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Characterization of a Kunitz trypsin inhibitor with a single disulfide bridge from seeds of *Inga laurina* (SW.) Willd.

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Abstract

Inga laurina is a tree that belongs to the Mimosoideae sub-family of the Leguminosae. A protein inhibitor of trypsin (ILTI) was isolated from its seeds by ammonium sulphate precipitation, ion-exchange chromatography and rechromatography on an HiTrap Q ion-exchange column. By SDS–PAGE, ILTI yielded a single band with a Mr of 20 kDa with or without reduction. ILTI was found to be a single polypeptide chain containing 180 amino acids, the sequence of which was clearly homologous to the Kunitz family of serine protease plant protein inhibitors, and it also showed significant similarity to the seed storage proteins, sporamin and miraculin. However, ILTI displayed major differences to most other Kunitz inhibitors in that it contained only one disulfide bridge, and did not have two polypeptide chains as for the majority of other Kunitz inhibitors purified from Mimosoideae species. ILTI inhibited bovine trypsin with an equilibrium dissociation constant (K_i) of 6×10^{-9} M, but did not inhibit chymotrypsin, papain and α-amylase. Its amino acid sequence contained a Lys residue at the putative reactive site (position 64). ILTI was stable over a wide range of temperature and pH and in the presence of DTT.

Keywords: *Inga laurina*; Mimosoideae; Leguminosae; Kunitz inhibitor; Primary structure

1. Introduction

Proteinase inhibitors (PIs) occur in reproductive organs, storage organs and vegetative tissues of most plant families (Ryan, 1990; Richardson, 1991; Shewry and Lucas, 1997). These inhibitors form stable complexes with target proteases, blocking, altering or preventing access to the enzyme active site. The inhibitors discovered so far have been found to be specific for each of the four mechanistic classes of proteolytic enzymes, and based on the active amino acid in their reactive site are classified as serine, cysteine, aspartic, and metallo-protease inhibitors (Richardson, 1977). Among these, serine proteinase inhibitors are the most widely studied, and have been isolated from various Leguminosae seeds (Macedo and Xavier-Filho, 1992; Sampaio et al., 1996; Macedo et al., 2000; Mello et al., 2001, Pando et al., 2001; Garcia et al., 2004; Bhattacharyya et al., 2006).

In plants, proteinase inhibitors may represent a form of storage protein (Richardson, 1991; Mosolov, 1995; Valueva and Mosolov, 1999) or may be involved in plant defense mechanisms against pests and diseases (Ryan, 1990; Macedo et al., 2003; Haq et al., 2004; Tamhane et al., 2005; Dunaevsky et al., 2005). They may be synthesized constitutively during normal development or may be induced in response to insect and pathogen attacks (Ryan, 1990; Ryan and Pearce, 1998). In addition to their natural

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biological functions, proteinase inhibitors may also have a role in the treatment of human pathologies such as blood clotting, hemorrhage, inflammation and cancer (Oliva et al., 2000; Chen and Shaw, 2003; Fook et al. 2005; Mello et al., 2006).

Based on the homologies evident in their primary structures, the location of disulfide bridges and position of their reactive sites, the plant inhibitors of serine proteinases have been grouped into distinct families (e.g. Bowman–Birk, Kunitz, Potato I, Potato II, Cucurbit, Cereal, Thaumatin-like and Ragi A1) (Richardson, 1991). These families mainly differ in molecular weight, disulfide bond content, three-dimensional structure, and stability to heat and denaturing agent (Teles et al., 2004).

The plant Kunitz inhibitors are proteins (Mr ~ 18,000–24,000 Da) with one or two polypeptide chains and low cysteine content, usually with four Cys residues arranged into two disulfide bridges. PIs are capable of inhibiting primarily proteases (mammalian) such as trypsin, chymotrypsin, subtilisin and human plasma kallikrein. However, while found in all kinds of plants, they are present in higher quantities in legume seeds (Pandya et al., 1996). Bowman–Birk type inhibitors, on the other hand, have Mr ~ 8000–10,000 Da, with a high cysteine content and two reactive sites (Richardson, 1991). Previous studies on the distribution pattern of PIs among seeds of leguminous trees clearly suggested an evolutionary relationship between the PI family and the legume sub-families (Norioka et al., 1988; Macedo et al., 2000). Interestingly, Kunitz type PIs, which are usually single polypeptide chains, cleave at Cys residue 140 or in this vicinity, there are also have two polypeptide chains in the inhibitors obtained from the Mimosoideae subfamily, following reduction of their disulfide bridges (Oliva et al., 2000; Bhattacharyya et al., 2006).

