

Storage and affinity properties of *Murraya koenigii* trypsin inhibitor

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Abstract

The *Murraya Koenigii* trypsin inhibitor was found to be the major protein component of the seed extract. The quantity of protein was determined to be approximately 20% of total protein extracted by simple buffer extraction. During different stages of seed development after flowering, the protein concentrations were found to be 5.27, 5.5, 8.5, 18.8 and 20% in 7, 19, 25, 37 and 55 days, respectively. During seed germination, protein degradations were observed from 20% to 12, 7 and 2% in 13, 16 and 22 days, respectively. This inhibitor, earlier purified using ion-exchange and gel filtration chromatography, was purified in single step by affinity column, using Cibacron blue 3GA, with substantial increase in yield. In partial internal sequencing by MALDI-TOF-TOF, six peptides of varying length, totalling 98 amino acid residues, exhibited similarities to the sequences from protease inhibitors, storage proteins and homeodomain-like proteins.

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Keywords: Trypsin inhibitor; Storage protein; Cibacron blue affinity; Protein profile; Partial sequencing

1. Introduction

Seed proteins play important roles in plants, maintaining viability of seeds, providing nutrition during the early seedling stage, and protecting the seeds from microbes and insects (Millerd, 1975). Seed proteins also play an important role in human and animal nutrition by providing the major share of dietary protein. These proteins may be classified as storage, structural, and biologically active proteins (Fukushima, 1991). The major biologically active proteins include lectins, enzymes and enzyme inhibitors (e.g. trypsin inhibitor). The seed storage proteins can be distinguished from other proteins by some of their characteristics; e.g. (1) these accumulate in high amounts in seed during the mid-maturation stage of seed development and are used up during germination; (2) these are synthesized only in the seed (in cotyledon or in endosperm) and not in other tissues (Mandal & Mandal, 2000); (3) in gen-

eral, a protein can be considered as a storage protein when this protein is present in amounts of 5% or more of the total protein content (Derbyshire, Wright, & Boulter, 1976). The amount of protein present in seeds varies from ~10% (in cereals) to ~40% (in legumes), forming a major source of dietary protein (Ramakrishna & Ramakrishna, 2005). Many plant storage organs, such as seeds and tubers, contain from 1 to >10% of their soluble proteins as trypsin inhibitors (Mandal, Kundu, Roy, & Mandal, 2002). Besides their storage function, by providing nitrogen and a sulphur source required during germination, trypsin inhibitors have been ascribed other functional roles, such as regulating endogenous plant proteinases to prevent precocious germination, inhibiting trypsin during passage through as animal's gut, thus helping in seed dispersal, and protecting plants against pests and diseases (Laskowski & Kato, 1980).

Although storage proteins and protease inhibitors are placed in two separate groups, as classified by Fukushima (1991), there are many reports of overlapping functions (Mosolov, Grigoreva, & Valueva, 2001). The proposed role for protease inhibitors as storage proteins was first suggested by Pusztai (1972).

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Murraya koenigii (L) Spreng. (*Rutaceae*) is a small tree commonly used as a spice throughout India for its medicinal and aromatic value. Earlier, we have reported the purification of a Kunitz-type trypsin inhibitor from the seeds of *M. koenigii* (Curry leaf tree) using ion-exchange and gel filtration chromatography. The approximate molecular mass of the protein was determined to be 27 kDa, from SDS-PAGE analysis. The purified protein inhibited bovine pancreatic trypsin at a molar ratio of 1:1.1, with a dissociation constant of 7×10^{-9} M. The maximum inhibition was observed at pH 8.0 (Shee & Sharma, 2007). Recently, *M. koenigii* trypsin inhibitor (MKTI) has been crystallized and preliminary crystallographic studies have been reported (Shee, Singh, Kumar, & Sharma, 2007). In the present study, we have demonstrated that *M. koenigii* trypsin inhibitor is a major seed storage protein by monitoring the relative concentrations during seed development and germination. Also, we report the single step purification on an affinity column using Cibacron blue 3GA and partial internal amino acid sequencing of MKTI.

2. Materials and methods

2.1. Materials

Seeds of *M. koenigii* were collected locally. Cibacron blue 3GA, trypsin, *N*-benzoyl-L-arginine *p*-nitronilide (BAPNA) and BSA were purchased from Sigma-Aldrich Pvt. Ltd. Centricon and Centriprep were purchased from Amicon (Beverly, MA). Acrylamide, bis-acrylamide, molecular weight standards and other chemicals for SDS-PAGE were obtained from Bio-Rad, Australia. All other chemicals were purchased from Qualigens fine chemicals.

2.2. Determination of soluble protein concentration

Mature seeds (10 g) were crushed with a mortar and pestle 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 12,000g for 1 h and the supernatant was used to determine the soluble protein concentration by the method of Bradford, using BSA as standard (Bradford, 1976).

2.3. Relative concentration of protein during seed development and germination

During different stages of development, seeds were collected after seven days of flowering at intervals of six days and immediately frozen in liquid nitrogen. The seeds were stored at -80 °C until the collection was completed. After complete collection, 250 mg of seeds, obtained in each stage of development, were crushed and soaked overnight in 1 ml of 50 mM Tris-HCl, pH 7.5, separately. Homogenates were centrifuged at 12,000g for 1 h and supernatant was run on a 15% SDS-PAGE for analysis. Gels were stained with Coomassie brilliant blue R-250 and the relative concentrations of each band were analyzed using

Quantity One 1D gel analysis software (version 4.5.2; Biorad).

