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Purification and characterization of a trypsin inhibitor from *Putranjiva roxburghii* seeds

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ABSTRACT

A highly stable and potent trypsin inhibitor was purified to homogeneity from the seeds of *Putranjiva roxburghii* belonging to *Euphorbiaceae* family by acid precipitation, cation-exchange and anion-exchange chromatography. SDS–PAGE analysis, under reducing condition, showed that protein consists of a single polypeptide chain with molecular mass of approximately 34 kDa. The purified inhibitor inhibited bovine trypsin in 1:1 molar ratio. Kinetic studies showed that the protein is a competitive inhibitor with an equilibrium dissociation constant of 1.4×10^{-11} M. The inhibitor retained the inhibitory activity over a broad range of pH (pH 2–12), temperature (20–80 °C) and in DTT (up to100 mM). The complete loss of inhibitory activity was observed above 90 °C. CD studies, at increasing temperatures, demonstrated the structural stability of inhibitor at high temperatures. The polypeptide backbone folding was retained up to 80 °C. The CD spectra of inhibitor at room temperature exhibited an α, β pattern. N-terminal amino acid sequence of 10 residues did not show any similarities to known serine proteinase inhibitors, however, two peptides obtained by internal partial sequencing showed significant resemblance to Kunitz-type inhibitors.

1. Introduction

Plant proteinase inhibitors are widely distributed in plant seeds and are most studied class of inhibitors (Richardson, 1991; Mello and Silva-Filho, 2002). The molecular mass of these inhibitors can vary from 4 to 85 kDa, with majority in the range of 8–20 kDa (Hung et al., 2003). In higher plants, several gene families of these protease inhibitors have been characterized. The plant serine proteinase inhibitors, particularly trypsin inhibitors, have been extensively studied (Laskowski and Kato, 1980; Barrett and Salvesen, 1986). They play an important role as plant defense agent against insects and pests attack (Sampaio et al., 1996; Shewry and Lucas, 1997; Walker et al., 1997; Franco et al., 2003) and are known to be involved in many biological functions, such as blood coagulation, platelet aggregation and anti-carcinogenesis (Kennedy, 1998; Oliva et al., 2000). Plant protease inhibitors have been described as endogenous regulators of proteolytic activity (Ryan, 1990; Kato, 2002) and as storage proteins (Xavier-Filho, 1992). These inhibitors have been grouped mainly into Kunitz, Bowman-Birk, Potato I and II and squash, cereal superfamily and thaumatin-like inhibitors (Richardson, 1991). Most serine proteinase inhibitors from seeds have been isolated and characterized from *Leguminosae*, *Cucurbitaceae*, *Solanaceae* and *Graminae* families (Garcia-Olmedo et al., 1987). There are not many reports of purification and characterization of these inhibitors from other plant families. Other families where an inhibitor has been purified and characterized include *Rutaceae* (Shee and Sharma, 2007; Shee et al., 2007a,b) and *Euphorbiaceae* (Sritanyarat et al. 2006) families.

Putranjiva roxburghii belonging to *Euphorbiaceae* family is an ornamental tree of tropical India known as child life tree. Roxburghonic keto acid and some flavonoids, terpenoids and triterpines has been purified and characterized from the leaf and trunk bark of this plant (Garg and Mitra, 1968, 1971a,b; Sengupta et al., 1967). To date, no protein has been characterized from this plant. This paper describes the purification and characterization of a highly stable and potent trypsin inhibitor from the seeds of *P. roxburghii*.

2. Results and discussion

2.1. Purification of *P. roxburghii* trypsin inhibitor (PRTI)

P. roxburghii trypsin inhibitor (PRTI) was purified to homogeneity in three steps by acid precipitation, CM-sepharose cation

