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Primary Structure and Reactive Site of a Novel Wheat Proteinase Inhibitor of Subtilisin and Chymotrypsin

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The proteinase inhibitor WSCI, active in inhibiting bacterial subtilisin and a number of animal chymotrypsins, was purified from endosperm of exaploid wheat (*Triticum aestivum*, c.v. San Pastore) by ion exchange chromatography and its complete amino acid sequence was established by automated Edman degradation. WSCI consists of a single polypeptide chain of 72 amino acid residues, has a molecular mass of 8126.3 Da and a pI of 5.8. The inhibition constants (K_i) for *Bacillus licheniformis* subtilisin and bovine pancreatic α-chymotrypsin are 3.92×10^{-9} M and 7.24×10^{-9} M, respectively. The inhibitor contains one methionine and of tryptophan residue and has a high content of essential amino acids (41 over a total of 72 residues), but no cysteines. The primary structure of WSCI shows high similarity with barley subtilisin-chymotrypsin isoinhibitors of the CI-2 type and with maize subtilisin-chymotrypsin inhibitor MPI. Significant degrees of similarity were also found between sequences of WSCI and of other members of the potato inhibitor I family of the serine proteinase inhibitors. The wheat inhibitor WSCI has a single reactive site (the peptide bond between methionyl-48 and glutamyl-49 residues) as identified by affinity chromatography and sequence analysis.

Key words: Enzyme inhibitor / Homology / Reactive site / Sequence analysis / Serine proteinases / Wheat.

Introduction

Protein proteinase inhibitors are widely distributed in the plant kingdom. These proteins, together with plant amylase inhibitors and a few more proteins that have no known inhibitory activity, have been tentatively assigned to 10–11 different protein families on the basis of their structural features (molecular dimension, primary structure, disulfide bridge pairing, position and number of reactive site(s), peculiar sequence traits) (Garcia-Olmedo *et al.*, 1987; Richardson, 1991). Members of these families have been found to be specific for each of the four mechanistic classes of proteolytic enzymes; however, the majority of known proteinase inhibitors, found in a variety of tissues of a wide number of plant species, are specific for serine proteinases.

One of the most widespread families of the serine proteinase inhibitors is the potato inhibitor I family. Members of this family have been found in Solanaceae (Melville and Ryan, 1972; Grahm *et al.*, 1985; Margossian *et al.*, 1988; Wingate and Ryan, 1991; Criqui *et al.*, 1992), Gramineae (Svendsen *et al.*, 1980; Williamson *et al.*, 1987; Cordero *et al.*, 1994), Leguminosae (Svendsen *et al.*, 1984; Nozawa *et al.*, 1989; Katayama *et al.*, 1994), Amaranthaceae (Valdés-Rodriguez *et al.*, 1993), Cucurbitaceae (Zeng *et al.*, 1988; Krishnamoorthi *et al.*, 1990), Linaceae (Lorenc-Kubis *et al.*, 2001), Polygonaceae (Pandya *et al.*, 1996; Belozersky *et al.*, 2000) and Compositae (Konarev *et al.*, 2002), as well as from the lower animal *Hirudo medicinalis* (Seemüller *et al.*, 1980). All these inhibitors share high levels of sequence similarity with the subunits of the oligomeric chymotryptic inhibitor I isolated from potato tubers (Richardson and Cossins, 1974) and have the capacity to interact with one or more serine proteinases through a specific peptide bond (reactive site). In a few cases (Jonassen and Svendsen, 1982) the existence of two distinct reactive sites has been invoked.

The physiological role of plant serine proteinase inhibitors has not been fully elucidated yet; however, various experimental evidence strongly suggests a role of plant defence against pest attacks (Bowles, 1990; Ryan, 1990; Koiwa *et al.*, 1997) as well as of regulation of endogenous proteolytic activities (Richardson, 1991).

The present report describes purification, determination of the primary structure and identification of the reactive site of a novel member of the potato inhibitor I family of serine proteinase inhibitors, isolated from endosperm of *Triticum aestivum*. This inhibitor has been named WSCI and its capability to interact with mammalian, insect and bacterial serine proteinases, as well as its sequence homology with other plant inhibitors, is discussed.

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Results

Inhibitor Purification

The main inhibitory activities of serine proteinase (anti-trypsin, anti-subtilisin and anti-chymotrypsin), present in the water soluble protein fraction of wheat endosperm, were partially separated by means of cation exchange chromatography at pH 7.8. Under these conditions, the anti-tryptic activity was retained, while the anti-subtilisin and anti-chymotrypsin activities were detected in the fraction eluted with the equilibration buffer. The latter fraction, chromatographed on the same column, at acidic pH, yielded two distinct active fractions: the first (fraction 1) eluted during the washing step and contained both anti-proteinase activities; the second (fraction 2) eluted with the saline gradient and exhibited anti-chymotrypsin activity only. The SDS-electrophoretic pattern of fraction 1 consisted of five main protein bands with apparent M_r values ranging from 70 000 to 8000 (Figure 1). At this stage, the specific activity calculated for both anti-subtilisin and anti-chymotrypsin activities was 300 units/mg and 285 units/mg, respectively. Fraction 1 was then submitted to a third chromatographic step on a strong anion-exchange resin at pH 8.4. The elution profile shows a number of peaks absorbing at 280 nm (Figure 2). In peak 1, anti-subtilisin activity was detected at a very low level; peak 2 eluted with the saline gradient and was active in inhibiting both proteinase activities; no anti-proteinase activity was detected in the other fractions. SDS-PAGE, carried out on the active peak 2, in the presence or absence of a reducing agent, showed a single protein band with an apparent M_r of 8000 (Figure 1). The homogeneity of the sample was also assessed by native PAGE under both acidic and alkaline conditions. The specific activities