To monitor the degradation of a major seed storage protein during germination, mature *M. koenigii* seeds were prepared for healthy germination. The freshly harvested seeds were washed with tap water to remove soft pulp attached to seeds before planting. The seeds were planted at 3/4" depth in well draining soil mixture. For germination, bright light, warmth and high humidity were maintained and the soil was allowed to dry to 1/2" depth before watering again. Germinated seeds were collected at three day intervals and extracts were prepared to run on a 15% SDS-PAGE for analysis, as described earlier.

Trypsin inhibitory activity was monitored during different stages of seed development and germination by taking a fixed volume of crude extract (3 µl diluted to 200 µl) from different samples prepared during the protein profiling experiment.

2.4. Purification on Cibacron blue 3GA

Mature seeds (10 g) were crushed with a mortar and pestle and soaked overnight at 4 °C in 50 ml of 20 mM Tris-HCl buffer, pH 8.0. The homogenate was cleared by centrifugation at 12,000g for 1 h and the supernatant was loaded onto a Cibacron blue 3GA affinity column (1.5 × 20 cm Econo-column, Bio-Rad) equilibrated with 20 mM Tris-HCl buffer, pH 8.0. The unbound molecules were washed extensively with absorbance at 280 nm was less than 0.05, to avoid any non-specific binding. The elutions of bound proteins were performed with 0.2 and 0.5 M NaCl in same buffer. The loosely bound proteins were further washed out along with the some amount of MKTI at 0.2 M NaCl. The fractions eluted at 0.5 M NaCl were concentrated and desalted by using 10 kDa cutoff Centriprep, followed by Centricon (Amicon, Beverly, MA). The purity of the protein was determined by 15% SDS-PAGE and MALDI-TOF analysis. Trypsin inhibitory activity of purified protein was determined by incubating different concentrations of purified protein to a fixed amount of trypsin.

2.5. SDS-PAGE analysis

Sodium dodecyl sulfate-polyacrylamide gel (15%) electrophoresis (SDS-PAGE), under both reducing and non-reducing conditions, was done as described by Laemli (1970). 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.

2.6. Mass spectrometry analysis

MALDI and MALDI-TOF-TOF studies were performed at The Center for Genomic Application (TCGA), New Delhi, India. For MALDI-TOF analysis, 1 μ l of the sample was mixed with 1 μ l of a saturated solution of matrix (sinnapinic acid in TA (0.1% TFA:acetonitrile, 2:1) and spotted on a MALDI target plate. The instrument (Bruker Daltonics Ultraflex TOF/TOF) was calibrated using Protein Calibration Standard II, Bruker Daltonics. The spectra were acquired using flexcontrol 2.2 software with ion source voltage 25 kV and accelerating voltage 23.2 kV in linear mode. The spectra were processed using FLEXANALYSIS™ 2.2 software.

For MALDI-TOF-TOF studies, the purified protein was run on a 15% SDS-PAGE and the band was cut from the gel on a clean surface (wiped with alcohol) using a clean scalpel or razor blade and transferred to an eppendorf tube. Gel plugs were washed with 150–200 μ l of 50 mM ammonium bicarbonate/50% acetonitrile (ACN) three times for 20 min and then washed with 100% ACN for 10 min. Gel pieces were shrunken and appeared opaque-white with a clear colourless supernatant. Then, ACN was removed and gel pieces were dried in a speed vac for 15 min. Gel pieces were reduced using 150 μ l of 10 mM dithiothreitol (DTT) in 100 mM ammonium bicarbonate, 5% ACN, for 1 h at 55 °C. Supernatant was removed and gel pieces were dehydrated in steps, using 100 μ l of 100 mM ammonium bicarbonate for 10 min and 100 μ l of 100% ACN for 20 min. For alkylation, 100 μ l of 50 mM iodoacetamide in 100 mM NH_4HCO_3 were added to the gel pieces. Tubes were incubated in the dark at room temperature for 30 min. Supernatant was removed and gel pieces were washed with 100 μ l of 100 mM NH_4HCO_3 for 10 min and 100 μ l of 100% ACN for 20 min. The gel pieces were dried in speed vac for 15 min and swollen in digestion buffer (50 mM NH_4HCO_3 with enzyme trypsin) on an ice for 45 min. The minimum volume (20–30 μ l for most gel pieces) of buffer necessary to swell the gel pieces was used and 5–25 μ l of 50 mM ammonium bicarbonate added to keep the pieces wet. Tubes were incubated at 37 °C for 16 h and then supernatant of the digestion solution was collected. The gel plugs were extracted 1 X with 100 μ l of 20 mM NH_4HCO_3 for 20 min and supernatant was collected. All the supernatants were pooled. Gel plugs were extracted 2 X with 100 μ l of 1% TFA in 50% ACN for 20 min. Gel plugs were extracted 1 X with 100 μ l of 100% ACN for 20 min. Combined supernatants were concentrated in a speed vac. 1 μ l of sample was mixed with 1 μ l of matrix solution and spotted on a MALDI target plate and dried. A saturated solution (~10 g/l) of matrix (HCCA) was prepared by adding one volume of acetonitrile and two volumes of 0.1% TFA to an aliquot of matrix dry powder, vortexing for 30 s and centrifuging to precipitate insoluble material. Supernatant was used in subsequent steps. The MALDI probe was inserted into the mass spectrometer (Bruker Ultraflex) and a mass spectrum

was acquired using flex control 2.2 software and processed using flexanalysis 2.2 software. MS/MS of the required peaks were done and spectra were processed using flexanalysis 2.2 software and searched against the database.

