PURIFICATION AND CHARACTERIZATION OF A TRYPSIN INHIBITOR FROM *PUTRANJIVA ROXBURGHII*

A THESIS

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled, **"PURIFICATION AND CHARACTERIZATION OF A TRYPSIN INHIBITOR FROM *PUTRANJIVA ROXBURGHII*"** in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during a period from July 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 any other Institute.

(NAVNEET SINGH CHAUDHARY)

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

Dated- 23.12.2008

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Signature of Supervisor

Signature of External Examiner

ABSTRACT

Protein proteinase inhibitors have been found in many plant tissues especially in legume seeds and other storage organ, numerous animal tissue and fluids and in microorganism. 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. There are not many reports of purification and characterization of these inhibitors from other plant families. Other families where an inhibitor has been purified and characterized include *Rutaceae* and *Euphorbiaceae*.

Putranjiva roxburghii belonging to *Euphorbiaceae* family is an ornamental tree of tropical India, known as child life tree. Deseeded fruits are used against cough, cold and sprue. Rosaries of hard stones are used for protecting children from infections. The seed kernel on steam distillation yield 0.5% of a sharp-smelling essential oil of the mustard oil type. The oil contains isopropyl and 2-butyl isothiocyanates as the main constituents and 2-methyl-butyl isothiocyanate as a minor component. Roxburghonic keto acid and some flavonoids, terpenoids and triterpines has been purified and characterized from the leaf and trunk bark of this plant. To date, no protein has been characterized from this plant. This work describes the purification and characterization of a highly stable and potent trypsin inhibitor from the seeds of *Putranjiva roxburghii*.

Chapter 1 reviews the literature in the area of plant proteinase inhibitors particularly serine proteinase inhibitors.

Chapter 2 describes the purification of a Kunitz-type trypsin inhibitor from the seeds of *Putranjiva roxburghii*. The *Putranjiva roxburghii* trypsin inhibitor (PRTI) was purified to homogeneity in three steps by acid precipitation, CM-sepharose cation exchange and DEAE-sepharose anion exchange chromatography. In acid precipitation step, low molecular mass proteins were precipitated along with some other proteins. The trypsin inhibitory activity was retained in supernatant. In second step on CM-sepharose column, all the low molecular mass proteins were bound to the column while trypsin inhibitory activity was found in flow through. In last step, protein with trypsin inhibitory activity was bound to a DEAE-sepharose column. After washing the column extensively, bound proteins were eluted with step gradient of NaCl. The fractions with trypsin inhibitory activity were eluted at 50 and 100 mM NaCl. The purity of the protein in above fractions was analyzed by SDS-PAGE. The fraction eluted at 100 mM NaCl showed single band on SDS-PAGE. The protein was further subjected to size exclusion chromatography column on HPLC. The purified protein showed single band on SDS-PAGE. The SDS-PAGE analysis under both reducing and non-reducing conditions showed that PRTI is a single polypeptide chain with a molecular mass of approximately 34 kDa.

Chapter 3 describes the biochemical characterization of *Putranjiva roxburghii* trypsin inhibitor. Amino acid sequence analysis was performed by Edman degradation and mass spectrometry studies. In N-terminal sequencing of PRTI, first 10 residues from the N-terminal were obtained. The sequence determined was Arg-Pro-Pro-Gln-Ala-Gly-Tyr-Ile-Gly-Val. The N-terminal sequence of PRTI showed no similarities with any of the known trypsin inhibitors. In partial internal sequencing, sequences of four peptides were obtained. In separate experiments, one peptide (Peptide 1) was obtained from LC-MS/MS and three peptides from MALDI-TOF/TOF studies. The peptide sequenced by LC-MS/MS analysis showed identity to

winged bean chymotrypsin inhibitor-3. The inhibitory activity of PRTI against trypsin and chymotrypsin were determined by measuring the hydrolytic activity toward BAPNA and BTEE respectively. The protein completely inhibited trypsin at a molar ratio of 1:1 but did not show any significant inhibition against α-chymotrypsin. The analysis of Dixon plot showed that the PRTI is a competitive inhibitor where two lines corresponding to each substrate intersect above the x-axis, a characteristic of competitive inhibition. The dissociation constant (Ki) value was found to be 1.4×10^{-11} M. In stability studies, effect of temperature, pH and DTT was examined on inhibitory activity of PRTI. In thermal stability studies, trypsin inhibitory activity of PRTI was completely retained up to 70 °C. Above 70 °C, there was a slight decrease in the inhibitory activity with PRTI retaining almost 85% inhibitory activity up to 80 °C. The inhibitory activity of PRTI fell sharply above 80 °C with a loss of almost 80% inhibitory activity at 90 °C. In pH stability studies, PRTI was highly stable under conditions ranging from highly acidic to highly alkaline. PRTI showed maximum inhibition at pH 8.0 and maintained over 95% of its inhibitory activity through a pH gradient of 2-12. In presence of DTT, PRTI was found completely stable with no loss in inhibitory activity when incubated for 1 h up to 100 mM DTT. Only a slight decrease of 5% in inhibitory activity was observed when PRTI was incubated for 2 h at 100 mM DTT. Purified PRTI proteins were used for proteolysis studies with different proteases. Purified protein was incubated with five different proteinases, namely trypsin, chymotrypsin, papain, pepsin and proteinase K, separately using a protease/PRTI molar ratio of 1:50 for different time periods ranging from 30 min to 24 h at room temperature. All samples were analyzed on a 15% SDS-PAGE. PRTI is very stable against trypsin, chymotrypsin and pepsin and the results obtained did not show any cleavage. Although, PRTI is a serine proteinase inhibitor but proteolytically it is very stable against aspartate proteinases like pepsin. PRTI is partially

cleaved by papain and completely cleaved by proteinase k enzyme. Time dependant proteolytic cleavage studies were also performed to determine any domain structure of PRTI but there was no such difference observed in the cleavage pattern. PRTI retained almost 90% inhibitory activity after one year storage at -20 °C.

Chapter 4 describes the biophysical characterization of PRTI by circular dichroism and fluorescence studies. Far-UV CD spectroscopy studies (240 -200 nm wavelength range) were carried out to analyze the secondary structure and conformational stability of PRTI at different temperatures from 20 to 100 °C. Analysis of CD spectra of native PRTI showed that it is an α, β protein with negative peaks at around 217 nm and 208 nm. Although, negative ellipticity was present but no clear negative peak characteristic of α-helical structures was observed at 222 nm. These results strongly suggest that PRTI is α, β protein rather than predominantly β protein. CD studies at increasing temperature demonstrated the thermo stability of PRTI structure. The PRTI retained the back bone protein folding with no significant change in CD spectra up to 70 °C. A significant loss in ellipticity was observed at and above 90 °C. This correlates well with the results of inhibitory activity where 15% loss was observed at 80 °C and 80% at 90 °C. The inhibitory activity and CD studies at increasing temperatures showed that transition midpoint for PRTI lies close to 88 °C. Fluorescence spectroscopy experiments were performed in different physicochemical conditions to monitor the extent of changes in native structure of PRTI and relate them to the inhibitory activity. Extrinsic (ANS) and intrinsic (tryptophan) fluorescence monitoring studies of conformational stability exhibited that PRTI gradually unfolds as the concentration of GuHCl and Urea increases and above 8M, completely unfolded molten globule structure present. PRTI lost its native conformation after incubation in the range of 3-5% SDS and 200-1000 mM HCl concentration. Fluorescence emission spectra analysis significantly

correlates the structure-activity relationship when studied as a function of DTT, pH and temperature denaturation. PRTI structure and inhibitory activity was retained up to 100 mM DTT, 80 °C temperature and in highly alkaline and acidic pH ranging from 2.0-12. In comparison to alkaline pH, PRTI exhibited little higher unfolding at acidic pH.

