each synthetic degenerate primer and 1 µg of total wheat RNA. The reverse transcription reaction was carried out at 50°C for 60 min in the presence of oligo-dT, using PowerScript™ reverse transcriptase purchased from BD Biosciences Clontech (Palo Alto, CA, USA). The reaction mixture was then submitted to 40 cycles of amplification (94°C for 30 s, 50°C for 30 s and 68°C for 40 s) and to an extension step for 3 min at 68°C. Following analysis on agarose gel, the amplified fragment was cloned into pGEM-T easy vector (Promega Italia S.r.l., Milano, Italy) and sequenced.

Nucleotide sequence analysis

Sequence analyses were carried out on both DNA strands and performed on automated apparatus (ABI Prism Genetic Analyzer; Applied Biosystems, Foster City, CA, USA).

Subcloning of *wsci* into *E. coli* expression vectors

To clone *wsci* into pGEX-2T expression vector (Amersham Biosciences), we designed two primers: the forward primer FBamHI (5'-GCG **GAT CCA** CGG ACA CGG GCG AC-3'), containing the BamHI restriction site at the 5'-end (in bold) and the reverse primer REcoRI (5'-CGG **AAT TCT** TAG CCG ACC CTG GGG-3') exhibiting the EcoRI restriction site at the 5'-end (in bold) and the stop codon (underlined). Following an amplification step, the product was directly digested and cloned into the expression vector. Alternatively, in order to obtain an unfused recombinant WSCI, the cDNA was cloned in pET-3a expression vector (Novagen, Merck Biosciences, Darmstadt, Germany). In this case the forward primer FNdel (5'-**CAT ATG** ACG GAC ACG GGC GAC-3') was designed on the basis of the first five codons of *wsci*. FNdel presented an extra nucleotide sequence at the 5'-end, containing both the Ndel restriction site (in bold) and the starting translation codon (underlined). The reverse primer RBamHI (5'-**GGA TCC** TAG CCG ACC CTG GGG-3') was designed on the basis of the 3'-region of the *wsci* sequence; this primer exhibited an extra nucleotide sequence at the 5'-end, containing both the BamHI restriction site (in bold) and the stop codon (underlined). The recombinant expression vectors (pGEX-2T-*wsci* and pET-3a-*wsci*) were employed to transform the *E. coli* BL21-DE3 strain.

Expression of WSCI recombinant protein

Both types of transformed bacterial cells were grown at 37°C in a culture medium (1 l of LB broth) containing 0.1 mg/ml ampicillin. When the culture absorption ($A_{600 \text{ nm}}$) reached a value of 1.0, the cells were induced with 1 mM isopropyl β-D-thiogalactoside (IPTG; final concentration) and allowed to grow for a further 3 h at 37°C. Bacterial cells were collected by low-speed centrifugation and resuspended at 6°C in 0.050 M Tris-HCl,

pH 8.0, containing 1 mM EDTA and 20% (w/v) sucrose. After 20 min, the suspensions were centrifuged and the recovered cells were washed twice (with 50 mM Tris-HCl, 1 mM EDTA, pH 8.0), sonicated and submitted to a centrifugation step. The proteins present in both supernatants were visualized by SDS-PAGE and compared with those extracted from uninduced *E. coli* control cultures.

Purification of GST-WSCI

The soluble protein fraction obtained from the *E. coli* pGEX-2T-*wsci* transformants was applied to a glutathione-Sepharose 4B (Amersham Biosciences) affinity column. After an appropriate number of washing steps, the fusion protein was eluted with reduced glutathione buffer, according to the manufacturer's instructions. The glutathione was then removed from the eluted GST-WSCI sample by ultrafiltration on a YM-3 Amicon membrane (nominal cut-off 3500 Da). The purity of the preparation was assessed by SDS-PAGE.

Cleavage of the fusion protein

An aliquot (1 mg) of the pure GST-WSCI sample was submitted to enzymatic hydrolysis with thrombin (Amersham Biosciences). Digestion was carried out in 0.050 M Tris-HCl, pH 8.0 for 16 h at 25°C. The enzyme/substrate ratio was 12 U of thrombin per mg of fusion protein. After digestion, the composition of the final mixture was analyzed by SDS-PAGE. The cleaved inhibitor, named WSCI*, differs from the native WSCI by an extra dipeptide at its N-terminus; it was separated by chromatography from both GST and uncleaved GST-WSCI.

