PURIFICATION AND CHARACTERIZATION OF A TRYPSIN INHIBITOR FROM *MURRAYA KOENIGII*

A THESIS

Submitted in partial fulfilment of the requirements for the award of the degree

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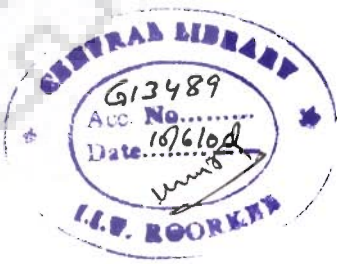
in

BIOTECHNOLOGY

by

CHANDAN SHEE



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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled, **PURIFICATION AND CHARACTERIZATION OF A TRYPSIN INHIBITOR FROM *MURRAYA KOENIGII*** in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during a period from July 2003 to May 2007 under the supervision of Dr. Ashwani Kumar Sharma, Assistant Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other Institute.

Chandan Shee

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This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

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The Ph.D. Viva-Voce Examination of Chandan Shee, Research Scholar has been held on .....

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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 on DEAE sepharose column 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. However, MALDI-TOF analysis revealed the exact molecular mass of protein to be 21.4 kDa. This protein showed high affinity towards Cibacron blue 3GA and therefore, was purified in single step by affinity column using Cibacron blue 3GA with substantial increase in yield. The inhibitory activity of purified protein at different concentration against a fixed trypsin concentration was determined by measuring the hydrolytic activity towards synthetic substrate N-benzoyl-L-arginine ethyl ester (BAEE) and N-benzoyl-L-arginine p-nitronilide (BAPNA) and by natural substrate BSA. The purified protein inhibited bovine pancreatic trypsin completely at a molar ratio of 1:1.1. The trypsin inhibitory activity was also determined at different pH buffers by using BAEE as substrate and the maximum inhibition was observed at pH 8.0. 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 and the mode of inhibition was determined to be a competitive inhibitor. The inhibitory assay of purified protein against chymotrypsin was also performed by measuring the hydrolytic activity towards synthetic substrate N-benzoyl-L-tyrosine ethyl ester (BTEE) and natural substrate BSA. Although, the protein showed proteolytically resistant against chymotrypsin but it did not show any chymotrypsin inhibitory activity.

Murraya koenigii trypsin inhibitor (MKTI) was found to be the most abundant protein in the mature seeds of *Murraya koenigii* plant. The quantity of protein was determined to be

approximately 20% of total protein extracted by simple buffer extraction. The relative concentrations of the inhibitor, monitored during seed development and germination periods were obtained from variable band intensities on SDS-PAGE gels under reducing condition using Quantity One 1D gel analysis software (version 4.5.2; Biorad). The electrophoretic patterns of different stages of seed development and germination showed most intense protein band at 27 kDa which expressed rapidly during mid maturation stage of seed development and was utilized during seed germination. Trypsin inhibitory activity, in soluble protein extract of seeds, was monitored during different stages of seed development and germination. During seed developmental stages, the residual trypsin activity decreased approximately from 94% to 6.8% against soluble extract of seeds collected at 7 and 55 days respectively and during the different stages of germination process the residual trypsin activity increased approximately from 6.9 to 94.5% against the soluble extract of seeds obtained after seeding at 1 and 22 days respectively. Considering the highest protein expression at mid-maturation stage and degradation during germination correlating with similar trypsin inhibitory activity patterns demonstrate the storage property of MKTI.

Amino acid sequence analysis was performed by Edman degradation and MALDI-TOF-TOF studies. The N-terminal amino acid sequence analysis was performed by Edman degradation and the sequence of first 15 amino acids (Ile-Asp-Pro-Leu-Leu-Asp-Ile-Asn-Gly-Asn-Val-Val-Glu-Ala-Ala), in short sequence search, showed significant homology to a Kunitz-type chymotrypsin inhibitor from *Erythrina variegata*. The partial internal amino acid sequencing of MKTI was also performed by MALDI-TOF-TOF studies after tryptic digestion and alkyl reduction. In partial internal sequencing by MALDI-TOF-TOF, six peptides of varying length totaling 98 amino acid residues were obtained. These peptides exhibited

similarities to the sequences from proteinase inhibitors, storage proteins and homeodomain like proteins. The maximum sequence homology of MKTI was found to be with miraculin like protein from *Citrus jambhiri*, a member of Kunitz family.

Solubility studies of *Murraya koenigii* trypsin inhibitor (MKTI) were carried out under different physicochemical conditions including temperature, pH, salts, detergents and organic solvents. For solubility studies, the protein (1 mg/ml) was incubated at different physicochemical conditions for different time periods and then centrifuged and supernatant and precipitate were collected separately for determination of protein concentration and inhibitory activity. The thermal stability studies showed that protein gradually precipitates irreversibly after heating above 50°C and around 50% of protein is precipitated on heating at 100°C for 30 minutes. Addition of salt further lowered the solubility of protein at higher temperatures. 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 the protein concentration of 1 mg/ml. The solubility of protein decreases below pH 7.5 to around 15% at pH 4.0. The presence of increasing amounts of salts (both divalent and monovalent) helped in resolubilization of protein at lower pH. Only, $(NH_4)_2SO_4$ was showing the salting out effect above 2 M concentration in pH 8.0. The inhibitory activity of precipitated protein at lower pH and high molar $(NH_4)_2SO_4$ was completely regained after resolubilization of protein in buffer of pH 8.0. Except ethylene glycol, all other organic solvents like ethanol, methanol, acetonitrile, dioxane and MPD precipitated MKTI at different percentages. Interestingly, the inhibitory activity of precipitated protein was almost completely regained after resolubilization in buffer of pH 8.0.

The thermal stability of this protein under *in-vitro and in-vivo* condition was investigated by incubating the purified protein and mature intact seeds respectively in different temperatures

ranging from 30 to 100°C. The inhibitory activity of purified MKTI decreased continuously with increasing temperature by 15% at 40°C to 95% at 90°C and sharp decline in inhibitory activity was observed above 50°C . However, the inhibitory activity of MKTI was not affected under *in-vivo* conditions at high temperature, even after heating to 100°C for 30 minutes.

The structural stability of MKTI was examined by proteolysis studies with five proteases, trypsin, papain, proteinase K, chymotrypsin and pepsin. Native MKTI was found to be completely resistant to proteolysis for all times tested from 30 minutes to 24 hours at protease/protein molar ratio of 1:50. Bovine Serum Albumin, used as positive control, was quite susceptible to proteolysis against all five proteases at 1:100 molar ratios. The thermal stability of MKTI towards proteolytic degradation was also explored by incubating the MKTI at range of temperatures from 30 to 90°C for 30 minutes prior to digestion with proteases. The results demonstrated that the heat treated MKTI at all temperatures was completely resistant to proteolytic degradation by trypsin, chymotrypsin, pepsin and papain but was found susceptible to proteolysis by proteinase K when heat treated at 90°C.

Circular dichroism studies, under native conditions, showed MKTI contains approximately 30.1% α-helix, 46% β-strand, 16.2% turn and 6.9% random-coil structure. There was remarkable change in the conformation of MKTI at higher temperatures. A gradual loss in α-helical content of MKTI was observed with increasing temperatures while β-strand structure almost remained unchanged. The loss of inhibitory activity and α-helical content of the inhibitor with rising temperatures may be correlated suggesting a possible role for α-helical structure in inhibitory function of the protein. These results suggest a compact structure for this inhibitor with stable β-sheet structure forming the core while α-helical structure is present on surface.

ANS fluorescence experiments were performed in different physicochemical conditions to monitor the extent of changes in native structure of MKTI and relate them to the inhibitory activity. The ANS fluorescence study showed a linear increase in fluorescence intensity with increase in temperature above 50°C was observed without showing any melting transition up to 90°C. In different pH conditions ranging from pH 2-12, the fluorescence intensity of ANS at 525 nm was found to be highest at pH 2.0 with sharp decrease from pH 3.0 – 5.0 and then remains almost constant till pH 12.0. In presence of different concentration of NaCl at pH 2.0, the fluorescence intensity decreased till 1.5 M and then remained stable up to 2.5 M NaCl concentration. However, further increase in salt concentration above 2.5 M resulted in an increase in fluorescence intensity. The addition of salts, therefore, solubilizes and stabilizes the MKTI till a particular concentration only. A minor increase in fluorescence intensity was observed with increasing concentration of urea (0 to 9 M) and guanidine hydrochloride (0 to 8 M), suggesting that overall MKTI structure remains intact with only a very slight relaxation.

Murraya koenigii has been crystallized by sitting-drop vapor diffusion method using PEG 8000 as precipitating agent. The crystals belong to the tetragonal space group $P4_32_12$, with unit-cell parameters a = b = 75.8, c = 150.9 Å. The crystals contain two molecules in asymmetric unit with a V_M value of 2.5 $Å^3$ Da^{-1}. Diffraction was observed to 2.65 Å resolution and a complete data set was collected to 2.9 Å resolution. The structure was solved by molecular replacement method using the structure of *Erythrina caffra* Kunitz type trypsin inhibitor as a search model. The model fitted well in electron density and was refined to R factor of 41.7% with overall correlation coefficient of 49.2%.

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CONTENTS

LIST OF PUBLICATIONS

1. **Chandan Shee**, Ashwani K. Sharma. Purification and characterization of a trypsin inhibitor from seeds of *Murraya koenigii. J Enzym Inhib Med Chem,* 2007, 22(1): 115-120.

2. **Chandan Shee**, Tej P. Singh, Pravindra Kumar, Ashwani K. Sharma. Crystallization and preliminary X-ray crystallographic study of *Murraya koenigii* trypsin inhibitor. *Acta Crystallograph Sect F Struct Biol Cryst Commun.,* 2007, F63: 318-319.

3. **Chandan Shee**, Ashwani K. Sharma. Storage and affinity properties of *Murraya koenigii* trypsin inhibitor. Communicated

4. **Chandan Shee**, Asimul Islam, Faizan Ahmad, Ashwani K. Sharma. Structure-function studies of *Murraya koenigii* trypsin inhibitor revealed a stable core beta sheet structure surrounded by α-helices with a possible role for α-helix in inhibitory function. Communicated.

5. **Chandan Shee**, Shilpy Kochhar, Sneha Chatterjee, Ashwani K. Sharma. Solubility and stability studies on *Murraya koenigii* trypsin inhibitor under different physicochemical conditions. Communicated.

INTRODUCTION

INTRODUCTION

Understanding the structure-function relationship of proteins greatly contribute to many practical applications in medicine and agriculture. Formation of specific complexes between antigen and antibody, hormone and receptor, or enzyme and inhibitor are classic examples of highly complementary and specific interactions vital to living organisms. These interactions are completely dependent on their structural and functional stability in different physicochemical conditions. The proteinase-inhibitor interaction is one of the most important targets to study the specificity of protein-protein interaction and molecular recognition. The proteinase inhibitors play an important role in regulating many physiological processes by inhibiting proteinases, such as inflammation, coagulation, fibrinolysis, intracellular protein breakdown, cell cycle, transcription and apoptosis. They also act as anti-cancer and anti-HIV agents. Their role as natural plant defense agents against pathogens and pests are well established.

Serine proteinase inhibitors are widely distributed in nature and have been isolated from many sources including animals, plants and microorganisms. Plant seeds are rich source of inhibitors. Many plant serine proteinase inhibitors have been purified and characterized particularly from the seeds of *Leguminosae*, *Cucurbitaceae*, *Solanaceae* and *Gramineae* 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, and cereal superfamily (Laskowski and Kato, 1980).

The Kunitz type inhibitors of serine proteinases are divided into two subfamilies; the Kunitz bovine pancreatic trypsin inhibitor (BPTI), with molecular mass of about 6.5 kDa and three disulfide bridges and the Kunitz soybean trypsin inhibitor (STI), with molecular mass of

about 20 kDa containing two disulfide bridges (Wlodawer et al., 1987). The crystallographic and circular dichroism studies have shown that Kunitz soybean trypsin inhibitor (STI) family members are predominantly β-sheet proteins with little or no α-helical structure. It consists of 12 crisscrossed antiparallel β-strands largely stabilized by hydrophobic side chains, random coil and turn structure. The spectroscopic and biochemical studies have shown that the Kunitz type inhibitors are highly stable over a broad range of temperature and pH and are remarkably resistant to proteolytic degradation (Sweet et al., 1974; Onesti et al., 1991; Roychadhuri et al., 2003, 2004; Azarkan et al., 2006).

Murraya koenigii (L) Spreng. (Rutaceae) is an aromatic more or less deciduous shrub or 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 stimulant, stomachache, antidysenteric and carminative activities (Pruthi, 1998, Yusuf et al., 1994, Satyavaty et al., 1987). Traditionally this plant is also consumed by diabetics in southern part of India (Yadava et al., 2002). Previous biochemical studies on *M. koenigii* (Curry leaf) have revealed the occurrences of alkaloids (Reisch et al., 1994; Bhattacharyya et al., 1994; Chakraborty et al., 1997), quinones (Saha and Chowdhury, 1998), terpenoids (Kureel et al., 1969). The plant also reported to have hypoglycemic (Khan et al., 1995), antihyperglycemic (Yadava et al., 2002), antidiabetic (Arulselvan et al., 2006) and anti-fungal effects (Das et al., 1965). Till date, no protein has been characterized from this plant.

In this work, a Kunitz type trypsin inhibitor has been purified by ion exchange chromatography, gel filtration chromatography on HPLC and affinity chromatography on cibacron blue 3 GA. The purity and molecular mass of this protein was determined by SDS-PAGE and MALDI-TOF analysis. The N-terminal and partial internal amino acid sequence was

determined by Edman degradation method and MALDI-TOF-TOF studies respectively. The sequence analysis was performed by NCBI - BLAST short sequence search tool and Clustal W. The trypsin inhibitory activity was performed by taking synthetic substrate BAEE and BAPNA and chymotrypsin inhibitory assay was performed by taking synthetic substrate BTEE. The kinetic study was performed by taking BAPNA as a substrate. The Ki value and mode inhibition was determined from Dixon plot. The storage property of protein was determined by monitoring the protein profile in different stages of seed development and germination. Solubility studies of *Murraya koenigii* trypsin inhibitor (MKTI) were carried out under different physicochemical conditions including temperature, pH, salts, detergents and organic solvents. The thermal stability of the protein under *in-vitro and in-vivo* condition was investigated by incubating the purified protein and mature intact seeds respectively in different temperatures. The structural stability of MKTI was examined by proteolysis studies with five different proteinases, trypsin, papain, proteinase K, chymotrypsin and pepsin. The thermal stability of MKTI towards proteolytic degradation was also explored by incubating the MKTI at range of temperatures prior to digestion with proteinases. Circular dichroism and fluorescence studies were performed under different physicochemical conditions for secondary structure analysis and stability studies of MKTI. Cystallization and preliminary X-ray diffraction study of MKTI was performed. The biochemical and biophysical characterization of MKTI helped in understanding the structure-function relationship of this protein.

CHAPTER 1

LITERATURE REVIEW

1.1. Introduction

Proteins are important functional elements of the cell, where in many cases their structural or mechanical functions are just as important as their biochemical roles. The chief characteristic of proteins that enables them to carry out their diverse cellular functions is their ability to bind other molecules specifically and tightly. This binding ability is mediated by the structure of the protein, by the chemical properties of the surrounding amino acids' side chains and by the physiological conditions. Formation of specific complexes between antigen and antibody, hormone and receptor, or enzyme and inhibitor/substrate in specific physiological conditions are classic examples of highly complementary and specific interactions vital to living organisms. These interactions are completely dependent on their structural and functional stability in different physicochemical conditions. The structural and functional stability of the proteins can be examined both *in-vitro* and *in-vivo* conditions. In order to perform *in-vitro* analysis, a protein must be purified away from other cellular components. This process usually begins with cell lysis, centrifugation, various chromatographic methods and various spectroscopic techniques. Purified protein can be characterized further biochemically by various bioassays and biophysically by various biophysical techniques like UV-Visible, circular dichroism, fluorescence spectroscopy and X-ray crystallography. The detailed characterization of a protein generally helps in understanding the structure-function relationship and therefore, can be controlled various cellular process in living organisms.

Proteolysis is a key process in all living organisms and must be carefully controlled in order not to be hazardous to the organism itself. It is, therefore, not surprising that a large number of naturally occurring proteinaceous proteinase inhibitors have been found in animals,

plants and microorganisms (Laskowski and Kato, 1980; Liener, 1979; Richardson, 1977; Mosolov and Valueava, 1993). Proteinase inhibitors are molecules that reduce the bio-availability of proteinases, a catalytic enzyme that catalyzes the hydrolysis of proteins to form smaller polypeptide units. Proteinases are mostly classified according to the main catalytic amino acid residue in their active site: (1) serine proteinases, with a serine and a histidine; (2) cysteine proteinases, with a cysteine; (3) aspartic proteinases, with an aspartate group and (4) metalloproteinases, with a metallic ion (Zn2+, Ca2+, or Mn2+) (Neurath, 1984), in their active site. They have evoked tremendous interest because of their pivotal role in the regulation of various physiological and pathological processes. Due to the essential nature of proteinases, proteinase inhibitors play a crucial role in developing therapeutics and as biocontrol agents. Proteinases are essential in the life cycles of organisms that cause mortal diseases such as malaria, cancer and AIDS (Billings et al., 1987; Seelmeir et al., 1988). Specific inhibition of these proteinases can be used as a strategy for drug design for the prevention of propagation of many causative agents and many proteinase inhibitors therefore, act as anti-cancer and anti-HIV agents. Proteinase inhibitors can be divided into two classes: small molecules and macromolecules (Stoop and Craik, 2003). Small molecule inhibitors use the chemical nature of the active site and extended substrate-binding pockets to bind and inhibit the proteinase. Because of their small size and limited recognition determinants, these inhibitors often cannot discriminate sufficiently between closely related serine proteinases (Sanderson, 1999 and Leung et al., 2000).

1.2. Serine proteinases and inhibitors

The serine proteinases are a large family of enzymes involved in a wide variety of vital biological processes. The crucial physiological functions of these enzymes in metabolism, blood

coagulation, fibrinolysis, complement pathways, viral maturation, apoptosis and cancer make them important targets for efforts to isolate, design and engineer potent and specific inhibitors. Macromolecular proteinase inhibitors bind to the target proteinase through a single loop that indicates the critical P1 residue. This residue fits into the binding pocket of the target proteinase in a substrate-like conformation to lock the enzyme in a complex formed between the proteinase and inhibitor (Yang and Craik, 1998).

1.2.1. Catalytic mechanism of serine proteinases

The three serine proteinases have been studied in greater detail are trypsin, chymotrypsin and elastase. Trypsin and chymotrypsin are structurally very similar, although they recognise different substrates. Trypsin acts on lysine and arginine residues, while chymotrypsin acts on large hydrophobic residues such as tryptophan, tyrosine and phenylalanine, both with extraordinary catalytic efficiency. Both enzymes have a catalytic triad of serine (Ser 195; hence the name serine proteinase), histidine (His 57) and aspartate (Asp 102) located near the heart of the enzyme and these three key amino acids each play an essential role in the cleaving ability of the proteinases.

As the polypeptide enters, the serine -OH group attacks the carbonyl carbon, the nitrogen of the histidine accepts the hydrogen from the -OH of the serine and a pair of electrons from the double bond of the carbonyl oxygen moves to the oxygen. As a result, a tetrahedral intermediate is generated.

The bond joining the nitrogen and the carbon in the peptide bond is now broken. The covalent electrons creating this bond move to attack the hydrogen of the histidine, breaking the connection. The electrons that previously moved from the carbonyl oxygen double bond move back from the negative oxygen to recreate the bond, generating an acyl-enzyme intermediate.

Now, water comes in to the reaction. Water replaces the N-terminus of the cleaved peptide, and attacks the carbonyl carbon. Once again, the electrons from the double bond move to the oxygen making it negative, as the bond between the oxygen of the water and the carbon is formed. This is coordinated by the nitrogen of the histidine, which accepts a proton from the water. Overall, this generates another tetrahedral intermediate.

In a final reaction, the bond formed in the first step between the serine and the carbonyl carbon moves to attack the hydrogen that the histidine just acquired. The now electron-deficient carbonyl carbon re-forms the double bond with the oxygen. As a result, the C-terminus of the peptide is now ejected.

1.2.2. Mechanism of serine proteinase inhibition

The binding of an inhibitor can stop a substrate from entering the enzyme's active site and/or hinder the enzyme from catalyzing its reaction. Inhibitor binding is either reversible or irreversible. Irreversible inhibitors usually react with the enzyme and change it chemically. These inhibitors modify key amino acid residues needed for enzymatic activity. In contrast, reversible inhibitors bind non-covalently and different types of inhibition are produced depending on whether these inhibitors bind the enzyme, the enzyme-substrate complex, or both.

Protein inhibitors of serine proteinases such as soybean trypsin inhibitor undergo the reaction, including formation of an acyl-enzyme, but have many interactions with the proteinase, so that the first product does not diffuse away and water has no room to attack the acyl-enzyme. A classical peptide inhibitor of serine proteinases that is hydrolyzed $\approx 10^7$ times more slowly than a good substrate is shown to form an acyl-enzyme intermediate rapidly. Despite this quick first step, further reaction is slowed dramatically because of tight and oriented binding of the cleaved peptide, the leaving group amine is poised for nucleophilic attack on the

acyl-enzyme and preventing acyl-enzyme hydrolysis and favoring the reverse reaction (Longstaff et al., 1990 and Shaw et al., 1995). The clogged gutter mechanism for proteinase inhibitor suggested that the combination of a hydrogen bond network, an acyl-enzyme, and the correct orientation of the religating amide can arrest the catalytic reaction of serine proteinases (Radisky and Koshland, 2002).

1.3. Plant proteinase inhibitors

Proteinaceous proteinase inhibitors are ubiquitously abundant in tubers and plant seeds (Ryan, 1977). In higher plants, several gene families of these proteinase inhibitors have been characterised, particularly the serine proteinase inhibitors from *Leguminosae, Cucurbitaceae, Solanaceae* and *Graminae* (Garcia-Olmeda et al., 1987). The classification of proteinaceous proteinase inhibitors (PIs) remains, however, unclear. The confusion in classification is resulting from the fact that PIs are often named after their first discovered biological origin (e.g: Potato Inhibitor II), the last name of the discoverer (e.g. Kunitz inhibitor from soybean) and/or the enzyme they inhibit (e.g. Serpins) (Birk, 2003a) and the type(s) of enzyme they inhibit (e.g. serine proteinase inhibitors) (Ryan, 1990; Bode and Huber, 2000). However, the serine proteinase inhibitors have been classified usually according to their sequence homology and structure of disulfide bridges and include Bowman-Birk type, squash inhibitor family, cereal superfamily, potato I, potato II and Kunitz-type inhibitors family (Laskowski and Kato, 1980).

1.3.1. The Bowman-Birk family

The Bowman-Birk inhibitor from soybeans (BBI) serves as the prototype for a family of inhibitors that are predominant in legume seeds (Birk, 1961; Birk et al., 1963; Birk, 1985). BBI has a molecular mass of about 8,000 Da with a high content of half cystines forming seven disulfide bridges. The inhibitor consists of two tandem homology regions on the same

polypeptide chain, each with a reactive, inhibitory site (Odani and Ikenaka, 1973a). In aqueous solutions, the inhibitor undergoes self-association, which is concentration dependent. BBI inhibits human, bovine, porcine, dog and avian trypsin and chymotrypsin, at a 1:1 enzyme-inhibitor ratio (Birk, 1985). It inhibits carp trypsin and chymotrypsin with a 1:2 stoichiometry of inhibition, indicating the binding of two molecules of either trypsin or chymotrypsin to one molecule of inhibitor (Cohen et al., 1981a,b). BBI is also a potent inhibitor of trypsin and chymotrypsin from the digestive tracts of insects, such as *Tenebrio molitor* (Levinsky et al., 1977), *Locusta migratoria* (Sakal et al., 1988, 1989). Most of the members of the BBI family exhibit numerous isoinhibitor forms. Homologous inhibitors have been found in lima beans, garden beans, adzuki beans, mung beans, ground nuts, chickpeas, peas, cowpeas and also in plant sources other than legume seeds such as wheat germ, rice and barley (Odani et al., 1986; Lyons et al., 1987; Tashiro et al., 1987; Greagg et al., 1994). They are double-headed, binding simultaneously to two serine proteinases, such as trypsin and a-chymotrypsin (Bode and Huber, 1992).