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exchange and DEAE-sepharose anion exchange chromatography. In acid precipitation step, low molecular mass proteins were precipitated along with some other proteins. The trypsin inhibitory activity was retained in supernatant. This method has been earlier used for the purification of *Brassica nigra* seed trypsin and subtilisin inhibitor (Genov et al., 1997). In second step on CM-sepharose column, all the low molecular mass proteins were bound to the column while trypsin inhibitory activity was found in flow through. This resulted in complete removal of major low molecular mass protein. In last step, protein with trypsin inhibitory activity was bound to a DEAE-sepharose column. After washing the column extensively, bound proteins were eluted with step gradient of NaCl. The fractions with trypsin inhibitory activity were eluted at 50 and 100 mM NaCl. The purity of the protein in above fractions was analyzed by SDS–PAGE. The fraction eluted at 100 mM NaCl showed single band on SDS–PAGE (Fig. 1a). The protein was further subjected to size exclusion chromatography column on HPLC where it showed a single peak with a retention time of 8.2 min in 50 mM Tris–HCl buffer, pH 8.0 (Fig. 1b). The SDS–PAGE analysis under both reducing (Fig. 1b, inset) and non-reducing (data not shown) conditions showed that PRTI is a single polypeptide chain with a molecular mass of approximately 34 kDa. Interestingly, the molecular mass of PRTI is significantly higher than the typical Kunitz-type inhibitor (20 kDa). Earlier, trypsin inhibitors of 32.5, 33 and 43.5 kDa has been reported from *Crotalaria pallida* seeds (Gomes et al., 2005), *Ipomoea batatas* (sweet potato) root (Hou et al., 2001) and *Avena sativa* L. (Mikola and Kirsi, 1972), respectively.

2.2. N-terminal and partial internal sequencing

In N-terminal sequencing of PRTI, first 10 residues from the N-terminal were obtained. The sequence determined was Arg-Pro-Pro-Gln-Ala-Gly-Tyr-Ile-Gly-Val. The N-terminal sequence of PRTI showed no similarities with any of the known trypsin inhibitors. In partial internal sequencing, sequences of four peptides were obtained. In separate experiments, one peptide (peptide 1) was obtained from LC-MS/MS and three peptides from MALDI-MS/MS studies. The peptide sequenced by LC-MS/MS analysis showed only one match when searched against FASTA database within BioWorks 3.2 program (Thermo Fischer scientific). It showed 100% identity to winged bean chymotrypsin inhibitor-3 (Shibata et al., 1988) (Fig. 2). All the peptides obtained by MALDI-MS/MS analysis showed low score in MASCOT search. Among three peptides only one peptide (peptide 2) with MASCOT score of 23 showed 100, 73 and 73% identity to *Acasia confussa* trypsin inhibitor (Wu and Lin, 1993), *Prosopsis juliflora* trypsin inhibitor (Negreiros et al., 1991) and trypsin isoinhibitors DE5 of *Adenanthera pavonina* L. (Richardson et al., 1986), respectively (Fig. 2). Although the MASCOT score of the peptide 2 is lower than the significant value, it has been included here as no information is available about this

