STUDIES ON TRYPSIN INHIBITORS FROM PLANTS OF RUTACEAE FAMILY

A THESIS

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled, "**STUDIES ON TRYPSIN INHIBITORS FROM PLANTS OF RUTACEAE FAMILY**" 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 January 2005 to December 2008 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 in any other Institute.

(DEEPANKAR GAHLOTH)

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

Dated: 29.12.2008

(Ashwani Kumar Sharma)
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The Ph.D. Viva-Voce Examination of **Deepankar Gahloth**, Research Scholar has been held on April 30, 2009

Signature of Supervisor

Signature of External Examiner

ABSTRACT

Serine proteinase inhibitors are present in leaves, flowers, seeds and tubers of many plants as their defensive agent against insect pests. Many plant serine proteinase inhibitors have been purified and characterized particularly from the seeds of *Leguminosae*, *Cucurbitaceae*, *Solanaceae* and *Gramineae* family. Among them, the Kunitz trypsin inhibitor super-family has gained particular importance for its specific activity against trypsin-like serine proteinases. These proteinase inhibitors have been shown to inhibit the proteolytic activity of several lepidopteran pests which largely depends on presence of serine proteinases for protein digestion. Their role in retarding growth and development of insect pests fed on diets containing inhibitor has been demonstrated in many studies. However, insect pests overcome the effect of plant proteinase inhibitors by expressing new proteinases which are either insensitive or can degrade them. Due to adaptive strategy adopted by insect pest against host proteinase inhibitors, it is important to identify effective and potent inhibitors of insect proteinases from unrelated non-host plants. One of the major focuses of plant biotechnology is to develop crops resistant to particular insect pests. One of the strategies could be to express effective proteinase inhibitors against insect gut proteinases from non-host plants in affected crops.

A Kunitz-type trypsin inhibitor from the seeds of *Murraya koenigii* belonging to *Rutaceae* family has been purified earlier in our lab. Biochemical characterization of *Murraya koenigii* trypsin inhibitor (MKTI) has shown that it is highly resistant to proteolytic degradation by selected proteinases and is a major seed storage protein. In the present work, *in vitro* and *in vivo* effects of MKTI on two lepidopteran insect pests namely *Helicoverpa armigera* and *Spodoptera litura* were studied. Both are polyphagous lepidopteran insect pests and affects many important crops causing severe economic losses. This work demonstrated the inhibitory

potential of MKTI against gut proteinases and their effect on growth and development of the two pests. Also, the cloning, sequencing, expression and homology modeling of MKTI were performed. The MKTI gene was cloned form both genomic and cDNA. This work also includes the cloning, sequencing and homology modeling of another Kunitz-type trysin inhibitor from the plant *Murraya paniculata* belonging to *Rutaceae* family.

The thesis is divided into six chapters. Chapter 1 reviews the literature in the area of plant proteinase inhibitors particularly serine proteinase inhibitors.

Chapter 2 assesses the *in vitro* and *in vivo* effects of *Murraya koenigii* trypsin inhibitor on *Helicoverpa armigera*, a polyphagous lepidopteran insect pest. MKTI inhibited the trypsin-like and total proteinases activity of *H. armigera* gut proteinases (HGP) by 78.5% and 40% respectively. In feeding experiments, MKTI was found to adversely affect the growth and development of larvae, pupae and adult in dose dependent manner. A reduction of 69% in larval weight after 10 days of feeding and 40% larval mortality was observed in larvae fed with diets containing 200 µM inhibitor. Likewise, pupation, pupal weight, adult survival, adult emergence, fertility and fecundity were adversely affected. Also, malformed pupae and adults were observed because of abnormal development. The effect of MKTI feeding on endogenous levels of proteinases showed a substantial decrease in trypsin-like activity and similar increase in chymotrypsin-like activity after being fed with inhibitor supplemented diet. The MKTI was found to be completely stable against proteolysis by HGP up to 72 h with inhibitory activity intact. The results clearly demonstrated the efficacy of MKTI as a plant defense agent against *H. armigera*.

Chapter 3 evaluates the effect of *Murraya koenigii* trypsin inhibitor (MKTI) on digestive proteinases and growth of *Spodoptera litura*. MKTI inhibited the trypsin-like and total

proteinases activity of *S. litura* gut proteinases (SGP) by over 81% and 48% respectively. The effect of MKTI feeding on endogenous levels of proteinases showed an 8-fold decrease in trypsin-like activity and approximately 4-fold increase in chymotrypsin-like activity in fifth instar larvae after being fed with inhibitor supplemented diet. MKTI was found to be remarkably stable against proteolysis by SGP even after 72 h of incubation with total gut proteinases. Incorporation of MKTI in artificial diet adversely affected the growth and development of larvae, pupae and adult in dose dependent manner. Larval feeding studies showed a reduction of 44.8% in larval weight after 10 days of feeding and larval mortality increased to 43.3% when fed with diets containing 200 μM inhibitor. These results showed that MKTI can be an effective plant defense agent against *S. litura*.

Chapter 4 describes cloning, sequencing, expression and homology modeling of *Murraya koenigii* trypsin inhibitor. A 648 base pair open reading frame was obtained after sequencing of the MKTI PCR product. DNA sequence analysis of both cDNA and genomic DNA showed same nucleotide sequence in the coding region, implying that genomic clone lacks intervening sequences. The deduced amino acid sequence of MKTI comprises a polypeptide of 215 amino acid residues of which first 25 N-terminal residues form a signal peptide and remaining 190 residues form mature protein. The open reading frame of mature protein was overexpressed as the glutathione S-transferase (GST)-fusion protein in *E. coli* TG1 cells after subcloning it in-frame in pGEX-5X-3. The recombinant MKTI was obtained from GST-MKTI fusion protein after treatment with factor Xa. Both, the fusion protein and isolated reMKTI showed identical trypsin inhibitory activity.

Chapter 5 describes amino acid sequence analysis which showed that MKTI forms a distinct cluster with miraculin-like proteins, a Kunitz family member, in phylogenetic analyses.

It showed maximum homology (57% identity & 74% similarity) with miraculin-like proteins. The amino acid sequence of reactive loop of MKTI showed major differences from other Kunitz-type inhibitors. The MKTI sequence showed existence of possible glycosylation, phosphorylation and myristoylation sites. The structure prediction by homology modeling showed significant differences between MKTI and other soyabean Kunitz family inhibitor structures despite similar overall fold. The major differences were observed in intermolecular contacts between MKTI reactive loop and trypsin when compared to soyabean Kunitz inhibitor. The present study explores the unique features of MKTI structure based on the amino acid sequence and homology modeling.

Chapter 6 describes the cloning, sequencing and homology modeling of a trypsin inhbitor from *Murraya paniculata.* In comparative protein profiling, a corresponding band similar to MKTI was observed in *Murraya paniculata* seed extract. But weak trypsin inhibitory activity has been observed in the *M. paniculata* seeds extract compared to MKTI crude extract. A PCR fragment of 570 bp was obtained from genomic DNA amplification. Sequence analysis has shown that it belongs to kunitz family of trypsin inhibitor. *Murraya paniculata* trysin inhibitor (MPTI) shares 96% homology matching with the amino acid sequence of MKTI. MPTI also forms a distinct cluster with miraculin like protein and distantly related with kunitz type trypsin inhibitor. Structure prediction by homology modeling revealed that it has overall three fold internal symmetry with β- trefoil fold. MPTI is a weak trypsin inhibitor as compared to MKTI. This study analyses its differences with MKTI based on the amino acid sequence and homology modeling.

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CONTENTS

CHAPTER 2

BIOINSECTICIDAL ACTIVITY OF *MURRAYA KOENIGII* TRYPSIN INHIBITOR (MKTI) ON *HELICOVERPA ARMIGERA*

List of Publications

1. **Deepankar Gahloth**; Umesh Shukla; Ajanta Birah; Gorakh Gupta; P. Ananda Kumar; Harcharan S. Dhaliwal; Pravindra Kumar; Ashwani Kumar Sharma. Bioinsecticidal activity of *Murraya koenigii* trypsin inhibitor on the *Helicoverpa armigera*. (Communicated)

2. **Deepankar Gahloth**; Gorakh Gupta; P. Ananda Kumar; Harcharan S. Dhaliwal; Pravindra Kumar; Ashwani Kumar Sharma. Insecticdal activity and effect of *Murraya koenigii* trypsin inhibitor on the growth and development of *Spodoptera litura*.(Communicated)

3. **Deepankar Gahloth**; Chandan Shee; Ashwani K. Sharma. Identification and Characterization of protease inhibitory activity in some common fruit plants. (Communicated)

4. **Deepankar Gahloth**; P. Selva Kumar; Pravindra Kumar; Ashwani Kumar Sharma. MKTI is a miraculin-like protein with Aspargine at the active site: Cloning, Sequence analysis and Homology Modelling of *Murraya koenigii* trypsin inhibitor. (Communicated)

5. **Deepankar Gahloth**; P. Selva Kumar; Pravindra Kumar; Ashwani Kumar Sharma. Molecular Cloning, Phylogenetic analysis and Homology modeling of miraculin-like protein from *Murraya panniculata*. (Under preparation)

Gene Submitted in NCBI database

MKTI gene

- *Murraya koenigii* trypsin inhibitor cDNA sequence NCBI accession number: **FJ468001.**
- *Murraya koenigii* trypsin inhibitor Genomic DNA sequence NCBI accession number: **FJ468001.**

MPTI gene

- Gene submission under process

INTRODUCTION

INTRODUCTION

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 which is known as β-trefoil fold, a distinct folding pattern which is common to the kunitz family of protease inhibitors (Murzin et al., 1992). 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).

These plant proteinase inhibitors are one of the natural defense molecules against many insect pests (Gracia-olmedo et al., 1987; Ryan, 1990; Boulter, 1993; Yeh et al., 1997). It has

been shown that wounding of plant tissues by insects pests or microbial infection significantly enhances the level of proteinase inhibitors (PIs) in tissues (Green and Ryan, 1972; Schaller and Ryan, 1995). Among them, serine PIs and particularly Kunitz trypsin inhibitor super-family has been shown to inhibit the proteolytic activity of several lepidopteron pests which largely depends on presence of trypsin-like serine proteinases for protein digestion (Broadway and Duffey, 1986; Richardson, 1991; Giri et al., 2003). Their role in retarding growth and development of insect pest fed on diets containing inhibitor has been demonstrated in many studies (Harsulkar et al., 1999; Murdock and Shade, 2002; Telang et al., 2003; Srinivasan et al., 2005; Bhattacharyya et al., 2007). Soyabean Kunitz, *Archidendron ellipticum* and bitter gourd trypsin inhibitors (SKTI, AeTI and BGTI) showed inhibition of gut proteinases and adverse effect on growth and development of *Spodoptera litura* (McManus and Burgess, 1995; Bhattacharyya et al., 2007; Telang et al., 2003).

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).

Previously, a 21.4 kDa Kunitz type trypsin inhibitor protein has been purified by ion exchange chromatography, gel filtration chromatography on HPLC and affinity chromatography on cibacron blue 3 GA (Shee et al., 2007, 2008). The kinetic study have shown that MKTI is a competitive inhibitor having Ki 7×10^{-7} M. Solubility studies of *Murraya koenigii* trypsin inhibitor (MKTI) have shown that the solubility decreases below pH 7.5. Protein profiling shows that it is a major storage protein in the seed. MKTI was found stable in proteolysis studies with five different proteinases, trypsin, papain, proteinase K, chymotrypsin and pepsin (Shee et al., 2008).

In this work, we have extended the study on *Murraya koenigii* trypsin inhibitor. The *in vitro* and *in vivo* effects of *Murraya koenigii* trypsin inhibitor has been evaluated on polyphagous lepidopteron insect pests, *Helicoverpa armigera* and *Spodoptera litura*. MKTI inhibited the trypsin-like and total proteinases activity of both the insects gut proteinases. MKTI was found to adversely affect the growth and development of larvae, pupae and adult in dose dependent manner. Likewise, pupation, pupal weight, adult survival, adult emergence, fertility and fecundity were adversely affected. In order to use MKTI as a potent insecticidal candidate for the development of resistant transgenic crop, MKTI gene was cloned from both cDNA and genomic DNA. Also the expression and purification of recombinant MKTI was performed. Homology modeling of *Murraya koenigii* trypsin inhibitor has been done to understand the inhibitory mechanism. The amino acid sequence of reactive loop of MKTI showed major differences from other Kunitz-type inhibitors. MKTI forms a distinct cluster with miraculin-like proteins, a Kunitz family member, in phylogenetic analyses. A homologous trypsin inhibitor gene of MKTI has been cloned from *Murraya paniculata* genomic DNA. Despite the overall 96% similarity in amino acid sequence with MKTI, it shows some key

structural differences from MKTI. MPTI also forms a distinct cluster with miraculin like protein and distantly related with typical kunitz type trypsin inhibitor like MKTI. Structure prediction by homology modeling revealed that it has overall three fold internal symmetry with β-trefoil fold.

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 *invitro* and *invivo* conditions. In order to perform *invitro* 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; 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 (Figure 1.1). 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).

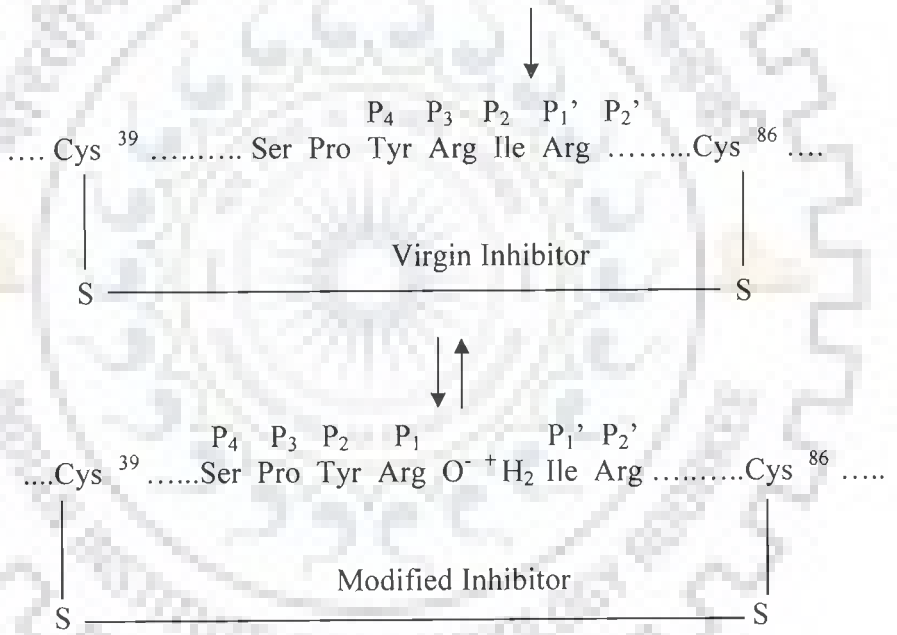

Figure 1.1: Schematic diagram of the reactive site of Kunitz's soybean trypsin inhibitor. Virgin inhibitor has all its peptide bonds intact. Modified inhibitor has the reactive site peptide bond hydrolyzed

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).

Shee and Sharma (2007) reported the purification and characterization of trypsin inhibitor from seeds of *Murraya koenigii*. Murraya koenigii trypsin inhibitor was a single polypeptide chain with 21.4 kDa molecular weight. It has an inhibitor constant of 7 nM. It has shown unusal solubility properties below pH 7.5. Comparative protein protein profiling during developmental stages have shown that this inhibitor is a major seed storage protein and has been consumed during the development of seed (Shee et al., 2008). CD studies showed that MKTI is α-β protein and on higher temperature it lost its trypsin inhibitory activity (Shee et al., 2007b). Priliminary crystallographic study has also been done on *Murraya koenigii* trypsin inhibitor (Shee et al. 2007b).

A highly stable and potent trypsin inhibitor was purified to homogeneity from the seeds of *Putranjiva roxburghii* belonging to *Euphorbiaceae* family by Chaudhary et al. (2008). SDS–PAGE analysis, under reducing condition, showed that protein consists of a single polypeptide chain with molecular mass of approximately 34 kDa. The purified inhibitor inhibited bovine trypsin in 1:1 molar ratio. Kinetic studies showed that the protein is a competitive inhibitor with an equilibrium dissociation constant of 1.4×10^{-11}.

1.3.6.2. Miraculin protein

The plant *Richadella dulcifica* bears fruits called miracle fruit, which have the unusual property of being able to change a sour taste into a sweet taste. It elicits lemon a sweet taste

after chewing the fruit. Itself pure miraculin has no taste. All the acids tested elicited a sweet taste if tasted after miraculin had been held in the mouth for a few min. The sweetness induced by 0. 1 M citric acid after tasting a 1 μM miraculin solution is equivalent to that of a 0.4 M sucrose solution; thus, the sweetness of a miraculin solution induced by 0.1 M citric acid solution is 40,0000 times that of a sucrose solution. The sweetness inducing activity of miraculin lasts for 2 h after the application of miraculin to the mouth. Purified native miraculin protein has an amino acid sequence of 191 residues with a molecular mass of 24,600 (Theerasilp et al., 1988), and a cDNA encoding miraculin also has been cloned and sequenced (Igeta et al., 1995). Miraculin in the fruit is present as a homodimer; but in the pure state, two miraculin dimers aggregate to form a tetramer. Both the dimer and the tetramer have taste-modlfying activity. There are two substituent sugar chains, which are connected to the 42^{nd} and 186^{th} asparagine residues.

Surprisingly, the amino acid sequence of miraculin has a high degree of homology with that of soybean trypsin inhibitor; the N-terminal sequence has 36.4% homology and C-terminal sequence has 51.1% homology. It is not known, why miraculin shows a high degree of homology with soybean trypsin inhibitor.

1.3.6.3 Miraculin like proteins

One of several cDNAs (designated LeMir) derived from tomato induced by a root-knot nematode (*Meloidogyne javanica*) showed sequence similarity to that of miraculin (Brenner et al.). It rapidly induced and localized to tomato root tips by nematode infection and it is predicted to secreted from root into the surroundings (Brenner et al., 1998).

Two full length cDNA encoding miraculin like proteins have been isolated from *Citrus jambhiri*, the product of both the sequence contains soyabean trypsin inhibitor motif and significant protease inhibitory activity. Nothing has been reported about the taste modifying properties of miraculin like proteins but these proteins have been considered as key molecule in the plant defense.

1.4. Structural analysis

Structural analysis is very important to understand the structure-function relationship and mechanism of action of protein. The Kunitz-type soybean trypsin inhibitor (STI), an archetypal member of its family, was characterized and crystallized as early as in 1947 (Kunitz, 1947a, b). The structural analysis have shown that Kunitz soyabean trypsin inhibitor (STI) family members proteins are predominantly β-sheet proteins with little or no helical structures. It consists of 12 criss-crossed antiparallel β-strands largely stabilized by hydrophobic side chains random coil and turn structure. This conformational pattern is known as β-trefoil fold. Its three- dimensional structure in complex was also determined (Sweet et al., 1974). The predominant conformation found in STI has been demonstrated to be approximate β-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 2.5Å, using the coordinates of the homologous *Erythrina* trypsin inhibitor as a search modal.

Onesti et al. (1991) studied the structure and properties of a Kunitz- type inhibitor from *Erythrina caffra* seeds (ETI). This inhibitor consists of 172 amino acid residues with two disulphide bridges. The three dimensional structure of ETI consists of 12 antiparallel β-strands joined by long loops. Six of the strands form a short antiparallel β-barrel that is closed at one

end by a "lid" consists of other six strands coupled inpairs termed as β-trefoil fold (Murzin et al., 1992). The scissile bond (Arg63- Ser64) of ETI is located on an external loop that protrudes from the suface the molecule. The overall structure of ETI is similar to the partial structure of STI, but scissile bond loop is displaced by 4Å.

Other Kunitz-type proteinase inhibitors from various sources show a high degree of homology to STI. Among them, three-dimensional structures have been reported for proteinase K/α-amylase inhibitor from wheat at 2.5Å (Zemke et al., 1991), and Kunitz-type chymotrypsin inhibitor from winged bean seeds (WCI) at 2.95Å (Dattagupta et al., 1996), all in the free state. Other homologous proteins include a trypsin inhibitor from winged bean seeds (Yamamoto et al., 1983), potato cathepsin D inhibitor (MaresÏ et al., 1989), inhibitors of subtilisin/endogeneous α-amylase from barley and wheat (Leah & Mundy, 1989), and winged bean albumin-1 (Kortt et al., 1989).

Soyabean trypsin inhibitors (STI) partial structure in complex with porcine pancreatic trypsin (PPT) was reported in 1974 (Blow et al., 1974; Sweet et al., 1974), and amino acid sequences were reported for the three isoforms of STI, Tia, Tib, and Tic (Kim et al., 1985).They all contain 181 amino acid residues with two disulphide bridges (Cys39-Cys86 and Cys136-Cys145). Between the sequences of Tia and Tic, there is only one substitution at position 55. On the other hand, eight amino acid substitutions exist between Tia and Tib (Kim et al., 1985).

In the refined structures of complex between bovine pancreatic trypsin inhibitor (BPTI) and bovine pancreatic trypsin (BPT), the scissile peptide bond remains nearly intact, with a slight out-of-plane deformation of the carbonyl oxygen atom being observed (Bode & Huber, 1992). However, the absence of a large upfield shift in the 13C of the P1 residue of STI upon

complex formation suggested that the STI:PPT complex is not a covalent, fully tetrahedral adduct (Baillargeon et al., 1980).

Suh et al., (1998) reported some minor differences between the interaction pattern between the orthorhombic and tetragonal crystal structures of the complex. Twelve amino acid residues out of the 181 in STI make contact with PPT in the orthorhombic crystal structure. They are: Asp1, Phe2, Asn13, Pro61 (P3), Tyr62 (P2), Arg63 (P1), Ile64 (P10), Arg65 (P20), His71, Pro72, Trp117 and Arg119. In the tetragonal crystal structure, the three residues His71, Pro72 and Arg119 do not interact with PPT. However, the pattern for the hydrogen bonding interaction involving the reactive site loop residues from Pro61 (P3) to Arg65 (P20) was well conserved between the two crystal forms, except that a hydrogen bond between Tyr62 and Gly96 was not present in the tetragonal crystal structure.

1.5. 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.

1.5.1. 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.

1.5.2. 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).

1.5.3. 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

BIOINSECTICIDAL ACTIVITY OF *MURRAYA KOENIGII* TRYPSIN INHIBITOR (MKTI) ON *HELICOVERPA ARMIGERA*

2.1. Introduction

Serine proteinase inhibitors are present in leaves, flowers, seeds and tubers of many plants as their defensive agent against insect pests (Gracia-olmedo et al., 1987 and Ryan et al., 1990). Among them, the Kunitz trypsin inhibitor super-family has gained particular importance for its specific activity against trypsin-like serine proteinases (Richardson 1991). These proteinase inhibitors (PIs) have been shown to inhibit the proteolytic activity of several lepidopteran pests which largely depends on presence of serine proteinases for protein digestion (Broadway and Duffey, 1986; Giri et al., 2003). Their role in retarding growth and development of insect pest fed on diets containing inhibitor has been demonstrated in many studies (Harsulkar et al., 1999; Murdock and Shade, 2002; Telang et al., 2003; Srinivasan et al., 2005; Bhattacharya et al., 2007). However, insect pests overcome the effect of plant PIs by expressing new proteinases which are either insensitive or can degrade PIs (Jongsma et al., 1995; Broadway, 1995; Jongsma and Bolter, 1997; Giri et al., 1998). Insects can also express proteinases with changed substrate specificity in response to PIs (Wu et al., 1997; Gatehouse et al., 1997). Another strategy adopted by insects for stability and enhanced activity against PIs is the compartmentalization of proteinases at specialized region inside the insect gut (Oppert et al., 2005). Due to adaptive strategy adopted by insect pest against host PIs, it is important to identify effective and potent PIs of insect proteinases from unrelated non-host plants (Harsulakar et al., 1999). The inhibitory effect of PIs from several non-host plants like winged bean (*Psophocarpus tetragonolobus*), potato (*Solanum tuberosum*), bitter gourd (*Momordica charantia*), capsicum (*Capsicum annum*) and groundnut (*Arachis hypogea*) against gut proteinases of *H. armigera* have been shown. These inhibitors have also been shown to have

adverse effects on growth and development of *H. armigera* (Harsulkar et al., 1999; Telang et al., 2003; Tamhane et al., 2005).

In the present chapter, we have studied the *in vitro and in vivo* effects of MKTI on *Helicoverpa armigera*. *H. armigera*, a polyphagous lepidopteran insect pest, is one of the most devastating field pests of many important crops causing severe economic losses (Manjunath et al. 1989). Here, we have demonstrated the inhibitory potential of MKTI against gut proteinases and their effect on growth and development of *H. armigera*.

2.2. Materials and Methods

2.2.1. Materials

Bovine pancreatic trypsin, chymotrypsin, N-benzoyl-L-arginine p-nitronilide (BAPNA), N-benzoyl-L-tyrosine ethyl ester (BTEE), Azocasein, SDS-PAGE chemicals were purchased from Sigma Aldrich Chemicals Co. (St. Louis, MO, USA). Gelatin was purchased from Fluka. All other chemical used were of analytical grade.

2.2.2. Purification of Murraya koenigii trypsin inhibitor (*MKTI)*

2.2.2.1. Purification on ion exchange and gel filtration column

Purification of MKTI was done as previously described (Shee and Sharma 2007). Briefly, seeds of *Murraya koenigii* were crushed and soaked overnight at 4°C in 30 ml of 50 mM Tris-HCl buffer, pH 7.5. The homogenate was cleared by centrifugation at 12,000g for 1 h and the supernatant was loaded onto a DEAE Sepharose column equilibrated with same buffer used for sample extraction. The unbound molecules were washed extensively and bound molecules were eluted with a NaCl step gradient from 0 to 500 mM in the same buffer.

Fractions with trypsin inhibitory activity were pooled and concentrated. The protein was further purified by HPLC using size exclusion chromatography column.

2.2.2.2. Single step purification on Cibacron blue 3GA

The inhibitor was also purified in single step by affinity chromatography using Cibacron blue 3 GA (Shee and Sharma, 2008). The purity of the protein was determined by 15% SDS-PAGE analysis (Laemmli, 1970).

2.2.3. Estimation of proteins

Protein contents were measured by dye binding method according to the procedure of Bradford (Bradford, 1976) with bovine serum albumin as protein standard (1 mg/ml).

2.2.4. Insect rearing

The *H. armigera* used for these experiments originated from a colony maintained in the laboratory of Dr. G.P.Gupta (Entomology Divison, Indian Agriculture Research Institute, New Delhi). The larvae were reared in rearing container with the photoperiod regime of 16h light–8h dark, 27°C±1°C temperature and 65% relative humidity. The composition of artificial diet on which the larvae were transferred and maintained throughout the experiment was similar to that followed by Gupta et al. (2004a) and chiefly composed of chickpea flour (*Cicer arietinum* L) (94.0 g), wheat germ, (*Triticum* sp) (13.0 g), distilled water (825 ml), agar-agar (15.0g), dried yeast powder (24.5 g), casein (15.0 g), ascorbic acid (6.0 g), formaldehyde 40% (1.0 ml), ABDEC drops (multivitamin solutions, 1.5 ml), multi-vitamin capsules (2) and α-tocopherol (Evion 400 mg, 1 capsule).

2.2.5. Preparation of H. armigera gut proteinase (HGP)

To assess the inhibitory effect of MKTI against *H. armigera* gut proteinase (HGP), midguts of the fifth instar larvae were carefully dissected on ice after decapitation. Each gut was transferred into an eppendorf tube maintained on ice at 4°C containing 40 µl of 1 mM HCl and then stored at -20°C. As trypsin can remain stable almost indefinitely when frozen in 1 mM HCl, the same is considered for gut serine proteinases (Walsh, 1970). For HGP extraction, the midgut was crushed with the glass rod and homogenates were centrifuged at 12,000g for 10 min at 4°C. The supernatants were used as crude enzyme for trypsin-like, chymotrypsin-like and total proteinase activity and were stored at -20°C.

2.2.6. Proteinase and proteinase inhibitory assay

Crude extract was evaluated for total proteinase, trypsin-like and chymotrypsin-like activity by estimating hydrolytic activity towards synthetic substrates azocasein, BAPNA and BTEE respectively.

2.2.6.1. Using BAPNA and BTEE as substrate

Trypsin-like and chymotrypsin-like activity assay were carried out by estimating the hydrolytic activity of supernatants towards the substrate BAPNA (1.25 mM) and BTEE (1.07 mM) respectively (Shibata et al., 1981). Reactions were performed in a UV-Vis spectrophotometer, set at 410 nm for hydrolysis of BAPNA and 256 nm for BTEE. The proteinase and trypsin inhibitory assay of MKTI was monitored after mixing variable concentrations of the inhibitor with the 20 µg of bovine pancreatic trypsin and with the volume of equivalent activity of the crude gut extract. The assays were run in the 1 ml of 50 mM Tris-

HCl buffer containing 20 mM $CaCl_2$, pH 8.0. As a control only the crude gut extract (without inhibitor) was used in the enzymatic assay. One proteinase unit was defined as the amount of enzyme that increased absorbance by 1 OD/min at 410 nm and one PI unit was defined as inhibition of 1 unit of proteinase activity under the given assay conditions. All assays were done in triplicate and averaged.

2.2.6.2. Using azocasein as a substrate

Total proteinase activity was evaluated by incubating crude gut extract with 500 µl of 1% azocasein in 50 mM Tris-Cl, pH 8.0 containing 20 mM $CaCl_2$ in total volume of 1 ml at 37°C for 30 min. After incubation, the reaction was stopped by adding 150 µl of 20% Trichloroacetic acid (TCA). The tubes were then centrifuged at 10,000g for 10 minutes, after centrifugation supernatant was collected and absorbance was measured at 366 nm (Saborowski et al. 2006.).

2.2.7. Proteolysis study of MKTI with HGP

To assess the stability of MKTI against HGP, equal amount of MKTI and HGP (w/w) were incubated in 50 mM Tris-HCl, pH 8.0 at 37°C for 0, 10, 30 min, 1, 3, 10, 24 and 72 h. The stability of MKTI was evaluated by running 12% SDS-PAGE and simultaneously trypsin inhibitory activity was also evaluated by inhibitory assay using synthetic substrate BAPNA as described earlier.

2.2.8. Larval gut activity in polyacrylamide gel containing 0.3% gelatin

The analysis was performed in SDS- PAGE (Laemmli, 1970) containing 0.3% gelatin (Silva et al. 2006). β-merceptoethanol was not included in the sample buffer. The gel was run at a constant current of 50 mA at 4°C. Gut homogenate of third, fourth and fifth instar larvae were used in gel activity assay. In gel inhibitory activity assay, gut homogenate of fifth instar larvae was used in the presence of MKTI. After electrophoresis, the gel was transferred to 2.5% Triton X-100 solution (v/v) for 30 min at room temperature. The gel was then equilibrated with developing buffer composed of 0.05 M Tris-HCl buffer, pH 8.0 containing 0.15 M NaCl and 0.005 M $CaCl_2$ with gentle agitation at room temperature for four hours. After incubation the reaction was stopped by transferring the gel to a staining solution (Commassie Brilliant Blue R-250 in methanol: acetic acid: water, 3:1:6, v/v/v) and then to a destaining solution (methanol: acetic acid: water, v/v/v). The proteinase activity of the gut extract was visualized by the clear zone on dark background.

2.2.9. Insect bioassay

2.2.9.1. Effect of MKTI on larval development

The *invivo* efficacy of MKTI was studied by feeding assay using the laboratory established culture of *H. armigera* on artificial diet supplemented with MKTI at concentrations of 25 µM, 50µM, 100µM, 150µM and 200µM. Ten early instar larvae were used for each treatment with three replicates. Larval weights were taken on 6^{th}, 8^{th} and 10^{th} day after treatment and percent weight reduction in the proteinase inhibitor fed larvae was compared to that of the control group. The larval mortality and larval period was recorded and averaged.

2.2.9.2. Effect of MKTI on pupal development

Pupation period, pupal weight, number of malformed pupae and adult emergence were recorded and compared with that of the control group.

2.2.9.3. Effect of MKTI on adult development

Total number of malformed adult were calculated and averaged and compared with that of control. The fecundity and fertility data was also collected by emerged male and female moth. Three pairs for each treatment was kept in rearing jar separately with the cotton plug immersed in 10% sucrose solution and number of eggs and number of hatching was counted for each treatment separately and average was taken to calculate the fecundity and fertility for single pair and compared with control group.

2.2.10. Determination of growth indices

Growth indices of larva, pupa and total development were calculated by the equation described by Gupta et al. (2004).

2.2.10.1. Larval growth index

The Larval growth index was calculated and compared with that of control group.

Growth Index (Larva): Percentage Pupation/Larval Period,

2.2.10.2. Pupal growth index

The pupal growth index was calculated by dividing total percentage of adult emergence with averaged pupal period. It signifies the overall growth of pupa.

Growth Index (Pupa): Percentage Adult Emergence/ Pupal Period

2.2.10.3. Developmental index

Developmental Index: Percentage Survival / Total Developmental Period.

2.2.11. Effect of MKTI feeding on endogenous level of proteinases

The effect of MKTI feeding was examined on the endogenous proteolytic activity of HGP at different stages of larval development. The trypsin and chymotrypsin-like activity was measured in larval guts obtained at different instar stages following inhibitor feeding as described earlier. A set of control without the presence of MKTI in the larval diet was maintained.

2.3. Results

2.3.1. Purification of MKTI

Pure MKTI was obtained from both the methods described earlier (Shee et al., 2007, 2008) (Figure 2.1 and 2.2).