2.7. Trypsin inhibitory activity

Trypsin inhibitory assays were performed by estimating the remaining hydrolytic activity of trypsin towards the substrate *N*-benzoyl-L-arginine *p*-nitroanilide (BAPNA). Trypsin was dissolved in 0.001 N HCl containing 20 mM CaCl_2 at a concentration of 200 μ g per ml. Then, 100 μ l of the enzyme solution were incubated with 200 μ l of the inhibitor solution in 50 mM Tris-HCl buffer, pH 7.5 at 30 °C for 10 min, and then 1 ml of 5 mM BAPNA solution in the same buffer containing 2.5% DMSO were added. After incubation at 30 °C a further 10 min, 200 μ l of 30% acetic acid were added to end the enzyme reaction, and the absorbance at 410 nm was measured on a spectrophotometer (Shibata, Hara, Ikenaka, & Abe, 1986).

3. Results

3.1. Total protein concentration

The percentage of total soluble protein concentration in mature *M. koenigii* seeds on a dry weight basis was determined to be 8%, as estimated by Bradford assay. The protein content of *M. koenigii* seeds was higher than that of guava seeds (7.2%) (Bernardino-Nicanor, Scilingo, Anon, & Davila-Ortiz, 2006) and lower than that of legume seeds (19–44%) (Lampart-Szczapa, 2001).

3.2. Relative protein concentration and trypsin inhibitory activity during seed development and germination

The protein physiology during seed development and germination, as monitored by SDS-PAGE method, was similar to that of other storage proteins. The relative protein concentrations of total soluble protein extracted by simple buffer extraction from *M. koenigii* seeds, during different stages of seed development and germination periods, were obtained from variable band intensities on SDS-PAGE gels under reducing conditions, using Quantity One 1D gel analysis software (version 4.5.2; Biorad). The electrophoretic patterns of different stages of seed development and germination showed the most intense protein band at 27 kDa which expressed rapidly during the mid-maturation stage of seed development (Fig. 1a) and was utilized during seed germination (Fig. 1b). At initial stages of seed development, the protein content of this band was estimated to be 5.27% of total soluble protein after seven days of flowering. After 19 days, during the mid-maturation stage, the protein concentration increased from 5.5% to 8.5% within 25 days and then peaked to 18.8% in 37 days. The final protein concentration of this band was estimated to be 20% of total soluble protein which was stored

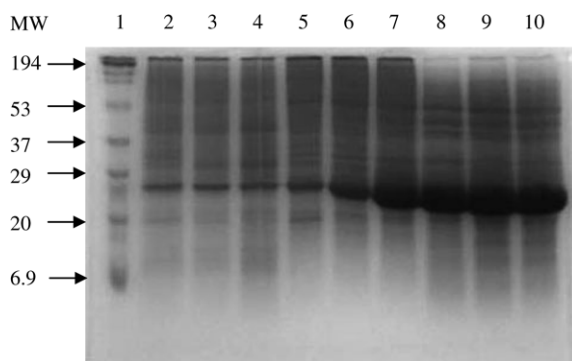


Fig. 1a. Protein profiling of *Murraya koenigii* seeds, at different stages of seed development after flowering, on a 15% SDS-PAGE; Lane 1, molecular weight marker; Lane 2, 7 days; Lane 3, 13 days; Lane 4, 19 days; Lane 5, 25 days; Lane 6, 31 days; Lane 7, 37 days; Lane 8, 43 days; Lane 9, 49 days; Lane 10, 55 days.

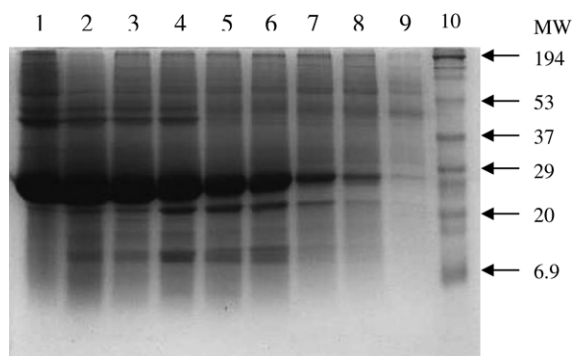


Fig. 1b. Protein profiling of *Murraya koenigii* seeds at different stages of seed germination on a 15% SDS-PAGE; Lane 1, mature seeds; Lane 2, 1 day; Lane 3, 4 days; Lane 4, 7 days; Lane 5, 10 days; Lane 6, 13 days; Lane 7, 16 days; Lane 8, 19 days; Lane 9, 22 days; Lane 10, molecular weight marker.

until germination. During the time of germination, this major protein degraded slowly from 20% to 12% in 13 days but, within the next three days, this protein decreased to 7% and then to 2% within the next six days (Fig. 2a).

Trypsin inhibitory activity, in soluble protein extract of seeds, was monitored during different stages of seed development and germination. During seed developmental

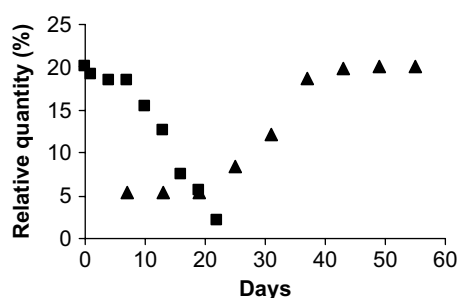


Fig. 2a. Relative quantity of MKTI protein band of 27 kDa from 15% SDS-PAGE at different stages of seed development and germination. (■) percent quantity at seed germination stage; (▲) percent quantity at seed developmental stage.

stages, the residual trypsin activity decreased approximately from 94% to 92.4, 89.2, 78.4, 59.2, 14.5, 8.8, 7.4 and 6.8% against soluble extracts of seeds collected at 7, 13, 19, 25, 31, 37, 43, 49 and 55 days, respectively. A sharp reduction in percentage of residual trypsin activity was observed during the mid-maturation stage. During the different stages of the germination process, it was observed that the residual trypsin activity increased gradually up to 13 days from the day of seeding and then a sharp increase in residual trypsin activity was observed. The residual trypsin activity increased approximately from 6.9% to 7.3, 12.5, 20.8, 35.6, 75, 89.1, 94.5% against the soluble extract of seeds obtained after seeding at 1, 4, 7, 10, 13, 16, 19 and 22 days, respectively (Fig. 2b).