1.3.2. The squash inhibitor (Cucurbit) family

This family was named following the discovery of *Momordica charantia* inhibitor 3 (MCI-3), a trypsin inhibitor from a *Cucurbitaceae* (Zeng et al., 1988). After that a series of inhibitors have been reported from the members of *Cucurbitaceae* family (Hamato et al., 1992; Hayashi et al., 1994; Lee and Lin, 1995; Haldar et al., 1996; Huang et al., 1999). The striking characteristic of this family is that its member inhibitors are very small, molecular mass is about 3,000 Da (Wieczorek et al., 1985). They consist of 27 – 33 amino acid residues and are cross-linked by three disulfide bridges. The reactive site peptide bond ($P_1 - P'_1$) is between residue 5 (Lys, Arg or Leu) and 6 (always Ile), and they inhibit proteinases via the standard mechanism.

1.3.3. The "Cereal superfamily" family

This is a small group of PIs extracted from cereals such as barley, rye, wheat, maize, rice and ragi (Kashlan and Richardson, 1981; Campos and Richardson, 1983). They are proteins of single polypeptide chain and molecular mass of about 12-13 kDa containing 4-5 S-S bridges (Odani et al., 1983). The highly homologous trypsin inhibitors from barley, rye and maize, as well as the bifunctional amylase-trypsin inhibitor from seeds of ragi, contain an Arg-Leu reactive site peptide bond in positions corresponding to the Arg33-Leu34 in the sequence of the barley inhibitor (Lyons et al., 1987).

1.3.4. The Potato inhibitor I (PI-1) family

Potato inhibitor I family was first described by Ryan and Balls (1962), is a multimeric protein with a molecular mass of 40-45 kDa (Melville and Ryan, 1972). This inhibitor family also referred to as chymotrypsin inhibitor I because its specificity is directed most strongly towards chymotrypsin than trypsin, subtilisin, pronase and alkaline microbial proteinases. The inhibitors belonging to this family are noncovalent tetramers of four different subunits. Each subunit (10,000 Da) comprised of a single chain with a single intra-chain disulfide bridge. One mole of tetramer inhibits 4 moles of chymotrypsin, which suggest that each of the subunits possesses a chymotrypsin-binding site.

1.3.5. The Potato inhibitor II (PI-2) family

Members of the potato proteinase inhibitor II (PI-2) family have been shown to inhibit serine proteinases, such as trypsin, chymotrypsin, subtilisin, oryzin and elastase (Pearce et al., 1982 and Plunkett et al., 1982). Inhibitor II has a molecular mass of 20,000 Da and is composed of dimmers of four distinctly different protomers, containing 16 cysteine residues per subunit, of which only 6 form intramolecular disulfide bridges. Each dimmer inhibits two molecules of

proteinases, indicating that each of the subunits has a binding site for proteinases (Iwasaki et al., 1974).

1.3.6. The Kunitz-type family

This is a large group of proteins sharing common criteria. Kunitz-type inhibitors are mostly monomeric. Dimeric members, in which the subunits are linked by a disulfide bridge, have also been described (Richardson, 1991). Kunitz-type inhibitors are proteins with 170-190 amino acids, which usually contain 4 cysteine residues that form two disulfide bridges. Kunitz-type inhibitors mostly contain one active site (single headed) that is located in the loop formed by the S-S bridge close to the N-terminus (Richardson, 1991).

1.3.6.1. Isolation, purification and general properties

The first plant proteinase inhibitor to be isolated and characterized was Kunitz soybean trypsin inhibitor (STI). STI is primarily an inhibitor of trypsin, but also weakly inhibits chymotrypsin. It is inactivated by heat and by gastric juice. The purification, crystallization, kinetics of the interaction and complex formation of STI with trypsin comprise a major landmark in the study of protein proteinase inhibitors (Kunitz, 1947a, b). The numerous studies on STI concerning specificity, stability, physical, kinetic and other properties have been compiled and summarized by Kassell (1970) and Birk (1976). STI consists of 181 amino acid residues and includes two disulfide bridges. The precursor for STI has a molecular mass of ~24,000 Da and the mature STI has a molecular mass of ~20,000 Da (Kim et al., 1989).

The Kunitz type inhibitors from other than soybean, the *Erythrina* seeds received the greatest amount of attention. The inhibitors have similar molecular mass (~20,000 Da) and share many other chemical characteristics. They are divided into three groups on the basis of their relative abilities to inhibit chymotrypsin, trypsin and tissue plasminogen activator (tPA). Group

(a) inhibitors were relatively specific for chymotrypsin; they were poor inhibitors of trypsin and had no apparent effect on tPA. Group (b) proteins inhibited trypsin strongly and chymotrypsin slightly less effectively. They had no effect upon tPA. Group (c) inhibitors inhibited trypsin, chymotrypsin and tPA. The sequence of these inhibitors shows a high degree of homology to those of Kunitz-type trypsin inhibitors from soybean and winged bean seeds (Joubert et al., 1985; Joubert and Dowdle, 1987). Onesti et al. (1992) also investigated the effects of pH and temperature on kinetic and thermodynamic parameters for the binding of ETI to different serine proteinases and defined their perspective affinities.

Studies on *Erythrina variegata* trypsin and chymotrypsin inhibitors were reported by Kouzuma et al. (1992). The stoichiometry of trypsin inhibitors with trypsin was 1:1, while that of the chymotrypsin inhibitor with chymotrypsin was 1:2 molar ratio. According to Kouzuma et al. (1992) the inhibitors show structural features characteristic of the Kunitz-type soybean trypsin inhibitor and exhibit a significant homology to the storage proteins, sporamin in sweet potato and the taste-modifying protein, miraculin, in miracle fruit.

Caldwell et al. (1990) determined the primary structure of a Kunitz-type trypsin inhibitor from winged bean seeds (*Psophocarpus tetragonolobus*). It consists of a single polypeptide chain of 182 amino acids, including four half-cystine residues and an N-terminal residue of pyroglutamic acid. The sequence comparisons to other Kunitz-type proteinase inhibitors indicate that the winged bean and *Erythrina* inhibitors are more closely related to each other than to other members of the Kunitz inhibitor family.

Negreiros et al. (1991) studied the activity and amino acid sequence of the major Kunitz type trypsin inhibitor from the seeds of *Prosopis juliflora*. The inhibitor has a molecular mass of

~20,000 Da, it consists of two polypeptide chains, of 137 residues and 38 residues, linked together by a single disulfide bond. The protein inhibited trypsin in the stoichiometric ratio of 1:1, but had only weak activity against chymotrypsin.

Lin et al. (1991) and Wu and Lin (1993) isolated and sequenced a trypsin inhibitor from seeds of small Philippine acacia (*Acacia confuse*). The inhibitor consists of 175 amino acid residues in two chains, 136 residues and 39 residues, linked by a disulfide bridge. The molecular mass of the inhibitor was found to be ~21,000 Da.

A proteinase inhibitor from *Enterolobium contortisiliquum* seeds was isolated, characterized and sequenced by Batista et al. (1996). The inhibitor contains 174 amino acid residues in two polypeptide chains, an alpha-chain consisting of 134 residues and a beta-chain made up of 40 residues, linked by a disulfide bridge. The inhibitor displays a high degree of sequence identity with other Kunitz-type proteinase inhibitors isolated from the *Mimosoideae* subfamily.

Di-Ciero et al. (1998) isolated, characterized and sequenced trypsin inhibitors from two varieties of *Bauhinia variegata* seeds: *B. variegate candida* and *B. variegate lilac*. In both varieties, three isoforms with molecular mass of ~20,000 Da were detected. Homology studies with other trypsin inhibitors show that *B. variegate* trypsin inhibitors belong to the Kunitz-type family, with active sites at Arg63-Ile64.

Polikarpov et al. (1999) reported the purification, crystallization and preliminary crystallographic study of a Kunitz-type trypsin inhibitor from *Delonix regia* seeds. Pando et al.

(1999) isolated and characterized a Kunitz-type trypsin inhibitor from *Crotalaria paulina* seeds. The inhibitor has a molecular mass of ~20,000 Da and it comprises 177 amino acid residues.

A trypsin inhibitor from *Dimorphandra mollis* seeds was isolated to apparent homogeneity by a combination of ammonium sulfate precipitation, gel filtration, ion-exchange and affinity chromatographic techniques. SDS-PAGE analysis gave an apparent molecular mass of 20 kDa. This inhibitor, which inhibited trypsin activity with a Ki of 5.3×10^{-10} M, is formed by a single polypeptide chain (Macedo et al., 2000).

Silva et al. (2001) purified, analyzed and characterized two trypsin inhibitors from seeds of the native Brazilian tree *Copaifera langsdorffii*. There was no inhibitory effect on chymotrypsin. The molecular mass of the inhibitors was ~24,000 Da. The molecular masses of the two inhibitors under reducing conditions were 11,456 and 10,008 Da. Based on their N-terminal sequences, one of them belongs to the Kunitz family of trypsin inhibitors, whereas the second one showed no homology to known inhibitors.

A novel trypsin inhibitor was purified from the seeds of *Peltophorum dubium* (Spreng.). *P. dubium* trypsin inhibitor (PDTI) was purified by extraction in 100 mM phosphate buffer (pH 7.6), ammonium sulfate precipitation (30–60% saturation), gel filtration on Sephadex G-75, ion-exchange chromatography on DEAE-Sepharose and affinity chromatography on trypsin-Sepharose. SDS-PAGE under reducing conditions showed that the inhibitor consisted of a single polypeptide chain (20 kDa). The dissociation constants of 4×10^{-10} and 1.6×10^{-10} M were obtained with bovine and porcine trypsin, respectively (Rodrigues Macedo et al., 2003).

A novel trypsin inhibitor (PPTI) was purified from the seeds of the native Brazilian tree *Poecilanthe parviflora* (Benth) (*Papilioinodeae, Leguminosae*) by gel filtration chromatography on a Sephadex G-100 followed by Superdex G75 chromatography (FPLC), Sepharose 4B-Trypsin column, and fractionated by reversed-phase HPLC on a C-18 column. SDS-PAGE showed that PPTI consisted of a single polypeptide chain with molecular mass of about 16 kDa. The dissociation constant of 1.0×10^{-7} M was obtained with bovine trypsin (Garcia et al., 2004).

Leguminous plants in the tropical rainforests are a rich source of proteinase inhibitors and this work illustrates isolation of a serine proteinase inhibitor from the seeds of *Archidendron ellipticum* (AeTI), inhabiting Great Nicobar Island, India. AeTI was purified to homogeneity by acetone and ammonium sulfate fractionation, and ion exchange, size exclusion and reverse phase chromatography (HPLC). SDS–PAGE of AeTI revealed that it is constituted by two polypeptide chains (α-chain, M_r 15,000 and β-chain, M_r 5000), the molecular mass being ~20 kDa. N-terminal sequence showed high homology with other serine proteinase inhibitors belonging to the *Mimosoideae* subfamily. Both Native-PAGE as well as isoelectric focussing showed four isoinhibitors (p*I* values of 4.1, 4.55, 5.27 and 5.65). Inhibitory activity of AeTI remained unchanged over a wide range of temperatures (0–60°C) and pH (1–10). The protein inhibited trypsin in the stoichiometric ratio of 1:1, but lacked similar stoichiometry against chymotrypsin. Also, AeTI-trypsin complex was stable to SDS unlike the SDS unstable AeTI-chymotrypsin complex. AeTI, possess inhibition constants (K_i) of 2.46×10^{-10} and 0.5×10^{-10} M against trypsin and chymotrypsin activity, respectively (Bhattacharyya et al., 2006).

A trypsin/chymotrypsin inhibitor (JSTI) was isolated from jack fruit seeds (*Artocarpus integrifolia* Hook f) by ammonium sulphate fractionation and chromatography on DEAE-cellulose and Sephadex G-100 (Sai Annapurna and Siva Prasad, 2006).

1.4. Storage properties

Many plant storage organs such as seeds and tubers contain from 1 to >10% of their soluble proteins as trypsin inhibitors (Mandal et al., 2002). Besides their storage function by providing nitrogen and 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 and Kato, 1980; Ryan, 1990; Hou et al., 1999; Admovskaya et al., 2000). 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 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 and Mandal, 2000). (3) In general, a protein can be considered as a storage protein when this protein is present in amounts of 5% and more of the total protein content (Derbyshire et al., 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 and Ramakrishna, 2005).

Although storage proteins and proteinase inhibitors are placed in two separate groups as classified by Fukusima but there are many report of having overlapping functions (Mosolov et al., 2001; Birk, 2003; Shewry, 2003, Haq et al., 2004). The proposed role for proteinase inhibitors as storage proteins was suggested first by Pusztai (1972). Papastoitsis and Wilson

(1991) showed that during seed germination and early seedling growth there is a degradation of STI in soybean cotyledons. McGrain et al., (1992) found that during soybean seed maturation there is a slow phase of STI accumulation, followed by a rapid phase when the seeds have reached half their maximum fresh weight.

1.5. Solubility properties

To be biologically active, proteins must adopt specific folded three-dimensional, tertiary structures in soluble condition. Protein solubility is a complex function of the physiochemical nature of the proteins, pH, temperature, organic solvents, detergents and the concentration of the salts used. Protein solubility is highly dependent upon the ionic strength (Arakawa & Timasheff, 1985; Ries-Kautt & Ducruix, 1997). In general, at low concentrations, the protein solubility exhibits a 'salting-in' behavior, with the protein solubility increasing with increasing ionic strength. At high ionic strengths, the protein solubility decreases with increased ionic strength in a 'salting-out' behavior. The denaturation of proteins leading to aggregation occurs due to the environmental changes such as temperature, pH, and addition of denaturants like urea and guanidine hydrochloride (Tanford, 1968). At lower pH, carboxyl groups are protonated and hydrophobic interactions dominate; this leads to precipitation of the polymer (Roy and Gupta, 2003). Protein stability can be measured directly using calorimetric methods, and indirectly by different spectroscopic methods. Upon unfolding, the polypeptide chain becomes less compact, more solvated and much more flexible and resulting more susceptible towards proteolytic degradation (Tanford, 1968). Unfolding of a protein can be easily performed by using extreme pHs, denaturants and by increase of temperature. The spectroscopic and biochemical studies have shown that the Kunitz type inhibitors are highly stable over a broad range of temperature

and pH and are remarkably resistant to proteolytic degradation. (Sweet et al., 1974; Onesti et al., 1991; Roychaudhuri et al., 2003, 2004; Azarkan et al., 2006).

1.6. Circular dichroism

Circular dichroism (CD) spectroscopy is a well-established method to understand the structure function relationship of proteins. The sensitivity of far-UV protein CD spectra to protein secondary structure is used in one of the most successful applications of CD to determine the secondary structure composition of a protein (Yang et al., 1986; Greenfield, 1996; Venyaminov and Yang, 1996; Sreerama et al, 2000; Sreerama and Woody, 2000, 2004). CD spectroscopic method depends upon the differential absorption of left- and right-circularly polarized light by optically active molecules. Alpha-helix, beta-sheet, and random coil structures each give rise to a characteristic shape and magnitude of CD spectrum. The far UV CD spectra of alpha-helical proteins are characterized by three peaks, a negative peak at ~222 nm, at ~208 nm and a stronger positive peak at ~192 nm. However, CD at 222 nm displays the strong concentration dependence characteristic of dissociative unfolding and usually the negative value at 222 nm is considered to measure the helix content (Holtzer et al., 2000). In general, spectra arising from β-sheets are characterized by a small negative peak near 217 nm and a positive peak near 195 nm that has approximately half the intensity of the α-helix peak in this region. β-Sheets give rise to considerably less intense signals than helices and show far more variation in spectral characteristics; the latter is partly attributable to the fact that β-sheets are much more structurally diverse than α -helices, with strands which may run parallel or antiparallel to each other, and with sheets displaying differing degrees of twisting. The weak β-sheet signal makes it more susceptible to distortions caused by the CD signals of aromatic side chains, which can produce peaks or dips between 225 nm and 235 nm. CD spectra are usually

presented in molar ellipticity [θ] units (degrees-cm^2 dmol^{-1}). For proteins, the mean residue ellipticity (*MRE*) can be calculated as follows: $MRE = (MRW)\theta/10cl.$ where *MRW* is the mean residue (amino acid) weight, *c* is the concentration of protein and *l* is the pathlength in cm. The α-helical content for each protein was calculated using the standard equation for [θ] at 222 nm (28): $[\theta]_{222} = -30{,}300f_H - 2{,}340$, where f_H is the fraction of α -helical content (f_H x 100, expressed in %). Spectra are presented as the mean residue ellipticity. Thermal stability is assessed using CD by following changes in the spectrum with increasing temperature.

Based on circular dichroism spectra, soybean Kunitz trypsin inhibitor (SKTI) has been classified in the β-II or the 'disordered' class of all antiparallel β-sheet proteins. Thermal transition studies done with SKTI at neutral and alkaline pH are reversible (Roychaudhuri et al., 2003). CD spectra of SKTI during denaturation in the far-UV region show decreased ellipticity, indicating loss of secondary structure (Roychaudhuri, 2004). Far-UV CD study of *Schizolobium parahyba* chymotrypsin inhibitor (SPCI) revealed that between 20 and 70°C the CD spectra is typical of β-structure and unordered structure protein. At temperature above 70°C the CD spectra decrease the minimum at 200 nm with a partial loss of signal. Analysis of temperature progress curve at pH 7.0 revealed that the native conformation of SPCI is thermally stable at temperatures below 70°C with partial unfolding of secondary elements (Teles et al., 2005). CD spectroscopic analysis of the Kunitz-type pigeonpea inhibitor has revealed the absence of any α-helix and the CD spectrum is representative of the β-II class of polypeptides and the proteinase/amylase inhibitor isolated from mung bean is a helical protein (Haq and Khan, 2005).

1.7. Fluorescence

Fluorescence spectroscopy is a very powerful technique to monitor conformational changes in proteins. The fluorophores can be either intrinsic (tryptophan) or extrinsic (ANS: 8-

anilino-1-naphthalene sulfonate) probes. The fluorescence property of tryptophan has long been utilized to understand the folding/unfolding, substrate binding and conformational heterogeneity in different physicochemical conditions. The tryptophan fluorescence usually varies in somewhat unpredictable manner in various proteins. Some proteins exhibit large increases in their intensities and lifetimes and others, decreases, on unfolding of the chain. Proteins with heme prosthetic groups typically exhibit an increase in intensity on unfolding due to loss of the heme group. The average energy of the emission of the tryptophan residues usually shifts to the red on unfolding because the solvent exposure and thus solvent relaxation is augmented in the unfolded state (Royer, 1995). The ANS anion (8-anilino-1-naphthalene sulfonate) is a much utilized 'hydrophobic probe' for proteins (Slavik, 1982). ANS in water is nonfluorescent but it fluoresces upon binding to a nonpolar (hydrophobic) site of proteins. In fact, ANS binding to proteins depends on protein cationic charge and solution pH, and occurs largely through the ANS sulfonate group (Matulis and Lovrien, 1998). The dependence of ANS binding on electrostatic interaction between the sulfonate group and protein cationic groups indicate that ANS binding does not require preexisting hydrophobic site on or in protein molecules to start the binding reaction. With increasing conformational unfolding state in different physicochemical conditions, the ANS binding fluorescence intensity increases. Thus, the increase in fluorescence intensity is a characteristic of relaxed or molten state where more access of ANS to hydrophobic core of a protein is allowed (Semisotnov et al., 1991; Price, 2000).

1.8. X-ray crystallography

Protein crystallography is very important and essential technique to determine the three dimensional structure at molecular level. The crystallographic studies help in understand the structure-function relationship and mechanism of action of protein. The interaction between the macromolecules which is important for molecular recognition can be done by determining the complex structures. Many proteins including antigen, antibody, hormone, receptors and various enzymes like proteinases and substrate/inhibitors have been characterized by X-ray crystallography.

The crystallographic studies have shown that Kunitz soybean trypsin inhibitor (STI) family members are predominantly β-sheet proteins with little or no α-helical structure. It consists of 12 crisscrossed antiparallel β-strands largely stabilized by hydrophobic side chains, random coil and turn structure. Its three-dimensional structure in complex with porcine trypsin was also determined (Sweet et al., 1974). The predominant conformation found in STI has been demonstrated to be approximate beta-sheet structures, with a small amount of regular sheet (Sweet et al., 1974; Ventura, 1989). De-Meester et al. (1998) studied the structure of STI in its free form, determined by molecular replacement to 2.5 Å, using the coordinates of the homologous *Erythrina* trypsin inhibitor as a search model.

Shieh et al. (1990) reported the crystallization of a chymotrypsin inhibitor from *Erythrina caffra* seeds into crystals that belong to the rhombohedral space group. Onesti et al. (1991) studied the structure and properties of a Kunitz-type trypsin inhibitor from *Erythrina caffra* seeds (ETI). This inhibitor consists of 172 amino acid residues with two disulfide bridges. The three-dimensional structure of ETI consists of 12 antiparallel beta-strands joined by long loops. Six of the strands forma short antiparallel beta-barrel that is closed at one end by a "lid"

consisting of the other six strands coupled in pairs. The scissile bond (Arg63-Ser64) of ETI is located on an external loop that protrudes from the surface of the molecule. The overall structure of ETI is similar to the partial structure of STI, but the scissile bond loop is displaced by about 4 Å.

1.9. Importance of plant proteinase inhibitors

As PIs are naturally present in many tubers and plant seeds (Ryan, 1977), they have been proposed to be storage proteins, and/or to control endogenous proteinases and/or to act as a defense mechanism.

1.9.1. Role in plant defense

Plants are inevitably subjected to a variety of pest and pathogen infestations, which substantially contribute to the overall loss in crop yield. Agricultural industries employ several chemical pesticides to combat this problem. However, they have serious drawbacks owing to their lack of specificity, development of resistance upon extended use and the ecological hazards inherent with residual toxicity. Thus, biodegradable biocontrol agents are advantageous alternatives as they are devoid of contaminating residues and have reduced incidence for development of resistance. However, proteinase inhibitors regulate the action of proteinases and play a significant role in the protection of plants from pest and pathogen invasion by virtue of their antinutritional interaction. Serine proteinase inhibitors from plants have been shown to have potential usefulness as defense tools to protect the plants from invading pests (Green and Ryan, 1972; Ryan, 1990). A few plant proteinase inhibitors have been shown to possess fungicidal activity (Lorito et al., 1994; Joshi et al., 1999).The presence of a "defense response" of the plant to the attack of insects was first reported by Green and Ryan (1972). Studies on the effects of dietary plant proteinase inhibitors on the development of insects were first reported by

Lipke et al. (1954) and the inhibition of insects own digestive proteinases by the ingested plant proteinase inhibitors was also observed by Broadway and Duffey (1986). Most of the plant proteinase inhibitors relevant to plant defense are members of the serine proteinase inhibitor families. Johnston et al. (1993) reported that Kunitz-type inhibitors (KTI) and Bowman-Birk inhibitors (BBI) from soybean in diets of newly molted third-instar larvae of the maize earworm *Helicoverpa armigera* reduced total larval biomass, mean larval weight, and trypsin-like activity in their gut contents. The effect on larval growth was greater with dietary KTI than with BBI. Burgess et al. (1994) showed that the efficiency of several proteinase inhibitors (KTI, potato II proteinase inhibitors, cowpea trypsin inhibitor, wheat germ inhibitor, and bovine pancreatic trypsin inhibitor) in reducing growth of the black field cricket *Teleogryllus commodus* was strongly influenced by the concentration of the inhibitors. The possible involvement of plant proteinase inhibitors as a defense mechanism against insects was supported by the discovery that insect attacks on tomato or potato plants induced a rapid accumulation of proteinase inhibitors in leaves (Green and Ryan, 1972). The signal by which tissues distant from the injury are induced to respond by synthesizing proteinase inhibitors was named "Proteinase Inhibitor Inducing Factor" (PIIF). Plant proteinase inhibitors can be induced not only by pathogen attack, but also by different biological and physiological conditions. Downing et al. (1992) isolated a drought-induced 22,000 Da protein from *Brassica napus* that is related to the Kunitz family of proteinase inhibitors.

Extremely low levels of several serine proteinase inhibitors, including KTI and BBI from soybeans, cowpea and squash inhibitors, enhanced the insecticidal activity of the insect control proteins from several varieties of *Bacillus thuringiensis* by 2-20 fold against their target

insects, tobacco budworm and other lepidopterans, Colorado potato beetle, and mosquito (Macintosh et al., 1990).

Confalonieri et al. (1998) regenerated *Populus nigra* transgenic plants that expressed a Kunitz-type proteinase inhibitor gene. The trypsin-like digestive proteinases of the polyphagous moth *Lymantria dispar* and *Clostera anastomosis* were inhibited in vitro by the Kunitz proteinase inhibitor from the transgenic plants.

Gatehouse et al. (1999) compared the susceptibility of the tomato moth (*Lacanobia oleracea*) to KTI expressed in transgenic potato plants, to KTI incorporated in potato leaf-based artificial diet. The presence of KTI in the artificial diet at 2% of total protein, decreased larval survival and growth and retarded development. However, when KTI was expressed in transgenic potato plants as ~0.5% of total protein, only marginal effects on *L. oleracea* larvae were observed, which decreased with time.

