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A TRYPSIN INHIBITOR FROM THE WATER-SOLUBLE PROTEIN FRACTION OF WHEAT KERNEL

ELIA POERIO, LUCIA CARRANO, ANNA MARIA GARZILLO and VINCENZO BUONOCORE*

Dipartimento di Chimica Organica e Biologica, Università di Napoli, Via Mezzocannone 16, 80134 Napoli, Italy; *Dipartimento di Agrobiologia e Agrochimica, Università della Tuscia, 01100 Viterbo, Italy

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Key Word Index—*Triticum aestivum*; Gramineae; wheat kernel; albumins; protein inhibitors; trypsin inhibitor.

Abstract—A protein inhibitor which is active against both exogenous and endogenous trypsin has been purified from wheat endosperm. The inhibitor is a single-chain protein of M_r 8500 and isoelectric point 8.7. It contains 10 half-cystine residues per mol, all of them linked in disulphide bridges. The *N*-terminal sequence, 25 residues long, has been determined. The inhibitor is very active against bovine, porcine, cod and wheat trypsin, and inactive against animal chymotrypsin and bacterial subtilisin. The inhibitor binds bovine trypsin with a 1:1 molar stoichiometry.

INTRODUCTION

Proteins which act as strong and specific inhibitors of hydrolytic enzymes are widely distributed in plant tissues [1, 2]. Depending on their localization and relative abundance in the plant and on the specificity of inhibition for endogenous and/or exogenous enzymes, these inhibitors have been thought to be either regulatory or protective proteins.

In particular, α-amylase inhibitors have been found as major components of the soluble proteins in wheat, bean and in other species of the Graminae and Leguminosae [2]. Since the specificity of these inhibitors is primarily directed toward digestive α-amylases found in animals, and only a few are known that inhibit endogenous enzymes, one major role of these proteins might be to protect the plant by interfering with the digestive processes of invading pests. On the other hand, amylase inhibitors, being thermostable and resistant to mild technological processes, have been found in active form in many commonplace cereal- and legume-based foods, thus giving rise to concern about the nutritional significance of these factors and their effects on animals and humans.

Unlike the α-amylase inhibitors, protease inhibitors from wheat have received limited attention. A number of authors reported the occurrence in wheat kernel of either alkaline or acidic protein factors active against trypsin and/or chymotrypsin; however, only a few of these proteins have been purified and characterized and their role is still obscure [1, 3–8]. In this study, we report the purification and the characterization of a wheat endosperm inhibitor active towards both endogenous and animal trypsin and inactive against chymotrypsin.

RESULTS

Inhibitor purification

Fractionation on Sephadex G-100 of the water-soluble proteins (albumins) extracted from endosperm flour gave the typical five-peak pattern made up of proteins with M_rs of >60000, 50000, 26000, 13000 and <7000 [9]. According to Petrucci *et al.* [9], antitryptic and antichymotryptic activities have been found in the protein fractions with M_r between 8000 and 26000. Similar results were obtained by submitting whole wheat flour to gel filtration. These findings are in agreement with those of Mikola and Kirsi [10], who observed that the endospermal inhibitor was the dominant component in whole grains. On applying the inhibitor fraction eluted from Sephadex G-100 to a CM-Sepharose column, two antitryptic activities were obtained. The summary of the purification of the major antitryptic inhibitor is reported in Table 1. When submitted to disc-gel electrophoresis at acidic pH, the purified trypsin inhibitor behaved as a homogeneous protein with mobility, relative to Methyl Green, of 0.45. The homogeneity of the inhibitor preparation was also supported by its behaviour on SDS-gel electrophoresis, gel-filtration chromatography and equilibrium sedimentation.

The inhibitor was code named WTI.

Molecular properties

As observed by disc-electrophoresis in the presence of SDS, the trypsin inhibitor is a single polypeptide chain of M_r 8000. A similar value (9000) was obtained by gel-filtration chromatography on a TSK SWG 3000 column. By using a partial specific volume of 0.723, a M_r of 8900 for WTI was calculated from the data obtained from equilibrium-sedimentation experiments.