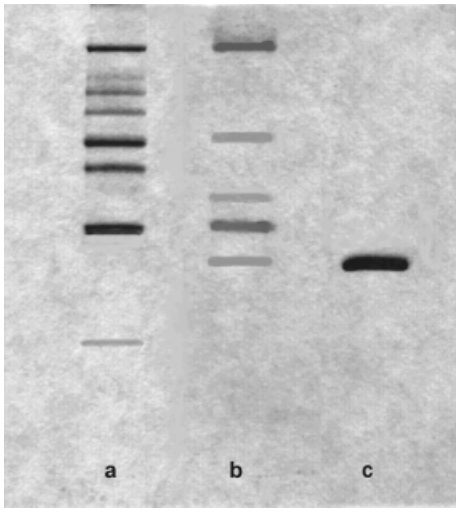

Fig. 1 SDS-PAGE Pattern of the Inhibitor Sample at Different Stages of Purification.
Lane a, markers (see Materials and Methods); lane b, inhibitor sample after cation-exchange chromatography at pH 5.0 (7 µg); lane c, purified inhibitor (4 µg).

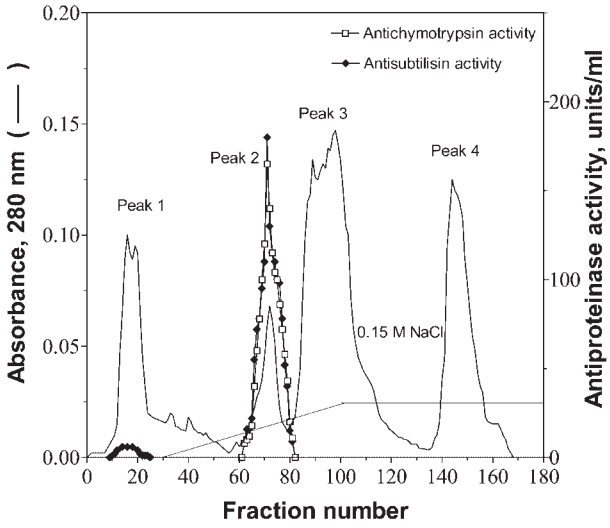

Fig. 2 Elution Profile from Anion-Exchange Chromatography at pH 8.4.

determined for anti-subtilisin and anti-chymotrypsin activities of the purified inhibitor were determined to be 2300 units/mg and 2200 units/mg, respectively. This monomeric inhibitor, on the basis of its inhibitory specificity, was named WSCI (wheat subtilisin/chymotrypsin inhibitor).

Molecular Mass and Isoelectric Point Determination

ES-MS and electric focusing analyses, carried out on the purified inhibitor, yielded an M_r value of 8126.3 ± 0.5 and a pI value of 5.8.

Inhibitory Properties

The purified WSCI, kept in 20 mM Tris-HCl, pH 8.0, at a concentration of 0.5 mg/ml, fully retained its inhibitory activity for several months when stored at –20 °C. The same inhibitor sample appeared fully stable when stored at 6 °C for 48 h; after 72 h at 6 °C, the residual inhibitory activity was 75%. In the pH interval 4.0 – 8.5, samples of purified inhibitor (0.2 – 0.6 mg/ml) stored for 24 h at 6 °C completely retained their inhibitory activity.

WSCI was very effective in inhibiting subtilisin from *Bacillus licheniformis* and *Bacillus subtilis* as well as chymotrypsin activities isolated from a number of different sources (Table 1). In addition to the different forms (α, β, γ, δ) of bovine and porcine chymotrypsins, WSCI strongly interacted with chymotrypsin-like activities isolated from the digestive tract of insect larvae such as *Tenebrio molitor*, *Plodia interpunctella* and *Helicoverpa armigera*. No inhibitory activity was detected under the reported assay conditions against bovine pancreatic trypsin, porcine pancreatic elastase or human leukocyte elastase. Inhibition of bacterial subtilisin and pancreatic chymotrypsin was determined to be both temperature and pH dependent: maximal inhibition was observed at 25 °C when both pre-incubation and incubation steps were

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Table 1 WSCI Activity against a Number of Exogenous Proteinases.

Proteinase	Inhibition	pmol of WSCI inhibiting 30% of proteinase activity
Bacillus licheniformis subtilisin	+	50
Bacillus subtilis subtilisin	+	58
Bovine pancreas α-chymotrypsin	+	70
Porcine pancreas α-chymotrypsin	+	74
Bovine pancreas trypsin	–	
Porcine pancreas elastase	–	
Human leukocyte elastase	–	
Tenebrio molitor L. chymotrypsin-like activity	+	320
Helicoverpa armigera L. chymotrypsin-like activity	+	200
Plodia interpunctella L. chymotrypsin-like activity	+	280

The inhibition of proteinase activities was evaluated and quantified by using 1 unit of enzyme (1 U.E.) in the assay volume under standard conditions (see Materials and Methods). In the inhibition assays of human leukocyte elastase 0.2 units of enzyme were employed.

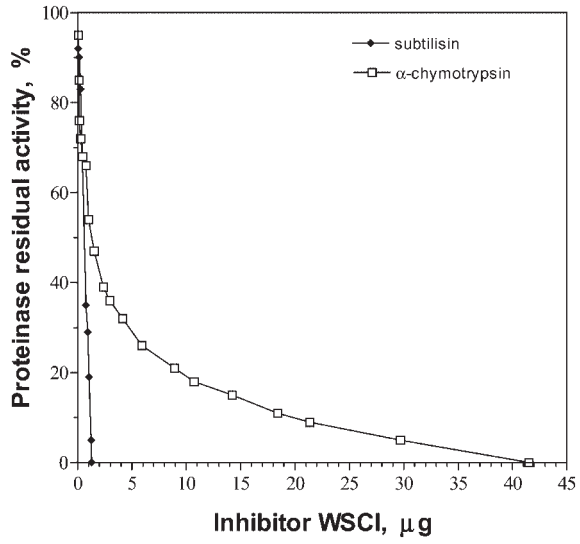

Fig. 3 Effect of WSCI Concentration on Bovine α-Chymotrypsin and Bacterial Subtilisin Activity.
Final concentrations of chymotrypsin and subtilisin in the assay were 18 nM and 53.3 nM, respectively.

carried out in the pH range 7.0 – 9.0. The extent of the complex formation during the pre-incubation step appeared not to be time dependent.