Inga laurina SW. (Willd.), is a tree belonging to the subfamily Mimosoideae of the Leguminoseae. It is a tropical plant with a widespread distribution in Central and South America. In Brazil, where it is known locally as inga branco, it has been widely planted as a shade tree in urban environments. The fruit of *I. laurina* is consumed by humans and also by birds, with the latter acting as seed dispersers. Although *I. laurina* is an important part of the Brazilian flora, very little is known about its biochemistry. In this work, we describe the purification, characterization and complete amino acid sequence of a trypsin inhibitor from *I. laurina* seeds.

2. Results and discussion

2.1. Purification and characterization of ILTI

I. laurina trypsin inhibitor (ILTI) was purified by extraction in 100 mM phosphate buffer (pH 7.6), followed by ammonium sulphate precipitation, ion-exchange chromatography on DEAE-Sepharose and rechromatography on an ion-exchange column of HiTrap Q Sepharose (Fig. 1).

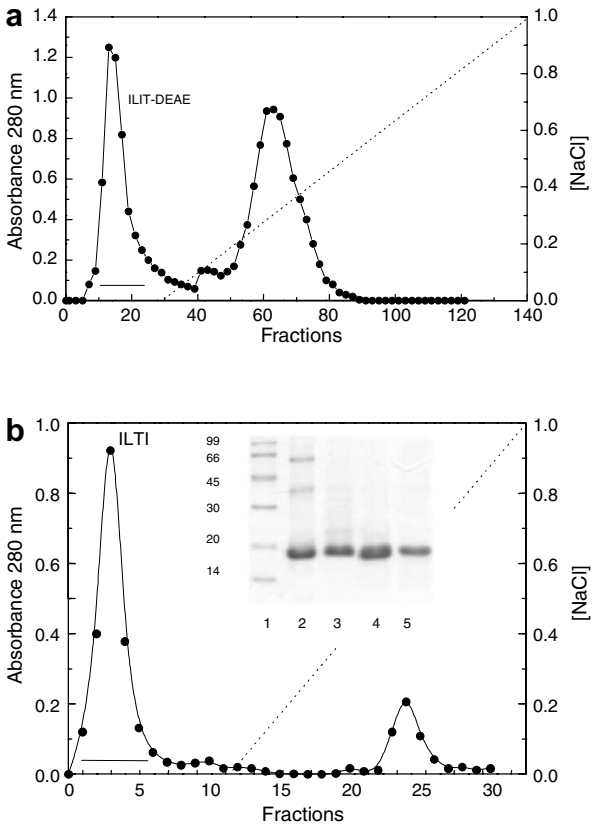

Fig. 1. (a) Ion-exchange chromatography (DEAE-Sepharose column) (2 × 20 cm), equilibrated with 0.05 M Tris–HCl buffer, pH 8.0, of the 30–60% ammonium sulfate precipitation fraction. (b) Fraction ILIT-DEAE resubjected to ion-exchange chromatography (HiTrap Q Sepharose column) (5 ml) equilibrated in 20 mM of Tris–HCl, pH 8.0 Insert: SDS–PAGE showing all fractions obtained during purification: Lane (1) Molecular mass markers, (2) crude extract, (3) fraction ILIT-DEAE, (4) fraction ILIT and (5) fraction ILIT reduced with 0.1 M DTT.

This procedure yielded ILTI with a 24-fold purification, and a yield of 22% (Table 1). SDS–PAGE (Fig. 1b, insert) showed that ILTI consisted of a single polypeptide with a molecular mass of approximately 20 kDa, which was also confirmed by gel filtration on Superdex G-75 and from its amino acid sequence. A further important finding is that ILTI gave a single band by SDS–PAGE under reducing conditions (Fig. 1b, insert), indicating the presence of only one polypeptide chain. This was also confirmed by amino acid sequence analysis, where no second chain was detected. The Mimosoideae usually contain proteinase