The inhibitor is a basic protein with a pI of 8.7. The amino acid composition is reported in Table 2 and compared with those of other trypsin inhibitors purified from wheat [4–7]. As indicated by DTNB titration, no free thiol groups were present in the inhibitor molecule; 10 half-cystine residues have been determined either as cysteic acid or carboxymethylcysteine. These findings are consistent with a content of five cystine residues per molecule. The high content of disulphide bridges is characteristic of a number of plant protease inhibitors as

Table 1. Purification of the trypsin inhibitor from wheat endosperm

Purification step	Vol (ml)	Protein (mg)	Specific activity (U/mg)	Yield (%)
Saline extraction	250	1630	25	100
$(NH_4)_2SO_4$ fractionation	20	600	64	96
Sephadex G-100	27	240	141	85
CM-Sepharose CL 6B	2.2	11.7	1199	35
Mono S	1.0	1.14	4802	14

well as of α-amylase inhibitors from wheat. From the amino acid composition of the inhibitor, we calculated a M_r of 8188.

The N-terminal sequence of the inhibitor was as follows: H_2N-Glu-Glu-Ala-Met-Pro-Ser-Ala-Trp-Pro-Cys-Cys-Asp-Glu-Cys-Gly-Thr-Cys-Thr-Arg-Met-Ile-Pro-Pro-Arg-Cys-. The presence of serine at position 6 and of threonine at positions 16 and 18 was confirmed by mass spectrometry analysis of the corresponding PTH-amino acids.

Inhibiting properties

WTI was very active against trypsin from various sources (bovine, porcine, cod and wheat). About 0.024 nmol of inhibitor gave 30% inhibition of 0.3 mU of each animal protease, whereas a five-fold higher amount of inhibitor was needed to inhibit to the same extent 0.3 mU of the endogenous trypsin. Up to 2.4 nmol of WTI, no inhibition was observed toward other serine proteases such as bovine α,β,γ,δ chymotrypsin, *Bacillus subtilis* subtilisin and endogenous chymotrypsin. WTI was also inactive against α-amylase from various sources (*Tenebrio molitor*, chicken pancreas, human saliva).

The extent of inhibition of WTI toward bovine trypsin was dependent on pH and on preincubation time of enzyme and inhibitor; maximal inhibition was observed after 3-min preincubation at pH 8.2. The inhibition was linear with respect to the amount of inhibitor up to 100%; 79 pmol of inhibitor inactivated completely 81.2 pmol of trypsin with a molar ratio of inhibitor and enzyme of 0.97:1 (Fig. 1).

DISCUSSION

Several authors have reported on the occurrence of antitryptic activities in different wheat cultivars but only a few of them have isolated and characterized protein inhibitors from this source [3–7]. Mitsunaga [6] isolated from wheat germ two basic proteins (WGTI-I and WGTI-II) showing M_r of 16000 and 10000, respectively. A third trypsin inhibitor (WETI) was purified later from wheat endosperm in the same laboratory [4]; it weighed about 8000 and inhibited weakly chymotrypsin too. An inhibitor with a similar specificity (WFTI) was isolated at the same time by Boisen and Djurtoft [5]; however this protein appeared to be a little heavier (M_r about 12500). Hochstrasser and Werle [3] also reported the purification of a trypsin inhibitor from wheat germ weighing about 17000; however, the use in their purification procedure of an affinity-chromatography step on resin-bound trypsin led to the isolation of a modified inhibitor which had been subject to proteolytic cleavage so that a comparison of the properties of this protein with those of