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(NAVNEET SINGH CHAUDHARY)

CONTENTS

Page No.

CANDIDATE'S DECLARATION

ABSTRACT i-v

ACKNOWLEDGEMENT vi-vii

LIST OF PUBLICATIONS xii

INTRODUCTION 1-4

CHAPTER 1 5-34

LITERATURE REVIEW

1.1. Introduction 5

1.2. Serine proteinases and inhibitors 6

1.2.1. Catalytic mechanism of serine proteinases 7

1.2.2. Mechanism of serine proteinase inhibition 8

1.3. Plant proteinase inhibitors 9

1.3.1. The Bowman-Birk family 10

1.3.2. *The squash inhibitor (Cucurbit) family* 10

1.3.3. The "Cereal superfamily" family 11

1.3.4. *The Potato inhibitor I (PI-1) family* 11

1.3.5. The Potato inhibitor II (PI-2) family 12

1.3.6. The Kunitz-type family 12

1.3.6.1. Isolation, purification and general properties 12

1.4. Storage properties 17

1.5. Solubility properties 18

1.6. Circular dichroism spectroscopy 19

1.7. Fluorescence spectroscopy 21

1.8. X-ray crystallography 22

1.9. Importance of plant protease inhibitors 23

1.9.1. Role in plant defense 24

1.9.2. Protease inhibitors as storage proteins 26

1.9.3. Anticarcinogenic agent 27

1.10 General purification methods for trypsin inhibitors 28

1.10.1 Precipitation by alteration of the pH 29

1.10.2 Ion Exchange Chromatography 31

1.10.3 Size-Exclusion Chromatography 32

1.10.4 Estimation of protein and analysis of protein purity 33

CHAPTER 2 35-47

PURIFICATION OF PROTEIN

2.1. Introduction 35

2.2. Materials and Methods

2.2.1. Materials 37

2.2.2. Purification of Putranjiva roxburghii trypsin inhibitor (PRTI) 37

2.2.2.1 Preparation of supernatant with inhibitory activity 37

2.2.2.2 Acid precipitation method 38

2.2.2.3 Cation-exchange chromatography 38

2.2.2.4 Anion-exchange chromatography 38

2.2.2.5 HPLC size-exclusion chromatograph 39

2.2.3. Protein estimation 39

2.2.4. SDS-PAGE analysis 39

2.2.5 Trypsin inhibitory assay 40

2.2.5.1 Using BAPNA as a substrate 40

2.2.5.2 Using BAEE as substrate 41

2.3. Results 41

2.3.1. Purification of PRTI 41

2.3.2 Protein estimation and SDS-PAGE analysis 42

2.4. Discussion 45

CHAPTER 3 48-68

BIOCHEMICAL CHARACTERIZATION

3.1 Introduction 48

3.2 Materials and methods 50

3.2.1. Materials 50

3.2.2 N-terminal and partial internal sequence determination 50

3.2.3. Assay of inhibitory activity 51

3.2.4. Dissociation constant (Ki) Determination 52

3.2.5. Stability studies of PRTI 52

3.2.6. Proteolysis studies of PRTI 53

3.3 Results

3.3.1 N-terminal and partial internal sequencing 54

3.3.2 Inhibitory properties and Ki determination 54

3.3.3 Stability studies 58

3.3.4 Proteolysis study of PRTI 58

3.3.5 Storage stability of PRTI activity 62

3.4 Discussion 65

CHAPTER 4 69-100

BIOPHYSICAL CHARACTERIZATION

4.1. Introduction 69

4.2. Materials and Methods

4.2.1. Materials 72

4.2.2 Circular Dichroism spectroscopy studies 72

4.2.3 Fluorescence spectroscopy studies 73

4.2.4 PRTI inhibitory Activity assay 75

4.3 Results

4.3.1 CD spectroscopy studies 75

4.3.2 Fluorescence spectroscopy studies 76

4.4 Discussion 95

CONCLUSIONS 101-107

REFERENCES 108-131

LIST OF PUBLICATIONS

1. **Navneet S. Chaudhary**, Chandan Shee, Asimul Islam, Faizan Ahmad, Dinesh Yernool, Pravindra Kumar, Ashwani K. Sharma. Purification and characterization of a trypsin inhibitor from *Putranjiva roxburghii* seeds. ***Phytochemistry*** **2008**, 69: 2120-2126.

2. **Navneet S. Chaudhary**, Ashwani K. Sharma. Proteolysis and fluorescence monitoring stability studies of *Putranjiva roxburghii* trypsin inhibitor under different physiochemical condition. (Communicated).

3. **Navneet S. Chaudhary**, Ashwani K. Sharma. Purification and characterization of a plant defense protein from *Putranjiva roxburghii* seeds. (Manuscript in preparation)

INTRODUCTION

INTRODUCTION

Protein proteinase inhibitors have been found in many plant tissues especially in legume seeds and other storage organ, numerous animal tissue and fluids and in microorganism. Involvement of plant proteinase inhibitors in prevention of tumerogenesis may contribute to the nutritional utilization of valuable plant protein sources. The inhibitors are also used as valuable tools in medical research because of unique pharmacological properties that suggests clinical application. Plant proteinase inhibitors are widely distributed in plant seeds and are most studied class of inhibitors (Richardson, 1991; Mello et al., 2002). The molecular mass of these inhibitors can vary from 4 to 85 kDa, with majority in the range of 8 to 20 kDa (Hung et al., 2003). In higher plants, several gene families of these protease inhibitors have been characterized. The plant serine proteinase inhibitors, particularly trypsin inhibitors, have been extensively studied (Lawkowski and Kato, 1980; Barrett and Salvesen, 1986). They play an important role as plant defense agent against insects and pests attack (Sampaio et al., 1996; Shewry and Lucas, 1997; Walker et al., 1997; Franco et al., 2003) and are known to be involved in many biological functions, such as blood coagulation, platelet aggregation and anti-carcinogenesis (Kennedy, 1998; Oliva et al., 2000). Plant protease inhibitors have been described as endogenous regulators of proteolytic activity (Ryan, 1990; Kato, 2002) and as storage proteins (Xavier-Filho, 1992). The proteinase inhibitors play an important role in regulating many physiological processes by inhibiting proteinases, such as inflammation, coagulation, fibrinolysis, intracellular protein breakdown, cell cycle, transcription and apoptosis (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). They also act as anti-cancer

and anti-HIV agents. Their role as natural plant defense agents against pathogens and pests are well established.

These inhibitors have been grouped mainly into Kunitz, Bowman-Birk, Potato I and II, squash, cereal superfamily and thaumatin-like inhibitors (Richardson, 1991). Most serine proteinase inhibitors from seeds have been isolated and characterized from *Leguminosae*, *Cucurbitaceae*, *Solanaceae* and *Graminae* families (Garcia-Olmeda et al., 1987). There are not many reports of purification and characterization of these inhibitors from other plant families. Other families where an inhibitor has been purified and characterized include *Rutaceae* (Shee & Sharma, 2007; Shee et al., 2007a, b) and *Euphorbiaceae* (Sritanyarat et al. 2006) families.

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. Understanding the structure-function relationship of proteins greatly contribute to many practical applications in medicine and agriculture. Formation of specific complexes between antigen and antibody, hormone and receptor, or enzyme and inhibitor are classic examples of highly complementary and specific interactions vital to living organisms. These interactions are completely dependent on their structural and functional stability in different physicochemical conditions. The proteinase-inhibitor interaction is one of the most important targets to study the specificity of protein-protein interaction and molecular recognition.

