Isolation and Amino Acid Sequence of a Serine Proteinase Inhibitor from Common Flax (*Linum usitatissimum*) Seeds

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*LUTI (*Linum usitatissimum *trypsin inhibitor), a member of the potato inhibitor I family, has been isolated from seeds of flax by ethanol fractionation, ion exchange chromatography on CM-Sephadex C-25, affinity purification on immobilized methylchymotrypsin (α-chymotrypsin in which His 57 has been converted to 3-methylhistidine) in the presence of 5 M NaCl, and finally by reversed-phase HPLC. The 7655 Da inhibitor consists of a single polypeptide chain of 69 residues with one disulfide bridge. The molecule is acetylated at the N terminus. Its primary structure has been determined after limited proteolysis of the native molecule with trypsin at the reactive site, cleavage with cyanogen bromide or arginyl endopeptidase (Arg-gingipain), and alcoholytic deacetylation of the N-terminally blocked serine. The association constants (K_a) of LUTI with bovine β-trypsin and α-chymotrypsin are $3.58 \times 10^{10}\,M^{-1}$ and $5.02 \times 10^{5}\,M^{-1}$, respectively. High NaCl concentration (3 M) increased the association constant of LUTI with α-chymotrypsin to $6.64 \times 10^{7}\,M^{-1}$. To our knowledge, LUTI is the first serine-proteinase-type inhibitor isolated from a plant of the Linaceae family.*

KEYWORDS:

enzyme inhibitors · hydrolases · proteinases · proteins · sequence determination

Introduction

It has been known that protein proteinase inhibitors, widespread in the animal, plant, and microbial kingdoms, have a variety of inhibitory specificities and structures. They were classified into families according to Laskowski on the basis of their primary structure, topology of disulfide bridges, and the position of the reactive site.[1] Plant proteinase inhibitors seem to be part of the defense mechanisms that protect plants against attack by pests and pathogens, because they are mainly active against exogenous enzymes.

The known plant proteinase inhibitors belong to at least nine families: Kunitz (STI),[2] Bowman – Birk (BBI),[3] squash,[4] cereal,[5] rape seed,[6] arrowhead,[7] potato I,[8] potato II,[9] and barley.[10] Some of them seem to be restricted to only one botanical family, for example, inhibitors of the squash family (Cucurbitaceae). Representatives of others families, for example the potato inhibitor I, were isolated from Solanaceae,[8] Gramineae,[11] Leguminosae,[12] Amaranthaceae,[13] and Cucurbitaceae.[14]

Here, we report on the purification and the primary structure of the trypsin inhibitor from seeds of common flax (*Linum usitatissimum*), abbreviated as LUTI, a member of the Linaceae family. The purification procedure involved affinity chromatography on immobilized methylchymotrypsin (α-chymotrypsin in which His 57 has been converted to 3-methylhistidine) in the presence of 5 M NaCl, which enables adsorption of the strong trypsin inhibitor with no or only weak antichymotrypsin activity.[15]

Results and Discussion

Isolation of the inhibitor

The purification procedure outlined above resulted in the isolation of the homogenous protease inhibitor from common flax seeds. The removal of the seed coats rich in mucilage at the beginning of the purification procedure improved the preparation and increased the concentration of extracted proteins. The inhibitor was isolated from the ethanol-precipitated proteins by CM-Sephadex C-25 chromatography followed by chromatography on immobilized methylchymotrypsin.

In the ion exchange chromatography the inhibitor was eluted as a single, symmetrical peak (data not shown). In the affinity chromatography all antitrypsin activity was adsorbed on the column only in the presence of high salt concentration (5 M NaCl). Bound inhibitor was eluted in two peaks: the first with water and the second with 0.01 N HCl (Figure 1). Finally, both inhibitor peaks (I and II) were purified and rechromatographed

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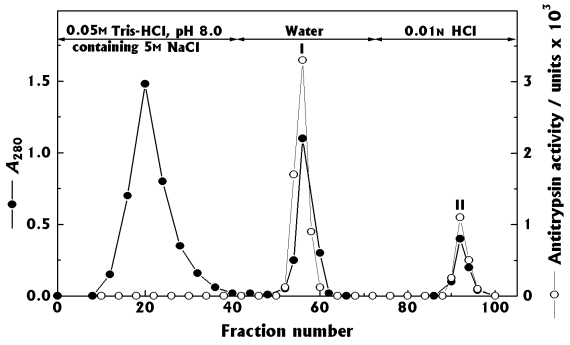

Figure 1. Affinity chromatography of trypsin inhibitor from flax on immobilized methylchymotrypsin. Samples of 50 mL (440 mg of protein) containing 5 M NaCl at pH 7.5 were loaded on the methylchymotrypsin – Sepharose 4B column (140 × 25 mm), equilibrated with 0.05 M Tris-HCl buffer, pH 7.5, containing 5 M NaCl. The column was washed intensively with equilibrating buffer containing 5 M NaCl until the A_{280} value dropped below 0.02. Finally the inhibitor was eluted with water (peak I) followed by 0.01 N HCl (peak II). Fractions of 8 mL were collected at a flow rate of 140 mL h^{-1}.

by reversed-phase (RP) HPLC on a C_{18} column (Figure 2). After this step the recovery was close to 40%, and the specific activity had increased 600-fold.