2.3.2. Inhibition of bovine pancreatic trypsin by MKTI

MKTI was evaluated against the bovine pancreatic trypsin as previously described (Shee at el. 2007). 20 µg of bovine pancreatic trypsin was completely inhibited by 25 µg of MKTI (Figure 2.3).

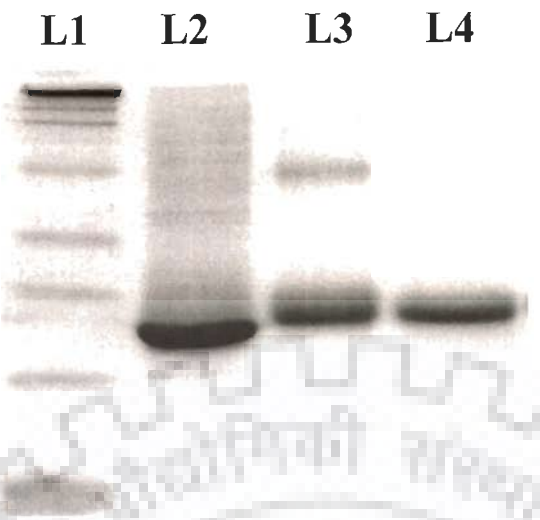

Figure 2.1: SDS- PAGE analysis of the protein. L1; molecular weight markers, L2; total protein in buffer extract, L3; 100 mM NaCl fraction after anion exchange chromatography, L4; purified protein after gel filtration chromatography on HPLC

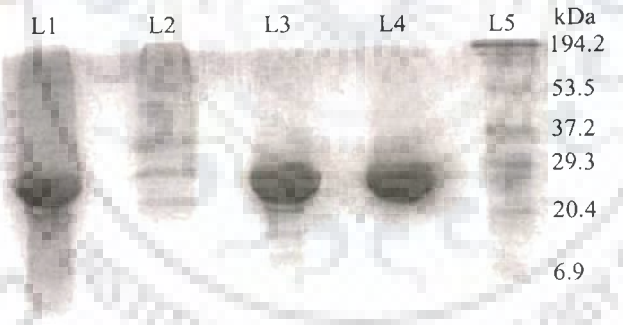

Figure 2.2: SDS- PAGE analysis of MKTI purified on Cibacron blue 3GA; L1, crude extract, L2; flow through, L3; 0.2 M NaCl fraction, L4; 0.5 M NaCl fraction, L5; molecular weight marker

2.3.3. Inhibition of proteinase activity of HGP by MKTI

MKTI was evaluated for its inhibitory activity against *H. armigera* gut proteinases (HGP) (Figure 2.3). The fifth instar larvae were used for the studies.

2.3.3.1. Using BAPNA and BTEE as a substrate

Trypsin-like and chymotrypsin-like activity was determined using synthetic substrates BAPNA and BTEE respectively. At 100% inhibition of bovine pancreatic trypsin, MKTI inhibited 78.5% of trypsin-like activity of HGP while the inhibitory effect of MKTI towards chymotrypsin was not observed (Figure 2.3).

2.3.3.2. Using azocasein as a substrate

Total proteinase inhibitory activity of MKTI against HGP was evaluated by using general synthetic substrate azocasein. At 78.5% inhibition of trypsin-like activity of HGP, MKTI inhibited 40% total proteinases activity (Figure 2.3).

2.3.4. Effect of MKTI on total gut protein content of H.armigera

Total protein concentration of insect gut was estimated from third, forth and fifth instar larva in various treatment along with control. The total protein content of gut in various instar has decreased as compared to control. The maximum protein concentration was found in 4^{th} instar larval gut, while the lowest concentration was found in 5^{th} instar indicating that the gut in 5^{th} instar become rudimentary. Table 2.1 concluded the protein concentration in different larval stages in various treatments.

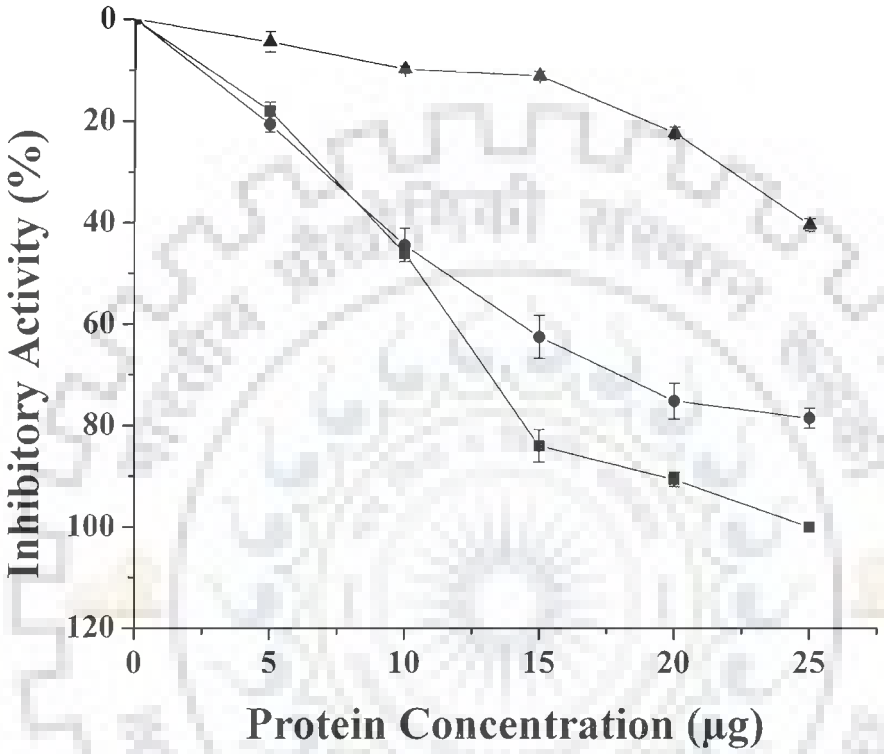

Figure 2.3: Inhibitory effect of MKTI on trypsin-like and total proteinase activity of SGP obtained from larvae fed on control diet. Inhibition assays were performed using BAPNA and azocasein as a substrate for trypsin-like (–●–) and total proteinase activity (–▲–) respectively. MKTI was titrated against HGP and bovine pancreatic trypsin (–■–). The experiment was repeated three times.

Table 2.1: Protein concenteration (mg/ml) in gut extract of different larval instar of *H. armigera* in various treatments

Treatment	3rd instar	4th instar	5th instar
Control	14.86 ± 0.6236	15.5833 ± 0.70829	11.92667 ± 0.56066
25 µM	13.67 ± 0.39	14.52 ± 0.35	10.18 ± 0.1
50 µM	13.02 ± 0.47	12.91 ± 0.50	9.65 ± 0.33
100 µM	12.59 ± 0.3407	11.29 ± 0.60893	7.66 ± 0.8567
150 µM	11.92 ± 0.57813	11.26 ± 0.63722	7.33 ± 0.5773
200 µM	11.645 ± 0.72	10.25 ± 0.58158	6.88 ± 1.00883

2.3.5. Resistance of MKTI towards proteolysis by HGP

The proteolytic stability of MKTI against gut proteinases was assessed. MKTI showed strong resistance against proteolysis by total gut proteinases of *H. armigera*. It was found to be stable even after 72 h of incubation with gut proteinases when equal amount of HGP and inhibitor were incubated (Figure 2.4). No loss in trypsin inhibitory activity of MKTI was observed.

2.3.6. Zymogram analysis of HGP inhibition by MKTI

The larval gut proteinase activity against MKTI was tested on gels containing 0.3% gelatin (Figure 2.5). The total gut proteinases of fifth instar larvae were used for testing in-gel inhibitory activity of MKTI. A clear zone against dark background was observed in the lanes where only gut proteinases from third, fourth and fifth instar larvae were run. A substantial reduction in zone of clearance was observed where sample contained both MKTI and gut

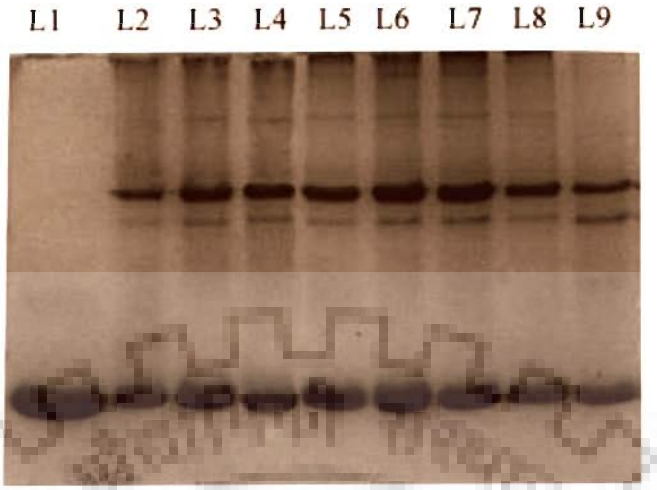

Figure 2.4: Proteolytic stability of MKTI against SGP on a 12% SDS-PAGE. MKTI was pre-incubated with HGP for different time intervals at 37°C. L1; Native MKTI, L2; 0 min, L3; 10 min; L4; 30min; L5; 1 h, L6; 3 h, L7; 10h, L8; 24 h, L9; 72 h

Figure 2.5: In gel inhibitory activity of MKTI against HGP on SDS- PAGE containing 0.3% gelatin as substrate. β-merceptoethanol was excluded from the sample buffer. L1-HGP incubated with equal amount of MKTI; L2-L4, gut extract of third, fourth and fifth instar larvae respectively

proteinases from fifth instar larvae. This clearly suggests that MKTI is able to inhibit partial gut proteinase activity by inhibiting trypsin like enzymes.

2.3.7. Effect of MKTI feeding on growth and development of H. armigera

Effect of MKTI on growth and development of larvae, pupae and adult of *H. armigera* was examined by incorporating different concentrations of MKTI (25–200 μM) in control diet.

2.3.7.1 Effects of MKTI feeding on larval growth

H. armigera larvae fed on diet containing MKTI showed substantial reduction in weight with increasing concentrations of inhibitor. At highest concentration of MKTI (200μM), approximately 88, 83 and 69% reduction in larval weight was observed after 6, 8 and 10 days of feeding when compared with larvae fed on control diet (Figure 2.6). Larval period also showed an increase from 15 to 20 days at above concentration of inhibitor (Figure 2.7). Larval mortality increased with increase in inhibitor concentration with 40% mortality in larvae fed on diet supplemented with 200 μM MKTI (Figure 2.8).

2.3.7.2 Effects of MKTI feeding on pupal growth

In case of pupal development, pupation decreased from 93% to 60% and pupal weight decreased marginally by 10% (Figure 2.9). The pupal period did not change substantially. In case of adult development, adult emergence decreased from 84 to 60% (Table 2.2), total survival reduced by 40% (Figure 2.10) and an increase from 26 to 33 days was observed in total development at highest dose. Also, malformed pupae (Figure 2.11 B) and adults (Figure 2.11 C) were observed due to abnormal development.

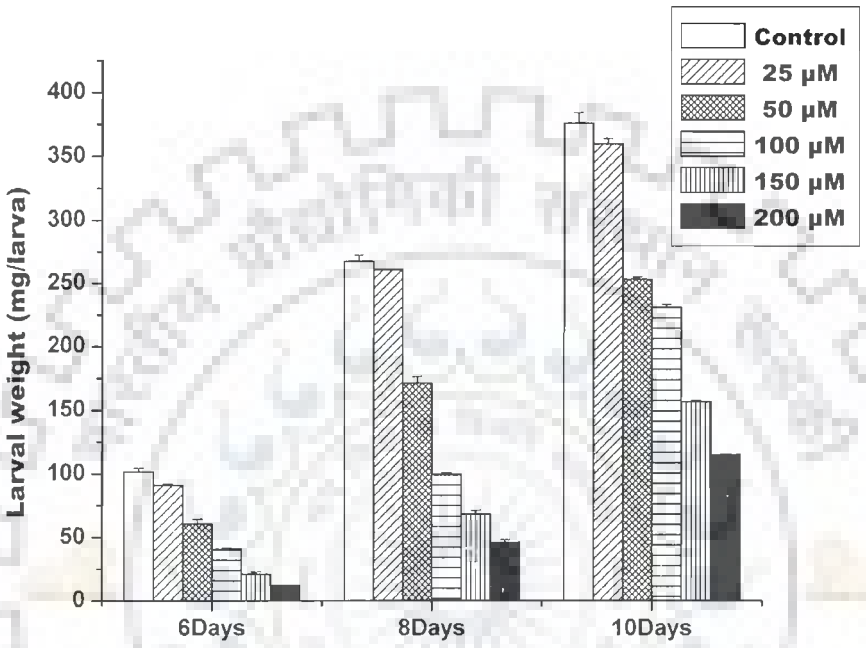

Figure 2.6: Effect of MKTI on larval weights of *H. armigera*. Different concentration (25 – 200 µM) of MKTI was incorporated in the standard diet. The weight (mg/larva) were taken at 6^{th}, 8^{th} and 10^{th} days after treatment and averaged. The Standard errors of mean is shown as bars

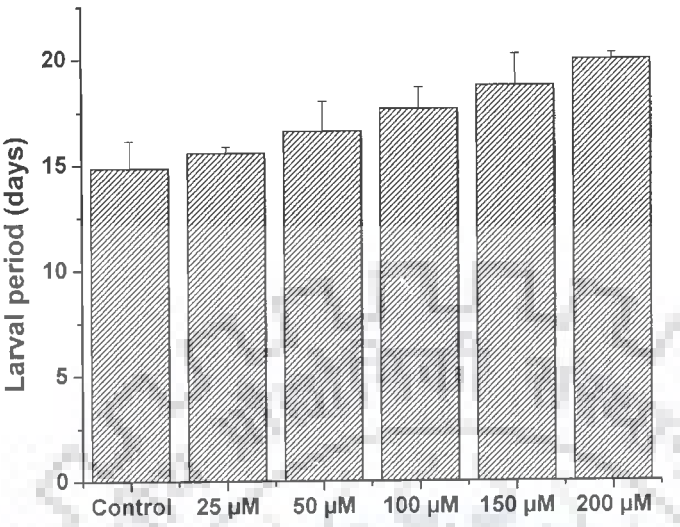

Figure 2.7: Larval period was calculated in different concentration of MKTI in standard diet. Means were calculated and standard errors are shown as bars

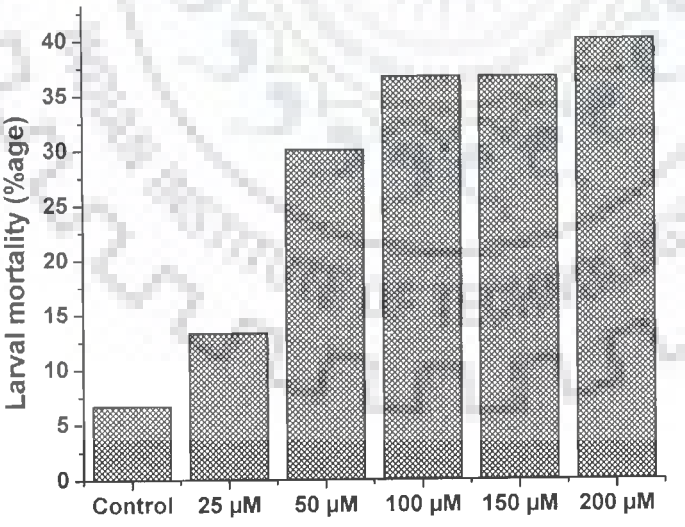

Figure 2.8: Larval mortality observed in different treatment of MKTI (25- 200 µM)

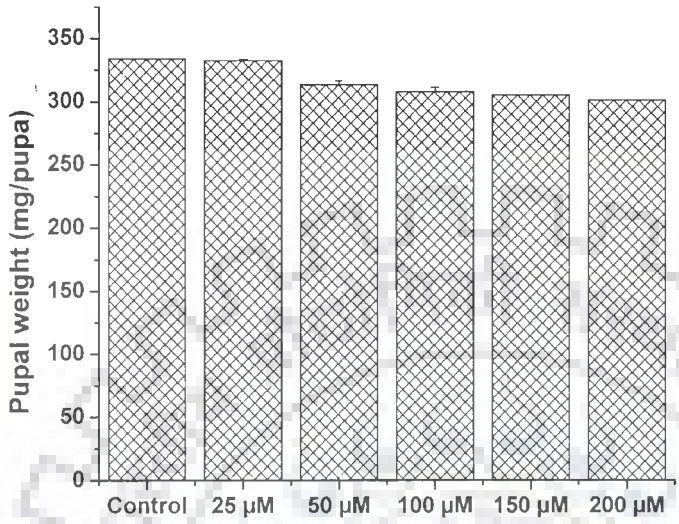

Figure 2.9: Pupal weight (mg/pupa) in various treatment of diet along with the control diet.

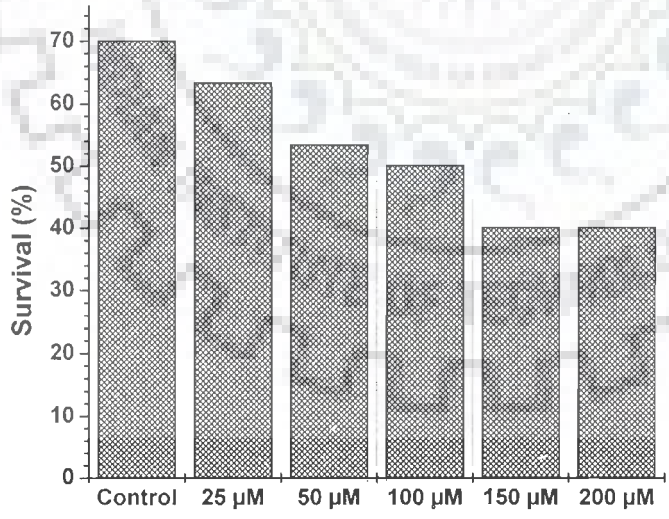

Figure 2.10: Total survival (%) of *H. armigera* in various treatments

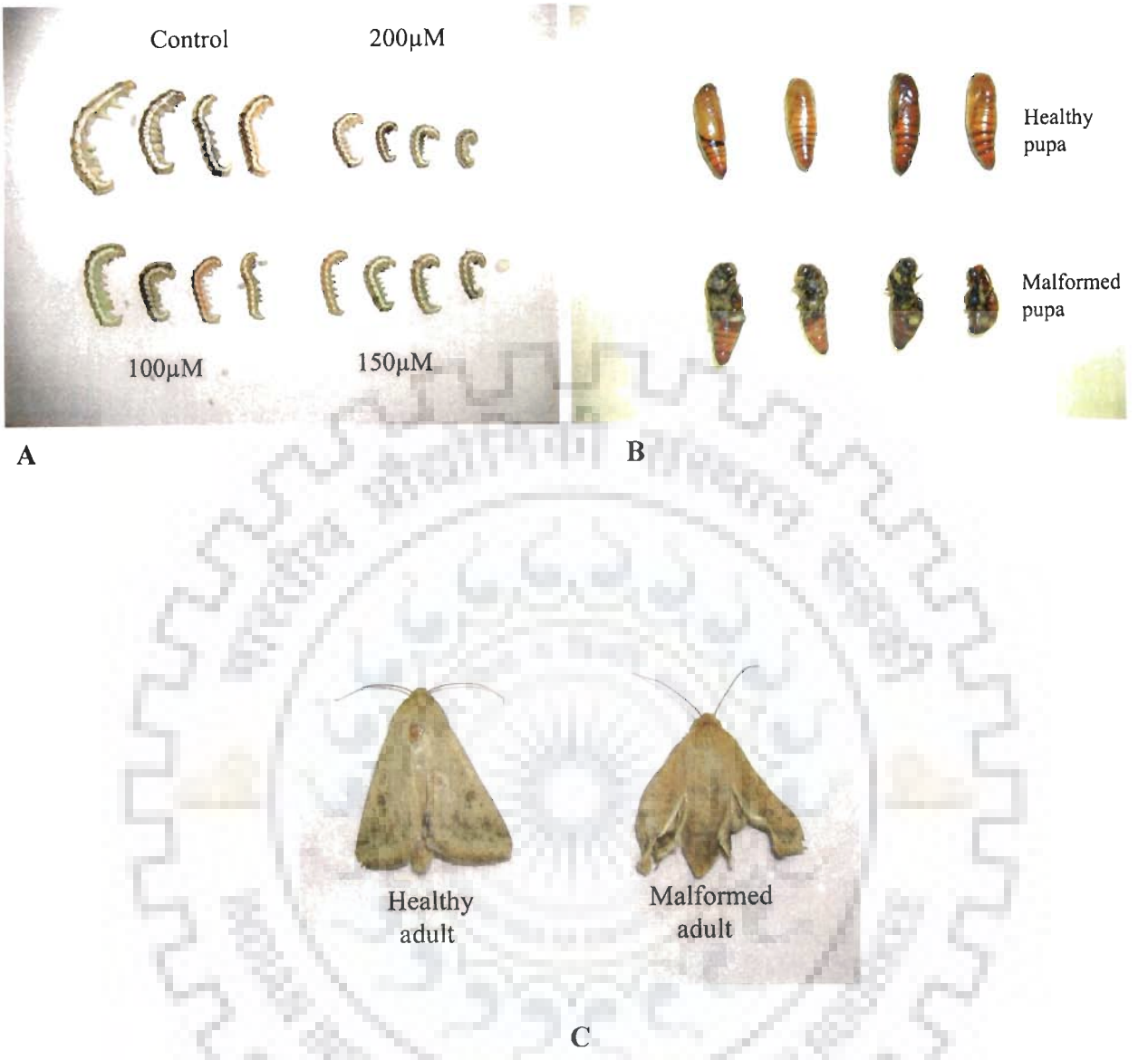

Figure 2.11: Representative photographs of effect of MKTI on growth and development of *H. armigera* larva and pupa. **(A)** Effect of MKTI on larval growth **(B)** Malformed pupa **(C)** Malformed adult

2.3.7.3. Effects of MKTI on adult development

Egg laying capacity of adult female (fertility) and hatching of larvae from eggs (fecundity) in adult insects fed on inhibitor diet was severely affected with reduction observed in dose dependent manner. Egg laying capacity of adult female (fertility) reduced from 822 eggs in adult fed on control diet to 109 eggs in those fed on diet containing 200 µM of inhibitor. Likewise, hatching of larvae from eggs (fecundity) reduced from 97% in control to 55% in MKTI fed insects (Table 2.2.).

2.3.8. Growth indices of H. armigera fed on MKTI

Analysis of growth and development indices showed substantial reduction in the growth index of larva and pupae and development index for the insects fed on diets containing MKTI as compared to the insects fed on control diet. The growth indices of larvae and pupae fed on MKTI (200 µM) decreased from 6.2 and 7.43 to 3.0 and 4.43 respectively. A lower development index of 1.2 was recorded for the insect fed on MKTI (200 µM) as compared to the insects fed on control diet (2.67) (Figure 2.12).

Table 2.2: Various parameters of the life-cycle of *H. armigera* at different concentrations of MKTI

	Malformed Pupa (%)	**Adult Emergence (%)**	**Malformed Adult**	**Developmental Days**	**Fecundity (Eggs/Female)**	**Fertility (%)**
Control	0	84.21	0	26.12	822.3± 18.28	97.08
25	0	78.94	0	27.12	461.6±67.29	90.03
50	0	75	0	29.06	96.7±24	85.38
100	0	68.42	0	30.65	237.3±20.8	81.18
150	10.52	65.21	16.33	31.91	157±38.63	63.06
200	16.66	60	11.11	33.51	109.6±18.93	55.54

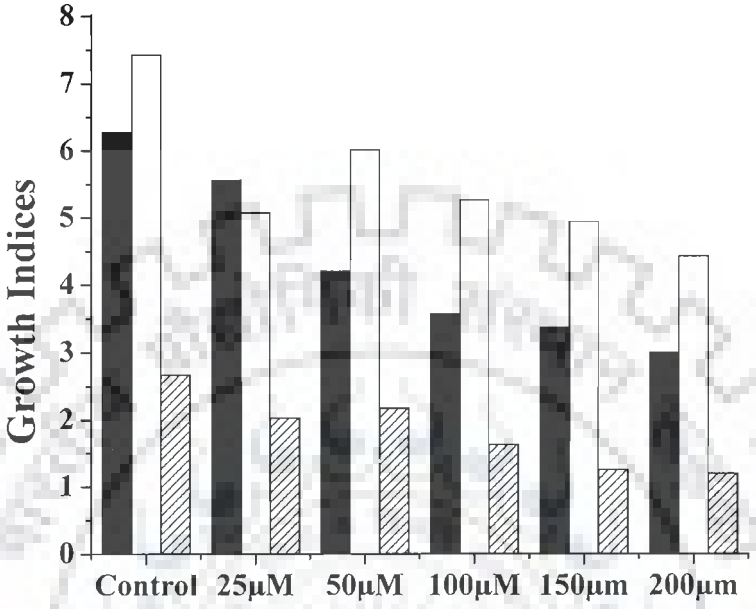

Figure 2.12: Effect of MKTI on growth and developmental indices of *H. armigera*. Growth index of larvae (▬); growth index of pupae (▭); developmental index (▨).

2.3.9. Effect of MKTI feeding on digestive proteinases of H. armigera

2.3.9.1 Trypsin-like activity

The effect of MKTI feeding on endogenous level of larval gut proteinases was studied (Table 2.3 A). The trypsin-like activity reduced continuously with increasing amounts of MKTI in larval diet. A significant decrease in trypsin-like activity was observed in larvae fed on diet containing MKTI as compared to those fed on control diet. A decline of 4, 5.5 and 3-fold was observed in the trypsin-like activity of HGP in third, fourth and fifth instars larvae fed on 200 µM MKTI respectively (Table 2.3 A).

2.3.9.2 Chymotrypsin-like activity

In contrast, chymotrypsin-like activity increased by 4.4, 3 and 3-fold in MKTI fed third, fourth and fifth instars larvae respectively (Table 2.3 B).

Table 2.3: The effect of MKTI feeding on endogenous level of proteinase activity in gut extracts from different instars of *H. armigera* larvae **(A)** Endogenous trypsin-like activity (µmol/ml/min). **(B)** Endogenous chymotrypsin-like activity (µmol/ml/min).

A

Instar	Control	25 µM	50 µM	100 µM	150 µM	200 µM
3rd	6.17±0.074	5.66±0.74	3.29±0.98	2.44± 0.19	1.59±0.65	1.49±0.09
4th	6.51±0.75	4.46±0.69	3.15±0.36	2.37±0.28	1.16±0.94	1.18±0.83
5th	2.18±0.02	1.97±0.37	1.18±0.77	0.94±0.10	0.76±0.35	0.68±0.25

B

Instar	Control	25 µM	50 µM	100 µM	150 µM	200 µM
3rd	2.22±0.042	2.55±0.04	3.83±0.39	6.16±1.51	7.97±0.02	9.64±0.62
4th	2.81±0.069	2.04±0.1	2.95±0.18	3.85±0.85	5.66±0.54	8.52±0.93
5th	2.37±0.08	2.56±0.19	3.05±0.54	7.53±1.09	8.14±0.28	8.3±0.4

2.4. Discussion

The present work evaluates the effectiveness of MKTI, a non-host Proteinase inhibitors (PIs), as a potential plant defense agent against *H. armigera*. It has been shown that insect pests usually adopt to host plant PIs (Jongsma et al., 1995; Broadway, 1995; Jongsma and Bolter, 1997; Giri et al., 1998) and non-host PIs can be potentially more effective plant defense agents (Harsulakar et al., 1999). Our studies have shown that MKTI is a stable and potent inhibitor of gut proteinases as well as have adverse effect on the growth and development of larvae, pupae and adult of *H. armigera* in dose dependent manner.

H. armigera gut proteinases are a complex mixture of serine proteinases having mainly trypsin and chymotrypsin like activity (Bown et al., 1997). MKTI showed significant inhibitory activity against HGP. It inhibited over 78% of trypsin-like activity and 40% of total activity. Comparing the results with other Kunitz-type inhibitors, MKTI showed higher trypsin-like activity. Soyabean Kunitz trypsin inhibitor (SKTI) exhibited 71% and 41% maximum inhibition of trypsin-like activity and total proteolytic activity against HGP respectively. Chickpea Kunitz trypsin inhibitor (CPTI) showed 60% maximum inhibition of trypsin-like activity which is less than MKTI and SKTI but exhibited 69% inhibition of total proteolytic activity against HGP, much higher than MKTI and SKTI (Srinivasan et. al., 2005).

The stability of a candidate PI against proteolysis by insect gut proteinases is an important criterion in developing it as a defense agent against the particular insect pest. It has been reported that many times insect gut proteinases can overcome the effect of proteinase inhibitors by degrading them (Giri et al., 1998; Girard et al., 1998). MKTI exhibited remarkable stability against proteolysis by HGP. It remained stable even after 72 h of incubation with total gut proteinases. The results support our earlier finding where native and heat-treated MKTI was found to be completely stable against proteolysis by selected proteinases (Shee et al, 2007a).

Both, *invitro* and *invivo* studies are required to assess the insecticidal effects of an inhibitor. It has been shown that inhibitors effective under *in vitro* conditions may not show anti-metabolic effect under *invivo* conditions (Edmonds et al., 1996). Insect feeding assays were, therefore, carried out to assess the effectiveness of MKTI as an insecticidal agent of *H. armigera*. The results clearly demonstrated the strong deleterious effect of MKTI on growth and development of *H. armigera*. MKTI adversely affected all three stages of development of *H. armigera* in dose dependent manner. A substantial reduction was observed in larval weight,

larval survival, pupation, adult emergence and adult survival in inhibitor fed insects. Both fertility and fecundity were severely affected and malformed pupae and adults were observed. A lower growth and developmental indices were recorded for the insects fed on inhibitor diet. The protein concentration in midgut of *H. armigera* larvae reduced substantially after feeding diet containing MKTI. The accumulation of nutrients during larval stage is important for growth and development of insect.

Effect of MKTI ingestion on endogenous level digestive proteinases showed a substantial decline in trypsin-like activity. Similar results have been observed for SKTI fed *H. armigera* larvae where significant decline in trypsin-like activity was observed (Johnston et al, 1993). However, a significant increase in chymotrypsin-like activity was also observed in MKTI treated larvae. The results are indicative of the phenomena where a decrease in trypsin-like activity in insect gut triggers an increase in production of other similar insensitive proteinases. It is interesting to note that the decline in trypsin-like and increase in chymotrypsin-like activity showed a noticeable reduction in the fifth instar larvae. The most reduction in trypsin-like activity was observed in fourth instar larvae whereas highest chymotrypsin-like activity was observed in third instar larvae. The results showed that initially a decrease in trypsin-like activity was compensated by an increase in chymotrypsin-like activity. However, in later stages, a reduction in trypsin-like activity was reversed to some degree by expressing MKTI insensitive trypsin-like proteinases. Analysis of *H. armigera* gut proteinases has revealed the complexities in their expression and specificities in response to different PIs (Bown et al., 1997; Patankar et al., 2001)

CHAPTER 3

BIOINSECTICIDAL ACTIVITY OF *MURRAYA KOENIGII* TRYPSIN INHIBITOR (MKTI) ON *SPODOPTERA LITURA*

3.1. Introduction

Among many lepidopteran insect pests, the cutworm *Spodoptera litura*, a polyphagous lepidopteran insect pest, is one of the most devastating field pests of many important crops causing severe economic losses (CAB International, 2002). One of the major focuses of plant biotechnology is to develop crops resistant to particular insect pests. One of the strategies could be to express effective proteinase inhibitors, against insect gut proteinases, from non-host plants in affected crops. Plant proteinase inhibitors are one of the natural defense molecules against many insect pests (Gracia-olmedo et al., 1987; Ryan, 1990; Boulter, 1993; Yeh et al., 1997).

Soyabean Kunitz, *Archidendron ellipticum* and bitter gourd trypsin inhibitors (SKTI, AeTI and BGTI) showed inhibition of gut proteinases and adverse effect on growth and development of *Spodoptera litura* (McManus and Burgess, 1995; Bhattacharyya et al., 2007; Telang et al., 2003).

In this chapter, we have demonstrated the inhibitory potential of MKTI against gut proteinases and their deleterious effect on growth and development of *S. litura*.

3.2. Materials and Methods

3.2.1. Materials

Bovine pancreatic trypsin, chymotrypsin, N-benzoyl-L-arginine p-nitronilide (BAPNA), N-benzoyl-L-tyrosine ethyl ester (BTEE), Azocasein, SDS-PAGE chemicals were purchased from Sigma Aldrich Chemicals Co. (St. Louis, MO, USA). Gelatin was purchased from Fluka. All other chemical used were of analytical grade.

3.2.2 Purification of MKTI

Purification of MKTI was done as previously described (Shee and Sharma, 2007). Briefly, seeds of *Murraya koenigii* were crushed and soaked overnight at 4°C in 30 ml of 50 mM Tris-HCl buffer, pH 7.5. The homogenate was cleared by centrifugation at 12,000g for 1 h and the supernatant was loaded onto a DEAE Sepharose column equilibrated with same buffer used for sample extraction. The unbound molecules were washed extensively and bound molecules were eluted with a NaCl step gradient from 0 to 500 mM in the same buffer. Fractions with trypsin inhibitory activity were pooled and concentrated. The protein was further purified by HPLC using size exclusion chromatography column. The inhibitor was also purified in single step by affinity chromatography using Cibacron blue 3 GA (Shee and Sharma, 2008). The purity of the protein was determined by 15% SDS-PAGE analysis (Laemmli, 1970).