3.3. Single-step purification of MKTI

The purification of protein was carried out in one step only by affinity chromatography, using Cibacron blue 3 GA. Homogeneous supernatant, collected after centrifugation of crude extract at 12,000g, was loaded onto a Cibacron blue 3 GA-packed column equilibrated with 20 mM Tris-HCl, pH 8.0. Many proteins, particularly high molecular mass proteins, and certain loosely bound proteins, were removed by extensive washing with the same buffer. After extensive washing, the bound proteins were eluted with 0.2 and 0.5 M NaCl in the same buffer. In the 0.2 M NaCl fraction, some MKTI and other low molecular mass proteins were eluted. The pure protein was eluted at 0.5 M NaCl concentration. The eluted sample was desalted and concentrated by using centriprep and centricon and the purity of the protein was confirmed by SDS-PAGE and MALDI-TOF analysis. SDS-PAGE analysis of MKTI, under both reducing and non-reducing conditions, showed that it was a single polypeptide chain. The molecular mass of the purified protein was determined to be approximately 27 kDa on 15% SDS-PAGE, as earlier reported for the protein purified by anion-exchange and gel filtration chromatography (Fig. 3a). However, MALDI-TOF analysis revealed the exact molecular weight of MKTI to be

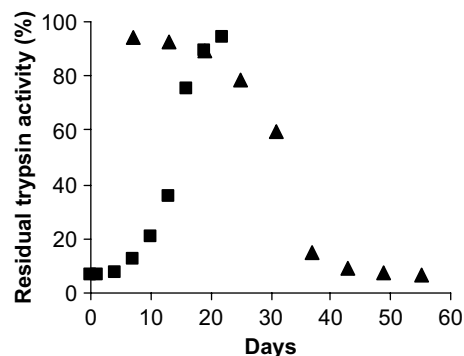


Fig. 2b. Residual trypsin activity against crude extract in *Murraya koenigii* seeds at different stages of seed development and germination; (■) seed germination; (▲) seed development. All experiments were done three times and averaged.

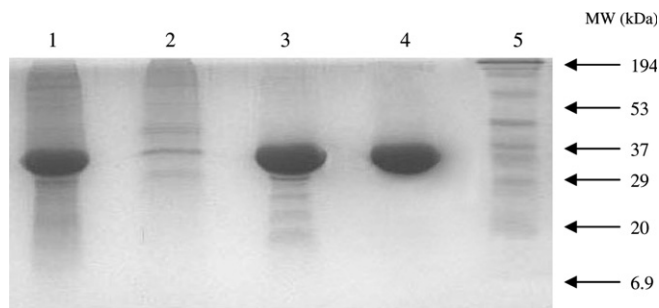


Fig. 3a. 15% SDS-PAGE analysis of MKTI purified by Cibacron blue 3GA; Lane 1, crude extract, Lane 2, flow through; Lane 3, 0.2 M NaCl fraction; Lane 4, 0.5 M NaCl fraction; Lane 5, molecular weight marker.

21.4 kDa (Fig. 3b). The inhibitory activity of purified protein against trypsin was determined by measuring the hydrolytic activity toward BAPNA (Fig. 3c). The trypsin inhibitory activity of the protein purified by affinity column was similar to the earlier reported protein purified using anion-exchange and gel filtration chromatography. However, the yield of the pure protein increased almost three-fold as compared to the earlier purification method.

3.4. Sequence analysis by MALDI-TOF-TOF

MALDI-TOF-TOF studies provided many new insights into the nature of MKTI. In total, six peptides of various lengths were obtained after performing the experiment twice. In the first attempt, three peptides were sequenced. In the second attempt, five peptides were sequenced, of which two were repeated. The peptides were randomly called peptides 1 to 6 and contained 19, 22, 22, 15, 9 and 11 amino acids, respectively. The homology search, under NCBI BLAST short sequence search for each fragment, separately showed significant homology to many serine protease inhibitors, storage proteins and homeodomain-like proteins. The peptide 1 showed significant homology

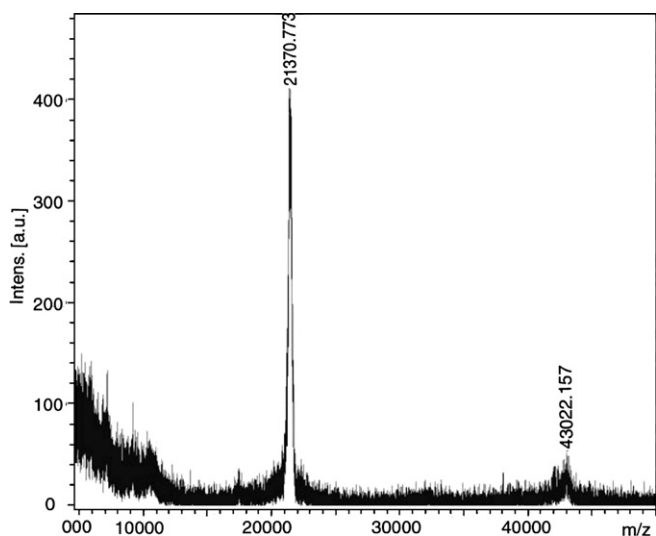


Fig. 3b. MALDI-TOF analysis, showing the molecular mass of MKTI to be 21.4 kDa.

