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Purification and characterization of a trypsin inhibitor from seeds of *Murraya koenigii*

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Abstract

A protein with trypsin inhibitory activity was purified to homogeneity from the seeds of *Murraya koenigii* (curry leaf tree) by ion exchange chromatography and gel filtration chromatography on HPLC. The molecular mass of the protein was determined to be 27 kDa by SDS-PAGE analysis under reducing conditions. The solubility studies at different pH conditions showed that it is completely soluble at and above pH 7.5 and slowly precipitates below this pH at a protein concentration of 1 mg/ml. The purified protein inhibited bovine pancreatic trypsin completely in a molar ratio of 1:1.1. Maximum inhibition was observed at pH 8.0. Kinetic studies showed that *Murraya koenigii* trypsin inhibitor is a competitive inhibitor with an equilibrium dissociation constant of 7×10^{-9} M. The N-terminal sequence of the first 15 amino acids showed no similarity with any of the known trypsin inhibitors, however, a short sequence search showed significant homology to a Kunitz-type chymotrypsin inhibitor from *Erythrina variegata*.

Keywords: Murraya koenigii, *trypsin inhibitor, solubility, proteinase inhibitor*

Abbreviations: *HPLC, high performance liquid chromatography; MKTI,* Murraya koenigii *trypsin inhibitor; BAEE, N-benzoyl L-arginine ethyl ester; BTEE, N-benzoyl-L-tyrosine ethyl ester; BSA, bovine serum albumin; BAPNA, N-benzoyl-L-arginine-p-nitroanilide*

Introduction

Proteinase inhibitors constitute an important group of proteins present in plants, animals and microorganisms [1–4]. The plant serine proteinase inhibitors, particularly trypsin inhibitors, have been extensively studied [1,5]. They play an important role in plant defense against pathogens and predators [6–7] and are known to be involved in many biological functions, such as blood coagulation, platelet aggregation and anti-carcinogenesis [8–9]. Many plant serine proteinase inhibitors have been purified and characterized particularly from the seeds of Leguminosae and Cucurbitaceae family. These proteinase inhibitors have been classified usually according to their sequence homology and structure of disulfide bridges and include Kunitz-type, Bowman-Birk type, potato I, potato II, squash, cereal superfamily and thaumatin-like inhibitors [10].

Murraya koenigii (L) Spreng. (*Rutaceae*) is a small tree, commonly used as a spice throughout India for its aromatic value. Apart from this it is well-known from ancient time for its many medicinal properties including stimulant, stomach ache, antidysenteric, antidiabetic and carminative activities [11–14]. Until now, no protein has been characterized from this plant. In this paper we report, for the first time, the purification and characterization of a trypsin inhibitor from seeds of *Murraya koenigii*.

Materials and methods

Materials

Seeds of *Murraya koenigii* were collected locally. DEAE-sepharose, trypsin, N-benzoyl-L-arginine ethyl ester (BAEE), N-benzoyl-L-arginine p-nitroanilide (BAPNA), chymotrypsin, N-benzoyl-L-tyrosine ethyl

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ester (BTEE) and BSA were purchased from Sigma-Aldrich Pvt. Ltd., Centricon and Centriprep were purchased from Amicon (Beverly, MA). Molecular weight standards were obtained from Bio-Rad, Australia. All other chemicals were purchased from Qualigens fine chemicals.

Purification of protein

Mature seeds (10 g) were crushed and soaked overnight at 4°C in 40 mL of 50 mM Tris-HCl buffer, pH 7.5. The homogenate was cleared by centrifugation at 12,000 × g for 1 h and the supernatant was loaded onto a DEAE Sepharose column (1.5 × 20 cm Econo-column, Bio-Rad) equilibrated with 50 mM Tris-HCl buffer, pH 7.5. The unbound molecules were washed out extensively and bound molecules were fractionated with a NaCl step elution from 0 to 500 mM (0, 50, 100, 150, 200, 300, 400 and 500 mM NaCl) in the same buffer. Fractions with trypsin inhibitory activity were pooled (100 mM NaCl) and concentrated by using Centriprep followed by Centricon with a 10 kDa cutoff (Amicon, Beverly, MA). The protein was further purified by HPLC using a size exclusion chromatography column (7.5 × 300 mm, Waters). The purity of the protein was determined by 15% SDS-PAGE analysis [15].

