

WCI, a novel wheat chymotrypsin inhibitor: purification, primary structure, inhibitory properties and heterologous expression

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Abstract A novel chymotrypsin inhibitor, detected in the endosperm of *Triticum aestivum*, was purified and characterized with respect to the main physical–chemical properties. On the basis of its specificity, this inhibitor was named WCI (wheat chymotrypsin inhibitor). WCI is a monomeric neutral protein made up of 119 residues and molecular mass value of 12,933.40 Da. Automated sequence and mass spectrometry analyses, carried out on several samples of purified inhibitor, evidenced an intrinsic molecular heterogeneity due to the presence of the isoform [des-(Thr)WCI], accounting for about 40% of the total sample. In vitro, WCI acted as a strong inhibitor of bovine pancreatic chymotryp-

sin as well as of chymotryptic-like activities isolated from the midgut of two phytophagous insects, *Helicoverpa armigera* (Hüb.) and *Tenebrio molitor* L., respectively. No inhibitory activities were detected against bacterial subtilisins, bovine pancreatic trypsin, porcine pancreatic elastase or human leukocyte elastase. The primary structure of WCI was significantly similar (45.7–89.1%) to those of several proteins belonging to the cereal trypsin/ α -amylase inhibitor super-family and showed the typical sequence motif of this crowd protein group. The cDNA of the inhibitor (*wci-cDNA*) was isolated from wheat immature caryopses and employed to obtain a recombinant product in *E. coli*. Experimental evidences indicated that the recombinant inhibitor was localized in the inclusion bodies from which it was recovered as soluble and partially active protein by applying an appropriate refolding procedure. WCI reactive site localization, as well as its inhibitory specificity, was investigated by molecular modeling approach.

This paper was dedicated by Elia Poerio to the memory of Dr. Hiroshi Taniuchi, distinguished scientist of remarkable intellectual integrity.

The protein and nucleotide sequence data reported in this paper were deposited in the UniProt Knowledgebase and NCBI data bank under the following accession numbers: P83207 and AJ422078, respectively.

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Abbreviations

BTEE	<i>N</i> -Benzoyl-L-tyrosine ethyl ester
CNBr	Cyanogen bromide
DTT	Dithiothreitol
IPTG	Isopropyl- β -D-1-thiogalactopyranoside
MALDI-TOF	Matrix-assisted laser desorption/ionisation-time of flight
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
SDS	Sodium dodecyl sulphate
TAME	<i>N</i> - α - <i>p</i> -Toluene-sulphonyl-L-arginine methyl ester

TFA	Trifluoroacetic acid
TLCK	<i>N</i> - <i>p</i> -tosyl-L-Lysine chloromethyl ketone
TPCK	<i>N</i> - <i>p</i> -tosyl-L-Phenylalanine chloromethyl ketone

Introduction

Plant seeds contain several proteins that, on the basis of a variety of criteria, have been grouped into different classes. The most commonly used classification identifies the seed proteins as storage, structural and biologically active proteins specialized in a multitude of physiological functions (Shewry 1995). Among the biologically active proteins, otherwise referred to as housekeeping proteins, a consistent number shows inhibitory properties towards a variety of enzymes. The investigations carried out on these inhibitors covered several aspects: structural and biological features, action mechanism, classification, relative presence and abundance in different organs and tissues, anti-nutritional effects and allergenic properties, roles of plant defense against pathogens, cancer prevention, and the use of their genes for plant transformation (Garcia-Olmedo et al. 1987; Richardson 1991; Bode and Huber 1992; Ussuf et al. 2001; Birk 2003; Rawlings et al. 2004a).

The plant protein inhibitors (of enzymes) comprise a very large number of proteins, including amylase inhibitors (AIs) and protease inhibitors (PIs); the latter being one of the most abundant and investigated group of plant proteins. Since the first report on a soybean Bowman–Birk inhibitor (Bowman 1948), several PIs have been isolated from organs and tissues of plants belonging to different botanical families, although most of them have been detected and isolated from Leguminosae and Gramineae. Plant PIs have been grouped into various families on the basis of their chemical–physical and biological properties; more than ten plant PIs families, specific for each of the four mechanistic classes of proteases (serine-, cysteine-, aspartic- and metallo-proteases), have been described (Garcia-Olmedo et al. 1987; Ryan 1990; Birk 2003). Among these families, the so-called “cereal trypsin/ α -amylase inhibitors” represents a crowded heterogeneous protein group including α -amylase inhibitors, protease inhibitors, bi-functional inhibitors, and storage proteins with no apparent inhibitory functions; striking evolutionary relationship between members of this group and those of the Kazal trypsin inhibitor family clearly demonstrated the existence of these molecules beyond the plant kingdom (Odani et al. 1983).

Several proteins isolated from common cereal grains (wheat, barley, finger millet and rye) and leguminosae seeds (i.e. soya beans) have been classified in the “cereal trypsin/ α -amylase inhibitors” family; in addition, some of them have also been designated as CM-proteins on the basis of their solubility in chloroform/methanol mixtures

(Barber et al. 1986; Garcia-Olmedo et al. 1987; Gautier et al. 1990). These protein inhibitors are characterized by molecular weight values ranging from 9 to 16 kDa with a typical sequence motif “C-X₄-[SAGDV]-X₄-[SPAL]-[LF]-X₂-C-[RH]-X-[LIVMFYA]₍₂₎-X_{3,4}-C” (Prosite code: PS00426). A specific pattern of five intra-chain disulphide bridges involved in the stabilization of the 3D structure is also considered distinctive for the members of this family (Garcia-Olmedo et al. 1987); in a number of cases, this stability is quite pronounced even at extreme values of pH and temperature (Garcia-Casado et al. 1994). The protein members of the “cereal trypsin/ α -amylase inhibitor” family are often referred to as anti-nutritional factors; in fact, a number of them are able to inhibit human and animal digestive enzymes, and/or to act as allergens (Birk 2003).

A number of authors have pointed out that some CM-proteins could be potentially useful for the transformation of plants of agronomic interest (Alfonso-Rubi et al. 2003); moreover, the role played by some proteins of this group in the process of beer fermentation and their impact on the final quality of this beverage has been highlighted (Robinson et al. 2007).

The aqueous protein fraction obtained from wheat endosperm contains several inhibitors belonging to different protein families and identified on the basis of their enzyme specificity (Poerio et al. 2003).

Here, we report on the isolation, purification and characterization (primary structure, inhibitory properties, molecular mass, *pI*, etc.) of a novel wheat protein coded WCI (wheat chymotrypsin inhibitor) on the basis of its inhibitory specificity. WCI is a strong inhibitor of pancreatic chymotrypsins as well as of chymotrypsin-like activities present in the digestive traits of two phytophagous insect larvae [*Tenebrio molitor* L. and *Helicoverpa armigera* (Hüb.)]. This work also describes the isolation of *wci*-cDNA, the expression of the recombinant inhibitor into *E. coli* cells, as His-tagged product, and its refolding procedure.

Materials and methods

Inhibitor extraction and purification

Wheat endosperms were obtained by removing manually the embryos from grains of *Triticum aestivum* (variety San Pastore). Wheat seeds, kindly supplied from the “Consiglio per la Ricerca e la Sperimentazione in Agricoltura” (via Cassia 176, I-00191 Roma, Italy), were cultivated in the “Azienda Agraria Nello Lupori” of the Università della Tuscia (Viterbo, Italy). Finely ground flour (250 g) was extracted overnight at 6°C with 1 L of 150 mM NaCl, under stirring. The suspension was centrifuged for 30 min at 10,000g and 6°C and the supernatant was freeze-dried.