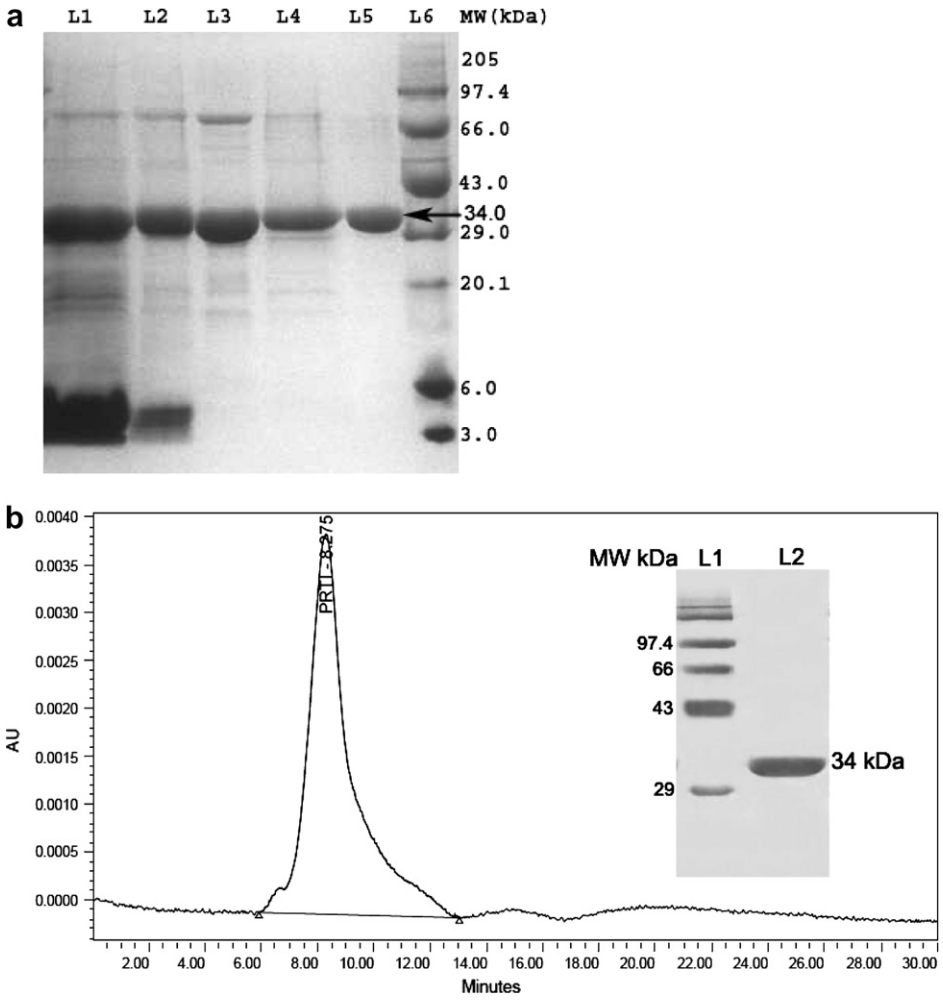

Fig. 1. (a) SDS–PAGE analysis of the protein. L1, crude extract; L2, supernatant after acid precipitation step; L3, CM flow-through; L4, 50 mM NaCl fraction after anion exchange column; L5, purified protein in 100 mM NaCl fraction after anion exchange chromatography; L6, molecular weight markers. (b) Elution profile of purified protein on HPLC gel-filtration column. Insert: SDS–PAGE analysis of HPLC purified PRTI.

A

Protein		Sequence		% Identity
Peptide1	1	FEKVSHSNIHVYK	13	
WCI-3	143	FEKVSHSNIHVYK	155	100%

B

Peptide2	1	YSTLPWKVEGESQEVK	16	
ACTI	90	YSTLPWKVEGESQEVK	105	100%
PJTI	90	-SSLQWKVEEESQQVK	104	73%
DE5	90	-SNLQWKVEEESQIVK	104	73%

Fig. 2. Amino acid sequence alignment of two internal peptides of PRTI with known serine proteinase inhibitors by NCBI BLAST short sequence search. WCI-3 (Winged bean chymotrypsin inhibitor-3; ACTI (*Acasia confussa* trypsin inhibitor); PJTI (*Prosopsis juliflora* trypsin inhibitor); DE5 (*Adenanthera pavonina* L. trypsin inhibitor).

protein. Other two peptides did not show resemblance to any of the protease inhibitors. These results suggest that PRTI may belong to Kunitz-type inhibitor family.

2.3. Inhibitory properties and K_i determination

The inhibitory activity of PRTI against trypsin and chymotrypsin were determined by measuring the hydrolytic activity toward BAPNA and BTEE, respectively. The protein completely inhibited trypsin at a molar ratio of 1:1 but did not show any significant inhibition against α-chymotrypsin (Fig. 3a). The dissociation constant (K_i) value and mode of inhibition of PRTI were determined from Dixon plot using BAPNA as a substrate (Fig. 3b). The analysis of Dixon plot showed that the PRTI is a competitive inhibitor where two lines corresponding to each substrate intersect above the *x*-axis, a characteristic of competitive inhibition. The dissociation constant (K_i) value was found to be 1.4×10^{-11} M which clearly indicates that PRTI is a highly potent inhibitor of bovine trypsin. Earlier, high K_i values of 5.3×10^{-10}, 4.0×10^{-10}, 2.5×10^{-10}, 1.7×10^{-9} M have been reported for plant trypsin inhibitors from *Dimorphandra mollis* (Macedo et al., 2000), *Peltophorum dubium* (Macedo et al., 2003), *Archidendron ellipticum* (Bhattacharyya et al., 2006) and *D. mollis* (Mello et al., 2001).