The Kunitz type inhibitors of serine proteinases are divided into two subfamilies; the Kunitz bovine pancreatic trypsin inhibitor (BPTI), with molecular mass of about 6.5 kDa and three disulfide-bridges and the Kunitz soybean trypsin inhibitor (STI), with molecular mass of about 20 kDa containing two disulfide-bridges (Wlodawer et al., 1987). The crystallographic and circular dichroism studies have shown that Kunitz soybean trypsin inhibitor (STI) family

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

Putranjiva roxburghii belonging to *Euphorbiaceae* family is an ornamental tree of tropical India known as child life tree. Deseeded fruits are used against cough, cold and sprue. Rosaries of hard stones are used for protecting children from infections. The seed kernel on steam distillation yield 0.5% of a sharp-smelling essential oil of the mustard oil type. The oil contains isopropyl and 2-butyl isothiocyanates as the main constituents and 2-methyl-butyl isothiocyanate as a minor component. Roxburghonic keto acid and some flavonoids, terpenoids and triterpines has been purified and characterized from the leaf and trunk bark of this plant. The leaves gave amentoflavone and its derivatives, beta-amyrin and its palmite, polyphenols, putranjiva saponin A, B, C, and D and stigmasterol. The bark contains friedelin, friedelanol, friedelanone, friedelan-3, 7-dione (putranjivadione), 3-alpha-hydroxy friedelan- 7-one (roxburgholone), carboxylic acid, putric acid, putranjivic acid (Garg and Mitra, 1968, 1971a, b; Sengupta et al., 1967). To date, no protein has been characterized from this plant. This work describes the purification and characterization of a highly stable and potent trypsin inhibitor from the seeds of *Putranjiva roxburghii.*

In this work, a trypsin inhibitor has been purified from the seeds of *Putranjiva roxburghii*. The *Putranjiva roxburghii* trypsin inhibitor (PRTI) was purified to homogeneity in three steps by acid precipitation, CM-sepharose cation exchange, DEAE-sepharose anion

exchange chromatography and size-exclusion chromatography on HPLC. The purified protein showed single band on SDS-PAGE. The SDS-PAGE analysis under both reducing and non-reducing conditions showed that PRTI is a single polypeptide chain with a molecular mass of approximately 34 kDa. N-terminal amino acid sequence analysis was performed by Edman degradation and partial internal sequencing analysis was performed by LC MS/MS and MALDI TOF/TOF mass spectrometry studies. In separate experiments, one peptide was obtained from LC-MS/MS and three peptides from MALDI-TOF/TOF studies. The inhibitory activity of PRTI against trypsin and chymotrypsin were determined by measuring the hydrolytic activity toward BAPNA and BTEE respectively. Kinetic studies over a range of PRTI concentrations were performed to determine the inhibition constant (Ki) from Dixon plot using BAPNA as a substrate. Studies were performed by adding a range of inhibitor concentrations to a fixed amount of trypsin at two different substrate concentrations. In stability studies, effect of temperature, pH and DTT was examined on inhibitory activity of PRTI. Purified PRTI protein was incubated to examine the proteolytic and structural stability with five different proteinases, namely trypsin, chymotrypsin, papain, pepsin and proteinase K. Purified PRTI protein was stored at -20 °C for one year period and inhibitory activity against trypsin was checked after three, six and one year interval. Far-UV CD spectroscopy studies (240 -200 nm wavelength range) were carried out to analyze the secondary structure and conformational stability of PRTI at different temperatures from 20 to 100 °C. Structure-function relationship studies were also performed with the help of CD and fluorescence spectroscopy. Extrinsic (ANS) and intrinsic (tryptophan) fluorescence monitoring studies were performed in different physicochemical conditions to monitor the extent of changes in native structure of PRTI and relate them to the inhibitory activity.

CHAPTER 1

LITERATURE REVIEW

1.1. Introduction

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

Proteolysis is a key process in all living organisms and must be carefully controlled in order not to be hazardous to the organism itself. It is, therefore, not surprising that a large number of naturally occurring proteinaceous proteinase inhibitors have been found in animals, plants and microorganisms (Laskowski and Kato, 1980; Liener, 1979; Richardson, 1977;

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

1.2. Serine proteinases and inhibitors

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

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

1.2.1. Catalytic mechanism of serine proteinases

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

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

The bond joining the nitrogen and the carbon in the peptide bond is now broken. The covalent electrons creating this bond move to attack the hydrogen of the histidine, breaking the connection. The electrons that previously moved from the carbonyl oxygen double bond move

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

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

1.2.2. Mechanism of serine proteinase inhibition

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

Protein inhibitors of serine proteinases such as soybean trypsin inhibitor undergo the reaction, including formation of an acyl-enzyme, but have many interactions with the proteinase, so that the first product does not diffuse away and water has no room to attack the acyl-enzyme. A classical peptide inhibitor of serine proteinases that is hydrolyzed $\approx 10^7$ times more slowly than a good substrate is shown to form an acyl-enzyme intermediate rapidly.

Despite this quick first step, further reaction is slowed dramatically because of tight and oriented binding of the cleaved peptide; the leaving group amine is poised for nucleophilic attack on the acyl-enzyme and preventing acyl-enzyme hydrolysis and favoring the reverse reaction (Longstaff et al., 1990 and Shaw et al., 1995). The clogged gutter mechanism for proteinase inhibitor suggested that the combination of a hydrogen bond network, an acyl-enzyme, and the correct orientation of the religating amide can arrest the catalytic reaction of serine proteinases (Radisky and Koshland, 2002).

1.3. Plant proteinase inhibitors

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

1.3.1. The Bowman-Birk family

The Bowman-Birk inhibitor from soybeans (BBI) serves as the prototype for a family of inhibitors that are predominant in legume seeds (Birk, 1961; Birk et al., 1963; Birk, 1985). BBI has a molecular mass of about 8,000 Da with a high content of half cystines forming seven disulfide bridges. The inhibitor consists of two tandem homology regions on the same polypeptide chain, each with a reactive, inhibitory site (Odani and Ikenaka, 1973a). In aqueous solutions, the inhibitor undergoes self-association, which is concentration dependent. BBI inhibits human, bovine, porcine, dog and avian trypsin and chymotrypsin, at a 1:1 enzyme-inhibitor ratio (Birk, 1985). It inhibits carp trypsin and chymotrypsin with a 1:2 stoichiometry of inhibition, indicating the binding of two molecules of either trypsin or chymotrypsin to one molecule of inhibitor (Cohen et al., 1981a, b). BBI is also a potent inhibitor of trypsin and chymotrypsin from the digestive tracts of insects, such as *Tenebrio molitor* (Levinsky et al., 1977), *Locusta migratoria* (Sakal et al., 1988, 1989). Most of the members of the BBI family exhibit numerous isoinhibitor forms. Homologous inhibitors have been found in lima beans, garden beans, adzuki beans, mung beans, ground nuts, chickpeas, peas, cowpeas and also in plant sources other than legume seeds such as wheat germ, rice and barley (Odani et al., 1986; Lyons et al., 1987; Tashiro et al., 1987; Greagg et al., 1994). They are double-headed, binding simultaneously to two serine proteinases, such as trypsin and a-chymotrypsin (Bode and Huber, 1992).

1.3.2. The squash inhibitor (Cucurbit) family

This family was named following the discovery of *Momordica charantia* inhibitor 3 (MCI-3), a trypsin inhibitor from a *Cucurbitaceae* (Zeng et al., 1988). After that a series of

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

1.3.3. The "Cereal superfamily" family

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

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

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

mole of tetramer inhibits 4 moles of chymotrypsin, which suggest that each of the subunits possesses a chymotrypsin-binding site.