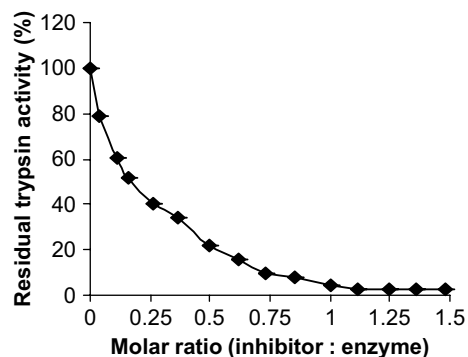


Fig. 3c. Trypsin inhibitory activity of MKTI, showing residual trypsin activity in percent as function of the inhibitor concentration at a fixed trypsin concentration, using BAPNA as substrate. The trypsin inhibition was observed at a molar ratio of 1:1.1, taking 27 kDa molecular mass. All experiments were done three times and averaged.

to a miraculin-like protein, a member of the Kunitz inhibitor family, isolated from various *Citrus* species, e.g. *Citrus shirauhi* (84% identity) and *Citrus jambhiri* (68% identity). Also, the sequence of this fragment showed significant homology to vegetative storage protein isolated from *Litchi chinensis* with 68% identities. The sequence of peptide 2 also showed homology to the miraculin-like protein from different *Citrus* species, e.g. *C. shirauhi* (100% identity), *C. jambhiri* (77% identity) and vegetative storage protein from *L. chinensis* (72% identity) and also additionally with many trypsin inhibitors, e.g. the Kunitz trypsin inhibitor 4 from *Populus balsamifera* subsp. *trichocarpa* x *Populus deltoids* (77% identity). Peptide 3 showed maximum homology to the trypsin inhibitors of many *Arabidopsis* species, e.g. *Arabidopsis lyrata* subsp. *Petraea* (100% identity) and *Arabidopsis thaliana* with 86% identity. It also showed significant homology, with 81% identity, to trypsin inhibitor from *Brassica napus*. Peptide 4 showed homology to protease inhibitor of the cereal super family isolated from various cereals, e.g. *Oryza sativa* (100% identity), *Triticum aestivum* (73% identity) and *Hordeum vulgare* (73% identity). The peptide 5 showed homology mostly to the squash family of inhibitors, with almost 100% identity to *Lagenaria leucantha*, *Lagenaria siceraria*, *Luffa acutangula*, *Citrus lanatus*, with the reactive site RI of the squash family inhibitor present in this stretch. Also, the two cysteine residues of peptide 5 were almost conserved in many members of the Kunitz inhibitor family. Interestingly, peptide 6 did not show significant homology to any of the trypsin inhibitors; rather it showed significant homology to homeodomain-like proteins from *Oryza sativa* (100% identity), *Medicago truncatula* (90% identity), *Lycopersicon esculentum* (90% identity) and *Solanum tuberosum* (90% identity) and many other DNA-binding proteins (Fig. 4a).

The six peptides, along with the N-terminal sequence obtained earlier, were assigned the relative positions in the MKTI sequence based on homology to the Kunitz family inhibitors with reference to conserved residues (Fig. 4b). Peptides 1 to 6 were placed at amino acid numbers (STI numbering) 34, 105 (first two residues WF present in pep-

Protein	Sequence	Identity (%)
A) P1	1 GRNELCPLDVVQLSSDLQK 19	
CSM	61 GRNELCPLDVVQLSSDSER 79	84%
LCSP	62 GRNERCPMDVFQHRSDLQR 80	68%
CJM	66 GRNGYCPLDVIQLPSDTQN 84	68%
B) P2	1 WFITTTGGVEGNPGAQTLKNWFK 22	
CSM	128 WFITTTGGVEGNPGAQTLKNWFK 149	100%
CJM	137 WFITTTGGLDGHPGAETLLNWFK 158	77%
PBTI	128 WFTTTGGEEGNPGIDTLTNWFK 149	77%
LCSP	135 WFITTTNGVIGNPGSKTIHNWFK 156	72%
C) P3	1 SVSTLAVSVILFLVIFEMPEIK 22	
ALTI	5 SVSTLAVSVILFLVIFEMPEIK 26	100%
ATTI	5 SVSTLAVFALFLVIVEMPEIK 26	86%
BNTI	7 SVSSLAAFLILFLVIFEMPEIE 27	81%
D) P4	1 TEWPELVGLTIEQAK 15	
HVCI	23 TEWPELVGKSVVEAK 37	73%
TAPI	23 TEWPELVGKSVVEAK 37	73%
OSSCI	14 TEWPELVGLTIEQAK 28	100%
E) P5	1 CPRIYMECK 9	
LLTI	3 CPRIYMECK 11	100%
LATI	3 CPRIYMECK 11	100%
LSTI	4 CPRIYMECK 12	100%
CLTI	4 CPRIYMECK 12	100%
F) P6	1 GLPESAVSVLR 11	
OSH	622 GLPESAVSVLR 632	100%
MTH	311 GLPERAVSVLR 321	90%
LEH	410 GLPERAVSVLR 420	90%
STH	397 GLPESSVSVLR 407	90%

Fig. 4a. Sequence homology studies of six peptides by NCBI BLAST short sequence search. CSM, *Citrus shirauhi* miraculin; CJM, *Citrus jambhiri* miraculin; LCSP, *Litchi chinensis* storage protein; PBTI, *Populus balsamifera* trypsin inhibitor; ALTI, *Arabidopsis lyrata* trypsin inhibitor; ATTI, *Arabidopsis thaliana* trypsin inhibitor; BNTI, *Brassica napus* trypsin inhibitor; HVCI, *Hordeum vulgare* chymotrypsin inhibitor; TAPI, *Triticum aestivum* proteinase inhibitor; OSSCI, *Oryza sativa* subtilisin-chymotrypsin inhibitor; LLTI, *Lagenaria leucantha* trypsin isoinhibitor; LATI, *Luffa acutangula* trypsin inhibitor; LSTI, *Lagenaria siceraria* trypsin inhibitor; CLTI, *Citrullus lanatus* trypsin inhibitor; OSH, *Oryza sativa* homeodomain; MTH, *Medicago truncatula* Homeodomain; LEH, *Lycopersicon esculentum* homeodomain; STH, *Solanum tuberosum* homeotic.

