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ISOLATION AND CHARACTERIZATION OF A PROTEASE INHIBITOR FROM SPINACH LEAVES

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Key Word Index—*Spinacia oleracea*; Chenopodiaceae; spinach; leaf; protease inhibitor; serine protease; trypsin.

Abstract—An acid-stable and heat-labile proteinous protease inhibitor which was found in spinach leaves but not in seeds was isolated by sequential chromatography and preparative isoelectric focusing. The isoelectric point of this inhibitor was 4.5. The inhibitor had a M_r of *ca* 18 000 and was rich in aspartic acid and glycine; it had 4 half-cystine, 2 tryptophan and no methionine residues. Its extinction coefficient ($E^{1\%}_{1cm}$) was 13.7 at 280 nm. The inhibition was competitive and the dissociation constant was 3.32×10^{-13} M. The inhibitor was specific to serine proteases and strongly inhibited trypsin and weakly inhibited α-chymotrypsin and kallikrein.

INTRODUCTION

It is known that proteinous protease inhibitors are widely distributed in micro-organisms, animals and higher plants [1–4]. In higher plants, many kinds of protease inhibitors have been purified, mainly from legume seeds and various tissues in the Solanaceae [5, 6]. The inhibitors from soybean seeds have been most thoroughly studied with respect to their structures and their protease–protease inhibitor interactions [7]. The present paper reports the results of the purification and characterization of a protease inhibitor from spinach leaves.

RESULTS

Distribution of protease inhibitors

The elution profiles on Sephadex G-75 chromatography of the acid-soluble crude extracts prepared from various spinach tissues are shown in Fig. 1. Inhibitors for trypsin were eluted in two peaks in seeds (Fig. 1, A) and in leaves (Fig. 1, B) and in three peaks in roots (Fig. 1, C). Chromatography of the inhibitors on a calibrated Sephadex G-75 column indicated apparent M_rs of *ca* 40 000 (termed I-40K), *ca* 20 000 (termed I-20K) and *ca* 7000 (termed I-7K), respectively. I-20K was found in leaves and roots, but not in seeds.

Purification of the protease inhibitor (I-20K) from leaves

The acid-soluble crude extract of spinach leaves, which was desalted with a Sephadex G-25 column, was applied to a DEAE-Sephacel column. As shown in Fig. 2, some of the inhibitory activities for trypsin eluted with the initial buffer and some were eluted with a salt gradient at

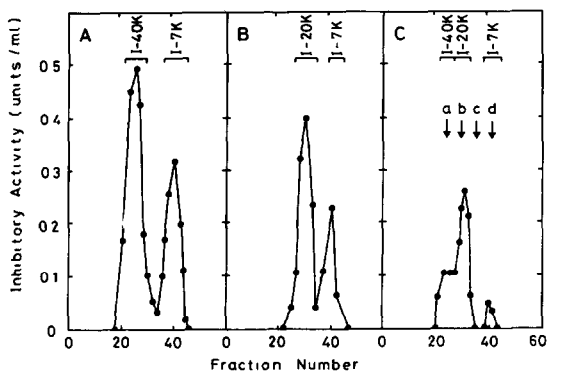

Fig. 1. Gel filtration of spinach trypsin inhibitors on Sephadex G-75. The acid-soluble materials were extracted from: 25 g seeds (A), 740 g leaves (B) and 562 g roots (C). Elution was done with Tris–HCl buffer, pH 7.4. Arrows a, b, c and d in the figure indicate the positions of the M_r markers of 45 000, 22 000, 12 500 and 6500, respectively.

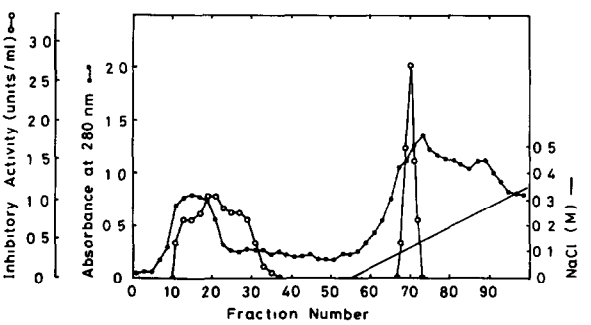

Fig. 2. Ion-exchange chromatography of the trypsin inhibitors extracted from spinach leaves on DEAE-Sephacel. The applied material was eluted by a linear gradient of NaCl. (●—●) Absorbance at 280 nm; (o—o) inhibitory activity for trypsin.