3.2.3. Estimation of proteins

Protein contents were measured by dye binding method according to the procedure of Bradford (Bradford, 1976) with bovine serum albumin as protein standard (1 mg/ml).

3.2.4. Insect rearing

The cultures of *S. litura* used for these experiments were maintained in the Division of Entomology, Indian Agriculture Research Institute, New Delhi. The larvae were reared with the photoperiod regime of 16h light–8h dark, 27°C±1 °C temperature and 65% relative humidity. The composition of artificial diet on which the larvae were transferred and maintained throughout the experiment was similar to that followed by Gupta et al. (2005) and chiefly composed of chickpea flour (*Cicer arietinum* L) (56.0 g), Kidney bean (*Phaseolus vulgaris*) (51.3 g) wheat germ, (*Triticum* sp) (26.0 g), agar-agar (16.4 g), dried yeast powder (31.6 g),

casein (15.20 g), ascorbic acid (3.2 g), formaldehyde 40% (2.0 ml), ABDEC drops (multivitamin solutions, 1.5 ml), multi-vitamin capsules (2) and α-tocopherol (Evion 400 mg, 1 capsule), Methyl-p-hydroxybanzoate (1.8 ml), distilled water (820 ml).

3.2.5. Preparation of Spodoptera litura gut proteinase (SGP)

Final instar larvae were used to assess the inhibitory potential of MKTI against *S. litura* midgut proteinases. The midguts of the fifth instar larvae were carefully dissected on ice after decapitation. Replicated sets of five guts were maintained and each gut was transferred into an eppendorf tube maintained on ice at 4°C containing 40 µl of 1 mm HCl and then stored at -20°C. As trypsin can remain stable almost indefinitely when frozen in 1 mM HCl, the same is considered for gut serine proteinases (Walsh, 1970). For SGP extraction, the midgut was crushed with the glass rod and homogenates were centrifuged at 12,000g for 10 min at 4°C. The supernatants were used as crude enzyme for trypsin-like, chymotrypsin-like and total proteinase activity and were stored at -20°C.

3.2.6. Proteinase and proteinases inhibitory assays

Crude extract was evaluated for total proteinase, trypsin-like and chymotrypsin-like activity by estimating hydrolytic activity towards synthetic substrates azocasein, BAPNA and BTEE respectively.

3.2.6.1. Using BAPNA and BTEE as substrate

Trypsin-like and chymotrypsin-like activity assay were carried out by estimating the hydrolytic activity of supernatants towards the substrate BAPNA (1.25 mM) and BTEE (1.07

mM) respectively (Shibata et al., 1981). Reactions were performed in a UV-Vis spectrophotometer, set at 410 nm for hydrolysis of BAPNA and 256 nm for BTEE. The proteinase and trypsin inhibitory assay of MKTI was monitored after mixing variable concentrations of the inhibitor with the 20 µg of bovine pancreatic trypsin and with the volume of equivalent activity of the crude gut extract. The assays were run in the 1 ml of 50 mM Tris-Cl buffer containing 20 mM $CaCl_2$, pH 8.0. As a control only the crude gut extract (without inhibitor) was used in the enzymatic assay. One proteinase unit was defined as the amount of enzyme that increased absorbance by 1 OD/min at 410 nm and one PI unit was defined as inhibition of 1 unit of proteinase activity under the given assay conditions. All assays were done in triplicate and averaged.

3.2.6.2. Using azocasein as a substrate

Total proteinase activity was evaluated by incubating crude gut extract with 500 µl of 1% azocasein in 50 mM Tris-Cl, pH 8.0 containing 20 mM $CaCl_2$ in total volume of 1 ml at 37°C for 30 min. After incubation, the reaction was stopped by adding 150 µl of 20% Trichloroacetic acid (TCA). The tubes were then centrifuged at 10,000g for 10 minutes, after centrifugation supernatant was collected and absorbance was measured at 366 nm (Saborowski et al. 2006.).

3.2.7. Proteolysis study of MKTI with SGP

The proteolytic stability of MKTI against SGP was evaluated by incubating equal amounts of MKTI and SGP (w/w) in 50 mM Tris-HCl, pH 8.0 at 37°C for 0, 10, 30 min, 1, 3, 10, 24 and 72 h. The stability of MKTI was determined by running 15% SDS-PAGE. Also,

trypsin inhibitory activity of MKTI was examined by performing inhibitory assay using synthetic substrate BAPNA as described earlier.

3.2.8. Larval gut activity in polyacrylamide gel containing 0.3% gelatin

The larval gut activity analysis was performed on a SDS- PAGE (Laemmli, 1970) containing 0.3% gelatin (Silva et al., 2006). β-merceptoethanol was not included in the sample buffer. The gel was run at a constant current of 50 mA at 4°C. *S. litura* Gut homogenate of third, fourth and fifth instar larvae were used in gel activity assay. In gel inhibitory activity assay, gut homogenate of fifth instar larvae was used in the presence of MKTI. After electrophoresis, the gel was transferred to 2.5% Triton X-100 solution (v/v) for 30 min at room temperature. The gel was then equilibrated with developing buffer composed of 0.05 M Tris-HCl buffer, pH 8.0 containing 0.15 M NaCl and 0.005 M $CaCl_2$ with gentle agitation at room temperature for four hours. After incubation the reaction was stopped by transferring the gel to a staining solution (Commassie Brilliant Blue R-250 in methanol: acetic acid: water, 3:1:6, v/v/v) and then to a destaining solution (methanol: acetic acid: water, v/v/v). The proteinase activity of the gut extract was visualized by the clear zone on dark background.

3.2.9. Insect feeding bioassays

3.2.9.1. Larval development

The effect of MKTI on growth and development of *S. litura* was examined by insect feeding bioassays. The assays were performed using the laboratory established culture of *S. litura* on artificial diet supplemented with MKTI at concentrations of 25 µM, 50µM, 100µM, 150µM and 200µM. Ten early instar larvae were used for each treatment in triplicate. Larval

weights were taken on 6^{th}, 8^{th} and 10^{th} day after treatment and percent weight reduction in the proteinase inhibitor fed larvae was compared to that of the control group. The larval period, larval mortality was recorded and averaged.

3.2.9.2. Pupal development

Pupation period, pupal weight, number of malformed pupae and adult emergence were recorded and compared with that of the control group

3.2.9.3. Adult development

The fecundity and fertility data was also collected by emerged male and female moth. Three pairs for each treatment were kept in rearing jar separately with the cotton plug immersed in 10% sucrose solution and number of eggs and number of hatching was counted for each treatment separately and average was taken to calculate the fecundity and fertility for single pair and compared with control group.

3.2.10. Determination of growth indices

Growth indices of larva, pupa and Total development were calculated by the equation described by Gupta et al. (2004).

3.2.10.1. Larval growth index

The Larval growth index was calculated and compared with that of control group.

Growth Index (Larva): Percentage Pupation/Larval Period,

3.2.10.2. Pupal growth index

The pupal growth index was calculated by dividing Total percentage of adult emergence with averaged pupal period. It signifies the overall growth of pupa.

Growth Index (Pupa): Percentage Adult Emergence/ Pupal Period

3.2.10.3. Developmental index

Total developmental index was determined by dividing total survival with averaged developmental period.

Developmental Index: Percentage Survival / Total Developmental Period.

3.2.11. Effect of MKTI feeding on SGP endogenous level of proteinases

The effect of MKTI feeding was evaluated on the endogenous proteolytic activity of SGP at different stages of larval development. The larval guts obtained at different instar stages following inhibitor feeding were used for measuring trypsin and chymotrypsin-like activity as described earlier. A set of control without the presence of MKTI in the larval diet was maintained. To determine endogenous proteolytic activity, three larvae each per instar stage per treatment were taken and mean was calculated.

3.3. Results

3.3.1. Purification of MKTI

Pure MKTI was obtained by the method described earlier. The purity of MKTI was assessed by the 15% SDS-PAGE.

3.3.2. Inhibition of bovine pancreatic trypsin by MKTI

MKTI was evaluated against the bovine pancreatic trypsin as previously described (Shee at el. 2007). 20 µg of bovine pancreatic trypsin was completely inhibited by 25 µg of MKTI (Figure 3.1).

3.3.3. Inhibition of proteinase activity of SGP by MKTI

The inhibitory potential of MKTI was evaluated against trypsin-like, chymotrypsin-like and total proteinase activity of *S. litura* gut proteinases (SGP) (Figure 3.1).

3.3.3.1. Using BAPNA and BTEE as a substrate

The trypsin-like, chymotrypsin-like and total proteinase activity was determined using synthetic substrates BAPNA, BTEE and azocasein respectively. A maximum inhibition of 81.75% for trypsin-like against SGP by MKTI were observed when it inhibited 100% activity of bovine pancreatic trypsin. No inhibition was observed by MKTI towards chymotrypsin-like enzymes in SGP (Figure 3.1).

3.3.3.2. Using azocasein as a substrate

Synthetic substrate azocasein was used to determine total proteolytic inhibition of SGP (Figure 3.1). At 100% inhibition of bovine pancreatic trypsin, MKTI inhibited 48% of total protease activity

3.3.4. Resistance of MKTI towards proteolysis by SGP

The stability of MKTI against proteolysis by *S. litura* gut proteinases was examined. MKTI exhibited remarkable stability against proteolysis by total total gut proteinases even after

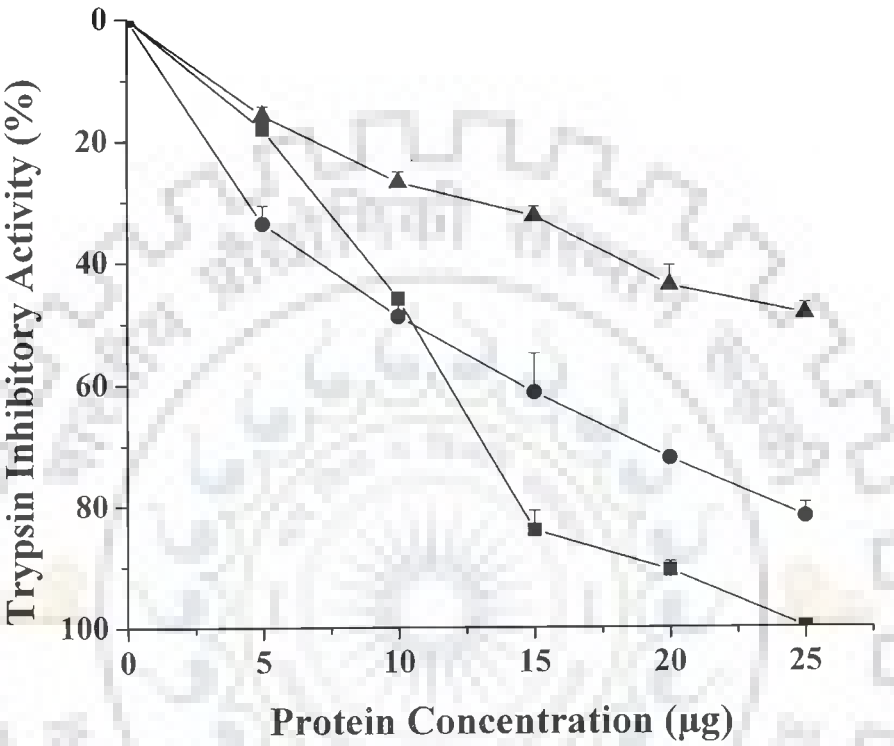

Figure 3.1: Inhibitory effect of MKTI on trypsin-like and total proteinase activity of SGP obtained from larvae fed on control diet. Inhibition assays were performed using BAPNA and azocasein as a substrate for trypsin-like (−●−) and total proteinase activity (−▲−) respectively. MKTI was titrated against SGP and bovine pancreatic trypsin (−■−). The experiment was repeated three times

72 h of incubation when equal amount of SGP and inhibitor were incubated (Figure 3.2). The trypsin inhibitory activity of MKTI remained unaffected in this period. The results support our earlier findings where native and heat-treated MKTI was found to be completely stable against proteolysis by selected proteinases (Shee et al., 2007a).

3.3.5. Zymogram analysis of SGP inhibition by MKTI

The larval gut proteinase activity against MKTI was tested on gels containing 0.3% gelatin (Figure 3.3). The total gut proteinases of fifth instar larvae were used for testing in-gel inhibitory activity of MKTI. A clear zone against dark background was observed in the lanes where only gut proteinases from third, fourth and fifth instar larvae were run. A substantial reduction in zone of clearance was observed where sample contained both MKTI and gut proteinases from fifth instar larvae. The results clearly indicate that MKTI is able to inhibit partial gut proteinase activity by inhibiting trypsin like enzymes.

3.3.6. Effect of MKTI on growth and development of S. litura

Insect feeding assays were carried out to assess the effectiveness of MKTI as an insecticidal agent of *S. litura*. Effect of inhibitor on growth and development of larvae, pupae and adult of *S. litura* was examined by incorporating MKTI (25–200 μM) in control diet.

3.3.6.1. Effect of MKTI feeding on larval growth

The larvae of *S. litura* showed reduction in weight in dose dependent manner when fed on diet containing MKTI, as compared to those fed on control diet. At highest concentration of MKTI (200μM), approximately 44, 53 and 44% reduction in larval weight was observed after 6,

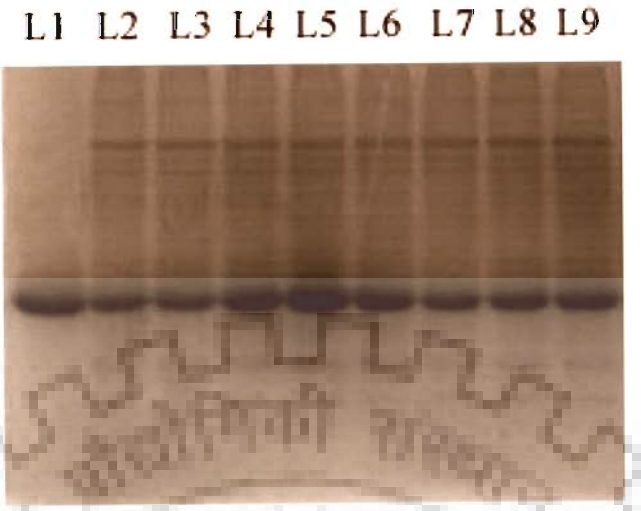

Figure 3.2: Proteolytic stability of MKTI against SGP on a 12% SDS-PAGE. MKTI was pre-incubated with SGP for different time intervals at 37 °C. L1: Native MKTI; L2: 0 min; L3: 10 min; L4: 30min; L5: 1 h; L6: 3 h; L7: 10h; L8: 24 h; L9: 72 h.

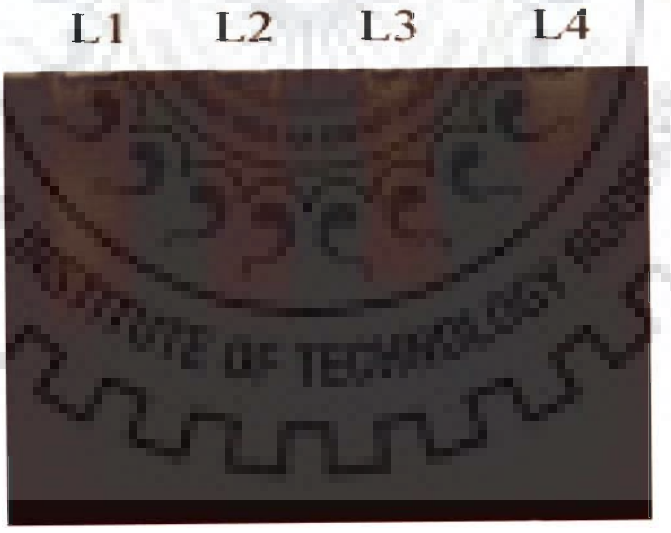

Figure 3.3: In gel inhibitory activity of MKTI against SGP on SDS- PAGE containing 0.3% gelatin as substrate. β- merceptoethanol was excluded from the sample buffer .; L1 – L3, gut extract of third, fourth and fifth instar larvae respectively. L4: SGP incubated with equal amount of MKTI.

8 and 10 days of feeding (Figure: 3.4). Larval period were marginally increased from 14.96 to 17.19 days at highest concentration of inhibitor (Figure 3.5). Larval mortality also showed an increase at higher inhibitor doses with 43.34% mortality observed in larvae (Figure 3.6).

3.3.6.2. Effect of MKTI feeding on pupal growth

Pupation decreased from 96.7% to 56.7% and pupal weight reduced by 20% at highest dose of inhibitor (Figure 3.8). Adult emergence decreased from 90 to 57%, pupal period did not change substantially when fed on diet supplemented with 200 µM MKTI (Table 3.1). Total survival was reduced by 33% and an increase from 22.5 to 26.4 days was observed in total development days at highest dose (Table 3.1). Malformed and small sized pupae (Figure 3.7 B & C) and adults were observed due to abnormal development during feeding assays (Table 3.1).

3.3.6.3. Effect of MKTI on adult development

Egg laying capacity of adult female (fertility) and hatching of larvae from eggs (fecundity) in adult insects fed on inhibitor diet was severely affected with reduction observed in dose dependent manner. Egg laying capacity of adult female (fertility) reduced substantially from 1945 eggs in control to 366 eggs in those fed on diet containing 200 µM of inhibitor. Hatching of larvae from eggs (fecundity) reduced from 91% in control to 64% in MKTI fed insects (Table 3.1).

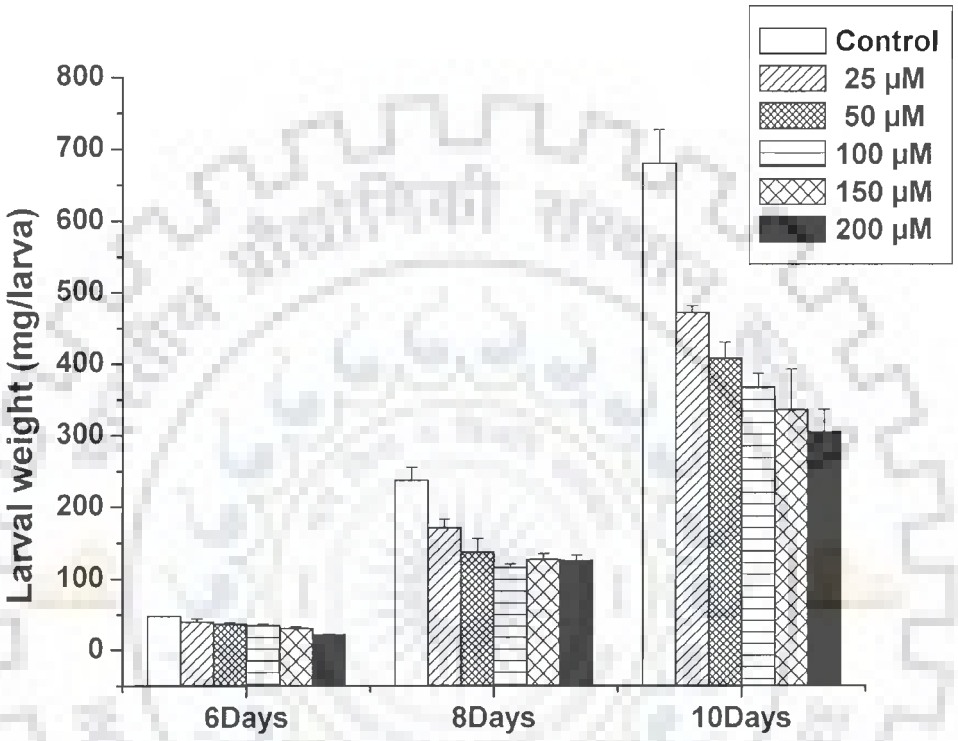

Figure 3.4: Effect of MKTI on larval weights of *S. litura.* Different concentration (25–200 µM) of MKTI was incorporated in the standard diet. The weight (mg/larva) were taken at 6^{th}, 8^{th} and 10^{th} days after treatment and averaged. The Standard error of mean is shown as bars.

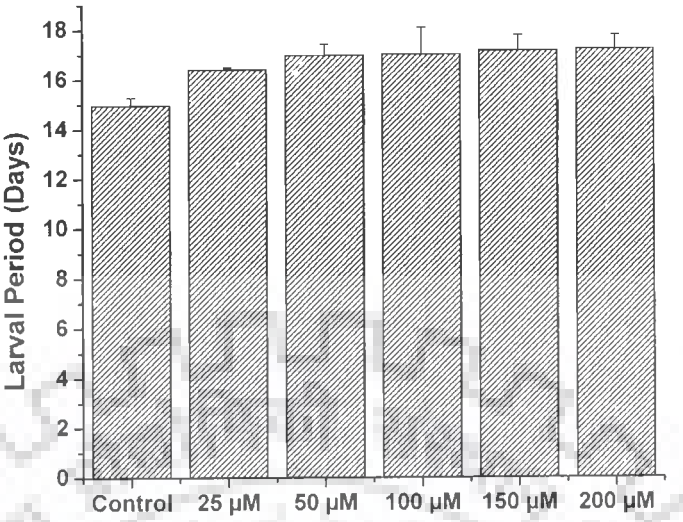

Figure 3.5: Effect of MKTI on larval period of *S.litura* in different treatments

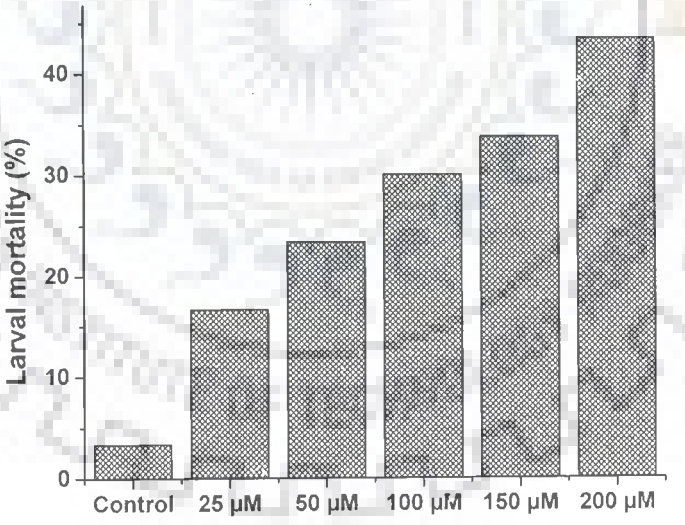

Figure 3.6: Larval mortality in the various MKTI treated diet compared to control group

Figure 3.7: Representative photographs of effect of MKTI on growth and development of *S. litura* larvae and pupae. **(A)** Effect of MKTI on larval growth. **(B)** Malformed pupae. **(C)** Reduced size pupa

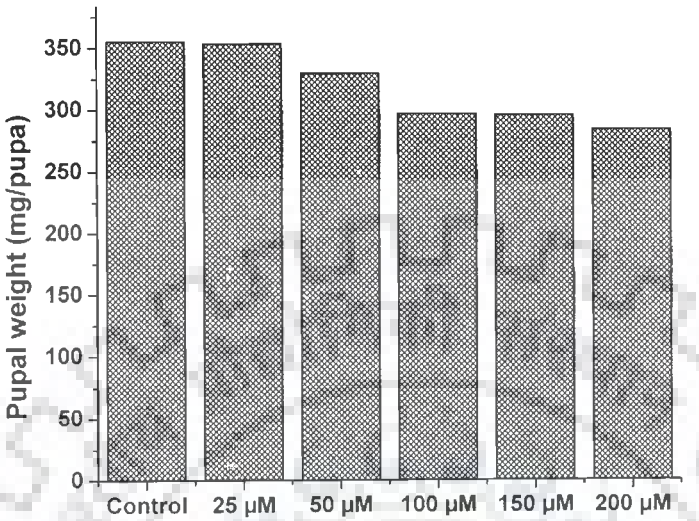

Figure 3.8: Pupal weight (mg/pupa) in various treatment of diet along with the control diet.

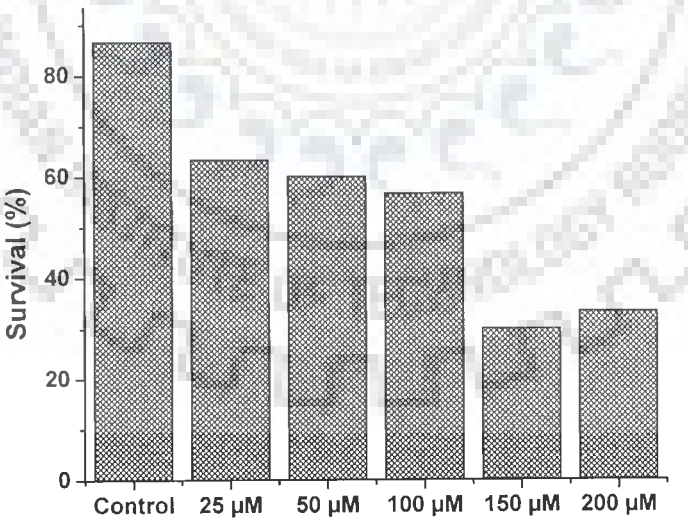

Figure 3.9: Total survival (%) of *S.litura* in various treatments

Table1 3.1: Effects of MKTI on growth and development of *S. litura*

	Malformed Pupa (%)	Adult Emergence (%)	Malformed Adult	Developmental Days	Fecundity (Eggs/Female)	Fertility (%)
Control	0	90	0	22.566	1945.33±24.82	91.75
25	0	75.92	0	25.01	1360 ± 45.36	87
50	0	79.16	0	26.158	666.67 ± 19.9	83
100	0	76.18	0	26.21	466.67 ± 54.24	72.99
150	16.19	44.88	0	26.09	450 ± 14.74	73.78
200	18.64	57.14	16.66	26.41	366.67 ± 28.54	64

3.3.7. Growth Indices of S. litura fed on MKTI

Analysis of growth and development indices showed substantial reduction in the growth index of larva and pupae and development index for the insects fed on diets containing MKTI as compared to the insects fed on control diet. The growth index of larvae and pupae fed on MKTI (200 µM) decreased from 6.45 and 11.18 to 3.30 and 6.69 respectively (Figure 3.10). A lower development index of 1.26 was recorded for the insect fed on MKTI (200 µM) as compared to the insects fed on control diet (3.84).

3.38. Effect of MKTI on digestive proteinases of S. litura

3.3.8.1 Trypsin-like activity

The effect of MKTI feeding on endogenous level of larval gut proteinases was studied. The trypsin-like activity reduced continuously with increasing amounts of MKTI in larval diet. A significant decrease in trypsin-like activity was observed in larvae fed on diet containing MKTI as compared to those fed on control diet. A decline of 8, 6.2 and 8-fold was observed in the trypsin-like activity of SGP in third, fourth and fifth instars larvae fed on 200 µM MKTI respectively (Table 3.2 A).

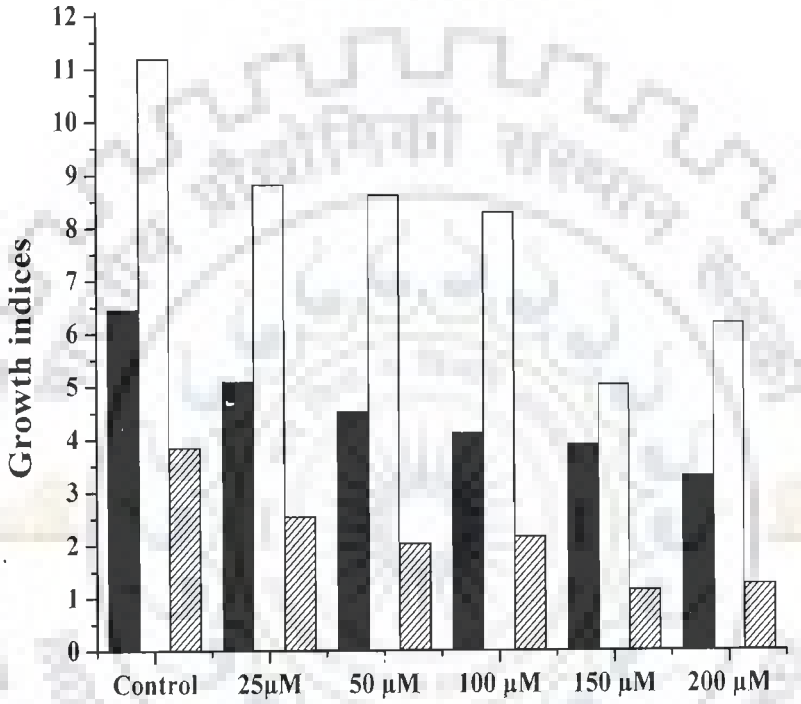

Figure 3.10: Effect of MKTI on growth and developmental indices of S. *litura*. Growth index of larvae (■); growth index of pupae (□); developmental index (▨).

Table 3.2: The effect of MKTI feeding on endogenous level of proteinase activity in gut extracts from different instars of *S. litura* larvae **(A)** Endogenous trypsin-like activity (µmol/ml/min). **(B)** Endogenous chymotrypsin-like activity (µmol/ml/min).

Instar	Control	25 µM	50 µM	100 µM	150 µM	200 µM
3rd	7.93±0.06	5.66±0.74	4.88±0.07	2.3± 0.27	1.22±0.27	0.94±0.04
4th	7.26±0.30	5.59±0.66	4.36±0.27	3.47±0.24	2.26±0.15	1.16±0.06
5th	5.87±0.58	3.80±0.39	3.03±0.09	1.92±0.03	0.96±0.17	0.73±0.09

A

Instar	Control	25 µM	50 µM	100 µM	150 µM	200 µM
3rd	2.76±0.06	3.52±0.22	4.70±0.08	4.48± 0.25	7.3±0.3	7.52±0.07
4th	1.81±0.1	2.53±0.29	3.65±0.14	3.7±0.10	5.75±0.05	6.25±0.26
5th	1.97±0.04	3.43±0.19	4.73±0.07	6.54±0.25	7.38±0.13	7.59±0.23

B

3.3.8.2 Chymotrypsin-like activity

In contrast, chymotrypsin-like activity increased by 2.7, 3.45 and 3.85-fold in MKTI fed third, fourth and fifth instars larvae respectively (Table 3.2 B).

3.4. Discussion

Lepidopteran insect pests depend mainly on serine proteinases particularly trypsin and chymotrypsin-like enzymes for digestion of food proteins (Bown et al., 1997). Developing pest resistant crops expressing effective trypsin inhibitors is one of the strategies of plant defense against lepidopteran insects. Earlier, our studies have shown deleterious effect of MKTI on one of the devastating polyphagous lepidopteran insect *Helicoverpa armigera*. This work evaluates

the *invitro* and *invivo* effects of MKTI on another polyphagous lepidopteran insect *S. litura*. MKTI is a non-host PI and it has been shown that non-host PIs can be potentially more effective plant defense agents (Harsulakar et al., 1999). The results of this study showed that MKTI is a potent inhibitor of gut proteinases and have adverse effect on the growth and development of *S. litura*.

In inhibitory assays, MKTI showed substantial amount of inhibition with over 81% inhibition of trypsin-like and 48% of total gut proteinase activity against SGP. Comparatively, the inhibitory activity of MKTI, with over 78% of trypsin-like and 40% of total proteinase activity, against *H. armigera* gut proteinases (HGP) was slightly lower than that of SGP. The results clearly indicate presence of MKTI insensitive trypsin-like enzymes in gut of lepidopteran insects. Similar in vitro inhibitory effects against SGP have been demonstrated for SKTI, AeTI and BGTI (McManus and Burgess, 1995; Bhattacharya et al., 2007; Telang et al., 2003).

One of the important aspects in developing a candidate PI as a potential plant defense agent is the proteolytic stability of the inhibitor against insect gut proteinases of that particular insect pest. It has been shown that many times insect gut proteinases can overcome the effect of proteinase inhibitors by degrading them (Giri et al., 1998; Girard et al., 1998). In proteolysis studies, MKTI was found to be remarkably stable against proteolysis by SGP even after 72 h of incubation with total gut proteinases. Similar results were obtained against *H. armigera* gut proteinases where MKTI was found completely stable against proteolysis. Earlier, it has been shown that native and heat-treated MKTI was completely stable against proteolysis by selected proteinases (Shee et al, 2007a). The results clearly indicate that MKTI can survive for longer time in the gut of lepidopteran insects without being digested.

To evaluate the bioinsecticidal activity of a candidate PI, it is important to study both *invitro* and *invivo* effects against particular insect pest. It has been demonstrated that inhibitors effective under *invitro* conditions may not show anti-metabolic effect under *invivo* conditions (Edmonds et al., 1996). *In vivo* studies were, therefore, carried out to evaluate the potential of MKTI as a bioinsecticidal agent of *S. litura*. The study clearly demonstrated the deleterious effect of MKTI on growth and development of larvae, pupae and adult in dose dependent manner. A significant decrease in larval weight, larval survival, pupation, adult emergence and adult survival was observed in MKTI fed insects. Both fertility and fecundity were severely affected in adults emerging from PI fed larvae. Also, malformed pupae and adults were observed. A lower growth index for larvae and pupae and a lower overall developmental index were recorded for the insects fed on inhibitor diet. The protein concentration in midgut of larvae reduced substantially after inhibitor fed diet. The accumulation of nutrients during larval stage is important for growth and development of insect. In our earlier studies, MKTI also showed adverse effect on growth of *H. armigera*. Although MKTI severely affected growth and development of both lepidopteran insects, there were some notable differences. There was significantly higher reduction in larval weight in MKTI fed larvae in case of *H. armigera* as compared to that of *S. litura*. Approximately 69% reduction in larval weight was recorded for *H. armigera* whereas only 44% reduction was recorded for *S. litura* after 10 days of PI feeding. The adverse effect of MKTI on pupation and adult emergence was slightly better in case of *S. litura* when compared to *H. armigera*. Clearly, MKTI showed deleterious effect on both lepidopteran insects.