The dried sample was suspended in 20 mM ammonium acetate, pH 7.8 (200 mL) and dialyzed against ten volumes of the same solution. After centrifugation (30 min at 10,000g and 6°C), the supernatant, containing anti-proteinase activities, was applied to a CM-Sepharose CL-6B column (2.5 × 28 cm; GE Healthcare Europe, Milano, Italy) equilibrated with 20 mM ammonium acetate, pH 7.8. The column was washed (flow rate 40 mL/h at 6°C) with the equilibration solution, until the absorbance value at 280 nm dropped below 0.01. The elution of the retained proteins was achieved with 300 mM ammonium acetate, pH 7.8 (400 mL). The eluted fractions were monitored for both proteinase inhibitory activities and absorbance at 280 nm. The fractions exhibiting anti-chymotrypsin activity (eluted during the washing step) were pooled, concentrated on a YM-3 Amicon membrane (nominal cut-off 3.500, Millipore, Milano, Italy) and equilibrated in 50 mM sodium acetate/acetic acid buffer, pH 5.0. The concentrated sample (60 mL) was applied to a CM-Sepharose CL-6B column (2.5 × 16 cm) equilibrated with the same buffer. The elution was performed at a flow rate of 40 mL/h at 6°C with the following program: (a) washing step with equilibration buffer (580 mL); (b) linear gradient from 0 to 150 mM NaCl, in the equilibration buffer (700 mL); and (c) isocratic step with 250 mM NaCl, in the equilibration buffer (300 mL). All fractions (7 mL each) were monitored both for proteinase inhibitory activity and absorbance at 280 nm. The anti-chymotrypsin activity was detected in the fractions collected during both the washing and linear gradient steps. The fractions eluted with the washing step presented anti-subtilisin and anti-chymotrypsin activities; they were pooled and used for the purification of the inhibitor WSCI (wheat subtilisin/chymotrypsin inhibitor) (Poerio et al. 2003). The fractions eluted with the linear gradient exhibited only anti-chymotrypsin activity; they were pooled, concentrated and equilibrated in 80 mM Tris/HCl, 100 mM CaCl₂, pH 7.8; this sample was then submitted to affinity chromatography on insoluble chymotrypsin (Sigma-Aldrich S.r.l., Milano, Italy) column (0.7 × 2.5 cm) equilibrated with the sample buffer. The unbound protein material was removed by washing the column with: (a) equilibration buffer (30 mL) and (b) equilibration buffer containing 1 M NaCl (10 mL). Finally, the chymotryptic inhibitor was recovered with 0.2 M KCl, pH 2.0. The fraction containing the inhibitor was dialyzed, concentrated, equilibrated in 80 mM Tris/HCl, 100 mM CaCl₂, pH 7.8 and then stored at −20°C.

Protein concentration, inhibitory activities and K_i determination

The protein concentration was determined with the Bio-Rad Protein Assay kit (Bio-Rad Laboratories S.r.l., Roma,

Italy) following the manufacturer's instructions and using bovine serum albumin (BSA) as the standard. Chymotrypsin and anti-chymotrypsin activities, as well as subtilisin and anti-subtilisin activities, were determined following the procedures already described (Di Gennaro et al. 2005) and using BTEE as synthetic substrate. Porcine pancreatic elastase, as well as anti-elastase activity assays, was performed by the same procedure. Lysosomal elastase from human leukocytes was assayed using *N*-t-BOC-L-alanine-*p*-nitrophenyl ester as synthetic substrate (Janoff 1969); its susceptibility to the WCI inhibitor was tested as previously reported (Poerio et al. 2003). Chymotrypsin-like activities isolated from larvae midguts of *H. armigera* (Hüb.), *T. molitor* L., *Plodia interpunctella* (Hüb.) and *Leptinotarsa decemlineata* (Say), as well as the extent of their inhibition were assessed according to the procedure adopted for chymotrypsin. Alpha-amylase and anti-alpha-amylase activities were determined following procedures already described (Buonocore et al. 1980). Trypsin and anti-trypsin activities were determined using TAME as synthetic substrate, following the procedure already described (Poerio et al. 1989). Proteinase and anti-proteinase activities were also assayed using azocasein (Sigma-Aldrich S.r.l.) as a substrate. All the enzymes and substrates employed for enzymatic assays were obtained from Sigma-Aldrich S.r.l.. The K_i value of the purified inhibitor, in its interaction with chymotrypsin, was estimated according to the theory proposed for tightly bound inhibitors (Cha 1975).

Isolation and determination of insect chymotrypsin-like activities

Insect larvae of the coleoptera *T. molitor* and *L. decemlineata* as well as of lepidoptera *P. interpunctella* and *H. armigera* were kindly supplied by the Dipartimento di Difesa delle Piante (University of Tuscia, Viterbo, Italy). In a typical preparation, 0.2 g of midguts dissected from fourth-instar larvae 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 12,000g for 5 min at 6°C and the clear supernatants were applied to a PD-10 column (GE Healthcare Europe). The eluted samples were tested for the presence of chymotrypsin-like activities; then, promptly used for the inhibition assays. The proteolytic activities present in the insect extracts were also characterized for their susceptibility to TPCK and TLCK reagents.

Electrophoretic analyses

The purity of the inhibitor preparation was assessed by SDS-PAGE (16.5% acrylamide) using a Mini Protean II apparatus (Bio-Rad Laboratories S.r.l.) (Schagger and von Jagow 1987). Reference proteins (Sigma-Aldrich S.r.l.)

were: bovine serum albumin (Mr 66,000), ovalbumin (Mr 45,000), glyceraldehyde-3-phosphate dehydrogenase (Mr 36,000), carbonic anhydrase (Mr 29,000), trypsinogen (Mr 24,000), soybean trypsin inhibitor (Mr 20,100), α -lactalbumin (Mr 14,200) and insulin β -chain (Mr 3,500).

Electrofocusing in the pH range 3.5–9.3 was performed on polyacrylamide gel (5% T, 3% C) on the Mini-IEF apparatus (Bio-Rad Laboratories S.r.l.). Carrier ampholytes (Ampholine) and reference proteins [amyloglucosidase (pI 3.50), soybean trypsin inhibitor (pI 4.55), β -lactoglobulin A (pI 5.20), bovine carbonic anhydrase B (pI 5.85), human carbonic anhydrase B (pI 6.55), myoglobin basic band (pI 7.35), lentil lectin acidic (pI 8.15), lentil lectin middle (pI 8.45), lentil lectin basic (pI 8.65), trypsinogen (pI 9.3)] were from GE Healthcare Europe.

Native polyacrylamide slab gel electrophoresis runs were performed both at acidic (pH 4.3) and alkaline (pH 8.8) conditions.

Desalting procedure

To perform automatic sequence and mass spectrometry analyses, the inhibitor sample was submitted to RP-HPLC on a 5 μ m particle size C-18 column (0.46 $\times$ 25 cm, Beckman Coulter, Milano, Italy). The elution system consisted of eluent A (aqueous 0.1% TFA) and eluent B (0.1% TFA in acetonitrile). Elution was accomplished with a linear gradient from 5 to 55% of B in 90 min (flow rate of 1 mL/min). We manually collected the fractions by monitoring the absorbance at 214 nm; they were then pooled and lyophilised.