Protein estimation

Protein concentration in crude extract and fractionated protein samples was estimated by standard dye-binding method using bovine serum albumin (Sigma) as standard [16]. Absorbances at 280 nm were also used to determine the protein content of different eluted fractions.

SDS-PAGE analysis

Sodium dodecyl sulfate-polyacrylamide gel (15%) electrophoresis (SDS-PAGE) under both reducing and non reducing conditions was done as described by Laemmli [15]. Relative molecular weight was determined by performing SDS-PAGE of protein with molecular weight standards under reducing condition calibrated on Tris-HCl gel. The molecular weight standards used were myosin (194.2 kDa), β-galactosidase (115.6 kDa), bovine serum albumin (97.3 kDa), ovalbumin (53.5 kDa), carbonic anhydrase (37.2 kDa), soyabean trypsin inhibitor (29.3 kDa), lysozyme (20.4 kDa) and aprotinin (6.9 kDa). The proteins were detected by staining the gel with 0.1% Coomassie brilliant blue R-250.

Solubility studies at different pH

For solubility studies, the purified protein was dissolved in buffers of different pH (50 mM sodium acetate pH 4.0, 4.5, 5.0, 5.5; 50 mM sodium phosphate pH 6.0, 6.5, 7.0; 50 mM Tris-HCl pH 7.5, 8.0, 8.5, 9.0) to a final concentration of 1 mg/mL. The protein solutions were incubated on ice for 1.5 h. After the incubation, samples were centrifuged at 15,000 × *g* for 30 min. The supernatants were collected and the protein concentration was estimated.

Standard trypsin assay

The inhibitory activities on bovine pancreatic trypsin were determined by measuring the hydrolytic activity towards the substrate N-benzoyl-L-arginine ethyl ester (BAEE) [17]. The inhibitor was incubated at 30°C for 5 min with 4.4×10^{-7} mol/L of trypsin in 2.5 mL of 50 mM Tris-HCl buffer, pH 8.0, containing 20 mM $CaCl_2$. Then 2 mL of this solution was pipetted into a cuvette and 0.1 mL of 10 mM BAEE solution in the same buffer was added. The change of absorbance at 253 nm was recorded at 30°C against a reference solution containing 2 mL of the buffer and 0.1 ml of the substrate solution with a Varian Cary 100 spectrophotometer. The amount of substrate hydrolysis by the enzyme was calculated using the molar extinction coefficient of 808 M^{-1} cm^{-1} at 253 nm. The trypsin inhibitory activity was also determined at different pH values (pH 7.5, 8.0, 8.4 & 8.8) using BAEE as substrate.

Determination of the Ki value

The inhibition constant (Ki) and the mode of inhibition was determined by the method of Dixon using BAPNA as substrate [18]. The kinetic studies were performed by adding various inhibitor concentrations to a fixed amount of trypsin (final concentration in the assay 1.38 nM) in the presence of two different substrate concentrations (final concentration in the assay 3 and 6 mM) in 50 mM Tris-HCl buffer, pH 8.0. The inhibitor concentrations in the assay were 11, 18.5, 37, 74, 110 and 138 nM. Reactions were performed by incubating 100 μL of the enzyme solution with 200 μL of inhibitor in 50 mM Tris-HCl buffer, pH 8.0 at 30°C for 10 min. Then 1 mL of the substrate, dissolved in the same buffer, was added. After incubation at 30°C for an additional 10 min, the reaction was stopped by adding of 200 μL 30% acetic acid [17]. The amount of product formation was calculated using a molar extinction coefficient of 8800 M^{-1} cm^{-1} at 410 nm.