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0.12 M NaCl. These activities corresponded to the I-7K and I-20K inhibitor activities, respectively, determined by rechromatography on Sephadex G-75. The I-20K fraction was applied to a trypsin-polyacrylamide affinity column. The inhibitor was eluted with 10 mM HCl as a single peak, and the elution peak of the inhibitory activity coincided with that of the A_{280} absorption. This fraction was collected, adjusted to 50 mM Tris–HCl by adding 1 M Tris–HCl buffer (pH 7.4) and subjected to preparative isoelectric focusing (Fig. 3). The inhibitor focused at pH 4.5. A summary of the typical purification procedures is presented in Table 1. An overall purification of *ca* 2500-fold was achieved. About 0.8 mg of the purified protease inhibitor was obtained from 2 kg of spinach leaves with 10.9% recovery.

SDS-polyacrylamide gel electrophoresis

The purified protease inhibitor (*ca* 5 μg) was assayed for purity by SDS-polyacrylamide gel electrophoresis (Fig. 4). A single band, stained with Coomassie Brilliant Blue R-250, was observed at the position of M_r 18 000.

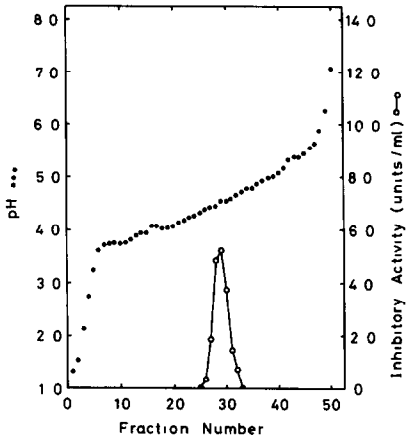

Fig. 3. Isoelectric focusing of the inhibitor eluted from an affinity column. The focusing was done for the first day at a constant voltage of 400 V, the second day at 600 V and the third and fourth days at 900 V. (●●●) pH; (o—o) inhibitory activity for trypsin.

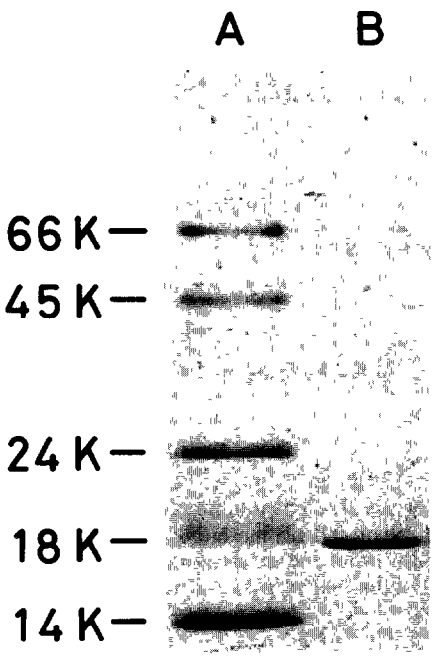

Fig. 4. SDS-polyacrylamide gel electrophoresis of the purified inhibitor. The electrophoresis was performed according to Laemmli [18], using 12% acrylamide gel. Proteins on the gel were stained with 0.1% Coomassie Brilliant Blue R-250 dissolved in 50% TCA. (A) Indicator: albumin (M_r 66 000), ovalbumin (M_r 45 000), trypsinogen (M_r 24 000), β-lactoglobulin (M_r 18 400) and lysozyme (M_r 14 300); (B) purified inhibitor (5 μg).

Amino acid composition

The results of amino acid analysis are given in Table 2. The calculation of amino acid residues was based on an M_r of 18 000. The inhibitor was composed of 166 residues and had a calculated M_r of 17 921. It was rich in aspartic acid and glycine, and had 4 half-cystine, 2 tryptophan and no methionine residues.

Table 1 Summary of the purification of protease inhibitor (I-20K)

Steps	Recovery (%)	Specific activity (units/mg)	Purification (times)
10 000 *g* sup.*	100.0	0.052	1.0
Perchloric acid† (f.c. 3%)	56.9	0.105	2.0
Ammonium sulfate* (70% sat.)	24.3	0.112	2.1
DEAE-Sephacel (pH 7.4)			
Non-charged	16.8	1.03	19.7
0.12 M NaCl		1.01	19.3
Sephadex G-75	16.4	4.20	80.8
Trypsin-PAG	11.5	117.1	2300
Isoelectric focusing	10.9	130.0	2500

*Activity was measured after desalting on Sephadex G-25.