Analytical methods

S-Pyridylethylation was performed as reported previously (Scudiero et al. 1995). The protein inhibitor WCI (100 μ g) was digested with thermolysin (Sigma-Aldrich S.r.l.) in 0.1 M *N*-ethyl-morpholine-acetate, pH 8.0, containing 2 mM CaCl_2 . The enzyme was added in two steps with a final enzyme-to-substrate ratio of 1:20 (w:w) and the reaction was carried out at 70°C for 4 h. Chemical (CNBr, Fluka, Milano, Italy) and enzymatic (endoproteinase Asp-N, Sigma-Aldrich S.r.l.) cleavages were performed as previously described (Di Maro et al. 2001).

Peptide separations

RP-HPLC separations of thermolysin, endoproteinase Asp-N and cyanogen bromide peptides were performed on a 5 μ m particle size C-18 column (0.46 $\times$ 25 cm, Beckman Coulter) mounted on a Breeze® Waters instrument (Milano, Italy). Peptide mixtures were chromatographed at a flow-rate of 1 mL/min, using a linear gradient of 0.1% TFA and

acetonitrile (Carlo Erba, Milano, Italy) containing 0.1% TFA, from 5 to 55%, over 90 min. Eluted peptides were monitored at 214 nm.

Sequence analyses

Samples of native inhibitor, S-pyridylethylated WCI and chemical/enzymatic-derived WCI peptides were sequenced by automated Edman degradation performed on pulsed phase sequencer (Procise Model 491C, Applied Biosystem Inc., Foster City, CA, USA) as previously reported (Di Maro et al. 2001).

Electrospray mass spectrometry (ESI-MS) and tryptic mapping by MALDI-TOF

Native WCI and relative peptide samples were analysed by ESI-MS using a Platform single-quadrupole mass spectrometer (Waters, Manchester, UK). Aliquots (20 μ L) of sample solution (1 μ g/10 μ L) were injected onto the ion source at a flow rate of 10 μ L/min. The WCI mass spectrum was scanned from m/z 1,000 to m/z 2,200 at 10 s per scan, using a capillary voltage of 3.6 kV and a cone voltage of 40 V.

Tryptic peptide mapping of the His-tagged WCI was performed by MALDI-TOF mass spectrometry. Briefly, the expressed fusion protein (37 kDa) was isolated by SDS-PAGE and submitted to reduction and alkylation; then, a tryptic digestion in 50 mM ammonium bicarbonate was performed on the alkylated sample, by adding two subsequent aliquots of enzyme (70 ng for 3 h at 37°C; then, 70 ng at 37°C, overnight); then, the mixture of extracted peptides was analyzed by MALDI-TOF LR spectrometer (Waters, Severino et al. 2010).

Homology studies

The search for sequence similarity and the multiple protein sequence alignment were carried out using BLASTp and ClustalW programs, respectively. Both programs are available at the NCBI (National Center for Biotechnology Information) web site. The alignment for similarity/identity matrix was obtained using the BOXSHADE software (<http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py?form=boxshade>).

Protein modeling

A model of the 3D structure of WCI was obtained using the automated I-TASSER service available at the site <http://zhang-lab.ccmb.med.umich.edu/I-TASSER/>. The online procedure yielded the 3D model on the basis of multiple-threading alignments by LOMETS and iterative TASSER simulations (Roy et al. 2010). The program, automatically selected the template

which corresponded to the 3D structures of the maize bi-functional inhibitor of trypsin and of the activated Hageman factor (pdb code: 1BEA) (Behnke et al. 1998).

RNA isolation, cDNA cloning and DNA sequencing

Developing caryopses of *T. aestivum* (collected 20 days post-anthesis) were finely ground in a mortar in the presence of liquid nitrogen and extracted with 50 mM Tris/HCl, 150 mM LiCl, 5 mM EDTA, 5% SDS, pH 9.0. Starch was removed by submitting the sample suspension to several steps of phenol/chloroform (v/v 1:1, pH 8.0) extraction (Prescott and Martin 1987). RNA samples were analyzed by electrophoresis on formaldehyde–agarose gel (1.5%) and monitoring their A_{260}/A_{280} ratio. The cDNAs were obtained by reverse transcription procedure; the reaction was carried out at 50°C for 60 min, using 1 µg of the total wheat RNA, oligo-dT and PowerScript™ reverse transcriptase (BD Biosciences Clontech, Palo Alto, CA, USA). wci-cDNA was amplified by PCR using 50 pmol of each specific primer [FW1: (5'-ACCTCCATCTACACCTGCTACG-3') and RW1: (5'-TGGAAGACGCAGTANGGGCC-3')], designed according to the CODEHOP program on the basis of the WCI amino acid sequence at N- and C-terminal regions, respectively (Rose et al. 1998). The PCR was carried out for 30 cycles at the following conditions: 94°C for 30 s, 64°C for 30 s and 68°C for 40 s; finally, an extension step was performed (68°C for 3 min). The amplified product was then cloned into pGEM-T easy vector (Promega Italia S.r.l., Milano, Italy) and sequenced. The nucleotide sequence analyses, carried out on both DNA strands, were performed on ABI Prism Genetic Analyzer (Applied Biosystems Inc.).

Subcloning of wci-gene into *E. coli* expression vector pET22b

To clone wci-cDNA into pET22b expression vector (AMS Biotechnology, Abingdon, UK), we designed two primers: (a) the forward primer FNdeI (5'-CGCCATATGACCTCCATCTACACCTGC-3'), containing the *Nde*I restriction site at the 5'-end (in bold); (b) the reverse primer RXhoI (5'-CCTCGAGTGGGAAGACGCAGTACG-3'), exhibiting the *Xho*I restriction site at the 5'-end (in bold). After amplification, the product was directly digested and cloned into the pET22b expression vector. Finally, the recombinant expression vector (pET22b-wci) was employed to transform *E. coli* cells (BL21-DE3 strain).

Expression of the recombinant WCI and refolding procedure

The transformed bacterial cells were grown, induced with IPTG and collected according to a previously reported

procedure (Di Gennaro et al. 2005). The refolding procedure was performed according to a previous procedure (Del Vecchio Blanco et al. 1998), with some modifications. Cells from 100 mL of induced cultures were harvested by centrifugation (5,000g 10 min, at 4°C), suspended in 8.0 mL TE (50 mM Tris/HCl, pH 8.0 containing 1 mM Na₂EDTA) and finally submitted to sonication (20 × 1 min cycle) on ice. The sonicated sample was centrifuged (5,000g for 20 min at 4°C); then, both supernatant and pellet were submitted to SDS-PAGE analysis. The insoluble fraction, characterized by the presence of a protein band with molecular mass of about 14.5 kDa, was suspended with 10 mL of TE (containing 2% Triton X-100 and 2 M Urea), sonicated (20 × 1 min cycle) and centrifuged at 5,000g for 20 min at 4°C. The recovered pellet was re-washed until a clear supernatant was obtained. A final wash was, then, performed with TE in the absence of chaotropic agents.

The clean insoluble sample was dissolved in 2 mL of denaturing/reducing buffer (0.1 M Tris/HCl pH 8.0, 6 M guanidine/HCl, 5 mM Na₂EDTA and 30 mM DTT) and the final solution was kept under N₂ for 16 h at 4°C.