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

Members of the potato proteinase inhibitor II (PI-2) family have been shown to inhibit serine proteinases, such as trypsin, chymotrypsin, subtilisin, oryzin and elastase (Pearce et al., 1982 and Plunkett et al., 1982). Inhibitor II has a molecular mass of 20,000 Da and is composed of di mers of four distinctly different protomers, containing 16 cysteine residues per subunit, of which only 6 form intramolecular disulfide bridges. Each di mer 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 (Macedo et al., 2003).

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

Leguminous plants in the tropical rainforests are a rich source of proteinase inhibitors and this work illustrates isolation of a serine proteinase inhibitor from the seeds of *Archidendron ellipticum* (AeTI), inhabiting Great Nicobar Island, India. AeTI was purified to homogeneity by acetone and ammonium sulfate fractionation, and ion exchange, size exclusion and reverse phase chromatography (HPLC). SDS–PAGE of AeTI revealed that it is constituted by two polypeptide chains (α-chain, M_r 15,000 and β-chain, M_r 5000), the molecular mass being ~20 kDa. N-terminal sequence showed high homology with other serine proteinase inhibitors

belonging to the *Mimosoideae* subfamily. Both Native-PAGE as well as isoelectric focussing showed four isoinhibitors (p*I* values of 4.1, 4.55, 5.27 and 5.65). Inhibitory activity of AeTI remained unchanged over a wide range of temperatures (0–60°C) and pH (1–10). The protein inhibited trypsin in the stoichiometric ratio of 1:1, but lacked similar stoichiometry against chymotrypsin. Also, AeTI-trypsin complex was stable to SDS unlike the SDS unstable AeTI-chymotrypsin complex. AeTI, possess inhibition constants (K_i) of 2.46×10^{-10} and 0.5×10^{-10} M against trypsin and chymotrypsin activity, respectively (Bhattacharyya et al., 2006).

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

1.4. Storage properties

Many plant storage organs such as seeds and tubers contain from 1 to >10% of their soluble proteins as trypsin inhibitors (Mandal et al., 2002). Besides their storage function by providing nitrogen and sulphur source required during germination, trypsin inhibitors have been ascribed other functional roles, such as regulating endogenous plant proteinases to prevent precocious germination, inhibiting trypsin during passage through as animal's gut, thus helping in seed dispersal, and protecting plants against pests and diseases (Laskowski and Kato, 1980; Ryan, 1990; Hou et al., 1999; Admovskaya et al., 2000). The seed storage proteins can be distinguished from other proteins by some of their characteristics, e.g. (1) these accumulate in

high amounts in seed during mid-maturation stage of seed development and are used up during germination. (2) These are synthesized only in the seed (in cotyledon or in endosperm) and not in other tissues (Mandal and Mandal, 2000). (3) In general, a protein can be considered as a storage protein when this protein is present in amounts of 5% and more of the total protein content (Derbyshire et al., 1976). The amount of protein present in seeds varies from ~10% (in cereals) to ~40% (in legumes), forming a major source of dietary protein (Ramakrishna and Ramakrishna, 2005).

Although storage proteins and proteinase inhibitors are placed in two separate groups as classified by Fukusima but there are many report of having overlapping functions (Mosolov et al., 2001; Birk, 2003; Shewry, 2003; Haq et al., 2004). The proposed role for proteinase inhibitors as storage proteins was suggested first by Pusztai (1972). Papastoitsis and Wilson (1991) showed that during seed germination and early seedling growth there is a degradation of STI in soybean cotyledons. McGrain et al., (1992) found that during soybean seed maturation there is a slow phase of STI accumulation, followed by a rapid phase when the seeds have reached half their maximum fresh weight.

1.5. Solubility properties

To be biologically active, proteins must adopt specific folded three-dimensional, tertiary structures in soluble condition. Protein solubility is a complex function of the physiochemical nature of the proteins, pH, temperature, organic solvents, detergents and the concentration of the salts used. Protein solubility is highly dependent upon the ionic strength (Arakawa & Timasheff, 1985; Ries-Kautt & Ducruix, 1997). In general, at low concentrations, the protein solubility exhibits a 'salting-in' behavior, with the protein solubility increasing with increasing ionic

strength. At high ionic strengths, the protein solubility decreases with increased ionic strength in a 'salting-out' behavior. The denaturation of proteins leading to aggregation occurs due to the environmental changes such as temperature, pH, and addition of denaturants like urea and guanidine hydrochloride (Tanford, 1968). At lower pH, carboxyl groups are protonated and hydrophobic interactions dominate; this leads to precipitation of the polymer (Roy and Gupta, 2003). Protein stability can be measured directly using calorimetric methods, and indirectly by different spectroscopic methods. Upon unfolding, the polypeptide chain becomes less compact, more solvated and much more flexible and resulting more susceptible towards proteolytic degradation (Tanford, 1968). Unfolding of a protein can be easily performed by using extreme pHs, denaturants and by increase of temperature. The spectroscopic and biochemical studies have shown that the Kunitz type inhibitors are highly stable over a broad range of temperature and pH and are remarkably resistant to proteolytic degradation. (Sweet et al., 1974; Onesti et al., 1991; Roychaudhuri et al., 2003, 2004; Azarkan et al., 2006).

1.6. Circular dichroism Spectroscopy

Circular dichroism (CD) spectroscopy is a well-established method to understand the structure function relationship of proteins. The sensitivity of far-UV protein CD spectra to protein secondary structure is used in one of the most successful applications of CD to determine the secondary structure composition of a protein (Yang et al., 1986; Greenfield, 1996; Venyaminov and Yang, 1996; Sreerama et al, 2000; Sreerama and Woody, 2000, 2004). CD spectroscopic method depends upon the differential absorption of left- and right-circularly polarized light by optically active molecules. Alpha-helix, beta-sheet, and random coil structures each give rise to a characteristic shape and magnitude of CD spectrum. The far UV

CD spectra of alpha-helical proteins are characterized by three peaks, a negative peak at ~222 nm, at ~208 nm and a stronger positive peak at ~192 nm. However, CD at 222 nm displays the strong concentration dependence characteristic of dissociative unfolding and usually the negative value at 222 nm is considered to measure the helix content (Holtzer et al., 2000). In general, spectra arising from β-sheets are characterized by a small negative peak near 217 nm and a positive peak near 195 nm that has approximately half the intensity of the α-helix peak in this region. β-Sheets give rise to considerably less intense signals than helices and show far more variation in spectral characteristics; the latter is partly attributable to the fact that β-sheets are much more structurally diverse than α -helices, with strands which may run parallel or antiparallel to each other, and with sheets displaying differing degrees of twisting. The weak β-sheet signal makes it more susceptible to distortions caused by the CD signals of aromatic side chains, which can produce peaks or dips between 225 nm and 235 nm. CD spectra are usually presented in molar ellipticity [θ] units (degrees-cm^2 dmol^{-1}). For proteins, the mean residue ellipticity (*MRE*) can be calculated as follows: $MRE = (MRW)\ \theta/10cl.$ where *MRW* is the mean residue (amino acid) weight, *c* is the concentration of protein and *l* is the pathlength in cm. The α-helical content for each protein was calculated using the standard equation for [θ] at 222 nm (28): $[\theta]_{222} = -30{,}300f_H - 2{,}340$, where f_H is the fraction of α -helical content (f_H x 100, expressed in %). Spectra are presented as the mean residue ellipticity. Thermal stability is assessed using CD by following changes in the spectrum with increasing temperature.