It has been shown that insect pests overcome the effect of plant PIs by expressing new proteinases which are either insensitive or with changed substrate specificity in response to PIs

(Jongsma et al., 1995; Jongsma and Bolter, 1997; Wu et al., 1997; Gatehouse et al., 1997). Effects of MKTI feeding on endogenous level of digestive proteinases in larvae of *S. litura* were examined. A very significant decline in trypsin-like activity and an increase in chymotrypsin-like activity were observed. Almost 8-fold reduction in trypsin-like activity and 3.8-fold increase in chymotrypsin-like activity was observed in fifth instar larvae. Similar results have been observed for AeTI fed *S. litura* larvae where significant decline in trypsin-like activity and increase in chymotrypsin-like activity was observed (Mazumadar et al, 2007). The results support the phenomena where a decrease in trypsin-like activity in insect gut triggers an increase in production of other similar insensitive proteinases in response to PI. MKTI-fed larvae of *H. armigera* also showed significant changes in trypsin-like and chymotrypsin-like activity (~ 3-fold in fifth instar larvae) in gut proteinases. Comparatively, the decline in trypsin-like activity was much lower in larvae of *H. armigera* as compared to that of *S. litura*. Notably, MKTI showed higher inhibition of gut proteinases of *S. litura* as compared to that of *H. armigera*. Also, it was interesting to note that increase in chymotrypsin-like activity reduced from 4.4-fold in third instar larvae to 3-fold in fifth instar larvae in case of *H. armigera*. In case of *S. litura,* however, chymotrypsin-like activity increased from 2.7-fold in third instar to 3.85 fold in fifth instar larvae. This clearly indicates a different response of MKTI feeding in digestive proteinases of two lepidopteran insects.

CHAPTER 4

CLONING, EXPRESSION AND PURIFICATION OF *MURRAYA KOENIGII* TRYPSIN INHIBITOR

4.1 Introduction

The presence of proteinase inhibitors in plants affects the development of insects, and insect attacks on plants induce a rapid accumulation of proteinase inhibitors in the leaves. The resistance in crop can be developed through genetic engineering. The basic idea was to increase the concentration of already existing proteinase inhibitors in the plant as well as to introduce them into plant devoid of proteinase inhibitors. Two main approaches to obtain insect-resistant plants, by expression of proteins in plants adverse to plant pathogens have been explored. The first one involves the use of delta-endotoxin coding sequences originating from the bacterium *Bacillus thuringiensis*. The second approaches uses plant derived genes, such as those encoding enzyme inhibitors or lectins.

MKTI has shown potent bioinsecticidal activity against the major lepidopteran pests, *H. armigera* and *S. litura*. MKTI gene can be a potential candidate which can be introduce into the crops for the insect resistance either alone or in combination with Bt gene. In the present study cloning of complete gene of MKTI including 5' and 3' UTR was done from cDNA and genomic DNA. Expression and purification of recombinant MKTI have also been performed.

4.2. Materials & methods

4.2.1. Materials

Isopropyl-1-thio-D-galactosidase (IPTG), Taq Polymerase, T4 DNA ligase, factor Xa, Trypsin and BAPNA were purchased from Sigma. The expression vector, pGEX-5X-3 and GST-sepharose affinity beads were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Restriction Enzymes, Z- competent *E. coli* cells- TG1 kit was purchased from Zymo Research (U.S.A), pGEM-T cloning kit were purchased from Promega (Madison, WI). Plasmid

Purification and Gel elution kit were purchased from Bangalore genei (Bangalore, India). All other chemicals used were of analytical grade.

4.2.2. Primer designing

On the basis of N-terminal sequence of MKTI previously reported (Shee et al. 2007), degenerate primers were designed to amplify the MKTI gene. The amino acid sequence of MKTI has shown significant identity with miraculin-like protein from *Citrus jambhiri*. Therefore, the gene sequence of miraculin-like protein was also used for designing forward primer except for the non-identical amino acids where mixed degenerate codon has been put. Table 4.1 lists all the primer used during this study.

4.2.3. Isolation of total RNA

4.2.3.1. Preparation of materials and samples

15 ml polypropylene centrifuge tubes were pretreated in 0.1% diethyl pyrocarbonate (DEPC) for one hour at room temperature and then autoclaved for 30 min to destroy residual DEPC. Freshly harvested *Murraya koenigii* seeds (500 mg) were freezed and grounded in the liquid nitrogen using ceramic mortar and pestle. Liquid nitrogen was allowed to evaporate and the grounded material was transferred to a sterile 15 ml centrifuge tube.

4.2.3.2. Isolation of total RNA

Total RNA was isolated from the seeds by following Guanidinium thiocynate method (Chomczynski and Sacchi, 1987; Sambrook et al., 2001) with minor modifications. In the grounded tissue, before adding solution D, 25 mg of polyvinyl polypyrrolidone (PVPP) per

gram of seeds was mixed (Malnoy et al., 2001). PVPP was used for the removal of pigment found in seeds. Sequentially, 0.1 ml of 2 M sodium acetate (pH 2.0), 1ml of phenol and 0.2 ml of chloroform-isoamyl alcohol per ml of Solution D was added. It was vortexed and incubated on ice for 15 minutes before centrifugation at 10,000g for 20 min at 4°C. Upper aqueous layer containing the RNA was transferred. RNA was precipitated for an hour with an equal volume of Isopropanol at -20°C. The precipitated RNA was collected after centrifugation, washed with 70% ethanol and air dried. The RNA pellet was dissolved in the DEPC treated water.

4.2.4. Isolation of genomic DNA

Murraya koenigii genomic DNA was extracted according to the Sambrook et al. (2001). Briefly, 1g of young leaves was powdered in liquid nitrogen, mixed with 5 ml of DEB and incubated at 65°C for one hour with frequent shaking in between. After one hour, equal volume of Chloroform: Isoamyl alcohol (24:1) was added and then the mixture was centrifuged at 5000g for 20 min for 20°C. Supernatant was collected, mixed with equal volume of ice cold Isopropanol and incubated at -20°C for 30 min for DNA precipitation. Precipitated DNA was collected, washed with 70% ethanol and stored for future use.

4.2.5. First strand cDNA synthesis or reverse transcription

Single-stranded cDNA was synthesized from total RNA using oligo$(dT)_{15}$ adaptor primer (Table 4.1) for reverse transcription in 20 µl reactions (Chomczynski and Sacchi, 1987; Sambrook et al, 2001). In brief 1 µg of total RNA was denatured by heating at 75°C in total volume of 20 µl for 5 min, followed by rapid chilling on ice. To the denatured RNA the following reaction mixture was added:

5x Reaction buffer	10.0 µl
10 mM mixed dNTPs	5.0 µl
20 mM $(dT)_{15}$ adaptor primer	5.0 µl
20 unit/ µl placental RNAs inhibitor	1.0 µl
100 unit/ µl Reverse Transcriptase	1.0 µl
H2O	to 50.0 µl

The above reaction mixture was incubated for 60 min at 37°C. After completion of reaction the reverse transcription product was kept at -20°C for future use.

4.2.6. Amplification of MKTI gene

Complete MKTI gene was cloned by using a combination of 3' Rapid amplification of cDNA ends PCR and 5' Rapid amplification of cDNA ends PCR (RACE PCR).

4.2.6.1. 3' Rapid amplification of complementary DNA ends of MKTI

4.2.6.1.1. Reaction mixture preparation

In a sterile 0.5 ml amplification tube, a series of PCR has been set up containing the following:

cDNA	0.5 µl
10x amplification buffer	2.5 µl
10 mM solution of mixed dNTPs	5.0 µl
20 mM oligo$(dT)_{15}$ adaptor primer	1.6 µl
20 mM Forward mixed primer	1.6 µl

5 unit/ µl Thermostable DNA polymerase	0.5 µl
H_2O	to 25.0 µl

4.2.6.1.2. Basic programming

3' RACE PCR was carried out using the reaction mixture with the following conditions: 95°C/4 min; 30 cycles of 95°C/60 s , 55°C/60 s, 72°C/60 s; and 72°C/10 min. The PCR product was detected on a 0.8% agarose gel.

4.2.6.1.3. Elution of DNA from agarose gel

The PCR product of 3'RACE PCR was eluted from low melting agarose gel according to the manufacturer's instruction of Gel Elution kit (Bangalore genei). In brief, agarose gel slice containing DNA fragment was cut out with clean scalpel and placed in fresh pre-weighed 1.5 ml eppendorf. Sample was incubated at 50°C after adding gel solubiliser. 10 µl of sodium acetate (pH 5.2) was added after the complete dissolution of gel. DNA was precipitated by adding 100 µl of Iso-propanol to every 100 mg of agarose gel slice. Complete solution of the tube was loaded on to the silica column provided with the kit and centrifuged. The column was washed with the wash buffer twice and eluted with the 50 µl of elution solution. The concentration measurement of eluted product was assessed either by measuring optical density on a spectrophotometer or by visualizing on an agarose gel.

4.2.6.1.4. Cloning of 3' RACE product in pGEM-T cloning vector

Cloning of 3'RACE product in pGEM-T vector was done according to the manufacturer's instruction (Promega). In brief, the eluted product was ligated with pGEM-T

vector in the ratio of 3:1 and incubated at 4°C overnight. The ligated product was transformed in chemically made competent *E.coli* cells DH5-α. The transformed cells were plated on the agar plates containing 80 μg/ml ampicillin. The plates were incubated overnight at 37°C, transformed colonies were picked and insertion was verified by PCR using isolated plasmid as DNA template.

4.2.6.1.5. Sequencing of cloned DNA by universal primers

pGEM-T construct from 3'RACE product was sequenced with universal primer T7 and SP6 by dideoxy method.

4.2.6.2. 5' Rapid Amplification of complementary DNA of MKTI

To get the upstream sequence of MKTI gene, 5' RACE was performed. 5' RACE involves three sequential enzymatic steps (reverse transcription, addition of homopolymeric tails and PCR). A nested primer was designed on the basis of 3' RACE product sequence, which has been used for the amplification of 5' RACE.

4.2.6.2.1. Reverse transcription with specific primers

Reverse transcription was done as previously described in this chapter, except the fact that the gene specific primer was used instead of oligo$(dT)_{15}$ adaptor primer.

4.2.6.2.2. Removal of excess of dNTPs and Primers

Unused primers and dNTPs were removed at the completion of the reverse transcription reaction, otherwise unused dNTPs will be incorporated into the tail added to the 3' end by

enzyme terminal transferase. The cDNA was precipitated twice in 2.5 M ammonium acetate and three volumes of ethanol. The precipitated cDNA was further dissolved in nuclease free water.

4.2.6.2.3. Terminal transferase reaction with cDNA

Terminal transferase adds the dNTP specifically at the 3' end of DNA. The dATP homopolymeric tail was added on to the 3' end of cDNA. The following reaction was set up:

cDNA	2.0 µl
5x Terminal transferase buffer	4.0 µl
10 mM dATP	2.0 µl
Terminal transferase	20.0 units
H_2O	to 10.0 µl

The reaction was incubated at 37°C for 15 min. Terminal transferase was inactivated by heating the reaction mixture for 3 min at 80°C.

4.2.6.2.4. Reaction mixture of 5' RACE

The 5' RACE reaction mixture contains the following:

Tailed cDNA	2.0 µl
10x amplification buffer	2.5 µl
10 mM solution of mixed dNTPs	5.0 µl
20 mM oligo$(dT)_{15}$ adaptor primer	1.6 µl
20 mM nested gene specific primer	1.6 µl
5 unit/ µl thermostable DNA polymerase	0.5 µl
H2O	to 25.0 µl

4.2.6.2.5. 5' RACE PCR programming

5' RACE PCR was carried out using the reaction mixture with the following conditions: 95°C/4 min; 30 cycles of 95°C/60 s , 58°C/60 s, 72°C/60 s; and 72°C/10 min. The PCR product was detected on 0.8% agarose gel.

4.2.6.2.6. Elution of 5'RACE product

The PCR product of 5'RACE PCR was eluted from low melting agarose gel according to the manufacturer's instruction of Gel Elution kit (Bangalore genei) as previously described in this chapter.

4.2.6.2.7. Cloning of eluted DNA in pGEM-T vector

Cloning of 5'RACE product in pGEM-T vector was done according to the manufacturer's instruction (Promega) as previously described in this chapter.

4.2.6.2.8. Sequencing of cloned 5'RACE product

Cloned 5' RACE product was sequenced by universal primer T7 and SP6.

4.2.6.3. Amplification of mature MKTI gene with specific primer

Gene specific primers were designed on the basis of complete nucleotide sequence of MKTI. These primers have been used to amplify the nucleotide sequence of mature MKTI protein.

4.2.7. Construction of expression vector in pGEX-5x-3 plasmid

Expression of open reading frame of mature MKTI protein was done in pGEX-5x-3 expression vector. It contains Glutathione-S-transferase tag at the N-terminus of the multiple cloning site. The MKTI has been expressed as a fusion protein with GST.

4.2.7.1. Primer designing for expression

Primers were designed for the expression of mature MKTI gene. BAM HI and Xho I restriction site has been put in forward and reverse primer respectively, for the directional cloning and expression. These primers have been used for the amplification of MKTI gene (Table 4.1).

4.2.7.2. Restriction digestion of MKTI gene and pGEX-5x-3 plasmid

Amplification product of MKTI and pGEX-5x-3 was digested with the BAM HI and Xho I. The total reaction mixture contains:

DNA template	1.0 µg
10x reaction buffer	10.0 µl
10 unit/µl BAM HI	1.0 µl
10 unit/µl Xho I	1.0 µl
100x BSA	1.0 µl
H_2O	to 100.0 µl

The reaction mixture was incubated overnight at 37°C. The digested DNA was purified by low melting agarose by using Gel extraction kit (Bangalore Genei) as previously described in this chapter.

4.2.7.3. Ligation and transformation of recombinant plasmid

Ligation of digested MKTI gene and pGEX-5x-3 was done according to the manufacturer's instruction of Ligation kit (Bangalore genei). Briefly, the digested MKTI DNA and pGEX-5x-3 has been ligated in 10 µl of reaction containing 1 unit of DNA ligase. The reaction mixture was incubated for 30 min on ice. The transformation of ligated product was done according to the manufacturer's instruction of Z-competent *E. coli*-TG1 cells kit (Zymo Research, USA). The transformed cells were plated on to the agar plates containing 50 µg/ml of ampicillin. The presence of insert was verified by PCR by using isolated plasmid with the gene specific primers of MKTI.

4.2.8. Expression and purification of GST fused and recombinant MKTI

4.2.8.1. Optimization of IPTG concentration, incubation time and temperature

pGEX-5x-3-MKTI was transformed freshly into the Z-competent *E. coli* cells TG1. The colony was picked and inoculated in 10 ml LB broth contained 50 µg/ml of ampicillin. The overnight grown culture was further inoculated in the one liter of LB broth contained 50 µg/ml of ampicillin. As O.D reached 0.5, the induction was done by adding IPTG at the final concentration of 1 mM. The induction was done at 37°C for four hours. Optimum conditions were screened by incubation for different time intervals at various concentrations of IPTG and temperature. Cells were harvested at 5000g, pellet was dissolved in 50 mM Tris- Cl, pH- 8.0 and incubated on ice with 0.2 µg/ml of lysozyme for 30 min. Cells were disrupted by sonication with six pulses for 15 seconds with regular interval of one minute. The supernatant was

collected after centrifugation at 13,000g for 30 min at 4°C. The overexpression was checked by 12% SDS-PAGE and trypsin inhibitory activity evaluation of crude lysate.

4.2.8.2. GST-sepharose and Cibacron blue-3GA affinity column purification of recombianant MKTI

The crude lysate was loaded on to the pre-equilibrated GST-sepharose beads with 50 mM Tris-Cl, pH 8.0. The column was washed with 50 mM Tris-Cl, pH 8.0 for the complete removal of unbound proteins. The GST-fused MKTI was eluted with 5 mM of reduced glutathione. The eluted fusion protein was further dialysed against 50 mM Tris-Cl, pH 8.0. The dialysed fused protein was cleaved by incubating with factor Xa overnight on ice in the molar ratio of 1:100. Recombinant MKTI was purified by loading the cleaved product on to the Cibacron blue-3GA. The recombinant MKTI remained bound to Cibacron blue-3GA while, GST protein comes out in flow through. The recombinant MKTI was eluted with 500 mM NaCl.

4.2.9. Comparison of trypsin inhibition of reMKTI with native MKTI

Trypsin inhibitory activity of purified recombinant MKTI was assessed 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 the various inhibitor concentrations in total volume of 300 µl in 50 mM Tris-HCL buffer, pH 7.5 at 30°C for 10 min and then 1 ml of 1.5 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.

4.3. Results

4.3.1 Primer designing

Table 4.1: List of all the primers used during this study.

Primer specification	**Tm (°C)**	**Sequence 5'- 3'**
N-terminal Forward degenerate primer	62	GATCCTTTGCTHGATATHAATGGC
3' RACE Adaptor primer	64.2	CTGATCTAGAGGTACCGGATCCTTTTTTTTTTTTTT
5'RACE Adaptor primer	64.2	CTGATCTAGAGGTACCGGATCCTTTTTTTTTTTTTT
Adaptor primer	72	CTGATCTAGAGGTACCGGATCCT
Reverse Specific		TCAAGACACGCATGAG
5'RACE Nested Primer	60	GGTTTCCTTCAACTCCACCA
Expression Primers		
Forward Primer	60.7	AAAAGGATCCGATCCTTTGCTTGATATT
Reverse Primer	63.2	AAAACTCGAGTCAAGACACGCATGAG

4.3.2. 3' Rapid amplification of cDNA ends PCR prouduct

The amplification was obtained with the forward degenerate primer which was identical to the gene sequence of miraculin-like protein except for the non-identical amino acids where mixed degenerate codon has been put. Approximately a 750 bp fragment was obtained after 3' RACE PCR (Figure 4.1B). The nucleotide sequence of 3' RACE PCR product includes the 3' UTR region (Figure 4.2). Table 4.2 shows all the constructs and strains used and made during this study.

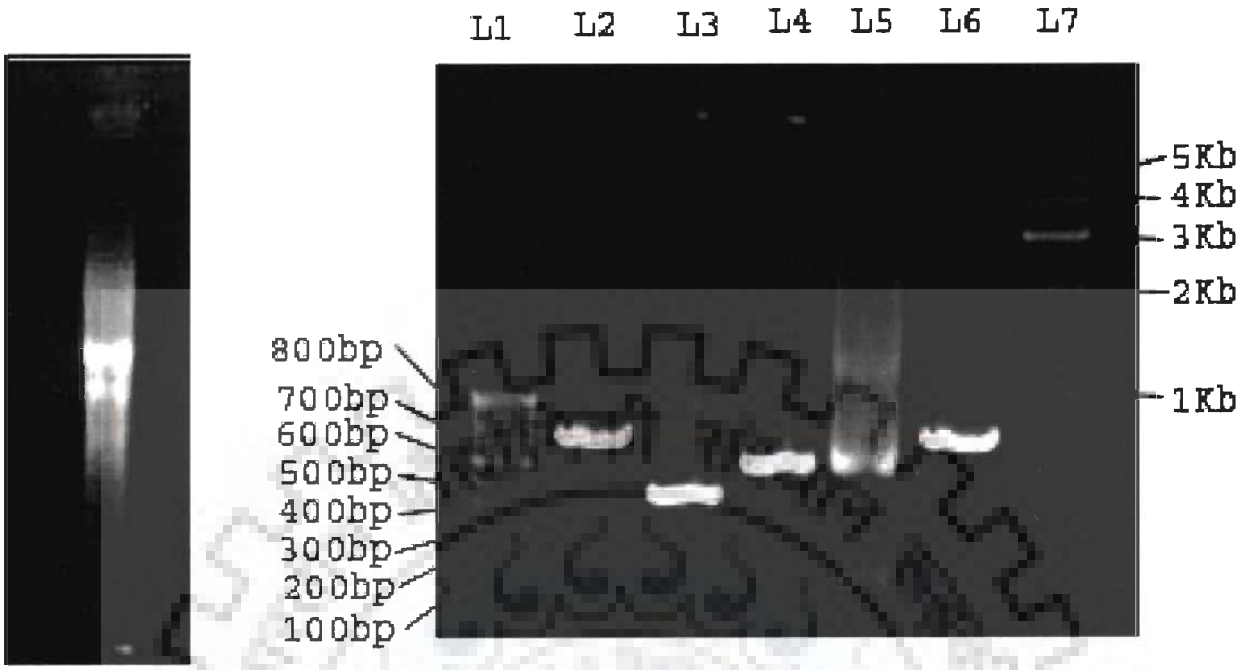

Figure 4.1: (A) Agarose gel electrophoresis of total RNA from seeds of *Murraya koenigii*. **(B)** Amplification of *Murraya koenigii* trypsin inhibitor (MKTI) genomic and cDNA. L1; 100 bp molecular weight marker, L2; 3' RACE PCR product, L3; 5' RACE PCR product, L4; Mature MKTI PCR product, L5; Genomic DNA amplification product, L6; MKTI gene with signal sequence, L7; 1 kbp molecular weight marker.

Table 4.2: Plasmids and strains used in this study

Plasmid	**Relative Characteristics**	**Reference**
pDG1	pGEM-T easy containing 750 bp fragment	This study
pDG2	pGEM-T easy containing 450 bp fragment with upstream sequence	This study
pDG3	pGEM-T east containing 573 bp MKTI gene	This study
pDG4	pGEM-T easy containing 648 bp MKTI gene with upstream signal sequence	This study
pDG5	pGEX-5x-3-573 bp MKTI gene BAMHI-XhoI fragment with N-terminal GST-Tag	This study
Strains	**Basic Characteristics**	**Reference**
DH5-α	F- endA1 glnV44 thi-1 relA1 gyrA96 deoR nupG lacZdeltaM15 hsdR17	Novagen
TG-1	F'traD36 $lacI^q$ Δ(lacZ) M15 $proA^+B^+$ /supE Δ(hsdM-mcrB)5 (r_k - m_k - $McrB^-$) thi Δ(lac-proAB)	Zymo Research
DG1	E.coli DH5-α containing pDG1 plasmid	This study
DG2	E.coli DH5-α containing pDG2 plasmid	This study
DG3	E.coli DH5-α containing pDG3 plasmid	This study
DG4	E.coli DH5-α -containing pDG4 plasmid	This study
DG5	E.coli TG1 containing pDG5 plasmid	This study

5'-

GATCCTTTGCTTGATATCAATGGCAACGTGGTCGAAGCAAGCCGAGACTAC

TATTTGGTTTCAGTAATTGGAGGAGCAGGCGGCGGTGGCCTCACCCTCTAC

AGGGGCAGAAACGAACTCTGCCCACTTGATGTTATCCAACTAAGCCCGGAT

CTACATAAAGGAACCCGTTTAAGATTCGCAGCGTATAATAACACTAGTATT

ATCCATGAAGCCGTGGATCTGAACGTGAAATTCTCAACAGAGACTAGCTGC

AACGAGCCAACAGTATGGAGAGTCGACAACTATGATCCATCGAGAGGAAAA

TGGTTTATAACAACTGGTGGAGTTGAAGGAAACCCTGGCGCACAAACTTTG

AAAAACTGGTTTAAGCTTGAGAGAGTTGGAACAGATCAAGGTACGTACGAG

ATTGTTCACTGTCCTTCCGTTTGCAAATCCTGTGTATTTTTATGCAATGATG

TTGGGGTTTCTTACGATTATCGTCGACGTTTGGCTCTCACTGCTGGTAATG

AGCGCGTCTTTGGTGTTGTTATAGTCCCGGCTAATGAGGGATCAGCCTCAT

GCGTGTCTTGA*ACAACATCATAATTTATTTTCTCCGCTTAAAACTGACAATTTTTT*

TTTT – 3'

Figure 4.2: Nucleotide sequence of 3' RACE PCR product. The product was ligated in the pGEM-T cloning vector and sequenced by universal primers. Red text is showing the nucleotide sequence used for designing of forward degenerate primer. Italics portion represent the 3' UTR region. Underlined portion has been used for the designing of nested reverse primer for 5' RACE PCR.

5'-

CCTTACACTGAAAACACCTCTGCGAAAACAAA

ATGAATACTCCTTTTGTGATTGCAATCTCCTTCCTTCTTCTTGCCTTTGCCACA

AAACCTCTTGTGGGACGCCCTGATCCTTTGCTTGATATCAATGGCAACGTGGT

CGAAGCAAGCCGAGACTACTATTTGGTTTCAGTAATTGGAGGAGCAGGCGGCG

GTGGCCTCACCCTCTACAGGGGCAGAAACGAACTCTGCCCACTTGATGTTATC

CAACTAAGCCCGGATCTACATAAAGGAACCCGTTTAAGATTCGCAGCGTATAA

TAACACTAGTATTATCCATGAAGCCGTGGATCTGAACGTGAAATTCTCAACAG

AGACTAGCTGCAACGAGCCAACAGTATGGAGAGTCGACAACTATGATCCATCG

AGAGGAAAATGGTTTATAACAACTGGTGGAGTTGAAGGAAACC

Figure 4.3: Nucleotide sequence of 5' RACE PCR product. Upstream portion of MKTI gene has been deduced from 5' RACE PCR product. The start codon located 75 bp upstream from the mature MKTI gene sequence. The underlined portion represents 5' UTR region includes ribosomal binding site given in green

5'-

CCTTACACTGAAAACACCTCTGCGAAAACAAA

ATGAATACTCCTTTTGTGATTGCAATCTCCTTCCTTCTTCTTGCCTTTGCCACA

AAACCTCTTGTGGGACGCCCTGATCCTTTGCTTGATATCAATGGCAACGTGGT

CGAAGCAAGCCGAGACTACTATTTGGTTTCAGTAATTGGAGGAGCAGGCGGCG

GTGGCCTCACCCTCTACAGGGGCAGAAACGAACTCTGCCCACTTGATGTTATC

CAACTAAGCCCGGATCTACATAAAGGAACCCGTTTAAGATTCGCAGCGTATAA

TAACACTAGTATTATCCATGAAGCCGTGGATCTGAACGTGAAATTCTCAACAG

AGACTAGCTGCAACGAGCCAACAGTATGGAGAGTCGACAACTATGATCCATCG

AGAGGAAAATGGTTTATAACAACTGGTGGAGTTGAAGGAAACCCTGGCGCACA

AACTTTGAAAAACTGGTTTAAGCTTGAGAGAGTTGGAACAGATCAAGGTACGT

ACGAGATTGTTCACTGTCCTTCCGTTTGCAAATCCTGTGTATTTTTATGCAATG

ATGTTGGGGTTTCTTACGATTATCGTCGACGTTTGGCTCTCACTGCTGGTAATG

AGCGCGTCTTTGGTGTTGTTATAGTCCCGGCTAATGAGGGATCAGCCTCATGC

GTGTCT**TGA***ACAACATCATAATTTATTTCTCCGCTTAAAACTGACAATTTTTTTTT*

– 3'

Figure 4.4: Complete gene sequence of MKTI deduced by the nucleotide sequence of 3' and 5' RACE PCR product. The complete gene of MKTI includes 5' and 3' UTR region. The open reading frame of MKTI consist of 648 bp which starts 75 base pair upstream from mature MKTI gene sequence.

4.3.3. 5' Rapid amplification of cDNA ends PCR prouduct

The gene specific primers were designed on the basis of 3'RACE sequence and nested 5'RACE PCR was performed. A single band of 450 bp was obtained (Figure 4.1B), which was cloned in pGEM-T vector and sequenced. The sequence of 5'RACE product was matched with 3' RACE-PCR product (Figure 4.3). The upstream portion of MKTI was deduced by comparing both the sequences and the complete open reading frame of MKTI were obtained (Figure 4.4). The complete gene of MKTI contains 5' and 3' UTR. The open reading frame of MKTI is 648 bp long.

4.3.4. Genomic DNA amplification

Genomic DNA template has been used to amplify the MKTI gene by using gene specific primers. A band of 573 bp was obtained with forward primer specific for mature MKTI and 648 bp with forward primer specific for upstream sequence (Figure 4.1B). The sequence analysis of both MKTI cDNA and genomic DNA were found completely identical. The results clearly showed that the gene sequence of MKTI does not contain introns. Table 4.2 shows all the strain used and made during this study.

4.3.5. Overexpression of reMKTI

MKTI cDNA was expressed as the GST fusion protein (GST-MKTI fusion protein) in *E. coli* TG1 cells transformed with pGEX-5x-3-MKTI by adding 1 mM IPTG to the culture. The presence of MKTI in construct was verified by PCR by using MKTI gene specific primers (Figure 4.5). The molecular masses of the MKTI and GST proteins were estimated to be 20.7 and 26.0 kDa, respectively, based on the deduced amino acid sequence. The molecular mass of

~47kDa for the expressed protein, which was produced by the cells transformed with pGEX-5x-3-MKTI, was in good agreement with the expected molecular mass of the GST-MKTI fusion protein. In SDS-PAGE gel, although the GST-MKTI fusion protein was detected from the cells cultured at 37°C (Figure 4.8). But the yield of the GST-MKTI fusion protein was significantly improved when the *E. coli* cells were cultured at 30°C rather than 37°C, and the yield was increased by increasing the induction time up to 12 h. Therefore, we determined the optimal culture conditions for the GST-MKTI fusion protein to be 30°C with a 12 h induction, and thus, we obtained 4 mg of the protein from 1000 ml of bacterial culture.

4.3.6. Purification of recombinant MKTI

A ~47 kDa protein was expressed in the TG1 E.coli strain of competent cells (Figure 4.6). The recombinant MKTI protein was purified to homogeneity by two step procedure. The first step was performed on an affinity column (GST-Sepharose). The crude lysate of cells were loaded and eluted with 5 mM reduced glutathione. A single peak of protein was obtained. The molecular weight of eluted peak was ~47 kDa, confirmed by SDS-PAGE analysis. The trypsin inhibitory activity was found in the GST tagged fusion protein. In the second step, the fused protein was dialysed against the 50 mM Tris-Cl, pH 8.0. The N- terminal GST tag was removed by factor Xa cleavage. reMKTI was obtained by Cibacron blue 3GA after eluting with 500 mM NaCl (Figure 4.7)

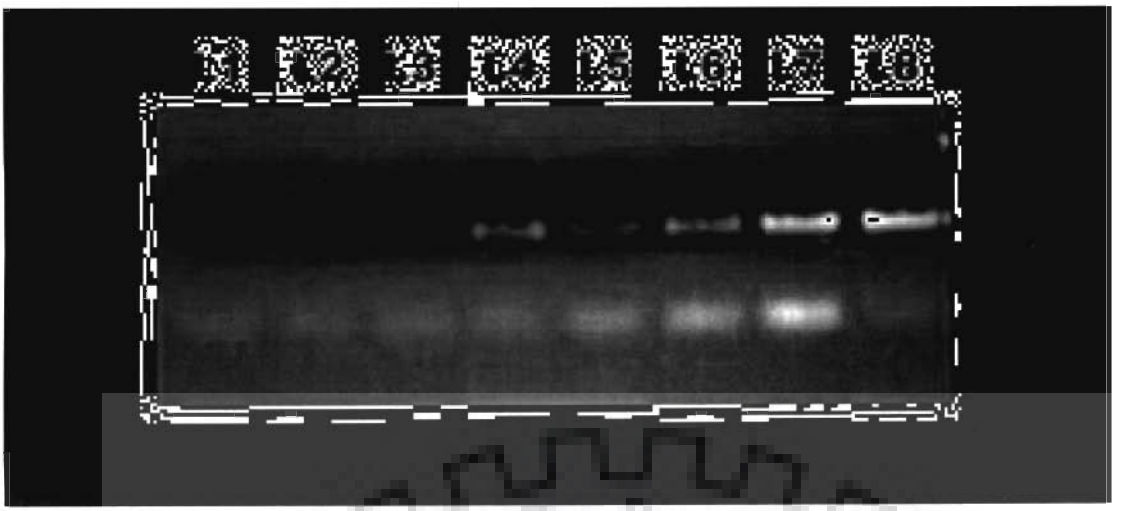

Figure 4.5: Confirmation of the MKTI coding DNA in the pGEX-5x-3 expression vector by PCR using gene specific primers of MKTI with the plasmid isolated from screened colonies as a template. L1- L3; No amplification, L4- L7; Positive amplification, L8; Control MKTI amplification

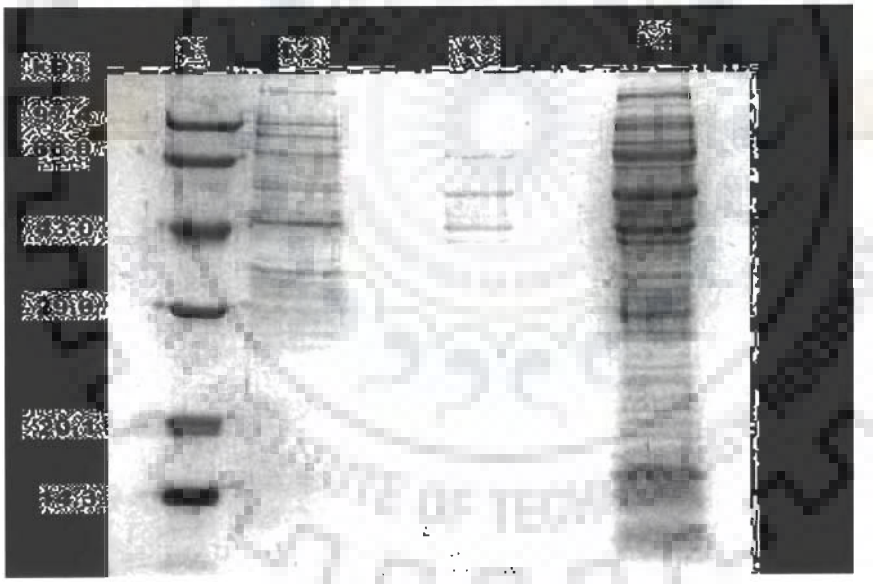

Figure 4.6: Analysis of recombinant MKTI expression as a fusion protein on 12% SDS- PAGE. L1; Molecular weight marker, L2; Blank bacterial lysate with only pGEX-5x-3, L3; Uninduced supernatant, L4; Induced supernatant

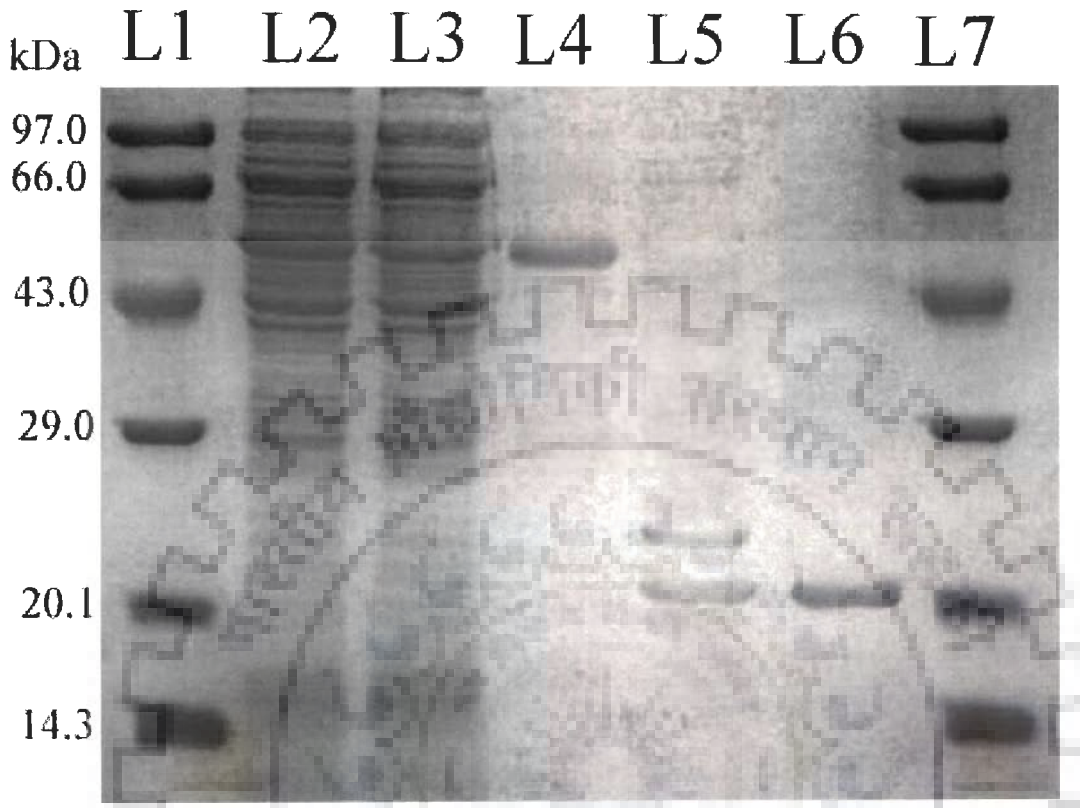

Figure 4.7: Purification of recombinant MKTI. L1 and L7; Molecular weight marker, L2 and L3; Crude Supernatant, L4; Purified GST- fused MKTI, L5; Fusion protein treated with factor Xa, L6; Purified reMKTI

4.3.7. Comparison of trypsin inhibitory activity of reMKTI with native MKTI

A comparison was made between the trypsin inhibitory activities of recombinant MKTI with native MKTI. The trypsin inhibitory activity in recombinant MKTI was found identical to native MKTI.