Electrospray mass spectrometry (ES-MS)

The recombinant inhibitor WSCI* was analyzed by ES-MS using a Platform single-quadrupole mass spectrometer (Micromass, Manchester, UK). Aliquots (20 µl) of the sample solution (approx. 1 µg per 10 µl) were injected onto the ion source at flow rate of 10 µl/min. The mass spectrum was scanned from m/z 1000 to m/z 2200 at 10 s per scan, using a capillary voltage of 3.6 kV and a cone voltage of 40 V.

Isolation of chymotrypsin-like activities from insects

Insect larvae of the coleopteron *Tenebrio molitor* L. and of lepidoptera *Plodia interpunctella* (Hüb.) and *Helicoverpa armigera* (Hüb.) were kindly supplied by the Department of Difesa delle Piante (University of Tuscia, Viterbo, Italy). In a typical preparation, 0.2 g of midguts dissected from fourth-instar larvae of *T. molitor* and of *H. armigera* and 0.2 g of whole fourth-instar larvae of *P. interpunctella* were frozen in liquid nitrogen and homogenized with 0.8 ml of 80 mM Tris-HCl, 100 mM CaCl₂, pH 7.8. The suspensions were centrifuged at 12 000 g for 5 min at 6°C and the clear supernatants were applied to a PD-10 column (Amersham Biosciences). The samples eluted were tested for the presence of chymotrypsin-like activity and promptly used for inhibition assays. The proteolytic activities present in the insect extracts were also tested for their susceptibility to *N*-p-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and *N*-p-tosyl-L-lysine chloromethyl ketone (TLCK) reagents.

Isolation of chymotrypsin-like activity from developing wheat caryopses

Immature wheat caryopses (10 g), collected 20 days post-anthesis and immediately frozen in liquid nitrogen, were homogenized in a mortar with 15 ml of 50 mM Tris-HCl, 60 mM CaCl₂, pH 7.5. The suspension was gently stirred at 6°C for 1 h, then centri-

fuged at 10 000 *g* for 15 min. The clear supernatant was tested for the presence of chymotrypsin-like activity and for its susceptibility to TPCK and TLCK reagents. The active samples were promptly used for inhibition assays or stored for up to 3 days at -20°C or 6°C without significant loss of their chymotrypsin-like activity. After 8 days at -20°C or 6°C, the samples exhibited residual activity of 95% and 85%, respectively.

Determination of protein concentration

Protein concentration was determined with the Bio-Rad Protein Assay kit (Bio-Rad Laboratories S.r.l., Milano, Italy) following the manufacturer's instructions and using bovine serum albumin (BSA) as the standard.

Proteinase and anti-proteinase activity assays

Pancreatic bovine and porcine chymotrypsins (EC 3.4.21.1) treated with TLCK (Sigma-Aldrich S.r.l., Milano, Italy), subtilisins (EC 3.4.21.62) from *Bacillus subtilis* and *B. licheniformis* (Sigma-Aldrich), and chymotrypsin-like activities isolated from insects and from immature wheat caryopses were assayed in 80 mM Tris-HCl (pH 7.8), 100 mM CaCl₂ according to a procedure described previously (Poerio et al., 1989) and in the presence of 0.5 mM *N*-benzoyl-L-tyrosine ethyl ester (BTEE) (Hummel, 1959). By definition, 1 U of pancreatic chymotrypsin, bacterial subtilisin or insect chymotrypsin-like activity, is the amount of enzyme producing an increase in absorbance at 256 nm of 0.05/min under the assay conditions described above. Due to the very low levels of proteinase activity found in immature wheat caryopses, 1 U of wheat chymotrypsin-like activity has been defined as the amount of enzyme, under the assay conditions reported, responsible of an increase in absorbance at 256 nm of 0.008/min. The native WSCI, the recombinant fusion product (GST-WSCI) and the thrombin-cleaved recombinant fusion product (WSCI*) were tested for their inhibitory properties against all proteolytic enzymes/activities mentioned above. The inhibition assays were carried out by preincubating 1 U of each enzyme activity with increasing amounts of the inhibitor sample. Preincubation of the enzyme/inhibitor mixture was carried out for 2 min at 25°C in 80 mM Tris-HCl, 100 mM CaCl₂, pH 7.8. The residual enzyme activity was measured, after addition of the synthetic substrate, by following the increase in absorbance at 256 nm.

SDS-PAGE analysis

SDS polyacrylamide slab gel electrophoresis runs were performed according to a previously reported procedure (Schägger and von Jagow, 1987) using 16.5% acrylamide. Reference proteins were: bovine serum albumin, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsinogen, soybean trypsin inhibitor, and α -lactalbumin.