Based on circular dichroism spectra, soybean Kunitz trypsin inhibitor (SKTI) has been classified in the β-II or the 'disordered' class of all antiparallel β-sheet proteins. Thermal transition studies done with SKTI at neutral and alkaline pH are reversible (Roychaudhuri et al., 2003). CD spectra of SKTI during denaturation in the far-UV region show decreased ellipticity,

indicating loss of secondary structure (Roychaudhuri, 2004). Far-UV CD study of *Schizolobium parahyba* chymotrypsin inhibitor (SPCI) revealed that between 20 and 70°C the CD spectra is typical of β-structure and unordered structure protein. At temperature above 70°C the CD spectra decrease the minimum at 200 nm with a partial loss of signal. Analysis of temperature progress curve at pH 7.0 revealed that the native conformation of SPCI is thermally stable at temperatures below 70°C with partial unfolding of secondary elements (Teles et al., 2005). CD spectroscopic analysis of the Kunitz-type pigeonpea inhibitor has revealed the absence of any α-helix and the CD spectrum is representative of the β-II class of polypeptides and the proteinase/amylase inhibitor isolated from mung bean is a helical protein (Haq and Khan, 2003; Haq et al. 2005).

1.7. Fluorescence Spectroscopy

Fluorescence spectroscopy is a very powerful technique to monitor conformational changes in proteins. The fluorophores can be either intrinsic (tryptophan) or extrinsic (ANS: 8-anilino-1-naphthalene sulfonate) probes. The fluorescence property of tryptophan has long been utilized to understand the folding/unfolding, substrate binding and conformational heterogeneity in different physicochemical conditions. The tryptophan fluorescence usually varies in somewhat unpredictable manner in various proteins. Some proteins exhibit large increases in their intensities and lifetimes and others, decreases, on unfolding of the chain. Proteins with heme prosthetic groups typically exhibit an increase in intensity on unfolding due to loss of the heme group. The average energy of the emission of the tryptophan residues usually shifts to the red on unfolding because the solvent exposure and thus solvent relaxation is augmented in the unfolded state (Royer, 1995). The ANS anion (8-anilino-1-naphthalene sulfonate) is a much

utilized 'hydrophobic probe' for proteins (Slavik, 1982). ANS in water is nonfluorescent but it fluoresces upon binding to a nonpolar (hydrophobic) site of proteins. In fact, ANS binding to proteins depends on protein cationic charge and solution pH, and occurs largely through the ANS sulfonate group (Matulis and Lovrien, 1998). The dependence of ANS binding on electrostatic interaction between the sulfonate group and protein cationic groups indicate that ANS binding does not require preexisting hydrophobic site on or in protein molecules to start the binding reaction. With increasing conformational unfolding state in different physicochemical conditions, the ANS binding fluorescence intensity increases. Thus, the increase in fluorescence intensity is a characteristic of relaxed or molten state where more access of ANS to hydrophobic core of a protein is allowed (Semisotnov et al., 1991).

1.8. X-ray crystallography

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

The crystallographic studies have shown that Kunitz soybean trypsin inhibitor (STI) family members are predominantly β-sheet proteins with little or no α-helical structure. It consists of 12 crisscrossed antiparallel β-strands largely stabilized by hydrophobic side chains, random coil and turn structure. Its three-dimensional structure in complex with porcine trypsin

was also determined (Sweet et al., 1974). The predominant conformation found in STI has been demonstrated to be approximate beta-sheet structures, with a small amount of regular sheet (Sweet et al., 1974; Ventura, 1989). De-Meester et al. (1998) studied the structure of STI in its free form, determined by molecular replacement to 2.5 Å, using the coordinates of the homologous *Erythrina* trypsin inhibitor as a search model.

Shieh et al. (1990) reported the crystallization of a chymotrypsin inhibitor from *Erythrina caffra* seeds into crystals that belong to the rhombohedral space group. Onesti et al. (1991) studied the structure and properties of a Kunitz-type trypsin inhibitor from *Erythrina caffra* seeds (ETI). This inhibitor consists of 172 amino acid residues with two disulfide bridges. The three-dimensional structure of ETI consists of 12 antiparallel beta-strands joined by long loops. Six of the strands forma short antiparallel beta-barrel that is closed at one end by a "lid" consisting of the other six strands coupled in pairs. The scissile bond (Arg63-Ser64) of ETI is located on an external loop that protrudes from the surface of the molecule. The overall structure of ETI is similar to the partial structure of STI, but the scissile bond loop is displaced by about 4 Å.

1.9. Applications of plant proteinase inhibitors

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

1.9.1. Role in plant defense

Plants are inevitably subjected to a variety of pest and pathogen infestations, which substantially contribute to the overall loss in crop yield. Agricultural industries employ several chemical pesticides to combat this problem. However, they have serious drawbacks owing to their lack of specificity, development of resistance upon extended use and the ecological hazards inherent with residual toxicity. Thus, biodegradable biocontrol agents are advantageous alternatives as they are devoid of contaminating residues and have reduced incidence for development of resistance. However, proteinase inhibitors regulate the action of proteinases and play a significant role in the protection of plants from pest and pathogen invasion by virtue of their antinutritional interaction. Serine proteinase inhibitors from plants have been shown to have potential usefulness as defense tools to protect the plants from invading pests (Green and Ryan, 1972; Ryan, 1990). A few plant proteinase inhibitors have been shown to possess fungicidal activity (Lorito et al., 1994; Joshi et al., 1999).The presence of a "defense response" of the plant to the attack of insects was first reported by Green and Ryan (1972). Studies on the effects of dietary plant proteinase inhibitors on the development of insects were first reported by Lipke et al. (1954) and the inhibition of insects own digestive proteinases by the ingested plant proteinase inhibitors was also observed by Broadway and Duffey (1986). Most of the plant proteinase inhibitors relevant to plant defense are members of the serine proteinase inhibitor families. Johnston et al. (1993) reported that Kunitz-type inhibitors (KTI) and Bowman-Birk inhibitors (BBI) from soybean in diets of newly molted third-instar larvae of the maize earworm *Helicoverpa armigera* reduced total larval biomass, mean larval weight, and trypsin-like activity in their gut contents. The effect on larval growth was greater with dietary KTI than with BBI. Burgess et al. (1994) showed that the efficiency of several proteinase inhibitors (KTI, potato II

proteinase inhibitors, cowpea trypsin inhibitor, wheat germ inhibitor, and bovine pancreatic trypsin inhibitor) in reducing growth of the black field cricket *Teleogryllus commodus* was strongly influenced by the concentration of the inhibitors. The possible involvement of plant proteinase inhibitors as a defense mechanism against insects was supported by the discovery that insect attacks on tomato or potato plants induced a rapid accumulation of proteinase inhibitors in leaves (Green and Ryan, 1972). The signal by which tissues distant from the injury are induced to respond by synthesizing proteinase inhibitors was named "Proteinase Inhibitor Inducing Factor" (PIIF). Plant proteinase inhibitors can be induced not only by pathogen attack, but also by different biological and physiological conditions. Downing et al. (1992) isolated a drought-induced 22,000 Da protein from *Brassica napus* that is related to the Kunitz family of proteinase inhibitors.

Extremely low levels of several serine proteinase inhibitors, including KTI and BBI from soybeans, cowpea and squash inhibitors, enhanced the insecticidal activity of the insect control proteins from several varieties of *Bacillus thuringiensis* by 2-20 fold against their target insects, tobacco budworm and other lepidopterans, Colorado potato beetle, and mosquito (Macintosh et al., 1990).

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

Gatehouse et al. (1999) compared the susceptibility of the tomato moth (*Lacanobia oleracea*) to KTI expressed in transgenic potato plants, to KTI incorporated in potato leaf-based artificial diet. The presence of KTI in the artificial diet at 2% of total protein decreased larval

survival and growth and retarded development. However, when KTI was expressed in transgenic potato plants as ~0.5% of total protein, only marginal effects on *L. oleracea* larvae were observed, which decreased with time.