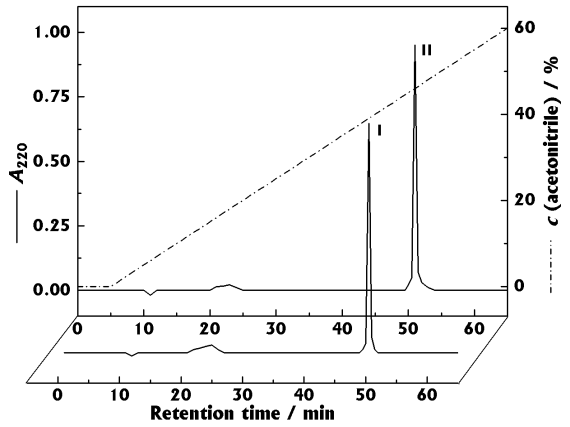

Figure 2. Reversed-phase HPLC rechromatography of the inhibitor peaks I and II from the previous affinity chromatography (see Figure 1). The proteins were applied to a Nucleosil-100 C_{18} column (10 μm; 250 × 8.0 mm i.d.) and eluted with acetonitrile (linear gradient in 0.1% TFA).

Recently, we applied 5 M NaCl for the purification of trypsin inhibitors from Cucurbitaceae family seeds and porcine pancreas on immobilized chymotrypsin.[15] Only in the presence of a high concentration of salt were these trypsin-specific inhibitors adsorbed on the affinity column and later eluted in their virgin forms (i.e., with the reactive-site peptide bond not split) either with water or 0.01 N HCl. In the case of LUTI we were not able to use the immobilized, catalytically active chymotrypsin because this enzyme, in both the presence and the absence of 5 M NaCl, inactivated the inhibitor (data not shown). Instead, we used immobilized, catalytically inactive methylchymotrypsin for the purification of the inhibitor from the flax seeds.

Characterization

On the basis of the similar mobility in both reducing (Figure 3) and native (data not shown) gel electrophoresis, the elution conditions in RP-HPLC (Figure 2), identical molecular weight of

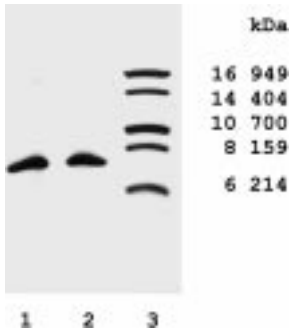

Figure 3. SDS-PAGE of HPLC-purified inhibitor. Lane 1 and 2: inhibitor from peak I (10 μg) and peak II (10 μg), respectively; lane 3: molecular weight markers.

7655 ± 2 Da (Figure 4), and similarity in amino acid compositions (Table 1), we assumed the inhibitors of peak I and II to be the same protein. To support this suggestion we rechromatographed the pure inhibitor of peaks I and II on immobilized methylchymotrypsin under the conditions described in the legend of Figure 1. Both inhibitor preparations were adsorbed in the presence of 5 M NaCl and then eluted in two peaks: the first with water and the second with 0.01 N HCl (data not shown). The proportions between peaks I and II in both cases were similar to the ones shown in Figure 1.

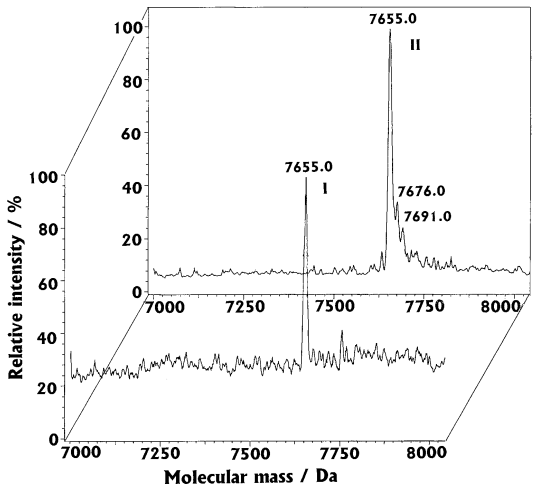

Figure 4. Mass spectra (electrospray ionization) of HPLC-purified peaks I and II of LUTI.