Wang et al. (1999) reported the introduction of a soybean KTI insecticidal gene, transferred into four upland cotton (*Gossypium hirsutum*) cultivars via Agrobacterium-mediated transformation and the transgenic plants were significantly resistant to the larvae of cotton bollworm (*Heliothis armigera*).

Lee et al. (1999) introduced a recombinant plasmid-containing cDNA of KTI into rice protoplasts and resulting transgenic plants are more resistant to the destructive insect pest of rice brown plant hopper (*Nilaparvata lugens*) than the control plants.

1.9.2. Proteinase inhibitors as storage proteins

The proposed role for proteinase inhibitors as storage proteins was suggested first by Pusztai (1972), who showed that during the germination of kidney bean the period of maximum proteinase inhibitor content coincided with the period of maximum proteolysis. In general, a

protein can be considered as a storage protein when this protein is present in amounts of 5 % and more of the total protein content (Derbyshire et al., 1976). However, since PIs in tubers and plant seeds belongs to different classes and families and that their concentration varies during the maturation, it would not fit the definition of a storage protein. Pusztai (1972) proposed that PIs may serve as a source of important sulphur-containing amino acids for the germination of the seed. PIs are in general considered to be proteins rich in cysteine residues (Jongsma, 1995). However, it can be observed that this postulate is true mainly for PIs with a small molecular mass (3-13 kDa) which exhibit a proportion of cysteine residues >20%. For PIs with a higher molecular mass (>13 kDa), only Thaumatin and PI-2 families (20 kDa) contain a high number of cysteine residues (lower than 10%). Knowing that most of the PIs in plants belong to the Kunitz-type of inhibitors, in which the cysteine residues consist of approximately 2%, it can be concluded that the proportion of cysteine residues is rather small. The potency of PIs as retarding insect proteinases is clear, but the assessment of PIs as storage proteins is complicated by the fact that maturation is accompanied by the expression of different PIs (McManus et al., 1999).

1.9.3. Anticarcinogenic agent

PIs are well established as a class of cancer chemopreventive agents (Kennedy, 1998). While PIs from different families have been shown to prevent the carcinogenic process, the most potent of the known anticarcinogenic PIs are those with the ability to inhibit chymotrypsin-like proteinases. The Bowman-Birk inhibitor (BBI) from soybean is until now the PI that has been most studied as an anticarcinogenic agent. However, also potato proteinase inhibitors have been shown to be potent anticarcinogenic agents, especially PI-1 and PI-2 (Frenkel et al., 1987; Billings et al., 1989; Huang et al., 1997). The interest for the other classes

of potato PIs, e.g. PIs active against cysteine proteinases and carboxypeptidase has grown as well (Billings et al., 1989; Blanco-Aparicio et al., 1998; Laurent-Matha et al., 1998).

Apart from these the serine proteinase inhibitors have been described as endogenous regulators of proteolytic activity (Ryan, 1991; Kato, 2002) and play an important role in regulating many physiological processes, such as inflammation, coagulation, fibrinolysis, complement activation intracellular protein breakdown, cell cycle, transcription and apoptosis (Silverman et al., 2001; Stein and Carrell, 1995; Huber and Carrell, 1989; Katoaka et al., 2002; Thompson and Palmer, 1998; Fumagalli et al., 1996; Kato, 1999). Because of all these advantages, the proteinase inhibitors can have many potential applications in different fields of biotechnology. Apart from this, the serine-proteinase-inhibitor complex is an important model for studying protein-protein interaction.

To use proteinase inhibitors in various biotechnological applications, it is important to study different aspects of inhibitor in relation to understanding the inhibitor-enzyme interaction, determination of different physicochemical parameters characterizing structural and functional stability in different physiological conditions and their three-dimensional structure.

CHAPTER 2

BIOCHEMICAL CHARACTERIZATION

2.1. Introduction

Seed proteins play important roles in the plants to maintain viability of seeds, providing nutrition during the early seedling stage, and protecting the seeds from microbes and insects (Millerd, 1975). 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 (Fukusima, 1991). The major biologically active proteins include lectins, enzymes and enzyme inhibitors (e.g. trypsin inhibitor).

Amino acid sequence determination is very essential to identify and characterize an unknown protein. There are many techniques available to determine the sequence, like Edman degradation method, mass spectroscopic analysis and cDNA cloning. The Edman degradation method generally applies for N-terminal amino acid sequence determination. The mass spectroscopic technique applies for internal sequence analysis. The experiment can be performed by different instruments like ESI-MS/MS, MALDI-TOF/TOF etc. In molecular biology, cDNA cloning is one of the best techniques to determine the complete and accurate amino acid sequence of corresponding protein. The complete process can be followed through isolating RNA, reverse transcription, polymerase chain reaction and nucleotide sequencing. Isolating RNA from plant seeds can be difficult because of a number of interfering compounds like storage lipids, storage proteins, polysaccharides, and polyphenolics present in seeds. However, high-quality RNA preparations are required to perform the successful RT-PCR reaction. The yield of total RNA depends on the tissue or cell source, but it is generally in the range of 4-7 μg/mg of starting tissue. The $A_{260/A280}$ ratio of the extracted RNA is generally 1.8-2.0.

Protein solubility is a complex function of the physiochemical nature of the proteins, pH, temperature, organic solvents, detergents and the concentration of the salts used. Protein solubility is highly dependent upon the ionic strength (Arakawa & Timasheff, 1985; Ries-Kautt & Ducruix, 1997). In general, at low concentrations, the protein solubility exhibits a 'salting-in' behavior, with the protein solubility increasing with increasing ionic strength. At high ionic strengths, the protein solubility decreases with increased ionic strength in a 'salting-out' behavior. The denaturation of proteins leading to aggregation occurs due to the environmental changes such as temperature, pH, and addition of denaturants like urea and guanidine hydrogen chloride (Tanford, 1968, 1970). At lower pH, carboxyl groups are protonated and hydrophobic interactions dominate; this leads to precipitation of the polymer (Roy and Gupta, 2003). Proteolysis is an important means to study structural stability of a protein. It has been reported that protein resistance to proteolytic attack increases with its conformational rigidity and susceptibility to proteolysis reflects the segmental mobility (Fontana, 1988; Fontana et al., 1997; Hubbard, 1998).

Murraya koenigii (L) Spreng. (*Rutaceae*) is a small tree, commonly used as a spice throughout India for its aromatic value. Till date, no protein has been characterized from this plant. For the first time, we have purified and characterized a trypsin inhibitor from seeds of *Murraya koenigii* by ion exchange chromatography on DEAE column and gel filtration chromatography on HPLC. In this chapter, purification, amino acid sequencing, solubility properties and proteolysis studies of MKTI has been described.

2.2. Materials and Methods

2.2.1. 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 ester (BTEE), Cibacron blue 3GA, and BSA were purchased from Sigma-Aldrich Pvt. Ltd., Centricon and Centriprep were purchased from Amicon (Beverly, MA). Acrylamide, bis-acrylamide, molecular weight standards were obtained from Bio-Rad, Australia. All other chemicals were purchased from Qualigens fine chemicals.

2.2.2. Purification of protein

2.2.2.1. Purification on ion exchange and gelfiltration column

Mature seeds (10 g) were crushed with mortal 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 12,000 x g for 1 hour and the supernatant was loaded onto a DEAE Sepharose column (1.5 x 20 cm Econo-column, Bio-Rad) equilibrated with 50 mM Tris-HCl buffer, pH 7.5. The unbound molecules were washed 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 size exclusion chromatography column (7.5 x 300 mm, Waters). The purity of the protein was determined by 15% SDS-PAGE analysis (Laemli, 1970).

2.2.2.2. Single step purification on Cibacron blue 3GA

Mature seeds (10 g) were crushed with mortal 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 12,000 x g for 1 hour and the supernatant was loaded onto a Cibacron Blue 3GA affinity column (1.5 x 20-cm Econo-column, Bio-Rad) equilibrated with 20 mM Tris-HCl buffer, pH 8.0. The unbound molecules were washed extensively till 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 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.

2.2.3. Protein estimation

Protein concentration in crude extract and fractionated protein samples were estimated by standard dye-binding method using bovine serum albumin (Sigma) as standard. For crude extract, mature seeds (10 g) were crushed with mortal 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 12,000 x g 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). Absorbances at 280 nm were also used to determine the protein content of different eluted fractions.

2.2.4. 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 (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.

2.2.5. MALDI-TOF analysis

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

2.2.6. Trypsin inhibitory assay

2.2.6.1. Using BAEE as substrate

The inhibitory activities on bovine pancreatic trypsin were determined by measuring the hydrolytic activity towards the substrate N-benzoyl-L-arginine ethyl ester (BAEE) (Shibata et al., 1986). The inhibitor was incubated at 30°C for 5 min with 4.4 x 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) by using BAEE as substrate.

2.2.6.2. Using BAPNA as substrate

Trypsin inhibitory assays were also 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 the concentration of 200 µg per ml. Then 100 µl of the enzyme solution was incubated with 200 µl 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 was added. After incubation at 30°C for another 10 min, 200 µl of 30% acetic acid was added to end the enzyme reaction, and the absorbance at 410 nm was measured on a spectrophotometer (Shibata et al., 1986).

2.2.7. 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 (Dixon, 1953). 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

minutes. Then 1 ml of the substrate, dissolved in the same buffer, was added. After incubation at 30°C for additional 10 min, the reaction was stopped by adding of 200 µl 30% acetic acid (Shibata et al., 1986). The amount of product formation was calculated using a molar extinction coefficient of 8800 M^{-1} cm^{-1} at 410 nm.

2.2.8. Chymotrypsin inhibitory assay

Different concentration of purified protein was 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 minutes (Smirnoff et al., 1976; Tsybina et al., 2004).

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 minutes. The samples were analyzed on a 15 % SDS-PAGE under reducing condition.

2.2.9. Protein profiling during seed development and germination

2.2.9.1. Seed development

During different stages of development, seeds were collected after seven days of flowering at an interval 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 12,000 x g for 1 hour and

supernatant was run on a 15% SDS-PAGE for analysis. Gels were stained with Coomassie brilliant blue R-250 and the relative concentration of each band were analyzed using Quantity One 1D gel analysis software (version 4.5.2; Biorad).

2.2.9.2. Seed germination

To monitor the degradation of a major seed storage protein during germination, mature *Murraya 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 was maintained and allowed the soil to dry to 1/2" depth before watering again. Germinated seeds were collected at three days interval and extracts were prepared to run on a 15% SDS-PAGE for analysis as described carlier.

2.2.9.3. Trypsin inhibitory activity during seed development and germination

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 protein profiling experiment.

2.2.10. Amino acid sequence determination

2.2.10.1. N-terminal sequencing

Pure protein was subjected to SDS-PAGE (15%) and electroblotted onto a PVDF membrane (Immobilone-P ^{SQ} Millipore) in 100 mM Caps-buffer, pH 11. (Matsudaira, 1987). 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.

2.2.10.2. Internal sequencing by MALDI-TOF-TOF studies

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 clean scalpel or razor blade and transferred to eppendorf tube. Gel plugs were washed with 150 – 200 µl of 50 mM ammonium bicarbonate / 50% acetonitrile (ACN) three times for 20 minutes and then washed with 100% ACN for 10 minutes. Gel pieces were shrunken and appeared opaque white with a clear colorless supernatant. Then ACN was removed and gel pieces were dried in speed vac for 15 minutes. Gel pieces were reduced using 150 µl of 10 mM dithiothreitol (DTT) in 100 mM ammonium bicarbonate, 5% ACN for 1 hour at 55°C. Supernatant was removed and gel pieces were dehydrated in steps using 100 µl of 100 mM ammonium bicarbonate for 10 minutes and 100 µl of 100% ACN for 20 minutes. For alkylation, 100 µl of 50 mM iodoacetamide in 100 mM NH_4CO_3 was added to the gel pieces. Tubes were incubated in dark at room temperature for 30 min. Supernatant was removed and gel pieces were washed with 100 µl of 100 mM NH_4CO_3 for 10 minutes and 100 µl of 100% ACN for 20 minutes. The gel pieces were dried in speed vac for 15 minutes and swollen in digestion buffer (50 mM NH_4CO_3 with enzyme trypsin) on ice for 45 minutes. The minimum volume (20 – 30 µl for most gel pieces) of buffer necessary to swell the gel pieces is used and 5 – 25 µl of 50 mM ammonium bicarbonate is added to keep the pieces wet. Tubes are incubated at 37°C for 16 hours and then supernatant of the digestion solution was collected. The gel plugs were extracted 1 X with 100 µl of 20 mM NH_4CO_3 for 20 minutes and supernatant was collected. All the supernatants were pooled. Gel plugs were extracted 2 X with 100 µl of 1% TFA in 50% ACN for 20 minutes. Gel plugs were extracted 1 X with 100 µl of 100% ACN for 20 minutes. Combined supernatants were

concentrated in speed vac. 1µl of sample was mixed with 1 µl of matrix solution and spotted on MALDI target plate and dried. 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, vortex for 30 seconds and centrifuged to precipitate insoluble material. Supernatant was used in subsequent steps. MALDI probe was inserted into mass spectrometer (Bruker Ultraflex) and mass spectrum was acquired using Flex Control™ 2.2 software and proceesesd 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.

2.2.11. cDNA cloning

2.2.11.1. Preparation of materials and samples

Pretreated the 15 ml polypropylene centrifuge tubes in 0.1% diethyl pyrocarbonate (DEPC) for one hour at room temperature and then autoclaved the tubes for 30 minutes to destroy and residual DEPC. Freezed the 500 mg freshly harvested *Murraya koenigii* seeds in liquid nitrogen and ground under liquid nitrogen using ceramic mortar and pestle. Allowed the liquid nitrogen to evaporate and transferred the ground tissues to a sterile 15 ml centrifuge tube. Added the 6 ml of denaturing solution containing guanidine thiocyanate, CSB (Citrate/Sarcosine/β-Mercaptoethanol) and two potent inhibitors of RNase, as provided with RNAgents@ Total RNA isolation system (Promega) and homogenized by vigorously shaking for 5 minutes.

2.2.11.2. RNA extraction

Added 600 µl of 2 M sodium acetate (pH 4.0) and mixed thoroughly by inverting the tube 4-5 times. Then added 6 ml of phenol:chloroform:isoamyl alcohol to the tube and carefully mixed by inversion 3-5 times and then shake vigorously for 10 seconds and chilled on ice for 15 minutes. Centrifuged the tube at 10,000 x g for 20 minutes at 4°C and carefully removed the top

aqueous phase, containing RNA and proteins were remaining in the organic phase and at the interface.

2.2.11.3. RNA precipitation

Added an equal volume of the isopropanol to the collected aqueous phase and incubated the sample at -20°C for 5 minutes to precipitate the RNA and centrifuged the tube at 10,000 x g for 10 minutes at 4°C to get the pellet.

2.2.11.4. Washing of RNA

Washed the pellet by adding a minimum of 10 ml of ice-cold 75% ethanol and centrifuged at 10,000 x g for 10 minutes at 4°C. Air dried the pellet in an RNase-free environment for 20 minutes and dissolved the RNA with nuclease-free water.

2.2.11.5. mRNA isolation

In a sterile, RNase-free 1.5 ml tube, brought 0.1 – 1.0 mg of total RNA to final volume of 500 µl in RNase-free water and placed the tube in a 65°C heating block for 10 minutes. Then added 3 µl of the biotinylated-oligo (dT) probe and 13 µl of 20 x SSC to the RNA and mixed gently and incubated at room temperature until completely cooled.

2.2.11.6. Stock solution preparation

Prepared 1.2 ml of sterile 0.5 x SSC by combining 30 µl of 20 x SSC with 1.17 ml of RNase-free water in a sterile, RNase-free tube and prepared 1.4 ml of sterile 0.1 x SSC by combining 7 µl of 20 x SSC with 1.393 ml of RNase-free water in a sterile, RNase-free tube.

2.2.11.7. Washing of streptavidin-paramagnetic particles

Resuspended one tube of the streptavidin-paramagnetic particles (SA-PMPs) per isolation by gently flicking the bottom of the tube until they are completely dispersed, then captured them by placing the tube in the magnetic stand until the SA-PMPs have collected at the

side of the tube (approx. 30 seconds). Carefully removed the supernatant and washed the SA-PMPs three times with 0.5 x SSC (300 µl per wash), each time captured them using the magnetic stand and carefully removed the supernatant and finally resuspended the washed SA-PMPs, in 100 µl of 0.5 x SSC.

2.2.11.8. Capture and washing of annealed oligo (dT) mRNA hybride

Added the entire content of annealing section (500 µl total RNA + 3 µl biotinylated-oligo dT probe + 13 µl of 20 x SSC) to the tube containing the washed SA-PMPs. Incubated at room temperature for 10 minutes and gently mixed by inverting every 1-2 minutes. Captured the SA-PMPs using magnetic stand and carefully remove the supernatant without disturbing the SA-PMPs pellet. Washed the particles four times with 0.1 x SSC (300 µl per wash) by gently flicking the bottom of the tube until all of the particles are resuspended and after the final wash, removed supernatant completely.

2.2.11.9. Elution of mRNA

Resuspended the final SA-PMP pellet in 100 µl of theRNase-free water and gently resuspended the particles by flicking the tube. Magnetically captured the SA-PMPs and transferred the eluted mRNA to a sterile, RNase-free tube. Repeated the elution step by resuspending the SA-PMPs pellet in 150 µl of RNase-free water, repeated the capture step and pooled the eluate separately. Stored the mRNA eluate at -20°C for 10 minutes and concentrated by freeze-drier.

2.2.11.10. Reverse transcription

The reverse transcription reaction was performed with the enzyme MMLV (Molony murine leukemia virus) reverse transcriptase. The first strand cDNA synthesis reaction was performed by taking oligo-$(dT)_{26}$ with T_m value 59.4°C as a down stream primer. Prepared 15 µl

of the reverse transcription reaction mix containing 6.8 µl nuclease-free water, 4.0 µl 5 x reaction buffer, 1.2 µl 25mM $MgCl_2$, 1.0 µl 10 mM dNTP mix, 1.0 µl ribonuclease inhibitor, 1.0 µl MMLV reverse transcriptase in 0.2 ml PCR tube and 5 µl of RNA and primer mix added to it, giving the final reaction volume of 20 µl. Placed the tube in a controlled temperature heat block equilibrated at 25°C and incubated for 5 minutes. Then incubated the tube in a controlled temperature heat block at 42°C for up to one hour and finally incubated the reaction tube in a controlled temperature block at 70°C for 15 minutes to inactivate the reverse transcriptase.

2.2.11.11. Polymerase chain reaction

Polymerase chain reaction (PCR) is a common method of creating copies of specific fragments of DNA. Immediately after completion of reverse transcription reaction the possible resulting cDNA was used to amplify it through polymerase chain reaction using gene specific primer, based on the N-terminal sequence of purified protein. The six gene specific primers were designed based on the overlapping nucleotide sequence, percentage of GC content and melting temperature. The six different oligo nucleotides sequence were GATCCTTTACTCGACATC with Tm value of 51°C and percentage of GC content 44%, GACCCACTGTTAGACATC with Tm value of 52°C and GC content 50%, GATCCGTTGCTAGACATC with Tm value 55°C and GC content 50%, GACCCTTTACTGGATATC with Tm value 51°C and GC content 44%, GATCCACTGTTGGACATT with Tm value 55°C and GC content 44%, GACCCGTTACTCGATATA with Tm value 52°C and GC content 44%. PCR reactions were also performed after designing degenerate primer sequence GAICCICTITTIGAIATIAAIGGI with Tm value 58 °C. Prepared 50 µl of reaction mix containing 32.05 µl of nuclease-free water, 4.95 µl 10 x reaction buffer, 3.9 µl 25 mM MgCl2, 1.0 µl 10 mM dNTP, 3.3 µl gene specific

upstream primer, 3.3 µl oligo (dT)26 down stream primer, 0.5 µl Taq polymerase and 1.0 µl RT reaction. Placed the reaction mix in a thermal cycler that has been prewarmed to 94°C and the following PCR program was used: an initial denaturation at 95°C for 2 minutes and then 95°C for 30 seconds; 45-55°C for 30 seconds and 72°C for 1 minute over 30 cycles, the run ended with an extension at 72°C for 10 minutes followed by cooling to 4°C.

For the post-PCR analysis 5 µl from the PCR-reaction was mixed with 2 µl gel loading solution (GLS) and 3 µl TE-buffer pH 8.0 (10 mM Tris HCl and 1 mM EDTA) and loaded on 1% agarose gel containing ethidium bromide.

2.2.12. Solubility studies of MKTI

2.2.12.1. Solubility at different pH

For solubility studies, 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 hours. After the incubation, samples were centrifuged at 15,000 x g for 30 minutes. The supernatants were collected and the protein concentration was estimated.

2.2.12.2. Effect of temperature

For all experiment, unless otherwise specified, protein was dissolved in 50 mM Tris-HCl, pH 8.0 to a final concentration of 1mg/ml.

The solubility of MKTI was examined by incubating the protein at different temperatures ranging from 30-100°C for 30 minutes. After incubation, each aliquot was kept on ice for 15 minutes and centrifuged at 15,000 x g for 30 minutes. Supernatant was taken for the estimation of protein concentration and inhibitory activity. The effect of salt (NaCl) on thermal

stability of MKTI was studied by dissolving the protein in buffer containing different concentrations of salt before incubation in each temperature condition.

2.2.12.3. Effect of salts

To study the effect of salt on solubility at low pH, purified protein was dissolved in acetate buffer of pH 4.0 as described previously (Shee and Sharma, 2007). The precipitate was redissolved in same buffer containing different concentrations of salt (0 to 1000 mM of NaCl, KCl and $MgSO_4$). After incubation for 30 minutes, solutions were centrifuged at 15,000 x g for 30 minutes and protein concentrations were determined in supernatant.

The effect of salts like NaCl, $MgCl_2$, $(NH_4)_2SO_4$ on protein solubility was determined by incubating protein overnight in different concentration of salt solutions (1 – 4 M) in buffer of pH 8.0 at 4°C. After overnight incubation, solutions were centrifuged at 15,000 x g for 30 minutes and protein concentrations were determined in supernatant. The effect of NaCl on inhibitory activity of MKTI at pH 8.0 was also examined.

2.2.12.4. Effect of denaturants

Solubility properties of MKTI in different concentration of denaturant like guanidine hydrochloride (1 – 8 M) and urea (1 – 9 M) were determined by incubating the protein overnight at 20°C. After overnight incubation, solutions were centrifuged at 15,000 x g for 30 minutes. The protein concentrations and inhibitory activity were determined in supernatant.

2.2.12.5. Effect of organic solvents

Effect of organic solvents like ethanol, methanol, acetonitrile, ethylene glycol, dioxane, MPD on solubility of MKTI was determined by incubating the protein overnight in different percentages of organic solvents (0 – 90%) at 4°C. After overnight incubation, all aliquots were centrifuged at 15,000 x g for 30 minutes. The supernatant was taken for protein concentration

and inhibitory activity determination. The remaining precipitates in different samples were redissolved in the buffer of pH 8.0 for inhibitory activity measurements.

2.2.13. Effect of temperature on stability

All inhibitory activity measurements for stability studies were performed by hydrolysis of substrate BAPNA. Trypsin was dissolved in 0.001 M HCl containing 0.02 M $CaCl_2$ at the concentration of 200 µg per ml. Then 100 µl of the enzyme solution was incubated with 200 µl of the inhibitor solution in 0.05 M Tris-HCl buffer, pH 7.5, at 30°C for 10 min, and then 1 ml of 0.01 M BAPNA solution in the same buffer was added. After incubation at 30°C for another 10 min, 200 µl of 30% acetic acid was added to end the enzyme reaction, and the absorbance at 410 nm was measured on a spectrophotometer (Shibata et al., 1986). For thermal stability studies, purified protein was dissolved in 50 mM Tris-HCl, pH 8.0 to a final concentration of 1mg/ml and incubated at different temperatures (30 - 90°C) for 30 minutes. After incubation, each aliquot was kept on ice for 15 minutes and centrifuged at 15,000 x g for 30 minutes. Supernatant was taken for the estimation of trypsin inhibitory activity.

2.2.14. In-vivo thermal stability studies

The stability of this protein under *in-vivo* condition was investigated by incubating the mature intact seeds in different temperatures and comparing the trypsin inhibitory activity with untreated seeds. The single intact seeds with 250 mg of dry weight were taken in different eppendorf tubes and incubated at 30, 50, 70, 90 and 100°C in water bath for 30 minutes. After incubation, each tube was kept on ice for 15 minutes. Both heat treated and untreated seeds were crushed separately and soaked overnight in 1 ml of 50 mM Tris-HCl, pH 7.5 at 4°C. After overnight incubation, each aliquot was centrifuged at 15,000 x g for 30 minutes. Supernatant were taken for the estimation of trypsin inhibitory activity using substrate BAPNA as described

earlier. Supernatant was then heated to 90°C for 30 minutes and inhibitory activity was estimated.