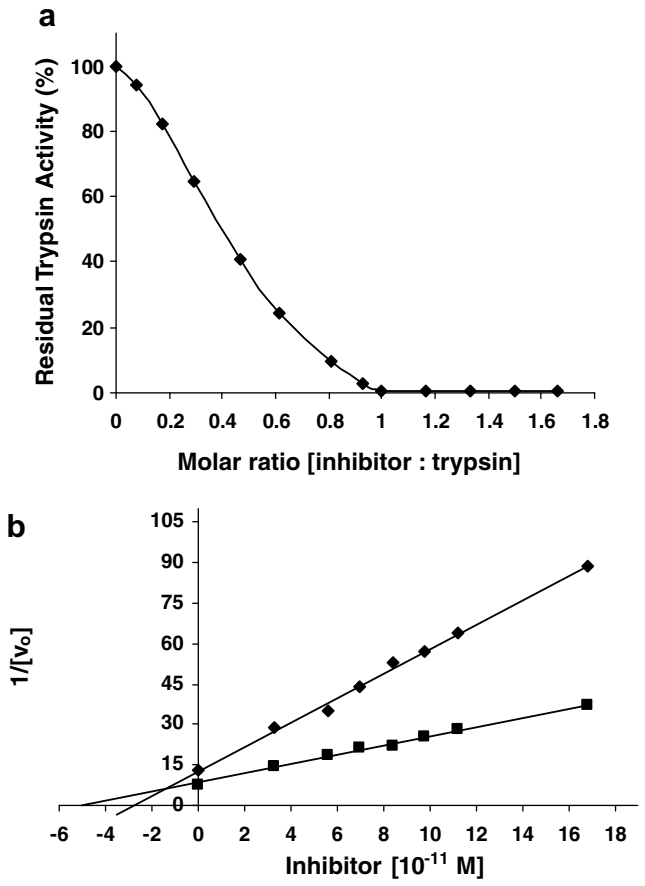

Fig. 3. Kinetic analysis of trypsin inhibitory activity of PRTI. (a) Trypsin inhibitory activity of PRTI showing residual trypsin activity in percent as function of the inhibitor concentration at a fixed trypsin concentration using BAPNA as substrate. All experiments were done three times and averaged. (b) Dixon plot for the determination of the dissociation constant (K_i) value of PRTI at two different concentration of BAPNA. Final concentrations of substrate were 0.001 M (■) and 0.005 M (◆). The reciprocals of velocity were plotted against different concentrations of PRTI.

2.4. Stability studies

In thermo stability studies, trypsin inhibitory activity of PRTI was determined at temperatures ranging from 20 to 100 °C. In thermal stability studies, trypsin inhibitory activity of PRTI was completely retained up to 70 °C. Above 70 °C, there was a slight decrease in the inhibitory activity with PRTI retaining almost 85% inhibitory activity up to 80 °C. The inhibitory activity of PRTI fell sharply above 80 °C with a loss of almost 80% inhibitory activity at 90 °C (Fig. 4b). The transition midpoint for PRTI lies close to 88 °C. Similar results have been reported for trypsin inhibitors from seeds of *Carica papaya* (Azarkhan et al., 2006) and *P. dubium* (Macedo et al., 2003). The transition midpoint for papaya trypsin inhibitor is approximately 85 °C and it retains almost 71% of inhibitory activity at 80 °C, whereas *P. dubium* inhibitor retains more than 80% of activity at 80 °C. In pH stability studies, PRTI was highly stable under conditions ranging from highly acidic to highly alkaline. PRTI showed maximum inhibition at pH 8.0 and maintained over 95% of its inhibitory activity through a pH gradient of 2–12 (Fig. 4a). Similar results has been reported for other trypsin inhibitors including from *Igna laurina* (Macedo et al., 2007), *C. pallida* (Gomes et al., 2005), *C. papaya* (Azarkhan et al., 2006) and *P. dubium* (Macedo et al., 2003). The inhibitory activity of PRTI was examined in the presence of DTT. PRTI was found completely stable with no loss in inhibitory activity when incubated for 1 h up to 100 mM DTT. Only a slight decrease of 5% in inhibitory activity was observed when PRTI was incubated for 2 h at 100 mM DTT (Fig. 4c). This was in contrast to most trypsin inhibitors which lose inhibitory activity to different extent at high DTT concentration (Azarkhan et al., 2006; Macedo et al., 2007). Similar results have been earlier reported for a Kunitz-type trypsin inhibitor from *Erythrina caffra* (ETI) which retained inhibitory activity after reduction with DTT (Lehle et al., 1996). This clearly suggests that the inhibitory activity of PRTI like ETI is not affected by the presence of disulfide bridges. The crystal structure of ETI showed that the disulfide bridges are far apart from the active site residues involved in protease binding. The reactive loop in ETI is stabilized by hydrogen bonds and is not constrained by secondary structural elements or disulfide bridges (Onesti et al, 1991).