Table 1
Purification protocol of ILTI from *Inga laurina* seeds

Steps	Total protein (mg)	Total activity (IU)	Specifity activity (IU/mg)	Purification (fold)	Yield (%)
Crude extract	21035	4238.6	0.20	1	100
PII precipitate	7824	3205.8	0.41	2.0	75.6
DEAE-Sepharose	355	1251.6	3.52	17.6	29.5
HiTrap Q	198	950.4	4.8	24	22.4

inhibitors formed by two polypeptide chains linked by a disulphide bridge, thus differing from the other Kunitz-type single-chain inhibitors from the Caesalpinoideae and Papilonoideae subfamilies (Richardson et al., 1986; Richardson, 1991; Araújo et al., 2005). In this respect, ILTI is similar to trypsin inhibitors from *Dimorphandra mollis* seeds (Macedo et al., 2000; Mello et al., 2001), which belong to the Mimosoideae, but which also have only one polypeptide chain. The inhibitory activity of Kunitz-type proteinase inhibitors varies. A few inhibitors of this family are specific for chymotrypsin and do not inhibit trypsin (Joubert et al., 1981). Some Kunitz-type inhibitors are potent inhibitors of trypsin but also inhibit chymotrypsin to varying degrees (Odani et al., 1979). The inhibitory activity of ILTI was assessed by using different enzymes. The inhibitory activity against trypsin was measured over a range of concentrations of the inhibitor, using *N*-benzoyl-DL-arginyl-*p*-nitroanilide (BAPNA) as substrate (Fig. 2). The K_i value was calculated using the equation for slow-tight binding inhibition (Morrison, 1982) and was found to be 6×10^{-9} M for bovine trypsin. The K_i value thus establishes a high affinity between the enzyme and the inhibitor, in agreement with data reported for other plant trypsin inhibitors (Batista et al., 1996; Macedo et al., 2000; Mello et al., 2001; Bhattacharyya et al. 2006). ILIT, however, showed no activity against chymotrypsin, papain and α-amylase (data not shown).

The reactive site of a protease inhibitor is defined as that part of the molecule entering into direct contact with the active center of the enzyme to form an enzyme-inhibitor complex (Tscheche, 1974). When ILIT was incubated with trypsin at a 1:1 molar ratio, a complex with a molecular mass of approximately 40 kDa was observed by gel filtration (Fig. 3), indicating the presence of a single reactive site of trypsin. The estimated molecular mass of ILTI was ca. 20 kDa, in agreement with the SDS–PAGE results.

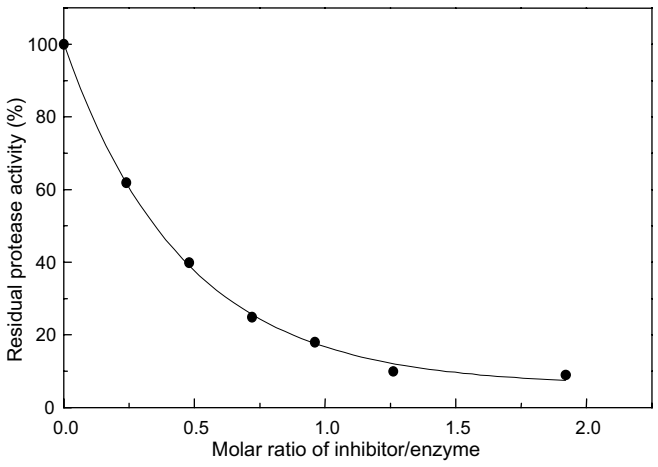

Fig. 2. Titration curve of trypsin inhibition by PPTI. Increasing concentrations of inhibitor were added to a fixed concentration of enzyme (4.2 nM). Residual enzyme activity was determined by using BAPNA as substrate. Each point is the mean of three assays.