Chymotrypsin assay

Different concentrations of purified protein were incubated with 10 μg of chymotrypsin dissolved in 1 mM HCl containing 20 mM $CaCl_2$ at 30°C for 10 min. Enzyme-protein mixtures (100 μL) were added to a solution of 900 μL in a cuvette containing a 1 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 7.5, containing 2.5% methanol. The substrate hydrolysis was monitored by measuring the absorbance at 256 nm for 5 min [19,20].

The inhibitory effect of MKTI against trypsin and chymotrypsin was also determined by taking BSA as natural substrate. BSA was incubated with trypsin and chymotrypsin at 100:1 molar ratio in the presence of MKTI in 50 mM Tris-HCl, pH 7.5 for 10 min. The samples were analyzed on a 15% SDS-PAGE under reducing conditions.

N-terminal amino acid sequence determination

Pure protein was subjected to SDS-PAGE (15%) and electroblotted on to a PVDF membrane (Immobilone-P ^{SQ} Millipore) in 100 mM Caps-buffer, pH 11. [21]. The N-terminal amino acid sequence analysis was performed by Edman degradation on a Shimadzu Automated Protein Sequencer (PPSQ-20) at the Biophysics Department, All India Institute of Medical Sciences, New Delhi, India.

Results

Purification of protein

We found that a protein with a trypsin inhibitory property was the major constituent of the supernatant after a simple buffer extraction from the seeds of *Murraya koenigii*. The protein was purified to homogeneity by a two step procedure. The first step was performed on an anion exchange column (DEAE), followed by a HPLC gel filtration. Two products were obtained from the HPLC gel filtration, whereas the peak 2 (retention time 15.675 min.) with the trypsin inhibitory activity was used for further studies (Figure 1 A). The purity of the protein was confirmed by SDS-PAGE analysis under both reducing and non-reducing conditions, MKTI is a single polypeptide chain with a molecular mass of approximately 27 kDa (Figure 1 B).

Solubility studies at different pH

The solubility of the protein was examined at different pH conditions in different buffers. These studies showed that the solubility of the protein decreases