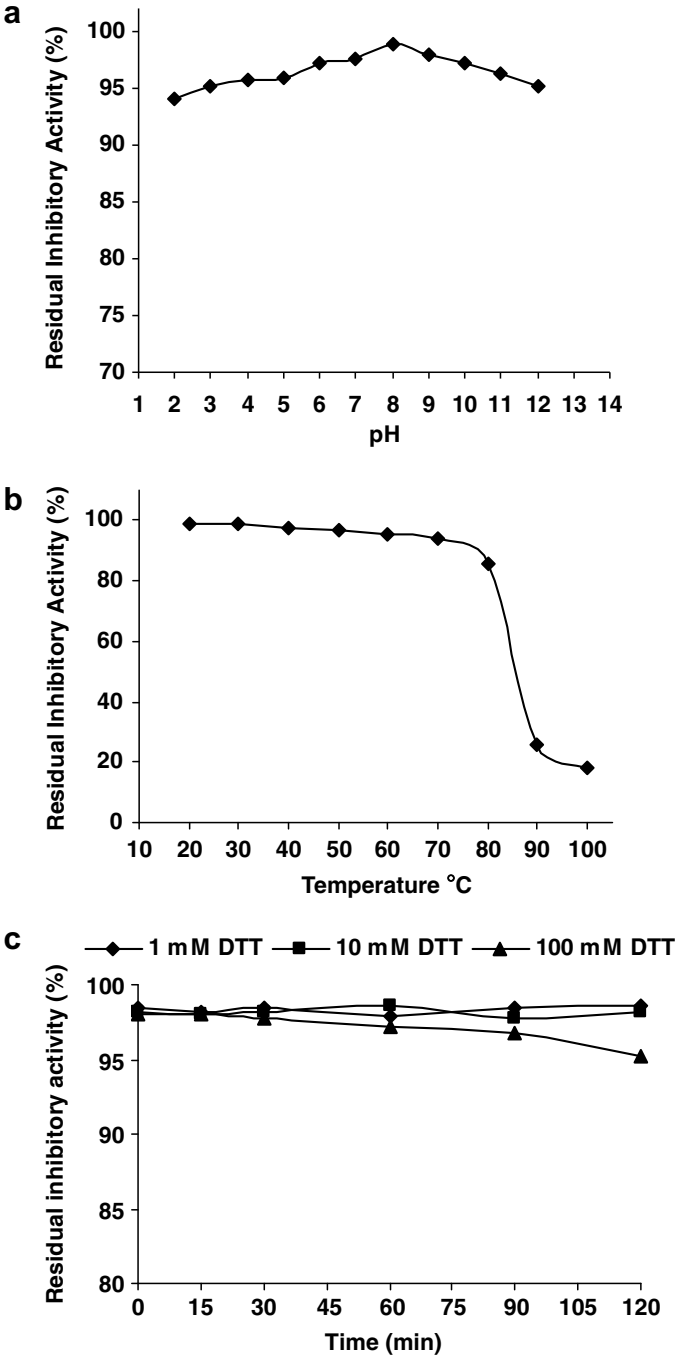

Fig. 4. Stability studies of PRTI. (a) pH stability of inhibitory activity of PRTI in pH range 2–12 after incubation for 30 min at 37 °C. (b) Temperature stability of the inhibitory activity of PRTI after incubation for 30 min at pH 8.0 in the range of 20–100 °C. (c) Effect of DTT on the stability of PRTI at final concentrations of 1, 10 and 100 mM after incubation for 15–120 min at 37 °C. The residual trypsin inhibitory activity for all experiments was measured using BAPNA as substrate and all experiments were done three times and averaged.