†Activity was measured in the 10 000 *g* supernatant fraction after neutralization with 5 M KOH

Table 2. Amino acid composition of spinach trypsin inhibitor (I-20K)

Amino acid	nmol	Residues per molecule
Aspartic acid	39.4	20
Threonine	30.0	16
Serine	28.3	15
Glutamic acid	23.7	12
Proline	17.9	9
Glycine	35.7	19
Alanine	15 7	8
Valine	24.3	13
Half-cystine*	7.4	4
Methionine†	0.4	0
Isoleucine	18.5	10
Leucine	23.1	12
Tyrosine	14.1	7
Phenylalanine	10.8	6
Lysine	11.0	6
Histidine	1.8	1
Arginine	12.0	6
Tryptophan‡	3.9	2
Total		166
M_r		17921

Calculations were based on an M_r of 18000.
*Determined as cysteic acid [19].
†Determined as methionine sulfone [19].
‡Determined by hydrolysis in the presence of thioglycollic acid [20].

Absorption spectrum and extinction coefficient

The UV absorption spectrum of the protease inhibitor in 50 mM Tris–HCl (pH 7.4) showed a maximum at 280 nm and a minimum at 245 nm. The A_{280}/A_{260} ratio of the protein was 1.92 and the extinction coefficient ($E^{1\%}_{1cm}$) was 13.7 at 280 nm.

Stoichiometric experiments

Inhibition of trypsin activity at pH 7.8 by increasing the level of inhibitor is shown in Fig. 5. The titration curve

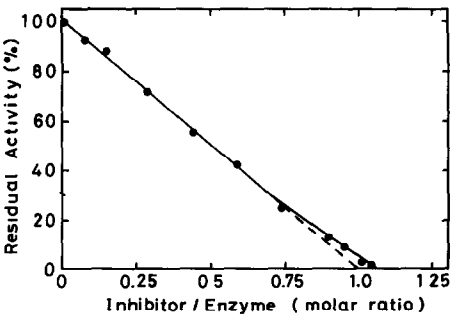

Fig. 5. Titration curve of trypsin activity with the inhibitor. The inhibitor was first added to the trypsin solution and incubated for 10 min at 25° and then the reaction was started by the addition of BAEE.

was linear up to 80% inhibition of trypsin activity and the extrapolation of the curve indicated that 1 mol of the inhibitor reacted with 1 mol of trypsin to form a 1:1 inhibitor–enzyme complex. The dissociation constant estimated by the method of Green and Work [8] was 3.32×10^{-13} M.

Enzyme specificity of the inhibitor

The specificity of the inhibitor was tested against several proteases from serine, –SH and acid classes (Table 3). The inhibition was highly specific for trypsin. α-Chymotrypsin and kallikrein were only slightly inhibited. The inhibitor exerted its inhibitory effect on both the esterase and amidase activities of trypsin and kallikrein, using benzoyl-L-arginine ethyl ester and H-D-valyl-L-leucyl-L-arginine-*p*-nitroanilide as substrates, respectively. On the other hand, no inhibition was observed for thiol proteases papain, chymopapain and bromelain, or for an acid protease, pepsin.

Heat stability of the inhibitor

The purified inhibitor was treated for 10 min at 80° and 90° in 50 mM Tris–HCl (pH 7.4). The treatments reduced the activity by 40 and 100%, respectively. More than 80% of the inhibitory activity remained after treatment at 70° for 10 min.

Table 3. Substrate specificity of the inhibitor (I-20K)

Enzyme	Substrate	Final concn of substrate (mM)	pH of reaction mixture	Inhibition (%)
Trypsin	BAEE	0.3	7.8	44.1
Trypsin	H-D-Val-Leu-Arg-*p*NA	0.6	7.8	34.1
α-Chymotrypsin	ATEE	1.5	7.8	8.6
Kallikrein	BAEE	0.3	8.7	9.1
Kallikrein	H-D-Val-Leu-Arg-*p*NA	0.6	8.7	12.5
Papain	Bz-Phe-Val-Arg-*p*NA	0.7	5 6	0
Cymopapain	Bz-Phe-Val-Arg-*p*Na	0.7	5.6	0
Bromelain	Bz-Phe-Val-Arg-*p*NA	0.7	5.6	0
Pepsin	Hemoglobin	*	†	0

The concentration of all enzymes in the reaction mixture was 100 nM.
*2% hemoglobin was used as the substrate.
†The reaction was carried out in 0.06 M HCl.