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References

- Amirhusin, B., Shade, R.E., Koiwa, H., Hasegawa, P.M., Bressan, R.A., Murdoch, L.L., and Zhu-Salzman, K. (2004). Soya cystatin N inhibits proteolysis of wheat α -amylase inhibitor and potentiates toxicity against cowpea weevil. *J. Econ. Entomol.* 97, 2095–2100.
- Birk, Y. (2003). Plant protease inhibitors: significance in nutrition, plant protection, cancer prevention and genetic engineering (Berlin, Germany: Springer-Verlag).
- Bown, D.P., Wilkinson, H.S., and Gatehouse, J.A. (1998). Midgut carboxypeptidase from *Helicoverpa armigera* (Lepidoptera: Noctuidae) larvae: enzyme characterization, cDNA cloning and expression. *Insect Biochem.* 28, 739–749.
- Broadway, R.M. and Duffey, S.S. (1986). Plant proteinase inhibitors: mechanism of action and effect on the growth and digestive physiology of larval *Heliothis zea* and *Spodoptera exigua*. *J. Insect Physiol.* 32, 827–833.
- Cleveland, T.E., Thornburg, R.W., and Ryan, C.A. (1987). Molecular characterization of a wound-inducible inhibitor I gene from potato and the processing of its mRNA and protein. *Plant Mol. Biol.* 8, 199–207.
- Cordero, M.J., Raventos, D., and San Segundo, B. (1994). Expression of a maize proteinase inhibitor gene is induced in response to wounding and fungal infection: systemic wound-response of a monocot gene. *Plant J.* 6, 141–150.
- Criqui, M.C., Plesse, B., Durr, A., Marbach, J., Parmentier, Y., Jamet, E., and Fleck, J. (1992). Characterization of genes expressed in mesophyll protoplasts of *Nicotiana glauca* before the re-initiation of the DNA replicational activity. *Mech. Dev.* 38, 121–132.
- Di Giaimo, R., Riccio, M., Santi, S., Galeotti, C., Ambrosetti, D.C., and Melli, M. (2002). New insights into the molecular basis of progressive myoclonus epilepsy: a multiprotein complex with cystatin B. *Hum. Mol. Genet.* 11, 2941–2950.
- Dominguez, F. and Cejudo, F.J. (1996). Characterization of the endoproteases appearing during wheat grain development. *Plant Physiol.* 112, 1211–1217.
- Garcia-Olmedo, F., Salcedo, G., Sanchez-Monge, R., Gomez, L., Royo, J., and Carbonero, P. (1987). Plant proteinaceous inhibitors of proteinases and alpha-amylases. In: *Oxford Surveys of Plant Molecular and Cell Biology*, Vol. 4, B.J. Mifflin, ed. (Oxford, UK: Oxford University Press), pp. 275–334.
- Gatehouse, A.M.R., Boulter, D., and Hilder, V.A. (1992). Potential of plant-derived genes in the genetic manipulation of crops for insect resistance. In: *Biotechnology in Agriculture No 7: Plant genetic manipulation for crop protection*, A.M.R. Gatehouse, V.A. Hilder and D. Boulter, eds. (CAB International, Wallingford, UK), pp. 155–181.
- Green, T.R. and Ryan, C.A. (1972). Wound-induced proteinase inhibitors in plant leaves: a possible defense mechanism against insects. *Science* 175, 776–777.
- Hilder, V.A. and Boulter, D. (1999). Genetic engineering of crop plants for insect resistance – a critical review. *Crop Prot.* 18, 177–191.
- Hilder, V.A., Gatehouse, A.M.R., and Boulter, D. (1992). Transgenic plants conferring insect tolerance: protease inhibitor approach. In: *Transgenic Plants*, S. Kung and R. Wu, eds. (New York, USA: Academic Press) pp. 317–338.
- Hummel, B.C.W. (1959). A modified spectrophotometric determinations of chymotrypsin, trypsin and thrombin. *Can. J. Biochem. Physiol.* 37, 1393–1399.
- Jongsma, M.A., Bakker, P.L., Peters, J., Bosch, D., and Stiekema, W.J. (1995). Adaptation of *Spodoptera exigua* larvae to plant proteinase inhibitors by induction of gut proteinase activity insensitive to inhibition. *Proc. Natl. Acad. Sci. USA* 92, 8041–8045.
- Lawrence, P.K. and Koundal, K.R. (2002). Plant protease inhibitors in control of phytophagous insects. *Electron. J. Biotechnol.* 5, 93–109.
- MacIntosh, S.C., Kishore, G.M., Perlak, F.J., Marrone, P.G., Stone, T.B., Sims, S.R., and Fuchs, R.L. (1990). Potentiation of *Bacillus thuringiensis* insecticidal activity by serine protease inhibitors. *J. Agric. Food Chem.* 38, 1145–1152.