Table 2. Amino acid composition of wheat tryptic inhibitors with similar M_r

Amino acid	WTI (residues %)	Residues/mol				
		WTI	WETI [4]	WGTI-II [6]	WFTI [5]	II-4 [7]
Asx	8.1	6	6	8	9	5
Thr	8.1	6	6	5	9	5
Ser	5.4	4	4	6	5	4
Glx	8.1	6	6	14	9	4
Pro	9.4	7	7	6	11	4
Gly	8.1	6	5	11	7	2
Ala	6.7	5	5	10	7	5
1/2Cys	13.5	10	6	3	18	10
Val	4.0	3	3	5	5	2
Met	4.0	3	4	2	4	4
Ile	2.7	2	2	3	3	1
Leu	2.7	2	2	4	4	—
Tyr	—	—	2	1	1	1
Phe	2.7	2	2	2	3	2
Lys	2.7	2	2	5	4	4
His	1.3	1	2	2	2	1
Arg	6.7	5	8	7	10	5
Trp	5.4	4	—	1	1	2
Total residues		74	72	95	112	61

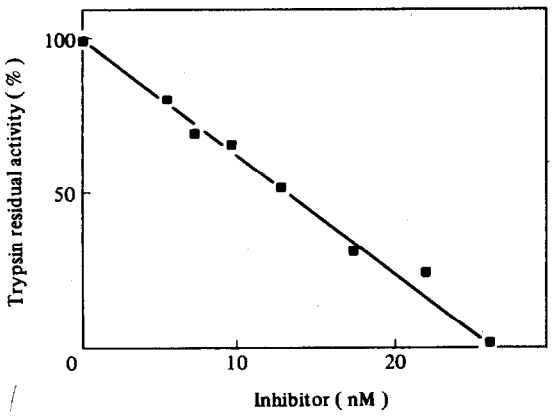

Fig. 1. Effect of inhibitor concentration on bovine trypsin inhibition. Trypsin concentration was 27.07 nM.

the other aforementioned inhibitors is hard to make. Recently, Odani *et al.* [7] isolated, with the same technique, a number of trypsin inhibitors falling into two families of different M_r (14 500 and 7000); also in this case, a number of minor components might be the products of tryptic degradation occurring during the preparation.

Apparently, all the trypsin inhibitors so far isolated from wheat flour are distinct molecules, even though they share some common properties. All of them are single-chain basic proteins with pI values ranging from 8.7 to 9.3 and inhibit trypsin with a 1:1 stoichiometry; chymotrypsin is either weakly or not inhibited at all. On the other hand, marked differences have been observed among the molecular properties of the various inhibitors. Amino acid compositions are significantly different and M_rs range from 7000 [7] to 10 000–12 500 (WGTI-II and WFTI, respectively) up to 16 000 (WGTI-I).

The inhibitor described in this paper (WTI) has a M_r similar to that of the endosperm inhibitor (WETI) isolated by Mitsunaga *et al.* [4], but other properties are distinctly different. WTI has a pI value of 8.7, as compared to that of 9.3 of WETI, and does not inhibit chymotrypsin; the amino acid composition shows that the level of four residues (1/2 Cys, Tyr, Arg and Trp) differs significantly in the two proteins (Table 2). The presence of tryptophan residues in WTI has been confirmed by *N*-terminal sequence analysis and the lower content of arginine in WTI as compared to WETI may account for the lower pI of the former inhibitor. The occurrence in wheat cultivars of several functionally related protein components with different molecular properties could be attributed to protein evolution from a common ancestor.

The N-terminal amino acid sequence of WTI has been compared with the known sequences of a large number of protease inhibitors from various sources and of other related cereal proteins [11]. The *N*-terminal region of WTI is highly homologous (40–56%) to that of eight Bowman–Birk type protease inhibitors occurring either in Leguminous plants [12, 13] or in cereals [7, 14, 15]. In particular, in all these homologous proteins there are seven invariant amino acids which include four half-cystine residues out of the five found (Table 3).