Subtilisin inhibition was linear with respect to the amount of inhibitor up to 100%; 140 pmol of WSCI inactivated completely 160 pmol of commercial subtilisin (from *B. licheniformis*) with an E/I molar ratio 1:0.87 (Figure 3). The inhibition of bovine α-chymotrypsin was linear with respect to the amount of inhibitor up to 50%; the complete inhibition of the bovine enzyme was obtained with a 40-fold molar excess of the inhibitor.

The above reported inhibition data were fully confirmed when the proteinase and anti-proteinase assays were carried out using azocasein as substrate. When BTEE was used as a synthetic substrate, the inhibition constant values (K_i), calculated for WSCI in its interaction with *B. licheniformis* and *B. subtilis* subtilisins, were 3.92×10^{-9} M and 5.70×10^{-9} M, respectively; K_i values for bovine and porcine α-chymotrypsins were 7.24×10^{-9} M and 9.35×10^{-9} M, respectively.

Sequence Analysis and Identification of the Reactive Site

Automated sequence analysis performed on the native inhibitor (0.4 nmol) yielded the first 35 amino acid residues at the N-terminus. To complete the amino acid sequence of WSCI and to identify its reactive site, we submitted the purified and active inhibitor to an affinity chromatography step on a subtilisin-agarose column. Over a total of 1080 units (500 µg) of inhibitor retained on the column, we recovered, by elution with KCl 0.2 M, pH 2.0, only 20% of material in terms of protein and 10% of inhibitory activity. The eluate contained a small amount of uncleaved inhibitor, confirming the occurrence of a strong interaction between the two macromolecules. The mixture eluted from the affinity column was resolved by RP-HPLC in three main peaks (S1, S2 and S3) (Figure 4). Each peak, when analysed by automatic sequence, was found to contain a single molecular species (Table 2). Sequence analysis of the peptide fragment S1 was carried out and completed for 24 cycles of Edman degradation. Peak S2 contained the uncleaved inhibitor; in fact, it had an elution time, an N-terminal sequence (up to residue 15), and a molecular mass identical with native WSCI. Sequence analysis of peptide fragment S3 was carried out for 48 Edman degradation cycles, yielding the N-terminal sequence of the inhibitor molecule. However, amino acid residues at positions 45, 46 and 48 were not identified. To complete its amino acid sequence, we digested fragment S3 with endoproteinase Asp-N. The enzymatic hydrolysis generated two main fragments (A1 and A2) that were resolved by RP-HPLC (insert of Figure 4) and fully sequenced (Table 2). Peptide fragment A2 was 26 amino acid residues long and corresponded to

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Table 2 Automated Sequence and Mass Spectrometry Data Obtained for WSCI and for the Peptide Fragments Generated by Enzymatic Hydrolyses.

Sample	Amino acid sequence	Sequence position	M_r (ES-MS)
WSCI	1 TDTGDHHNQKTEWPELVGKSVEEAKKVILQDKSEA... 35	1–35	8126.3±0.5
S1	1 EYRIDRVRLFVDSLDKIAQVPRVG 24	49–72	2844.9±0.5
S2	1 TDTGDHHNQKTEWPE... 15	1–15	8126.3±0.5
S3	1 TDTGDHHNQKTEWPELVGKSVEEAKKVILQDKSEAQIVVLPVGTXXTX 48	1–48	5299.0±0.5[a]
A1	1 DKSEAQIVVLPVGTIVTM 18	31–48	–
A2	1 DHHNQKTEWPELVGKSVEEAKKVILQ 26	5–30	–

[a]In S3, the molecular species with the mass values of 5299.0+0.5 and 5316.1+0.5 account for about 75% and 25%, respectively.
S1, S2 and S3 were eluted from the subtilisin-agarose column and purified by RP-HPLC (Figure 4); A1 and A2 are peptide fragments generated by Asp-N endoproteinase digestion of S3 and purified by RP-HPLC (insert of Figure 4).

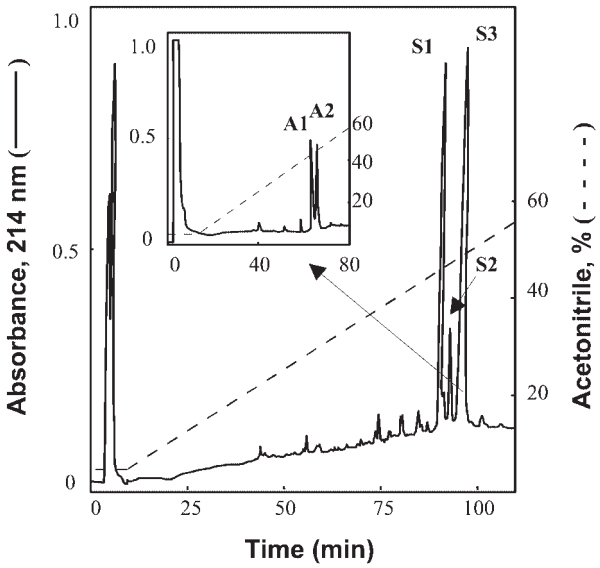

Fig. 4 RP-HPLC Separation of the Sample Eluted from a Subtilisin-Agarose Column with 0.2 M KCl, pH 2.0.
The insert reports the RP-HPLC re-chromatography of the peptide mixture generated by Asp-N digestion of fragment S3.

position 5–30 of the peptide fragment S3. The amino acid sequence of the peptide fragment A1 (18 amino acid residues long) was useful in identifying the amino acid residues at positions 45, 46 and 48 of the peptide fragment S3.