Because these results indicated that the proteins of both peaks were identical, further characterization was performed only for the inhibitor of peak I (LUTI). We are not yet able to explain why LUTI has a tendency to elute in two protein peaks in the chromatography on immobilized methylchymotrypsin after its adsorption in the presence of 5 M NaCl. To our knowledge,

Table 1. *Amino acid compositions of LUTI and its fragments obtained after proteolytic and chemical cleavage.*[a]

Amino acid	LUTI I	LUTI II	T_2	C_2	C_3	R_2	R_4
Asp	7.77 (8)	8.41	3.94 (4)	2.07 (2)	4.07 (4)	2.07 (2)	1.15 (1)
Ser	3.82 (4)	3.82	0.95 (1)	1.03 (1)	1.08 (1)	1.11 (1)	1.29 (1)
Glu	4.48 (4)	4.23	0.00 (0)	3.08 (3)	1.09 (0)	2.04 (2)	1.27 (1)
Gly	5.76 (5)	5.60	1.01 (1)	1.27 (1)	1.28 (1)	3.33 (3)	1.78 (1)
His	2.44 (3)	2.83	1.85 (2)	0.85 (1)	1.99 (2)	0.00 (0)	0.87 (1)
Arg	5.90 (6)	6.43	2.02 (2)	2.08 (2)	2.20 (2)	0.00 (1)	0.00 (0)
Thr	4.25 (4)	3.88	1.99 (2)	0.91 (1)	2.96 (3)	1.12 (1)	0.91 (1)
Ala	6.33 (6)	6.19	0.00 (0)	5.45 (5)	0.66 (0)	3.20 (4)	1.89 (2)
Pro	3.58 (3)	3.16	0.94 (1)	0.00 (0)	1.08 (1)	2.00 (2)	0.00 (0)
Tyr	0.00 (0)	0.00	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)
Val	7.51 (10)	8.40	4.54 (6)	2.54 (3)	4.67 (6)	1.85 (2)	1.76 (2)
Met	1.47 (2)	1.99	0.00 (0)	n.d. (1)	0.00 (0)	0.00 (1)	0.00 (1)
Lys	3.84 (4)	4.08	0.00 (0)	1.06 (1)	1.08 (1)	2.42 (2)	2.19 (2)
Ile	2.60 (3)	2.41	1.67 (2)	0.57 (1)	1.67 (2)	0.00 (0)	0.72 (1)
Leu	2.23 (2)	2.38	0.00 (0)	1.03 (1)	0.24 (0)	1.05 (1)	1.17 (1)
Phe	1.09 (1)	1.16	1.00 (1)	0.00 (0)	0.97 (1)	0.00 (0)	0.00 (0)
Cys[b]	2.15 (2)	1.73	n.d. (1)	n.d. (0)	n.d. (1)	n.d. (1)	n.d. (0)
Trp[c]	2.13 (2)	2.42	n.d. (1)	n.d. (0)	n.d. (1)	n.d. (1)	n.d. (0)
total	(69)		(24)	(23)	(26)	(24)	(15)

[a] Numbers in parentheses are calculated from the corresponding sequence of LUTI. LUTI I and LUTI II represent inhibitor eluted from immobilized methylchymotrypsin with water and 0.01 N HCl, respectively. [b] Cysteine was determined after oxidation of LUTI with performic acid.[48] [c] Tryptophan was determined according to ref. [47].

only Ryan and Feeney applied immobilized methylchymotrypsin for the purification of serine proteinase inhibitors. They purified avian ovomucoids, which, contrary to LUTI, were strong chymotrypsin inhibitors and adsorbed in batch procedure on methylchymotrypsin – Sepharose resin at low salt concentration, and were then eluted as single active fractions at pH 2.0.[16]

Inhibitory activity

LUTI was found to be a strong inhibitor of bovine β-trypsin ($K_a = 3.58 \times 10^{10}\,\text{M}^{-1}$). It also weakly inhibited α-chymotrypsin, subtilisin BPN′, subtilisin Carlsberg, and cathepsin G, while human leukocyte elastase (HLE) and porcine pancreatic elastase (PPE) were not affected. At 3 M NaCl the K_a value for α-chymotrypsin, subtilisin BPN′, subtilisin Carlsberg, and, to a lesser extent, the other enzymes was significantly increased (Table 2). For the inhibition of chymotrypsin by LUTI we studied the dependency of the association constants on the NaCl concentration. In the absence of NaCl the association constant was $K_a = 5.02 \times 10^5\,\text{M}^{-1}$, whereas NaCl concentrations of 1, 2, and 3 M increased its value to $1.74 \times 10^6\,\text{M}^{-1}$, $1.54 \times 10^7\,\text{M}^{-1}$ and $6.64 \times 10^7\,\text{M}^{-1}$, respectively. Recently, we observed that at a high NaCl concentration the trypsin-specific inhibitors (Kazal-type inhibitor from bovine pancreas and inhibitors from the squash family) also exhibited antichymotrypsin activity.[15]

Incubation of LUTI with α-chymotrypsin in 30 mol % excess for 72 h at pH 8.0 and 25 °C caused a loss of antitrypsin activity of the inhibitor in either the presence or the absence of 4.5 M NaCl.