2.2.15. Proteolysis studies

Native and heat treated proteins were taken for proteolysis studies. Purified protein was incubated with five different proteinases namely trypsin, papain, proteinase K, pepsin and chymotrypsin separately using a proteinase/MKTI molar ratio of 1:50 for different time periods ranging from 30 minutes to 24 hours at 30°C. Trypsin, chymotrypsin and proteinase K were incubated with MKTI in 50 mM Tris-HCl, pH 8.0, papain was incubated with MKTI in 50 mM sodium phosphate buffer, pH 6.9 and pepsin was incubated with MKTI in 100 mM acetate buffer, pH 3.5. BSA was taken as a positive control and incubated with proteinases in respective conditions at 1:100 molar ratios for 30 minutes. Also, MKTI and BSA were incubated in similar conditions without proteinases as a negative control. Proteolysis studies of heat treated MKTI were carried out after incubation of MKTI at temperatures ranging from 30 to 90°C for 30 minutes. Heat treated samples were incubated with enzymes using proteinase/MKTI molar ratio of 1:50 for 1 hour at 30°C. All samples were analyzed on a 15% SDS-PAGE.

2.3. Results

2.3.1. 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 (Shee and Sharma, 2007). 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 2.1A). 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 2.1B).

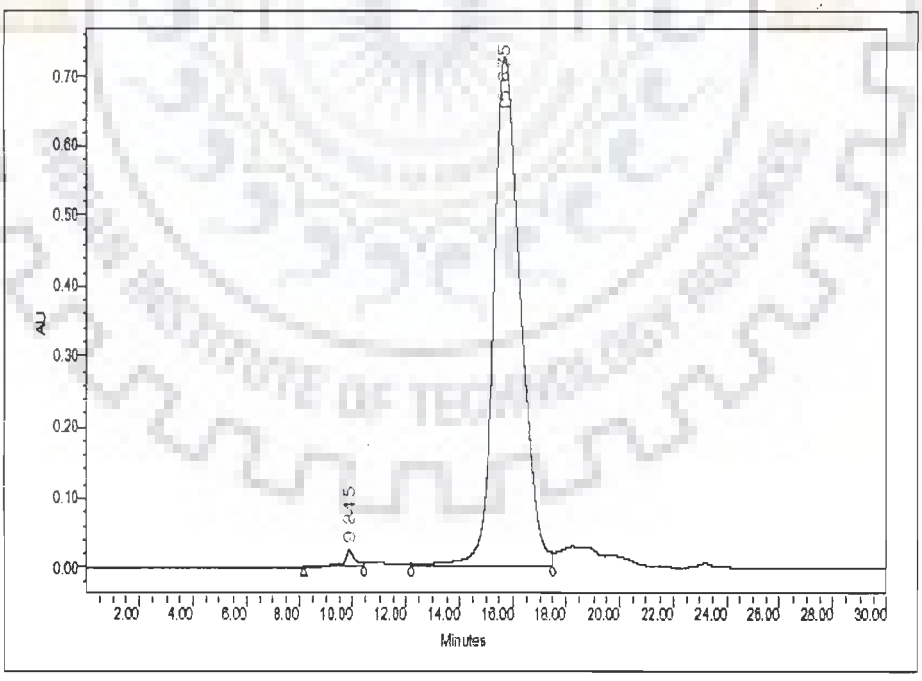


Figure 2.1A: Elution profile on HPLC gel filtration column

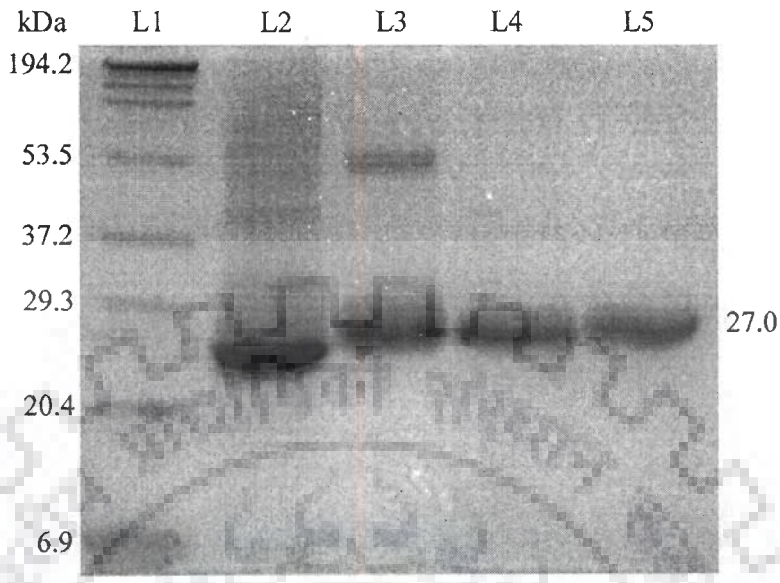


Figure 2.1B: SDS-PAGE analysis of the protein. L1, molecular weight markers; L2, total protein in buffer extract; L3, 100 mM NaCl fraction after anion exchange chromatography; L4, purified protein after gel filtration chromatography on HPLC.

2.3.2. Single-step purification of MKTI

The purification of protein was also carried out in one step only, by affinity chromatography using Cibacron blue 3 GA. Homogeneous supernatant collected after centrifugation of crude extract at 12000 x g was loaded on to a Cibacron blue 3 GA packed column equilibrated with 20 mM Tris-HCl, pH 8.0. Many proteins particularly high molecular mass proteins came in flow through and certain loosely bound proteins were removed by extensive washing with same buffer. After extensive washing, the bound proteins were eluted with 0.2 and 0.5 M NaCl in same buffer. In 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 is a single polypeptide chain. The Molecular mass of the purified protein was determined to be approximately 27 kDa on a 15% SDS-PAGE as earlier reported for the protein purified by anion-exchange and gel filtration chromatography (Figure 2.2).

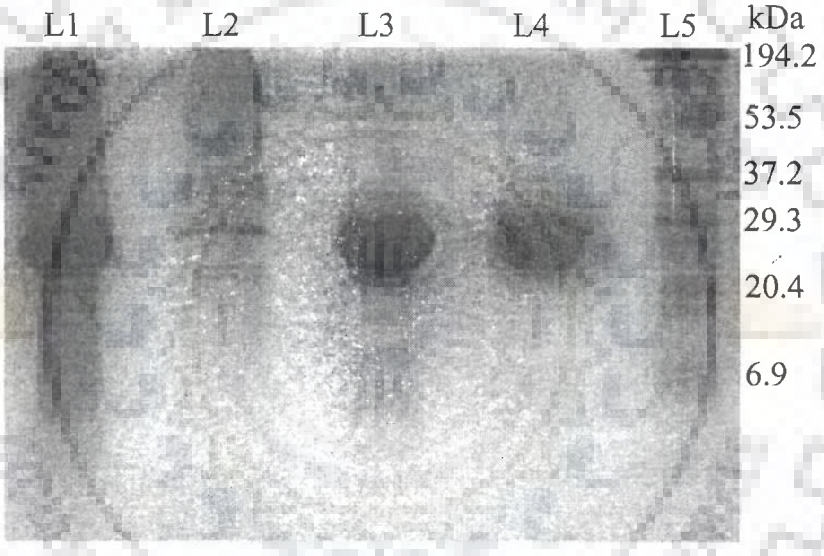


Figure 2.2: 15 % SDS-PAGE analysis of MKTI purified by Cibacron blue 3GA; Lane 1, crude extract, Lane 2, flow through; Lane 3, 0.2 M NaCl fraction; Lane 4, 0.5 M NaCl fraction; Lane 5, molecular weight marker.

2.3.3. MALDI-TOF

MALDI-TOF studies were performed at The Center for Genomic Application (TCGA), New Delhi, India. For MALDI-TOF analysis, 1 µl of the sample was mixed with 1 µl of saturated solution of matrix (sinnapinic acid in TA (0.1% TFA:Acetonitrile, 2:1) and spotted on MALDI target plate. MALDI-TOF analysis revealed the exact molecular mass of MKTI to be 21.4 kDa (Figure 2.3). In SDS-PAGE the molecular mass was determined to be approximately 27 kDa which may be because the protein runs higher in SDS-PAGE.

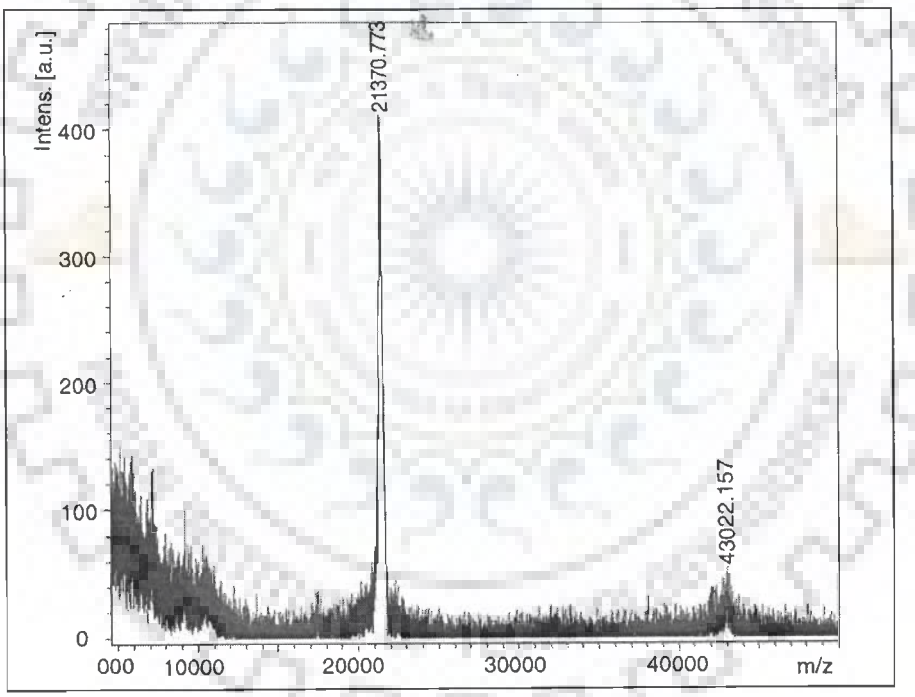


Figure 2.3: MALDI-TOF analysis showed the molecular mass of MKTI to be 21.4 kDa.

2.3.4. 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 2.4). In addition, the trypsin inhibition was determined at different pH conditions at a 1:1 molar ratio (Figure 2.5). A maximal trypsin inhibition was observed at pH 8.0, whereas the trypsin inhibition was reduced above and below this pH (Shee and Sharma, 2007).

The inhibitory activity of purified protein against trypsin was also determined by measuring the hydrolytic activity toward BAPNA. The protein completely inhibited bovine pancreatic trypsin at a molar ratio of 1:1.1 (Figure 2.6).

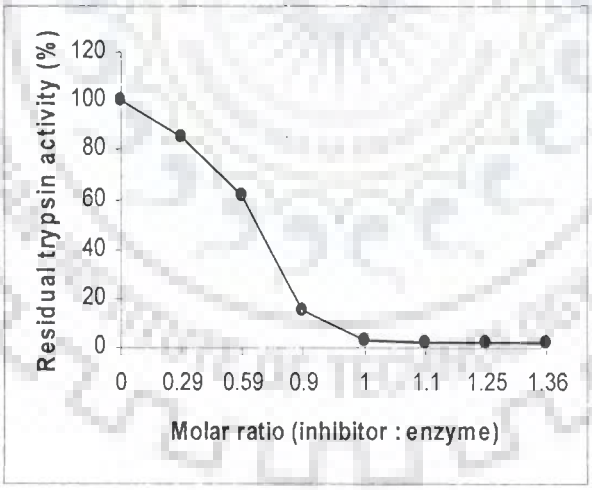


Figure 2.4: Trypsin inhibitory activity of *M. koenigii* trypsin inhibitor showing residual trypsin activity in percent as function of the inhibitor concentration at a fixed trypsin concentration using BAEE as substrate. All experiments were done three times and averaged.

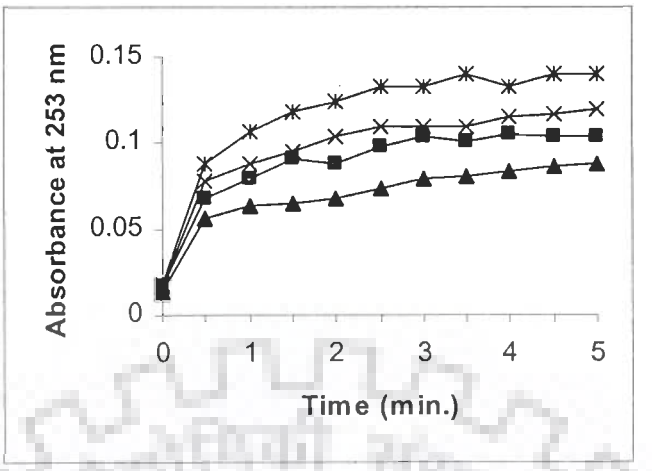


Figure 2.5: Trypsin inhibitory activity of *M. koenigii* trypsin inhibitor at different pH conditions in 50 mM Tris-HCl using BAEE as substrate. The concentration of trypsin and inhibitor were 4.4 x 10^{-7} M. The different pH taken were (✳) pH 8.8, (×) pH 7.5, (■) pH 8.4, and (▲) pH 8.0. All experiments were done three times at 30°C and averaged.

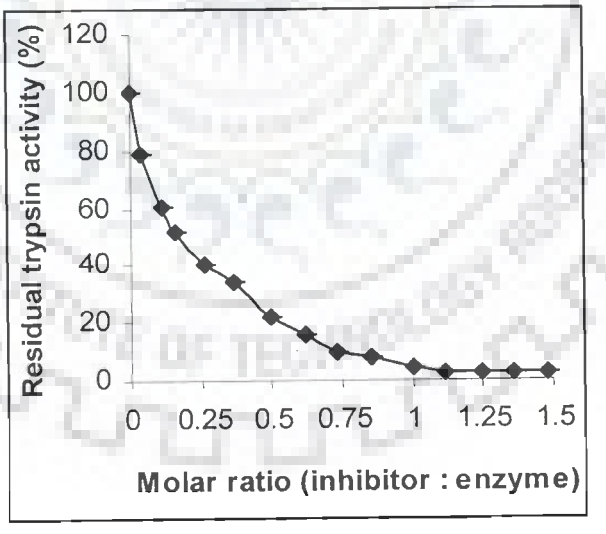


Figure 2.6: Trypsin inhibitory activity of *M. koenigii* trypsin inhibitor showing residual trypsin activity in percent as function of the inhibitor concentration at a fixed trypsin concentration using BAPNA as substrate. All experiments were done three times at 30°C and averaged.

2.3.5. Ki determination

The Ki value and mode of inhibition of the inhibitor was determined using BAPNA as a substrate (Shee and Sharma, 2007). The Ki value obtained from a Dixon plot was found to be 7 x 10^{-9} M. The analysis of Dixon plot showed that the two lines corresponding to each substrate intersect above the x-axis, which is a characteristic of competitive inhibition (Figure 2.7).

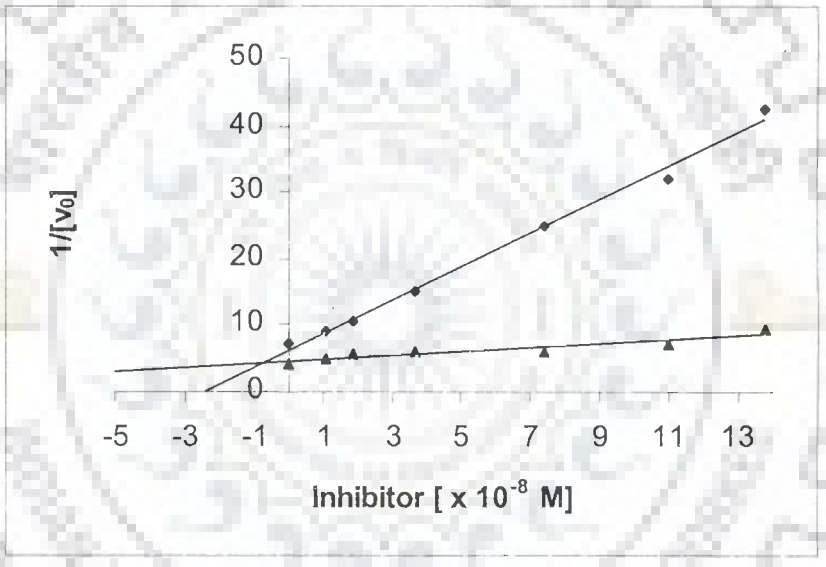


Figure 2.7: Dixon plot for the determination of the dissociation constant (Ki) value of MKTI at two different concentration of BAPNA. Final concentrations of substrate were 0.003 M (■) and 0.006 M (▲). The reciprocals of velocity were plotted against different concentrations of MKTI.

2.3.6. Chymotrypsin inhibition

MKTI did not show any inhibitory activity against chymotrypsin. The studies with BSA also confirmed our results. Though proteolytically resistant against chymotrypsin and trypsin, it did not show any inhibitory effect on the hydrolysis of BSA by chymotrypsin (Figure 2.8).

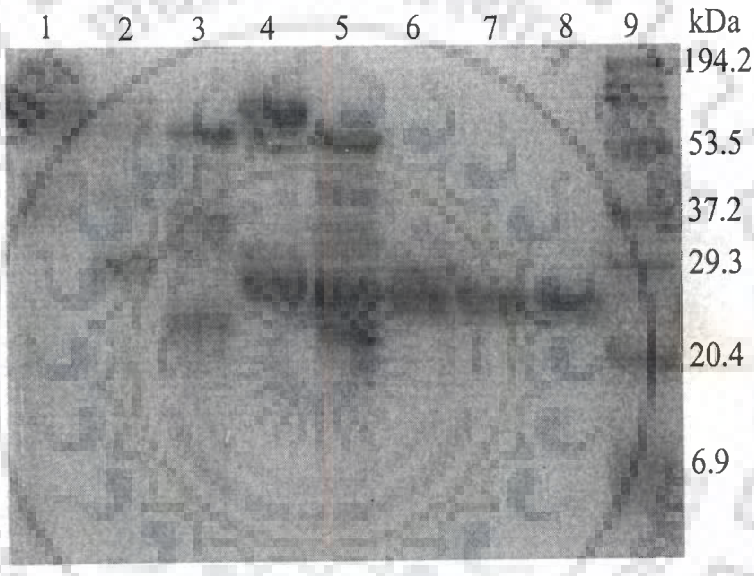


Figure 2.8: Inhibitory effect of MKTI against trypsin and chymotrypsin using BSA as substrate on 15 % SDS-PAGE. Lane 1, BSA; 2, BSA incubated with trypsin; 3, BSA incubated with chymotrypsin; 4, BSA incubated with trypsin and MKTI complex; 5, BSA incubated with chymotrypsin and MKTI complex; 6, MKTI incubated with trypsin; 7, MKTI incubated with chymotrypsin; 8, pure MKTI; 9, Molecular weight marker.

2.3.7. Total protein concentration

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

2.3.8. 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 *Murraya koenigii* seeds, during different stages of seed development and germination periods, were obtained from variable band intensities on SDS-PAGE gels under reducing condition using Quantity One 1D gel analysis software (version 4.5.2; Biorad). The electrophoretic patterns of different stages of seed development and germination showed most intense protein band at 27 kDa which expressed rapidly during mid maturation stage of seed development (Figure 2.9A) and was utilized during seed germination (Figure 2.9B). At initial stages during seed development, the protein content of this band was estimated to be 5.27% of total soluble protein after seven d of flowering. After 19 days, during mid-maturation stage, the protein concentration increased from 5.5% to 8.5% within 25 days and then peaked to 18.75% in 37 days. The final protein concentration of this band was estimated to be 20% of total soluble protein which is stored till germination. During the time of germination, this major protein degrades slowly from 20% to 12% in 13 days but with in next three days this protein decreased to 7% and then to 2% with in next six days (Figure 2.10A).

Trypsin inhibitory activity, in soluble protein extract of seeds, was monitored during different stages of seed development and germination. During seed developmental 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 extract of seeds collected at 7, 13, 19, 25, 31, 37, 43, 49 and 55 days respectively. The sharp reduction in percentage of residual trypsin activity was observed during mid maturation stage. During the different stages of germination process, it was observed that the residual trypsin activity increased gradually upto13 days from day of seeding and then a sharp increase in residual trypsin activity was observed after that. 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 (Figure 2.10B).

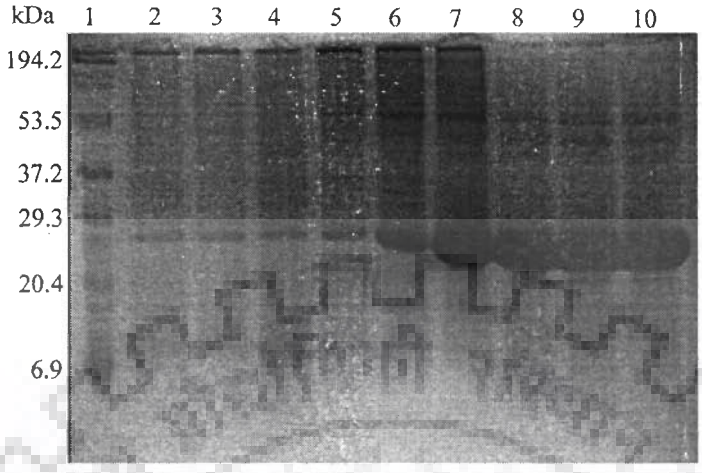


Figure 2.9A: Protein profiling of *Murraya koenigii* seeds at different stages of seed development after flowering on a 15% SDS-PAGE; Lane 1, molecular weight marker; Lane 2, 7 days; Lane 3, 13 days; Lane 4, 19 days; Lane 5, 25 days; Lane 6, 31 days; Lane 7, 37 days; Lane 8, 43 days; Lane 9, 49 days; Lane 10, 55 days.

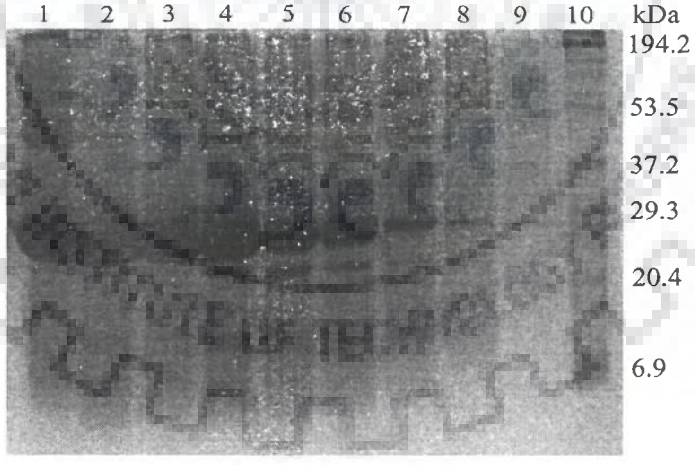


Figure 2.9B: Protein profiling *Murraya koenigii* seeds at different stages of seed germination on a 15% SDS-PAGE; Lane 1, mature seeds; Lane 2, 1 day; Lane 3, 4 days; Lane 4, 7 days; Lane 5, 10 days; Lane 6, 13 days; Lane 7, 16 days; Lane 8, 19 days; Lane 9, 22 days; Lane 10, molecular weight marker.

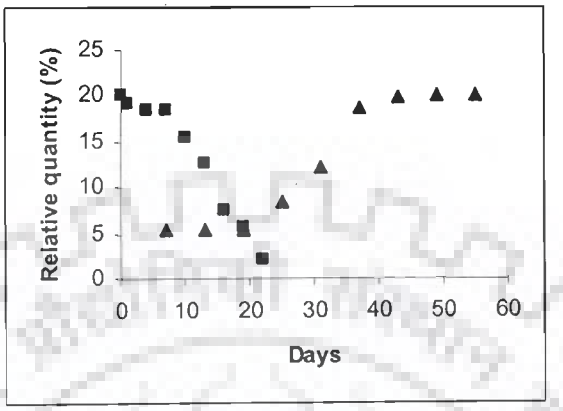


Figure 2.10A: Relative quantity of MKTI protein band of 27 kDa from 15% SDS-PAGE at different stages of seed development and germination. (■) percent quantity at seed germination stage; (▲) percent quantity at seed developmental stage.