4.4. Discussion

MKTI has been shown to be an effective proteinase inhibitor against insect gut proteinases and can be developed as a potential plant defense agent. It is important to obtain the complete gene of MKTI in order to express the protein in crops affected from particular insect. Therefore, the complete MKTI gene was obtained from both cDNA and genomic DNA. cDNA of MKTI was cloned by 3' and 5' RACE PCR amplification with the synthesized mixed degenerate and oligo$(dT)_{15}$ adaptor primer and genomic DNA of *Murraya koenigii* as the template. The forward degenerate primer was designed according to the N-terminal sequence of MKTI (Shee at al. 2007)

Electrophoresis of 3' RACE product indicated that the cDNA of about 750 bp had been obtained. The nucleotide sequence result contains the whole coding region of mature MKTI gene with 3' UTR region. To get the compiete gene of MKTI including 5' UTR region, the 5' RACE was performed. The nested primer was designed on the basis of nucleotide sequence of 3' RACE PCR product. The analysis of 5'RACE product was indicated approximately 450 bp size. After comparing the 3' and 5' RACE PCR product nucleotide sequence the whole gene of MKTI was deduced. The complete gene of MKTI contains 5' and 3' UTR region. The 5' UTR region contains a ribosomal binding site just upstream of start codon (ATG). The open reading frame of MKTI was 648 bp. The open reading frame of MKTI starts 75 bp upstream than the mature MKTI, which indicates the presence of a signal sequence in the gene of MKTI. 573 bp

size product was obtained with the gene specific primers of MKTI when genomic DNA was used for the amplification. In genomic DNA also the presence of signal sequence was indicated by the amplification of approximately 648 bp when the primer designed for the upstream sequence has been used. The nucleotide sequence of genomic DNA and cDNA was matching perfectly which indicated that the MKTI gene did not contain intervening sequences. Other inhibitor from leguminosae family including *Delonix regia* (DrTI) and *Soyabean trypsin inhibitor* (STI) have also been reported to contain intronless gene (Huang et al., 2007; Song et al., 1993).

Mature MKTI gene was expressed as ~47 kDa GST-fused protein in pGEX-5x-3 expression vector. The optimum concentration of recombinant MKTI was induced with 1 mM IPTG concentration for 12 h incubation. The purification of protein utilized the GST-sepharose beads which is an affinity matrix for the purification of GST tagged protein. Native MKTI was purified on affinity matrix Cibacron blue 3GA. After cleaving with factor Xa, the recombinant MKTI was released and purified on Cibacron blue 3GA. This is in good agreement with the previously reported data about the MKTI (Shee et al., 2008). The MKTI cDNA and genomic DNA amplification has been submitted in the NCBI data base. The accession number assigned to the cDNA and genomic DNA sequence of MKTI gene is **FJ468001** and **FJ468002** respectively. Figure 4.9 and 4.10 shows the representative pages to be displayed on NCBI database after the release of data.

```
LOCUS       bankit1153727           648 bp    DNA     linear       12-NOV-2008
DEFINITION  Murraya koenigii trypsin inhibitor (MKTI)gene, complete cds.
ACCESSION   1153727
VERSION
KEYWORDS    .
SOURCE      Murraya koenigii. This plant belongs to: Superdivison
            Spermatophyta, Class- Magnoliopsida and Family- Rutaceae.
  ORGANISM  Murraya koenigii. This plant belongs to: Superdivison
            Spermatophyta, Class- Magnoliopsida and Family- Rutaceae.
            Unclassified.
REFERENCE   1  (bases 1 to 648)
  AUTHORS   Gahloth,D., Kumar,S.P., Kumar,P. and Sharma,A.K.
  TITLE     Cloning, Expresion, Purification and Homology modelling of Murraya
            koenigii trypsin inhibitor
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 648)
  AUTHORS   Gahloth,D., Kumar,S.P., Kumar,P. and Sharma,A.K.
  TITLE     Direct Submission
  JOURNAL   Submitted (12-NOV-2008) Biotechnology, Indian Institute of
            Technology Roorkee, Roorkee, Uttranchal 247667, India
COMMENT     Bankit Comment: Murraya koenigii. This plant belongs to: Kingdom-
            Plantae, Sub- kingdom- Tracheobionata, Superdivison- Spermatophyta,
            Divison- Magnoliophyta, Class- Magnoliopsida, Sub-class- Rosidae,
            Order- Sapindales, Family- Rutaceae, Genus- Murraya, Species-
            koenigii (Curry leaves).
            aksbsfbs@iitr.ernet.in.
FEATURES             Location/Qualifiers
     source          1..648
                     /organism="Murraya koenigii. This plant belongs to:
                     Superdivison Spermatophyta, Class- Magnoliopsida and
                     Family- Rutaceae."
                     /mol_type="genomic DNA"
BASE COUNT      168 a    141 c    156 g    183 t
ORIGIN
        1 atgaatacta ctttgtgat tgcaatctcc ttccttcttc ttgcctttgc cacaaaacct
       61 cttgtgggac gccctgatcc tttgcttgat atcaatggca acgtggtcga agcaagccga
      121 gactactatt tggtttcagt aattggagga gcaggcggcg gtggcctcac cctctacagg
      181 ggcagaaacg aactctgccc acttgatgtt atccaactaa gcccggatct acataaagga
      241 acccgtttaa gattcgcagc gtataataac actagtatta tccatgaagc cgtggatctg
      301 aacgtgaaat tctcaacaga gactagctgc aacgagccaa cagtatggag agtcgacaac
      361 tatgatccat cgagaggaaa atggtttata acaactggtg gagttgaagg aaaccctggc
      421 gcacaaactt tgaaaaactg gtttaagctt gagagagttg gaacagatca aggtacgtac
      481 gagattgttc actgtccttc cgtttgcaaa tcctgtgtat ttttatgcaa tgatgttggg
      541 gtttcttacg attatcgtcg acgtttggct ctcactgctg gtaatgagcg cgtctttggt
      601 gttgttatag tcccggctaa tgagggatca gcctcatgcg tgtcttga
//
```