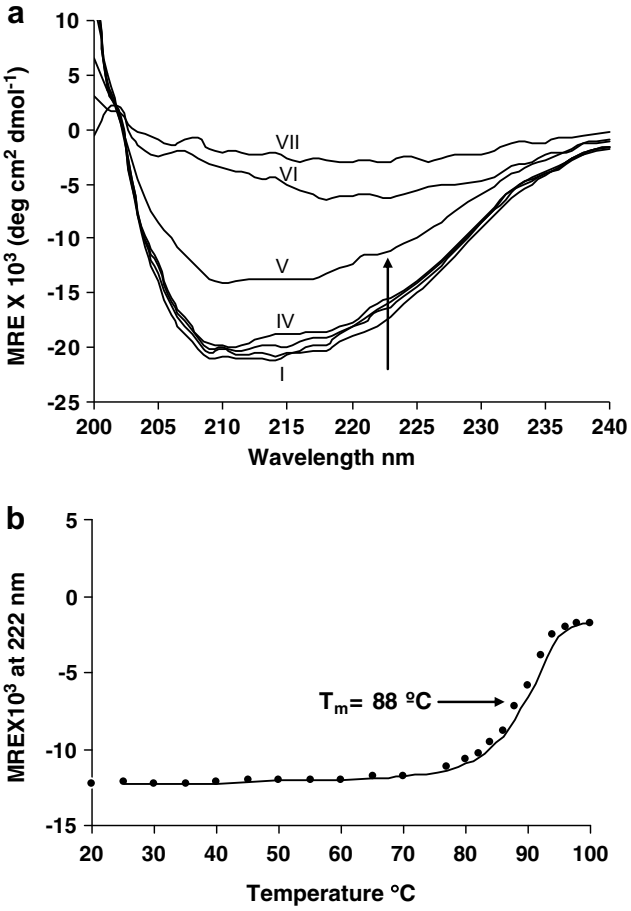

Fig. 5. CD studies of PRTI. (a) Far-UV CD spectra (200–240 nm) measurements of PRTI in 50 mM sodium phosphate buffer, pH 7.0 as a function of temperature ranging from 20 to 100 °C (I–IV – 20, 30, 50, 70 °C; V – 80 °C; VI – 90 °C; VII – 100 °C). (b) CD melting curve by measuring change in ellipticity at 222 nm during thermal denaturation of PRTI.

2.5. CD spectroscopy

Far-UV CD spectroscopy studies (240–200 nm wavelength range) were carried out to analyze the secondary structure and conformational stability of PRTI at different temperatures from 20 to 100 °C (Fig. 5a). Analysis of CD spectra of native PRTI showed that it is an α, β protein with negative peaks at around 217 and 208 nm. Although, negative ellipticity was present but no clear negative peak characteristic of α-helical structures was observed at 222 nm. These results strongly suggest that PRTI is α, β protein rather than predominantly β protein. Most trypsin inhibitors particularly Kunitz family inhibitors are predominantly β-sheet proteins with little helical content (Sweet et al., 1974; Onesti et al, 1991; Azarkhan et al., 2006). CD studies at increasing temperature demonstrated the thermo stability of PRTI structure. The PRTI retained the back bone protein folding with no significant change in CD spectra up to 70 °C. A significant loss in ellipticity was observed at and above 90 °C. This correlates well with the results of inhibitory activity where 15% loss was observed at 80 °C and 80% at 90 °C. The inhibitory activity and CD studies at increasing temperatures showed that transition midpoint for PRTI lies close to 88 °C (Fig 5b). The results were similar to other trypsin inhibitors which exhibited significant structural stability at high temperatures (Roychaudhuri et al., 2003; Azarkhan et al., 2006; Macedo et al., 2003).

3. Conclusions

The results in this work demonstrated that PRTI, a single chain protein with molecular mass of approximately 34 kDa, is a very stable and highly potent trypsin inhibitor and may belong to Kunitz family of inhibitors. The K_i value of 1.4×10^{-11} M demonstrated a high affinity between enzyme and inhibitor. PRTI displayed a remarkable stability at a wide range of pH (pH 2–12) and temperature (up

to 80 °C). PRTI retained almost 95% of inhibitory activity when incubated with 100 mM DTT for 2 h which demonstrated that disulfide bridges do not have any direct role on inhibitory activity. CD analyses of PRTI revealed that it is an α, β protein rather than predominantly β-sheet protein and the polypeptide backbone folding of the protein was retained up to 80 °C.

4. Experimental

4.1. Materials

Seeds of *P. roxburghii* were collected locally. DEAE-sepharose, CM-sepharose, trypsin, *N*-benzoyl-L-arginine *p*-nitroanilide (BAPNA), chymotrypsin, *N*-benzoyl-L-tyrosine ethyl ester (BTEE) and BSA were purchased from Sigma–Aldrich Pvt. Ltd., PVDF membrane (Immobilon-P^{SQ} Millipore, USA), Centricon and Centriprep were purchased from Amicon (Beverly, MA). Reagents for SDS–PAGE were purchased from Sigma–Aldrich Pvt. Ltd. Protein molecular weight standards were obtained from Bangalore GeNei™ India. All other reagents were of analytical or HPLC grade.