DISCUSSION

The purification and characterization of protease inhibitors have been carried out mainly from legume seeds, which are rich in inhibitors for serine proteases [3]. These inhibitors for serine proteases have been classified into two families: the first is designated 'Kunitz-Inhibitor', characterized by a higher M_r (*ca* 20000), a low cysteine content, a single head (having a binding site against proteases) and competitive inhibition; and the second as 'Bowman–Birk-Inhibitor', characterized by a lower M_r (*ca* 8000), a high cysteine content (*ca* 20%), a double head (having two binding sites against proteases) and non-competitive inhibition. The well-known 'Soybean Trypsin Inhibitor' belongs to the former.

The inhibitor isolated from spinach leaves was specific for trypsin (Table 3). The facts that the inhibitor formed a 1:1 inhibitor–enzyme complex with trypsin (Fig. 5) and slowly displaced the substrate indicate that this inhibitor has a single reaction site. Moreover, the inhibitor has a M_r of 18000 and possesses 4 cysteine and 2 tryptophan residues per mol (Table 2). These results indicate that the protease inhibitor isolated from spinach leaves may belong to the Kunitz-Inhibitor family. This inhibitor is somewhat different from the Soybean Trypsin Inhibitor, having a much lower K_i value against trypsin.

The existence of a chymotrypsin inhibitor in the leaves was also observed in some Solanaceae plants, which was induced by wounding [9, 10]. However, the molecular characteristics of the inhibitor are different from those of the spinach inhibitor [11].

Two possible functions have been postulated for the proteinous protease inhibitors in plants: one is the protection against insects and the other is the regulation of endogenous proteases. The former was proposed because the inhibitors were induced in leaves by insect attacks [12], and because the endogenous proteases which combine with the inhibitors are rarely found in plants. The second function was proposed because some protease inhibitors in seeds combine with endogenous trypsin-like proteases, and when the inhibitor contents have fallen sufficiently during germination, the trypsin-like proteases become active [13–15].

We showed that the protease inhibitor (M_r 18000) was present in leaves but not in seeds. Whether the inhibitor is induced in leaves by wounding to inhibit the proteases of insects or plays a role in the regulation of undiscovered endogenous serine proteases in leaves are topics that require further investigation.

EXPERIMENTAL

Plant material. Seeds, roots and leaves of spinach (*Spinacia oleracea* L. cv New Asia) were obtained from plants grown in a Phytotron in the National Institute for Environmental Studies. Plants were grown under short-days (8 hr light/16 hr dark) for 25 days followed by long-days (14 hr light/10 hr dark) for 25 days (20° during the day and 15° at night with 70% relative humidity).

Extraction procedure. Seeds (2000 grains, 25 g fr. wt) were milled and then homogenized with 250 ml H_2O. Leaves (2 kg fr. wt) and roots (562 g fr. wt) were homogenized for 5 min in a Polytron homogenizer (Kinematica, Switzerland) at *ca* 20000 rpm in 2 l. and 1.6 l. of H_2O, respectively. Each homogenate was filtered through 6 layers of gauze and then centrifuged at 10000 *g* for 20 min. To the supernatant was added 60% $HClO_4$ to give a final concn of 3%. The soln was stirred for 1 hr and then centrifuged at 10000 *g* for 10 min. The supernatant was neutralized with 5 M KOH and centrifuged to remove the resulting $KClO_4$. The supernatant was brought to 70% saturation with solid $(NH_4)SO_4$, stirred for 2 hr and centrifuged in 15 ml 50 mM Tris–HCl buffer (pH 7.4), dialysed overnight against 2 l. of the same buffer and then centrifuged at 10000 *g* for 10 min to remove the insoluble materials. The resulting supernatant was used as the acid-soluble crude extract. The entire extraction procedure was carried out at 4°.

Column chromatography. Sephadex G-25 and G-75 (Pharmacia Fine Chemicals) were packed into 4 × 20 cm and 2.64 × 91 cm columns, respectively, and pre-equilibrated with 50 mM Tris–HCl buffer (pH 7.4). The applied materials were eluted with the same buffer at a flow rate of 50 ml/hr for G-25 and 20 ml/hr for G-75. Fractions (10 ml each) were collected. DEAE-Sephacel (Pharmacia) was also pre-equilibrated with the same buffer while in the column (2.2 × 25 cm). The applied materials were eluted with 300 ml of a linear gradient of NaCl in 50 mM Tris–HCl (pH 7.4); the limiting buffer contained 0.5 M NaCl. The flow rate was 10 ml/hr, and 5 ml was collected in each fraction. The M_rs of the inhibitors were estimated by the following standard proteins: albumin (Boehringer; M_r 45000), soybean trypsin inhibitor (Sigma; 22000), cytochrome *c* (Boehringer; 12500) and aprotinin (Sigma; 6500). Column chromatography was carried out at 4°. The concn of NaCl was estimated by titration with standard solns of $AgNO_3$ and K_2CrO_4.