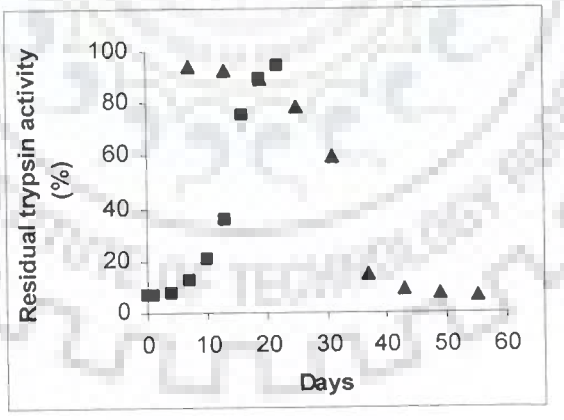


Figure 2.10B: Residual trypsin activity against crude extract in *Murraya koenigii* seeds at different stages of seed development and germination; (■) seed germination; (▲) seed development. All experiments were done three times and averaged.

2.3.9. N-terminal sequencing

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, short sequence search showed significant homology with 72% identity (8/11) to a Kunitz-type *Erythrina variegata* chymotrypsin inhibitor (ECI) precursor (Figure 2.11).

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 2.11: 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.

2.3.10. Internal sequence analysis by MALDI-TOF-TOF

The partial internal amino acid sequencing of MKTI was performed at The Center for Genomics Applications, New Delhi, India (TCGA) by MALDI-TOF/TOF studies after tryptic digestion and alkyl reduction. MALDI-TOF-TOF studies provided many new insights into the nature of MKTI and also supported our findings. In total, six peptides of various lengths were obtained after performing the experiment two times. In first attempt, three peptides were sequenced. In second attempt, five peptides were sequenced of which two were repeated. The peptides were randomly called peptide 1 to 6 and contained 19, 22, 22, 15, 9, 11 amino acids respectively. The homology search under NCBI BLAST short sequence search for each fragment separately showed significant homology to many serine proteinase inhibitors, storage proteins and homeodomain like proteins. The peptide 1 showed significant homology to miraculin-like protein, a member of Kunitz inhibitor family, isolated from various *Citrus* species, like *Citrus shiranuhi* (84% identity), *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 miraculin like protein from different *Citrus* species like *Citrus shiranuhi* (100% identity), *Citrus jambhiri* (77% identity) and vegetative storage protein from *Litchi chinensis* (72% identity) and also additionally with many trypsin inhibitors like 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 like *Arabidopsis lyrata subsp. Petraea* (100% identity), *Arabidopsis thaliana* with 86% identity. It also showed significant homology with 81% identity to trypsin inhibitor from *Brassica napus*. Peptide 4 showed

homology to proteinase inhibitor of cereal super family isolated from various cereals like *Oryza sativa* (100% identity), *Triticum aestivum* (73% identity), *Hordeum vulgare* (73% identity). The peptide 5 showed homology mostly to squash family of inhibitors with almost 100% identity to *Lagenaria leucantha*, *Lagenaria siceraria*, *Luffa acutangula*, *Citrulus lanatus* with the reactive site RI of squash family inhibitor present in this stretch. Also, the two cysteine residues of peptide 5 were almost conserved in many members of Kunitz inhibitor family. Interestingly, peptide 6 did not show significant homology to any of the trypsin inhibitor; rather it showed significant homology to homeodomain like protein from *Oryza sativa* (100% identity), *Medicago truncatula* (90% identity), *Lycopersicon esculentum* (90% identity) and *Solanum tuberosum* (90% identity) and many other DNA binding proteins (Figure 2.12). The six peptides along with N-terminal sequence obtained earlier (Shee and Sharma, 2007) were assigned the relative positions in MKTI sequence based on homology to Kunitz family inhibitors with reference to conserved residues. Sequence comparison of partial MKTI sequence with other Kunitz family inhibitors showed certain residues or motifs conserved despite lot of variations in rest of the sequence. There were only 8 out of 113 residues found completely conserved in MKTI sequence. Peptide 1 to 6 were placed at amino acid numbers (STI numbering) 34, 105 (first two residues WF present in peptide 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 similarity. In peptide 2, sequence stretches WFITTGG and WFK were completely identical to miraculin like protein CJI, a member of Kunitz family inhibitors but did not show any resemblance to other members of Kunitz family. In peptide 3 and 4, stretch FLVIEF and TEW showed resemblance to many Kunitz family inhibitors respectively. 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 (Figure 2.13).

	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	WFITTGGVEGNPGAQTLKNWFK	22	
	CSM	128	WFITTGGVEGNPGAQTLKNWFK	149	100%
	CJM	137	WFITTGGLDGHPGAETLLNWFK	158	77%
	PBTI	128	WFVTTGGEEGNPGIDTLTNWFK	149	77%
	LCSP	135	WFITTNGVIGNPGSKTIHNWFK	156	72%
C)	P3	1	SVSTLAVSVILFLVIFEMPEIK	22	
	ALTI	5	SVSTLAVSVILFLVIFEMPEIK	26	100%
	ATTI	5	SVSTLAVFAILFLVIVEMPEIK	26	86%
	BNTI	7	SVSSLAAFLILFLVIFEMPEIE	27	81%
D)	P4	1	TEWPELVGLTIEQAK	15	
	HVCI	23	TEWPELVGKSVEEAK	37	73%
	TAPI	23	TEWPELVGKSVEEAK	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%

Figure 2.12: Sequence homology studies of six peptides by NCBI BLAST short sequence search. CSM, *Citrus shiranuhi* 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* subitilisin-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.


PTI MKST---LLAWFTFLLFAFVLSVPSIEASTEPVLDIQGEELKAGTEYIISSIFWGAGGGD 57
TCI MKTATAVVLLLFAFTSKSYFFGVAN--AANSPVLDTDGDELQTGVQYYVLSSISGAGGGG 58
CJI MKISLATTLSFLILALASNSLLVLGTSSVPEPLLDVNGNKVESTLQYYIVSAIWGAGGGG 60
ETI ----------VLLDGNGEVVQN-GGTYYLLPQVWAQGGG 28
WTI ----------EPLLDSEGELVRN-GGTYYLLPDRWALGGG 29
STI ----------DFVLDNEGNPLEN-GGTYYILSDITAFGG- 28
BVI ----------DTLLDTDGEVVRNNGGPYYIIPAFRGNGGG 30
ATI ---MTKTTKTMNPKFYLVLALTAVLASNAYGAVVDIDGN-AMFHE-SYYVLPVIRGRGGG 55
OSI ------MVSLRLPLILLSLLAISFSCSAAPPPVYDTEGH-ELSADGSYYVLPASPGHGGG 53
MTI --MKHFLSLTLSFFIFVFITNLSLATSNDVEQVLDINGNPIFPGGQYYILPALRGPGGGG 58
MKTI ----------IDPLLDINGNVVEAAXXXXXXXXXXXXXXXX 31
PTI VA---ATNKTCPDDVIQYSLDLLQGLPVTFSPASSEDDVIRVSTDLNIKFS-----IKKA 109
TCI LALGRATGQSCPEIVVQRRSDLDNGTPVIFSNADSKDDVVRVSTDVNIEFVPI---RDRL 115
CJI VSLHGGRNGYCPLDVIQLPSDTQNGIKLTLSPYNN-STIVRESADLNLRFSVLL--SGRD 117
ETI VQLAKTGEETCPLTVVQSPNELSDGKPIRIESRLR-SAFIPDDDKVRIG------FAYAP 81
WTI IEAAATGTETCPLTVVRSPNEVSVGEPLRISSQLR-SGFIPDYSLVRIG------FANPP 82
STI IRAAPTGNERCPLTVVQSRNELDKGIGTIISSPYR-IRFIAEGHPLSLKFDS-FAVIMLC 86
BVI LTLTRVGSETCPRTVVQASSEHSDGLPVVISALPR-SLFISTSWRVTIQ------FVEAT 83
ATI LTLAGRGGQPCPYDIVQESSEVDEGIPVKFSNWRLKVAFVPESQNLNIETDV-----GAT 110
OSI LTMAPR-VLPCPLLVAQETDERRKGFPVRFTPWGGAAAPEDRTIRVSTDVRIRFNAATII 112
MTI VRLGRTGDLKCPVTVLQDRREVKNGLPVKFTIPGISPGIIFTGTPLEIEYTK-----KPS 113
MKTI XXXXXGRNELCPLDVVQLSSDLQKGLPESAVSVLRXXXXXXXXXXXXXXXXXXXXXXXX 91
PTI CDRSSVWKIQKSSNSEVQ-WLVTTGGEEGNPGCDTFTNWFKIEKAGVLG---YKLVYCPE 165
TCI CSTSTVWR-LDNYDNSAGKWWVTTDGVKGEPGPNTLCSWFKIEKAGVLG---YKFRFCPS 171
CJI YCNEQPLWKVDNYDAASGKWFITTGGLDGHPGAETLLNWFKLEKIGNFPGT-YKIVHCPS 176
ETI KCAPSPWWTVVEDEQEGLS----VKLSEDES-TQFDYPFKFEQVS-DQLHS-YKLLYCEG 134
WTI KCAPSPWWTVVEDQPQQPS----VKLSELKSTKFD-YLFKFEKVT-SKFSS-YKLKYCA- 134
STI VGIPTEWSVVEDLPEGPA---VKIGENKDAMDG-----WFRLERVSDDEFNNYKLVFCPQ 138
BVI -CIPKPSFWHIPQDSELEGA---VKVGAS-DERFP-LEFRIERVS---EDT-YKLMHCSS 133
ATI CIQSTYWRVG--EFDHERRQYFVVAGPKPEGFGQDSLKSFFKIEKSGEDA--YKFVFCPR 167
OSI CVQSTEWHVGDEPLTGARR---VVTGP-LIGPSPSGRENAFRVEKYG-GG--YKLVSCR- 163
MTI CAASTKWLIFVDNVIGKACIGIGGPENYPGVQTLKGKFNIQKHASGFG----YNLGFCVT 169
MKTI XXXXTEWPELVGLTIEQAKWFITTGGVEGNPGAQTLKNWFKXXXXXXXXXXXXXXXXCPR 151
PTI DICPSVGLCRDVGIYFESNRGRILSLSDKLSPFLVVFKKVGPLSSSI----- 212
TCI VCDSCTTLCSDIGRHSDDDGQIRLALSD--NEWAWMFKKASKTIKQVVNAKH 221
CJI VCESCVKLCNNVGRSFEDGVRRLVLVRDDEPAFPVVLIPATERSTSV----- 223
ETI KHEKCAS----IGINRDQK-GYRRLVVTEDYPLTVVLKKDESS--------- 172
WTI KRDTCKD----IGIYRDQK-GYARLVVTDENPLVVIFKKVESS--------- 172
STI QAEDDKCG--DIGISIDHDDGTRRLVVSKNKPLVVQFQKLDKESL------- 181
BVI TSDSCRD----LGISIDEE-GNRRLVVRDENPLLVRFKKANQDSEK------ 174
ATI TCDSGNPK--CSDVGIFIDELGVRRLAISDKPFLVMFKKANVTEVSSKTM-- 215
OSI --DS------CQDLGVSRDGARA-WLGASQPPHVVVFKKARPSPPE------ 200
MTI GSPTCLDIG-----RFDNDEAGRRLNLTEHEVYQVVEVDAATYEAEYIKSVV 216
MKTI IYMECKXXXXXXXXXXXXXXXSVSTLAVSVILFLVIFEMPEIKXX------- 195

Figure 2.13: Multiple sequence alignment of Kunitz family inhibitors with stretches of MKTI sequences based on homology with reference to conserved residues using. 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 variegate* inhibitor; MKTI, *Murraya koenigii* trypsin inhibitor

2.3.11. cDNA cloning

Attempts at isolating RNA from the seeds of *Murraya koenigii* have been successful. The $A_{260/280}$ ratio was approximately 1.5. The messenger RNA was also purified by using oligo-dT magnetic beads. Both total RNA and mRNA were used for RT PCR experiment using oligo-dT and several genespecific primers but the reaction was unsuccessful.

2.3.12. 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 below pH 7.5, while it is completely soluble at and above pH 7.5 (Figure 2.14). 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.

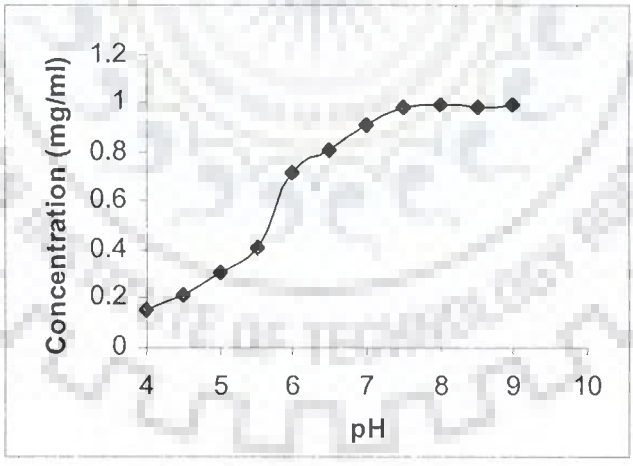


Figure 2.14: Solubility studies of *M. koenigii* trypsin inhibitor at different pH conditions. The protein was dissolved in buffers of different pH (50 mM sodium acetate pH 4.0, 4.5, 5.0, 5.5; 50 mM sodium phosphate pH 6.0, 6.5, 7.0; 50 mM Tris-HCl pH 7.5, 8.0, 8.5, 9.0) to a final concentration of 1 mg/ml. All experiments were done three times and averaged.

2.3.13. Effect of salts on solubility of MKTI at different pH conditions

Earlier, we have reported that the solubility of MKTI decreases constantly below pH 7.5 (Shee and Sharma, 2007). Here, the effect of salts was examined on the MKTI solubility at pH 4.0 where around 85% of protein precipitation was observed. The protein precipitate gradually redissolved with the increasing amount of different salts. The solubility of MKTI increased with increasing concentration of salt at pH 4.0. Solubility of MKTI increased from 15% at 0 mM NaCl to 35% at 100 mM NaCl and the 100% solubility was observed at 1000 mM NaCl (Figure 2.15). Similar results were obtained with $MgCl_2$. The addition of NaCl and $MgCl_2$ up to 4 M did not show any salting out effect at pH 8.0. However, $(NH_4)_2SO_4$ showed the salting out effect above 2 M with around 97% of soluble protein getting precipitated at and above 3 M of $(NH_4)_2SO_4$ (Figure 2.16).

There was no significant effect on activity of this protein in the presence of different concentration of salt (0 – 1000 mM NaCl) at pH 8.0. The increasing concentration of salt slightly reduced the inhibitory activity of MKTI.

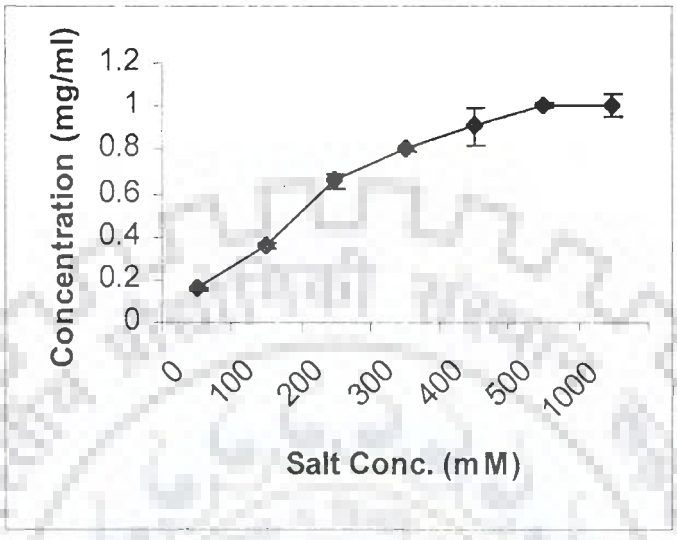


Figure 2.15: Effect of NaCl on solubility of MKTI at pH 4.0.

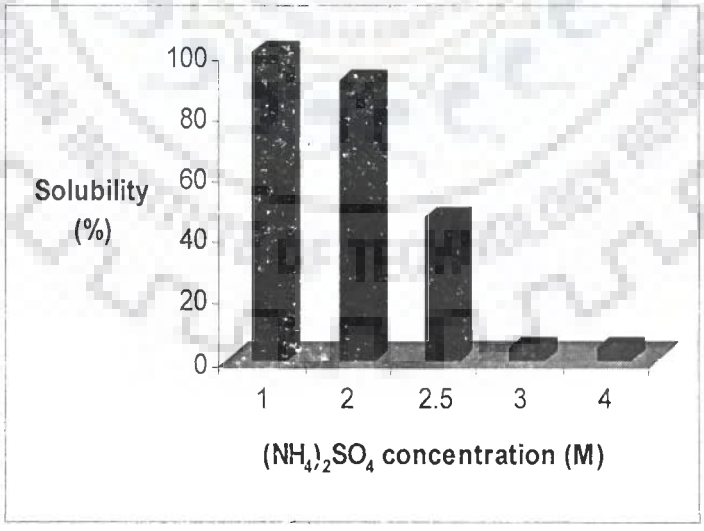


Figure 2.16: Salting-out effect of $(NH_4)_2SO_4$ on solubility of MKTI at pH 8.0.

2.3.14. Effect of denaturant and organic solvents

Although the denaturant like guanidine hydrochloride and urea did not show any effect on solubility of MKTI, however, the stability of MKTI was affected. The inhibitory activity of MKTI in urea and guanidine hydrochloride was retained till 6.0 M concentration. In 8.0 M urea and guanidine hydrochloride, almost 50 and 40% activity was retained respectively.

All organic solvents, except ethylene glycol, precipitated MKTI to different degrees at different concentrations. No protein precipitation was observed with ethylene glycol even at 90% concentration. In ethanol, only around 7% of protein precipitation was observed up to 50% concentration but the degree of precipitation increased sharply to 92% at 70% concentration. Similar results were obtained with methanol and MPD where 22 and 35% protein precipitation was observed at 50% concentration and 72 and 99% precipitation was observed at 70% concentration respectively. In acetonitrile, the precipitation of MKTI increased from 17% at 30% acetonitrile to 72 and 99% at 50% and 90% acetonitrile concentration respectively. Highest level of precipitation was observed in dioxane where almost 87% of protein was precipitated at 30% dioxane concentration (Figure 2.17). In all cases, the inhibitory activity of precipitated protein was almost completely regained after redissolving the precipitate in a buffer of pH 8.0.

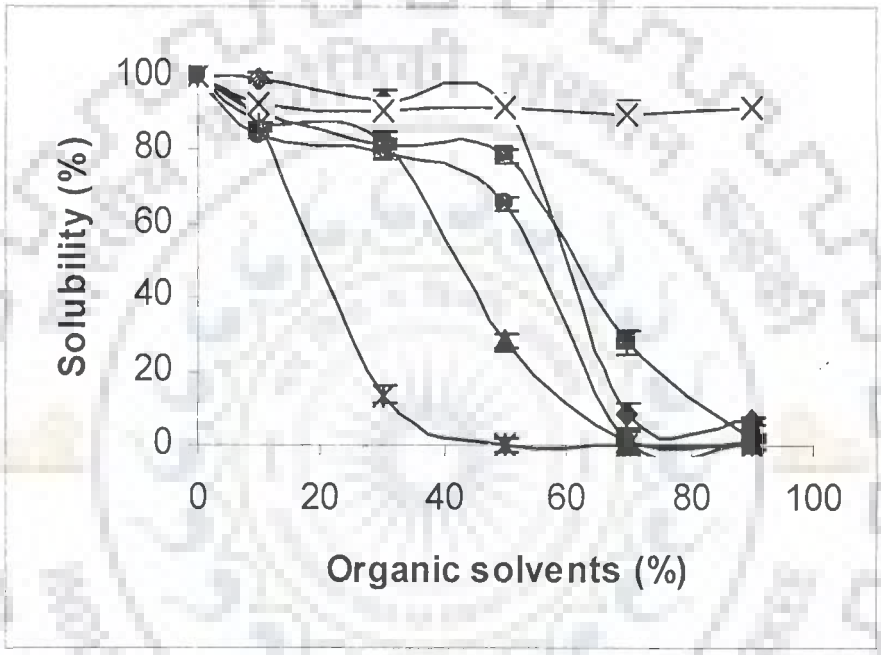


Figure 2.17: Solubility of MKTI as function of different organic solvents. (×) Ethylene glycol, (◆) Ethanol, (■) Methanol, (●) MPD, (▲) Acetonitrile, (Ж) Dioxane

2.3.15. Effect of temperature on solubility of MKTI

The solubility of MKTI was examined at different temperatures ranging from 30 to 100°C in the absence and presence of different concentrations of NaCl. The results showed that the solubility of MKTI decreases above 50°C with solubility decreasing to 75, 60 and 50% at temperatures of 70, 90 and 100°C respectively. Efforts to resolubilize the precipitate were not successful. Interestingly, the presence of different concentrations of NaCl (0-1 M) enhanced the rate of precipitation at higher temperatures (Figure 2.18).

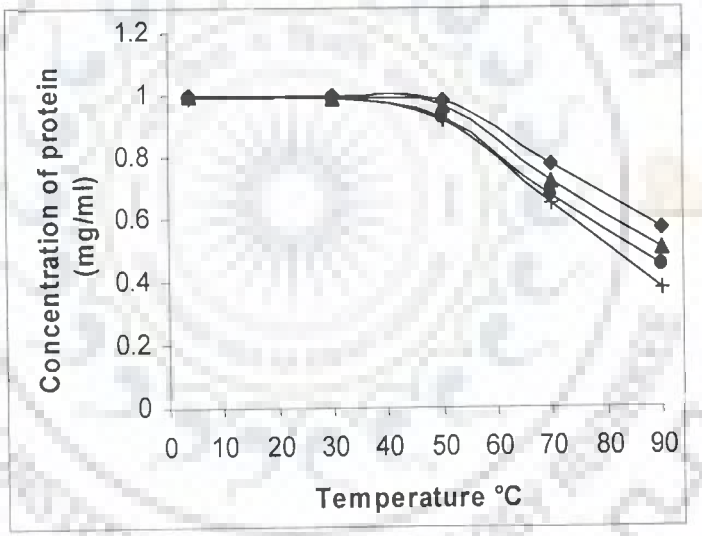


Figure 2.18: Effect of NaCl on solubility of MKTI at increasing temperatures. (◆) 0 M NaCl (▲) 0.2 M NaCl (●) 0.5 M NaCl (×) 1 M NaCl.

2.3.16. Effect of temperature on stability

The trypsin inhibitory activity of purified MKTI was determined at different temperatures ranging from 30°C to 90°C. The inhibitory activity measurements of heat treated protein showed that the inhibitory activity reduced continuously with increasing temperature with a sharp reduction above 50°C. Inhibitory activity reduced by 15, 28, 53 and 95% at temperatures of 40, 50, 70 and 90°C respectively (Figure 2.19).

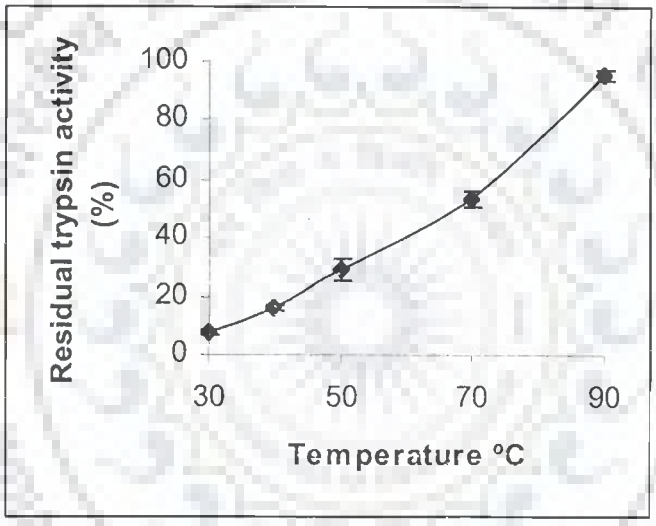


Figure 2.19: Effect of temperature on inhibitory activity of MKTI. Purified inhibitor was pre-incubated at different temperature and inhibitory activity was determined at equimolar concentration.