S1, S2, and S3, as well as the native inhibitor, were also analysed by ES-MS (Table 2). Molecular mass values of the peptide fragments S1 (2844.9±0.5) and S3 (5299.0±0.5) clearly indicated that they were generated by a specific hydrolysis of the inhibitor molecule (8126.3±0.5), which occurred at the peptide bond located between amino acids Met48-Glu49. For S3 a second peak (Mr 5316.1±0.5), accounting for about 25%, was also found; this peak is likely due to the peptide fragment 1–48 containing the sulfoxide form of methionine at the C-terminus. The complete amino acid sequence of the inhibitor WSCI, reconstructed on the basis of sequence analyses and ES-MS data, is presented in Figure 5. Furthermore, enzymatic cleavage experiments and sequence studies allowed the localisation and the identification of the inhibitor reactive site that corresponds to the peptide bond between Met48 and Glu49.

The amino acid composition of WSCI is characterised by the absence of cysteinyl residues and by the presence of only one methioninyl and tryptophanyl residue, respectively. Moreover, this inhibitor molecule is characterised by having a high content in essential amino acids (41 residues over a total of 72).

The amino acid sequence of WSCI exhibits a peculiar segment (from residues 13 to 24) that is consistent with the potato inhibitor I family signature reported in the Prosite database: [FYW]-P-[EQH]-[LIV](2)-G-x(2)-[STAGV]-x(2)-A.

The WSCI sequence data appear in the Swiss-Prot Protein Data Bank under the accession number P82977.

Homology Studies

In order to classify the inhibitor WSCI and to gain insight into its biological function, we performed a search for sequence homology. Among the numerous sequences showing a degree of identity, ranging from about 90% to about 30%, we focused our interest on those reported in Figure 5. The barley chymotrypsin inhibitors CI-2A and CI-2B, both specific for pancreatic chymotrypsin and bacterial subtilisin (Svendsen *et al.*, 1980; Williamson, *et al.*, 1987), showed the highest degree of identity with WSCI (88% and 84%, respectively). A sequence identity of about 60% was found for the wound-induced maize proteinase inhibitor MPI, specific for both elastase and chymotrypsin (Cordero *et al.*, 1994). Identities ranging from about 40% to 30% were found for: (i) the potato proteinase inhibitor PI-I, subunits D and A (Richardson and Cossins, 1974); (ii) the adzuki bean subtilisin inhibitor ASI-I (Nozawa *et al.*, 1989); (iii) the *Vicia faba* subtilisin inhibitor VSI (Svendsen *et al.*, 1984); (iv) the barley chymotrypsin inhibitors CI-1a and CI-1c, both specific for chymotrypsin and subtilisin (Svendsen *et al.*, 1982; Williamson *et al.*, 1988); (v) the *Nicotiana sylvestris* trypsin inhibitor NSTI-I (Criqui *et al.*, 1992); (vi) the tomato in-

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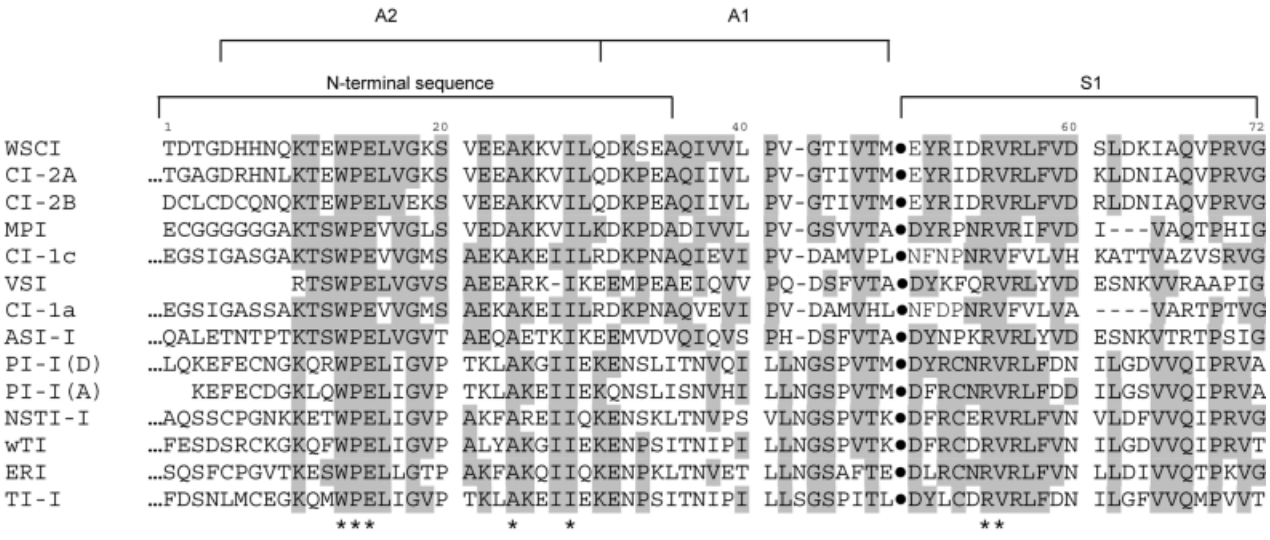

Fig. 5 Comparison of the Amino Acid Sequences of Inhibitor WSCI and of Proteinase Inhibitors of the Potato Inhibitor I Family Isolated from Gramineae, Fabaceae and Solanaceae.