Figure 4.9: cDNA sequence of MKTI submitted in the NCBI database. The accession number assigned to the cDNA sequence of MKTI is **FJ468001.**

```
LOCUS       bankit1153629            648 bp    mRNA    linear       12-NOV-2008
DEFINITION  Murraya koenigii trypsin inhibitor (MKTI)mRNA, complete cds.
ACCESSION   1153629
VERSION
KEYWORDS    .
SOURCE      Murraya koenigii. This plant belongs to: Kingdom- Plantae, Sub-
            kingdom- Tracheobionata, Superdivison- Spermatophyta, Divison-
            Magnoliophyta, Class- Magnoliopsida, Sub-class- Rosidae, Order-
            Sapindales, Family- Rutaceae, Genus- Murraya, Species- koenigii
            (Curry leaves).
  ORGANISM  Murraya koenigii. This plant belongs to: Kingdom- Plantae, Sub-
            kingdom- Tracheobionata, Superdivison- Spermatophyta, Divison-
            Magnoliophyta, Class- Magnoliopsida, Sub-class- Rosidae, Order-
            Sapindales, Family- Rutaceae, Genus- Murraya, Species- koenigii
            (Curry leaves).
            Unclassified.
REFERENCE   1  (bases 1 to 648)
  AUTHORS   Gahloth,D., Kumar,S.P., Kumar,P. and Sharma,A.K.
  TITLE     Cloning, Expresion, Purification and Homology modelling of Murraya
            koenigii trypsin inhibitor
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 648)
  AUTHORS   Gahloth,D., Kumar,S.P., Kumar,P. and Sharma,A.K.
  TITLE     Direct Submission
  JOURNAL   Submitted (12-NOV-2008) Biotechnology, Indian Institute of
            Technology Roorkee, Roorkee, Uttranchal 247667, India
COMMENT     Bankit Comment: aksbsfbs@iitr.ernet.in.
FEATURES             Location/Qualifiers
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                     /organism="Murraya koenigii. This plant belongs to:
                     Kingdom- Plantae, Sub- kingdom- Tracheobionata,
                     Superdivison- Spermatophyta, Divison- Magnoliophyta,
                     Class- Magnoliopsida, Sub-class- Rosidae, Order-
                     Sapindales, Family- Rutaceae, Genus- Murraya, Species-
                     koenigii (Curry leaves)."
                     /mol_type="mRNA"
BASE COUNT      168 a    141 c    156 g    183 t
ORIGIN
        1 atgaatactc cttttgtgat tgcaatctcc ttccttcttc ttgcctttgc cacaaaacct
       61 cttgtgggac gccctgatcc tttgcttgat atcaatggca acgtggtcga agcaagccga
      121 gactactatt tggtttcagt aattggagga gcaggcggcg gtggcctcac cctctacagg
      181 ggcagaaacg aactctgccc acttgatgtt atccaactaa gcccggatct acataaagga
      241 acccgtttaa gattcgcagc gtataataac actagtatta tccatgaagc cgtggatctg
      301 aacgtgaaat tctcaacaga gactagctgc aacgagccaa cagtatggag agtcgacaac
      361 tatgatccat cgagaggaaa atggtttata acaactggtg gagttgaagg aaaccctggc
      421 gcacaaactt tgaaaaactg gtttaagctt gagagagttg gaacagatca aggtacgtac
      481 gagattgttc actgtccttc cgtttgcaaa tcctgtgtat ttttatgcaa tgatgttggg
      541 gtttcttacg attatcgtcg acgtttggct ctcactgctg gtaatgagcg cgtctttggt
      601 gttgttatag tcccggctaa tgagggatca gcctcatgcg tgtcttga
//
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Figure 4.10: Genomic DNA sequence of MKTI gene submitted in the NCBI database. The accession assigned to the gene is **FJ468001**

CHAPTER 5
SEQUENCE ANALYSIS AND HOMOLOGY MODELING OF *MURRAYA KOENIGII* TRYPSIN INHIBITOR

5.1 Introduction

There are an intricate number of factors that contribute to the specificity and the efficiency of proteinase inhibitors. The composition and the conformation of their reactive loop site are clearly important, but various other aspects of the molecular structure must be considered as well. This is because not only the inhibitor reactive loop interacts with the enzyme, but there are other important areas of contact between the two proteins. In a way, the entire molecule must be considered. The global architecture, for example, is essential to the inhibitor stability, rigidity, and efficiency. The reactive loop site of Kunitz (STI) family of inhibitors is located between the repeats A and B of β-strands. The cleavage of scissile peptide bond theoretically could provoke the separation of the motif A from the motifs B and C of β-strands. However, the motifs A–C are held together by an extensive intramolecular hydrogen bonding network.

There has been substantial evidence that this network, which does not exist in a normal substrate vulnerable to proteolysis, remains intact in the cleaved inhibitor (Shaw et al., 1995). These numerous contacts stabilize the newly formed N-terminus and maintain it in an optimal orientation for nucleophilic attack on the acyl–enzyme, favoring the religation. Moreover, the positioning of the amine also sterically hinders the hydrolytic water molecule from achieving the necessary proximity to the histidine base for nucleophilic activation. The composition of the reactive loop determines the specificity of the inhibitor. The amino acid residue in the P1 position, which fits in the enzyme reactive site pocket, is of particular importance. Besides the specificity, the loop conformation also determines the efficiency of the inhibitor as in the substrate-like inhibitors, in which much closer to the canonical conformation the reactive loop is, the more effective as the inhibitor.

In the present chapter, amino acid sequence analysis of MKTI has been done. To understand the mode of trypsin inhibition, three dimensional structure of MKTI was predicted by homology modeling. Its possible modes of interaction with trypsin were also studied. Some functional motifs such as phosphorylation, glycosylation and myristoylation were predicted in three-dimensional structure of MKTI.

5.2. Materials and methods

5.2.1. Deduction of amino acid sequence of MKTI

3' RACE and 5' RACE PCR sequences were matched and the complete nucleotide sequence of MKTI gene was deduced. The amino acid sequence of complete MKTI gene was deduced by ORF finder tool on NCBI online server. The amino acid sequence of MKTI was aligned against the triplet nucleotide codon.

5.2.2. BLAST of deduced amino acid sequence

The similarity search for deduced amino acid sequence of MKTI was done by using NCBI online server protein BLAST taking non-redundant (nr) database and protein data base (PDB).

5.2.3. Multiple sequence alignment

Phylogenetic analysis was performed to analyze evolutionary relationship between the similar sequences. MKTI sequence was compared with other Kunitz inhibitors by multiple sequence alignment.

5.2.4. Identification of various functional motifs

Probable motifs involved in post translational modifications such as glycosylation, phosphorylation and myristoylation site were predicted by "Motif Scan Server". The Kunitz family signature pattern was also observed by Scan prosite server. The prediction about presence of signal sequence was made by using online server iPSORT.

5.2.5. Theoretical determination of isoelectric point and hyrdropathy index of MKTI

Theoretical determination of isoelectric point and hydropathy index of MKTI amino acid sequence was made by online proteomic *EXPASY* server.

5.2.6. Secondary structure prediction

The secondary structure predictions of MKTI were carried out using the PHD method (http://www.predictprotein.org/).

5.2.7. Tertiary structure predicition by homology modeling

The three-dimensional model of MKTI was constructed using the crystal coordinates of *Delonix regia* trypsin inhibitor, (PDB id: 1R8N) done at a resolution of 1.75 Å. Automated homology model building was performed using protein structure modeling program MODELLER 9 (Sali and Blundell, 1993) which models protein tertiary structure by satisfaction of spatial restraint. The input for the program MODELLER consisted of the aligned sequence of MKTI and DrTI, and a steering file which gives all the necessary commands to the Modellar for generating the homology model of MKTI on the basis of its alignment with crystal coordinates of the template. Many runs of model building were carried out in order to obtain most plausible model.

5.2.7.1. Validation of tertiary structure

The evaluation of the predicted MKTI model, i.e., analysis of geometry, stereochemistry, and energy distribution in the models, was performed using either the ENERGY commands of MODELLER or using Program "PROCHECK" and Whatcheck. In addition the variability in the predicted model, i.e., RMSD was calculated by superposition of Cα traces and backbone onto the template crystal structure. The protein structures were visualized and analyzed on PYMOL (http://www.pymol.org) and WINCOOT.

5.2.8. Interaction study of MKTI model with trypsin

In attempt to find a structural rationale why MKTI is an effective inhibitor of trypsin with Ki 7nM. Putative interactions of MKTI with trypsin were studied by Homology modeling. Firstly, MKTI model was superimposed with the STI-trypsin complex structure (PDB id: 1AVW) and the putative contacts between MKTI-trypsin were analyzed. The intermolecular contacts involve not only residues of reactive site, but also residues in other parts of the molecules forming a molecular interface between the enzyme and the inhibitor.

5.3. Results

5.3.1. Amino acid sequence deduction

A 648 base pair ORF was obtained after sequencing of the MKTI PCR product which coded for a polypeptide of 215 amino acid residues. N-terminal sequencing of the protein done earlier showed that it starts with Asp (D) residue which signifies the existence of signal sequence (Figure 5.1).

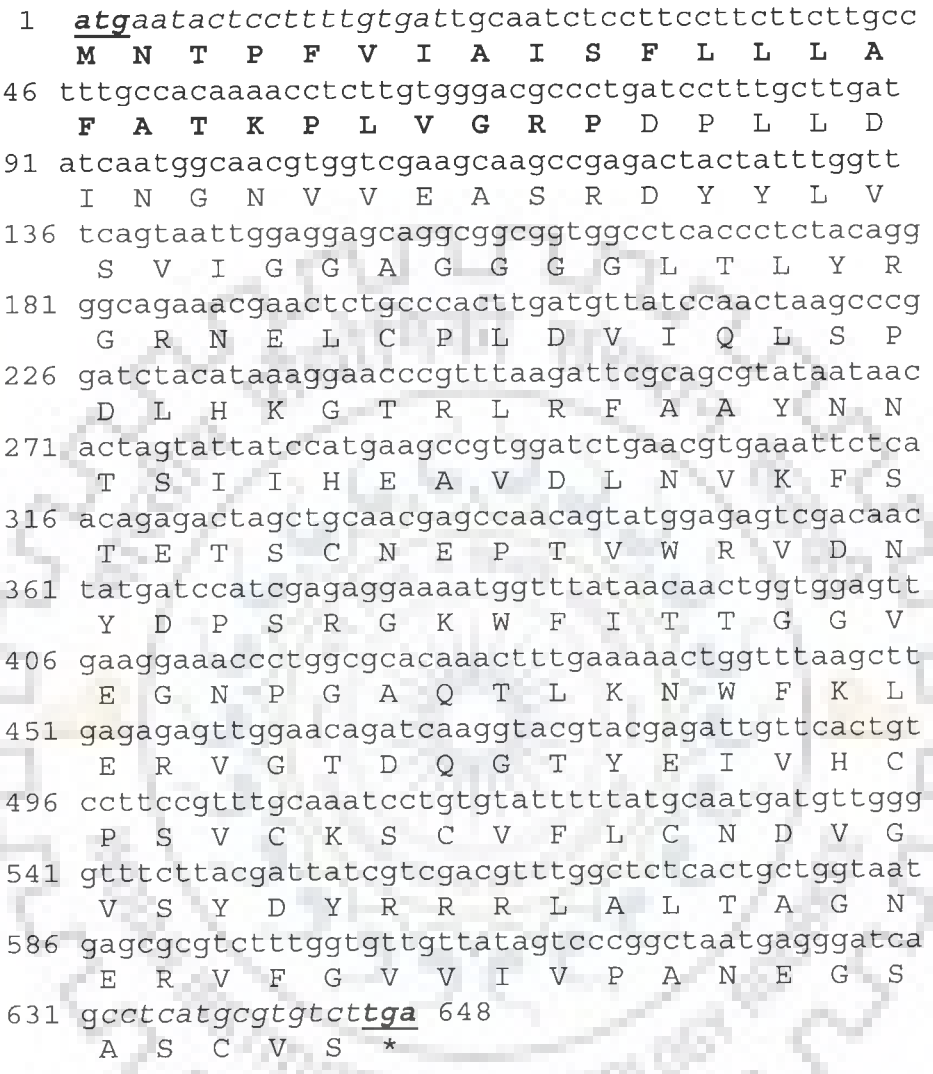


Figure 5.1: Nucleotide and deduced amino acid sequence of MKTI. The nucleotide sequence for the start codon and stop codon are underlined and shown in bold. The nucleotide sequences of forward and reverse primers used for full cDNA cloning are italics. The translated sequence of MKTI includes a 25-amino acid signal peptide, followed by a 190-amino acid mature protein

5.3.2. Similarity search by NCBI- BLAST

A homology search using NCBI BLAST showed significant similarity to miraculin-like proteins isolated from various Citrus species plants belonging to Rutaceae family against the nr-database. The MKTI showed maximum sequence homology to reported Kunitz-type inhibitors with highest similarity with *Delonix regia* against the PDB database. MKTI shared 74% similarity and 57% identity with the Miraculin-like protein from *Citrus jambhiri* belongs to Rutaceae family. In Kunitz family inhibitor MKTI matches with DrTI, shared 53% similarity and 32% identity (Figure 5.2).

5.3.3 Multiple sequence alignment

Multiple sequence alignment of MKTI sequence with that of other Kunitz-type trypsin inhibitors by CLUSTALW analysis revealed significant differences. The sequence of MKTI possesses three extra cysteine residues when compared to most Kunitz-type trypsin inhibitors. Similar to miraculin-like proteins, there are seven cysteine residues in MKTI sequence as opposed to four cysteines residues in other Kunitz-type inhibitors. The sequence comparison of residues of reactive site loop of MKTI with other Kunitz-type inhibitors showed that it comprises from the residue Ala62(P1) to the residue Ile69(P4'). There is a marked variation in the reactive site residue in MKTI, wherein a conventional Arg or Lys has been replaced by Asn 65(P1) (Figure 5.3). Miraculin-like protein from *C. jambhiri* having most similarity with MKTI also contains Asn at corresponding position (Figure 5.3). Earlier, the variation in reactive site residue has been reported in Kunitz inhibitors from *Cicer arietinum* where conserved Arg or Lys has been replaced by a Gly/Glu-Ile-Ser motif (Srinivasan et al., 2005).

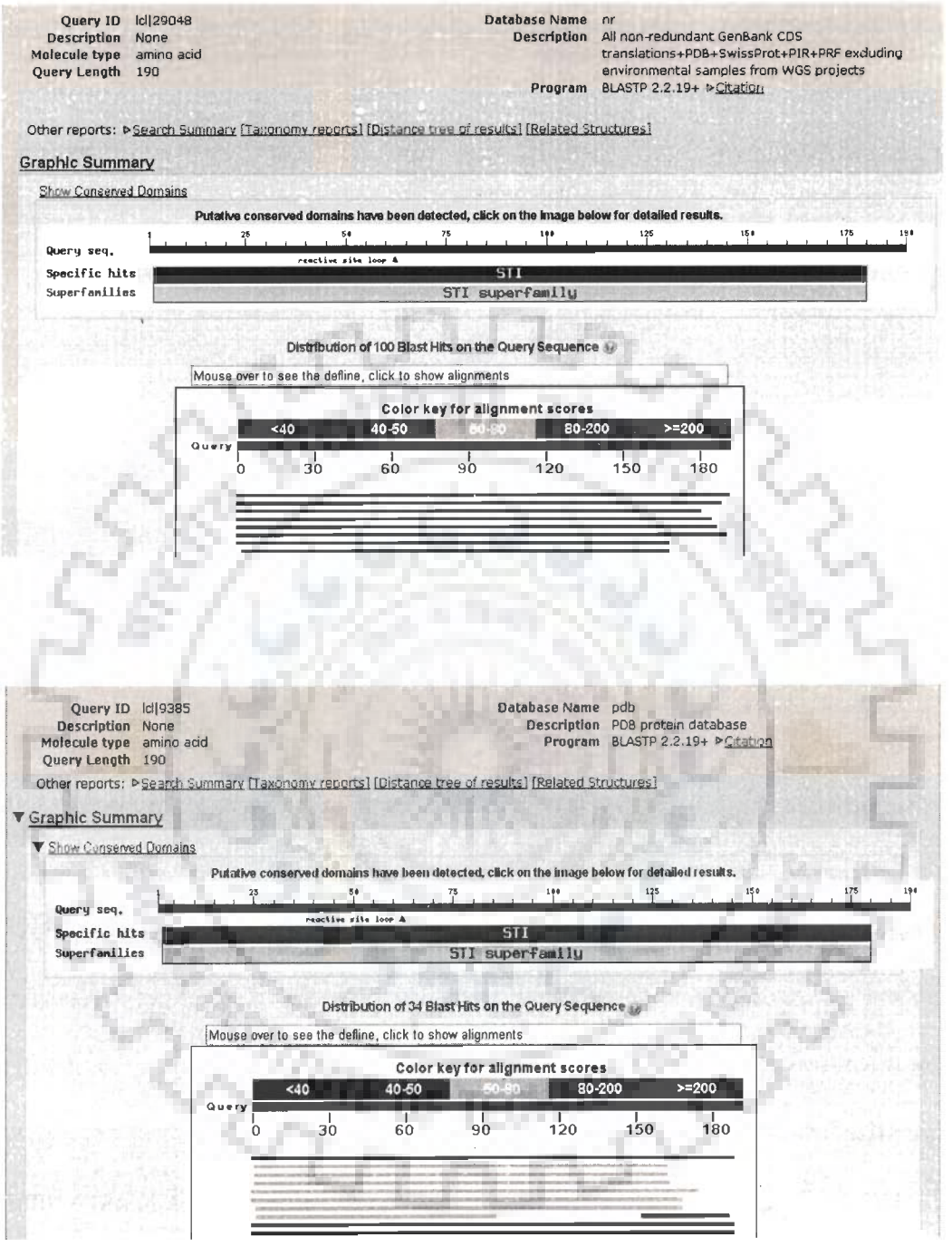


Figure 5.2: Sequence Similarity search was done with the NCBI BLAST search program. **(A).** Search done against the non- redundant database. **(B)**. Search done against the protein database

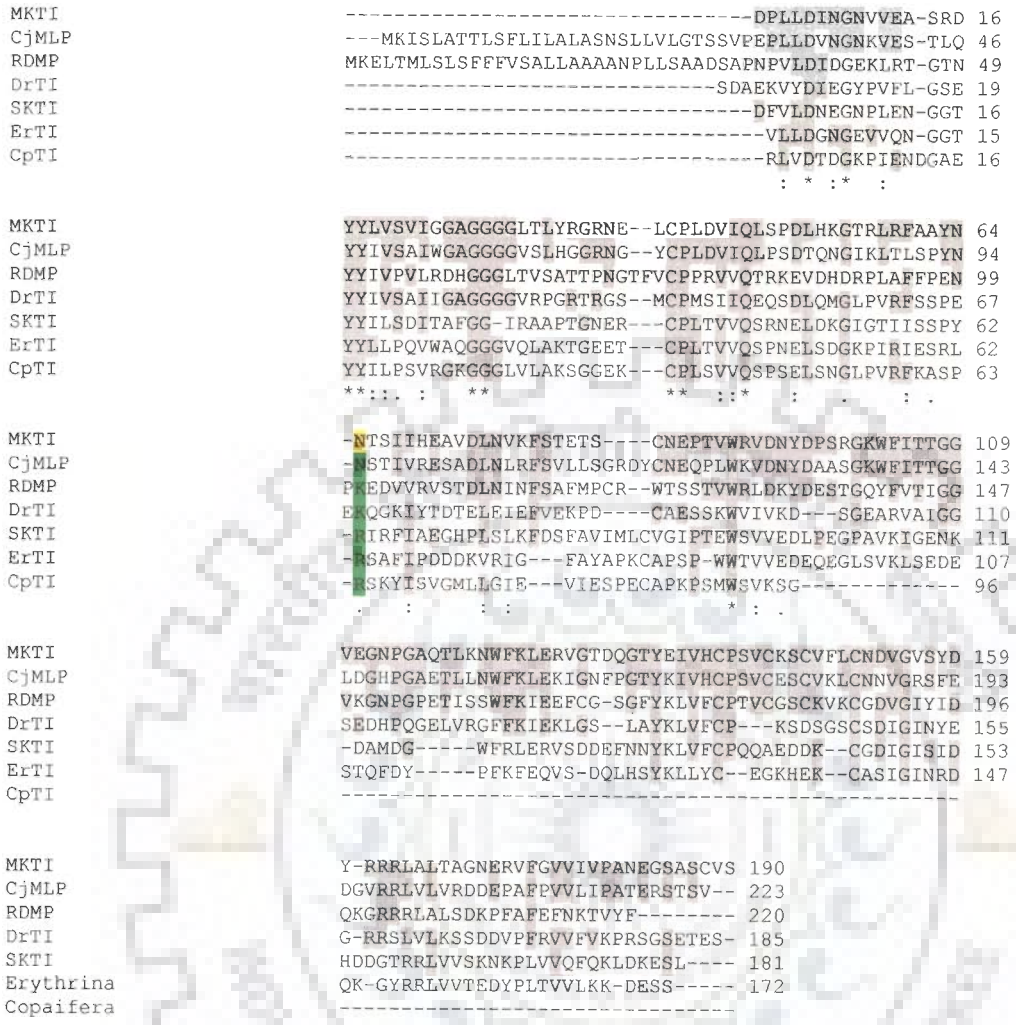


Figure 5.3: Comparison of amino acid sequences of various trypsin inhibitors and miraculin like proteins stored in NCBI and Uniport databases using CLUSTALW program. MKTI; *Murraya koenigii* trypsin inhibitor, CjMLP; *Citrus jambhiri* miraculin like protein (gi| 87299377), RDMP; *Richedella dulcifica* miraculin protein (Uniport database, sp|P13087), DrTI; *Delonix regia* trypsin inhibitor (gi|49258681), SKTI; Soyabean Kunitz trypsin inhibitor (gi|3318878), ErTI; *Erythrina caffra* trypsin inhibitor (gi|157833954), CpTI; *Copaifera langsdorffii* trypsin inhibitor (gi|49258682). Conserved residues were highlighted in gray. Residue at the active sites are highlighted in green

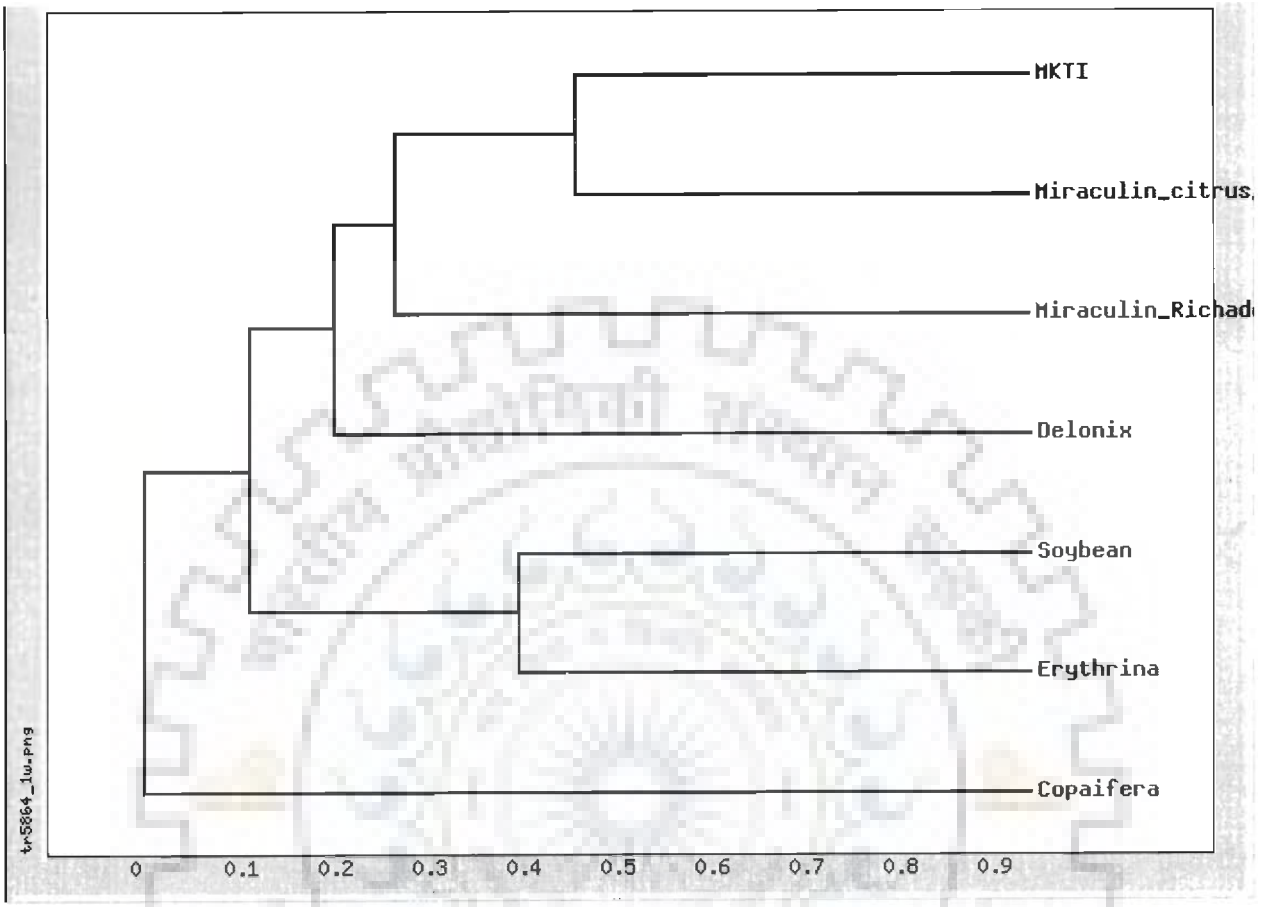
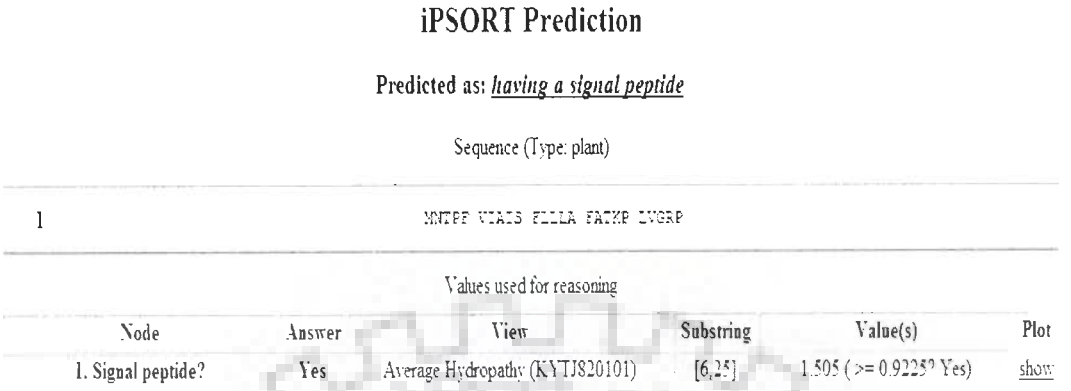


Figure 5.4: Phylogenetic tree derived from MKTI related sequences isolated so far. Phylogenetic analysis of these sequences using the neighbor joining distances method within phylip package. MKTI; *Murraya koenigii* trypsin inhibitor, Miraculin_citrus; Miraculin like protein from *Citrus jambhiri* (gi|87299377), Miraculin_Richade; Miraculin protein from *Richadella dulcifica*(Uniport database, sp|P13087), Delonix; *Delonix regia* trypsin inhibitor (DrTI) (gi|49258681), Soyabean; Soyabean Kunitz trypsin inhibitor (SKTI) (gi|3318878), Erythrina; *Erythrina caffara* trypsin inhibitor (ErTI) (gi|157833954), Copaifera; *Copaifera lagsdorffii* trypsin inhibitor (CpTI) (gi|49258682).

Table 5.1: Determination of different functional motifs in the amino acid sequence of MKTI. Observed Kunitz Inhibitor Family Signature pattern is shown in the shaded text. Glycosylation site is underlined, Phosphorylation sites are shown in bold and Myristoylation site is shown in bold, italic and underline. N-terminal signal sequence of MKTI has shown in last row

Kunitz Inhibitor Signature Pattern	[LIVM]-x-D-x-[EDNTY]-[DG]-[RKHDENQ]-x-[LIVM]-(x)5-Y-x-[LIVM]
Observed pattern	L-L-D-I-N-G-N-V-V-E-A-S-R-D-Y-Y-L
MKTI mature sequence	DPLLDINGNVVEASRDYYLVSVIGGAGGGGLTLYRGRNELCPLDVIQLSPDLHKGTRL RFAAYNNTSIIHEAVDLNVKFSTET"**SCNE**P"TVWRVDNYDPSRGKWFITT***GGVEGN***P GAQ**TLK**NWFKLE**RVGTDQGTY**EIVHCPSVCKSCVFLCNDVGVSYDYRRRLALTAGNER VFGVVIVPANEGSAS CVS
N-terminal Signal Sequence	N T P F V I A I S F L L L A F A T K P L V G R P



iPSORT Prediction

Predicted as: *having a signal peptide*

Sequence (Type: plant)

1 MMTPF VIAIS FLLLA FATKP IVGRP

Values used for reasoning

Node	Answer	View	Substring	Value(s)	Plot
1. Signal peptide?	Yes	Average Hydropathy (KYTJ820101)	[6,25]	1.505 (>= 0.92252 Yes)	show

Figure 5.5: Prediction of N- terminal signal sequence in MKTI by online server iPSORT

Phylogenetic analysis of representative Kunitz-type inhibitors from different plants was performed to understand evolutionary relationship among these inhibitors. The phylogenetic analysis showed that MKTI sequence formed a distinct cluster with inhibitors from miraculin-like proteins (Figure 5.4).

5.3.4. Identification of various motifs in MKTI amino acid sequence

The MKTI sequence was probed for presence of various functional motifs. Probable locations of motifs predicted from motif scan server showed existence of possible glycosylation, phosphorylation and myristoylation sites in MKTI. N-linked glycosylation site (Asn-Asn-Thr-Ser) was identified at position 64-67. The result was supported by the fact that molisch test used for identifying glycoprotein gave positive result for MKTI (Table 5.1).

N-terminal sequence of native MKTI done earlier showed that the it starts with Asp(D) residue and which signifies the existence of signal sequence. The observation was confirmed using signal iPSORT server which showed the existence of cleavage site between amino acids

25-26 and existence of signal sequence located upstream of mature MKTI (Figure 5.5). The mature MKTI, therefore, is a 190 amino acid residues polypeptide. The primary amino acid sequence of MKTI showed a conserved motif [LIVM]-x-D-x-[EDNTY]-[DG]-[RKHDENQ]-x-[LIVM]-(x)5-Y-x-[LIVM] found in soyabean trypsin inhibitor family when queried in 'Scan-Prosite' server. The observed pattern (LLDINGNVVEASRDYYL) was located at the N-terminal of the sequence (Table 5.1).

The phosphorylation sites were located at three positions in MKTI sequence. These are Ser-Cys-Asn-Glu (position 84-87, Thr-Leu-Lys (position 118-120) and Arg-Val-Gly-Thr-Asp-Gln-Gly-Thr-Tyr (position 127-135). A possible myristoylation site Gly-Gly-Val-Glu-Gly-Asn was identified at position 108-113 (Table 5.1).

5.3.5. Theoretical determination of isoelectric point and hyrdropathy index of MKTI

Theoretical isoelectric point and hydropathy index of amino acid sequence of MKTI was calculated by protparam program of online proteomic server EXPASY. The calculated isoelectric point was 5.62 and the grand average of hydropathy (GRAVY) index of total amino acids was -0.168. Hydropathy index value of MKTI determined its cytosolic nature.

5.3.6. Secondary structure of MKTI

Secondary structure distribution pattern of MKTI shows that this protein consists of approximately 47% β- sheet, joined by β- turns with little or almost no alpha helical part. Table 5.2 summarized the secondary structure distribution of MKTI. The secondary structure alignment of MKTI with *Delonix regia* trypsin inhibitor revealed that it consists of approximately three fold internal symmetry (Figure 5.6).

Table 5.2: Secondary structure distribution of MKTI

β Strands		Loops	No. of residues	Disulphide bond
		N- terminal	1-16	
A1	17- 21	A1-A2	22-28	
A2	29-34	A2-A3	35-43	Cys41-Cys 85
A3	44-48	A3-A4	49-57	Cys140-Cys151
A4	58-61	A4-B1	62-74	Cys144- Cys147
B1	75-79	B1-B2	80-90	Reactive site- 63-66
B2	91-94	B2-B3	95-103	3_{10} Helix- 115-117
B3	104-107	B3-B4	108-120	
B4	121-128	B4-C1	129-133	
C1	134-141	C1-C2	142-151	
C2	152-157	C2-C3	158-162	
C3	163-166	C3-C4	167-175	
C4	176-179	C4-C5	180- 190	

5.3.7. Prediction and evaluation of three-dimensional structure of MKTI

The three-dimensional structure of MKTI was modeled using the crystal structure of Kunitz-type trypsin inhibitor from seeds of *Delonix regia* (PDB code: 1R8N) as the sequence homology (32% identity) was highest between these two proteins among typical Kunitz-type inhibitors. A good quality model was generated with 88% residues in most favored region, 12% residues in the allowed region (Figure 5.9). The overall geometry of the MKTI structure was similar to other soyabean Kunitz-type inhibitors. The MKTI possess seven cysteine residues, three extra as compared to reference structure. The two disulfide bridges (Cys41-Cys85 and Cys 140-151) in the model align well with the reference structure (Figure 5.8A). However, there is an extra disulfide bridge (Cys144-Cys147) in the model and one cysteine at position 188 remains free and there is no evidence that it forms interchain bridge. The three intrachain

Alignment with Delonix regia trypsin inhibitor (1r8nA):

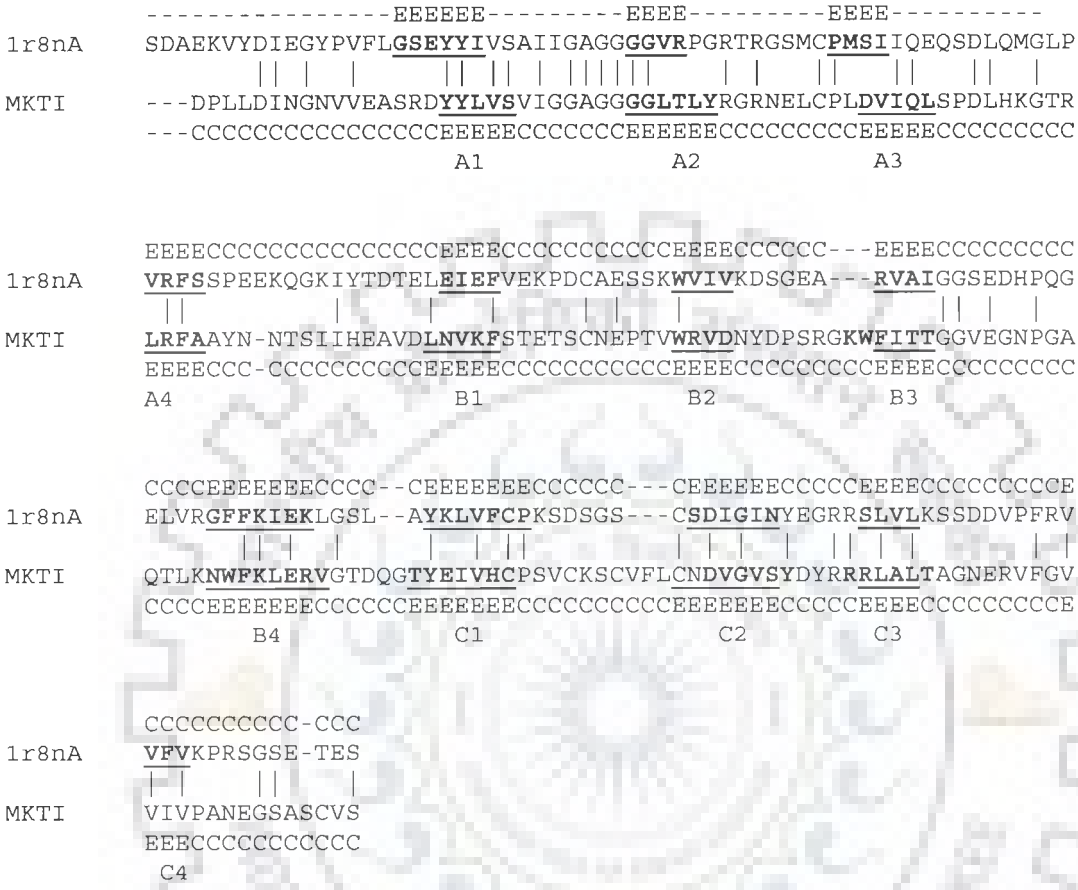


Figure 5.6: Multiple sequence alignment of template DrTI (PDB id: 1R8N) and target (MKTI). MKTI has three fold internal symmetry. β-Sheet in this subunit is labeled as An, Bn and Cn indicated number of sheets.

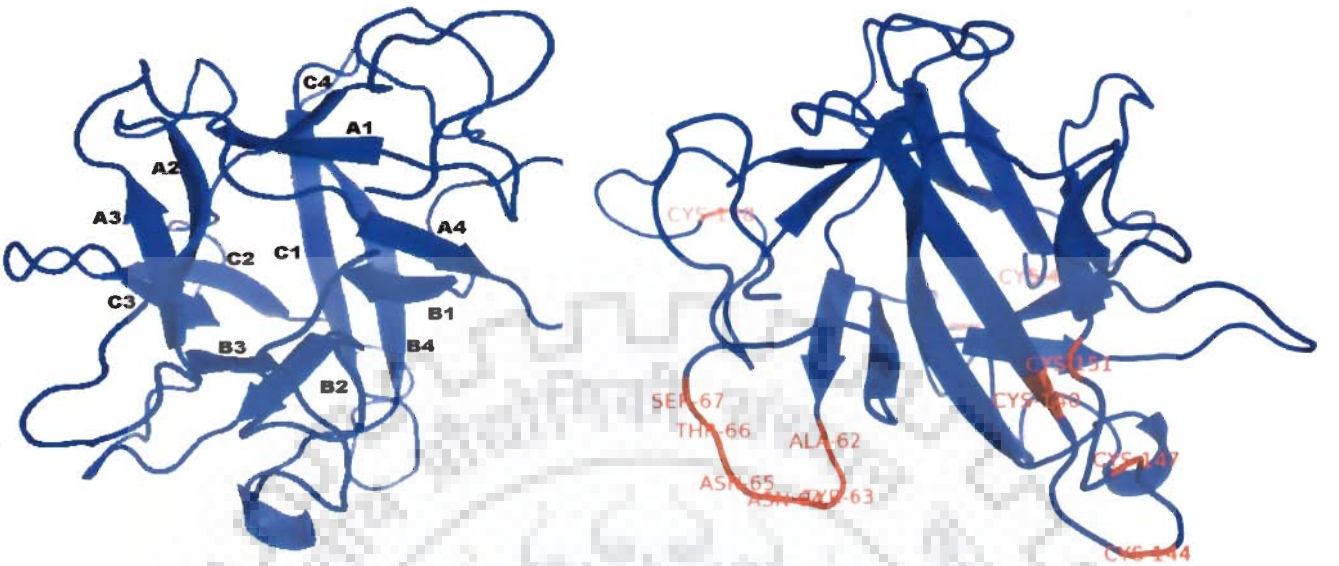


Figure 5.7: (A) Schematic representation of the predicted model of MKTI showing the arrangements of α-helices, β-sheets and loop regions in the predicted three dimensional structure of MKTI. **(B)** Schematic representation of the inhibitory loop in the predicted three dimensional model of MKTI.

A B

Figure 5.8: (A) Three disulphide bond in the predicted model of MKTI. **(B)** The superposition of predicted MKTI structure onto crystal structure of DrTI (PDB id: 1R8N). The rms deviation between the two structures was 1.19 Å

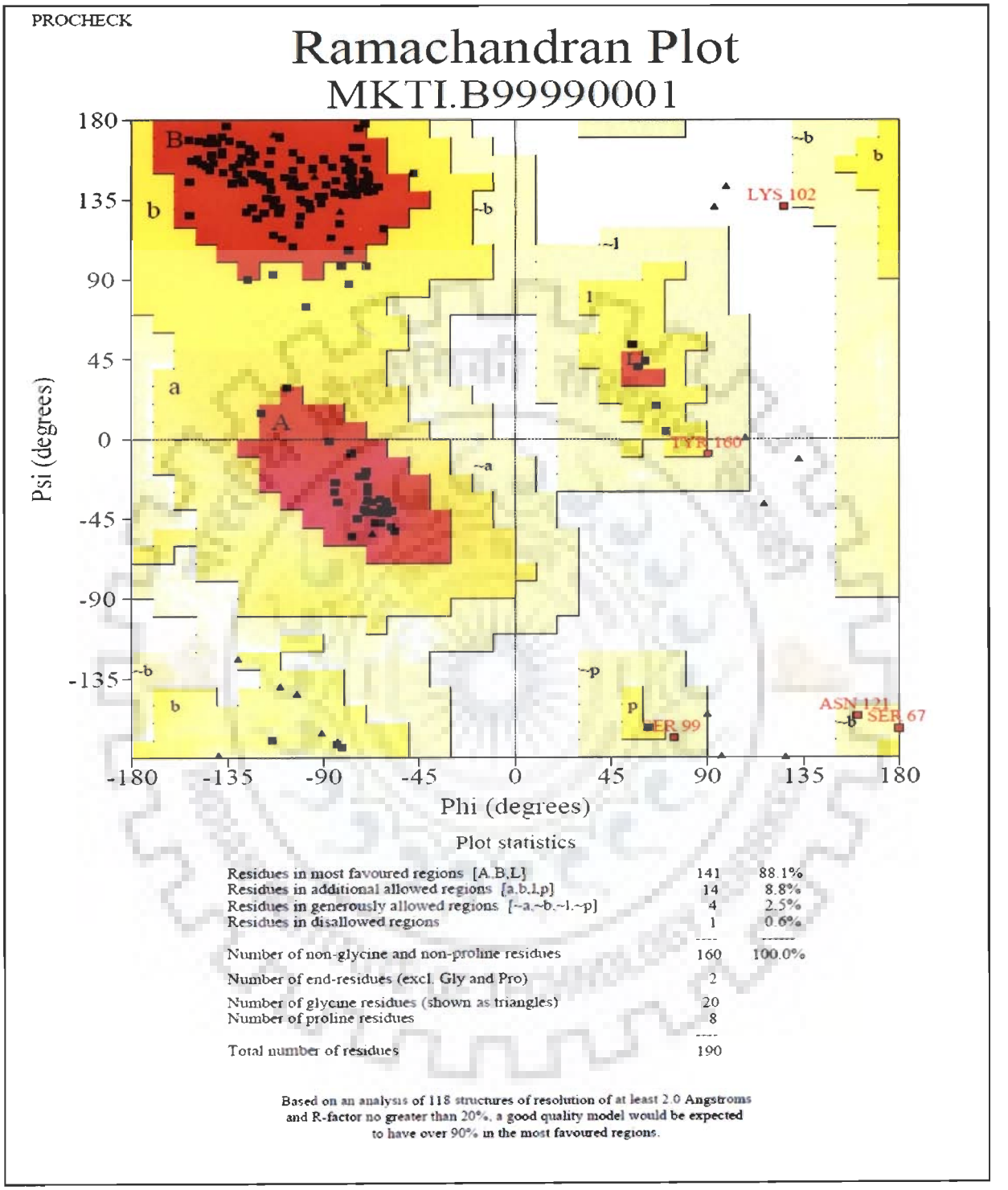


Figure 5.9: Validation of predicted three dimensional structure of MKTI has shown that only one residue lies in the disallowed region.

disulfide bridges limit the flexibility of loops and therefore, confer stability and rigidity to MKTI structure. The MKTI structure, like the template structure, is a β-trefoil fold made of 12 anti parallel β-strands with six of the strands arranged in a barrel structure and other six forms a triangular lid on the barrel. The predicted structure contains around 47.9% of β-stands connected by long loops and a small 3_{10} helix (Figure 5.7A). Secondary structure prediction was in agreement with predicted structure except for the absence of helical content. The analysis of conformational angles of some loops revealed that the values to be approximately close to that of distorted helices. Previously, the CD spectra of MKTI have shown it to be α-β protein. Superposition of modeled MKTI with reference structure gave rmsd values of 1.19 Å for Cα atoms and secondary structure regions (Figure 5.8B). The values suggest that the main difference lies in the loop regions. The superposition of the reactive site loop (Ala62-Ile69) of MKTI with that of DrTI showed more or less similar conformation. The superposition of two loops gave the rmsd value 2.40 Å. The reactive site loop of MKTI is not constrained by any secondary structural elements or disulfide bridges (Figure 5.7B). One of the residues in STI (Asn13) which plays an important role in stabilizing reactive loop conformation is absent in MKTI.

The predicted structure of MKTI exhibits three-fold internal symmetry with a four-stranded motif as the repeating unit. Around 63 amino acids in each unit are structurally organized as L-β1-L-β2-L-β3-L-β4 (L denotes loop connecting consecutive β-strands). The superposition of three domains shows a high degree of similarity for the β-strands and not for the loops. Strand β1 and β4 from the same motif are adjacent, wherease strand β1 is hydrogen bonded to the strand β4 of the previous motif and strand β4 is hydrogen bonded to the strand β1 of the following one.

5.3.8. Interaction studies of MKTI model with Trypsin

5.3.8.1. Superimposition of MKTI model on Trypsin-STI complex

The MKTI structure was superimposed on to the STI-trypsin complex structure (PDB code: 1AVW) to analyze the intermolecular contacts between MKTI reactive loop and trypsin.

5.3.8.2 Interactions of MKTI with trypsin

The MKTI structure was superimposed on to the STI-trypsin complex structure (PDB code: 1AVX) to analyze the intermolecular contacts between MKTI reactive loop and trypsin. In MKTI structure, thirteen residues namely Pro2, Ala13, Ser14, Ala62 (P4), Tyr63 (P3), Asn64 (P2), Asn65 (P1), Thr66 (P1'), Ser67 (P2'), His70, Asp74, Asn121 and Trp122 interacts with trypsin (Figure). The difference in interactions is mainly due to the changes in the reactive site residues of MKTI. Asn65 (P1) makes contact with Ser195, Val213 and Gln192 of trypsin. It occupies the same position as Arg or Lys at P1 position in S1 subsite of trypsin but it is too short to interact with Asp189, a key residue in binding pocket of trypsin. The geometry of the carbonyl group at the P1 position, important for the interaction between inhibitor and proteinase during catalysis, showed that carbonyl carbon atom is within van der waals distance from Ser195 of trypsin. Also, carbonyl group forms a hydrogen bond with NH of Ser195 of trypsin. The side chain of Asn65 (P1) also makes one hydrogen bond with Ser214 of trypsin. Asn64 at P2 position can make two hydrogen bonds with His57 of trypsin and interacts with His57, Asp102, Ser195 and Ser214 of trypsin. Tyr63 at P3 position makes hydrogen bond with Gly96 of trypsin and interacts with four residues, Gly216, Leu99, Gly96 and Try211, of trypsin Gly96, Leu99, Ser214 and Trp215 of trypsin. Thr66 at P1' position in MKTI interacts only with His57

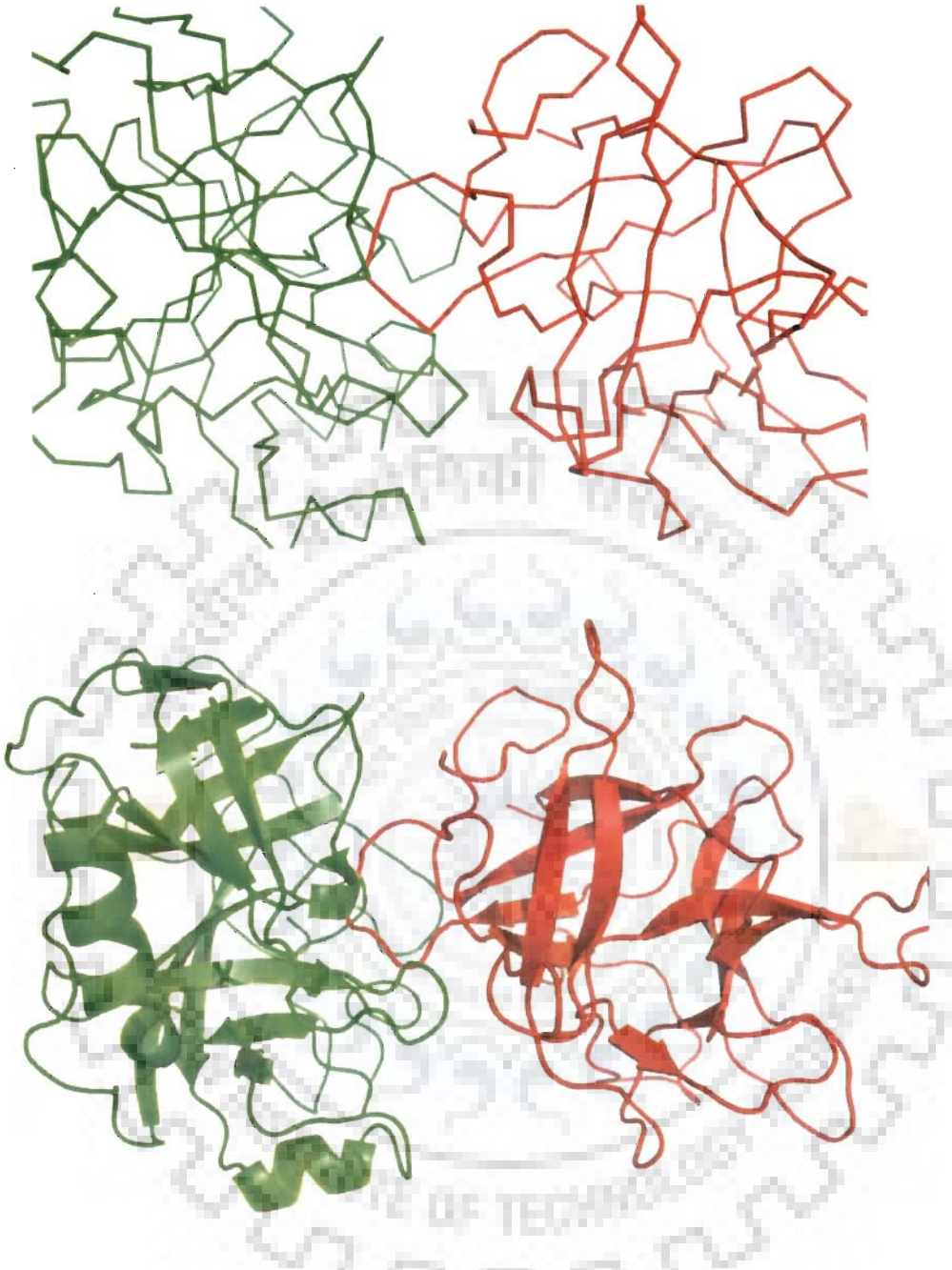


Figure 5.10: MKTI-trypsin complex showing intermolecular interactions. The predicted three dimensional structure of MKTI was superimposed onto the template crystal structure of STI-trypsin complex (PDB id: 1AVX). Inhibitor and trypsin molecule is shown in red and green color backbone respectively **(A)** MKTI-trypsin complex model shown in Cα traces. **(B)** Schematic representation of MKTI-trypsin complex in cartoon.

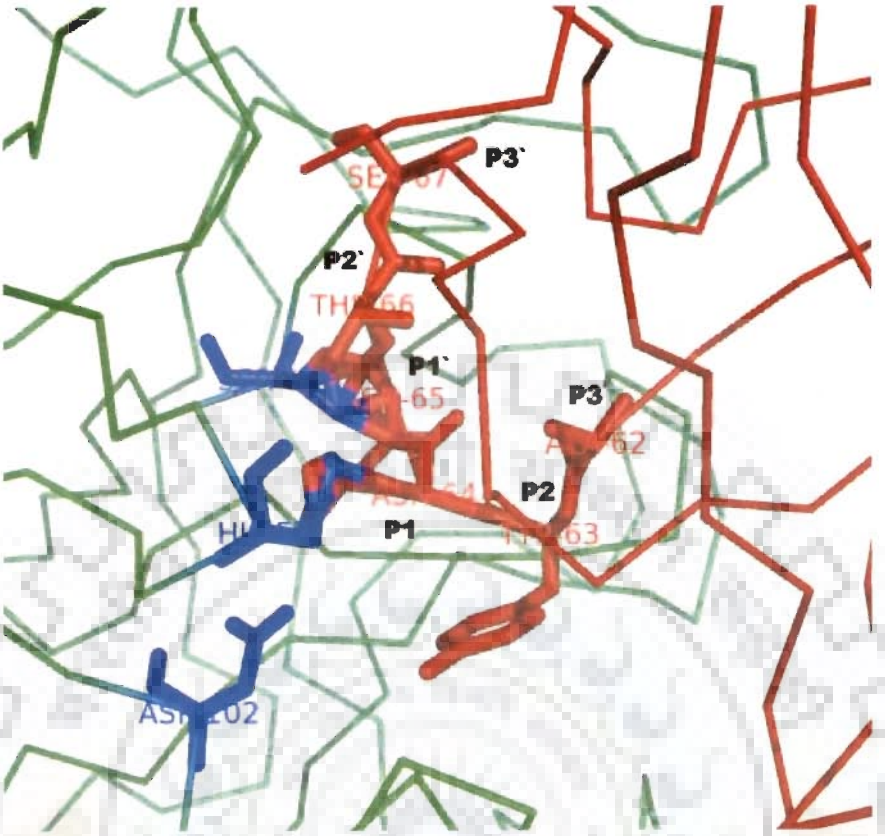


Figure 5.11: The interaction of inhibitory loop of predicted three dimensional model of MKTI with active site of trypsin. Residues present at the different positions (P1, P2, P3, P1`, P2` and P3`) of the principal binding loop of MKTI are depicted as stick representation in red. Active site residues of trypsin are shown in blue stick representation

Table 5.2: List of all the interactions between predicted structure of MKTI and porcine pancreatic trypsin. Possible hydrogen bond distances are shown

MKTI	PPT	H bond	Distance (Å)
Proline2	Lysine60	-	-
Alanine13	Gln192	-	-
Ser14	Gln192	-	-
Ala62	Gln192	-	-
Tyr63	Gly96	OH-O	2.24
	Leu99	-	-
	Trp215	-	-
	Ser214	-	-
Asn64	His57	ND2-O	2.99
	His57	OD1-NE3	0.87
	Ser195	-	-
	Ser214	-	-
	Asp102	-	-
Asn65	Ser195	O-N	2.25
	Ser214	ND2-O	3.05
	Val213	-	-
Thr66	His57	-	-
Ser67	Gly193	-	-
	Phe41	-	-
	His40	OG-O	3.19
His70	Lys60	-	-
Asp74	Gly96	-	-
	Asn97	-	-
Asn121	Asn97	ND2-OD1	3.17
Trp122	Asn97	-	-

Table 5.3: Comparative chart of residues from MKTI and STI (Suh et al., 1998) involved in intermolecular contacts with porcine pancreatic trypsin

MKTI	SKTI (Orthorhombic)	SKTI (Tetragonal)
Pro2	Asp1	Asp1
Ala13	Phe2	Phe2
Ser14	Asn13	Asn13
Ala62 (P3)	Pro61 (P3)	Pro61 (P3)
Tyr63 (P2)	Tyr62 (P2)	Tyr62 (P2)
Asn64 (P1)	Arg63 (P1)	Arg63 (P1)
Asn65 (P1`)	Ile64 (P1`)	Ile64 (P1`)
Thr66 (P2`)	Arg65 (P2`)	Arg65 (P2`)
Ser67	His71	-
His70	Pro72	-
Asp74	Trp117	Trp117
Asn121	Arg119	-
Trp122		

of trypsin. In P2' position, Ser67 of MKTI makes one hydrogen bond with His40 and interacts with His40, Phe41 and Gly193 of trypsin. Three N-terminal residues Pro2, Ala13 and Ser14 interact with one residue each namely Lys60, Gln192 and Gln192 respectively of trypsin. His70, Asp102, Asn121 and Trp122 of MKTI interact with Lys60, Gly96, Asn97 and Asn97 of trypsin respectively. Asn121 is involved in hydrogen bonding with Asn97 of trypsin (Table 5.2 and 5.3). Therefore, six of the total seven hydrogen bonds between MKTI and trypsin involve residues from reactive site loop.

5.4. Discussion

The purification and biochemical characterization of MKTI, in previous studies, showed many notable differences when compared to other similar inhibitors. It showed differences in structural and functional stability. Unlike other Kunitz-type inhibitors which are stable over a broad range of pH and temperature, MKTI was stable structurally but not functionally at higher temperature. At pH below 7.5, protein gradually precipitates. The partial amino acid sequence of MKTI showed maximum similarity to miraculin-like protein rather than typical soyabean Kunitz inhibitor. The cloning and sequencing of MKTI gene was performed in order to get more insights into nature of this protein. Also, structure prediction by homology modeling was carried out in order to analyze the structure of MKTI and its interaction with trypsin.

The cloning and sequencing of MKTI gene from both genomic and cDNA showed that it is a single polypeptide chain made of 215 amino acid residues. The sequencing of genomic clones demonstrated that MKTI gene does not contain introns. There are reports where intronless genes have been observed in Kunitz trypsin inhibitors from *Delonix regia*, Soyabean, (Huang et al., 2007; Song et al., 1993). The first N-terminal 25 amino acid residues form the signal sequence and therefore mature protein is made of 190 amino acid residues with a calculated molecular mass of 20738.3 Da. The N-terminal signal sequence has a possible membrane-spanning motif for plasma membrane as predicted from hydropathy index of iPSORT program. This indicates the translocation of protein from the site of synthesis. It has been shown that plant defense proteins are secreted and transported to the site of their action (Giri et al., 1998; Tsukuda et al., 2006). The amino acid sequence analysis and homology modeling of MKTI revealed great insights into the nature of MKTI. The inhibitor belongs to soyabean Kunitz family as evident from the presence of conserved motif typical of Kunitz

family at N-terminal of the primary amino acid sequence. The structure prediction of MKTI by homology modeling showed that overall fold is similar to the soyabean Kunitz-type trypsin inhibitors. Despite being a member of soyabean Kunitz family, MKTI exhibited significant differences from other members. Sequence homology studies unambiguously demonstrated that MKTI is a miraculin-like protein, also a member of soyabean Kunitz family. It showed 57% identity and 74% similarilty in amino acid sequence with miraculin-like proteins whereas only 29% identity and 43% similarity with STI. Interestingly, like MKTI, miraculin-like protein also belongs to Rutaceae family. In phylogenetic analysis, MKTI is distinctly clustered with miraculin-like proteins demonstrating that evolutionarily it is closer to miraculin-like protein rather than typical Kunitz family members like STI. Comparison of the amino acid sequence of MKTI with that of original miraculin showed 56% identity and 39% similarity. There are seven cysteines forming three disulfide bridges in MKTI which imparts rigidity and stability to the molecule. Biochemical analysis has shown that native and heat treated MKTI is remarkably stable against proteolysis by wide range of proteases (Shee et al., 2008). Interestingly, four cysteines forming two disulfide bridges are conserved in almost all the members of Kunitz family but the position of the extra disulfide bridge and free cysteine is not identical in miraculin and miraculin-like proteins. It has been reported that free cysteine in miraculin forms intermolecular disulfide bridge. It is not clear whether free cysteine of MKTI is involved in formation of inter-chain disulfide bridge as a single band was observed in non-reducing SDS-PAGE (Shee et al., 2007). Miraculin is a taste modifying protein and the taste modifying properties has been attributed by the two key residues, His29 and His59. The very preliminary studies on a small sample demonstrated that MKTI possess property to modify sour into sweet taste (unpublished results). The sequence comparison with miraculin showed that only one

histidine residue, His53, is present in the corresponding loop in MKTI. Interestingly, miraculin-like proteins lack both histidine residues and to the best of our knowledge there is no report regarding their taste modifying activity.

The comparison of exposed reactive site loop of MKTI showed that it superposes well with that of STI and exhibits almost similar conformation with a rms deviation (P4-P4') of 2.33 Å. respectively. In most soyabean Kunitz-type inhibitors like STI, reactive site loop is not constrained by disulfide bridges and its conformation is stabilized through a network of hydrogen bonds (Song et al., 1998). The reactive site loop of MKTI, like STI, is also not constrained by any disulfide bridge but no hydrogen bonds were observed for stabilization of its conformation. In MKTI, Asn13 which plays an important role in stabilizing the reactive loop conformation in STI is replaced by Ala13. No other interaction between the reactive site loop and rest of the molecule were observed in predicted MKTI structure. This structural feature could explain functional instability of MKTI where a sharp decline in inhibitory activity was observed at higher temperature (Shee et al., 2007a). Certainly, loop conformation required for inhibitory activity is not retained in MKTI at higher temperature. This is in contrast to other Kunitz-type trysin inhibitors which exhibit a high degree of structural and functional stability at range of pH and temperature. The comparative analysis of amino acid residues at reactive site loop revealed a major alteration in MKTI as compared to other soyabean Kunitz trypsin inhibitors. In MKTI, conventional Arg/Lys at P1 position has been replaced by Asn residue. Except for some miraculin-like proteins, no other known Kunitz-type inhibitor possess Asn as active site residue. Even native miraculin and many miraculin-like proteins possess Lys as active site residue (Theerasilp et al., 1989; Igeta et al., 1991; Tsukuda et al., 2006). The results suggest that the scissile peptide bond to be cleaved by trypsin is absent in MKTI as it lacks

Arg/Lys as active site residue essential for trypsin specificity. It seems that like most Kunitz family members, MKTI may not act as a typical substrate like inhibitor where peptide bond cleavage occurs during inhibition. There is a RLRF sequence 5 amino acids N-terminal to active site residue but homology modeling showed that it is a part of β-strand and is not exposed enough to interact with trypsin. Apart from primary specificity which is most important for interaction with trypsin, there are differences at all the secondary specificity sites in MKTI when compared to soyabean Kunitz trypsin inhibitor.

In MKTI, Tyr63 (P3), Asn64 (P2), Thr66 (P1'), Ser67 (P2') are present in place of Pro61 (P3), Tyr62 (P2), Ile64 (P1'), Arg65 (P2') in STI. Therefore, the mode of interaction observed in the MKTI-trypsin complex at both primary and secondary specificity sites are significantly different from that of interactions in STI-trypsin complex. Asn65 at P1 position in MKTI is not able to interact extensively like Arg63 (P1) in STI with the residues forming S1 subsite of trypsin. It mainly interacts with Ser195, Serine 214 and Valine 213 of trypsin whereas Arg in STI interacts additionally with Asp189, Ser190, Cys191, Gln192, Gly193, Asp194, Trp215, Gly216, Gly219, Cys220 and Gly226 of trypsin. Asn65 is too short to interact with side chain of Asp189 of trypsin, a key residue of primary binding pocket. Asn64 at P2 position in MKTI makes contacts with His57, Asp102, Ser195 and Ser214 of trypsin whereas Tyr62 (P2) in STI also interacts with Gly96, Glu99, Gln192 and Trp215 of trypsin in addition to the interactions present in MKTI. Tyr63 at P3 position in MKTI interacts with Gly96, Leu99, Ser214 and Trp215 of trypsin whereas Pro61 (P3) in STI only interacts with Trp215 and Gly216 of trypsin. Thr66 at P1' position in MKTI interacts only with His57 of trypsin whereas Ile64 (P1') in STI makes contacts with Phe41, Cys42, His57, Gln192, Gly193 and Ser195 of trypsin. In P2' position, Ser67 in MKTI interacts with His40, Phe41 and Gly193 whereas Arg65 also

interacts with Tyr151 in addition to the interaction made by MKTI. Comparing the hydrogen bonds in inhibitor-trypsin complex, MKTI makes only seven hydrogen bonds in total whereas STI makes fourteen and eleven hydrogen bonds in orthorhombic and tetragonal crystal structures of the complex respectively. Although MKTI makes less number of hydrogen bonds with trypsin as compared to STI, it interacts extensively with trypsin. In MKTI, thirteen residues are involved in interaction with trypsin whereas in STI twelve and nine residues are involved in interaction with trypsin in orthorhombic and tetragonal crystal structures of the complex respectively. Earlier, the variation in reactive site residue has been reported in Kunitz-type trypsin inhibitors. In *Cicer arietinum*, conserved Arg or Lys active site residue has been replaced by a Gly/Glu-Ile-Ser motif (Srinivasan et al., 2005) Other variations include amino acid insertion between P1 and P2 position in *Delonix regia* kunitz trypsin inhibitor (Huang et al., 2007).

MKTI is a major protein of seed and has been shown to have additional properties (Shee et al., 2008). The amino acid sequence analysis for possible functional motifs showed glycosylation, phosphorylation and myristoylation sites in MKTI. A possible N-linked glycosylation site (Asn-Asn-Thr-Ser) has been identified at position 64-67 in MKTI. The fact that calculated molecular mass from amino acid sequence (20738.3 Da) is different from that of native MKTI obtained from MALDI-TOF analysis (21370.8 Da) supports this observation. This difference may account for possible glycosylation in MKTI. The three possible phosphorylation sites were observed in the sequence of MKTI which may be recognized by Casein kinase, Protein kinase C and Tyrosine kinase. Ser87, Thr118 and Thr121 could be the probable residues for phosphorylation. Thses motifs have also been observed in *Leucaena leucocephala* trypsin inhibitor (LTI) and Soyabean trypsin inhibitor (Abbasi et al., 2003). Also, a possible

myristoylation site at position 108-113 (Gly-Gly-Val-Glu-Gly-Asn) was observed which can be significant for protein-protein and protein-lipid interaction.

CHAPTER 6

CLONING, SEQUENCE ANALYSIS AND HOMOLOGY MODELING OF *MURRAYA PANICULATA* TRYPSIN INHIBITOR

6.1. Introduction

The Kunitz inhibitor family constitutes the protease inhibitors isolated from various plant families. The major inhibitors are reported from Leguminosae, Graminae and Cucurbitaceae family. Despite the different origin and sources these inhibitor constitutes some conserved characteristics which imparts the inhibitory properties in these molecules. The miraculin like proteins also shares some homology with the Kunitz inhibitor family and possesses the protease inhibitory property. Two homologous miraculin like protein have been reported previously from *Citrus* species (Tsukuda et al., 2006).

In the comparative protein profiling of seed extracts of common plants belonging to Rutaceae family, a corresponding protein band similar to MKTI was found in the seeds extract of *Murraya paniculata*. The weak trypsin inhibitory activity was found in the seed extract of *Murraya paniculata*, which indicates that the protein may belong to same class and may be conserved in nature.

In the present chapter, *Murraya paniculata* trypsin inhibitor (MPTI) gene homologue to MKTI has been cloned and also, sequence analysis and structure prediction by homology modeling was carried out.

6.2. Materials and Methods

6.2.1. Cloning of Murraya paniculata trypsin inhibitor (MPTI) gene

6.2.1.1. Primer designing

5'GATCCTTTGCTTGATATT 3' and 5'TCAAGACACGCATGAG 3', primers have been used for the amplification of MPTI as forward and reverse primer respectively. 5'

ATGAATACTCCTTTTGTGATTGCAATCTCC 3' primer sequence has been used as a forward primer for the amplification of MPTI gene with signal sequence.

6.2.1.2. Isolation of genomic DNA

Leaves was powdered in liquid nitrogen, mixed with 5 ml of DEB and incubated at 65°C for one hour with frequent shaking in between. After one hour, equal volume of Chloroform: Isoamyl alcohol (24:1) was added and then the mixture was centrifuged at 5000g for 20 min at 20°C. Supernatant was collected, mixed with equal volume of ice cold isopropanol and incubated at -20°C for 30 min for DNA precipitation. Precipitated DNA was collected, washed with 70% ethanol and stored for future use.

6.2.1.3. Amplification of Murraya paniculata trypsin inhibitor gene

MPTI gene was amplified by using gene specific primer of MKTI taking the genomic DNA as a template. The following reaction mixture was used for the amplification of MPTI:

Genomic DNA template		100 ng
Amplification Buffer		2.5 µl
dNTPs		5.0 µl
10 mM Forward primer		1.0 µl
10 mM Reverse primer		1.0µl
5 U/µl DNA polymerase		0.5 µl
H2O	up to	25.0 µl

PCR was carried out using the reaction mixture with the following conditions: 95°C/4 min; 30 cycles of 95°C/60 s , 58°C/60 s, 72°C/60 s; and 72°C/10 min. The PCR product was detected on 0.8% agarose gel. The PCR product was eluted from low melting agarose gel

according to the manufacturer's instruction of Gel Elution kit (Bangalore Genei) as previously described. The eluted product was cloned in pGEM-T vector according to the manufacturer's instruction (Promega) and sequenced by using universal primers T7 and SP6.

6.2.2. Deduction of amino acid sequence of MPTI

The nucleotide sequences of MPTI were matched and the complete nucleotide sequence of MPTI gene had been deduced. The amino acid sequence of complete MKTI gene was deduced by ORF finder tool on NCBI online server. The amino acid sequence of MPTI was aligned against the triplet nucleotide codon.

6.2.3 Similarity search by BLAST

The similarity search of deduced amino acid sequence of MPTI was done by using NCBI online server protein BLAST taking non- redundant (nr) database and protein data base (PDB).

6.2.4 Multiple sequence alignment

Phylogenetic analysis was performed to analyze evolutionary relationship between the similar sequences. MPTI sequence was compared with other Kunitz inhibitors by multiple sequence alignment

6.2.5 Identification of various functional motifs

Probable motifs involved in post translational modifications such as glycosylation, phosphorylation and myristoylation site were predicted by "Motif Scan Server". The Kunitz

family signature pattern was also observed by Scan prosite server. The prediction about presence of signal sequence was made by using online server iPSORT.

6.2.6 Theoretical determination of isoelectric point and hyrdropathy index of MPTI

Theoretical determination isoelectric point and hydropathy index of MPTI amino acid sequence was made by online proteomic *EXPASY* server.

6.2.7 Secondary structure prediction

The secondary structure predictions of MPTI were carried out using the PHD method (http://www.predictprotein.org/).

6.2.8. Tertiary structure prediction by homology modeling

The three-dimensional model of MPTI was constructed using the crystal coordinates of *Delonix regia* trypsin inhibitor (DrTI), (PDB id: 1R8N) done at a resolution of 1.75 Å. Automated homology model building was performed using protein structure modeling program MODELLER 9 (Sali and Blundell, 1993) which models protein tertiary structure by satisfaction of spatial restraint. The input for the program MODELLER consisted of the aligned sequence of MKTI and DrTI, and a steering file which gave all the necessary commands to the Modellar for generating the homology model of MKTI on the basis of its alignment with crystal coordinates of the template. Many runs of model building were carried out in order to obtain most plausible model.

6.2.8.1. Validation of tertiary structure

The evaluation of the predicted MPTI model, i.e., analysis of geometry, stereochemistry, and energy distribution in the models, was performed using either the ENERGY commands of

MODELLER or using Program "PROCHECK" and Whatcheck. In addition the variability in the predicted model, i.e. RMSD was calculated by superposition of Cα traces and backbone onto the template crystal structure. The protein structures were visualized and analyzed on PYMOL (http://www.pymol.org) and WINCOOT.

6.3. Results

6.3.1. Cloning of Murraya paniculata trypsin inhibitor (MPTI)

Comparative protein profiling showed a protein band comparable to MKTI in the crude extract of *Murraya paniculata* (Figure 6.1). Gene specific Forward and Reverse primers of MKTI were used for the amplification of MPTI gene by using *Murraya paniculata* genomic DNA template. A single band of 570 bp was detected on 0.8% agarose gel with gene specific forward primer specific for mature MKTI used. To get the upstream sequence with signal sequence of MPTI gene, forward primer specific for upstream sequence of MKTI was used. A single band of 645 bp was obtained and detected on 0.8% agarose gel (Figure 6.2). The nucleotide sequence analysis of MPTI gene amplified from genomic DNA has shown that it encode 189 amino acids (Figure 6.3). MPTI amino acid sequence showed 96% homology with the MKTI amino acid sequence. The results clearly showed that the genomic DNA of MPTI does not contain introns. The presence of N-terminal signal sequence in MPTI was indicated by the amplification product with the forward primer specific for upstream signal sequence of MKTI. The calculated molecular weight of mature MPTI from amino acid sequence is 20692.2 Da.

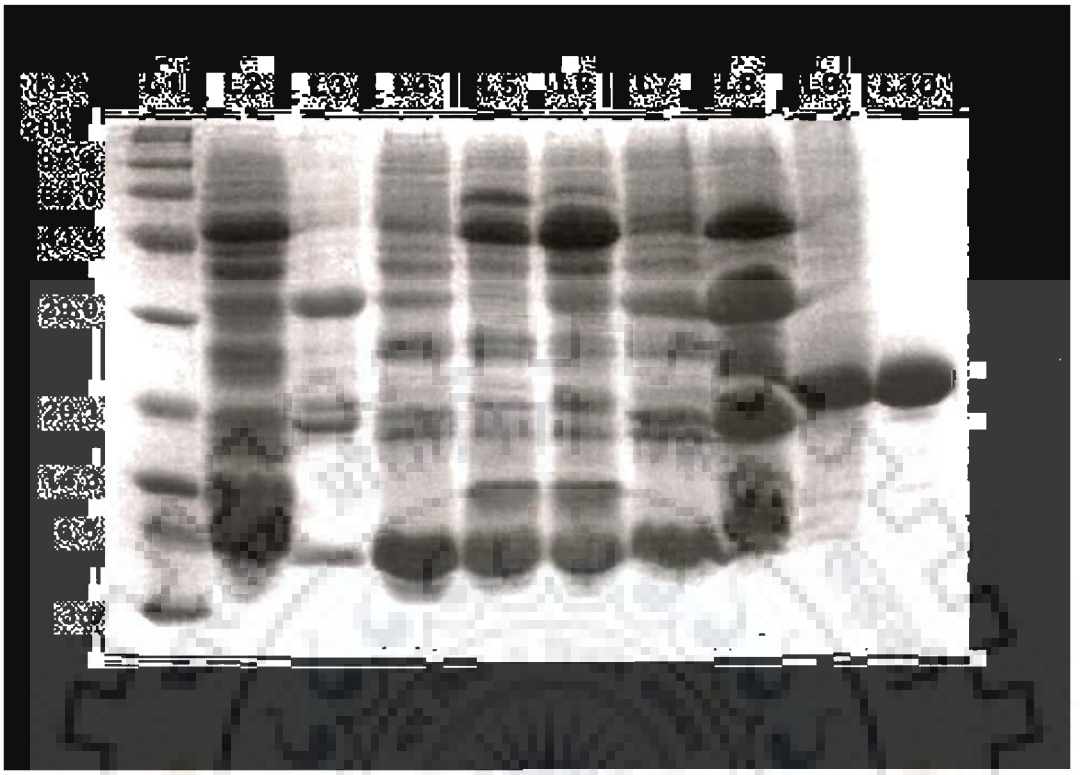


Figure 6.1: Comparative protein profiling of seeds extracts from various plants. L1; Molecular weight marker, L2; *Pyrus malus* (Apple), L3; *Aegle marmilos* (Stone apple), L4; *Citrus aurentium* (Orange), L5; *Citrus lemon* (Lemon), L6; *Citrus sinensis* (Bitter orange), L7; *Citrus reticulate* (Mandarin orange), L8; *Murraya paniculata*, L9; *Murraya koenigii* (Curry leaves), L10; MKTI

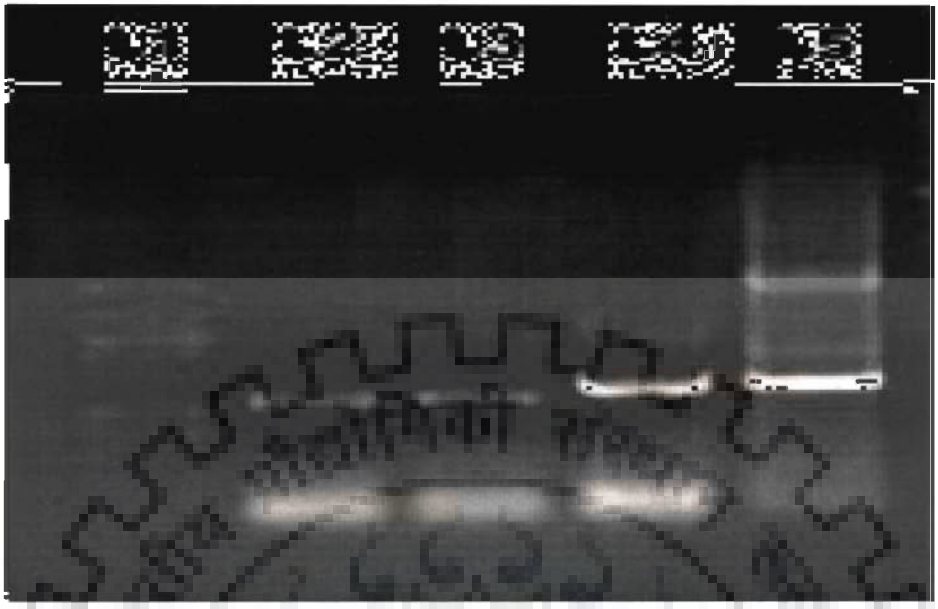


Figure 6.2: PCR amplification product of *Murraya paniculata* trypsin inhibitor with genomic DNA used a template. L1; 100bp molecular weight marker, L2; *Murraya paniculata* trypsin inhibitor gene with MKTI specific primer, L3; Control mature MKTI gene, L4; *Murraya paniculata* gene with signal sequence, L5; Control MKTI gene with signal sequence