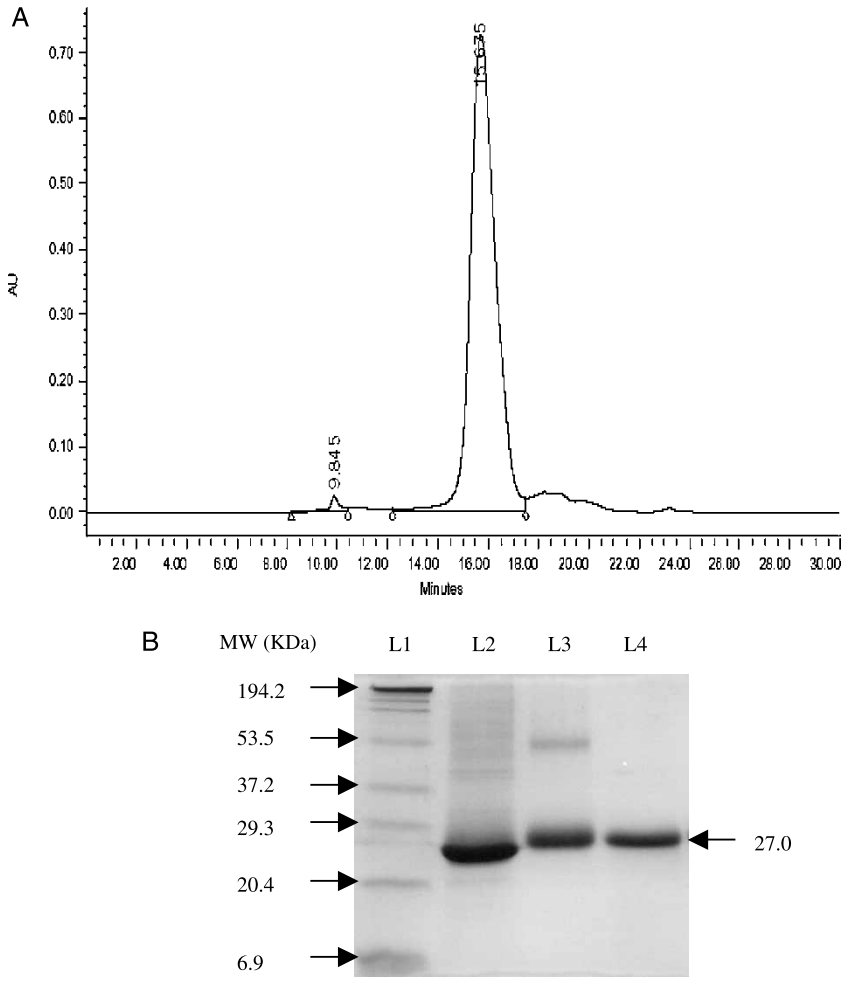

Figure 1. Purification of *M. koenigii* trypsin inhibitor (A) Elution profile on HPLC gel filtration column; (B) SDS-PAGE analysis of the protein. L1, molecular weight markers; L2, total protein in buffer extract; L3, 100 mM NaCl fraction after anion exchange chromatography; L4, purified protein after gel filtration chromatography on HPLC.

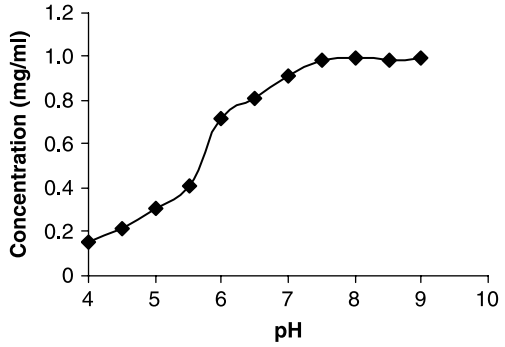

Figure 2. Solubility studies of *M. koenigii* trypsin inhibitor at different pH conditions. The protein was dissolved in buffers of different pH (50 mM sodium acetate pH 4.0, 4.5, 5.0, 5.5; 50 mM sodium phosphate pH 6.0, 6.5, 7.0; 50 mM Tris-HCl pH 7.5, 8.0, 8.5, 9.0) to a final concentration of 1 mg/mL. All experiments were in triplicate and averaged.

below pH 7.5, while it is completely soluble at and above pH 7.5 (Figure 2). It was observed that around 85% of the total protein is precipitated at pH 4.0, 70% at pH 5.0 and 30% at pH 6.0.

Trypsin inhibitory properties

The inhibitory activity of MKTI against trypsin was determined by measuring the hydrolytic activity toward BAEE. The protein completely inhibited bovine pancreatic trypsin at a molar ratio of 1:1.1 (Figure 3). In addition, the trypsin inhibition was determined at different pH conditions at a 1:1 molar ratio (Figure 4). A maximal trypsin inhibition was observed at pH 8.0, whereas the trypsin inhibition was reduced above and below this pH.

The Ki value and mode of inhibition of the inhibitor was determined using BAPNA as a substrate. The Ki value obtained from a Dixon plot was found to be 7×10^{-9} M. The analysis of Dixon plot showed that

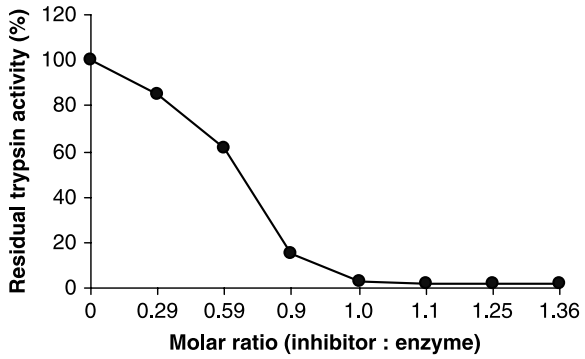

Figure 3. Trypsin inhibitory activity of M. *koenigii* trypsin inhibitor showing residual trypsin activity in per cent as function of the inhibitor concentration at a fixed trypsin concentration using BAEE as substrate. All experiments were in triplicate and averaged.