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

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

1.9.2. Proteinase inhibitors as storage proteins

The proposed role for proteinase inhibitors as storage proteins was suggested first by Pusztai (1972), who showed that during the germination of kidney bean the period of maximum proteinase inhibitor content coincided with the period of maximum proteolysis. In general, a protein can be considered as a storage protein when this protein is present in amounts of 5 % and more of the total protein content (Derbyshire et al., 1976). However, since PIs in tubers and plant seeds belongs to different classes and families and that their concentration varies during the maturation, it would not fit the definition of a storage protein. Pusztai (1972) proposed that PIs may serve as a source of important sulphur-containing amino acids for the germination of the seed. PIs are in general considered to be proteins rich in cysteine residues (Jongsma, 1995). However, it can be observed that this postulate is true mainly for PIs with a small molecular mass (3-13 kDa) which exhibit a proportion of cysteine residues >20%. For PIs with a higher

molecular mass (>13 kDa), only Thaumatin and PI-2 families (20 kDa) contain a high number of cysteine residues (lower than 10%). Knowing that most of the PIs in plants belong to the Kunitz-type of inhibitors, in which the cysteine residues consist of approximately 2%, it can be concluded that the proportion of cysteine residues is rather small. The potency of PIs as retarding insect proteinases is clear, but the assessment of PIs as storage proteins is complicated by the fact that maturation is accompanied by the expression of different PIs (McManus et al., 1999).

1.9.3. Anticarcinogenic agent

PIs are well established as a class of cancer chemopreventive agents (Kennedy, 1998). While PIs from different families have been shown to prevent the carcinogenic process, the most potent of the known anticarcinogenic PIs are those with the ability to inhibit chymotrypsin-like proteinases. The Bowman-Birk inhibitor (BBI) from soybean is until now the PI that has been most studied as an anticarcinogenic agent. However, also potato proteinase inhibitors have been shown to be potent anticarcinogenic agents, especially PI-1 and PI-2 (Frenkel et al., 1987; Billings et al., 1987; 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, 1990; 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.

1.10 General purification methods for trypsin inhibitors

The analysis of proteins, whether on a small or large scale, requires methods for the separation of protein mixtures into their individual components. Protein separation methods can be placed on a sliding scale from fully selective to fully nonselective. Selective methods aim to isolate individual proteins from a mixture usually by exploiting very specific properties such as their molecular weight, charge, binding specificity or biochemical function. In protein purification, it is important to adopt procedures that do not cause denaturation of proteins, especially the protein of interest. The successful separation of a protein from a complex mixture usually involves exploiting some of its inherent properties; be it size, charge, solubility or the presence of specific binding sites. The initial stage of purification requires the removal of cell debris and contaminating proteins co-localizing during the extraction phase. Peptidase enzymes are present in low concentrations and can easily be inactivated by endogenous inhibitors present in the cell extract. Hence the initial fractionation of the cell extract to obtain a protein rich fraction is an important prerequisite towards purification of a protein inhibitor. Table 1.1

provides a brief overview of the different types of pre-fractionation methodologies used in the purification of protein. A protein may be purified by a single step (for example, affinity chromatography), or by a combination of several steps (for example, acid precipitation, salt fractionation, ion exchange chromatography, gel filtration chromatography etc.). In general, anion-exchange chromatography is employed for the purification of an acidic protein. Similarly, for the purification of a basic protein, cation-exchange chromatography is the better choice. Reverse-phase chromatography is suitable for a family of active proteins of similar charge.

1.10.1 Precipitation by alteration of the pH

One of the easiest methods of precipitating a protein and achieving a degree of purification is by adjusting the pH of the solution to close or equal to the pI of the protein (termed isoelectric precipitation). The surface of protein molecules is covered by both negatively and positively charged groups. Above the pI the surface is predominantly negatively charged, and therefore like-charged molecules will be repelled from one another; conversely below the pI the overall charge will be positive and again like-charged molecules will repel one another. However, at the pI of the protein the negative and positive charges on the surface of a molecule cancel one another out, electrostatic repulsion between individual molecules no longer occurs, and electrostatic attraction between molecules may occur, resulting in formation of a precipitate. Isoelectric precipitation is often used to precipitate unwanted proteins, rather than to precipitate the protein of interest, since denaturation and inactivation can occur on precipitation.

Table 1.1 - Protein fractionation techniques employed in protein purification.

Fractionation Step	Method	Application
Precipitation	This procedure involves changing the solubility of the protein either by changing the pH or the ionic strength. Common precipitation agents include high salt concentrations such as ammonium sulphate. Alternatively, decreasing the dielectric constant using water miscible solvents also cause the precipitation of large, charged molecules or sometimes by the acid precipitation.	This technique results in selective denaturation and can result either in the inactivation or reduction of protein activity. However, numerous enzymes on resuspension and renaturation demonstrate retained activity.
Centrifugation	Large organelles can be sedimented by high centrifugal speeds (30,000 g), generated by ultracentrifugation. More often it is used to separate insoluble material generated during the homogenization of the sample.	Depends on a variety of factors such as size and shape of the protein and viscosity of the sample. This method is not usually used to separate samples containing multiple proteins in a single mixture.
Dialysis or Ultra filtration	Protein separation method involving size based fractionation through a semi-permeable membrane, which selectively retains large globular proteins of a specific molecular range. It is used more often in the removal of salts, organic solvents and inhibitors of low molecular weight. Ultrafiltration involves a similar process, under pressure. It is useful in reducing the volume of the sample and concentrating the proteins.	Cannot separate between complex mixtures of enzyme. Dialysis membranes usually can get clogged due to precipitating proteins.
Lyophilisation	Used to effectively reduce the solvent component of the protein solution. Used when the protein sample is relatively pure and is used for storage purposes.	Re-solubilization of proteins can sometimes be an issue.

1.10.2 Ion Exchange Chromatography

Ion exchange chromatography is widely used in protein purification due to its dynamic range and high resolution capabilities. Protein separation by this method is based on differences in electric charge (Roe, 2001). Since proteins fundamentally differ in their amino acid sequence, they vary in their net charge at any given pH other than their isoelectric point. The solid phase in ion-exchange chromatography consists of modified derivatives of support materials such as cellulose, sepharose, sephadex, etc. When a protein sample consisting of different ionic species passes through the column, the proteins are distributed between the mobile phase and the solid phase. The strength of their interaction is dependent upon the degree of counter ion exchange. These interactions are reversible (Glod, 1997) by progressively increasing the ionic strength of the mobile phase, so that the counter ions compete with the solutes for the interaction sites on the solid phase (Headon and Walsh, 1994). Ion exchange chromatography wherein the stationary phase carries positive charges is known as "anion-exchange chromatography" whereas a negatively charged solid phase is known as "cation-exchange chromatography". These exchanges allow for the separation of a wide range of differentially charged proteins. Protein and enzymes are usually applied to an ion exchange column in a solution of low ionic strength and pH. The elution of the protein is usually performed by one of two mechanisms, (i) Changing the pH of the mobile phase, resulting in the alteration of the charges of the ionic species bound; i.e. lower pH for anionic exchange and increasing pH for cationic exchange or (ii) Increasing the ionic strength, thereby weakening the electrostatic interactions between the proteins and the adsorbent material. Commonly used ion exchange solid phases and their optimal pH range for anion-exchanger are Quarternary ammonium group (pH range 2-12), Diethylaminoethyl (DEAE) group (pH range 2-9), Quarternary aminoethyl group (pH range 2-

12) and for cation-exchanger are Sulphopropyl groups (pH range 2-12), Methyl sulphonate groups (pH range 2-12), Carboxymethyl groups (pH range 6-11) (Karlsson et al, 1998).