Table 2. *Inhibition of serine proteinases by LUTI.*

Enzyme		Substrate		Equilibrium association constant (K_a [M^{-1}])		
Name	Conc. [M]	Compd.	Conc. [M]	no NaCl	0.5 M NaCl	3 M NaCl
bovine β-trypsin (EC 3.4.21.4)	8.05×10^{-10}	Bz-Val-Gly-Arg-pNA	2.41×10^{-5}	3.58×10^{10}	n.d.	n.d.
subtilisin BNP′ (EC 3.4.21.14)	2.80×10^{-7} (1.72×10^{-8})[a]	Suc-Ala-Ala-Ala-pNA	2.46×10^{-4}	5.74×10^{7}	n.d.	2.00×10^{9}
subtilisin Carlsberg (EC 3.4.21.62)	2.30×10^{-7} (9.06×10^{-9})[a]	Suc-Ala-Ala-Ala-pNA	1.64×10^{-4}	4.47×10^{6}	n.d.	1.25×10^{9}
cathepsin G (EC 3.4.21.20)	7.10×10^{-8}	Suc-Ala-Ala-Pro-Phe-pNA	1.74×10^{-4}	n.d.	1.72×10^{6}	3.56×10^{6}
bovine α-chymotrypsin (EC 3.4.21.1)	3.93×10^{-7}	Suc-Phe-pNA	1.24×10^{-4}	5.02×10^{5}	n.d.	6.64×10^{7}
human leukocyte elastase (EC 3.4.21.37)	4.84×10^{-9}	MeO-Suc-Ala-Ala-Pro-Val-pNA	1.39×10^{-5}	n.d.	9.12×10^{4}	2.88×10^{5}
porcine pancreatic elastase (EC 3.4.21.36)	7.57×10^{-9}	Suc-Ala-Ala-Ala-pNA	1.64×10^{-4}	8.56×10^{3}	n.d.	2.93×10^{4}

[a] Numbers in parentheses are the concentrations of subtilisins used for determination of K_a in the presence of 3 M NaCl. Bz = benzoyl; MeO = methoxy; pNA = *p*-nitroanilide; Suc = succinyl.

The antitrypsin activity of LUTI was found to be unaffected when the inhibitor was incubated with trypsin in 30 mol% excess under analogous conditions (data not shown).

The antitryptic activity of LUTI was not affected by modification of guanidyl groups of arginine residues with 1,2-cyclohexanedione (CHD), whereas acetylation of free amino groups with acetic anhydride led to its inactivation. This indicated the involvement of a lysine residue in the reactive site of the inhibitor.

Sequence studies

Attempts to determine the amino acid sequence of LUTI revealed that it has a blocked N terminus. Alcoholytic deblocking of the native inhibitor with trifluoroacetic acid (TFA) in methanol permitted the determination of an N-terminal sequence of 24 residues (SRRXPGKNAXPELVGXSGNMAAAT). This suggested that LUTI has an *N*-acetylamino acid at the N-terminal end. The presence of *N*-acetylserine at the N terminus was confirmed both by NMR spectroscopic studies and by mass spectrometry (see above). The ^{1}H NMR spectrum shows a sharp singlet peak at $\delta =$ 2.0 corresponding to the CH_3 moiety of the acetyl group. In addition, a strong NOE signal was observed between the amide proton of Ser 1 and the methyl protons at $\delta = 2.0$.[17]

It would be interesting to know why only some inhibitors belonging to the potato I family have an N terminus that is blocked by acetylation.[14, 18] A similar phenomenon is observed in the squash inhibitor family where also only some of their members possess a blocked N terminus, but in this case it is a pyroglutamic acid residue.[19] For antiproteolytic activity it is not relevant whether inhibitors have a free or blocked N terminus, so it seems to be unimportant for their function as defensive proteins that can protect plants against insects and pathogens. It is possible that proteinase inhibitors in plants play multiple roles that might be connected with more than only the inhibition of proteolytic enzymes. Products of their proteolytic degradation can appear in the process of germination and perform important regulatory functions. These functions might depend on whether the peptides have blocked or free N termini. Enzymatic degradation of the basic pancreatic trypsin inhibitor (BPTI) with clostripain is an example of the generation of bioactive peptides with antimicrobial activity from an inhibitory protein.[20]

The remaining part of the amino acid sequence of LUTI was determined in several steps. Incubation of the protein with a catalytic amount of trypsin at pH 3.2 converted part of the virgin inhibitor into a modified form (with the reactive-site peptide bond split). The modified form of LUTI (Figure 5) was reduced and alkylated, and the resulting peptides were separated by RP-HPLC (data not shown). Peptide T_2 was submitted to sequence analysis without further treatment. Peptide T_1 was digested with Arg-gingipain, the product mixture obtained by enzymatic cleavage was separated by RP-HPLC (data not shown), and the amino acid sequences of peaks R_2 and R_4 were elucidated.