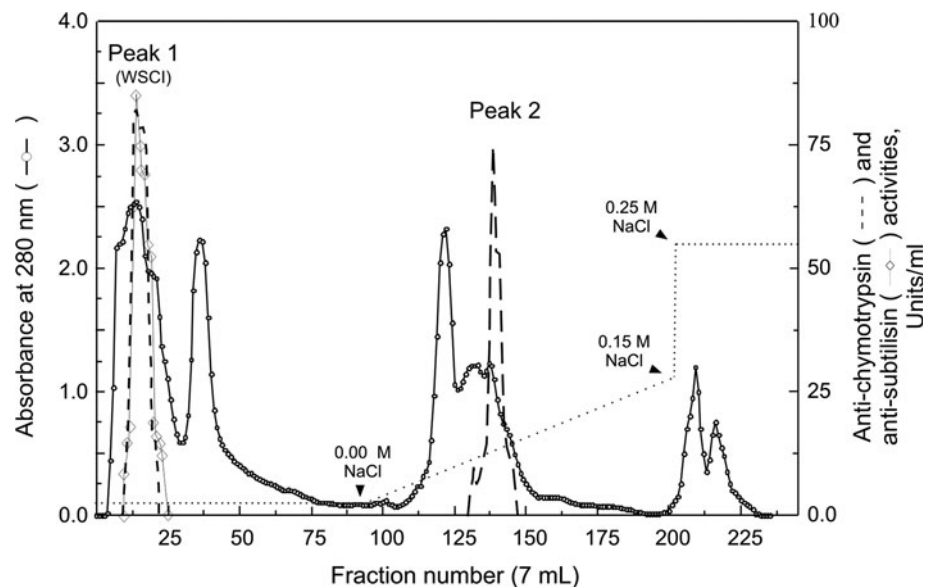
Renaturation process was performed by diluting the solubilised recombinant protein sample with renaturation solution (0.1 M Tris/HCl pH 8.0, containing 0.5 M L-arginine, 2 mM oxidized glutathione, 3 mM reduced glutathione and 5 mM Na₂EDTA) up to a final protein concentration of about 0.05 mg/mL. The diluted solution was purged with N₂ and incubated for 48 h at 15°C under N₂. After a centrifugation step, the clear supernatant (14 mL) was dialyzed at 15°C against: (a) 250 mL of 50 mM Tris/HCl, pH 8.0 (twice); (b) 250 mL of 50 mM Tris/HCl, pH 8.0 containing 0.5 mM DTT. The sample after a concentration step (Amicon membrane YM-3; cut-off: 3,500) was tested against chymotrypsin.

Results

Inhibitor purification

The aqueous protein fraction extracted from wheat flour with saline buffer (150 mM NaCl) was submitted to cation-exchange chromatography at pH 7.8. Under these conditions, most of the proteins loaded onto the column was eluted with the equilibration buffer during a washing step; this fraction, containing anti-chymotrypsin, anti-subtilisin, anti-alpha-amylase and beta-amylase activities, was re-chromatographed on the same column equilibrated at pH 5.0 (Fig. 1). Anti-chymotrypsin activity detected in the fraction eluted with the equilibration buffer (peak 1) was due to the subtilisin/chymotrypsin inhibitor WSCI, whose purification and characterization has been already reported

Fig. 1 Elution profile of wheat anti-proteinase activities from a cation-exchange chromatography at pH 5.0. The sample submitted to this purification procedure was that obtained from the previous chromatographic step and eluted with the equilibration buffer (pH 7.8). *Peak 1* refers to fractions containing the subtilisin/chymotrypsin inhibitor WSCI; the fractions corresponding to *peak 2* exhibited only anti-chymotrypsin activity. One unit of inhibitory activity is defined as the amount of the inhibitor giving 30% inhibition of a fixed amount of the enzyme (chymotrypsin or subtilisin) used in the assay mixtures



(Poerio et al. 2003). The fraction eluted with the saline gradient (peak 2), contained 17% of the total anti-chymotrypsin activity initially loaded into the column. The specific activity of the inhibitor present in the peak 2, expressed as units of anti-chymotrypsin activity per mg of protein (Di Gennaro et al. 2005), was 218 units/mg. The sample peak 2 was then submitted to affinity chromatography on chymotrypsin-agarose (data not shown). The protein with anti-chymotrypsin activity was specifically retained and successively eluted as reported in “Materials and methods”. The inhibitor sample was characterized by SDS-PAGE, carried out either in the presence or in the absence of reducing agent. For the single protein band we calculated an apparent M_r of 13 kDa (Fig. 2a). PAGE runs, carried out both at alkaline and acidic pH, confirmed the purity of the WCI sample (data not shown). The specific activity of the purified inhibitor WCI was 2,937 units/mg. The inhibitor WCI was present in the aqueous protein extract at a concentration of 1.0–1.5% (w/w) and the overall yield of the purification procedure was about 10%.

Molecular mass and isoelectric point

ESI mass spectra of purified WCI samples showed two series of molecular ions, corresponding to molecular masses of 12,933.40 and 12,832.53 Da, respectively (Fig. 2b). IEF analysis yielded a single protein band with a pI value of 7.4 (data not shown).

Temperature and pH stability, and inhibitory properties

Purified samples of native WCI (0.5–1.0 mg/mL) in 80 mM Tris/HCl pH 8.0 remained fully active for several months when stored at -20°C ; these samples retained 75% of their

anti-chymotrypsin activity after 72 h, at 6°C . At the above indicated concentrations, WCI samples were stable in the pH range 4.0–10.0, when stored for 24 h at 6°C .

Inhibitory specificity of native WCI was assessed by analyzing its capability to interact with a number of commercial hydrolases and proteinase-like activities isolated from the digestive tracts of some insect larvae (Table 1). Inhibitions of pancreatic chymotrypsins (α , β , γ , δ) were temperature- and pH-dependent; maximal inhibition was observed at 25°C , when the pre-incubation of the enzyme/inhibitor mixtures and the subsequent incubation step (following the addition of substrate) were performed at pH range 7.0–9.0. The formation of E–I complex appeared rapid; a pre-incubation step of 5 min led to a stable enzyme–inhibitor interaction. Chymotrypsin inhibition was linear with respect to the amount of the inhibitor, up to 75% inhibition; the complete inactivation of the bovine enzyme was accomplished with 80-fold molar excess of the inhibitor (data not shown).

Proteinase and anti-proteinase assays were also performed with azocasein as substrate. When BTEE was used as synthetic substrate, the inhibition constant value (K_i) calculated for WCI in its interaction with bovine chymotrypsin was 0.6 nM.

Sequence analysis

The primary structure of WCI was assessed by performing the following experimental steps: (a) molecular mass determination of native WCI, by ESI-MS, (b) N-terminal sequence analysis performed on *S*-pyridylethylated inhibitor sample, (c) specific hydrolysis of *S*-pyridylethylated WCI, by chemical (CNBr) and enzymatic (thermolysin and Asp-N endoproteinase) procedures, (d) chromatographic