1.10.3 Size-Exclusion Chromatography

Size-exclusion chromatography is a convenient and highly reproducible method of separating simple protein mixtures whose components differ sufficiently in their molecular weight (Andrews 1965). Smaller proteins require at least 10% difference in their molecular weights for efficient resolution by this method whereas larger molecules such as enzymes require at least two fold differences to resolve efficiently (Porath 1997; Paulsen et al. 2002). This chromatography uses an aqueous mobile phase and hydrophilic matrix to separate the proteins in a mixture. The solid phase consists of a matrix made up beads, with pores of defined size, packed in a column through which the mobile phase flows. In principle, large molecular weight proteins that cannot permeate the beads flow through the column faster and elute earlier while smaller proteins within the pore size range are retained within the beads and hence take longer to flow, eluting later. The primary factors affecting the resolution of protein separation in size-exclusion chromatography are column volume, particle size, pore size distribution, flow rate of the mobile phase, protein conformation, temperature and solvent viscosity. The resolution of separation is directly proportional to the size of the column. However, larger columns result in increased separation/analysis time since effective separation of the proteins requires slow flow rates (Scopes 1993). These shortcomings have been circumvented with the development of more effective particulate solid phase materials that can withstand the high back pressures associated with faster flow rates. These include cross-linked dextrans such as Sephadex, polystyrene packing materials and polyacrylamide based matrices (Bio-gel), etc.

Size-exclusion chromatography is well suited for enzyme purification due to its gentle binding and elution techniques and its ability to retain biological activity. However the limitations on its efficiency make it more suitable for purification stages where the mixture is not highly complex.

1.10.4 Estimation of protein and analysis of protein purity

Proteins show maximal ultraviolet (UV) absorption at about 280 nm arising from the contribution made by aromatic residues (of tryptophan, tyrosine) and to some extent from cystine groups. Absorption at 280 nm can, therefore, be used to provide an approximate protein assay directly without the addition of any reagents (Stoscheck, 1990). The quantitation of protein by this method can only be applied to pure protein. Nonetheless, absorbance is widely used for monitoring purification progress and for generating a protein elution profile during column chromatography. The elution profile provides a guide for pooling fractions containing proteins. For A280, the amino acids containing aromatic rings, such as tryptophan, tyrosine, phenylamine, and histidine, are involved. The method is simple, rapid, and non-destructive. The relationship between protein concentration and absorbance is linear. The Bradford protein assay (Bradford, 1976) has become the preferred method for many investigators, because it is simple and rapid compared to the Lowry method. Moreover, this assay is comparatively free from interference by common reagents except detergents. The assay involves the use of Coomassie Brilliant Blue G-250, which reacts primarily to basic (especially arginine) and aromatic amino acids (Congdon et al, 1993). The Bradford protein assay is performed in two formats: standard assay (0.1-1.0 mg/ml) and a microassay (5-40 μg/ml). Denaturing PAGE in the presence of sodium dodecyl sulfate (better known as SDS-PAGE) is a low-cost, reproducible, and rapid method for analyzing protein purity and for estimating protein molecular weight (Laemmli,

1970). SDS-PAGE is also employed for the following: (a) monitoring protein purification; (b) verification of protein concentration; (c) detection of proteolysis; (d) detection of protein modification; and (e) identification of immunoprecipitated proteins. Protein subunits can be determined by using the β-mercaptoethanol with the SDS-PAGE.

CHAPTER 2

PURIFICATION OF PROTEIN

2.1. Introduction

Plant seeds are the rich source of the protein and these protein play an important role in protecting the seeds from microbes and insects, providing the nutrition during early seedling development stages and also in maintain the seed viability (Millerd, 1975). Plant seed proteins also play an important role in human and animal nutrition by providing the major share of dietary protein. These proteins may be classified as storage, structural and biologically active proteins (Fukusima, 1991). The major biologically active proteins include lectins, enzymes and enzyme inhibitors (e.g. trypsin inhibitor)

The analysis of proteins, whether on a small or large scale, requires methods for the separation of protein mixtures into their individual components. Protein separation methods can be placed on a sliding scale from fully selective to fully nonselective. Selective methods aim to isolate individual proteins from a mixture usually by exploiting very specific properties such as their molecular weight, charge, binding specificity or biochemical function. In protein purification, it is important to adopt procedures that do not cause denaturation of proteins, especially the protein of interest. The successful separation of a protein from a complex mixture usually involves exploiting some of its inherent properties; be it size, charge, solubility or the presence of specific binding sites. The initial stage of purification requires the removal of cell debris and contaminating proteins co-localizing during the extraction phase

One of the easiest methods of precipitating a protein and achieving a degree of purification is by adjusting the pH of the solution to close or equal to the pI of the protein (termed isoelectric precipitation). The surface of protein molecules is covered by both negatively and positively charged groups. Above the pI the surface is predominantly negatively charged, and therefore like-charged molecules will be repelled from one another; conversely

below the pI the overall charge will be positive and again like-charged molecules will repel one another. However, at the pI of the protein the negative and positive charges on the surface of a molecule cancel one another out, electrostatic repulsion between individual molecules no longer occurs, and electrostatic attraction between molecules may occur, resulting in formation of a precipitate. Isoelectric precipitation is often used to precipitate unwanted proteins, rather than to precipitate the protein of interest, since denaturation and inactivation can occur on precipitation The Bradford protein assay is performed in two formats: standard assay (0.1-1.0 mg/ml) and a microassay (5-40 µg/ml). Denaturing PAGE in the presence of sodium dodecyl sulfate (better known as SDS-PAGE) is a low-cost, reproducible, and rapid method for analyzing protein purity and for estimating protein molecular weight (Laemmli, 1970). SDS-PAGE is also employed for the following: (a) monitoring protein purification; (b) verification of protein concentration; (c) detection of proteolysis; (d) detection of protein modification; and (e) identification of immunoprecipitated proteins. Protein subunits can be determined by using the β-mercaptoethanol with the SDS-PAGE.

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

2.2. Materials and Methods

2.2.1. Materials

Seeds of *Putranjiva roxburghii* were collected from the premises of Indian Institute of Technology Roorkee. Trypsin, N-benzoyl-L-arginine p-nitroanilide (BAPNA), N-benzoyl-L-tyrosine ethyl ester (BTEE), DEAE-Sepharose and CM-Sepharose and BSA were purchased from Sigma-Aldrich Pvt. Ltd. Centricon and Centriprep were purchased from Amicon (Beverly, MA). Reagents for SDS-PAGE were purchased from Sigma-Aldrich Pvt. Ltd. Protein molecular weight standards were obtained from Bangalore GeNei^{TM} India. All other reagents were of analytical or HPLC grade.

2.2.2. Purification of Putranjiva roxburghii trypsin inhibitor (PRTI)

2.2.2.1 Preparation of supernatant with inhibitory activity

Putranjiva roxburghii is a tree of *Euphoebiaceae* family and the mature seeds were used for the purification of *Putranjiva roxburghii* Trypsin Inhibitor (PRTI). Mature seeds (25 g), after removing the hard seed coat, were grounded and soaked overnight at room temperature in 200 ml of distilled water. After overnight (12 h) soaking the extract was filtered with muslin cloth and filtered extract was centrifuged at 12000 rpm for 45 minutes to obtain a clear supernatant. The supernatant was checked for the trypsin inhibitory activity with the method used by Shibata et al, (1986). Then the supernatant was kept at 4 °C for two hours and again centrifuged at 12,000 rpm for 45 minute and obtained clear supernatant with trypsin inhibitory activity was kept at 4 °C for further purification procedures.