ptide 2 were absent in STI), 158, 91, 136 and 53, respectively. The sequence stretch CPL in peptide 1 was found conserved in most Kunitz family inhibitors while stretch VVQ showed significant homology. In peptide 2, sequence stretches WFITTTGG and WFK were completely identical to the miraculin-like protein CJI, a member of the Kunitz family inhibitors but did not show any resemblance to other members of the Kunitz family. In peptides 3 and 4, stretches FLVIEF and TEW showed homology to many Kunitz family inhibitors. In peptide 5, two cysteine residues were found conserved in almost all Kunitz inhibitors. The sequence stretch GLP of peptide 6 showed homology to other Kunitz-family inhibitors with Gly in GLP completely conserved. The sequence identity of MKTI, for 113 residues obtained, with STI, ETI and CJI, was found to be 28.3, 24.78, and 39.89%, respectively.

4. Discussion

The results presented in this study clearly indicate that MKTI possesses properties other than trypsin inhibitory activity. These are storage and affinity properties. The seed

storage proteins play an important role in providing nutrition during seed germination. Our studies showed that this protein possesses certain characteristics which distinguish seed storage proteins, e.g. accumulation in high amounts during seed maturation and degradation during seed germination. Many trypsin inhibitors have been earlier reported to be seed storage proteins (Yeh, Chen, Lin, Chen, & Lin, 1997; Mandal et al., 2002). Single step purification of MKTI on Cibacron blue 3GA resin demonstrated the affinity properties mostly exhibited by DNA-binding proteins. There are only a few reports of trypsin inhibitors which have been purified on an affinity column (Potempa, Kwon, Chawla, & Travis, 1989; Kuehn, Rutschmann, Dahlmann, & Reinauer, 1984). The partial internal sequencing further supported the results of storage and affinity properties of MKTI.

The protein physiology during seed development and germination, as monitored by SDS-PAGE method, was similar to that of any other storage protein. The protein expression and degradation patterns, taken at different stages of development and germination, clearly showed that the MKTI band at 27 kDa was the major protein in

PTI	MKST---	LLAWFTFLLLFAFVLSVPSIEASTEPEVLDIQGEEELKAGTEYIISSIFWGAGGGD	57
TCI	MKTATAVVLFFF	FAFTSKSYFFGVAN--AANSFVLDTDGDELQTVQYVVLSSISGAGGGG	58
CJI	MKISLATTL	SFLILALASNSLLVLGTSSVPEPLLDVNGNKVESTLQYYIVSAIWGAGGG	60
ETI	-----	-----VLLDNGEVVQN-GGTYLLPQVWAQGGG	28
WTI	-----	-----EPLLDSEGEIVRN-GGTYLLPDRWALGGG	29
STI	-----	-----DFVLDNEGNPLEN-GGTYIILSDITAFGG-	28
BVI	-----	-----DTLLDGDGEVVRNNGGPYYIIPAFRGNNGG	30
ATI	---MKT	TTKTMNPKFYLVLALAVLASNAYGAVVDIDGN-AMFHE-SYYVLPVIRGRGG	55
OSI	-----	MVSLRPLILLSLLAISFSCSAAPPVYDTEGH-ELSDGSYYVLPASPGHGG	53
MTI	--MKH	FLSLTSLFFIFVFITNLSLATSNDVEQVLDINGNPIFPGGQYYILPALRGPGGG	58
MKTI	-----	-----IDPLLDINGNVVEAAXXXXXXXXXXXXXXXXXX	31
PTI	VA---	ATNKTCPDDVIQYSLDLLQGLPVTFSPASSEDVIRVSTDLNIKFS-----	IKKA 109
TCI	LALGRAT	QSCPEIVVQRSDLDNGTPVIFSNADSKDDVVRVSTDVNIEFVPI---	RDRL 115
CJI	VSLHGG	RNGYCPDVDIQLPSDTQNGIKLTLSPYNN-STIVRESADLNLRFVSL--	SGRD 117
ETI	VQLAKT	GEETCPLTVVQSPNELSDGKPIRIESRLR-SAFIPDDDKVRIG-----	FAYAP 81
WTI	IEAAAT	GTETCPLTVVRSNPEVSVGEPLRISSQLR-SGFIPDYSLVRIG-----	FANPP 82
STI	IRAAPT	GNERCPLTVVQSRNELDKGIGTIISSPYR-IRFIAEGHPLSLKFDS-FAVIMLC	86
BVI	LTLTRV	GSETCPRTVVQASSEHSDGLPVVISALPR-SLFISTSWRVTIQ-----	FVEAT 83
ATI	LTLAGR	GQPCPYDIVQESSEVDEGIPVKFSNWRLLKVAFVPESQLNLIETDV-----	GAT 110
OSI	LTMAPR	-VLPCLLVAQETDERRKGFVRFPTPWGAAAPEDRTIRVSTDVIRFNAATII	112
MTI	VRLGR	TGDLKCPVTVLQDRREVKNGLPVKFTIPGISPGIIFTGTPLEIEYTK-----	KPS 113
MKTI	XXXXX	GRNELCPLDVVQLSSDLQKGLPESAVSVLRXXXXXXXXXXXXXXXXXXXXXXXXX	91
PTI	CDRSSV	WKIQKSSNSEVQ-WLVTTGGEEGNPGCDFTFNWFKIEKAGVLG---	YKLVYCP 165
TCI	CSTSTV	WR-LDNYDNSAGKWWVTTDGVKGEPPNLTCSWFKIEKAGVLG---	YKFRFCPS 171
CJI	YCNEQ	PLWKVDNYDAASGKWFITTTGGLDGHGPAETLLNWFKLEKIGNFPGT-YKIVHCPS	176
ETI	KCAPSP	WWTVVVEDEQEGLS----VKLSEDES-TQFDYPPKFEQVS-DQLHS-YKLLYCEG	134
WTI	KCAPSP	WWTVVVEDEQPPQS----VKLSELKSTKFD-YLFKFEKVT-SKFSS-YKLYKA-	134
STI	VGIPTE	WSVVEDLPEGPA---VKIGENKDAMDG----WFRLERVSDDFNNYKLVFCPQ	138
BVI	-CIPK	PSFWHIPQDSELEGA---VKVGAS-DERFP-LEFRIERSV---EDT-YKLMHCSS	133
ATI	CIQSTY	VRVG--EFDHERRQYFVVAGPKPEGFGQDSLKSPFKIEKSGEDA--YKFVFCPR	167
OSI	CVQSTE	WHVGDDEPLTGARR---VVTGP-LIGPSPSGRENAPRVEKYG-GG--YKLVSCR-	163
MTI	CAASTK	WLIFVDNVIGKACIGIGGPEENYPGVQTLKGFKNIQKHASGFG---YNLGFVCT	169
MKTI	XXXXT	EWPELVGLTIEQAKWFITTTGGVEGNPGAQTLKNWFXXXXXXXXXXXXXXXXXXCPR	151
PTI	DICPSV	GLCRDVGIFYESNRGRILSLDKLSPFLVVFVKVGPLSSSI-----	212
TCI	VCDSCT	TLCSDIGRHSDDDGQIRLALSD--NEWAWMFKKASKTIKQVNAKH	221
CJI	VCESCV	KLCNNVGRSFDGVRRLVLRDEPAFPVVLIPATERSTSV-----	223
ETI	KHEKCA	S-----IGINRDQK-GYRRLVVTEDYPLTVVLKDESS-----	172
WTI	KRDTCK	D----IGIYRDQK-GYARLVVTDENPLVVIKKEVSS-----	172
STI	QAEDDK	CG--DIGISIDHDDGTRRLVVSKNKPLVVQFQKLDKESL-----	181
BVI	TSDSCR	D---LGISIDEE-GNRRLVVRDENPLVRFKKANQDSEK-----	174
ATI	TCDSGN	PK--CSDVGFIDELGVRRLALSDKPLVLMFKKANVTEVSSKTM--	215
OSI	--DS-	-----CQDLGVSRDGARA-WLGASQPPHVVFVKKARSPPE-----	200
MTI	GSPTCL	DIG-----RFDNDEAGRRLNLTHEVEYQVVFVDAATYEAEYIKSVV	216
MKTI	IYMECK	XXXXXXXXXXXXXXXXXSVSTLAVSVILFLVIFEMPEIKXX-----	195