```
  1 gatcctttgcttgatattaatggcccagtaggtcaacagcgagac
     D  P  L  L  D  I  N  G  P  V  G  Q  Q  R  D
 46 tactatttggtttcagtaattggaggagcaggcggcggtggcctc
     Y  Y  L  V  S  V  I  G  G  A  G  G  G  G  L
 91 accctctacaggggcagaaacgaactccgcccacttgatgttatc
     T  L  Y  R  G  R  N  E  L  R  P  L  D  V  I
136 caactaagctcggatctacataaaggaacccgtttaagattcgca
     Q  L  S  S  D  L  H  K  G  T  R  L  R  F  A
181 gcgtataatgacactagtattatccatgaagccgtggatctgaac
     A  Y  N  D  T  S  I  I  H  E  A  V  D  L  N
226 gtgaaattctcaacagagactagctgcaacgagccaacagtatgg
     V  K  F  S  T  E  T  S  C  N  E  P  T  V  W
271 agagtcgacaactatgatccatcgagaggaaaatggtttataaca
     R  V  D  N  Y  D  P  S  R  G  K  W  F  I  T
316 actggtggagttgaaggaaaccctggcgcacaaactttgaaaaac
     T  G  G  V  E  G  N  P  G  A  Q  T  L  K  N
361 tggtttaagcttgagagagttggaacagatcaaggtacgtacgag
     W  F  K  L  E  R  V  G  T  D  Q  G  T  Y  E
406 attgttcactgtccttccgtttgcaaatcctgtgtatttttatgc
     I  V  H  C  P  S  V  C  K  S  C  V  F  L  C
451 aatgatgttggggtttcttacgattatcgtcgacgtttggctctc
     N  D  V  G  V  S  Y  D  Y  R  R  R  L  A  L
496 actgctggtaatgagcgcgtctttggtgttgttatagtcccggct
     T  A  G  N  E  R  V  F  G  V  V  I  V  P  A
541 aatgagggatcagcctcatgcgtgtcttga
     N  E  G  S  A  S  C  V  S
```

Figure 6.3: Nucleotide and deduced amino acid sequence of MPTI. The nucleotide sequences of forward and reverse primers used for cloning are in bold and italics. The translated sequence of MKTI includes a 189- amino acid mature protein

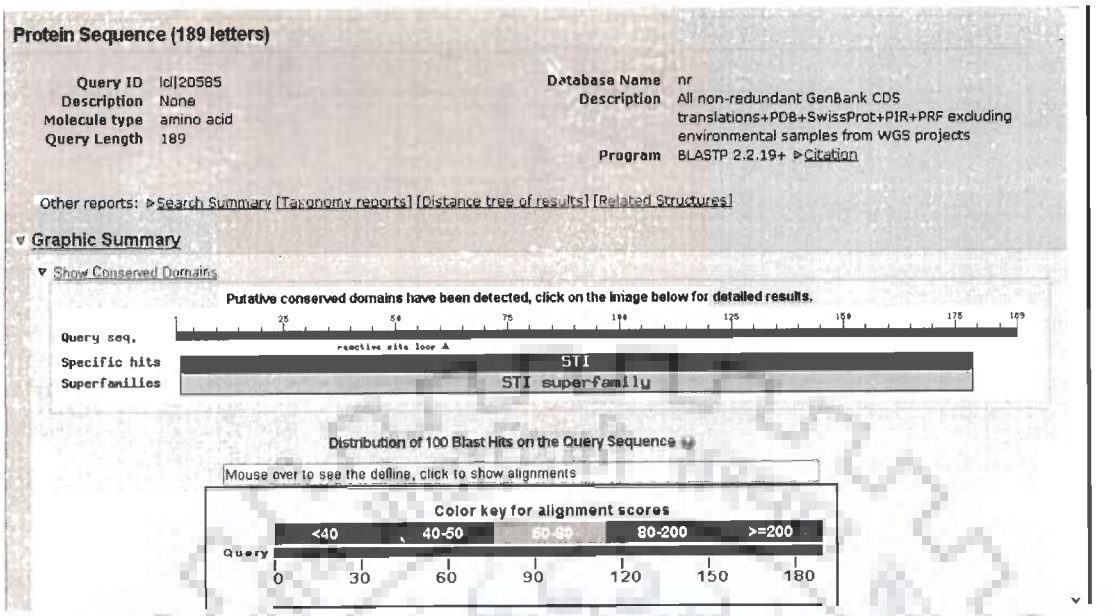
6.3.2. Similarity search by NCBI-BLAST

Amino acid sequence of MPTI showed 96% similarity and 95% identity with MKTI sequence. Homology search using NCBI BLAST showed significant similarity, like MKTI, to miraculin-like proteins isolated from various Citrus species plants belonging to Rutaceae family against the nr- database. The MPTI showed maximum sequence homology to reported Kunitz-type inhibitors with highest similarity with *Delonix regia* against the PDB database.

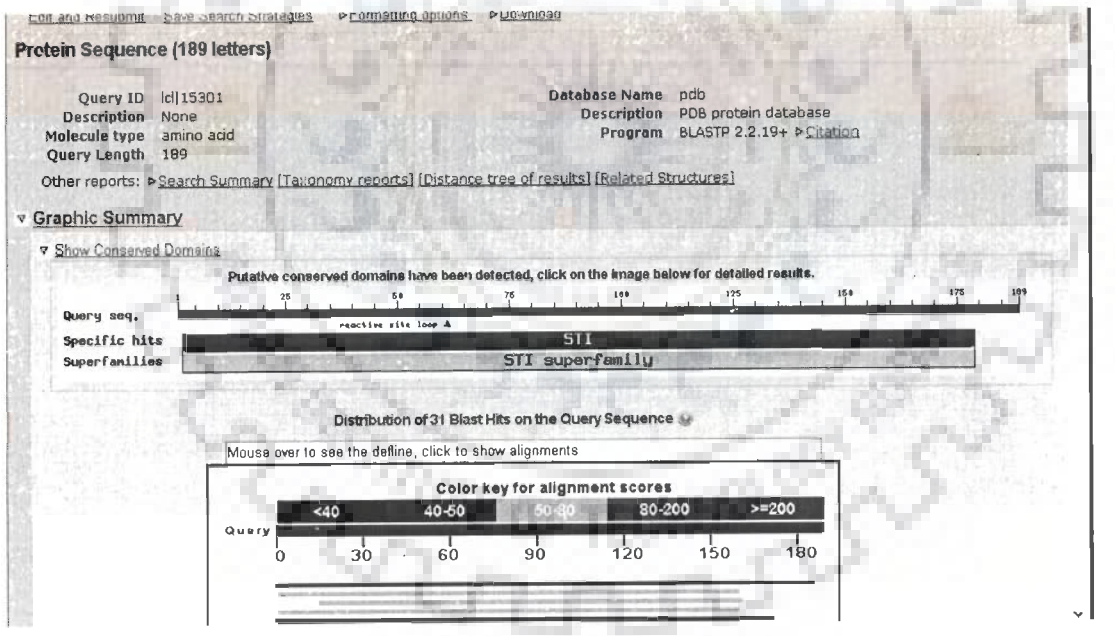
MPTI shared 72% similarity and 56% identity with the Miraculin-like protein from *Citrus jambhiri* belonging to Rutaceae family. In Kunitz family inhibitors MPTI matches with DrTI, shared 54% similarity and 33% identity (Figure 6.4).

6.3.3. Multiple sequence analysis

Multiple sequence alignment of MPTI sequences with that of other Kunitz-type trypsin inhibitors by CLUSTALW analysis revealed significant differences. The sequence of MPTI possesses two extra cysteine residues when compared to most Kunitz- type trypsin inhibitors. There are six cysteine residues in MPTI sequence as opposed to four cysteines residues in other Kunitz-type inhibitors. Also, miraculin-like proteins and MKTI contains seven cysteine residues. The sequence comparison of residues of reactive site loop of MPTI with other Kunitz-type inhibitors showed that it comprises from the residue Ala61(P1) to the residue Ile68(P4'). There is a marked variation in the reactive site residue in MPTI, wherein a conventional Arg or Lys has been replaced by Asp 64(P1) (Figure 6.5).



A



B

Figure 6.4: Sequence Similarity search was done with the NCBI BLAST search program. **(A).** Search done against the non- redundant database. **(B).** Search done against the protein database

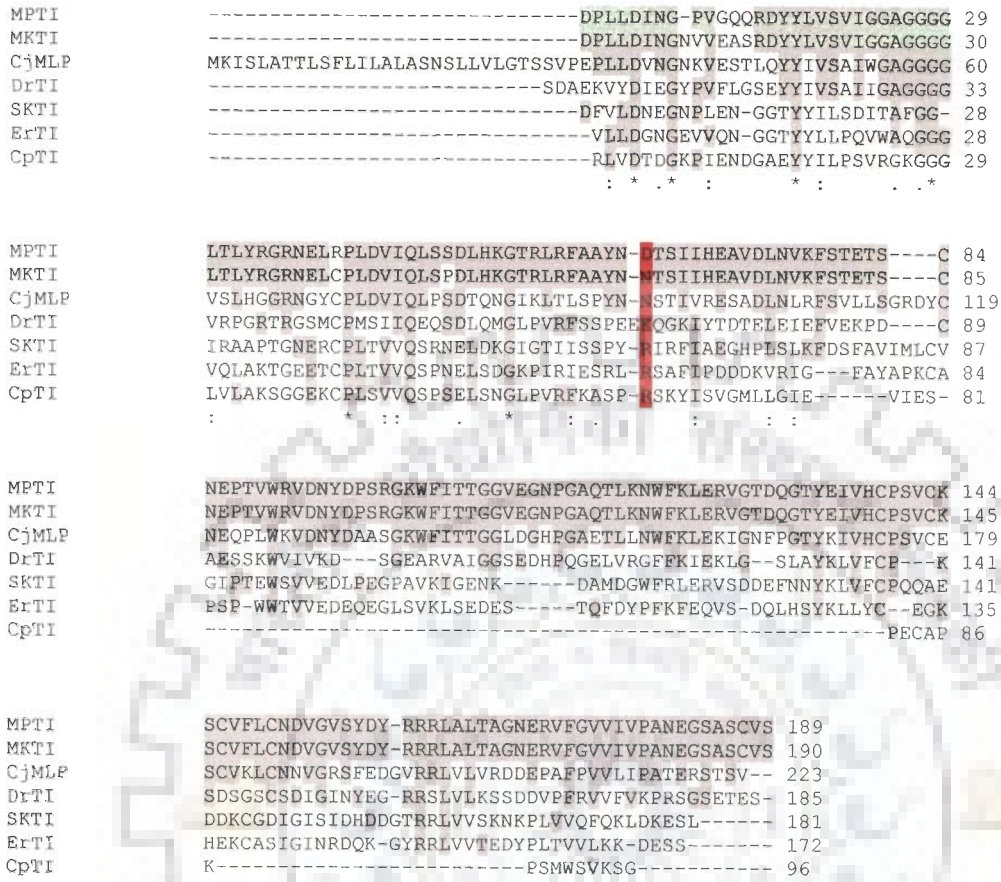


Figure 6.5: Comparison of amino acid sequences of various trypsin inhibitors and miraculin like proteins stored in NCBI and Uniport databases using CLUSTALW program. MPTI; *Murraya paniculata* trypsin inhibitor, MKTI; *Murraya koenigii* trypsin inhibitor, CjMLP; *Citrus jambhiri* miraculin like protein (gi|87299377), DrTI; *Delonix regia* trypsin inhibitor (gi|49258681), SKTI; Soyabean Kunitz trypsin inhibitor (gi|3318878), ErTI; *Erythrina caffra* trypsin inhibitor (gi|157833954), CpTI; *Copaifera langsdorffii* trypsin inhibitor (gi|49258682). Conserved residues are shown in highlighted in gray. Residues at the active site are highlighted in red

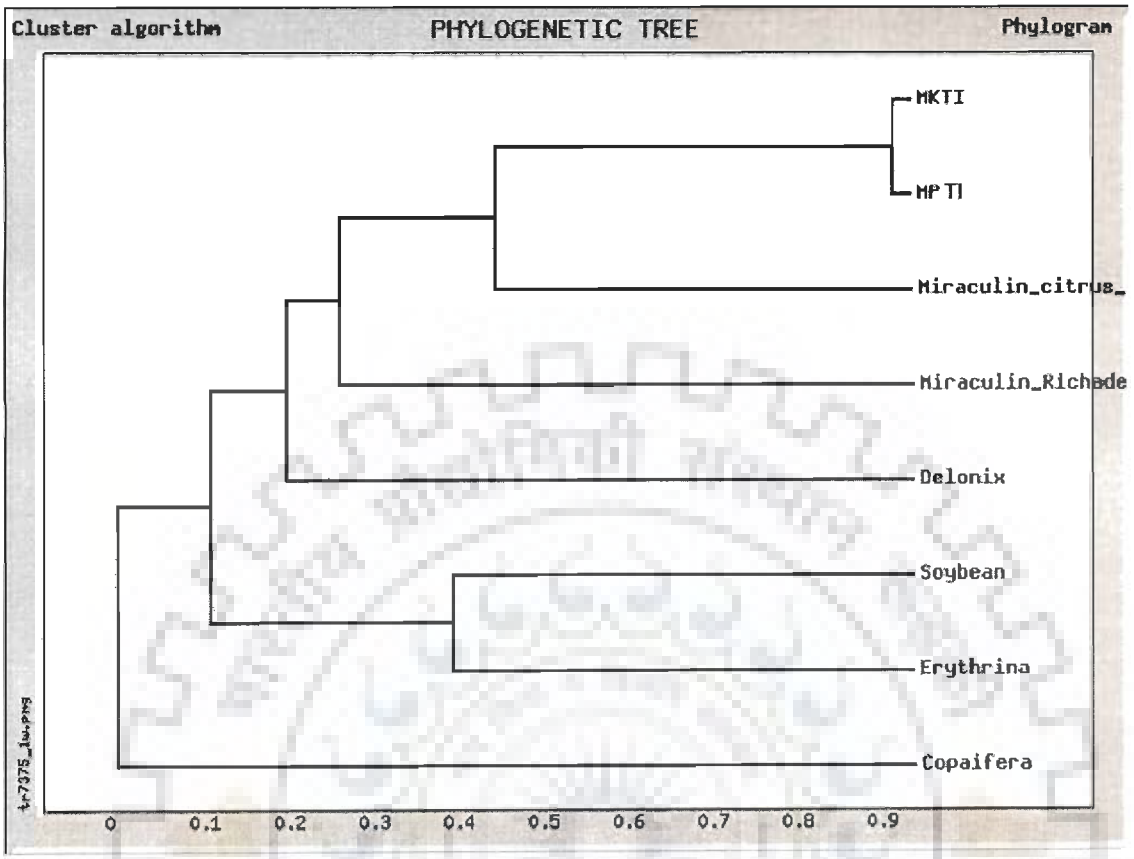


Figure 6.6: Phylogenetic tree derived from MKTI related sequences isolated so far. Phylogenetic analysis of these sequences using the neighbor joining distances method within phylip package. MKTI; *Murraya koenigii* trypsin inhibitor, MPTI; *Murraya paniculata* trypsin inhibitor, Miraculin_citrus; Miraculin like protein from *Citrus jambhiri*, Miraculin_Richade; Miraculin protein from *Richadella dulcifica*, Delonix; *Delonix regia* trypsin inhibitor (DrTI), Soyabean; Soyabean Kunitz trypsin inhibitor (SKTI), Erythrina; *Erythrina caffara* trypsin inhibitor (ErTI), Copaifera; *Copaifera lagsdorffii* trypsin inhibitor (CpTI).

Phylogenetic analysis of representative Kunitz-type inhibitors from different plants was performed to understand the any evolutionary relationship among these inhibitors. The phylogenetic analysis showed that MPTI sequence formed a distinct cluster with miraculin-like proteins (Figure 6.6).

6.3.4. Sequence comparison of MPTI with MKTI

MPTI amino acid sequence showed 96% similarity and 95% identity with the amino acid sequence of MKTI. In MPTI a deletion has been observed at the 9th position where Asn was present in the case of MKTI. MPTI constitute 189 residues long polypeptide chain.

The significant difference lies in the N-terminal region of MPTI (Figure 6.6). Major differences in amino acid sequence of MPTI and MKTI include the replacement of Val (11), Glu (12), Ala (13), Ser (14), Cys (41), Pro (50), and Asn (65) in MKTI to Pro, Gly, Gln, Gln, Arg, Ser and Asp respectively. It is to be noted that Asp64 is active site residue based on sequence similarity search (Figure 6.7).

6.3.5. Identification of various motifs in MPTI amino acid sequence

The MPTI sequence was probed for presence of various functional motifs. Amino acid sequence analysis by Scan Prosite Server demonstrated that MPTI lacks Kunitz family signature pattern. Probable locations of motifs predicted from motif scan server showed existence of possible glycosylation, phosphorylation and myristoylation sites in MKTI. N-linked glycosylation site (Asn-Asp-Thr-Ser) was identified at position 63-66. The structural motif of phosphorylation was picked up specific for Casein kinase II, Protein kinase C and Tyrosine kinase. In MKTI three sequence pathches, i.e., Ser-Cys-Asn-Glu (position 83-86), Thr-Leu-Lys