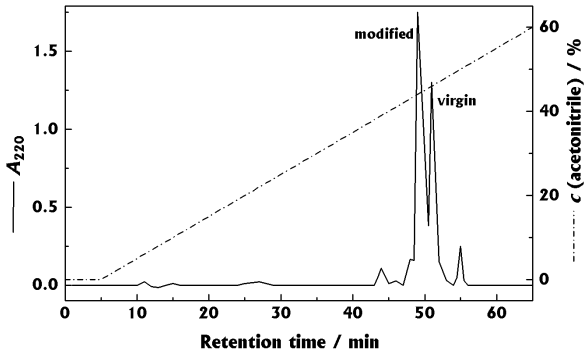

Figure 5. *Reversed-phase HPLC of trypsin-modified LUTI (150 μg) on a Nucleosil-100 C_{18} column (10 μm; 250 × 8.0 mm i.d.). The proteins were eluted with acetonitrile (linear gradient in 0.1% TFA).*

On the basis of the finding that LUTI has two methionine residues (Table 1) the reduced and alkylated inhibitor after purification on RP-HPLC (data not shown) was cleaved with CNBr. The resulting peptides were separated by RP-HPLC, and peaks C_2 and C_3 (data not shown) were sequenced. The amino acid sequences of the isolated peptides afforded the entire primary structure of LUTI. The sequence of LUTI coincided well with the amino acid compositions of the isolated peptides and the intact protein (Table 1). The molecular weight of LUTI was determined to be 7655 ± 2 Da by mass spectrometry, which is in good agreement with the theoretical value of 7653.4 Da, which was calculated according to Equation (1).

$$m_{\text{protein}} = \{\Sigma m_{\text{aa}} - 68\, m_{\text{H}_2\text{O}}\} + m_{\text{CH}_3\text{CO}} - 1 \quad (1)$$

The complete amino acid sequence of the LUTI is shown in Figure 6. The protein consists of a single polypeptide chain of 69 amino acids and contains one disulfide bridge. The protein

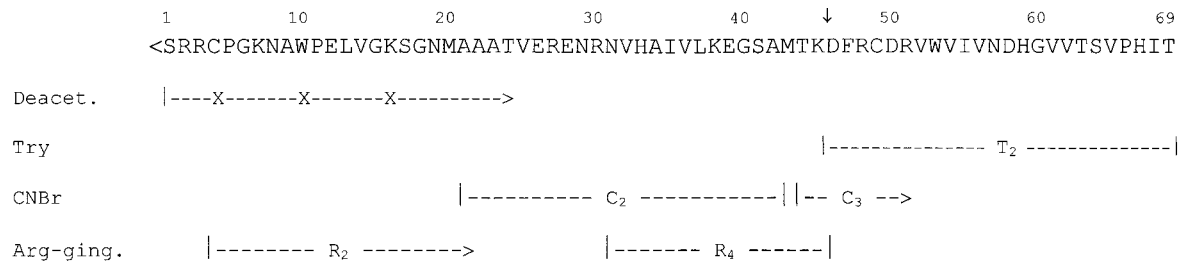

Figure 6. *Amino acid sequence of common flax trypsin inhibitor (LUTI). Deacet. indicates the amino acid residues determined after alcoholytic deacetylation of N-terminally blocked inhibitor. Try, CNBr, and Arg-ging. stand for the peptides derived from trypsin, CNBr, and Arg-gingipain cleavage, respectively. X indicates non-identified amino acid residues. The arrow indicates the reactive-site peptide bond.*