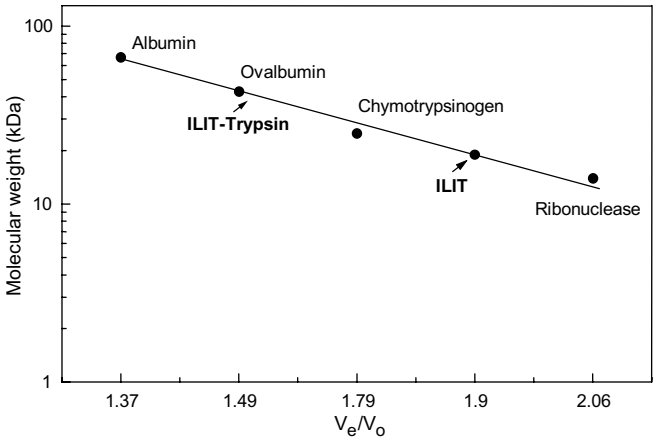

Fig. 3. Molecular weight estimation of ILTI and ILTI-trypsin complex on a Superdex 75 column (1.5 × 50 cm). The column was equilibrated with 50 mM Tris–HCl buffer, pH 8.0, containing 100 mM NaCl, and was calibrated using bovine serum albumin (67 kDa), ovalbumin (45 kDa), chymotrypsinogen (24 kDa) and cytochrome c (12 kDa). Flow rate, 0.3 ml/min.

The intramolecular disulfide bridges are presumably responsible for the functional stabiltiy of certain Kunitz-type inhibitors in the presence of various physical and chemical denaturants (Broze et al., 1990) such as temperature, pH, and reducing agents. ILTI lost ca. 20% of its activity when incubated at 80 °C for 30 min. When heated to 100 °C, a greater decrease in activity was observed (Fig. 4a). However, the inhibitory activity was not sensitive to pH over the range 2.0–10.0 (Fig. 4b); a similar result was reported for an *Enterolobium contortisiliqumm* trypsin inhibitor (ECTI) (Batista et al., 2001) and a *Dimorphandra mollis* trypsin inhibitor (DMTI) (Mello et al., 2001. DTT up to 10 mM had no effect on the activity or stabiltiy of ILTI (Fig. 4c), in contrast to the findings of Ramasarma et al. (1998) who studied a Bowman–Birk inhibitor from *Dolichos biflorus*. ILTI with 100 mM DTT lost ca. 56% of its inhibitory activity during the last 2 h. Macedo et al. (2003) observed that PDTI, a Kunitz type trypsin inhibitor from *Peltophorum dubium*, had its inhibitory activity lowered after reduction with 100 mM DTT. Similar results were obtained by Garcia et al. (2004) with PPTI, a trypsin inhibitor from *Poecilanthe parviflora*. Lehle et al. (1996) observed that ETI, a Kunitz-type trypsin inhibitor from *Erythrina caffra*, retained its inhibitory activity after reduction with DTT. Thus it appears that the stability of some inhibitors is apparently unrelated to the presence of disulfide bridges.

2.2. Determination of amino acid sequence of ILTI

The complete primary structure of ILTI (Fig. 5) was determined by the automated sequencing of the intact protein and peptides derived from the *S*-pyridyl-ethylated molecule following separate digestions with trypsin, chymotrypsin and the GLU-specific endoproteinase from *S. aureus*. The sequence of ILTI was shown to contain