4.2. Purification of *P. roxburghii* trypsin inhibitor (PRTI)

Mature seeds (25 g), after removing the hard seed coat, were grounded and soaked overnight at room temperature in 200 ml of distilled water. The extract was centrifuged at 12,000 rpm for 45 min to obtain a clear supernatant. The pH of the supernatant was adjusted to 4.5 with 5 N HCl. After 12 h incubation at 4 °C, precipitated proteins were removed by centrifugation at 12,000 rpm for 30 min and discarded. The supernatant was dialyzed against distilled water at 4 °C, freeze-dried, and dissolved in 0.05 M phosphate buffer, pH 6.0. The supernatant was then applied to a CM-sepharose column (1.5 × 20 cm Econo-column, Bio-Rad) pre-equilibrated with 0.05 M phosphate buffer pH 6.0. The flow through having trypsin inhibitory activity was collected. It was dialyzed against distilled water at 4 °C, freeze-dried, and dissolved in 0.05 M Tris–HCl buffer pH 8.0. The CM flow through was then loaded on to a DEAE-sepharose column, (1.5 × 20 cm Econo-column, Bio-Rad) pre-equilibrated with 50 mM Tris–HCl buffer, pH 8.0. The column was washed extensively to remove unbound molecules and bound proteins were eluted with a NaCl step gradient from 0 to 0.5 M (0, 0.05, 0.1, 0.15, 0.2, 0.3, 0.4 and 0.5 M NaCl) at room temperature in the same buffer. Fractions with trypsin inhibitory activity at 0.1 M NaCl were pooled and concentrated by using Centriprep of 10 kDa cutoff (Amicon, Beverly, MA). The protein was further purified by HPLC using a size exclusion chromatography column (7.5 × 300 mm, Waters) with the 50 mM Tris–HCl buffer at pH 8.0. The elution was monitored at 280 nm wavelength at the flow rate of 0.75 ml/min. The homogeneity of the protein was determined by 15% SDS–PAGE analysis.

4.3. Protein quantification

Protein contents were estimated by Coomassie blue staining (dye-binding method) (Bradford, 1976) with bovine serum albumin as the standard (1 mg/ml).

4.4. SDS–PAGE analysis

Gel (15%) electrophoresis under both reducing (SDS–PAGE) and non reducing conditions was done as described by Laemmli (1970). Relative molecular mass was determined by performing SDS–PAGE of protein with molecular weight standards under reducing condition. The molecular weight standards used were myosin [rabbit muscles] (205 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa), lysozyme (14.3 kDa), aprotinin (6.5 kDa) and insulin [α and β chain] (3.0 kDa). The proteins were detected by staining the gel with 0.1% Coomassie brilliant blue R-250.

4.5. N-terminal and partial internal sequence determination

For N-terminal amino acid sequencing, pure protein sample was subjected to a 15% SDS–PAGE and electroblotted onto a PVDF membrane (Immobilon-P^{SQ} Millipore, USA) using 100 mM CAPS buffer, pH 11 (Matsudaria, 1987). The N-terminal amino acid sequencing was performed by Edman degradation on an automated sequencer (model 494; Applied Biosystems) at the protein sequencing facility of Columbia University, New York, USA. Partial internal sequencing was performed by MS/MS fragmentation analysis studies. For MS/MS studies, purified protein was run on a 15% SDS–PAGE and protein band was cut from the gel. The cut gel was processed, tryptic digested and peptides obtained were analyzed by MALDI-TOF–TOF (Bruker Daltonics Ultraflex TOF/TOF) and LC-MS/MS (Thermo Fischer scientific) experiments at proteomics facility of The Center for Genomic Application (TCGA), New Delhi. For MALDI-MS/MS analysis, spectra were searched against the MASCOT database. For LC-MS/MS analysis, spectra were searched against FASTA database within BioWorks 3.2 program (Thermo Fischer scientific).