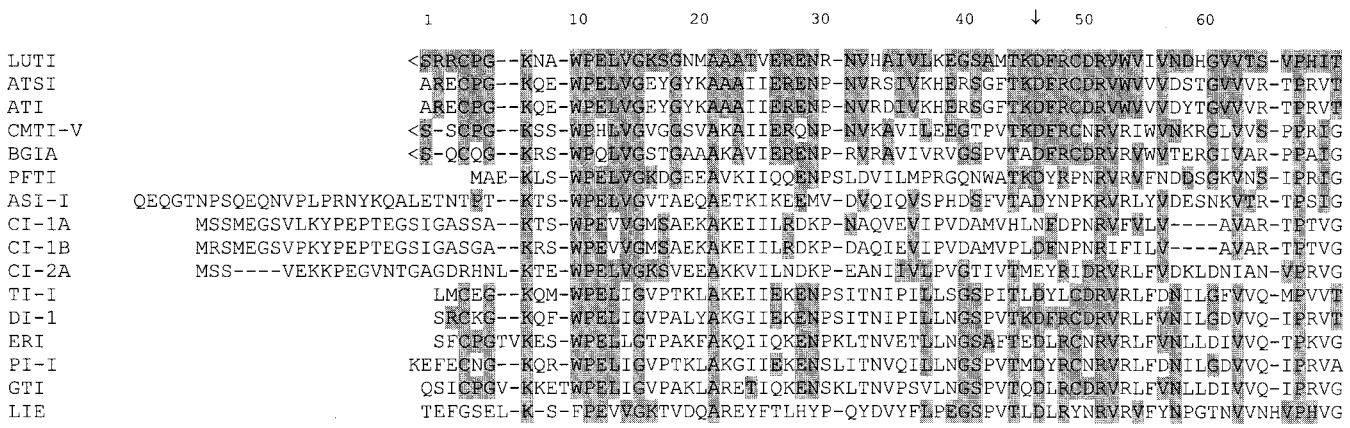

Figure 7. *Alignment of the amino acid sequence of LUTI (trypsin inhibitor from common flax (*Linum usitatissimum*) seeds with those of inhibitors belonging to the potato inhibitor I family. ATSI = trypsin and subtilisin inhibitor from amaranth seeds (*Amaranthus caudatus*);[21] ATI = trypsin inhibitor from amaranth (*Amaranthus hypochondriacus*) seeds;[13] CMTI-V = inhibitor of trypsin and Hageman factor from pumpkin (*Cucurbita maxima*);[14] BGIA = inhibitor of* Streptomyces griseus *protease from seeds of bitter gourd (*Momordica charantia*);[18] PFTI = trypsin inhibitor from pumpkin fruit phloem exudate;[22] ASI-I = subtilisin inhibitor from seeds of adzuki beans (*Vigna angularis*);[12] CI-1 and CI-2 = chymotrypsin inhibitors from barley (*Hordeum vulgare*) seeds;[23, 24] TI-I = wound-induced inhibitor from tomato (*Lycopersicon peruvianum*) leaves;[8] DI-1 = trypsin inhibitor from fruit of wild tomato;[25] ERI = tomato ethylene-responsive inhibitor;[26] PI-I = potato inhibitor I;[27] GTI = inhibitor from a tobacco genetic tumor;[28] LIE = eglin C from leech (*Hirudo medicinalis*).[29] Gaps inserted into a sequence to preserve homology are indicated by dashes (--); the P_1 residue of the reactive site is indicated by an arrowhead; shaded areas indicate residues that are identical with the sequence of LUTI.*

revealed significant homology to the potato I inhibitor family, particularly to inhibitors ATSI and ATI from Amaranthaceae (Figure 7). It seems that the presence of a disulfide bridge in some potato I family members is not required for their antiproteolytic activity. Plunkett and Ryan have shown that the reduction and carboxymethylation of a single disulfide bond in proteinase inhibitor I from potato tubers had no effect on its inhibitory function.[30] To our knowledge LUTI is the first serine proteinase inhibitor isolated from the Linaceae plant family.

Experimental Section

Materials: The seeds of *Linum usitatissimum* were supplied by Garden Seed Company (Wrocław, Poland). α-Chymotrypsin (EC 3.4.21.1), subtilisin Carlsberg (EC 3.4.21.62), *N*-α-benzoyl-DL-Arg-*p*-nitroanilide (BAPNA), *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide, *N*-methoxy-succinyl-Ala-Ala-Pro-Val-*p*-nitroanilide, *N*-succinyl-L-Phe-*p*-nitroanilide, *N*-benzoyl-Val-Gly-Arg-*p*-nitroanilide, Tris(hydroxymethyl)aminomethane (Tris), 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), dimethylsulfoxide (DMSO), phenylmethylsulfonyl fluoride (PMSF), divinyl sulfone, and iodoacetic acid were from Sigma (St. Louis, MO, USA); porcine pancreatic elastase (EC 3.4.21.36) and *p*-nitrophenyl *p*-guanidinobenzoate·HCl (NPGB) from Merck (Darmstadt, Germany); methyl *p*-nitrobenzenesulfonate, cyanogen bromide, reagents for SDS-PAGE and trifluoroacetic acid (TFA) from Fluka (Buchs, Switzerland); subtilisin BNP′ (EC 3.4.21.14); dithiothreitol (DTT) and 1,2-cyclohexanedione (CHD) were purchased from ICN Biomedicals (Costa Mesa, CA, USA). Sepharose 4B, DEAE-Sephadex A-25, and molecular weight markers for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were from Pharmacia LKB Biotechnology (Uppsala, Sweden). The reversed-phase Nucleosil-100 C_{18} (10 μm; 250 × 8.0 mm i.d.) column was from Knauer (Berlin, Germany); YM 3 membrane filter was purchased from Amicon (Danvers, MA, USA).