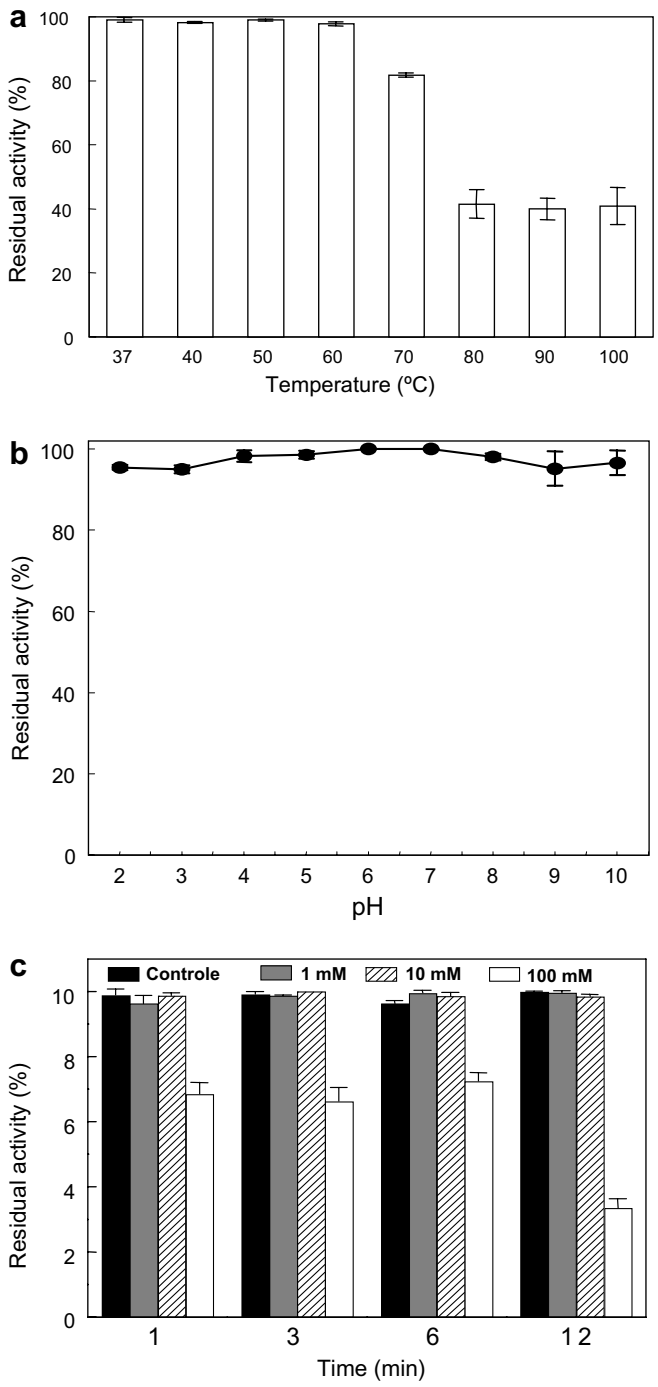

Fig. 4. Stability of ILTI. (a) Temperature stability of the inhibitory activity of ILTI after incubation for 30 min at the indicated temperatures; (b) pH stability of ILTI, after incubation at the indicated pH for 30 min at 37 °C; (c) effect of DTT on the stability of ILTI. The inhibitor was treated with different final concentrations (1, 10 and 100 mM) of DTT for 15–120 min at 37 °C. The residual trypsin inhibitory activity was measured using BAPNA as substrate.

180 amino acid residues and was compared with various protein databases using both FASTA and BLAST programmes. This comparison showed that these proteins share 30–40% identical residues and have long runs of highly conserved sequences (Fig. 5). Based upon the similarity of its primary amino acid sequence, ILTI should be assigned to the Kunitz family from plants. ILTI has just one disulfide bridge (in position Cys42–Cys86), which is located in the homologous position for the two other Kunitz inhibitors which also have only one disulfide bridge (Fig. 5). These are the *Swartzia pickellii* trypsin inhibitor-SWTI (Do Socorro et al., 2002) (35% identity), the glycosylated protein from *Bauhinia rufa* which inhibits elastase-gBrEI (Sumikawa et al., 2006) (15% identity) and the trypsin inhibitor from *Copaifera langsdorffii*-CTI (Krauchenco et al., 2004) that contains its disulfide bridge located between residues A40 and A84. Thus, ILTI is the fourth Kunitz inhibitor described in the literature which contains only one disulfide bridge. The second disulfide bond, absent in these trypsin inhibitors, is conserved in the majority of other Kunitz-type inhibitors. However, there are also Kunitz-type inhibitors with no disulfide bonds in the corresponding region of the polypeptide chain (Odani and Ikenaka, 1977; Kim et al., 1985; Oliva et al., 2000). These observations indicate that the second disulfide bond appears not to be essential for inhibitory activity.

The requirement of either arginine or a lysine in the P1 position of trypsin inhibitors is well established (Haldar et al., 1996). We have identified Lys-64 in ILIT as the putative P1 residue, on the basis of sequence alignment with other plant inhibitors (Richardson, 1991). The trypsin inhibitor from *Acacia confusa* (Lin et al.,1991), Mimosoideae subfamily, also has lysine in its reactive site and high inhibitory specificity for trypsin.

Furthermore, ILTI shows a significant homology to the storage proteins, sporamin, in sweet potato and the taste-modifying protein, miraculin, in miracle fruit, having about 30% identity in the amino acid residues (data not shown). It is perhaps interesting to note that whilst all Kunitz trypsin inhibitors are from members of the Leguminoseae, the storage proteins are not. Other Kunitz type trypsin inhibitors, such as the *Erythrina variegata* trypsin inhibitors (Kouzuma et al., 1992) and the *Prosopsis juliflora* Kunitz trypsin inhibitor (Negreiros et al., 1991) also have homology with these storage reserves. These facts substantiate the idea that the Kunitz family of protease inhibitors, sporamin and miraculin, are encoded by a family of related genes.