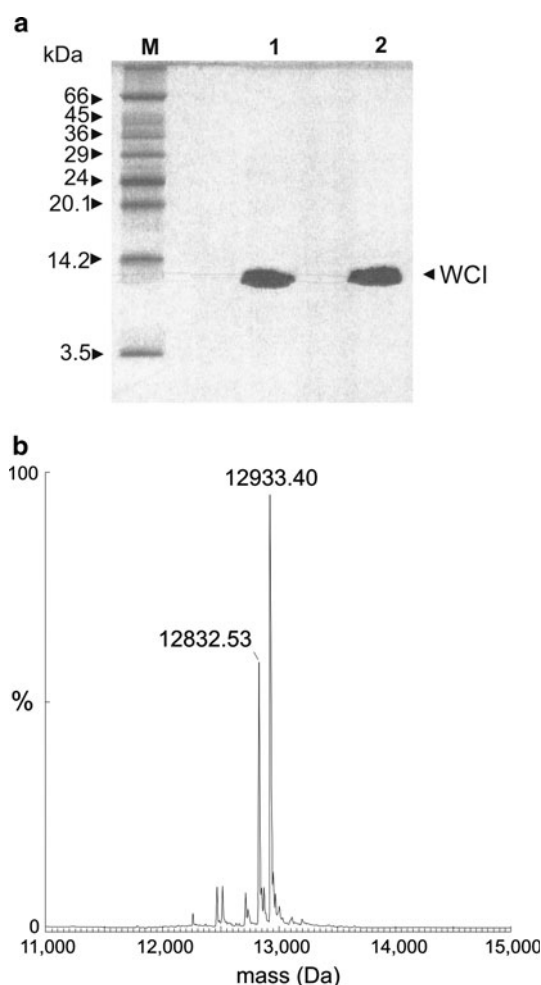


Fig. 2 SDS-PAGE and ESI-MS analyses of the inhibitor WCI sample. **a** Electrophoretic patterns obtained for the purified chymotrypsin inhibitor in presence (lane 1) and in absence (lane 2) of the reducing agent 2-mercaptoethanol. **b** Deconvoluted mass spectrum (ESI-MS) of the purified inhibitor. This spectrum is characterized by two molecular mass peaks of 12,933.40 and 12,832.53 Da, respectively

separation of peptide fragments (RP-HPLC) and their sequence analysis by Edman degradation, (e) alignment of CNBr peptide fragments by means of thermolysin and Asp-N peptide fragments (overlapping procedure).

N-Terminal sequence analysis (35 cycles), performed on S-pyridylethylated protein sample, yielded the two sequences reported in Table 2. The first sequence, accounting for approximately 60% of the total protein sample, was TSIYTCYEGV... and the pertinent protein was named WCI; the second was SIYTCYEGVG... and was named des-(Thr)WCI. The automated Edman degradation procedure performed on CNBr-, Asp-N- and thermolysin-fragments provided the peptide-sequences reported in Table 2. The complete amino acid sequences of the two inhibitor isoforms [WCI and des-(Thr)WCI] are shown in Fig. 3a. The WCI sequence is reported in the Swiss-Prot Protein Data Bank under the accession number P83207.

The purified native inhibitor sample did not react with DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)] (data not shown); this result indicated the absence of free sulfhydryl groups in both isoforms (Parente et al. 1985). The absence of free thiol groups in the polypeptide chain, and consequently the presence of five S–S bridges, was confirmed by assessing the molecular mass values of the two isoforms, by ESI–MS analysis (Fig. 2b). The primary structure of WCI was employed to perform a search for sequence homology with the protein sequences present in currently known databases. In Fig. 4a, WCI sequence is aligned with those of a number of cereal proteins (wheat, barley, ragi, and maize) exhibiting a significant degree (%) of sequence identity/similarity (Fig. 4b). On the basis of this study, we identified WCI as a member of the cereal trypsin/ α -amylase inhibitor family (Gautier et al. 1990). A further detailed observation of sequences reported in Fig. 4a highlighted that: (a) WCI exhibits the consensus pattern, C-X₄-[SAGDV]-X₄-[SPAL]-[LF]-X₂-C-[RH]-X-[LIVMFYA]₂-X_{3,4}-C, typical of this protein family (Prosite signature PS00426); (b) all cysteinyl residues of WCI are localized at sequence positions compatible with the formation of the typical network of five S–S bridges (Prosite documentation PDOC00350), which characterizes the members of this protein family.

Cloning of *wci*-cDNA and its expression in *E. coli*

The cDNA encoding for the chymotrypsin inhibitor was cloned and sequenced as reported in “Materials and methods”; it consisted of 357 nucleotides coding for a polypeptide chain of 119 amino acid residues, corresponding to the inhibitor isoform WCI (Fig. 3b). The cDNA of *wci* nucleotide sequence is recorded in NCBI data bank under the accession number AJ422078. In order to obtain a recombinant form of the inhibitor, we cloned the *wci*-cDNA into pET22b expression vector; the resulting plasmid pET22b-*wci* was then employed to transform *E. coli* cells. Under IPTG induction, the *E. coli* transformants expressed a large amount of a recombinant protein, as evidenced by SDS-PAGE (Fig. 5). The band at 14.5 kDa was unambiguously identified as due to the recombinant protein of interest by submitting it to a tryptic mapping procedure (data not shown).

The recombinant product was recovered from the inclusion bodies using the refolding protocol described in “Materials and methods”. The specific activity of the refolded inhibitor was in the range of 290–400 units/mg.

Prediction of the 3D model of WCI

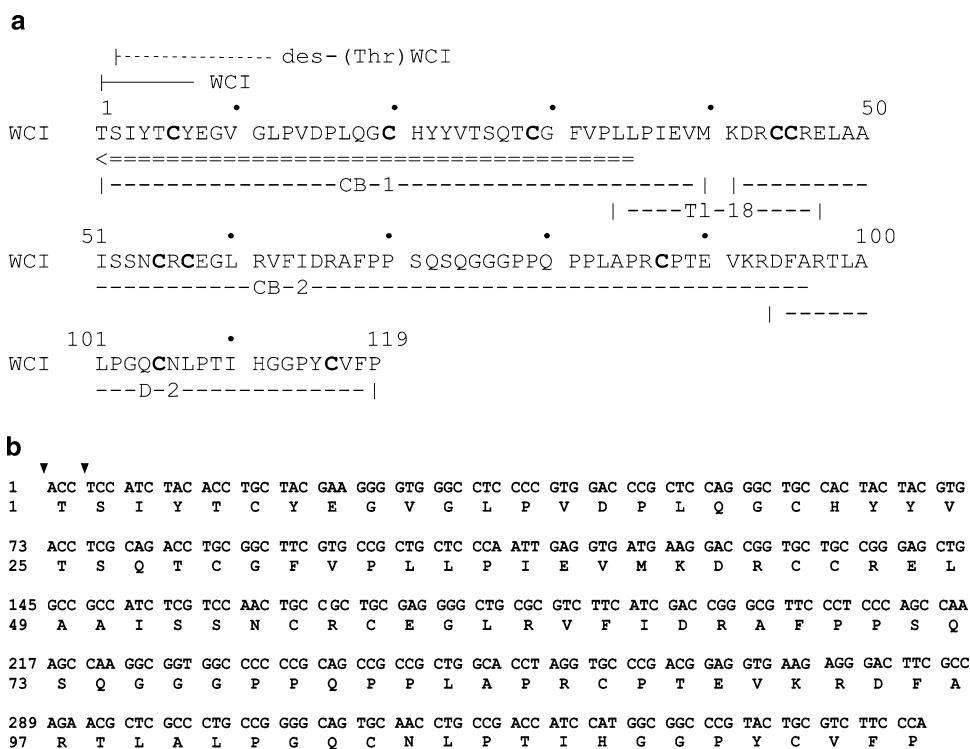
The sequence of WCI was analyzed to search for homologous proteins present in the currently known databases and to perform structure prediction studies. The observation of a

Table 1 Native WCI activity against a number of commercial hydrolases (proteases and α -amylases) and proteinase-like activities