Since most trypsin inhibitors occurring in seeds and storage organs are active against exogenous trypsin, they are thought to play a defence role in the plant against attack from micro-organisms, insects and higher animals [1]. The defence role has been questioned for those inhibitors present at low levels in tissues or plants. However, the findings that, at least in tomato and potato, leave wounding by pest attack induces a rapid accumulation of protease inhibitors in the plant [16], provides still more support to the possible involvement of protease inhibitors in plant protection. WTI levels in the kernel appear low (*ca* 0.5% of water-soluble proteins) and preliminary investigations did not suggest the presence of any wound-induced mechanism of inhibitor accumulation in the plant [E. Poerio *et al.*, unpublished results]; moreover, WTI is fully active against endogenous trypsin. These findings suggest a regulatory role for this inhibitor which might control protein metabolism during development and germination of the kernel by forming inactive trypsin-inhibitor complexes. A similar conclusion was arrived at by Preston and Kruger, who followed the activity of proteolytic enzymes during development of wheat grains [17]. It is also worth recalling that in the endosperm of barley, a species taxonomically closely related to wheat, protease inhibiting activities disappear during germination [18].

Table 3. Comparison of N-terminal amino acid sequences of trypsin inhibitors from various sources

	1								10										20									
WTI		E	E	A	M	P	S	A	W	P	C	C	D	E	C	G	T	C	T	R	M	I	P	P	R	C
DE-3	. . .	D	E	P	S	E	S	S	K	P	C	C	D	E	C		A	C	T	K	S	I	P	P	Q	C
DE-4	. . .	D	E	S	S	E	S	S	K	P	C	C	D	L	C		T	C	T	K	S	I	P	P	Q	C
MBI	. . .	D	E	P	S	E	S	S	E	P	C	C	D	S	C		D	C	T	K	S	K	P	P	Q	C
BBI				D	D	E	S	S	K	P	C	C	D	Q	C		A	C	T	K	S	N	P	P	Q	C
II-4			A	T	R	P			W	K	C	C	D		R	A	I	C	T	K	S	F	P	P	M	C
I-2b			A	A	K	K	R	P	W	K	C	C	D		Q	A	V	C	T	R	S	I	P	P	I	C
Coix	. . .	D	E	K	R	P			W	E	C	C	D		I	A	M	C	T	R	S	I	P	P	I	C
RBI	. . .	T	P		R	P		W	G	D	C	C	D	K		A	F	C	N	K	M	N	P	P	T	C

Single-letter codes for amino acids are: A = Ala; C = Cys; D = Asp; E = Glu; F = Phe; G = Gly; H = His; I = Ile; K = Lys; L = Leu; M = Met; N = Asn; P = Pro; Q = Gln; R = Arg; S = Ser; T = Thr; V = Val; W = Trp; Y = Tyr. Areas of high homology in sequences are boxed.

DE-3, DE-4 [12], MBI [13] and BBI [7] are double-headed Bowman–Birk-type inhibitors from Leguminosae; II-4 and I-2b [7] are from wheat grains; Coix [14] is from seeds of Jobs' tears; RBI [15] is from rice bran.

It is well documented that the trypsin inhibitors are the major factors responsible for the adverse nutritional effects of raw soybean and other inhibitor-rich products on experimental animals. The primary physiological effects of these inhibitors are growth inhibition and pancreatic hypertrophy and hyperplasia, accompanied by an increase in protein synthesis and exocrine secretion from the pancreas [19]. These effects are usually manifested in animals which have been fed large amounts of raw materials or purified inhibitors for short period of time, but the question is still open as to whether or not the ingestion of low levels of active trypsin inhibitors over a prolonged period of time can lead to adverse effects [19]. Studies are now in progress to ascertain the stability of wheat tryptic inhibitors to heat, pH and animal proteases and to verify the possibility that they could reach the animal duodenum in an active form.

EXPERIMENTAL

Materials. Triticum aestivum, pure variety S. Pastore, was kindly supplied by Istituto Sperimentale per la Cerealicoltura (Roma, Italy). Exogenous proteases and their substrates, and standard proteins for M_r and pI determination were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Mono S prepacked HR 5/5 column, Sephadex G-100 and CM-Sepharose CL 6B were the products of Pharmacia Fine Chemicals (Uppsala, Sweden). Carrier ampholytes (Ampholine) and TSK SWG 3000 column were supplied from LKB (Bromma, Sweden). All other reagents were of analytical grade.