Fig. 4b. Multiple sequence alignment of Kunitz family inhibitors with stretches of MKTI sequences based on homology with reference to conserved residues. All other residues in MKTI were taken as alanine. ETI, *Erythrina* trypsin inhibitor; STI, Soybean trypsin inhibitor; CJI, *Citrus jambhiri* inhibitor; ATI, *Arabidopsis thaliana* inhibitor; OSI, *Oryza sativa* inhibitor; WTI, Winged bean inhibitor; MTI, *Medicago truncatula* inhibitor; PTI, *Populus tremula* inhibitor; TCI, *Theobroma cacao* inhibitor; BVI, *Bauhinia variegata* inhibitor; MKTI, *Murraya koenigii* trypsin inhibitor.

the seed. The expression of this protein started slowly and then peaked at the mid-maturation stage to approximately 20% of total soluble protein. During seed germination, the protein degraded gradually in first thirteen days from around 20% to 12% and then degraded rapidly to 7% in the next three days and finally to 2% in the next six days. The highest protein expression at the mid-maturation stage and degradation during germination correlating with similar trypsin inhibitory activity patterns demonstrate the storage property of MKTI.

MKTI was purified in a single step on an affinity column, Cibacron blue 3GA resin, which improved the yield almost three-fold. It is interesting to note that only this protein bound to the column with high affinity. It is well known that many proteins with DNA-binding properties have been purified on this column (Emlen & Burdick, 1983). Interestingly, one of the peptides, peptide 6, showed a strong resemblance to homeodomain-like proteins, which are DNA-binding proteins, but not to the trypsin inhibitors. This may be one possible explanation for the high

affinity of the protein for this column. Most Kunitz-family inhibitors do not exhibit this property.

MALDI and MALDI-TOF-TOF studies provided many new insights into the nature of MKTI and also supported our findings. MALDI-TOF analysis revealed the molecular weight to be 21.4 kDa as opposed to 27 kDa obtained by SDS-PAGE analysis. This clearly indicates that the protein runs higher in SDS-PAGE for unknown reasons. This observation has also been made in the case of a miraculin-like protein, a Kunitz-family member, where SDS-PAGE analysis showed molecular mass to be 28 kDa while amino acid analysis showed it to be 24 kDa (Theerasilp et al., 1989). The new molecular mass of MKTI is typical of the Kunitz family inhibitors. In partial internal sequencing, 6 peptides, totalling 98 residues, were obtained. Including N-terminal 15 residues, a total of 113 residues was obtained. Sequence comparison of partial MKTI sequence with other Kunitz family inhibitors showed certain residues or motifs conserved despite a lot of variations in the rest of the sequence (Fig. 4b). There were only 8 out of 113 residues found completely conserved in the MKTI sequence. Apart from the Kunitz family inhibitors, individual peptides also showed high degree of homology to other proteins. Peptides 1 and 2 showed high degree of homology to seed storage proteins while peptide 6 showed resemblance to homeodomain-like proteins and some other DNA-binding proteins. Though no definite conclusions can be drawn, these results are indicative of seed storage function and affinity binding of MKTI. Peptides 3, 4 and 5 showed homology to trypsin inhibitors from *Arabidopsis* sp. and *B. napus*, cereal superfamily and squash family inhibitors, respectively. Another interesting feature was that peptide 2 showed homology only to Kunitz inhibitors from miraculin-like proteins and not to any other typical Kunitz inhibitors. It is interesting to note that MKTI showed maximum sequence homology to CJI (39.89% identity) rather than STI or ETI (28.31 and 24.78% identity) which implies that it may be close to a miraculin-like protein. The fact that both have been isolated from the Rutaceae family supports this observation.