Hydrolases	Inhibition	Hydrolases inhibition (%)
Bovine pancreas α -chymotrypsin	+	100
Bovine pancreas β , γ , δ -chymotrypsin	+	100
Larvae of <i>Helicoverpa armigera</i> Hub chymotrypsin-like activity	+	95
Larvae of <i>Tenebrio molitor</i> L. chymotrypsin-like activity	+	70
Larvae of <i>Plodia interpunctella</i> Hub chymotrypsin-like activity	–	~0
Larvae of <i>Leptinotarsa decemlineata</i> Say chymotrypsin-like activity	–	~0
Bovine pancreas trypsin	–	~0
Porcine pancreas elastase	–	~0
<i>Bacillus subtilis</i> subtilisin	–	~0
<i>Bacillus licheniformis</i> subtilisin	–	~0
Human salivary α -amylase	–	~0
Larvae of <i>Tenebrio molitor</i> L. α -amylase	–	~0
Larvae of <i>Tenebrio molitor</i> L. trypsin-like activity	–	~0

Anti-hydrolase activities were assessed using 1 unit of each hydrolytic enzyme, defined as in Buonocore et al. (1980), Poerio et al. (2003) and Di Gennaro et al. (2005) and in the standard assay conditions described in “Materials and methods”

Fig. 3 a Complete amino acid sequence of the wheat chymotrypsin inhibitor isoforms WCI and des-(Thr)WCI. The peptide fragments utilized in the overlapping procedure were the following: CNBr fragments CB-1 and CB-2; thermolysin fragment Tl-18; endoproteinase Asp-N fragments D-2; N-terminal sequence (35 cycles) of the S-pyridylethylated inhibitor sample. **b** cDNA sequence of *wci*-gene. The deduced amino acid sequence (here reported with the standard one-letter code) was identical with that experimentally obtained (see above)



selected set of protein sequences (Fig. 4) showed that the primary structure of the wheat inhibitor WCI exhibited significant values of identity (from 37.9 to 85.7%) with those of a number of homologous cereal proteins which are currently classified as members of the protease inhibitor I6 (cereal trypsin/ α -amylase inhibitor) family (Rawlings et al. 2004b).

Ragi α -amylase/trypsin inhibitor (P01087; pdb code 1BIP) and maize trypsin/factor IIA inhibitor (P01088; pdb code 1BEA), whose sequences are reported in Fig. 4, have known tertiary structures (Strobl et al. 1995; Behnke et al. 1998; Gourinath et al. 2000). In order to carry out a struc-

ture prediction study on WCI, we used the online I-TASSER service; the program built a 3D model of WCI by selecting (automatically) the 3D structure of the maize inhibitor as template. Overlapping computational methods were employed to compare the sorted WCI model with the template structure; the superimposition procedure had the coverage of 0.96 with rms deviation value of 0.69. The overall fold of the WCI model, including secondary structure elements, is shown in Fig. 6a. The protein core consisted of a globular four helices motif with a simple “up-and-down” topology; the helices of this motif (h1–h4)

Table 2 Automated sequence data obtained for the *S*-pyridylethylated inhibitor sample and for the peptide fragments generated by chemical and enzymatic hydrolysis

Protein/peptide	Amino acid sequences ^a			Notes
<i>S</i> -Modified WCI/des-(Thr)WCI	(a) 1	TSIYTCYEGV GLPVDPLQGC HYYVTSQTCTG FVPLL	35	N-terminal sequences
	(b) 2	SIYTCYEGVG LPVDPLQGCH YYVTSQTCTGF VPLL	36	
Tl-1	24	VTSQTCTG	30	
Tl-2	95	FART	98	
Tl-3	5	TCYEG	9	
Tl-4	51	ISSNCRCEG	59	
Tl-5	23	YVTSQTCTG	30	
Tl-6	39	VMKDRCCREL A	49	
Tl-7	83	LAPRCPT	90	
Tl-8	110	IHGGPYC	116	
Tl-9	83	LAPRCPTVK RD	94	C-terminal peptide
Tl-10	37	IEVMKDRCCR E	47	
Tl-11	17	LQGCHYY	23	
Tl-12	64	IDRAFPPSQS QGGGPP plus	79	
	117	VFP	119	
Tl-13	64	IDRAFPPSQS QGGGPPQP	81	
Tl-14	10	VGLPVD	16	
Tl-15	99	LALPGQCNLP T	109	
Tl-16	60	LRVF	63	
Tl-17	10	VGLP	13	
Tl-18	34	LLPIEVMKDR CCRE	47	
Tl-19	10	VGLPVDPLQG	18	→ (119)
Tl-20	9	GVGLPVDPLQ GCHY	22	
Tl-21	10	VGLPVDPLQG CHYY	23	
Tl-22	3	IYTCYEGVGL PVDPLQGC	20	
CB-1 ^b	41	KDRCCRELAA ISSNCRCEGL RVFIDRAFPP SQSQGGGPPQ PPLAPRCPT VKRDFA	96	
CB-2	1	TSIYTCYEGV GLPVDPLQGC HYYVTSQTCTG FVPLLPIEVM	40	
D-1 ^b	65	DRAFPPSQSQ GGGPPQPPLA PRCPT	90	
D-2	94	DFARTLALPG QCNLP TIHGG PYCVFP	119	C-terminal peptide
D-3	1	TSIYTCYEGV GLPV	14	

The adopted acronyms were: *Tl* thermolysin peptides, *CB* cyanogen bromide fragments, *D* endoproteinase Asp-N peptides. The numerical progression of each group of peptides reflected their chromatographic (RP-HPLC) elution times from a C-18 Beckman column

^a Numbering of the sequence positions of the peptide fragments are referred to the isoform (a)

^b For CB-1 and D-1 only the sequenced amino acid residues have been reported. The full length of the peptide fragments CB-1 and D1 were 79 and 29, respectively

corresponded to the sequence traits Gln18-Gln27, Ile37-Ala50, Ser53-Ile64 and Thr89-Leu99.