4.6. Assay of inhibitory activity

The inhibitory activity on bovine pancreatic trypsin was determined by measuring the hydrolytic activity towards the substrate *N*-benzoyl-L-arginine *p*-nitroanilide (BAPNA) (Shibata et al, 1986). The different concentrations of inhibitor were incubated with 5.6×10^{-7} M trypsin (final concentration in 1.5 ml assay volume) at 30 °C for 15 min in 50 mM Tris–HCl buffer, pH 8.0 containing 20 mM $CaCl_2$ and 0.001 N HCl. After 15 min incubation, 1.0 ml of 0.5 mM BAPNA was added. After incubation of another 10 min at 30 °C, the reactions were stopped by adding 200 μl of 10% acetic acid. The changes in absorbance at 410 nm were recorded at 30 °C against a blank solution containing 1.5 ml of the substrate solution in same buffer with a Varian Cary 100 spectrophotometer. The amount of substrate (BAPNA) hydrolysis by the enzyme was calculated using the molar extinction coefficient of 8800 M^{-1} cm^{-1} at 410 nm. For chymotrypsin inhibitory assay, different concentrations of PRTI were incubated with 10 μg of chymotrypsin dissolved (final concentration was 4.0×10^{-9} M in 1.0 ml assay volume) in 1.0 mM HCl containing 20 mM $CaCl_2$ at 30 °C for 15 min. Enzyme–protein mixtures (100 μl) were added to a solution of 900 μl in a cuvette containing a 1.0 mM final concentration of substrate *N*-benzoyl-L-tyrosine ethyl ester (BTEE, molar extinction coefficient of 964 M^{-1} cm^{-1}) in 50 mM Tris–HCl, pH 8.0 containing 10% methanol. The substrate hydrolysis was monitored by measuring the absorbance at 256 nm for 5 min with a Varian Cary 100 spectrophotometer.

4.7. K_i determination

Kinetic studies over a range of PRTI concentrations were performed to determine the inhibition constant (K_i) from Dixon plot using BAPNA as a substrate (Dixon, 1953; Segel, 1975). Studies were performed by adding a range of inhibitor concentrations to a fixed amount of trypsin (final concentration was 0.0032 nM) at two different substrate concentrations of 1.0 and 5.0 mM. The final inhibitor concentrations used were 0.033, 0.056, 0.070, 0.084, 0.098, 0.11 and 0.21 nM. All the reactions were performed as described earlier. The reciprocal velocity ($1/v$) versus inhibitor

concentrations [I], for each substrate concentration, [S_1] and [S_2] were plotted (Dixon plots). A single regression line for each [S] was obtained, and the K_i was calculated from the intersection of the two lines.

4.8. Stability studies

In stability studies, effect of temperature, pH and DTT was examined on inhibitory activity of PRTI. For thermal stability studies, purified protein (1 mg/ml) in 50 mM Tris–HCl buffer, pH 8.0 was incubated at various temperatures (30–100 °C) for 30 min. After incubation, all the samples were kept on ice for 15 min and then centrifuged. Supernatants were taken for the estimation of trypsin inhibitory activity. In pH stability, trypsin inhibition assays under a range of pH (2–12) conditions were performed to assess the pH stability and pH optima of PRTI. Purified PRTI was incubated with buffers of pH 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 to a final concentration of 1 mg/ml. The buffers used were 0.1 M each of glycine–HCl (pH 2, 3), Na-acetate (pH 4, 5), Na-phosphate (pH 6, 7), Tris–HCl (pH 8, 9), glycine–NaOH (pH 10, 11, 12). After incubation of 30 min, the trypsin inhibitory activity was determined at pH 8.0. The effect of DTT on inhibitory activity was determined by incubating purified PRTI (1.0 mg/ml) with DTT at final concentrations of 1, 10 and 100 mM for 10–120 min at 37 °C. The reaction was stopped by adding twice the amount of iodoacetamide to each DTT concentration and then the residual trypsin inhibitory activity was measured. All experiments were done in triplicate and the results are the mean of three assays.

4.9. CD spectroscopy

Circular dichroism measurements were carried out on a JASCO J-715 spectropolarimeter, equipped with a peltier-type temperature controller (PTC-348 WI) and a thermostated cell holder, interfaced with a thermostatic bath. CD instrument was routinely calibrated with D-10-camphorsulfonic acid. Far-UV CD spectra, (200–240 nm), were recorded in 1 cm path length quartz cell at a protein concentration of 0.35 mg/ml in 50 mM sodium phosphate buffer at pH 7.0. The effect of temperature on conformational stability of PRTI was determined by measuring CD spectra at temperatures ranging from 20 to 100 °C. Three consecutive scans were accumulated and the average spectra stored. The results of all the CD measurements are expressed as mean residue ellipticity (MRE).

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