The sequencing data, along with experimental results, clearly demonstrate that MKTI has additional properties, as opposed to the typical trypsin inhibitor of the Kunitz family. Also, the high degrees of sequence homology of individual peptides to storage and DNA-binding proteins support our results of storage and affinity properties of MKTI.

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References

- Bernardino-Nicanor, A., Scilingo, A. A., Anon, M. C., & Davila-Ortiz, G. (2006). Guava seed storage protein: Fractionation and characterization. *LWT-Food Science and Technology*, *39*, 902–910.
- Bradford, M. (1976). A rapid and sensitive method for the quantification of microgram quantities of proteins utilizing the principle of protein dye binding. *Analytical Biochemistry*, *72*, 248–254.
- Derbyshire, E., Wright, D., & Boulter, D. (1976). Legumin and vicilin storage proteins in legume seeds. *Phytochemistry*, *15*, 3–24.
- Emlen, W., & Burdick, G. (1983). Purification of DNA antibodies using cibacron blue F3GA affinity chromatography. *Journal of Immunological Methods*, *62*, 205–215.
- Fukushima, D. (1991). Recent progress of soybean protein foods: Chemistry, technology, and nutrition. *Food Reviews International*, *7*, 323–351.
- Kuehn, L., Rutschmann, M., Dahlmann, B., & Reinauer, H. (1984). Proteinase inhibitors in rat serum: Purification and partial characterization of three functionally distinct trypsin inhibitors. *Biochemical Journal*, *218*, 953–959.
- Laemli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, *227*, 680–685.
- Lampart-Szczapa, E. (2001). Legume and oilseed proteins. In Z. E. Sikorski (Ed.), *Chemical and functional properties of food proteins* (pp. 407–434). New York: CRC Press.
- Laskowski, M., Jr., & Kato, I. (1980). Protease inhibitors of proteinases. *Annual Review of Biochemistry*, *49*, 593–626.
- Mandal, S., Kundu, P., Roy, B., & Mandal, R. K. (2002). Precursor of the inactive 2S seed storage protein from the Indian Mustard *Brassica juncea* is a novel trypsin inhibitor. *Journal of Biological Chemistry*, *277*, 37161–37168.
- Mandal, S., & Mandal, R. K. (2000). Seed storage proteins and approaches for improvement of their nutritional quality by genetic engineering. *Current Science*, *79*, 576–589.
- Miller, A. (1975). Biochemistry of legume seed proteins. *Annual Review of Plant Physiology*, *26*, 53–72.
- Mosolov, V. V., Grigoreva, L. I., & Valueva, T. A. (2001). Plant proteinase inhibitors as multifunctional proteins. *Applied Biochemistry and Microbiology (Moscow)*, *37*, 643–650.
- Potempa, J., Kwon, K., Chawla, R., & Travis, J. (1989). Inter- α -trypsin inhibitor: Inhibition spectrum of native and derived forms. *Journal of Biological Chemistry*, *264*, 15109–15114.
- Pusztai, A. (1972). Metabolism of trypsin-inhibitory proteins in the germinating seeds of kidney bean (*Phaseolus vulgaris*). *Planta*, *107*, 121–129.
- Ramakrishna, V., & Ramakrishna, R. P. (2005). Purification of acidic protease from the cotyledons of germinating Indian bean (*Dolichos lablab* L.var lignosus) seeds. *African Journal of biotechnology*, *4*, 703–707.
- Shee, C., & Sharma, A. K. (2007). Purification and characterization of a trypsin inhibitor from seeds of *Murraya koenigii*. *Journal Enzyme Inhibition and Medicinal Chemistry*, *22*, 115–120.
- Shee, C., Singh, T. P., Kumar, P., & Sharma, A. K. (2007). Crystallization and preliminary X-ray diffraction studies of *Murraya koenigii* trypsin inhibitor. *Acta Crystallograph Sect F Struct Biol Cryst Commun*, *63*, 318–319.
- Shibata, H., Hara, S., Ikenaka, T., & Abe, J. (1986). Purification and characterization of proteinase inhibitors from winged bean (*Psophocarpus tetragonolobus* (L.) DC.) seeds. *Journal of Biochemistry*, *99*, 1147–1155.
- Theerasilp, S., Hitotsuya, H., Nakajo, S., Nakaya, K., Nakamura, Y., & Kurihara, Y. (1989). Complete amino acid sequence and structure characterization of the taste-modifying protein, miraculin. *Journal of Biological Chemistry*, *264*, 6655–6659.
- Yeh, K. W., Chen, J. C., Lin, M. I., Chen, Y. M., & Lin, C. Y. (1997). Functional activity of sporamin from sweet potato (*Ipomoea batatas* Lam.): a tuber storage protein with trypsin inhibitory activity. *Plant Molecular Biology*, *33*, 565–570.