```
MKTI            DPLLDINGNVVEASRDYYLVSVIGGAGGGGLTLYRGRNELCPLDVIQLSPDLHKGTRLRF 60
MPTI            DPLLDING-PVGQQRDYYLVSVIGGAGGGGLTLYRGRNELRPLDVIQLSSDLHKGTRLRF 59
                ******** *  .************************** ********.**********

MKTI            AAYNNTSIIHEAVDLNVKFSTETSCNEPTVWRVDNYDPSRGKWFITTGGVEGNPGAQTLK 120
MPTI            AAYNDTSIIHEAVDLNVKFSTETSCNEPTVWRVDNYDPSRGKWFITTGGVEGNPGAQTLK 119
                ****:************************************************************

MKTI            NWFKLERVGTDQGTYEIVHCPSVCKSCVFLCNDVGVSYDYRRRLALTAGNERVFGVVIVP 180
MPTI            NWFKLERVGTDQGTYEIVHCPSVCKSCVFLCNDVGVSYDYRRRLALTAGNERVFGVVIVP 179
                ************************************************************

MKTI            ANEGSASCVS 190
MPTI            ANEGSASCVS 189
                **********
```

Figure 6.7: Sequence comparison of *Murraya paniculata* trypsin inhibitor with *Murraya koenigii* trypsin inhibitor

Table 6.1: Amino acid sequence of MPTI shows various functional motifs. Glycosylation site is underlined; Phosphorylation sites are shown in bold and myristoylation site is bold and italics

MPTI amino acid sequence	DPLLDINGPVGQQRDYYLVSVIGGAGGGGLTLYRGRNELRPLDVIQLSSDLH KGTRLRFAAYNDTSIIHEAVDLNVKFSTET**SCNE**PTVWRVDNYDPSRGKWFI TT***GGVEGN***PGAQ**TLK**NWFKLE**RVGTDQGTY**EIVHCPSVCKSCVFLCNDVGVS YDYRRRLALTAGNERVFGVVIVPANEGSASCVS

(position 117-119) and Arg-Val-Gly-Tyr-Asp-Gln-Gly-Thr-Tyr (position 126-134) have been identified as possible targets for the Phosphorylation possible by Casein kinase, Protein kinase C and Tyrosine kinase respectively with Ser83, Thr117 and Tyrosin134 being the most probable phosphorylation site. In MPTI, a possible myristoylation site, i.e., Gly-Gly-Val-Glu-Gly-Asn is seen at position 107-112 (Table 6.1).

6.3.6 Theoretical determination of isoelectric point and hyrdropathy index of MPTI

Theoretical isoelectric point and hydropathy index of amino acid sequence of MPTI was calculated by protparam program of online proteomic server EXPASY. The calculated isoelectric point was 5.93 and the grand average of hydropathy (GRAVY) index of total amino acids was -0.240. The calculated hydropathy index concludes that the MPTI is cytosolic in nature.

6.3.7. Secondary structure prediction of MPTI

Secondary structure prediction of MPTI has shown that it is a typical β-sheet protein. It constitutes approximately 47% β-sheet joined with β-turns with almost no helix. In the secondary structure of MPTI there were only two disulphide bond exists, as it lacks first Cysteine residue (Position 40), which could make a disulphide bond with the Cysteine at position (Figure 6.6). MPTI shares more or less similar overall three fold internal symmetry as in the case of *Delonix regia* and MKTI. The summary of secondary structure is given in Table 6.2.

Table 6.2: Secondary structure distribution of MPTI

β- Strands		Loops	No. of residues	Disulphide bond
		N- terminal	1-15	
A1	16- 20	A1-A2	21-27	
A2	28-33	A2-A3	34-42	Cys139-Cys150
A3	43-47	A3-A4	48-56	Cys143-Cys146
A4	57-60	A4-B1	61-73	Reactive site: 63-66
B1	74-78	B1-B2	79-89	3_{10} Helix: 114-116
B2	90-93	B2-B3	94-101	
B3	102-106	B3-B4	107-119	
B4	120-127	B4-C1	128-132	
C1	133-140	C1-C2	141-150	
C2	151-156	C2-C3	157-161	
C3	162-165	C3-C4	166-174	
C4	175-178	C4-C5	179- 189	

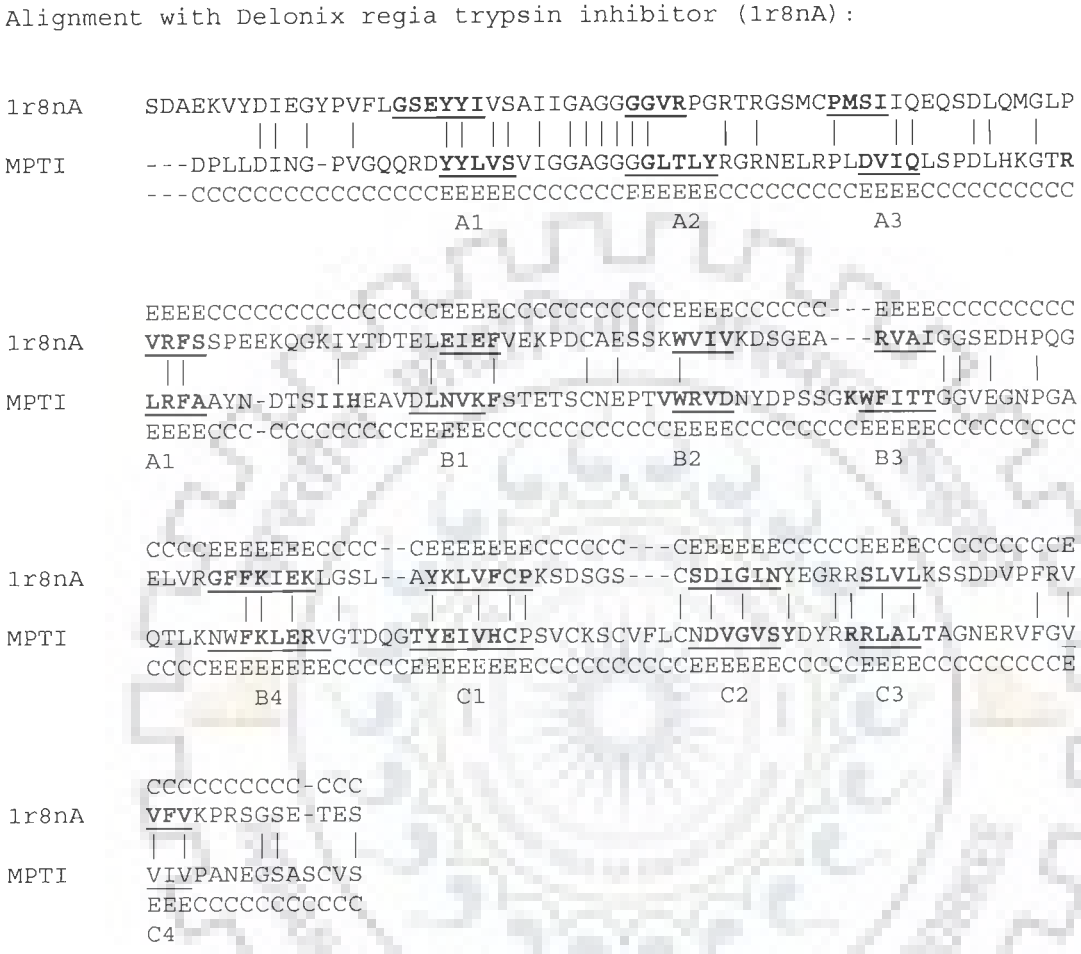


Figure 6.8: Multiple sequence alignment of template DrTI (PDB id: 1R8N) and target (MPTI). MPTI has three fold internal symmetry. β-Sheet in this subunit is labeled as An, Bn and Cn indicated number of sheets.

6.3.8. Prediction and evaluation of three-dimensional structure of MPTI

Homology modeling of MPTI was done using crystal co-ordinates of DrTI (PDB id: 1R8N) because MPTI shares 33% identity with the template (Figure 6.8). MPTI is made up of single polypeptide chain containing 189 amino acids. It forms a compact structure possessing feature characteristics of Kunitz type trypsin inhibitor as shown in Figure 6.9A.

The MPTI structure, like the template structure, is a β-trefoil fold made of 12 anti parallel β-strands with six of the strands arranged in a barrel structure and other six forms a triangular lid on the barrel. The structure of MPTI possesses three fold internal symmetry. The predicted structure contains around 47.9% of β-stands connected by long loops and a small 3_{10} helix. Procheck summary of MPTI shows that 86.2% residues are in the most favorable region, 13.1% in the allowed region and only one residue is in disallowed region (Figure 6.10). The reactive site of the predicted model of MPTI protrudes out from the structure (Figure 6.9B). The MPTI possesses six cysteine residue leading to two disulphide bridges, one between Cys139-Cys150 and the other between because Cys143-Cys146 (Figure 6.9C). MPTI have shown significant difference in the amino acid sequence with MKTI. The key difference between MPTI and MKTI exists between positions 41, where cysteine was replaced by Arg, which means one disulphide bond less as compared to MKTI. In all other Kunitz family inhibitors and even in MKTI this disulphide bond is conserved. The N- terminal residue Asn13 in other Kunitz family inhibitors stabilized the inhibitory loop. In MPTI, the Asn13 was replaced by Gln. But there was no interaction has been observed between Gln13 and the inhibitory loop in the predicted three-dimensional structure of MPTI. The other major difference lies in the N-terminal region where the Kunitz family signature was absent in MPTI.

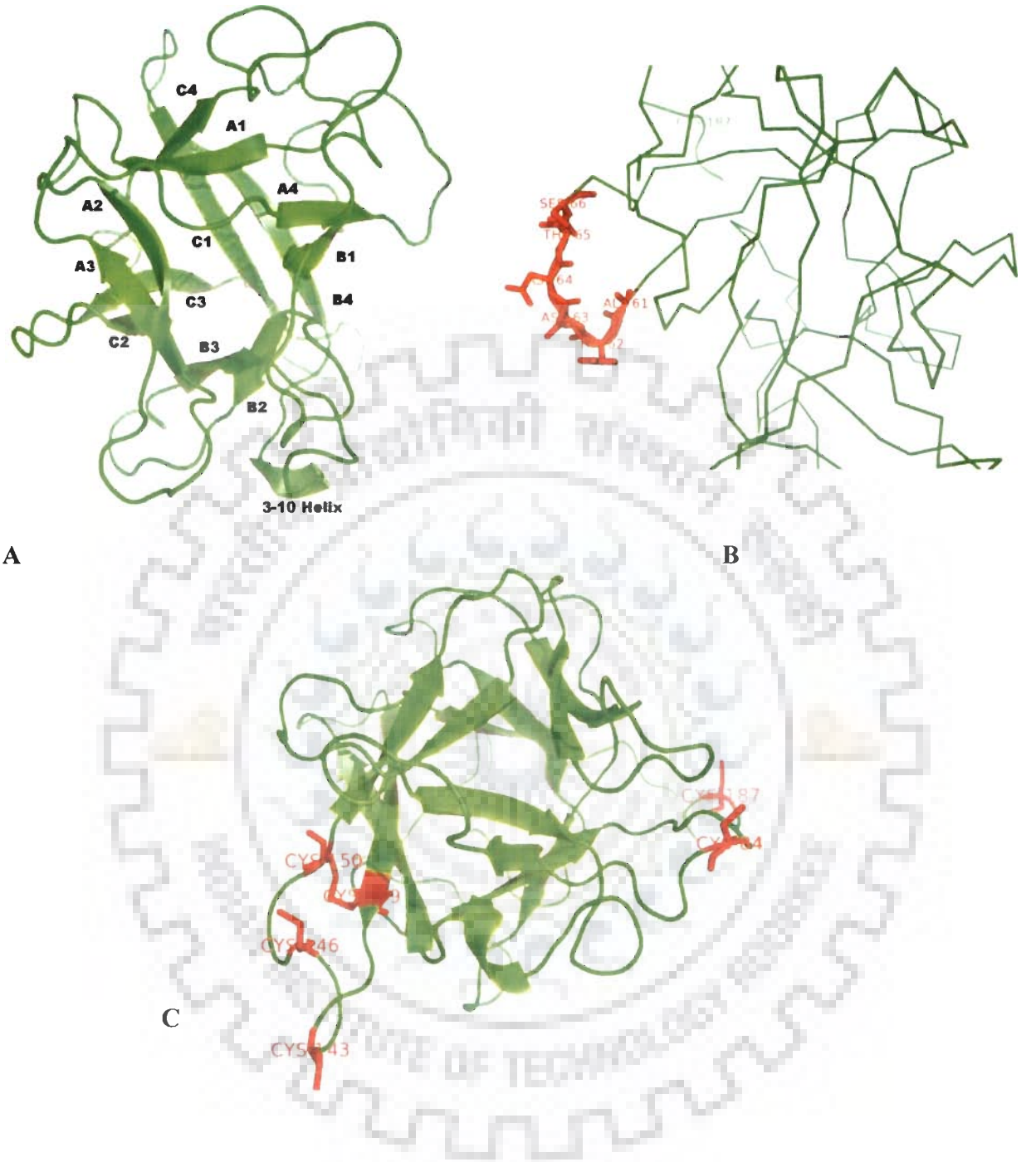


Figure 6.9: (A) Predicted model of MPTI showing the arrangements of α-helices, β-sheets and loop regions **(B)** Schematic representation of the inhibitory loop (red sticks) in the predicted three dimensional model of MPTI. **(C)** The positions of disulphide bonds and free cysteine residues in the predicted model of MPTI

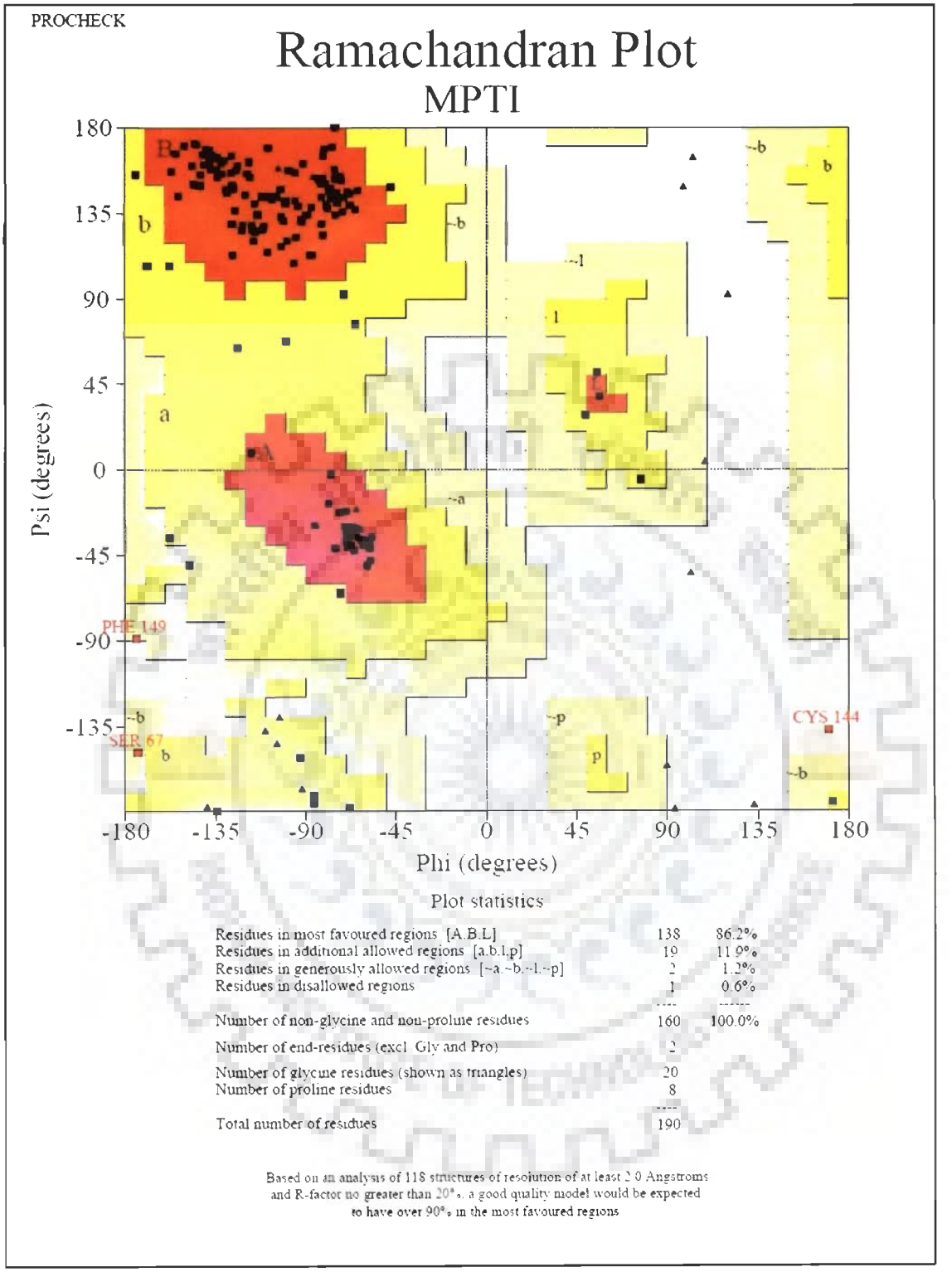


Figure 6.10: Ramachandran plot analysis of predicted three dimensional structure of MPTI

In MKTI three residues, Pro2, Ala13, Ser14 was found to interact with the trypsin as secondary interactions which can increase the specificity of the inhibitor. Like MKTI, the predicted structure of MPTI also exhibits three-fold internal symmetry with a four-stranded motif as the repeating unit. Around 63 amino acids in each unit are structurally organized as L-β1-L-β2-L-β3-L-β4 (L denotes loop connecting consecutive β-strands). The superposition of three domains shows a high degree of similarity for the β-strands and not for the loops. Strand β1 and β4 from the same motif are adjacent, wherease strand β1 is hydrogen bonded to the strand β4 of the previous motif and strand β4 is hydrogen bonded to the strand β1 of the following one, as in the case of typical Kunitz family inhibitors.

6.4 Discussion

In the comparative protein profiling of the seeds extract of *Murraya paniculata* a corresponding band to MKTI was present. Gene specific primers for the MKTI mature gene have been used for the amplification of MPTI gene which reveals that this protein is conserved in both the plants. Deduced amino acid sequence showed that MPTI polypeptide chain is one amino acid shorter than that of MKTI amino acid sequence and shares 96% homology with MKTI. There are significant difference between amino acid sequence of MPTI and MKTI. The similarity search of MPTI sequence showed that it is a member of Soyabean Kunitz inhibitor family with maximum homology to miraculin like protein from *Citrus jambhiri* as in the case of MKTI. Surprisingly, the prosite scan of MPTI amino acid sequence revealed the absence of Kunitz family inhibitor signature pattern which was present in MKTI. In Multiple sequence alignment MPTI forms a distinct cluster with miraculin-like proteins from the plants of Rutaceae family and distantly related with Kunitz inhibitor family members.

Protein kinases play a vital role in regulating and coordinating aspects of metabolism, gene expression, cell mortality, cell differentiation, cell division and protein activity by "switching on and off" mechanism allowing phosphorylation and dephosphorylation to proceed in an orderly fashion in cellular life. Some functional motif could be picked up in MPTI primary structure by the "PROSITE Search" indicating the possible Phosphorylation site. The structural motif correlates well with the pattern recognized by Protein kinase C, Casein kinase II and Tyrosine kinase. The myristoylation residue can bind with viral proteins and appears to play purely structural role. In addition to mediating protein-protein interactions, protein myristoylation is also known to enhanced protein-lipid interactions by targeting and binding polypeptide chains to different types of membranes. MPTI also possesses a putative glycosylation site like MKTI, which reveals the glycoprotein nature of MPTI.

MPTI shares a typical kunitz family inhibitor structure, roughly spherical consist of twelve antiparallel β-strands, long loop connecting these β-strands, and a 3_{10} helix. It shows same overall fold that had been described for the structure of DrTI (Polikarpov et al., 2003), SKTI (Suh et al., 1998) and WCI (Dattagupta et al., 1996). The reactive site protrudes out from the one end of structure. Six of the strand (A1, A4, B1, B4, C1 and C4) form a short anti parallel β-barrel, with one side of barrel being closed by a lid consisting of the other six strands. This common fold termed the β-trefoil fold has also been observed in the structures of nonhomologous proteins including including interleukin-1α (Graves et al., 1990), interleukin-1β (Priestle et al., 1988; Finzel et al., 1989), ricin B-chain (Rutenber and Robertus, 1991), hisactophilin (Habazetti et al., 1992), and fibroblast growth factors (Eriksson et al., 1991; Zhang et al., 1991; Zhu et al., 1991).

There are significant differences observed in the amino acid sequence of MPTI with MKTI. A deletion was observed in the sequence of MPTI at position 9, where Asn was present in the MKTI. The other differences include the replacement of Val (11), Glu (12), Ala (13), Ser (14), Cys (41), Pro (50), Asn (65), to Pro, Gly, Gln, Gln, Arg, Ser and Asp in MPTI sequence. The Kunitz family inhibitor signature pattern was not observed in the amino acid sequence of MPTI which was present in the MKTI.

MPTI possesses six cysteine residues one short than MKTI and forms two disulphide bridges. Arg has replaced the first cysteine (position 40) residue in the primary amino acid sequence of MPTI, which is almost conserved in all miraculin like proteins and other Soyabean kunitz inhibitor family members. It forms a disulphide bridge which stabilized the structure. Two Cysteine residues remain free at position 85 and 186. The role of free cysteine is needed to be explored. The two disulphide bridges provide stability and rigidity to the structure of MPTI. Disulphide bond between Cys41-Cys85 remained conserved in the single disulphide bond containing trypsin inhibitor isolated from seeds of *Swartzia pickellii* (Sampio et al., 2002). The other major difference between MPTI and MKTI is lies in inhibitory loop, where Asn65 has been replaced by a positively charged residue Asp. It is not clear whether presence of Asp in MPTI in place of Asn in MKTI may be responsible for weak inhibitory activity as compared to MKTI. It is to be noted that MPTI also lacks signature pattern of Kunitz family and other major differences in N-terminal region.

As in the case of DrTI, compared to other trypsin inhibitors, the reactive loop of DrTI is distorted due to an insertion of Glu68 between the residues P1 and P2. This weakens the inhibitory activity of DrTI against trypsin. DrTI inhibition constant Ki against trypsin is 21.9 nM, whereas the canonical inhibitors from the family have Ki close to 1 nM (Polikarpov et al.

2003). Other Kunitz family inhibitor isolated from the seeds of *Swartzia pickellii* also showed the insertion of Gln at position 65 (Sampio et al., 2002) with the Ki close to 50 nM. Interaction studies of MPTI have shown more or less similar pattern of interaction as MKTI because the overall symmetry of both the inhibitors were remain same. Sequence similarity shows that the MPTI and MKTI belong to the same class of proteins, but during the course of evolution MKTI became more advance in the function and serves as a key molecule in the plant defense system. As the *Murraya paniculata* is shrub in appearance while *Murraya koenigii* plant developed as a tree.

CONCLUSIONS

CONCLUSIONS

BIOINSECTICIDAL ACTIVITY OF *MURRAYA KOENIGII* TRYPSIN INHIBITOR (MKTI)

- MKTI inhibited 78.5% of trypsin-like activity and 40% total proteinases activity of HGP at 100% inhibition of bovine pancreatic trypsin, while the inhibitory effect of MKTI towards chymotrypsin was not observed
- In the case of *S. litura*, a maximum inhibition of 81.75% for trypsin-like and 48% of total proteinase activity was found against the gut extract.
- Substantial inhibition of proteinase activity of MKTI has been detected on gel zymogram analysis.
- Stability studies showed that the MKTI remains stable up to 72h with the trypsin inhibitory activity intact in both the insect's gut extract.
- *H. armigera* larvae fed on diet containing MKTI showed substantial reduction in weight with increasing concentrations of inhibitor. At highest concentration of MKTI (200μM), approximately 88, 83 and 69% reduction in larval weight was observed after 6, 8 and 10 days of feeding
- At highest concentration of MKTI (200μM), approximately 44, 53 and 44% reduction in larval weight was observed after 6, 8 and 10 days of feeding in the case of *S. litura*.
- 40% and 43.3% mortality was observed in larvae fed on diet supplemented with 200 μM MKTI in *H. armigera* and *S. litura* respectively.
- Larval period also showed an increase from 15 to 20 days and 14.9 to 17 days at the highest concentration of inhibitor in *H. armigera* and *S. litura* respectively.

- In case of *H. armigera*, pupation decreased from 93% to 60% and pupal weight decreased marginally by 10%. The pupal period did not change substantially. In case of adult development, adult emergence decreased from 84 to 60%. Adult survival reduced by 40% and an increase from 26 to 33 days was observed in total development at highest dose.
- Pupal weight reduced by 20% and pupation decreased from 96.7% to 56.7% at highest dose of inhibitor. Adult emergence decreased from 90 to 57%, total survival was reduced by 33% and an increase in total development days was observed at highest dose in the case of *S.litura.*
- Malformed pupae and Adults were observed in the life cycle of both the insects of treatment groups. Fecundity and fertility of both the lepidoteran insect were severely affected by the highest concentration of MKTI.
- Growth indices of larva, pupa and total development were decreased with the increasing concentration of MKTI compared to the control group.
- A significant decrease in the endogenous trypsin-like activity and increase in Chymotrypsin-like activity was observed in the treated group.
- A decline of 4, 5.5 and 3-fold was observed in the trypsin-like activity of HGP in third, fourth and fifth instars larvae fed on 200 μM MKTI respectively.Chymotrypsin-like activity increased by 4.4, 3 and 3-fold in MKTI fed third, fourth and fifth instars larvae of *H. armigera* respectively.
- Almost 8-fold reduction in trypsin-like activity and 3.8-fold increase in chymotrypsin-like activity was observed in fifth instar larvae of *S. litura.*

CLONING, EXPRESSION AND PURIFICATION OF *MURRAYA KOENIGII* TRYPSIN INHIBITOR

- Complete gene of MKTI with 3' and 5' UTR has been cloned from cDNA and genomic DNA. Open reading frame of MKTI contains 648 bp which encodes 215 amino acids.
- The nucleotide sequence of genomic DNA and cDNA was matching perfectly which indicated that the MKTI gene did not contain intervening sequence.
- Mature MKTI gene was expressed as ~47 kDa GST-fused protein in pGEX-5x-3 expression vector.
- The accession number assigned to the cDNA and genomic DNA sequence of MKTI gene is **FJ468001** and **FJ468002** respectively.

SEQUENCE ANALYSIS AND HOMOLOGY MODELING OF *MURRAYA KOENIGII* TRYPSIN INHIBITOR

- MKTI shared 74% similarity and 57% identity with the miraculin-like protein from *Citrus jambhiri* belongs to Rutaceae family. In Kunitz family inhibitors, MKTI showed maximum homology to DrTI with 53% similarity and 32% identity.
- Probable locations of motifs predicted from motif scan server showed existence of possible glycosylation, phosphorylation and myristoylation sites in MKTI.
- The observed Kunitz family signature pattern (LLDINGNVVEASRDYYL) was located at the N-terminal of the MKTI sequence.
- Secondary structure distribution pattern of MKTI shows that this protein consists of approximately 47% β-sheet, joined by β-turns with little or almost no alpha helical part.

- The overall geometry of the MKTI structure was similar to other soyabean Kunitz-type inhibitors. The MKTI possess seven cysteine residues, three extra as compared to reference structure. The three disulfide bridges (Cys41-Cys85, Cys 140-151 and Cys 144-Cys 147) was found in the MKTI model.
- In MKTI-trypsin complex predicted structure, thirteen residues namely Pro2, Ala13, Ser14, Ala62 (P4), Tyr63 (P3), Asn64 (P2), Asn65 (P1), Thr66 (P1') and Ser67 (P2'), His 70, Asp74, Asn121 and Try122 was found to be interacting with trypsin.

CLONING, SEQUENCE ANALYSIS AND HOMOLOGY MODELING OF *MURRAYA PANICULATA* TRYPSIN INHIBITOR

- A corresponding protein band similar to MKTI was found in the seeds extract of *Murraya paniculata*. Weak trypsin inhibitory activity was found in the seed extract of *Murraya paniculata.*
- MPTI gene has been amplified by using gene specific primer of MKTI with the genomic DNA as a template. A band of 570 bp was obtained with forward primer specific for mature MKTI and 645 bp with forward primer specific for upstream sequence.
- The nucleotide sequence analysis of MPTI genomic DNA has shown to encode 189 amino acids. The nucleotide sequence of MPTI does not encode any intervening sequence.
- Amino acid sequence of MPTI showed 96% similarity and 95% identity with MKTI sequence. Similar to MKTI, MPTI shared 72% similarity and 54% identity with the miraculin-like protein from *Citrus jambhiri* belongs to Rutaceae family. In Kunitz

family inhibitors, MPTI showed maximum homology to DrTI with 54% similarity and 33% identity.

- The significant difference lies in the N-terminal region of MPTI. Major differences in amino acid sequence of MPTI and MKTI include the replacement of Val (11), Glu (12), Ala (13), Ser (14), Cys (41), Pro (50) and Asn (65) to Pro, Gly, Gln, Gln, Arg, Ser and Asp respectively.
- Kunitz family signature pattern was absent in the amino acid sequence of MPTI. Probable locations of motifs predicted from motif scan server showed existence of possible glycosylation, phosphorylation and myristoylation sites in MKTI.
- The MPTI structure, like the template structure, is a β-trefoil fold made of 12 anti parallel β-strands with six of the strands arranged in a barrel structure and other six forms a triangular lid on the barrel.

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