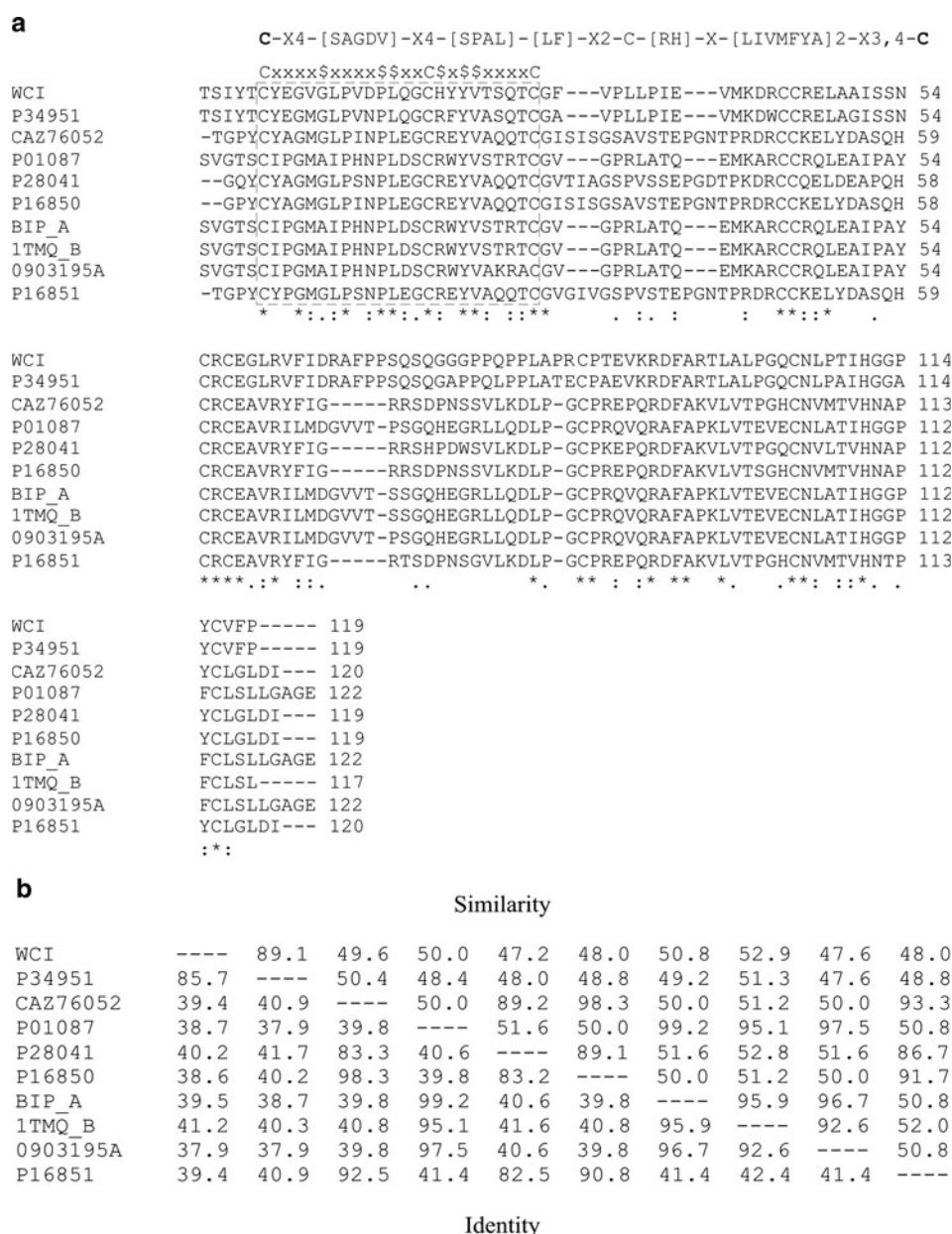
Among the proteins reported in Fig. 4, the barley trypsin inhibitor CMc (P34951) exhibited the highest value of sequence identity (85.7%) with WCI; consequently, when the sequence of the barley inhibitor CMc was submitted to I-TASSER online service, to perform a structure prediction, the program yielded a 3D model almost identical with that of WCI (Fig. 6a). In both models, the sequence traits (indicated as loop1) connecting the secondary structure elements h1 and h2 consisted of two peptide segments of the

same length and almost identical sequence (TCGFVPLL in WCI; TCGAVPLL in CMc). The only difference was present at the sequence position 31: in WCI, there is a phenylalanyl residue whereas in CMc was present an alanyl residue (insert of Fig. 6a, b).

Discussion

The presence of anti-chymotrypsin activity in hexaploid wheat grains was, firstly, reported nearly 30 years ago

Fig. 4 Sequence comparison of the inhibitor WCI with a number of homologous proteins present in databases. **a** Alignment of the protein sequences. **b** Matrix of identity/similarity values (%). The boxed area highlights the sequence traits corresponding to the consensus pattern typical of the “cereal trypsin/ α -amylase inhibitor family”. The standard one-letter code was used for the amino acid residues; *asterisk* identical, *colon* conserved and *dot* semi-conserved amino acid residues. P34951, CM protein inhibitor from *Hordeum vulgare*; CAZ76052, α -amylase inhibitor CM1 from *Triticum aestivum*; P16850, α -amylase/trypsin inhibitor from *Triticum aestivum*; P16851, α -amylase/trypsin inhibitor from *Triticum aestivum*; P28041, CMA protein from *Hordeum vulgare* with anti- α -amylase activity (subunit of the bifunctional tetrameric α -amylase/trypsin inhibitor); P01087, α -amylase/trypsin inhibitor from *Eleusine coracana*; 0903195A, α -amylase/trypsin inhibitor from *Eleusine coracana*; P01088, bifunctional trypsin/factor XIII inhibitor from *Zea mays* L.



(Boisen and Djurtoft 1981). Since then, a number of anti-proteinase activities have been detected in wheat extracts; however, the inhibitor WCI (we report on in this paper) represents the first case of a wheat protein, specifically active against chymotrypsin, that was isolated, as pure protein, from endosperm tissue and submitted to extensive characterization. This novel protein was active in inhibiting mammalian pancreatic chymotrypsins as well as two insect chymotrypsin-like activities. WCI exhibited chemical, physical and inhibitory activity distinct from those of the subtilisin/chymotrypsin inhibitor (WSCI) we already purified from endosperm of *T. aestivum* (Poerio et al. 2003; Di Gennaro et al. 2005); in fact, WSCI behaved mainly as a strong inhibitor of bacterial subtilisin, exhibited a weak

activity against pancreatic chymotrypsin and was identified as a member of the “potato inhibitor I family”.

Automated sequence and ESI-MS analyses, performed on samples of purified WCI, indicated the presence in those samples of two molecular isoforms named WCI and des-(Thr)WCI, respectively. The presence of these isoforms can be conjectured as result of two distinct proteolytic events occurring, at the N-terminus, during the endoplasmic reticulum-translocation process; however, in the absence of direct experimental evidences, we cannot exclude that the truncated protein [des-(Thr)WCI] can be generated by proteolysis of the main isoform WCI, during the purification steps.

WCI titration, performed with Ellman’s reagent (DTNB), excluded the presence of free thiol groups in both

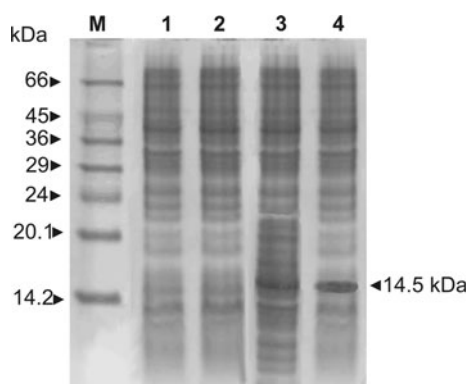


Fig. 5 SDS-PAGE analysis of the protein fraction obtained after lysis of non-induced and induced *E. coli* cells with IPTG. Lane M Molecular weight markers, lanes 1–2 aliquot of fraction recovered after lysis of non-induced *E. coli* cells, lanes 3–4 aliquot of protein fraction of *E. coli* induced. The arrow indicates a protein band of about 14.5 kDa. SDS-PAGE were run under reducing conditions and stained with Coomassie Brilliant Blue R-350

isoforms; on the basis of this data, we concluded that the native protein contained five disulphide bridges. In WCI isoforms, the existence of an intra-chain network of five S–S bridges was, unambiguously, demonstrated by ESI-MS analysis; in fact, the experimental molecular mass values (12,933.40 and 12,832.53 Da for WCI and des-(Thr)WCI,