3. Experimental

3.1. Materials

I. laurina seeds were obtained locally (Três Lagoas, MS, Brazil) and were identified by Prof. Maria José Neto, Universidade Federal de Mato Grosso do Sul (UFMS – Departamento de Ciências Naturais, Campus de Três Lagoas, Brazil). Bovine serum albumin (BSA), bovine pancreatic trypsin, bovine pancreatic α-chymotrypsin, papain, porcine pancreatic α-amylase, GLU-specific endoproteinase from *Staphylococcus aureus*, BAPNA and *N*-benzoyl-

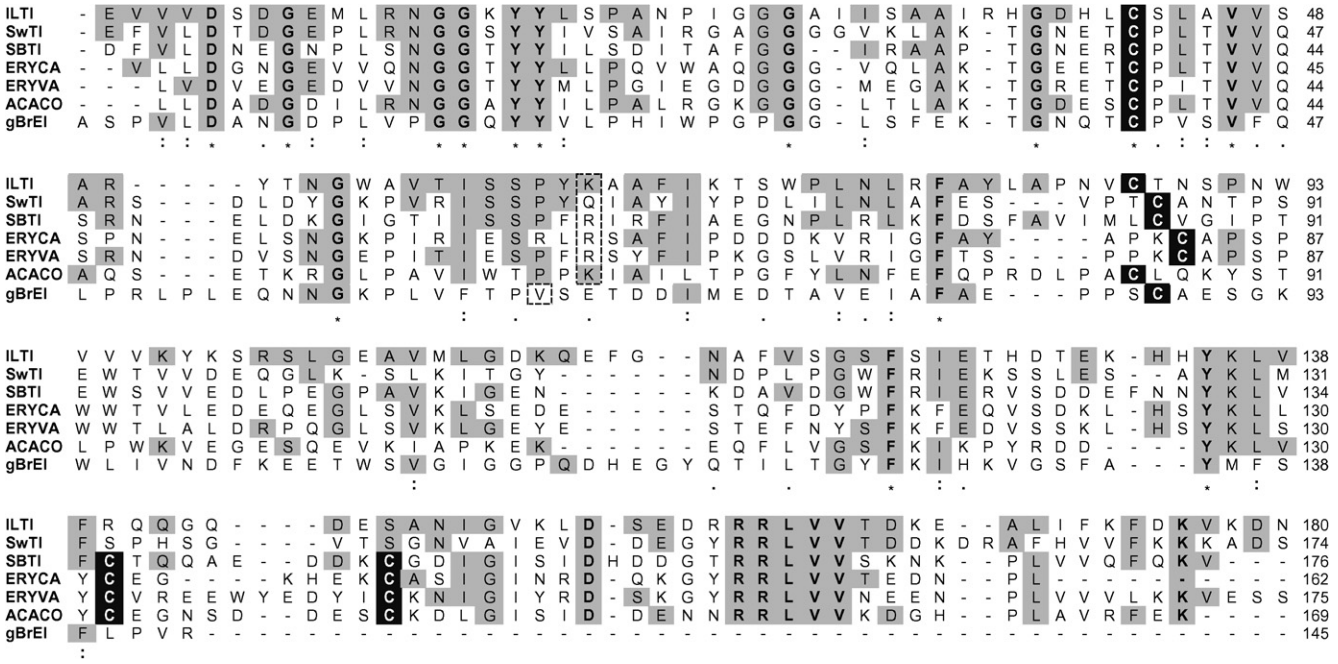

Fig. 5. Multiple alignments of similarity of the amino acid sequences and of the consensus regions of the proteins. ILTI, *I. laurina* inhibitor; SBTI, soybean trypsin inhibitor (Kim et al., 1985); ERYCA, *Erythrina caffra* trypsin inhibitor (Batista et al., 2001); *Erythrina variegata* trypsin inhibitor (Kouzuma et al., 1992); *Acacia confusa* trypsin inhibitor (Lin et al., 1991); MIR, Miraculin (Theerasilp et al., 1989); SPO, Sporamin (Hattori and Nakamura, 1988); SWTI, *S. pickellii* (Do Socorro et al., 2002) ; gBrEI, glycosylated *B. rufa* elastase inhibitor (Sumikawa et al., 2006). Identical residues are in blocks. In black box: cysteine residue. The dotted line indicate the reactive site. (∗) means that the residues of the column are identical; (:), means that conserved substitutions have been observed; (·), means that semi-conserved substitutions are conserved. Multiple alignments were performed using CLUSTAL W (1.82).