Inhibitor extraction and purification. Wheat grains (300 g) were treated for 3 hr at room temp. with 300 ml 9 M H_2SO_4; after washing out the acid with running H_2O, the grains were soaked for 3 hr in H_2O. Embryos and endosperms were separated by hand dissection and dried at room temp. under red. pres.

Finely ground flour (200 g) from endosperms was extracted for 3 hr at room temp. on a rotary shaker with 300 ml of 150 mM NaCl. The suspension was centrifuged for 30 min at 18 000 *g* and the fraction pptd from the supernatant between 10 and 80% $(NH_4)_2SO_4$ was collected by centrifugation, suspended in H_2O and dialysed against 100 mM NH_4OAc buffer (pH 6.8). *Ca* 0.5 g of protein were applied to a Sephadex G-100 column (5 × 80 cm) equilibrated and eluted, at room temp., with the acetate buffer at a flow rate of 42 ml/hr; the *A* of the eluate was monitored continuously at 280 nm. Fractions of 10 ml each were collected and tested for protease and antiprotease activities; the active fractions were pooled and lyophilized. The trypsin activity was localized in the albumin fraction with apparent M_r higher than 40 000; the antitryptic activity was eluted in the fraction with apparent M_r between 8000 and 26 000.

Ca 0.1 g of lyophilized material with antitryptic activity was suspended in 20 mM NH_4OAc buffer (pH 7.8), clarified by centrifugation and applied to a CM-Sepharose CL 6B column (1.5 × 18 cm) equilibrated with the same buffer. After washing the column with the equilibration buffer (90 ml), proteins were eluted with the following program: linear gradient of 20–70 mM NH_4OAc buffer, pH 7.8, (25 ml of each); washing with 50 ml of 70 mM acetate buffer; linear gradient of 70–200 mM acetate buffer (25 ml of each); washing with 50 ml of 200 mM acetate buffer. The fractions (1.2 ml each) were monitored for *A* at 280 nm and trypsin inhibitory activity; the active fractions eluted at about 190 mM acetate were pooled, concd and equilibrated in 10 mM Na-Pi buffer (pH 7.5) by ultrafiltration on an Y-M 5 Amicon membrane (nominal cut-off 5000). This inhibitor fraction was further chromatographed on a Mono S HR 5/5 column by using a FPLC apparatus from Pharmacia. The column was equilibrated at a flow rate of 0.5 ml/min with the phosphate buffer, and eluted with the following program: linear gradient of 0–30 mM NaCl in 10 mM Na-Pi buffer, pH 7.5 (3 ml of each); washing with 8 ml of the Pi buffer, 30 mM in NaCl; linear gradient 30–200 mM NaCl in the phosphate buffer (8 ml of each). Active fractions (0.25 ml each), eluted at about 60 mM NaCl, were pooled, ultrafiltered and equilibrated with 10 mM Pi buffer, pH 7.5.

Wheat trypsin purification. The albumin fraction with tryptic activity eluted from the aforementioned Sephadex G-100 column was utilized for a partial purification of the endogenous protease. Lyophilized material (0.1 g) was suspended in 50 mM NH_4OAc buffer (pH 4.5), clarified by centrifugation and applied to a CM-Sepharose CL 6B column (1 × 30 cm) equilibrated with the same buffer. The elution was carried out at a flow rate of 0.1 ml/min with a linear salt gradient of 0–0.4 M NaCl in the acetate buffer (50 ml of each). The fractions (1 ml each) were monitored for trypsin activity; the active fractions, eluted at *ca* 90 mM NaCl, were pooled and concd by ultrafiltration. This chromatographic step allowed a 30-fold purification of the tryptic activity.