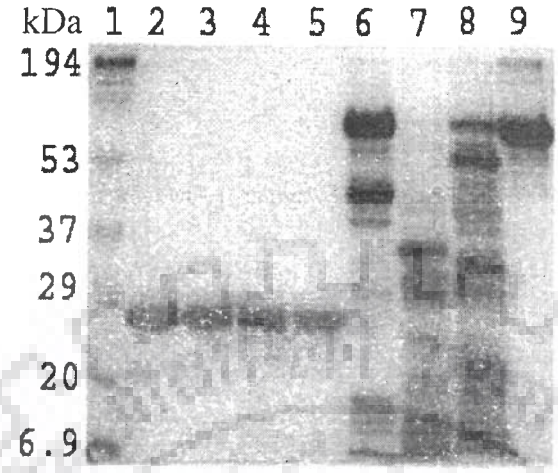
2.3.17. In-vivo thermal stability studies

In-vivo thermal stability studies were performed to observe the differences in inhibitory activity of MKTI at broad range of temperature under *in-vivo* and *in-vitro* conditions. Interestingly, no loss in inhibitory activity of heat treated seeds was observed, under *in-vivo* conditions, up to temperature of 100°C. In comparison, the inhibitory activity of purified MKTI was almost completely lost at 90°C. Also, the inhibitory activity of heat-treated supernatant obtained from seeds was completely lost at 90°C.

2.3.18. Proteolysis studies

The structural stability of MKTI was examined by proteolysis studies with five proteinases, trypsin, papain, proteinase K, chymotrypsin and pepsin. Native MKTI was found to be completely resistant to proteolysis for all times tested from 30 minutes to 24 hours at proteinase/protein molar ratio of 1:50. Bovine Serum Albumin, used as positive control, was quite susceptible to proteolysis against all five proteinases at 1:100 molar ratios (Figure 2.20). The thermal stability of MKTI towards proteolytic degradation was also explored by incubating the MKTI at range of temperatures from 30 to 90°C for 30 minutes prior to digestion with proteinases. The results demonstrated that the heat treated MKTI at all temperatures was completely resistant to proteolytic degradation by trypsin, chymotrypsin, pepsin and papain but was found susceptible to proteolysis by proteinase K when heat treated at 90°C (Figure 2.21).

A



B

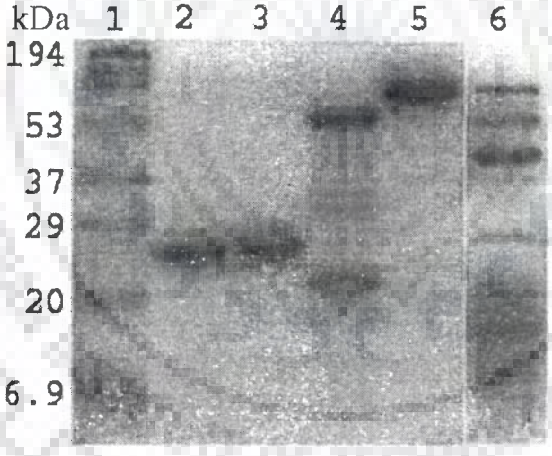


Figure 2.20: Proteolysis studies of native MKTI in presence of trypsin, proteinase K, papain, chymotrypsin and pepsin at 50:1 ratio as determined on 15% SDS-PAGE. BSA was taken as a positive control at 100:1 ratio. **A)** L1, MW marker; L2 – L4, MKTI with trypsin, proteinase K and papain respectively; L5, pure MKTI; L6 - L8, BSA with trypsin, proteinase K and papain respectively; L9, pure BSA. **B)** L1, MW marker; L2, MKTI with chymotrypsin; L3, MKTI with pepsin; L4, BSA with chymotrypsin; L5, BSA; L6, BSA with pepsin.

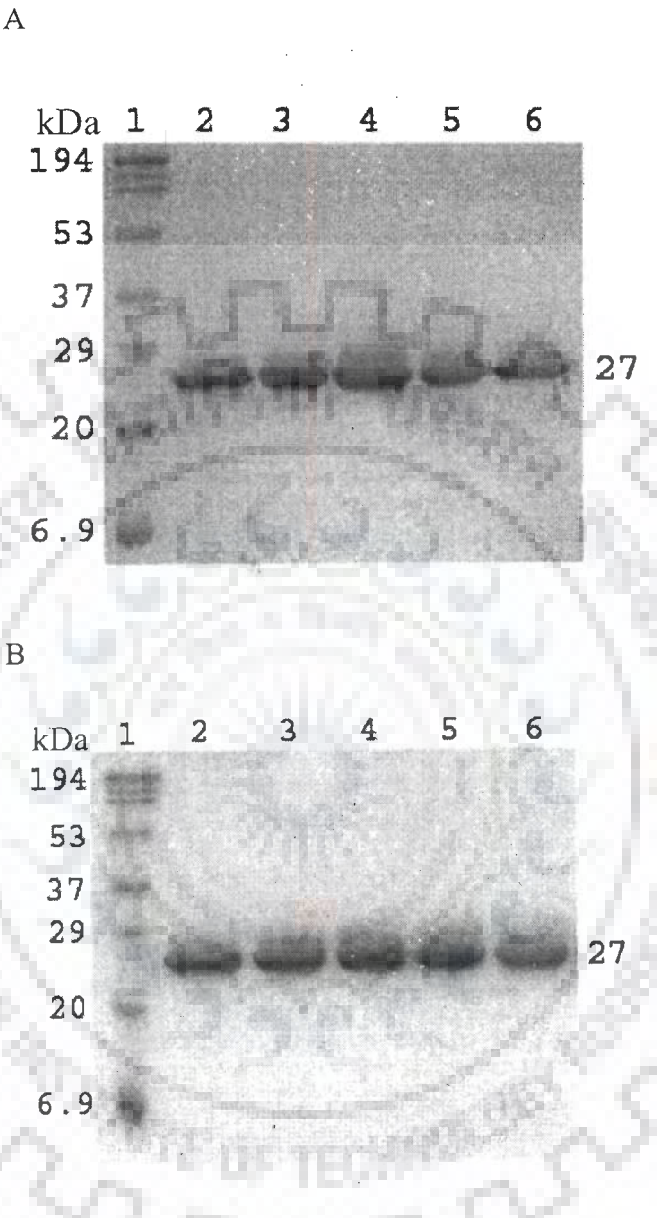


Figure 2.21: Temperature dependent proteolysis studies of MKTI in presence of different proteinases namely trypsin (A), papain (B) on a 15% SDS-PAGE. In each gel, L1, Molecular weight marker; L2, 90°C heated MKTI with proteinases; L3, 70°C heated MKTI with proteinases; L4, 50°C heated MKTI with proteinases; L5, 30°C heated MKTI with proteinases; L6, MKTI without proteinases.

Figure 2.21: Temperature dependent proteolysis studies of MKTI in presence of different proteinases namely proteinase K (C), pepsin (D) on a 15% SDS-PAGE. In each gel, L1, Molecular weight marker; L2, 90°C heated MKTI with proteinases; L3, 70°C heated MKTI with proteinases; L4, 50°C heated MKTI with proteinases; L5, 30°C heated MKTI with proteinases; L6, MKTI without proteinases.

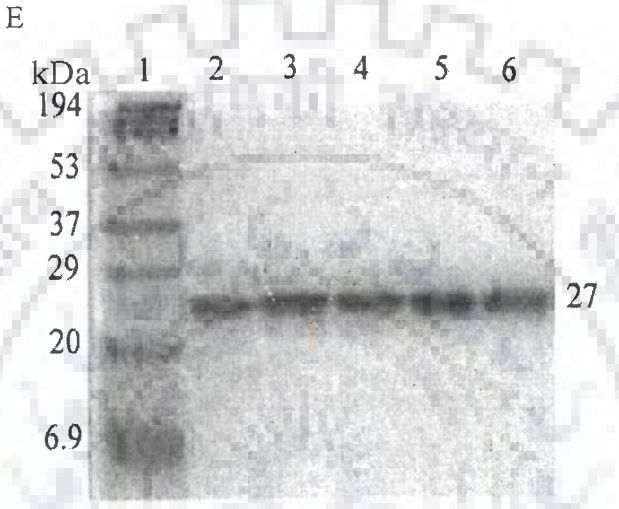


Figure 2.21: Temperature dependent proteolysis studies of MKTI in presence of chymotrypsin (E) on a 15% SDS-PAGE. L1, Molecular weight marker; L2, 90°C heated MKTI with proteinases; L3, 70°C heated MKTI with proteinases; L4, 50°C heated MKTI with proteinases; L5, 30°C heated MKTI with proteinases; L6, MKTI without proteinases.

2.4. Discussion

Murraya koenigii is a known herbal plant with many medicinal properties including anti-diabetic and is widely used in India in food preparations. Till date, no protein has been characterized from this plant. In this chapter, the isolation and biochemical characterization of a trypsin inhibitor from seeds of *Murraya koenigii* is described. The purification was achieved by two different methods. In first method, 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 second step was performed on HPLC gel filtration column. In second method, 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 and Burdick, 1983). Interestingly, one of the peptides, peptide 6, showed strong resemblance to homeodomain like proteins, which are DNA-binding proteins, but not to the trypsin inhibitors. This may be one possible explanation for high affinity of the protein to this column. There are only a few reports of trypsin inhibitors which have been purified on an affinity column (Potempa et al., 1989; Kuehn et al., 1984). The approximate molecular mass of the protein was determined to be 27 kDa from SDS-PAGE analysis. However, MALDI-TOF analysis revealed the molecular mass to be 21.4 kDa. This clearly indicates that protein runs higher in SDS-PAGE due to unknown reasons. This observation has also been made in the case of 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 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 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 precipitating. Kinetic studies demonstrate that, like other serine proteinase inhibitors, MKTI is a competitive inhibitor with a dissociation constant of 7×10^{-9} M.

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 patterns taken at different stages of development clearly showed that MKTI band at 27 kDa was the major protein in the seed. The expression of this protein started slowly and then peaked at mid-maturation stage to approximately 20% of total soluble protein. During seed germination, the protein degrades gradually in first thirteen days from around 20% to 12% and then degrades rapidly to 7% in next three days and finally to 2% in next six days. Comparing the two physiological processes of seed development and germination, the profiles looks opposite but similar. The trypsin inhibitory activity in the supernatants at different stages of either seed development or germination also showed respective patterns. The inhibitory activity increased during seed development as the relative protein concentration increased while it decreased during germination with decrease in amount of protein. Many trypsin inhibitors have been earlier reported to be seed storage proteins (Yeh et al., 1997; Mandal et al., 2002).

The amino acid sequence analysis revealed many insights into the nature of this protein. Though the protein has strong trypsin inhibitory activity, the N-terminal sequence showed no significant homology to any of the known trypsin inhibitors. However, a NCBI BLAST short sequence search showed homology to a chymotrypsin inhibitor from *Erythrina variegata*

belonging to the Kunitz-type proteinase inhibitor family. Despite the N-terminal sequence homology, MKTI did not show any chymotrypsin inhibitory activity. Considering the molecular weight of MKTI and its significant homology to one of the inhibitors belonging to the Kunitz-type inhibitors, this protein belongs to the Kunitz-type proteinase inhibitor family.

In partial internal sequencing by MALDI-TOF-TOF, 6 peptides totaling 98 residues were obtained. Including N-terminal 15 residues, total of 113 residues were obtained. Sequence comparison of partial MKTI sequence with other Kunitz family inhibitors showed certain residues or motifs conserved despite lot of variations in rest of the sequence. There were only 8 out of 113 residues found completely conserved in MKTI sequence. Apart from Kunitz family inhibitors, individual peptides showed high degree of homology to other proteins also. Peptide 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. Peptide 3, 4 and 5 showed homology to trypsin inhibitors from *Arabidopsis* sp and *Brassica napus*, cereal superfamily and squash family inhibitors respectively. The homology of 9 residues of peptide 5 was 100% with squash family inhibitors with the reactive site RI of squash family inhibitor present here, a feature unique to MKTI and completely absent in other members of Kunitz family. Another interesting feature was that peptide 2 showed homology to only 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 miraculin like protein. The fact that both have been isolated from Rutaceae family plant supports this observation. The sequencing data along

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

The solubility and stability studies at different physicochemical conditions demonstrated the differences in structural and functional stability of MKTI. On heating, the protein starts precipitating irreversibly after 50°C and approximately 50% of protein is precipitated at 100°C. The efforts to redissolve this protein at low temperature were not successful. It seems that protein aggregation along with denaturation leads to irreversible precipitation. It was also observed that the precipitation of MKTI increased in presence of different concentrations of salt at higher temperature. It has been shown that the loss of solubility is an indication of aggregation or denaturation (Shen, 1976). Also, protein aggregation occurs on heating and is enhanced by salt (Koshiyama, 1972; Wolf and Tanura, 1969). The inhibitory activity of purified MKTI decreased continuously with increasing temperature by 15% at 40°C to 95% at 90°C and a sharp decline in inhibitory activity was observed above 50°C which demonstrated that MKTI is functionally unstable at higher temperature. However, the inhibitory activity of MKTI was not affected under *in-vivo* conditions at high temperature. Interestingly, the inhibitory activity in seeds was completely retained even after heating to 100°C for 30 minutes. It seems that there are certain stabilizing factors in the seeds which preserve the conformation of MKTI required for activity. The loss of inhibitory activity in heat-treated supernatant obtained from seeds clearly indicated that inhibitor is stable only under *in-vivo* conditions.

The solubility studies at different pH conditions showed unusual solubility properties of this protein. MKTI was soluble at and above pH 7.5, but solubility decreased as pH was

lowered. Around 85% of protein was precipitated at pH 4.0 (Shee and Sharma, 2007). The inhibitory activity of the precipitated protein was regained completely when dissolved in buffer of pH 8.0. This observation demonstrates that though the protein is precipitated at lower pH, the relaxation in structure does not affect protein resolubilization and regaining of activity at pH 8.0. The precipitation at lower pH seems to be due to aggregation of protein molecules because of change in electrostatic interaction. It has been reported that the pH dependent effects in protein stability are mainly electrostatic in nature and originate from changes in the protonation status of acidic and basic residues (Yang & Honig, 1993; Schaefer et al., 1997; Ullmann and Knapp, 1999). It is interesting to note that the precipitation at lower pH is functionally reversibly as compared to the irreversible precipitation at higher temperatures where protein could not be resolubilized. This clearly demonstrates that the nature of aggregation is different in active and inactive form and thereby suggesting that there are certain differences in interactions among protein molecules in two conditions. The presence of salt at different concentrations helped in resolubilizing the protein at low pH. At pH 4.0, the solubility of precipitated protein increased with increase in the salt concentration with complete resolubilization at 1000 mM NaCl concentration. Here, we observe two contrasting effects of salt on this protein in two different conditions; one is slating-in effect in case of low pH, while other is salting-out effect at high temperature condition. In presence of different concentration of organic solvents like ethanol, methanol, acetonitrile, dioxane, MPD, the protein was precipitated reversibly to different degrees with almost complete precipitation at 90% of organic solvent concentration. The inhibitory activity of the protein was regained when it was redissolved in buffer of pH 8.0. Only ethylene glycol did not precipitate MKTI even at 90% of concentration.

Proteolysis studies of both native and heat treated MKTI showed that it is highly resistant to proteolytic degradation. It was found to be completely stable against proteinases trypsin, chymotrypsin, papain and pepsin up to temperature of 90°C. With proteinase K, MKTI was completely resistant to proteolytic degradation up to 70°C. Proteolysis results clearly suggest that the structure of MKTI is quite compact and rigid even at high temperatures. It seems that the conformational changes do occur in the protein at high temperature but the rigidity of molecular structure is still maintained. Relating the proteolysis results of native and heat treated MKTI to its inhibitory activity, it can be safely said that the conformational changes responsible for the inhibitory activity does not disrupt the overall structural stability of protein. These conformational changes in MKTI are not large enough up to 90°C for proteolysis by all the proteinases except for susceptibility to proteinase K, a non specific proteinase, above 70°C.

In summary, the results clearly indicate that MKTI possesses additional properties other than trypsin inhibitory activity. MKTI was found to be the most abundant protein in the seeds of *Murraya koen*igii plant. The protein profiling during seed maturation and germination demonstrated that *Murraya koen*igii trypsin inhibitor is a major seed storage protein. MKTI showed affinity to Cibacron Blue 3GA resin. The reason for the affinity of MKTI to Cibacron Blue 3GA resin is not very clear except for the homology of peptide 6 to DNA binding proteins. The partial internal amino acid sequencing, with six peptides totaling 98 residues, showed resemblance to trypsin inhibitors, storage proteins and homeodomain like proteins. The MKTI showed unusual solubility properties. Two important phenomenons were observed in the solubility studies of MKTI. One was the reversible precipitation at low pH and other precipitant and irreversible precipitation at high temperature. The inhibitory activity was regained in case of reversible precipitation at pH 8.0. Second was the salting-in effect at low pH and salting-out

effect at high temperature. The presence of salt helps in resolubilization of protein at low pH while it enhances the precipitation at high temperature. The MKTI is highly resistant to proteolytic degradation indicating a compact structure.

CHAPTER 3

BIOPHYSICAL CHARACTERIZATION

3.1. Introduction

To understand structure-activity relationship, it is important to study different aspects of inhibitor in relation to structural and functional stability in different physiological conditions and their three-dimensional structures. The stability of inhibitor can be studied as a function of temperatures, pH, salts and detergents using various biophysical methods. Circular dichroism and fluorescence spectroscopy are very important tools to evaluate the secondary structure and conformational stability under various physichochemical conditions. The X-ray crystallographic analysis provides conformational details of protein structure and its interaction at atomic level. To understand the structure-function relationship of Murraya koenigii trypsin inhibitor circular dichroism and fluorescence studies were performed under different physicochemical conditions. In CD studies secondary structure analysis were performed at different temperatures and in presence of guanidine hydrochloride and NaCl. In fluoresecence studies, both ANS (8-anilino-1-naphthalene sulfonate) and tryptophan fluorescence were performed at different temperature, pH, salts, and detergents to understand the folding/unfolding nature of this protein. Cystallization and preliminary X-ray diffraction study of MKTI was performed. The conformational changes occurring at different physiological conditions, as analyzed by circular dichroism and fluorescence helped in correlating structure to the function of this inhibitor.

3.2. Materials and Methods

3.2.1. Materials

Protein was purified as described earlier. ANS (8-anilino-1-naphthalene sulfonate) and PEG 8000 were purchased from Sigma-Aldrich Pvt. Ltd. All other chemicals were purchased from Qualigens fine chemicals.

3.2.2. Circular Dichroism spectroscopic studies

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. All solutions were filtered with a 0.22 μm pore nylon filter before performing the experiments. Far-UV CD spectra, (200 - 250 nm), were recorded in 1 cm path length quartz cell at a protein concentration of 0.25 mg/ml in 20 mM sodium phosphate buffer at pH 7.5. The effect of temperature on conformational stability of MKTI was determined by measuring CD spectra at temperatures ranging from 20 to 80°C at 10°C interval for 30 minutes. Far-UV CD spectra at increasing temperatures could not be recorded below 216 nm because of bad signal to noise ratio. The effect of different concentration of guanidine hydrochloride (0, 2, 4, 6 and 8 M) and NaCl (0, 1, 2, 3, 4 and 5 M) on conformational stability of MKTI was also determined by measuring the CD spectra in the range of 206 – 250 nm. Three consecutive scans were accumulated and the average spectra stored. The results of all the CD measurements are expressed as mean residue ellipticity [θ] in deg cm^2 $dmol^{-1}$ using the relation: $[\theta] = [\theta] Mo / 10cl$, where [θ] is the observed ellipticity in millidegrees, *Mo* is the mean residue weight of the protein (*Mo* = 110), c is the protein concentration (mg/cm^3), and l is the path length (cm). The percentages of different secondary structures (α-helix, β-sheet, turn and random coil) were estimated using the data provided by Yang et al., (1986) for the reference spectra.

3.2.3. Fluorescence measurements

Fluorescence measurements were performed on a Varian Cary Eclipse spectrofluorimeter at a protein concentration of 1.5 μM. The ANS fluorescence measurements were performed in different physicochemical conditions, like different HCl concentration ranging from 0 – 2 M,

different pH values, ranging from 2.0 to 12.0 (pH 2.0-3.0, glycine-HCl buffer; pH 4.0-5.0, sodium acetate buffer; pH 6.0-7.0, sodium phosphate buffer; pH 8.0-9.0, Tris-HCl buffer; pH 10.0-12.0, sodium carbonate buffer), different NaCl concentrations at pH 2.0 (0 – 4 M), $CaCl_2$ concentrations at pH 2.0 (0 – 2 M), $(NH_4)_2SO_4$ concentrations at pH 2.0 (0 – 2 M) and detergents like urea (0 – 9 M), guanidine hydrochloride (0 – 8 M) and β-mercaptoethanol (0 - 5 M). Proteins were incubated in these different conditions for overnight and the ANS was incubated 10 minutes before the measurement of fluorescence intensity. For ANS binding experiments, the molar ratio of protein and ANS was 1:50. The excitation wavelength was set at 360 nm and the emission spectra were measured in the range 400 – 650 nm using an excitation and emission slit widths of 5.0 and 10.0 nm, respectively.

ANS fluorescence studies as a function of different temperature was also performed. ANS (8-anilino-1-naphthalene sulfonate) dye was dissolved in 100 mM Tris-HCl, pH 8.7, to a concentration of 50 mM. The final concentration of protein in the reaction mixture was 1.5 μM and ANS concentration was 75 μM. The fluorescence was read immediately at different temperatures ranging from 20 to 90°C at 10°C interval for 15 minutes on Spectrofluorimeter (Horiba Jobin Yvon, Model FL3-21) controlled by peltier thermal accessories (Model LF1-3751) using an excitation wavelength of 360 nm and emission spectra were measured in the range 420-700 nm using an excitation and emission slit width of 5.0 nm.

The tryptophan fluorescence measurements were also performed in presence of SDS (0 – 9%) at a protein concentration of 1.5 μM. The excitation wavelength was set at 280 nm and the emission spectra were measured in the range 290 – 400 nm using an excitation and emission slit widths of 5.0 nm.

3.2.4. Crystallization and data collection

Crystallization experiments were performed using the sitting-drop vapor diffusion method at 293 K. Crystals of MKTI were grown in 4 µl drops, containing equal volumes of protein (25 mg ml^{-1} in 100 mM Tris-HCl, pH 8.0) and reservoir solution. The reservoir solution consists of 6% (w/v) PEG 8000, 100 mM Tris-HCl buffer pH 8.0, 80 mM NaCl, 80 mM $CaCl_2$ and 8% (v/v) glycerol. The crystals of MKTI appeared in 4 days and grew to a maximum dimension of 0.10 x 0.10 x 0.75 mm in 10 days. A single crystal was mounted in a 0.5 mm capillary using reservoir buffer. Diffraction data were collected on a MAR345 imaging-plate system using Cu Kα radiation generated by a RIGAKU rotating-anode generator at the Biophysics Department, All India Institute of Medical Sciences, New Delhi, India. The data collection was performed at 293 K with a crystal-to-detector distance of 220 mm and 1° oscillation per image. The data were processed with DENZO and SCALEPACK (Otwinowski and Minor, 1997).

3.2.5. Structure determination and refinement

The structure was solved by molecular replacement with the MOLREP program (Vagin and Teplyakov, 1997) from the CCP4 software suite (Collaborative Computational Project, Number 4, 1994; Dodson et al., 1997) using the *Erythrina caffra* Kunitz type trypsin inhibitor (PDB code 1TIE) as a search model (Onesti et al., 1991). The residues of search model were replaced with alanine using program PDBSET (Collaborative Computational Project, Number 4, 1994; Dodson et al., 1997). The rotation function was calculated with diffraction data from 25 to 2.9 Å resolution within a sphere radius of 20 Å. The first peak in the output of rotation function gave distinct solution. This solution was used for translation function calculations. It gave correlation coefficient of 21.2% and an R factor of 61.1%. Rigid-body refinement and restrained

refinement were carried out with REFMAC (Collaborative Computational Project, Number 4, 1994) and model building with the program O (Jones et al., 1991). The 5% of the total reflections selected randomly was used for free R calculations (Brünger, 1992).The partial sequence obtained from N-terminal and internal sequencing was fitted into the electron density. Only those residues were placed where the electron density was very clear.

3.3. Results

3.3.1. Circular dichroism spectroscopy

To get insight into the secondary structural elements of the MKTI, far-UV circular dichroism studies (wavelength range of 250 – 200 nm for native and 250 – 216 for heat treated protein) were performed in the temperature range of 20°C to 80°C (Figure 3.1). Efforts to record data below 216 nm at increasing temperatures were not successful because of excessive noise possibly due to protein aggregation. Analysis of circular dichroism spectra at pH 8.0 revealed that native MKTI contains approximately 30.1% α-helix, 46% β-strand, 16.2% turn and 6.9% random-coil structure. There was remarkable change in the conformation of MKTI at higher temperatures. A gradual loss in α-helical content of MKTI was observed with increasing temperatures while β-strand structure almost remained unchanged. The percentage of α-helices reduced to 29.6, 28.0, 23.1, 20.0, 17.8 and 11.1% at temperatures of 30, 40, 50, 60, 70 and 80°C respectively. There was only minor change observed in percentage of β-strand from 46.0% at 20°C to 41.8% at 80°C. The CD studies under different concentration of guanidine hydrochloride (0 – 8 M) showed that there was a slight decrease in molar ellipticity with increasing concentrations (Fgure 3.2). At different concentration of NaCl, it was observed that the CD spectra of MKTI were variable. With increasing concentration of NaCl upto 2 M, there was a

slight increase in the ellipticity while further increase in NaCl concentration upto 5 M decreased the CD content (Figure 3.3).