L-tyrosyl-*p*-nitroanilide (BTPNA) were purchased from Sigma (St. Louis, MO, USA), as were SDS–PAGE molecular weight markers, acrylamide, bis-acrylamide and other electrophoresis reagents. Chromatography supports were from Pharmacia (Uppsala, Sweden), All other chemicals and reagents used were of analytical grade.

3.2. Purification of *I. laurina* trypsin inhibitor (ILTI)

I. laurina seeds free of integument and defatted with hexane were ground in a coffee mill. A crude extract (CE) preparation was obtained by extraction of this meal with 0.1 M phosphate buffer, ph 7.6 (1:10, w/v) for 2 h at 25 °C with subsequent centrifugation at 7500*g* for 30 min. The supernatant was fractionated by $(NH_4)_2SO_4$ precipitation into three fractions, corresponding to 30%, 60% and 80% saturation. The three fractions were dialyzed against distilled water for 24 h at 4 °C and lyophilized. The fraction corresponding to 30–60% $(NH_4)_2SO_4$ saturation (PII) was selected for further purification because it contain the highest inhibitory activity.

Lyophilized PII was dissolved in 50 mM Tris–HCl buffer, pH 8.0, and applied to a DEAE-Sepharose column (2 × 20 cm) equilibrated in the same buffer, and eluted with a linear gradient of NaCl (0–1 M) in the same buffer. The peak containing inhibitory activity was rechromatographed by ion-exchange chromatography on a HiTrap Q Sepharose column (5 ml) equilibrated with 20 mM of Tris–HCl, pH 8.0, and eluted with the same buffer containing NaCl (0–1 M), at a flow rate of 30 ml/h. Proteins were detected by monitoring the absorbance at 280 nm.

3.3. Protein quantification

Protein contents were determined by Coomassie blue staining (dye-binding method) (Bradford, 1976) or from the absorbance at 280 nm. BSA (1 mg/ml) was used as standard.

3.4. Assay of inhibitory activity

Trypsin inhibitory activity was determined by measuring the residual hydrolytic activity of bovine trypsin and chymotrypsin towards the substrates BAPNA and BTPNA, respectively, at pH 8.0 after pre-incubation with inhibitor (Mello et al., 2001). The abiltiy to inhibit papain was assayed as described by Xavier-Filho et al. (1989). The inhibition of porcine pancreatic α-amylase was measured by the method of Bernfeld (1955). The assay employed soluble starch (1%) as the substrate. One inhibitor unit was defined as the amount of inhibitor that inhibited one unit of enzyme activity.

3.5. Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel (12.5%) electrophoresis (SDS–PAGE) in the absence and presence of

dithiothreitol (DTT, 100 mM) was carried out as described by Laemmli (1970). The proteins, used as molecular weight standards, were phosphorylase (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and ?-lactalbumin (14 kDa). The proteins were detected by staining with 0.1% Coomassie brilliant blue R-250.

3.6. K_i Determination

The dissociation constant (K_i) and the inhibitor concentration were determined for bovine trypsin by pre-incubating the enzyme with increasing concentrations of purified inhibitor in 50 mM Tris–HCl (pH 8.0), 37 °C, followed by measurement of the residual activity using the synthetic substrate BAPNA. The apparent K_i was determined by adjusting the experimental points to the equation for slow-tight binding (Knight, 1986), using a nonlinear regression with the help of the Enzfitter program.

3.7. Formation of ILTI-trypsin complex

The ILTI–trypsin complex was subjected to gel filtration (0.3 ml/min) on a Superdex 75 column (1.5 × 50 cm) equilibrated in 50 mM Tris–HCl buffer, pH 8.0, containing 100 mM NaCl. Trypsin, ILTI, and the trypsin/inhibitor complex (1:1 molar ratio) were preincubated for 10 min at 30 °C in 100 mM Tris–HCl buffer, pH 8.0, with 0.02% $CaCl_2$. As a control, isolated proteins were gel filtered as described above. The elution profiles were monitored based on absorbance at 230 nm, with the inhibitory activity followed by using BAPNA as substrate. The column was calibrated with albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsinogen (24 kDa), and cytochrome c (12 kDa).