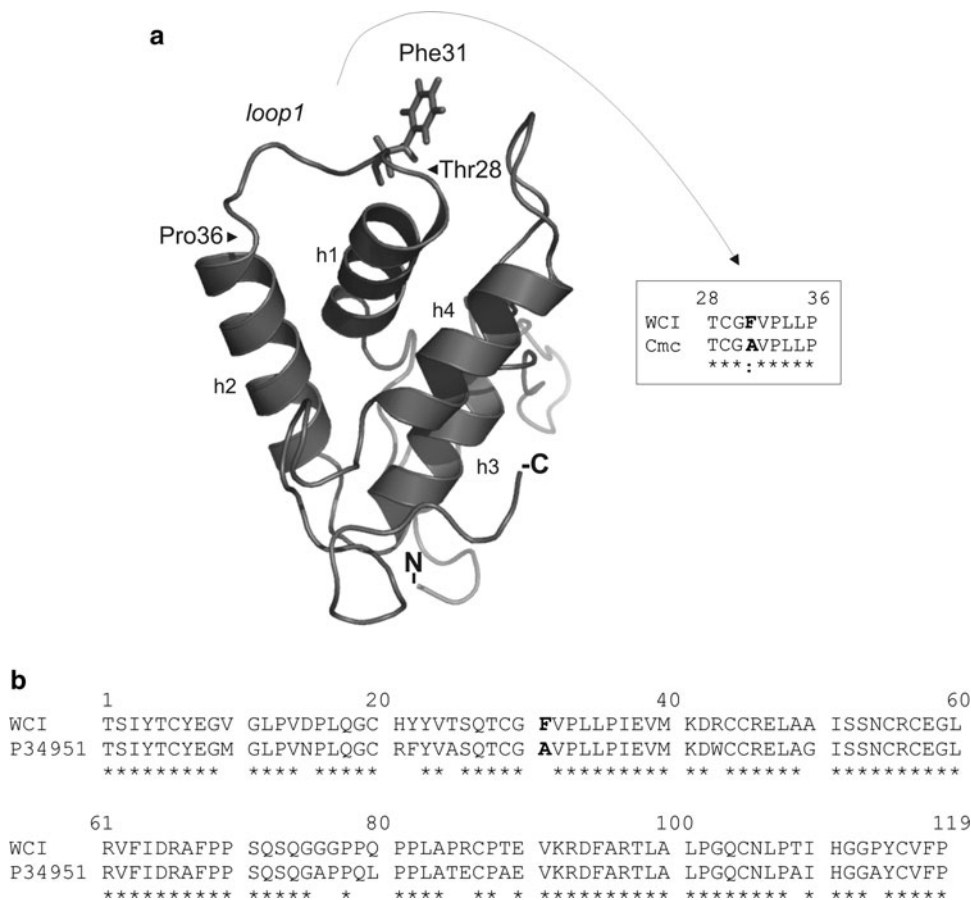
respectively) were about 10 Da lower than the theoretical mass values calculated from the primary structures of the two isoforms [12,944.10 and 12,842.99 Da for WCI and des-(Thr)WCI, respectively].

Sequence homology data supported classification of WCI among the members of the “cereal trypsin/ α -amylase inhibitors” family; such belonging was further confirmed by identification, in WCI sequence, of a typical consensus pattern of 24 contiguous residues (Prosite signature PS00426). According to these findings, the sequence positions of the cysteinyl residues resulted fully compatible with the specific network of five S–S bridges, which constitutes a common feature of this protein family (Prosite documentation PDOC00350).

Native WCI and its truncated form appeared quite resistant to proteolysis; this behaviour, which is common among the members of this family, was exploited to set up its final purification step consisting in an affinity chromatography on insoluble-chymotrypsin. This purification step yielded a protein inhibitor sample exhibiting a specific activity of 2,937 units/mg.

The cDNA of *wci* nucleotide sequence (NCBI data bank: AJ422078) is in agreement with that reported in PlantGDB which encode putative WCI variant (PUT-163b-Triticum_aestivum-104669); the only difference regards an

Fig. 6 **a** 3D-molecular model obtained for both WCI and CMc. In this model, reported as a backbone ribbon, the secondary structure topology is evidenced. In the insert we report the sequence alignment corresponding to loop1 of the wheat inhibitor WCI and barley inhibitor CMc. **b** Comparison of the complete sequences of WCI (P83207) and CMc (P34951)



additional signal peptide sequence, commonly found in this protein family.

A recombinant (His-tagged) form of the inhibitor was expressed in transformed *E. coli* cells and recovered from the inclusion bodies as a protein partially active. The specific activity of the refolded recombinant inhibitor was ten times lower than the specific activity of the native WCI; however, by considering the high number of disulfide bridges present in this small protein, the adopted refolding procedure was considered encouraging.

Among the proteins listed in Fig. 4, ragi α -amylase/trypsin inhibitor (P01087) and maize trypsin/factor IIA inhibitor (P01088) have known tertiary structures (Strobl et al. 1995; Behnke et al. 1998; Gourinath et al. 2000). The structures of these two inhibitors, as well as those of the wheat α -amylase/inhibitor 0.19 (Oda et al. 1997) and the binary complex *T. molitor* α -amylase/ragi bifunctional inhibitor (Strobl et al. 1998), have been utilized to propose a general model for the simultaneous interaction of the ragi inhibitor (P01087) with the two cognate enzymes, namely α -amylase and trypsin (Strobl et al. 1998). According to the structural features of this model, the core of the ragi inhibitor is made up of a four helices motif (h1–h4) with a simple “up-and-down” topology (also known as right-handed super-helix motif). It was suggested that the reactive site for the susceptible trypsin, lying between the helices h1–h2, exhibits the canonical substrate-like conformation and is located on the upper part of the super-helix motif; the binding site recognized by the α -amylase is instead located in the opposite side of the molecule; so the simultaneous inhibition of the two enzymes can take place, following the ternary complex (trypsin/ragi bifunctional inhibitor/ α -amylase) formation.

Barley trypsin inhibitor CMc (P34951) shares 85.7% sequence identity with wheat chymotrypsin inhibitor WCI (P83207); as expected, a structure prediction study performed with the online program (I-TASSER service) yielded very similar 3D models, for both proteins (Fig. 6a). In addition, these models shared the four helices motif (h1–h4) with 3D structure of ragi bifunctional inhibitor; moreover, the two 3D models (WCI and CMc) exhibited significant similarities (both in sequence and length) between the loops connecting the corresponding pair of helices of the above motif. According to these models and to the mechanism proposed for the simultaneous interaction of ragi bifunctional inhibitor with the two cognate enzymes (Strobl et al. 1998), we proposed that the binding reactive sites of barley (CMc) and wheat (WCI) inhibitors, likely, could lay on the loop connecting helices h1 and h2 (Fig. 6a). Sequence comparison of these connecting loops (loop 1 in both models) allowed to identify the residue (likely) responsible for the different inhibitory specificity of WCI and CMc: residue present at the sequence position 31 (insert of Fig. 6a, b). In the proteinase inhibitors, this

residue is commonly known as the P1 residue; moreover, according to the “Laskowski” (standard) inhibition mechanism, the chemical nature of this residue determines the specificity of the protein inhibitor. In the present case, the alanyl residue present at the sequence position 31 of CMc determines the anti-trypsin activity of the barley inhibitor; analogously, we suggest that the aromatic side chain of Phe31 in WCI could be invoked as responsible for the anti-chymotrypsin activity of the wheat inhibitor.

The data we accumulated on the inhibitor WCI, and reported in this paper, stimulated our curiosity with respect to the physiological role played in vivo by this protein; very often the physiological role of proteinase inhibitors of plant origin receives scarce attention, due to the difficulty to identify the proper target enzymes. In order to investigate, in vivo, WCI function we decided to identify, isolate and characterize the endogenous target enzyme(s), capable to interact with WCI. At moment, in our lab, a number of experiments are in progress; they are aimed to obtain active agarose-linked WCI, utilizable to fish out chymotrypsin-like activity present in extracts of germinating wheat grains.

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