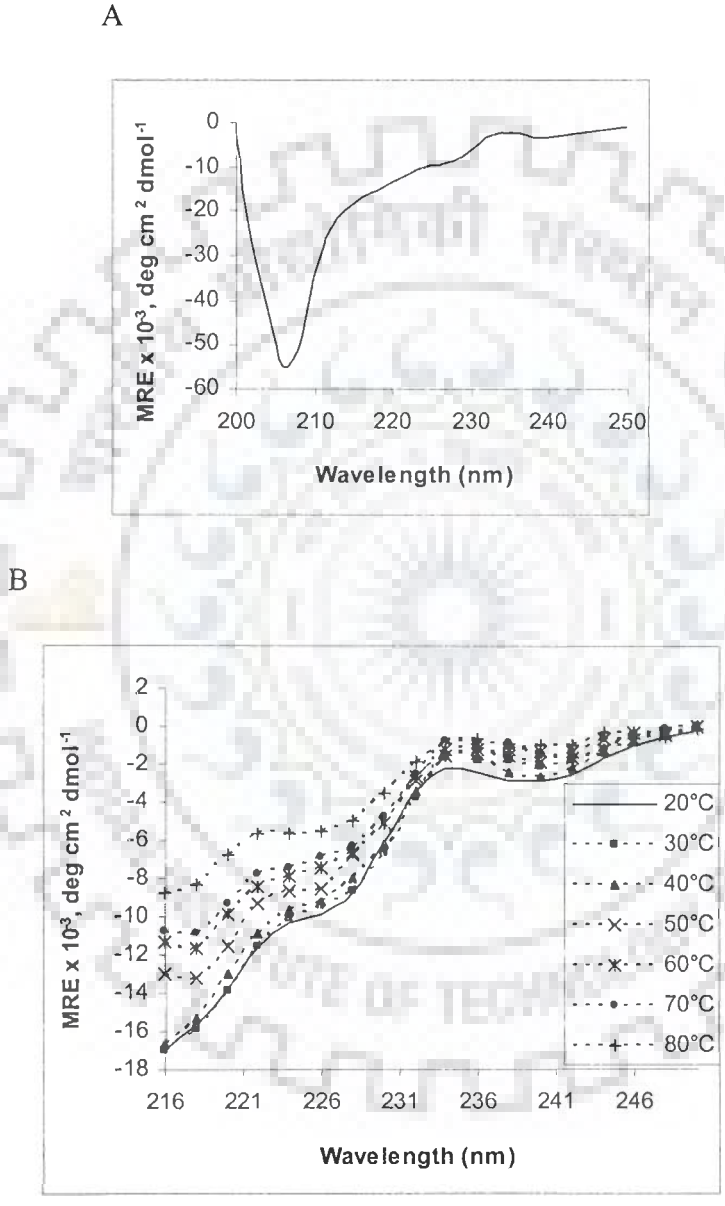


Figure 3.1: A) Far-UV CD spectra of native MKTI in 20 mM sodium phosphate buffer at pH 7.5. B) Far-UV CD spectra of MKTI in 20 mM sodium phosphate buffer at pH 7.5 as a function of temperature ranging from 20 to 80°C.

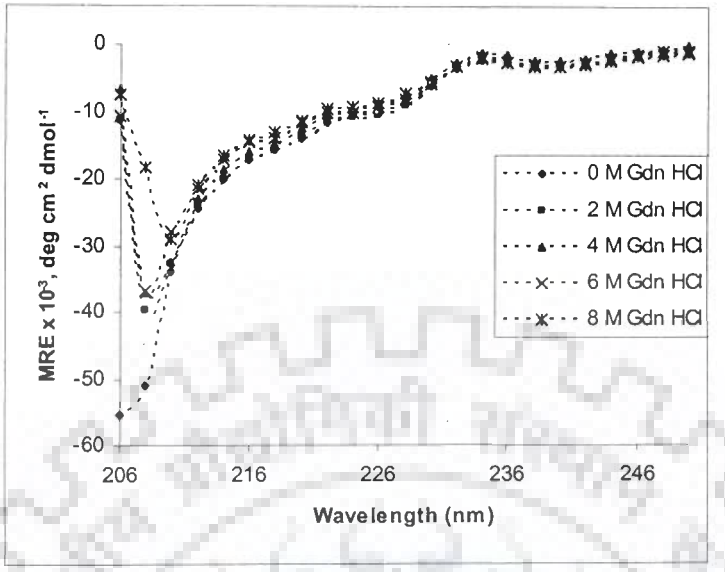


Figure 3.2: Far-UV CD spectra of MKTI in 20 mM sodium phosphate buffer at pH 7.5 as a function of different concentration of guanidine hydrochloride ranging from 0 to 8 M.

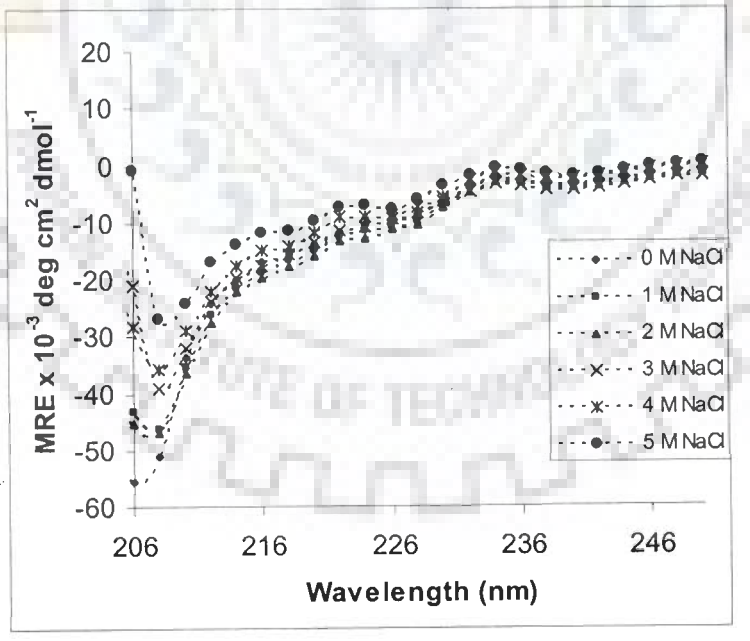


Figure 3.3: Far-UV CD spectra of MKTI in 20 mM sodium phosphate buffer at pH 7.5 as a function of different concentration of NaCl ranging from 0 to 5 M.

3.3.2. Fluorescence measurements

ANS fluorescence experiments were performed in different physicochemical conditions to monitor the extent of changes in native structure of MKTI and relate them to the inhibitory activity. ANS fluorescence intensity in the presence of MKTI during thermal denaturation study from 20 to 90°C is shown in Figure 3.4. There is negligible increase in fluorescence up to 50°C indicating minimal change in the structure of the native protein. However, a linear increase in fluorescence intensity with increase in temperature above 50°C was observed without any melting transition up to 90°C. The linear increase in fluorescence intensity at increasing temperatures indicates that protein may have partially relaxed at higher temperature.

In different pH conditions ranging from pH 2.0-12.0, the fluorescence intensity of ANS at 490 nm was found to be highest at pH 2.0 with sharp decrease from pH 3.0 – 5.0 and then remains almost constant till pH 12.0 with highest intensity at 525 nm (Figure 3.5). These results indicate a gradual relaxation in the structure of MKTI below pH 5.0 with substantial blue shift occurring at pH 2.0. In presence of different concentration of NaCl, $CaCl_2$ and $(NH_4)_2SO_4$ at pH 2.0, the decrease in fluorescence intensity was observed with increasing concentrations of salts. The fluorescence intensity decreased till 1.5 M and then remained stable up to 2.5 M NaCl concentration. However, further increase in NaCl concentration above 2.5 M resulted in an increase in fluorescence intensity also (Figure 3.6). The divalent salts like $CaCl_2$ and $(NH_4)_2SO_4$ also helped in stabilizing the structure of MKTI at pH 2.0 where a sharp decrease in ANS fluorescence intensity was observed up to 100 mM concentration and then gradual decrease in intensity was observed up to 2 M (Figure 3.7, 3.8). The ANS fluorescence measured at various concentration of HCl showed a constant increase in ANS fluorescence with increasing concentration of HCl up to 0.7 M indicating a gradual unfolding of the native structure. Above

0.7 M HCl, the intensity decreased significantly which indicate the complete denaturation of protein (Figure 3.9). At different concentrations of urea (0 to 9 M) and guanidine hydrochloride (0 to 8 M), a minor increase in fluorescence intensity was observed with increasing concentration of urea and guanidine hydrochloride (Figure 3.10, 3.11). At different concentration of β-mercaptoethanol, a sharp increase in fluorescence intensity was observed after 0.4 M concentration with blue shift in wavelength maxima from 525 to 508 nm (Figure 3.12).

The tryptophan fluorescence was performed in case of SDS as ANS fluorescence could not been done for the same. With increasing concentration of SDS from 0 to 9% the fluorescence intensity decreased continuously with significant red shift in wavelength maxima from 325 to 350 nm (Figure 3.13).

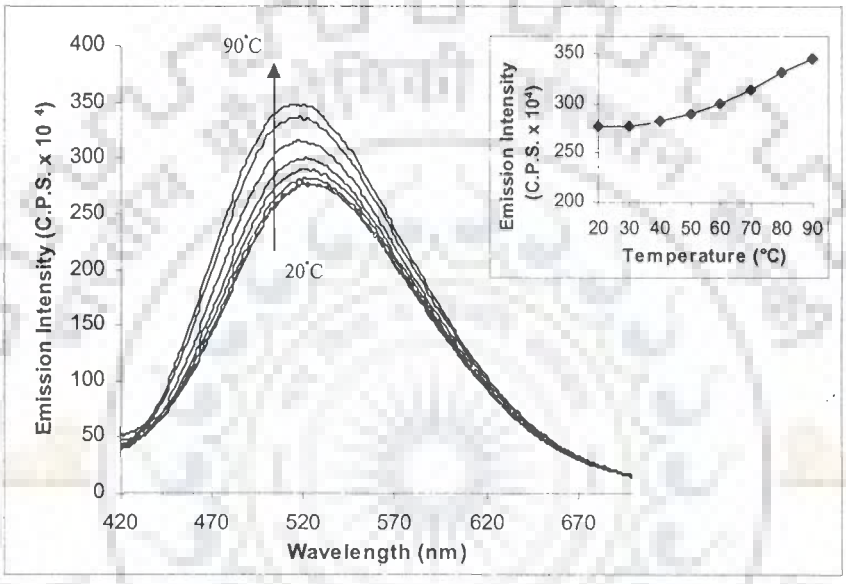


Figure 3.4: ANS fluorescence spectra of MKTI, as a function of temperature ranging from 20 to 90°C. Excitation was at 360 nm. The maximum emission intensity in counts per second (CPS) was in the range of 525 nm to 528 nm.

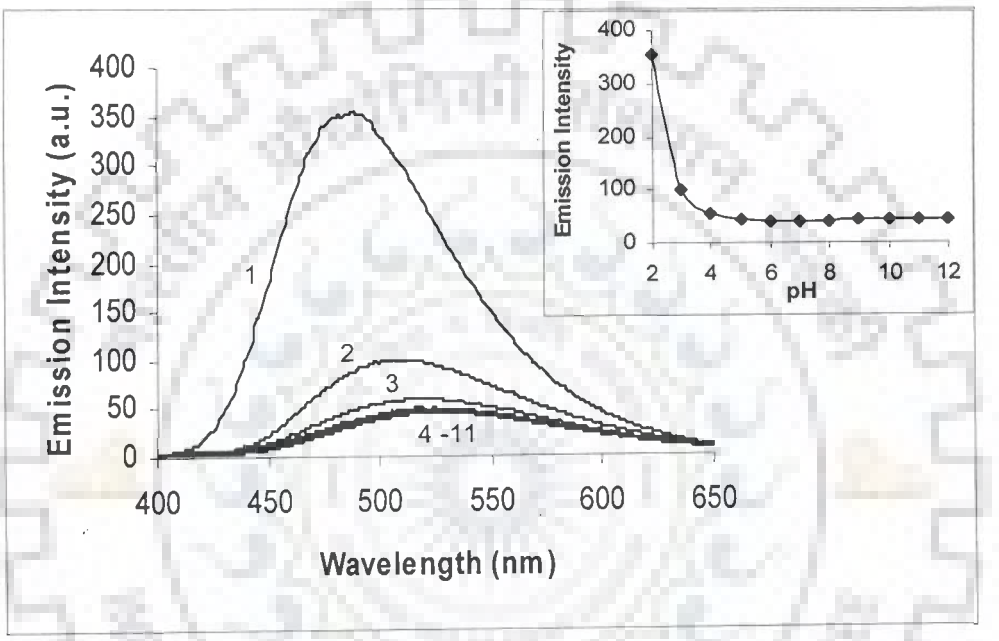


Figure 3.5: ANS fluorescence study of MKTI at different pH.

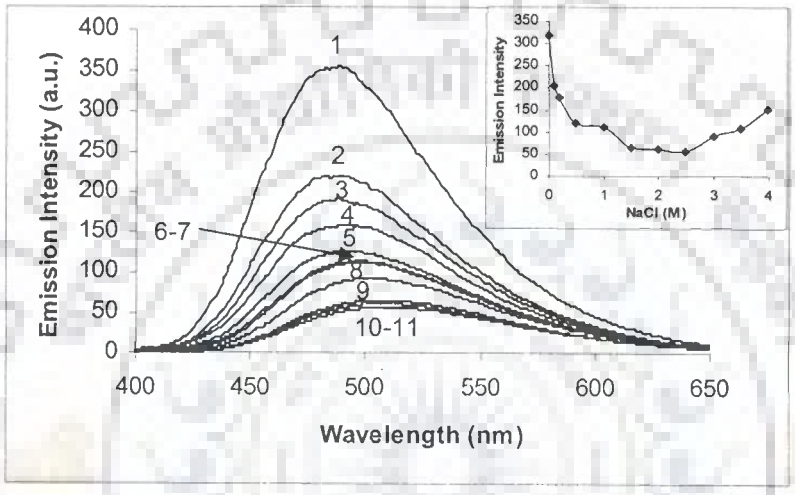


Figure 3.6: Effect of increasing concentration of NaCl on stability of MKTI at pH 2.0 by ANS fluorescence studies. ANS emission spectra were recorded after excitation at 360 nm. 1, 0 M; 2, 0.1 M; 3, 0.2 M; 4, 4.0 M; 5, 0.5 M; 6, 1.0 M; 7, 3.5 M; 8, 3.0 M; 9, 1.5 M; 10, 2.0 M; 11, 2.5 M NaCl.

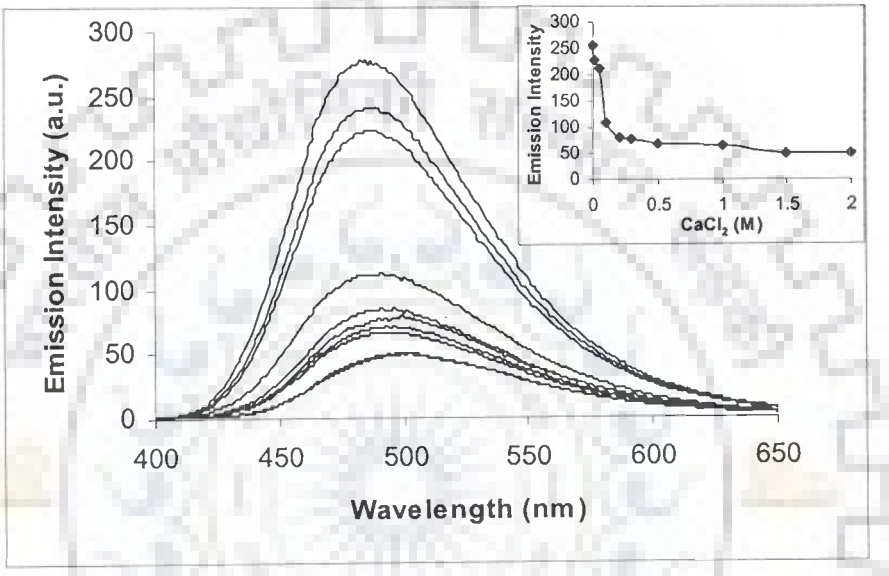


Figure 3.7: $CaCl_2$ induced refolding of MKTI at pH 2.0 as monitored by ANS fluorescence after excitation at 360 nm.

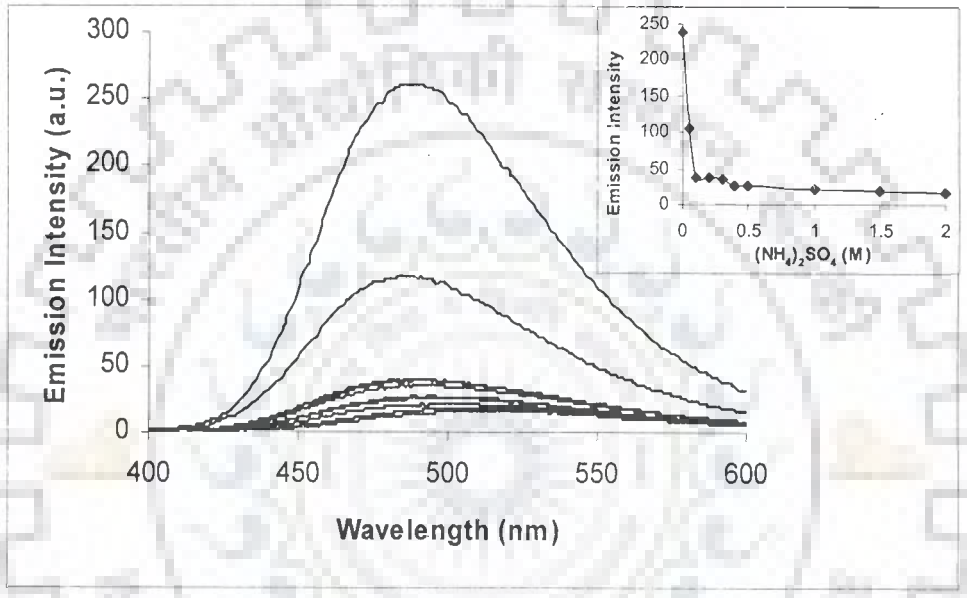


Figure 3.8: $(NH_4)_2SO_4$ induced refolding of MKTI at pH 2.0 as monitored by ANS fluorescence after excitation at 360 nm.

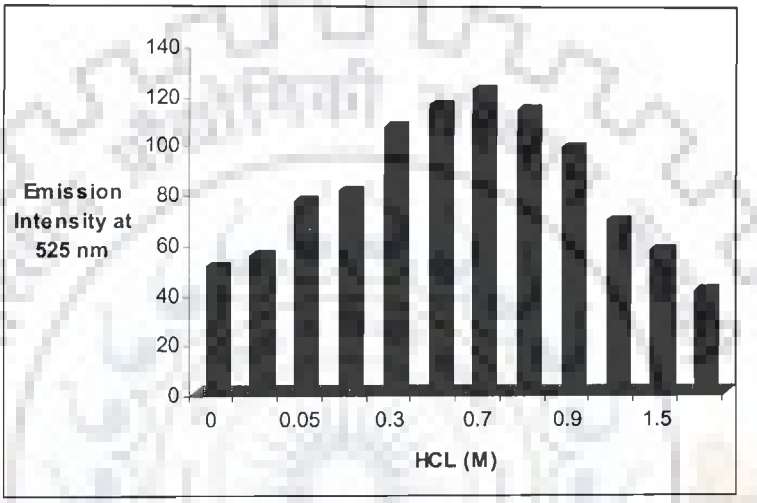


Figure 3.9: Variation of ANS fluorescence intensity as a function of increasing concentration of HCl on stability of MKTI. ANS emission spectra were recorded after excitation at 360 nm.

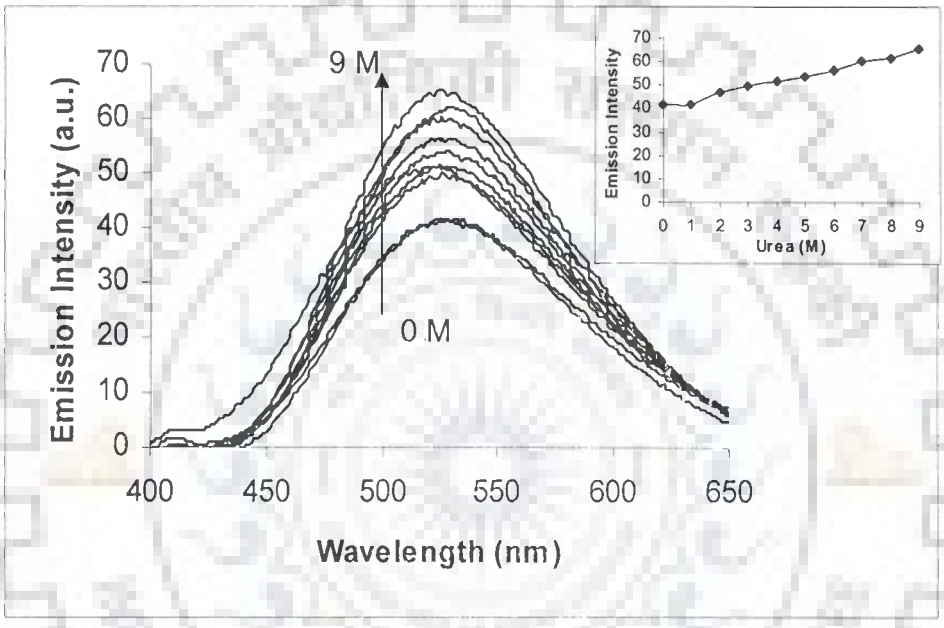


Figure 3.10: Effect of urea on stability of MKTI at pH 8.0 as monitored by ANS fluorescence after excitation at 360 nm.

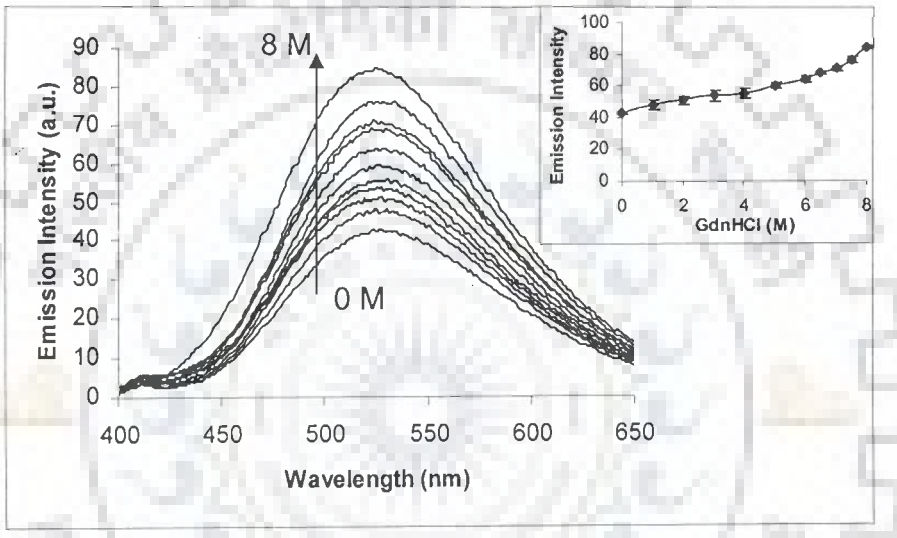


Figure 3.11: Effect guanidine hydrochloride on stability of MKTI at pH 8.0 as monitored by ANS fluorescence after excitation at 360 nm.