Methylchymotrypsin was prepared by the method of Nakagawa and Bender,[31] and traces of nonmodified enzyme were inactivated with PMSF. Bovine trypsin (EC 3.4.21.4) was prepared according to Wilimowska-Pelc and Mejbaum-Katzenellenbogen,[32] and bovine β-trypsin was isolated from this preparation as described by Liepniecks and Light.[33] Cathepsin G (EC 3.4.21.20) and human leukocyte elastase (EC 3.4.21.37) were purified by the method of Wątorek et al.[34] Arg-gingipain (EC 3.4.22.37) was a gift from Dr. J. Potempa from the Institute of Molecular Biology, Jagiellonian University (Kraków, Poland). *Cucurbita maxima* trypsin inhibitor I (CMTI I) was prepared according to Polanowski et al.[35] Turkey ovomucoid (OMTKY) was separated after Bogard et al.[36]

All other reagents were of analytical or HPLC-grade purity.

Preparation of trypsin inhibitor: Ground flax seeds were washed twice with five volumes (w/v) of acetone. Quickly sedimented coats and coarse particles were discarded and finely ground meal was collected by centrifugation and dried under vacuum. Defatted meal (600 g) was extracted with 0.1 M acetate buffer, pH 5.0 (6 L) for 1 h at 4 °C with mechanical stirring, and the supernatant solution was isolated by centrifugation for 30 min at 9000 × *g*. Mucilage from the supernatant was precipitated at room temperature with an equal volume of ethanol and removed by filtration. To the clear supernatant four volumes of cold ethanol (−20 °C) were added, left for 24 h at 4 °C, and then the crude protein precipitate was collected by centrifugation and dried under vacuum. The resulting powder was solubilized in distilled water (500 mL) and clarified by filtration. To the protein solution an equal volume of 0.1 M acetate buffer, pH 5.5, was added, and the mixture was applied to a CM-Sephadex C-25 column (260 × 35 mm) preequilibrated with 0.05 M acetate buffer, pH 5.5. The proteins were eluted with a gradient of 0 – 0.6 M NaCl in the same buffer at a flow rate of 60 mL h^{-1}. The inhibitor peak fractions were pooled and brought to pH 7.5 with 2 M Tris, and NaCl was added to a final concentration of 5 M. The solution was then applied to a column packed with immobilized methylchymotrypsin – Sepharose 4B (140 × 25 mm) and equilibrated with 5 M NaCl in 0.05 M Tris-HCl buffer, pH 7.5. Nonbound proteins were washed out with starting buffer. The adsorbed inhibitor was first eluted with water (peak I) and then with 0.01 N HCl (peak II). The inhibitor preparations of peaks I

and II were concentrated and desalted on a YM 1 membrane filter and finally chromatographed on a Nucleosil-100 C_{18} column (10 μm; 250 × 8.0 mm i.d.) with an HPLC system.

Protein assay: The amount of protein was estimated by either the microbiuret method of Goa[37] or by spectrophotometric measurement of the absorbance at 215 (A_{215}) and 225 nm (A_{225}) and calculating the concentration c by using Equation (2).[38]

$$c\,[\mathrm{mg\,mL^{-1}}] = 144\,(A_{215} - A_{225}) \quad (2)$$

Measurement of enzyme and inhibitory activities: Enzyme activities were measured spectrophotometrically at 410 nm after 10 min incubation at 25 °C in 0.1 M Tris-HCl buffer, pH 8.0, with 20 mM $CaCl_2$, by using BAPNA for β-trypsin [39] and *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide for α-chymotrypsin as substrates.[40] One unit of inhibitory activity was defined as the amount of protein required to reduce the activity of 2 μg of an enzyme to 50% of the original value.

Standardization of enzyme and inhibitor stock solutions: Bovine β-trypsin and α-chymotrypsin were dissolved in 1 mM HCl, 20 mM $CaCl_2$; subtilisin BPN′, subtilisin Carlsberg, and PPE in 0.02 M acetate buffer, 20 mM $CaCl_2$, pH 5.0; and cathepsin G and HLE in 0.1 M acetate buffer, 1 M NaCl, pH 5.6. Stock solutions of inhibitors were prepared in 1 mM HCl. β-trypsin and α-chymotrypsin concentrations were determined by spectrophotometric titration with NPGB.[41] The standardized trypsin solution was used to titrate LUTI, CMTI I, and OMTKY, which in turn served to determine the activity of other enzymes, namely cathepsin G (with CMTI I) and HLE, PPE, subtilisin BNP′, and subtilisin Carlsberg (with OMTKY).