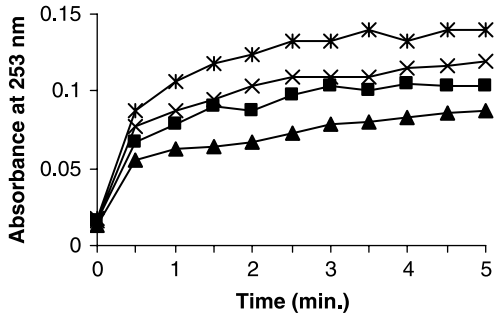

Figure 4. Trypsin inhibitory activity of *M. koenigii* trypsin inhibitor at different pH conditions in 50 mM Tris-HCl using BAEE as substrate. The concentration of trypsin and inhibitor were 4.4×10^{-7} M. The different pH taken were (*) pH 8.8, (×) pH 7.5, (■) pH 8.4, and (▲) pH 8.0. All experiments were done in triplicate at 30°C and averaged.

the two lines corresponding to each substrate intersect above the x-axis, which is a characteristic of competitive inhibition (Figure 5).

N-terminal sequencing and chymotrypsin inhibition

Sixteen cycles of sequence data were obtained from the analysis of the protein sample. The sequence of the first 15 N-terminal amino acids was Ile-Asp-Pro-Leu-Leu-Asp-Ile-Asn-Gly-Asn-Val-Val-Glu-Ala-Ala. The amino acid sequence was searched against the NCBI BLAST short sequence search protein data base. The N-terminal sequence of the first 15 amino acids showed no similarity with any of the trypsin inhibitors, however, a short sequence search showed significant homology with 72% identity (8/11) to a Kunitz-type *Erythrina variegata* chymotrypsin inhibitor (ECI) precursor (Figure 6).

However, MKTI did not show any inhibitory activity against chymotrypsin. The studies with BSA also confirmed our results. Though proteolytically

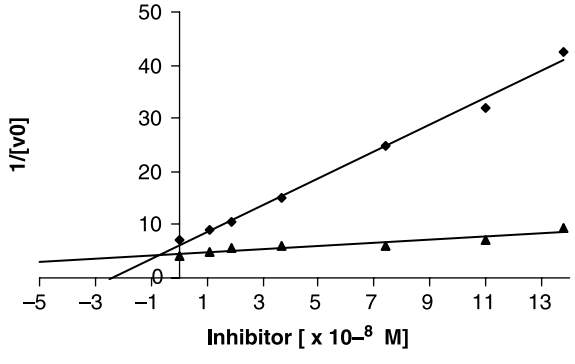

Figure 5. Dixon plot for the determination of the dissociation constant (Ki) value of MKTI at two different concentration of BAPNA. Final concentrations of substrate were 0.003 M (■) and 0.006 M (▲). The reciprocals of velocity were plotted against different concentrations of MKTI.

Name of protein		Sequence		Identity
Murraya koenigii trypsin inhibitor	3	PLLDINGNVVE	13	
		PLLD+ GN+VE		72%
Erythrina variegata Chymotrypsin inhibitor (ECI) precursor	26	PLLDVEGNLVE	36	

Figure 6. Sequence homology study by NCBI BLAST short sequence search showing the N-terminal amino acid sequence of MKTI (residues 3–13) having 72% identity with *Erythrina variegata* chymotrypsin inhibitor (ECI) precursor.

resistant against chymotrypsin and trypsin, it did not show any inhibitory effect on the hydrolysis of BSA by chymotrypsin (Figure 7).