Prefixes S and A, referring to the wheat inhibitor sequence, indicate the amino acid sequences of peptide fragments generated by subtilisin and Asp-N digestion, respectively (see Table 2). The primary structure of WSCI was aligned for maximum homology with traits of sequence of similar length; (…) denotes N-terminal regions that have not been reported. The standard one-letter code is used for amino acids; Z denotes either E or Q. Gaps (–) represent insertions/deletions. Numbering refers to the WSCI amino acid sequence in which a gap has been inserted between Val42 and Gly43 in order to get the best alignment with the other inhibitors. Amino acids residues common to six or more inhibitor sequences are highlighted in grey. •, Reactive site, either directly or indirectly identified; *, amino acid residues identical in all reported sequences. CI-2A and CI-2B (Svendsen *et al.*, 1980; Williamson *et al.*, 1987), as well as CI-1c and CI-1a (Svendsen *et al.*, 1982; Williamson *et al.*, 1988), are subtilisin/chymotrypsin inhibitors from barley (*Hordeum vulgare*); MPI is a wound-induced subtilisin/chymotrypsin inhibitor from maize (*Zea mays*) (Cordero *et al.*, 1994); VSI is a subtilisin inhibitor from broad bean seeds (*Vicia faba*) (Svendsen *et al.*, 1984); PI-I(D) and PI-I(A) are the subunits D and A of the wound-induced proteinase inhibitor I from potato leaves (*Solanum tuberosum*) (Richardson and Cossins, 1974); ASI-I is the subtilisin inhibitor I from adzuki beans (*Vigna angularis*) (Nozawa *et al.*, 1989); NSTI-I is a trypsin inhibitor from tobacco (*Nicotiana sylvestris*) (Criqui *et al.*, 1992); TI-I is the wound-induced proteinase inhibitor I from tomato (*Lycopersicon esculentum*) (Graham *et al.*, 1985); ERI is the ethylene-responsive proteinase inhibitor from *Lycopersicon esculentum* (Margossian *et al.*, 1988); wTI is a wound-induced proteinase inhibitor from a wild species of tomato (*Lycopersicon peruvianum*) (Wingate and Ryan, 1991).

hibitor TI-I (Graham *et al.*, 1985) and the ethylene-responsive inhibitor ERI (Margossian *et al.*, 1988), both from tomato (*Lycopersicon esculentum*); and (vii) the wound-induced proteinase inhibitor wTI from the wild species of tomato *Lycopersicon peruvianum* (Wingate and Ryan, 1991). The percentage of identities was calculated on the basis of the reported alignment by comparing the sequence (or sequence traits) of WSCI with corresponding sequence traits of other inhibitors. Comparison of the above inhibitor sequences shows the evident alignment of the WSCI reactive site with those of the other inhibitors (Figure 5).

Discussion

Although a number of authors have reported the presence of anti-chymotryptic (Boisen and Djurtoft, 1981) and anti-subtilisin (Hejgaard *et al.*, 1981; Mundy *et al.*, 1984; Mosolov and Shul'gin, 1986) activities in extracts of exaploid wheat kernels, up to now no proteins active against chymotrypsin or against both chymotrypsin and subtilisin have been isolated and purified from this source. Among the serine proteinase inhibitors isolated from this cereal, three are trypsin inhibitors of the Bowman-Birk type (Odani *et al.*, 1986; Poerio *et al.*, 1994) and one is a bifunctional endogenous alpha-amylase/subtilisin inhibitor that has been purified from endosperm and classified as member of the Kunitz family (Mundy *et al.*, 1984).

The inhibitor WSCI, whose characterisation is described in this report, represents the first case of a wheat protein inhibitor active in inhibiting animal chymotrypsins and bacterial subtilisin. This inhibitor has been purified from the aqueous protein extract of wheat endosperm where it is present at a concentration of 0.8 – 0.9% (w/w). WSCI was obtained as a pure protein with a 60-fold increase of its specific activity; the overall yield of the purification procedure was about 6%. As side products of this procedure, we isolated two more proteins: the anti-trypsin inhibitor WTI (Poerio *et al.*, 1994) and an anti-chymotryptic inhibitor coded WCI, whose characterisation is still in progress.

The primary structure of WSCI, determined by automated sequence and mass spectrometry analyses, was utilised to perform a search for sequence homology. The

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classification of WSCI as member of the potato inhibitor I family is supported by the following experimental evidence: (i) the amino acid sequence of WSCI shows a high degree of similarity with other members of this family isolated from barley (Svendsen *et al.*, 1980; Williamson *et al.*, 1987) and maize (Cordero *et al.*, 1994); (ii) a significant degree of similarity was also found with other inhibitors of the potato inhibitor I family isolated from Leguminosae (Svendsen *et al.*, 1984; Nozawa *et al.*, 1989) and Solanaceae (Richardson and Cossins, 1974; Graham *et al.*, 1985; Margossian *et al.*, 1988; Wingate and Ryan, 1991; Criqui *et al.*, 1992; Figure 5); (iii) WSCI shows subtilisin substrate-like behaviour and on subtilisin-agarose undergoes a single specific cleavage at its reactive site (Met48-Glu49); the same specific cleavage has been observed on a chymotrypsin-agarose column; (iv) the WSCI sequence trait located between residues 13 and 24 (Figure 5) is consistent with the potato inhibitor I family signature [FYW]-P-[EQH]-[LIV](2)-G-x(2)-[STAGV]-x(2)-A.

The presence of a single reactive site per polypeptide chain is a common feature in both monomeric and oligomeric inhibitors belonging to this family; however, for the barley isoinhibitors of the CI-1 type the presence of an extra susceptible peptide bond between Met30 and Ser31 (position 19 and 20 in Figure 5) has been claimed (Jonassen and Svendsen, 1982). These inhibitors often exhibit hydrophobic residues (methionine, alanine, leucine) at the P1 position of the reactive site. Occasionally, lysine (as in wTI and NSTI-I) or glutamic acid (as in ERI) have been found as the P1 residue. In almost all the reported homologous sequences the P1' position is occupied by an acidic residue; the barley isoinhibitors CI-1c and CI-1a appear unique in appearing at the two P1' positions, asparagine and serine, respectively. The wheat inhibitor exhibits valine, threonine, tyrosine and arginine at positions P3, P2, P2' and P3', respectively; similar patterns have been found at these positions in most members of this protein family.