Determination of equilibrium association constants: The equilibrium association constants (K_a) were determined in 0.05 M Tris-HCl buffer, 20 mM $CaCl_2$, 0.005% Triton X-100, pH 8.3 (in the case of cathepsin G and HLE, the buffer also contained 0.5 M NaCl) at 22 °C with the method of Empie and Laskowski,[42] as described by Otlewski et al.[43] Increasing amounts of inhibitor were added to a constant amount of enzyme, and after a suitable incubation time the residual enzyme activity was measured by using an appropriate turnover substrate (see Table 2 for details). Kinetic measurements were performed at 410 nm, and the residual enzyme concentration was calculated from initial slopes. The experimental data were fitted to Equation (3), where $[E_0]$ and $[I_0]$ are total enzyme and inhibitor concentrations, respectively, [E] is the residual enzyme concentration, and F is the enzyme–inhibitor equimolarity factor.

$$[\mathrm{E}] = \frac{1}{2}\left([\mathrm{E_0}] - F[\mathrm{I_0}] - K_a^{-1} + \sqrt{([\mathrm{E_0}] - F[\mathrm{I_0}] - K_a^{-1})^2 - 4[\mathrm{E_0}]F[\mathrm{I_0}]}\right) \quad (3)$$

Immobilization of methylchymotrypsin: Methylchymotrypsin (1 g) was immobilized on divinyl sulfone activated Sepharose 4B (70 mL) according to Pepper.[44]

Electrophoresis: Samples of inhibitor were subjected to electrophoresis on 16.5% (w/v) polyacrylamide gels in the presence of SDS according to Schägger and von Jagow.[45]

Amino acid analysis: Samples containing 1–3 μg of protein were hydrolyzed by vapor-phase HCl hydrolysis at 112 °C for 20 h in a protein hydrolyzer (Knauer, Germany). Hydrolysis was performed by manual derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate.[46] The derivatized amino acids were separated by HPLC on an AccQ·Tag column (160 × 3.9 mm i.d.; Waters, USA). Tryptophan was determined spectrophotometrically according to Beaven and Holiday.[47] Cysteine was determined after oxidation of LUTI with performic acid.[48]

Enzymatic modification and proteolytic digestion: The reactive-site peptide bond (P_1—P_1' according to the notation by Schechter and Berger)[49] of LUTI was hydrolyzed by incubation with 2 mol% of trypsin at pH 3.2 for 48 h at 25 °C. The N-terminal peptide T_1 (150 μg) obtained after selective hydrolysis of LUTI by trypsin at pH 3.2 was digested with about 1 mol% of Arg-gingipain for 20 h at 37 °C in 200 μL of 0.3 M Tris-HCl buffer, pH 7.6, containing 1 mM $CaCl_2$ and 10 mM cysteine. Prior to incubation the enzyme was activated for 15 min at 37 °C in 0.2 M HEPES, pH 8.0, containing 5 mM $CaCl_2$ and 10 mM cysteine.[50] Peptides resulting from enzymatic digestion were separated by RP-HPLC with a linear gradient of acetonitrile in 0.1% TFA. The susceptibility of LUTI to α-chymotrypsin degradation was examined by incubating the intact molecule (3.15×10^{-8} mol) for 72 h at 25 °C with the enzyme in 30 mol% excess in 0.05 M Tris-HCl, 20 mm $CaCl_2$, pH 8.0 (400 μL) in either the presence or the absence of 4.5 M NaCl. During incubation the antitrypsin activity of LUTI was monitored.

Chemical modifications: Free amino groups of lysine residues were acetylated with acetic anhydride.[51] Guanidyl groups of arginine were modified with CHD.[52] Carboxymethylation or alkylation of cysteine with acrylamide after reduction of LUTI with DTT was carried out according to refs. [48] and [53], respectively. The modified protein was separated by RP-HPLC with a linear gradient of acetonitrile in 0.1% TFA. Carboxymethylated LUTI was cleaved by cyanogen bromide according to Gross and Witkop,[54] and the resulting fragments were separated by RP-HPLC with a linear gradient of acetonitrile in 0.1% TFA. Alcoholytic deblocking of N-terminally acetylated LUTI was performed according to Gheorghe et al.[55]

Mass spectrometry: Mass spectra were recorded on a Finnigan MAT TSQ 700 triple-stage quadrupole mass spectrometer equipped with an electrospray ion source (ESI-MS). Samples were dissolved in methanol/water/acetic acid (50:45:5, v/v/v) and introduced into the electrospray needle by mechanical infusion through a microsyringe at a flow rate of 2 μL min^{-1}. A potential difference of 4.5 kV was applied between the electrospray needles. Nitrogen gas was used to evaporate the solvent from the charged droplets. At least twenty scans were averaged to obtain each spectrum. Transformations of the resulting spectra were performed with the *BioWorks* software package (Finnigan MAT).

Amino acid sequence determination: Sequence analyses were performed on a gas-phase sequencer (Model 491, Perkin–Elmer/Applied Biosystems, Foster City, CA, USA). The PTH-derivatives were analyzed by online gradient HPLC on a SPHERI-5 PTH column (5 μm C_{18}; 220 × 2 mm i.d.; Perkin–Elmer) using the microgradient delivery system Model 140C equipped with a programmable absorbance detector Model 785A (both from Perkin–Elmer/Applied Biosystems). The amino acid sequence of LUTI has been deposited in the SWISS-PROT protein data bank under the accession number P82381.

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