3.8. Stabiltiy of inhibitory activity against bovine trypsin

3.8.1. Effect of temperature

The inhibitor solution (1 mg/ml in 50 mM Tris–HCl buffer, pH 8.0) was heated for 30 min in a water bath at various temperatures (37°–100 °C), and then cooled to 0 °C before testing for residual inhibitory activity. All experiments were done in triplicate and the results are the mean of three assays.

3.8.2. Effect of pH

To measure pH stabiltiy, a solution of inhibitor (1 mg/ml) was diluted with an equal volume of various buffers (100 mM): sodium citrate (pH 2–4), sodium acetate (pH 5), sodium phosphate (pH 6–7), Tris–HCl (pH 8) and sodium bicarbonate (pH 9–10). After incubation in each buffer for 30 min at 37 °C, the pH was adjusted to pH 8.0 with the inhibitory activity on trypsin was assayed as described below. All experiments were carried out in triplicate and the results is the means of three assays.

3.8.3. Effect of DTT

The inhibitor (1 mg/ml) was incubated with the reducing agent DTT at final concentrations of 1, 10 and 100 mM for 15–120 min at 37 °C. The reaction was terminated by adding iodoacetamide to twice the amount of each DTT concentration, with the residual inhibitory activity on trypsin then determined.

After the treatment above, the residual inhibitory activity on trypsin was measured using BAPNA as substrate. Aliquots (50 μl of trypsin inhibitor solution) were mixed with a stock solution of bovine trypsin (50 μl, 0.33 mg/ml in 2.5 mM HCl) in 50 mM Tris–HCl buffer, pH 8.0. The mixture was incubated at 37 °C for 10 min followed by the addition of BAPNA (1 ml, 100 mM) to give a final volume of 1.5 ml. After 20-min incubation, the reaction was stopped by adding 200 μl of 30% (v/v) acetic acid. Substrate hydrolysis was followed by the increase in absorbance at 405 nm. All experiments were done in triplicate and the results are the mean of three assays.

3.9. S-Reduction and alkylation

The protein (ILTI, 3.5 mg) was *S*-reduced and alkylated with vinyl pyridine essentially as described by Henschen (1985). The material was dissolved 6 M guanidine-HCL (1 ml) in 0.1 M Tris–HCl, pH 8.6. To this solution, 2-mercaptoethanol (30 μl) was added under N_2, with the sample then incubated at 50 °C for 4 h. After this, vinyl pyridine (40 μl) was added and the sample was incubated at 37 °C for a further 2 h. The reduced and alkylated protein was recovered by desalting on a column (22 mM × 25 cm) of Vydac C4 (214TP54), using a gradient of 0–70% CH_3CN in 0.1% trifluoroacetic acid over 70 min at a flow rate of 1 ml/min. The collected protein was lyophilized.

3.10. Determination of amino acid sequence

Samples (1 mg) of the *S*-pyridyl-ethylated protein were dissolved in 0.1 M ammonium bicarbonate (1 ml, pH 7.9) and digested separately at 37 °C with trypsin (for 3.5 h), chymotrypsin (4 h) and the GLU-specific endoproteinase from *Staphylococcus aureus* (18 h) using 2% (w/w) enzyme/substrate. After lyophilisation, the peptides produced were separated by reversed phase HPLC on a column (22 mm × 25 cm) of Vydac C18 (small pore, 201SP54) using an extended gradient of 0–50% CH_3CN in 0.1% trifluoroacetic acid for 150 min at a flow rate of 1 ml/min.

The amino acid sequences of the S-pyridyl-ethylated intact protein (200 μg) and the peptides derived from it by the enzymatic digestions were determined by Edman degradation using a Shimadzu PPSQ-21A automated protein sequencer.

3.11. Sequence comparisons

The amino acid sequence of ILTI was compared with the sequences of other related proteins in the SWISS-

PROT/ TREMBL data bases using the FASTA 3 and BLAST programs.

3.12. Statistical analysis

All data were examined using one-way analysis of variance (ANOVA) (General Linear Models on GLM procedure). The Student–Neuman–Keul's test was used to identify the means which differed if the ANOVA test indicated significance. A P value < 0.05 was considered to be significant.

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