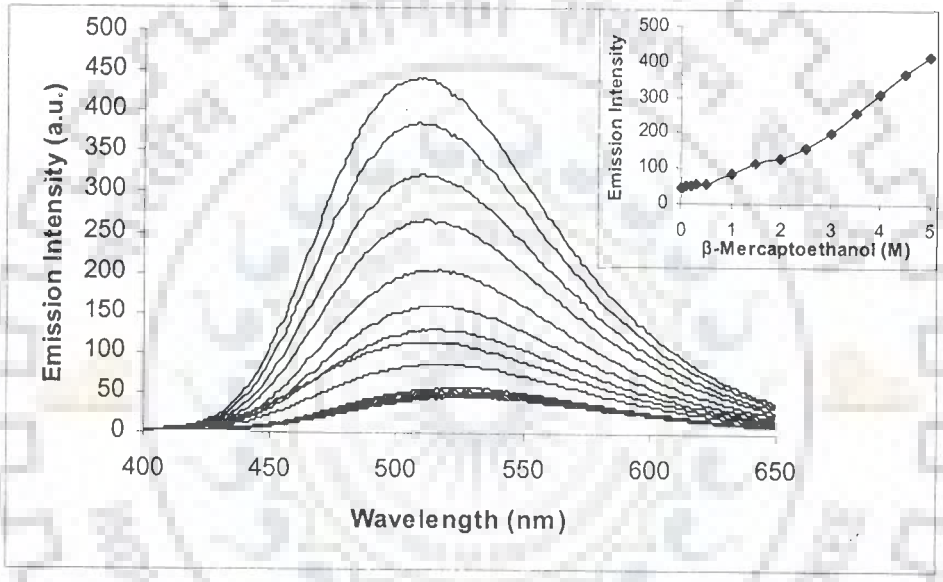


Figure 3.12: β-Mercaptoethanol induced unfolding of MKTI at pH 8.0 as monitored by ANS fluorescence after excitation at 360 nm.

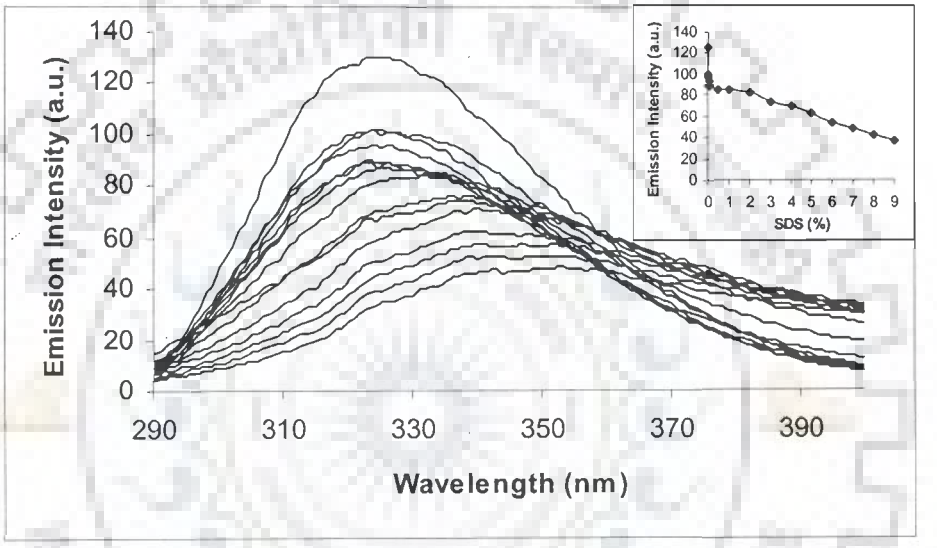


Figure 3.13: Tryptophan fluorescence of MKTI as a function of different SDS with distinct red shift at increasing concentration from 0 to 9%. The spectra were recorded after excitation at 280 nm.

3.3.3. X-ray crystallography

The crystals of MKTI appeared in 4 dyas and grew to a maximum dimension of 0.10 x 0.10 x 0.75 mm in 10 days (Figure 3.14). The diffraction data were collected in 85 images with 1° oscillation (Figure 3.15). The MKTI crystals belong to the tetragonal space group $P4_32_12$, with unit-cell parameters a = b = 75.8, c = 150.9 Å. The crystals diffracted to 2.65 Å resolution and a complete data set with 10,300 unique reflections was collected to a completeness of 96.1 % in resolution range 25 -2.9 Å with an R_{sym} of 14.6%. Based on the molecular mass of MKTI (21.4 kDa) and space group $P4_32_12$, it is assumed that the crystal contains two molecules per asymmetric unit. This assumption gives a V_M value (Matthews, 1968) of 2.5 $Å^3$ Da^{-1} and a solvent content of 51 %. The data-collection statistics are summarized in Table 3.1 (Shee at el., 2007).

The MKTI structure was solved by molecular replacement using the structure of *Erythrina caffra* Kunitz type trypsin inhibitor (PDB code 1TIE) (Onesti et al., 1991) as a search model. This solution gave correlation coefficient of 21.2% and an R factor of 61.1%. After rigidbody and restrained refinement, the correlation coefficient improved to 49.2% and R factor reduced to 41.7% and free R to 48.6%. The electron density map was calculated using FFT (Collaborative Computational Project, Number 4, 1994). The model was nicely fitted in 2 Fo – Fc map as shown in Figure 3.16. In refinement, some of the amino acids obtained from N-terminal and internal sequencing of MKTI were also fitted in the electron density. Only those residues were placed where the electron density was very clear. Due to the unavailability of complete sequence the further refinement was not possible.

Table 3.1:

Crystal data and intensity statistics.
Values in parentheses are for the last resolution shell.

Space group	$P4_32_12$
Unit-cell parameters (Å)	a = b = 75.8, c = 150.9
Temperature (K)	293
Resolution range (Å)	25 – 2.9 (2.95-2.9)
No. observed reflections	58581
No. of unique reflections	10300 (540)
Completeness	96.1 (99.2)
R_{sym} (%)*	14.6 (42.2)
Mean I/σ(I)	4.7 (1.6)

*$R_{sym} = (\Sigma_{hkl,I} |I_i - \langle I \rangle|)/(\Sigma_{hkl,i} I_i)$

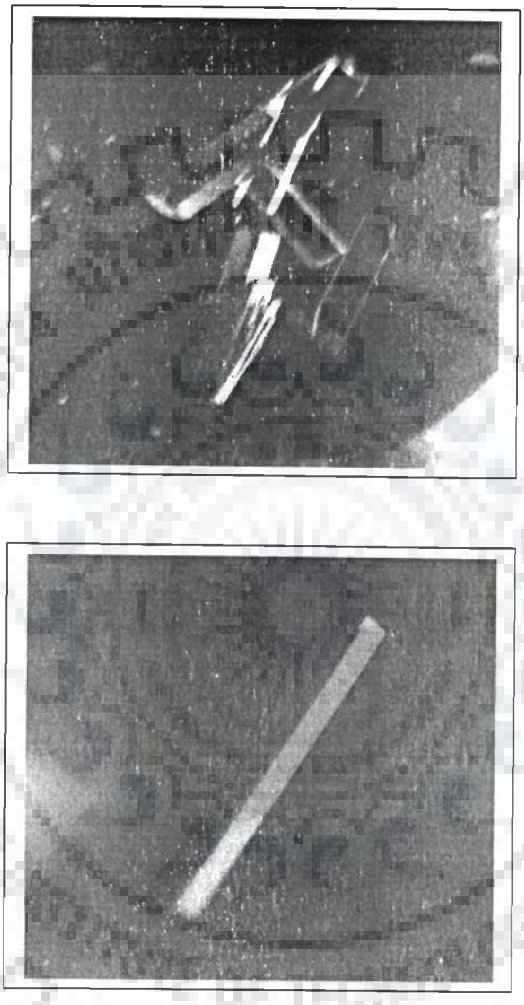


Figure 3.14: Crystal of *Murraya koenigii* trypsin inhibitor as grown by the sitting-drop method. The dimension of the crystal was 0.10 x 0.10 x 0.75 mm.

Figure 3.15: X-ray diffraction image from a *Murraya koenigii* trypsin inhibitor crystal. The crystal diffracted to 2.65 Å resolution.

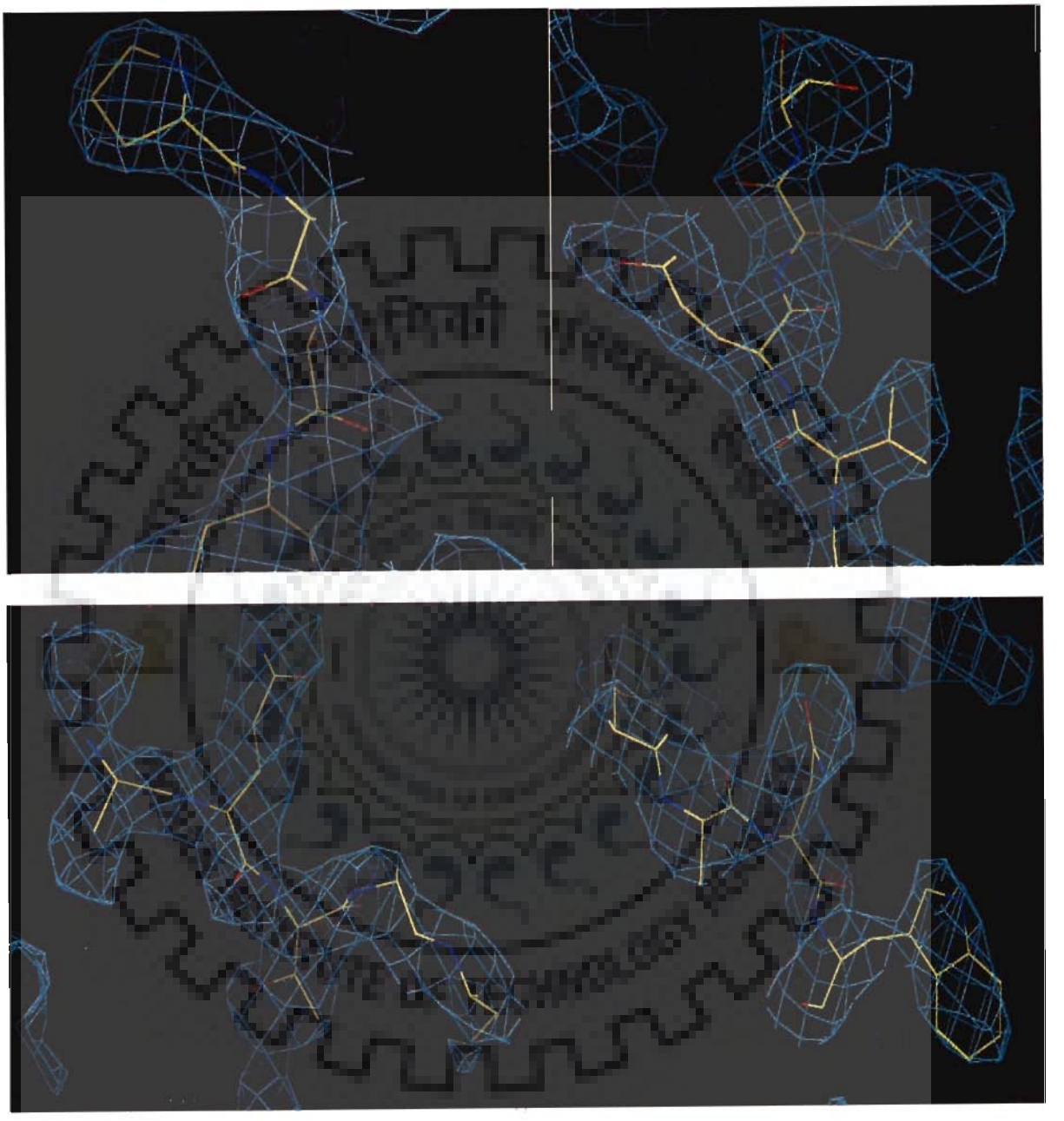


Figure 3.16: The 2Fo–Fc electron density map from different regions of model to show the electron density fitting. The map was contoured at 1.0σ.

3.4. Discussion

Here, we have described the stability studies and secondary structure analysis of *Murrya koenigii* trypsin inhibitor. These results demonstrated the differences in structural and functional stability of MKTI and also structure-activity relationship in terms of conformational changes occurring at the surface of the protein. CD spectra of MKTI at room temperature demonstrated α, β pattern with approximately 46% β-strand and 30% helical component. The CD spectra at increasing temperatures revealed a helix to coil transition with β-sheet component almost constant. Also, no melting transition was observed in thermal denaturation studies by CD spectroscopy up to 80°C. The CD studies under different concentration of guanidine hydrochloride also showed that there is a slight decrease in molar ellipticity at 8 M concentrations. At different concentration of NaCl, it was observed that the CD spectra of MKTI were variable and there was a slight decrease in the ellipticity at 4-5 M concentration. These results suggest that β-sheet structure in MKTI is highly stable and all the changes in conformation of MKTI at high temperatures are occurring in helical portion. Interestingly, the loss in inhibitory activity and reduction in helical content due to helix to coil transition at increasing temperatures demonstrated similar patterns. The two phenomena occurring at functional and structural level can be correlated with respect to structure and activity of MKTI and it can be safely said that α-helical component may have a role for inhibitory activity of this protein. These results, therefore, imply that the core structure of the protein is formed of beta sheet while helix is present on surface.

Analyses of stability and secondary structural studies together strongly suggest that structural stability of MKTI is due to a compact structure formed by central β-sheet surrounded by α-helix. The conformational change from α-helix to random coil at high temperature does not

make MKTI susceptible to protcolytic degradation suggesting that the compact nature of protein is retained. Interestingly, the inhibitory activity of MKTI, unlike purified inhibitor, was retained at high temperature under *in-vivo* conditions. The unique feature of MKTI is the presence of substantial helical content, lacking in the other similar Kunitz type inhibitors. The X-ray and CD studies of other kunitz inhibitors showed that they are mostly sheet structure with almost little or none helical content. These studies have shown that Soybean trypsin inhibitor (STI) contains approximately >25% of β-sheet, 16% of turn and 50% of random coil; Papaya proteinase inhibitor (PPI), ~ > 35% of β-sheet, 18% of turn and 42% of random coil; and Erythrina trypsin inhibitor (ETI), ~ > 35% of β-sheet, 18% of turn and 42% of random coil (Azarkan et al., 2006). On comparing the structure of MKTI with other Kunitz type inhibitors, it becomes clear that MKTI is a α, β protein rather than a predominantly β-protein.

Most members of this family exhibit a high degree of stability at a range of temperatures, pH and against various proteinases. However, our studies have clearly shown that MKTI is not soluble below pH 7.5 and functionally not stable at high temperatures but proteolytically very stable against various proteinases. Although, MKTI is a serine proteinase inhibitor but proteolytically it is very stable against cystein proteinases like papain and aspartate proteinases like pepsin and also there are high degree of stability was observed against non-specific proteinases proteinase k. The structure obtained by CD and X-ray crystallography of other members of this family showed that they are typical β-sheet proteins. In CD spectra, a minimum ellipticity at about 200 nm is obtained for these proteins which are indicative of random coils and β-sheet with little or no helical content. In case of MKTI, CD studies clearly showed that it is a α β protein rather than only beta protein structure. The CD spectra of MKTI are quite different from other members and minimum ellipticity was observed at 208 nm and ellipticity at around

222 nm. Taking into account all these factors, it is clear that MKTI has certain differences structurally and functionally when compared to other members and overall structure was found to be very stable.

The fluorescence studies revealed the conformational stability of MKTI at different physicochemical conditions. The ANS fluorescence studies at different pH conditions showed that the native structure of MKTI was completely retained up to pH 5.0 and partial relaxed state was observed only below this pH with significant unfolding at pH 2.0. Interestingly, addition of both monovalent and divalent salts at pH 2.0 helped in refolding of the protein as observed by the decrease in ANS fluorescence intensity. This clearly demonstrates the stabilizing effect of salts on MKTI structure. Although, $(NH_4)_2SO_4$ helps in precipitation of protein at pH 8.0 after 2 M concentration but at pH 2.0 it helps in resolubilization and refolding of MKTI up to 2 M concentration. In urea and guanidine hydrochloride, even at high concentration, a minor increase in ANS fluorescence intensity was observed indicating that overall MKTI structure remains intact with only a very slight relaxation. Also, no melting transition was observed in thermal denaturation studies by ANS fluorescence up to 90°C. The results indicate that the conformational changes occurring in the structure as a function of temperature, guanidine hydrochloride and urea, does not significantly expose the hydrophobic pockets for ANS binding. However, the significant unfolding of the MKTI structure was observed in presence of high concentration of β-mercaptoehtanol and SDS as determined by ANS and tryptophan fluorescence respectively. In presence of HCl gradual unfolding of MKTI structure was observed up to 0.7 M concentration with complete unfolding above this concentration.

The diffraction quality crystals of MKTI were obtained by sitting-drop vapor diffusion method using PEG 8000 as the precipitating agent. The crystals belong to the tetragonal space

group with two molecules in asymmetric unit. The structure was solved by using structure of *Erythrina caffra* trypsin inhibitor as search model. The model was fitting well in the electron density. The refinement of the structure improved the correlation coefficient and reduced the R factor. As complete amino acid sequence of MKTI is not known, the further refinement and model building could not be pursued.

In summary, analyses of stability and secondary structural studies together strongly suggest that structural stability of MKTI is due to a compact structure formed by central beta sheet surrounded by α-helix. Despite structural stability, MKTI is not stable functionally at high temperatures. The loss of inhibitory activity is possibly due to the conformational changes leading to gradual loss in helical component at the surface of MKTI with increasing temperature. The conformational change from α-helix to random coil at high temperature does not make MKTI susceptible to proteolytic degradation suggesting that the compact nature of protein is retained. Interestingly, the inhibitory activity of MKTI, unlike purified inhibitor, was retained at high temperature under *in vivo* conditions. The unique feature of MKTI is the presence of substantial helical content, lacking in the other similar Kunitz type inhibitors. Analyses of stability and secondary structural studies by CD spectroscopy revealed that the MKTI structurally is very stable protein made of central β-sheet surrounded by α-helix. Fluorescence studies to understand the conformational stability of protein showed partial relaxation to significant unfolding at different conditions. The protein significantly unfolds at pH 2.0 and addition of salts helped in refolding of the protein. A minor increase in ANS fluorescence intensity was observed in presence of urea and guanidine hydrochloride, however, the significant increase in intensity indicating the unfolding of MKTI structure was observed in presence of high concentration of β-mercaptoehtanol, SDS and HCl. The crystals of MKTI were obtained

and structure was solved by molecular replacement method taking Kunitz type trypsin inhibitor as search model. The model fitted well in 2 Fo – Fc electron density map and refined to the R factor of 41.7%.

CONCLUSIONS

CONCLUSIONS

Biochemical characterization

- The *Murraya koenigii* trypsin inhibitor was purified to homogeneity by two purification methods. In first method, the protein was purified by two step procedure, purification on an anion exchange column followed by HPLC gel filtration column. In second method, the MKTI was purified in a single step on an affinity column, Cibacron Blue 3GA resin, which improved the yield almost three-fold.
- The SDS-PAGE analysis under both reducing and non-reducing conditions confirmed that MKTI is a single polypeptide chain with approximate molecular mass of 27 kDa. However, the MALDI-TOF studies revealed the exact molecular mass of the protein to be 21.4 kDa.
- The complete trypsin inhibition at different concentrations of MKTI was achieved at a molar ratio of 1:1.1. The trypsin inhibition differs at different pH values and at 1:1 molar ratio, it was observed that the protein inhibits trypsin maximum at pH 8.0.
- Kinetic studies demonstrate that, like other serine proteinase inhibitors, MKTI is a competitive inhibitor with a dissociation constant of 7×10^{-9} M.
- N-terminal sequence analysis, with a NCBI BLAST short sequence search, showed homology to a chymotrypsin inhibitor from *Erythrina variegata* belonging to the Kunitz-type proteinase inhibitor family.
- The partial internal amino acid sequencing by MALDI-TOF-TOF, with six peptides totaling 98 residues, showed resemblance to trypsin inhibitors, storage proteins and homeodomain like proteins.

- The maximum sequence homology of MKTI was found to be with miraculin like protein CJI, a member of Kunitz family.
- The results clearly suggest that MKTI belongs to Soybean Kunitz family inhibitor.
- MKTI was found to be the most abundant protein in the seeds of *Murraya koeni*gii plant. The protein profiling during seed maturation and germination demonstrated that *Murraya koeni*gii trypsin inhibitor is a major seed storage protein and the relative protein concentration increased with highest concentration at mid-maturation stage during seed development and the protein was utilized during germination providing essential nutrition, a characteristic of a storage protein.
- The solubility studies at different pH conditions showed unusual solubility properties of this protein. The protein is completely soluble above pH 7.5 and solubility of the protein decreases with the pH below pH 7.5 at the protein concentration of 1 mg/ml. The inhibitory activity was regained completely after resolubilization of the protein in buffer at pH 8.0
- At pH 4.0, the solubility of precipitated protein increased with increase in the salt concentration with complete resolubilization at 1000 mM NaCl concentration.
- The effect of NaCl, $MgCl_2$ and $(NH_4)_2SO_4$ at high molar concentration showed that the protein was precipitated only in presence of high molar $(NH_4)_2SO_4$ concentration. The monovalent and divalent salts like NaCl and $MgCl_2$ respectively did not precipitate MKTI even at 4 M concentration in room temperature at neutral pH.
- In presence of different concentration of organic solvents like ethanol, methanol, acetonitrile, dioxane, MPD, the MKTI was found to be precipitated at different level. Although, at 90% of these organic solvents the MKTI was completely precipitated, but

the inhibitory properties of MKTI regained when it redissolved in buffer of pH 8.0. The ethylene glycol did not help in precipitation of MKTI even at 90% of concentration.

- Thermal stability studies of MKTI clearly demonstrate that the functional stability and solubility of the protein decreases with increasing temperature. On heating, the protein starts precipitating irreversibly and the efforts to redissolve this protein at low temperature were not successful. The activity of this protein reduced from 15% at 40°C to 95% at 90°C. It was also observed that the precipitation of MKTI increased in presence of different concentrations of salt at higher temperature.
- The inhibitory activity of MKTI, unlike purified inhibitor, was retained at high temperature under *in-vivo* conditions.
- Proteolysis studies revealed that the MKTI is very resistant to different proteinases like trypsin, chymotrypsin, proteinase k, papain and pepsin. Heat treated MKTI at all temperatures was also completely resistant to proteolytic degradation by trypsin, chymotrypsin, pepsin and papain but was found susceptible to proteolysis by proteinase K when heat treated at 90°C.

Biophysical characterization

- CD spectra of MKTI at room temperature demonstrated α, β pattern with approximately 46% β-strand and 30% helical component. The CD spectra at increasing temperatures revealed a helix to coil transition with β-sheet component almost constant.
- CD study in the presence of different concentration of guanidine hydrochloride and NaCl showed slight decrease in CD concent.

- The fluorescence studies revealed the conformational stability of MKTI at different physicochemical conditions. The native structure of MKTI was completely retained in the range of pH 5.0 to pH 12.0 and partial relaxed state was observed only below the pH 2.0 with significant unfolding.
- Addition of both monovalent and divalent salts at pH 2.0 helped in refolding of the protein. This clearly demonstrates the stabilizing effect of salts on MKTI structure.
- A minor increase in ANS fluorescence intensity in presence of 8 M urea and guanidine hydrochloride indicates the structural rigidy of MKTI.
- The significant unfolding of the MKTI structure was observed in presence of high concentration of β-mercaptoehtanol, SDS and HCl.
- Analyses of stability and secondary structural studies together strongly suggest that structural stability of MKTI is due to a compact structure formed by central beta sheet surrounded by α-helix. Despite structural stability, MKTI is not stable functionally at high temperatures. The loss of inhibitory activity is possibly due to the conformational changes leading to gradual loss in helical component at the surface of MKTI with increasing temperature. The conformational change from α-helix to random coil at high temperature does not make MKTI susceptible to proteolytic degradation suggesting that the compact nature of protein is retained. Interestingly, the inhibitory activity of MKTI, unlike purified inhibitor, was retained at high temperature under *in-vivo* conditions. The unique feature of MKTI is the presence of substantial helical content, lacking in the other similar Kunitz type inhibitors. On comparing the structure of MKTI with other Kunitz type inhibitors, it becomes clear that MKTI is a α, β protein rather than a predominantly β-protein with a possible role for helical structure in inhibitory function.

- MKTI was crystallized by the sitting-drop vapor diffusion method using PEG 8000 as the precipitating agent. The crystals belong to the tetragonal space group $P4_32_12$, with unit-cell parameters a = b = 75.8, c = 150.9 Å. The crystals contain two molecules in asymmetric unit with a V_M value of 2.5 $Å^3$ Da^{-1}. A complete data set was collected to 2.9 Å resolution.
- The structure was solved by molecular replacement method using the structure of *Erythrina caffra* Kunitz type trypsin inhibitor as a search model. The model fitted well in electron density and refined to R factor of 41.7% with overall correlation coefficient of 49.2%. In refinement, some of the amino acids obtained from N-terminal and internal sequencing of MKTI were also fitted in the electron density. Only those residues were placed where the electron density was very clear. Due to the unavailability of complete sequence the further refinement could not be done.

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