WSCI contains no free thiol groups or disulfide bridges; this is a feature that the wheat inhibitor shares with a number of inhibitors of this family, such as CI-2A (Svendsen *et al.*, 1980a), CI-1a (Williamson *et al.*, 1988), CI-1c (Svendsen *et al.*, 1982), VSI (Svendsen *et al.*, 1984) and ASI-I (Nozawa *et al.*, 1989). The inhibitors PI-I from potato, TI-I from tomato, CMTI-V (Cucurbita maxima trypsin inhibitor-V) from pumpkin (Krishnamoorthi *et al.*, 1990) and ATSI (Amaranth trypsin/subtilisin inhibitor) from amaranth (Valdes-Rodriguez *et al.*, 1993) do contain a single disulfide bridge in their structures; however, as suggested by several authors (Beuning *et al.*, 1994) the S-S bond does not appear to be involved in the inhibitory activity of these proteins.

The prolamine storage proteins of wheat and barley have a very low content of essential amino acids; this deficiency, which is particularly significant for lysine and threonine, determines the poor nutritional quality of these two cereals for livestock feeding as well as for human nutrition. WSCI has a high content of essential amino acid residues (41 over a total of 72); in particular, it contains 8.3 mol% lysine. Similarly, the barley chymotrypsin inhibitors (Figure 5) show a lysine content ranging from about 6.0 to about 9.5 mol%. Although characterised by favourable contents of essential amino acids, these barley and wheat inhibitors of chymotrypsin do not give a significant contribution to the nutritional quality of the grains. These proteins, in fact, account for less than 1% (w/w) of the total salt-soluble protein fraction and could also interfere, *in vivo*, with digestive proteolytic enzymes. In spite of these problematic aspects, the presence in normal and spontaneous high-lysine barley mutant Hiproly (Munck *et al.*, 1970) of high-lysine protein inhibitors CI-2 and CI-1 has stimulated a number of investigations (Williamson *et al.*, 1987, 1988; Roesler and Rao, 1999, 2001) directed at improving the nutritional quality of barley and other cereals. Isolation of the inhibitor WSCI and cloning of its gene (EMBL accession number: AJ422055) could provide a further important tool in the hands of scientists involved in research aimed at improving the nutritional quality of cereals.

The inhibitors WSCI, CI-2, CI-1 and MPI are active in inhibiting both bacterial subtilisin and chymotrypsins; however, in contrast with the barley inhibitor CI-2 (Roesler and Rao, 1999) and with the recombinant maize proteinase inhibitor rMPI (Tamayo *et al.*, 2000), no inhibitory activity toward porcine pancreatic elastase was detected for WSCI. The presence of a methioninyl residue at the P1 position of the WSCI reactive site justifies the absence of anti-elastase activity; in fact, it is well known that the pancreatic enzyme catalyses the hydrolysis of peptide bonds whose C=O groups belong preferably to small neutral residues (Ala, Gly, Ser, Val) (Hartley and Shotton, 1971). In order to test the role played by the methioninyl residue at the P1 position of the WSCI reactive site, we assayed the wheat inhibitor against human leukocyte elastase; in fact, the primary specificity pocket of this enzyme has been reported to be easily accessible to larger or branched residues (Bode *et al.*, 1989). However, we were not able to detect inhibition of the human enzyme even using a large molar excess of inhibitor. We can also state that the absence of inhibition of human leukocyte elastase is not due to oxidation of methionine 48 of the inhibitor molecule since no peaks with mass values corresponding to oxidised forms of the protein were detected in the mass spectrum of the native inhibitor.

The physiological role of plant proteinase inhibitors is still a matter of investigation. Depending on their abundance, and on the modality of their expression (constitutive or inducible) in plant tissues, as well as on their capability to interact with endogenous and/or exogenous proteolytic enzymes, a role has been proposed for these proteins in amino acid storage, a function in modulating the endogenous proteolytic activities and a role in plant defence against attacks of predatory animals, insect pests and pathogenic microorganisms (Richardson, 1991).

Wound-induced inhibitors are quite numerous in the

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potato inhibitor I family and the accumulation of these proteins or of their mRNAs, following a variety of biotic and abiotic stress conditions (attacks of insects, microorganisms and viruses, mechanical wounding, darkness, chemicals, *etc*.) have been documented in *Zea mays* (Cordero *et al.*, 1994; Tamayo *et al.*, 2000), *Lycopersicon esculentum* (Green and Ryan, 1972; Plunkett *et al.*, 1982; Margossian *et al.*, 1988; Pautot *et al.*, 1991; Bowles, 1998), *Solanum tuberosum* (Green and Ryan, 1972; Cleveland *et al.*, 1987) and *Nicotiana tabacum* (Kuo *et al.*, 1984; Geoffroy *et al.*, 1990; Linthorst *et al.*, 1993). At the moment we cannot state whether the expression of WSCI is induced by stress conditions or if it is tissue specific.

WSCI, in addition to bacterial subtilisin and mammalian chymotrypsin, is capable of interacting with chymotrypsin-like activities that we detected in crude extracts of midguts dissected from coleopteran larvae of *T. molitor* and from lepidopteran larvae of two insects (*H. armigera and P. interpunctella*). The inhibition of insect proteolytic activities could suggest a role in plant defence for the wheat inhibitor. Expression studies will be carried out to evaluate the levels of mRNA or of the mature protein following mechanical wounding mimicking insect chewing.

During the writing of this manuscript, we obtained preliminary data regarding the capability of WSCI to powerfully interact with a chymotrypsin-like activity we detected in crude extracts of germinating wheat embryos. This finding, if confirmed, could support the role of this small protein inhibitor in the regulation mechanisms of endogenous proteolytic activity.