- Margossian, L.J., Federman, A.D., Giovannoni, J.J., and Fischer, R.L. (1988). Ethylene-regulated expression of a tomato fruit ripening gene encoding a proteinase inhibitor I with a glutamic residue at the reactive site. *Proc. Natl. Acad. Sci. USA* 85, 8012–8016.
- Newell, C.A., Lowe, J.M., Merryweather, A., Rooke, L.M., and Hamilton, W.D.O. (1995). Transformation of sweet potato [*Ipomoea batatas* (L.) Lam.] with *Agrobacterium tumefaciens* and regeneration of plants expressing cowpea trypsin inhibitor and snowdrop lectin. *Plant Sci.* 107, 215–227.
- Patankar, A.G., Giri, A.P., Harsulkar, A.M., Sainani, M.N., Deshpande, V.V., Ranjekar, P.K., and Gupta, V.S. (2001). Complexity in specificities and expression of *Helicoverpa armigera* gut proteinases explains polyphagous nature of the insect pest. *Insect Biochem. Mol. Biol.* 31, 453–464.
- Poerio, E., Carrano, L., Garzillo, A.M., and Buonocore, V. (1989). A trypsin inhibitor from the water-soluble protein fraction of wheat kernel. *Phytochemistry* 28, 1307–1311.
- Poerio, E., Di Gennaro, S., Di Maro, A., Farisei, F., Ferranti, P., and Parente, A. (2003). Primary structure and reactive site of a novel wheat proteinase inhibitor of subtilisin and chymotrypsin. *Biol. Chem.* 384, 295–304.
- Prescott, A. and Martin, C. (1987). A rapid method for the quantitative assessments of levels of specific mRNA in plants. *Plant Mol. Biol. Rep.* 4, 219–224.
- Purcell, J.P., Greenplate, J.T., and Sammons, R.D. (1992). Examination of midgut luminal proteinase activities in six economically important insects. *Insect Biochem. Mol. Biol.* 22, 41–47.
- Ren, L., Chang, E., Makky, K., Haas, A.L., Kaboord, B., and Qoronfleh, M.W. (2003). Glutathione S-transferase pull-down assays using dehydrated immobilized glutathione resin. *Anal. Biochem.* 322, 164–169.
- Richardson, M. (1991). Seed storage proteins: the enzyme inhibitors. In: *Methods in Plant Biochemistry*, Vol. 5, L.J. Rogers, ed. (London, UK: Academic Press), pp. 259–305.
- Rose, T.M., Schultz, E.R., Henikoff, J.G., Pietrokovski, S., McCallum, C.M., and Henikoff, S. (1998). Consensus-degenerate hybrid oligonucleotide primers for amplification of distantly-related sequences. *Nucleic Acids Res.* 26, 1628–1635.
- Ryan, C.A. (1990). Protease inhibitors in plants: genes for improving defences against insects and pathogens. *Annu. Rev. Phytopathol.* 28, 425–449.
- Schägger, H. and von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 Da. *Anal. Biochem.* 166, 368–379.
- Schuler, T.H., Poppy, J.M., Kerry, B.R., and Denholm, I. (1998). Insect-resistant transgenic plants. *Trends Biotechnol.* 16, 168–175.
- Shewry, P.R. and Lucas, J.A. (1997). Plant proteins that confer resistance to pests and pathogens. *Adv. Bot. Res.* 26, 1351–1392.
- Terra, W.R., Ferreira, C., Jordão, B.P., and Dillon, R.J. (1996). Digestive enzymes. In: *Biology of the Insect Midgut*, M.J. Lehane and P.F. Billingsley, eds. (London, UK: Chapman and Hall), pp. 153–194.
- Williamson, M.S., Forde, J., Buxton, B., and Kreis, M. (1987). Nucleotide sequence of barley chymotrypsin inhibitor-2 (CI-2) and its expression in normal and high-lysine barley. *Eur. J. Biochem.* 165, 99–106.
- Wingate, V.P.M., Broadway, R.M., and Ryan, C.A. (1989). Isolation and characterization of a novel, developmentally regulated proteinase inhibitor I protein and cDNA from the fruit of a wild species of tomato. *J. Biol. Chem.* 264, 17734–17738.
- Zhang, J., Wang, C., and Qin, J. (2000). The interaction between soybean inhibitor and δ -endotoxin of *Bacillus thuringiensis* in *Helicoverpa armigera* larva. *J. Invertebr. Pathol.* 75, 259–266.

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