Discussion

Murraya koenigii is a known herbal plant with many medicinal properties including anti-diabetic and is widely used in India in food preparations. Until now, no protein has been characterized from this plant. We report, for the first time, the isolation and characterization of a trypsin inhibitor from the seeds of *Murraya koenigii*. The inhibitor was purified to homogeneity by a two step procedure. First step was done on an anion exchange column (DEAE) at pH 7.5 and the second step was performed on a HPLC gel filtration column. The approximate molecular weight of the protein was determined to be 27 kDa from SDS-PAGE analysis.

The solubility studies at different pH conditions showed the unusual solubility properties of this protein. The protein is completely soluble above pH 7.5, whereas its solubility was significantly decreased below pH 7.5. It is interesting to note that the solubility decreases at constant rate from pH 7.5 to pH 6.0, but falls sharply from 70% at pH 6.0 to 30% at pH 5.0 and then again decreases gradually. The instability of MKTI in acidic conditions may be due to certain interactions which lead to the precipitation of the protein. It is important to note that the inhibitory activity was regained completely after resolubilization of the protein in buffer at pH 8.0 (unpublished results). Also, the solubility of the protein at lower pH could be increased by adding different concentrations of salts (unpublished results).

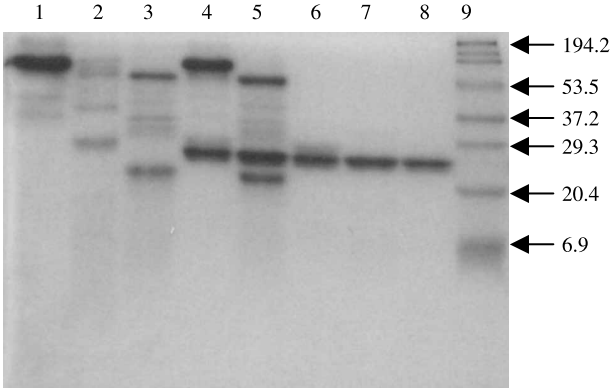

Figure 7. Inhibitory effect of MKTI against trypsin and chymotrypsin using BSA as substrate on 15% SDS-PAGE. Lane 1, BSA; 2, BSA incubated with trypsin; 3, BSA incubated with chymotrypsin; 4, BSA incubated with trypsin and MKTI complex; 5, BSA incubated with chymotrypsin and MKTI complex; 6, MKTI incubated with trypsin; 7, MKTI incubated with chymotrypsin; 8, pure MKTI; 9, Molecular weight markers.

The trypsin inhibition at different concentrations by MKTI demonstrated that a complete inhibition was achieved at a molar ratio of 1:1.1. In addition, the trypsin inhibition depends on pH, at a 1:1 molar ratio, the maximal trypsin inhibition is achieved at pH 8.0. The inhibition falls both above and below pH 8.0. The trypsin inhibition could not be determined below pH 7.5 as protein starts to precipitate. Kinetic studies demonstrated that, like other serine proteinase inhibitors, MKTI is a competitive inhibitor with a dissociation constant of 7×10^{-9} M.

Though the protein has strong trypsin inhibitory activity, the N-terminal sequence showed no homology to any of the known protein-like trypsin inhibitors. However, a NCBI BLAST short sequence search showed some homology to a chymotrypsin inhibitor from *Erythrina variegata* belonging to the Kunitz-type protease inhibitor family. However, despite this N-terminal sequence homology, MKTI did not show any chymotrypsin inhibitory activity. A high specificity of different protease inhibitors, which belong to the same family, is quite common among the various protease inhibitor types [22,23]. Considering the molecular weight of MKTI and its significant homology to one of the inhibitors belonging to the Kunitz-type inhibitors, this protein may belong also to the Kunitz-type protease inhibitor family.

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