Affinity chromatography. Trypsin-polyacrylamide gel (Sigma) was packed into a 0.8 × 4 cm column and pre-equilibrated with 50 mM Tris–HCl buffer containing 20 mM $CaCl_2$. The sample was adjusted to a final concn of 20 mM $CaCl_2$ and applied to the column. The applied materials were eluted with 10 mM HCl after washing the column with the same buffer containing 500 mM NaCl. Fractions (2 ml each) were collected. The procedure was carried out at room temp. and A_{280} was recorded using a flow cell system (ISCO UA-5, U.S.A.).

Isoelectric focusing was carried out by the method of ref. [16] employing a 22 ml preparative column composed of a gradient concn of sucrose. A mixture of 2 vol. of pH 3.5–10 and 8 vol. of pH 3.5–5 ampholine (LKB) was used as the carrier ampholyte. Focusing was carried out for the 1st day at a constant 400 V, the 2nd day at 600 V and the 3rd and 4th days at 900 V. After focusing, 0.5 ml fractions were collected and the pH of each fraction was measured with a micro-electrode (Fuji Kagaku Keisoku, Japan). The ampholine was removed after immobilizing the inhibitor by affinity chromatography on a trypsin-polyacrylamide gel.

SDS-polyacrylamide gel electrophoresis was performed as previously described [17] according to ref. [18] using 12% acrylamide gel. After electrophoresis, the gel was stained with 0.1% Coomassie Brilliant Blue R-250 (Bio-Rad), dissolved in 50% TCA.

Amino acid analysis. A 2 ml aliquot containing 1.8 nmol of the purified inhibitor was hydrolysed in 6 M HCl at 108° for 24 hr and analysed with a Durrum D-502 instrument. The cystine and cysteine contents were detected as cysteic acid, and methionine as methionine sulfone by analysis of the sample pretreated with 79% performic acid at 0° for 2 hr before hydrolysis [19]. The value for tryptophan was obtained by analysis of the sample hydrolysed in the presence of 4% (v/v) thioglycollic acid [20].

The protein concentration of the solns of trypsin and the purified inhibitor were calculated from the results of amino acid analysis. *A* at 280 nm was measured with a UV-200 spectrophotometer (Shimadzu, Japan) and was used for the determination of protein concn at each purification step of the inhibitor.

Assay of protease inhibitor. Protease inhibitor activities were estimated at 25° from the residual trypsin activity of benzoyl-L-

arginine ethyl ester (BAEE, Sigma) after addition of inhibitors. The assay system was composed of 0.5 ml sample, 0.2 ml 500 mM Tris–HCl buffer (pH 7.8), 0.1 ml 25 μg/ml trypsin (Worthington) containing 0.0025 M HCl and 0.02 M $CaCl_2$, and 0.2 ml 0.0015 M BAEE. The reaction was started by the addition of BAEE, and the increase in A_{253} was recorded. The activity of inhibitors was expressed as units of trypsin activity inhibited.

The enzyme specificity of the inhibitor was tested by using 3 groups of proteases: serine proteases [trypsin, α-chymotrypsin (Worthington) and kallikrein (Sigma)], thiol proteases [papain, chymopapain and bromelain (Sigma)] and an acid protease [pepsin (Worthington)]. The concn of all enzymes used in reaction mixture was 100 nM. The activity of the inhibitor was spectrophotometrically estimated from the residual hydrolysis activity of substrates [21, 22]. As substrates of proteases, H-D-valyl-L-leucyl-L-arginine-*p*-nitroanilide (H-D-Val-Leu-Arg-*p*NA, Kabi), *N*-benzoyl-L-phenylalanyl-L-valyl-L-arginine-*p*-nitroanilide (Bz-Phe-Val-Arg-*p*NA, Kabi), *N*-acetyl-L-tyrosine ethyl ester (ATEE, Sigma) and hemoglobin (Sigma) were used.

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