Amino acid analysis. Protein samples (8 μg) were hydrolysed *in vacuo* for 24, 48 and 72 hr in 6 M HCl and analysed with a Beckman 119 CL amino acid analyser as described in ref. [20]. Tryptophan content was evaluated on the same apparatus by hydrolysing the protein with 3 N mercaptoethansulphonic acid according to ref. [21].

The presence of free thiol groups was investigated with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) [22]; half-cystine residues were determined as cysteic acid [23] or carboxymethylcysteine [24].

Determination of protein and inhibitory activity. The protein concentration was determined by using the Bio-Rad Protein Assay kit, following the manufacturer's instructions and using bovine serum albumin as standard.

Trypsin activity was measured at 30° by monitoring the increase of absorbancy at 247 nm in a reaction mixture (3.0 ml) that contained 0.40 M Tris–HCl buffer (pH 8.1), 0.01 M $CaCl_2$, 0.1 mM *N-p*-toluen-sulphonyl-L-arginine methyl ester (TAME) as substrate and trypsin [25]. One trypsin unit is the amount of enzyme producing one μmol/min of product in the described assay conditions. The antitryptic activity was determined by preincubating in the same reaction mixture (2.7 ml) trypsin and inhibitor for 5 min at 30°; the residual tryptic activity was then tested after addition of the substrate. One unit of inhibitory activity is the amount of inhibitor giving 30% inhibition of 278 μU of protease.

Chymotrypsin activity was determined at 25° by monitoring the increase of absorbancy at 256 nm in a reaction mixture (3.0 ml) that contained 0.08 M Tris–HCl (pH 7.8), 0.1 M $CaCl_2$, 0.5 mM *N*-benzoyl-L-tyrosine ethyl ester (BTEE) and chymotrypsin [25]. The antichymotryptic activity was determined by preincubating in the same reaction mixture (2.86 ml) chymotrypsin and inhibitor for 5 min at 25°; the residual enzymatic activity was then tested after addition of the substrate.

The subtilisin and antisubtilisin activities were assayed according to ref. [25] using BTEE as substrate. The amylase and antiamylase activities were tested as already described [26].

Electrofocusing, electrophoresis and M_r *determination.* Electrofocusing in the pH range 7.5–10.5 was carried out on a polyacrylamide gel slab in a Multiphor apparatus from LKB by following the manufacturer's instructions. The mixture of ampholines contained 0.1% ampholine pH 7–9 and 2.4% ampholine pH 9–11. Ribonuclease A (pI 8.7), alcohol dehydrogenase (9.7) and cytochrome *c* (10.4) were used as standards.

SDS-polyacrylamide slab-gel electrophoresis was performed according to ref. [27]; reference proteins were egg albumin (M_r

43 000), chymotrypsinogen A (25 000), cytochrome *c* (12 500) and insulin β-chain (3000). Disc-gel electrophoresis at acidic pH (4.3) was performed according to ref. [28] at a concn of 10% acrylamide.

The TSK SWG 3000 column (0.75 × 60 cm) was equilibrated and eluted with 10 mM Na-Pi buffer, pH 7.5. Standard protein mixture was as that used in the SDS-polyacrylamide gel electrophoresis.

Equilibrium-sedimentation runs were performed at 4° in a Beckman E analytical ultracentrifuge for 48 hr at 8000 rev/min in 10 mM Pi buffer, 0.1 M KCl (pH 7.5); the protein concentration was 0.5 mg/ml.

Sequence determination. N-Terminal sequence of the reduced and carboxymethylated inhibitor (20 nmol) was determined on a Beckman 890/C sequencer, modified with a cold trap, using a 200 mM Quadrol and double-coupling single-cleavage program. Polybrene (Pierce, Rockford, IL) in 10 mM NaCl was used as carrier. Phenylthiohydantoin (PTH)-amino acids were analysed by HPLC as described in ref. [29]. PTH-derivatives of serine and threonine were identified on a Kratos MS80 mass spectrometer [30]. Amino acid sequences were compared by the 'Microgenie' sequence analysis program from Beckman.

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