Materials and Methods

Materials

Triticum aestivum, San Pastore variety, was originally supplied from Istituto Nazionale per la Cerealicoltura (S. Angelo Lodigiano, Milano, Italy) and cultivated in 'Azienda Agraria Nello Lupori' of University of Tuscia (Viterbo, Italy). Ion exchange resins (CM-Sepharose CL-6B and Q-Sepharose Fast Flow), carrier ampholytes (Ampholine) and IEF protein markers were all products of Amersham Pharmacia Biotech Italia. Cells and membranes for ultrafiltration were obtained from Millipore S.p.A. (Italy). Mini Protean II and Mini IEF apparatus for electrophoretic and isoelectric focusing analyses and Protein Assay kit for determination of protein concentration were purchased from Bio-Rad Laboratories S.r.l. (Italy). Subtilisin (EC 3.4.21.62, from *Bacillus subtilis* and from *Bacillus licheniformis*), α-chymotrypsins (EC 3.4.21.1) N-tosyl-L-lysine chloromethyl ketone (TLCK)-treated, and trypsins (EC 3.4.21.4) N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated both from bovine and porcine pancreas, elastase (EC 3.4.21.36) from porcine pancreas, lysosomal elastase (EC 3.4.21.37) from human leukocytes, α-amylase (EC 3.2.1.1) from human saliva, insoluble subtilisin (from *B. licheniformis*) and insoluble α-chymotrypsin (from bovine pancreas), N-benzoyl-L-tyrosine ethyl ester (BTEE), N-α-p-toluene-sulphonyl-L-arginine methyl ester (TAME), N-t-BOC-L-alanine-*p*-nitrophenyl ester, DL-dithiothreitol (DTT), azocasein and protein markers for SDS-PAGE, were purchased from Sigma-Aldrich S.r.l. (Italy). Insect chymotrypsin-like activities were isolated in heterogeneous forms from midguts of larvae (*Tenebrio molitor*, *Plodia interpunctella* and *Helicoverpa armigera*) supplied by the department of 'Difesa delle Piante' of the University of Tuscia (Viterbo, Italy). Sequence-grade endoproteinase Asp-N was from Boehringer Mannheim S.p.A. (Italy).

The HPLC system was a Beckman GOLD™ apparatus; reverse-phase analytical columns C_{18} and C_8 were obtained from Beckman Coulter S.p.A. (Italy) and Vydac (Hesperia, CA, USA), respectively. All HPLC solvents were HPLC grade and were purchased from Carlo Erba (Italy). A pulsed liquid-phase automatic sequencer (mod. 473A) equipped on-line with a phenylthiohydantoin (PTH) amino acid analyser, ProSorb cartridges and all the sequence-grade chemicals were from Applied Biosystems.

Inhibitor Extraction and Purification

Wheat endosperms were obtained by manually removing embryos from grains. Finely ground flour (250 g) was extracted, under stirring, overnight at 6 °C with 1 l of 150 mM NaCl. The suspension was centrifuged for 30 min at 10 000 g and 6 °C and the supernatant was freeze-dried. The dried sample was then suspended in 20 mM ammonium acetate, pH 7.8 (200 ml) and dialysed against 10 vol of the same solution. After centrifugation (30 min at 10 000 g and 6 °C), the supernatant, containing antiproteinase activities, was applied to a CM Sepharose CL-6B column (2.5×28 cm) equilibrated with 20 mM ammonium acetate, pH 7.8, and washed with the equilibration solution, until the absorbance at 280 nm was below 0.01. Eluted fractions (12 ml each) were monitored for both proteinase inhibitory activities and absorbance at 280 nm. Retained proteins were then eluted with 300 mM ammonium acetate, pH 7.8 (400 ml). Both elution steps were carried out at a flow rate of 40 ml/h, at 6 °C. The active fractions eluted during the washing step were concentrated on a YM-3 Amicon membrane (nominal cut-off 3500). The concentrated sample (60 ml), equilibrated in 50 mM sodium acetate/acetic acid buffer, pH 5.0, was applied to a CM Sepharose CL-6B column (2.5×16 cm), and equilibrated with the same buffer. The elution was performed at a flow rate of 40 ml/h at 6 °C with the following program: (i) washing step with equilibration buffer (580 ml); (ii) linear gradient from 0 to150 mM NaCl in the equilibration buffer (700 ml); (iii) 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 fractions active against both chymotrypsin and subtilisin (eluted during the washing step) were pooled and concentrated. The sample was then equilibrated in 50 mM N-methyl-diethanolamine·HCl buffer, pH 8.4 and loaded onto a Q-Sepharose Fast Flow column (1.0×19.5 cm). The column, equilibrated with the same buffer, was eluted at 6 °C and at 30 ml/h with the following program: (i) washing step with equilibration buffer (65 ml); (ii) linear gradient from 0 to 150 mM NaCl in equilibration buffer (120 ml); (iii) isocratic step with 150 mM NaCl in equilibration buffer (45 ml). The fractions (1.5 ml each), eluted with the linear gradient and exhibiting both inhibitory activities, were pooled and concentrated.

To perform automatic sequence and mass spectrometry analyses on the native inhibitor molecule, the active sample (obtained by Q-Sepharose Fast Flow column) was submitted to RP-HPLC on a C_8 Vydac column (0.46×25 cm). Eluent A was aqueous 0.05% trifluoroacetic acid and eluent B was 0.05% trifluoroacetic acid in acetonitrile. Elution, performed at a flow rate of 1 ml/min, was accomplished with a concave gradient from 20 to 60% of B in 100 min. Each peak was manually collected by monitoring absorbance at 214 nm, lyophilised and re-suspended in 20 mM Tris-HCl, pH 8.0.

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Determination of Protein Concentration and Inhibitory Activities

The protein concentration was determined with the Bio-Rad Protein Assay kit following the manufacturer's instructions and using bovine serum albumin (BSA) as the standard. Trypsin and anti-trypsin activities, as well as chymotrypsin and anti-chymotrypsin activities, were determined following a procedure already described (Poerio *et al.*, 1989) and using TAME and BTEE as substrates, respectively. Porcine pancreatic elastase and subtilisin activities, as well as anti-elastase and anti-subtilisin activities, were measured by the same procedure employed for chymotrypsin and anti-chymotrypsin activities. Lysosomal elastase from human leukocytes was assayed as already described (Janoff, 1969) using N-t-BOC-L-alanine-*p*-nitrophenyl ester as the synthetic substrate. The neutrophil elastase susceptibility to the WSCI inhibitor was evaluated by pre-incubating (for 2 min at 37 °C in 50 mM phosphate buffer, pH 6.5) 0.2 units of enzyme (defined according to the supplier) with increasing amounts of the wheat inhibitor (up to 10 µg); the residual enzymatic activity was then measured after addition of the synthetic substrate. Insect chymotrypsin-like activities, isolated from larvae midguts, were determined by using the same assay conditions already described for pancreatic chymotrypsins; their susceptibility to the inhibitor was evaluated with the same procedure adopted for chymotrypsin. Proteinase and anti-proteinase activities were also assayed using azocasein as a substrate. K_i values of the purified inhibitor were calculated according to the equation proposed for tightly-bound inhibitors (Cha, 1975).

Electrophoretic Analyses

Homogeneity of the inhibitor preparation was evaluated by SDS polyacrylamide slab gel electrophoresis (Schägger and von Jagow, 1987); a 16.5% T, 3% C was used as a separating gel overlaid by a 4% T, 3% C stacking gel. Reference proteins were: bovine serum albumin (M_r 66 000), ovalbumin (M_r 45 000), glyceraldehyde-3-phosphate dehydrogenase (M_r 36 000), carbonic anhydrase (M_r 29 000), trypsinogen (M_r 24 000), soybean trypsin inhibitor (M_r 20 100), α-lactoalbumin (M_r 14 200) and insulin β-chain (M_r 3500). Electric focusing in the pH range 2.5 – 7.0 was carried out on a polyacrylamide slab gel (5% T, 3% C) on the Mini-IEF apparatus. Carrier ampholytes were used in the following proportions: four parts of pH 2.5 – 4.0, three parts of 3.5 – 5.0, two parts of pH 4.0 – 6.0, one part of pH 5.0 – 7.0 and one part of pH 6.0 – 8.0. Reference proteins were pepsinogen (pI 2.8), amyloglucosidase (pI 3.5), glucose oxidase (pI 4.15), soybean trypsin inhibitor (pI 4.55), β-lactoglobulin A (pI 5.20), bovine B carbonic anhydrase (pI 5.85) and human B carbonic anhydrase (pI 6.55). Native polyacrylamide slab gel electrophoresis runs were performed at acidic (pH 4.3) and alkaline (pH 8.8) conditions.

Hydrolysis on Subtilisin-Agarose and Chymotrypsin-Agarose

Aliquots (0.5 mg) of active and purified inhibitor were equilibrated in 80 mM Tris-HCl, 100 mM $CaCl_2$, pH 7.8 and applied onto a subtilisin-agarose column (0.7×2.5 cm) equilibrated with the same buffer at 6 °C. The first washing step was performed with the equilibration buffer (6 ml) and continued with the same buffer containing 1 M NaCl (3 ml). The column was finally eluted with 200 mM KCl, pH 2.0. Fractions (0.5 ml each) were collected and their absorbance monitored at 220 nm. The same procedure was used for the affinity chromatography on an α-chymotrypsin-agarose column (0.7×1.0 cm). In both cases, fractions eluted at acidic pH were dialysed, concentrated and purified by RP-HPLC on an ultrasphere C_{18} column (0.46×25 cm, 5 µm particle size). Eluent A was aqueous 0.1% trifluoroacetic acid and eluent B was 0.1% trifluoroacetic acid in acetonitrile. Elution was accomplished at a flow rate of 1 ml/min with the following program: isocratic elution for 10 min at 5% B, followed by a linear gradient from 5 to 55% B in 100 min. The fractions corresponding to the eluted peaks were manually collected, monitoring the absorbance at 214 nm and freeze-dried.

Asp-N Hydrolysis

Peptide fragment S3 produced by the interaction of WSCI inhibitor with subtilisin-agarose support was digested with endoproteinase Asp-N. Hydrolysis was performed at 37 °C in 50 mM sodium phosphate buffer, pH 8.0 containing 10% acetonitrile. The enzyme:substrate ratios were: 1:100 for 4 hours; 1:50 overnight; 1:25 for a further 4 hours. The digestion mixture was stopped by adding few drops of formic acid and 8 M guanidine hydrochloride up to a final concentration of 2 M. The mixture was then resolved by RP-HPLC on an ultrasphere C_{18} column (0.46×25 cm, 5 µm particle size). Eluent A was aqueous 0.1% trifluoroacetic acid and eluent B was 0.1% trifluoroacetic acid in acetonitrile. Elution was accomplished at a flow rate of 1 ml/min with the following program: isocratic elution for 10 min at 5% B, followed by a linear gradient from 5 to 55% B in 100 min. The fractions corresponding to the peaks were manually collected by monitoring the absorbance at 214 nm and freeze-dried.

Sequence Analyses

All sequence analyses on the native protein inhibitor, as well as on the peptide fragments originated by enzymatic cleavages, were performed by automated Edman degradation as previously reported (Parente *et al.*, 1993). To clean up and concentrate the samples prior to sequence analysis they were absorbed on a ProSorb cartridge.

Electrospray Mass Spectrometry (ES-MS)

Protein and peptide samples were analysed by ES-MS using a Platform single-quadrupole mass spectrometer (Micromass, Manchester, UK). Aliquots (20 µl; 1 µg per 10 µl) of protein solution were injected onto the ion source at a flow rate of 10 µl min^{-1}. 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 40V.

Homology Studies

A sequence similarity search between WSCI and proteins present in databases was carried out by using the BLAST (Basic Local Alignment Search Tool) program available at NCBI (National Center for Biotechnology Information) web site.

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