2.2.2.2 Acid precipitation method

Acid precipitation method was applied for further purification of protein. The pH of the supernatant was adjusted to 4.5 with 5 N HCl. After 12 h incubation at 4 °C, precipitated proteins were removed by centrifugation at 12000 rpm for 30 min and discarded. The supernatant was dialyzed against distilled water at 4 °C, freeze-dried, and dissolved in 0.05 M phosphate buffer, pH 6.0. The supernatant was checked for the trypsin inhibitory activity.

2.2.2.3 Cation-exchange chromatography

The supernatant with trypsin inhibitory activity which was obtained after acid precipitation was then applied to a CM-Sepharose column (1.5 × 20 cm Econo-column, Bio-Rad) pre-equilibrated with 0.05 M phosphate buffer pH 6.0. The flow through having trypsin inhibitory activity was collected. It was dialyzed against distilled water at 4 °C, freeze-dried, and dissolved in 0.05 M Tris–HCl buffer pH 8.0.

2.2.2.4 Anion-exchange chromatography

The CM flow through which was dissolved in 0.05 M Tris-HCl buffer pH 8.0, was then loaded on to a DEAE-Sepharose column, (1.5 × 20 cm Econo-column, Bio-Rad) pre-equilibrated with 50 mM Tris-HCl buffer, pH 8.0. The column was washed extensively with the equilibriation buffer to remove unbound molecules and bound proteins were eluted with a NaCl step gradient from 0 to 0.5 M (0, 0.05, 0.1, 0.15, 0.2, 0.3, 0.4 and 0.5 M NaCl) at room temperature in the same buffer. Fractions with trypsin inhibitory activity at 0.1 M NaCl were pooled and concentrated by using Centriprep of 10 kDa cutoff (Amicon, Beverly, MA).

2.2.2.5 HPLC size-exclusion chromatography

The protein was further purified by HPLC using a size exclusion chromatography column (7.5 × 300mm, Waters) with the 50 mM Tris-HCl buffer at pH 8.0. The 100 mM pooled and concentrated fraction after DEAE anion-exchange chromatography was used as the sample for the size-exclusions column chromatography. The elution was monitored at 280 nm wavelength at the flow rate of 0.75 ml/min. The homogeneity of the purified protein was determined by 15% SDS-PAGE analysis.

2.2.3. Protein estimation

Protein concentration in crude extract and fractionated protein samples were estimated by standard dye-binding method using bovine serum albumin (Sigma) as standard (Bradford, 1976). For crude extract, mature seeds (25 g) were crushed with mortal-pestle and soaked overnight at 4 °C in 100 ml of 50 mM Tris-HCl buffer, pH 8.0. The homogenate was cleared by centrifugation at 12,000 rpm for 45 min and the supernatant was used to determine the soluble protein concentration by the method of Bradford, using BSA as standard (Brabford, 1976). Absorbance at 280 nm was also used to determine the protein content of different eluted fractions.

2.2.4. SDS-PAGE analysis

Sodium dodecyl sulfate-polyacrylamide gel (15%) electrophoresis under both reducing (SDS-PAGE) and non reducing conditions was done as described by Laemmli (1970). Relative molecular mass was determined by performing SDS-PAGE of protein with molecular weight standards under reducing condition. The molecular weight standards used were myosin [rabbit

muscles] (205 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa), lysozyme (14.3 kDa), aprotinin (6.5 kDa) and insulin [α and β chain] (3.0 kDa). The proteins were detected by staining the gel with 0.1% Coomassie brilliant blue R-250.

2.2.5 Trypsin inhibitory assay

2.2.5.1 Using BAPNA as a substrate

The inhibitory activity on bovine pancreatic trypsin was determined by measuring the hydrolytic activity towards the substrate N-benzoyl-L-arginine p-nitroanilide (BAPNA) (Shibata et al, 1986). Trypsin was dissolved in 50 mM Tris-HCl buffer, pH 8.0 containing 20 mM $CaCl_2$ and 0.001 N HCl. The 100 µl of the bovine pancreatic trypsin solution (final conc. in 1.5 ml reaction mixture was 5.6×10^{-7} M) was incubated with 200 µl of the inhibitor solution in 50 mM Tris-HCl buffer pH 8.0 at 30 °C for 15 min. After 15 min incubation, 1.0 ml of 0.5 mM BAPNA containing 2.5% DMSO was added. After incubation of another 10 min at 30 °C, the reactions were stopped by adding 200 µl of 10% acetic acid. The changes in absorbance at 410 nm were recorded at 30 °C against a blank solution containing 1.5 ml of the substrate solution in same buffer with a Varian Cary 100 spectrophotometer. The amount of substrate (BAPNA) hydrolysis by the enzyme was calculated using the molar extinction coefficient of 8800 M^{-1} cm^{-1} at 410 nm.

2.2.5.2 Using BAEE as substrate

The inhibitory activities on bovine pancreatic trypsin were determined by measuring the hydrolytic activity towards the substrate N-benzoyl-L-arginine ethyl ester (BAEE) (Shibata et al, 1986). The inhibitor was incubated at 30 °C for 15 min with 4.0×10^{-9} M in 2.5 ml of 50 mM Tris-HCl buffer, pH8.0, containing 20 mM $CaCl_2$. Then 2 ml of this solution was pipette into a cuvette and 0.1 ml of 10 mM BAEE solution in the same buffer was added. The change in absorbance at 253 nm was recorded at 30 °C against a reference solution containing 2 ml of the buffer and 0.1 ml of the substrate solution with a Varian Carry 100 spectrophotometer. The amount of substrate hydrolysis by the enzyme was calculated using the molar extinction coefficient of 808 M^{-1} cm^{-1} at 253 nm.

2.3. Results

2.3.1. Purification of PRTI

Putranjiva roxburghii trypsin inhibitor (PRTI) was purified to homogeneity in three steps by acid precipitation, CM-sepharose cation exchange and DEAE-sepharose anion exchange chromatography. In acid precipitation step, low molecular mass proteins were precipitated along with some other proteins (Lane L2 in Fig 2.1). The trypsin inhibitory activity was checked and found that inhibitory activity retained in supernatant. In second step on CM-Sepharose column (cation-exchanger), all the low molecular mass proteins were bound to the column while trypsin inhibitory activity was found in flow through (Lane L3 of Fig 2.1). These results clearly suggest that PRTI does not bind to cation-exchanger at pH 6.0 and inhibitor protein comes out in CM flow through. Some other protein of the supernatant bind to the CM

column and did not come out with the flow through, in this way it was possible to get rid of unwanted proteins, CM flow through was further used in next purification step. This resulted in complete removal of major low molecular mass protein.

In the next step, protein with trypsin inhibitory activity was bound to a DEAE-Sepharose column (anion-exchanger). After washing the column extensively, bound proteins were eluted with step gradient of NaCl. The fractions with trypsin inhibitory activity were eluted at 50 and 100 mM NaCl. The purity of the protein in above fractions was analyzed by SDS-PAGE. The fraction eluted at 100 mM NaCl showed single band on SDS-PAGE (Lane L5 of Fig. 2.1).

The protein was further subjected to size exclusion chromatography column on HPLC where it showed a single peak with a retention time of 8.2 min in 50 mM Tris-HCl buffer, pH 8.0 (Figure 2.2). The trypsin inhibitory activity of the PRTI protein was checked after every purification step and observed that inhibitor protein almost retained its activity and there was no significant loss of inhibitory activity found.

2.3.2 Protein estimation and SDS-PAGE analysis

PRTI was estimated by both dye-binding method and absorbance at 280 nm in the 100 mM DEAE pooled and concentrated fractions and was found up to 10 mg/ml. SDS-PAGE analysis on the 15% gel with protein molecular mass standards revealed that PRTI has the approximate size of 34 kDa. The SDS-PAGE analysis under both reducing (Fig. 2.2, inset) and non-reducing (data not shown) conditions showed that PRTI is a single polypeptide chain.

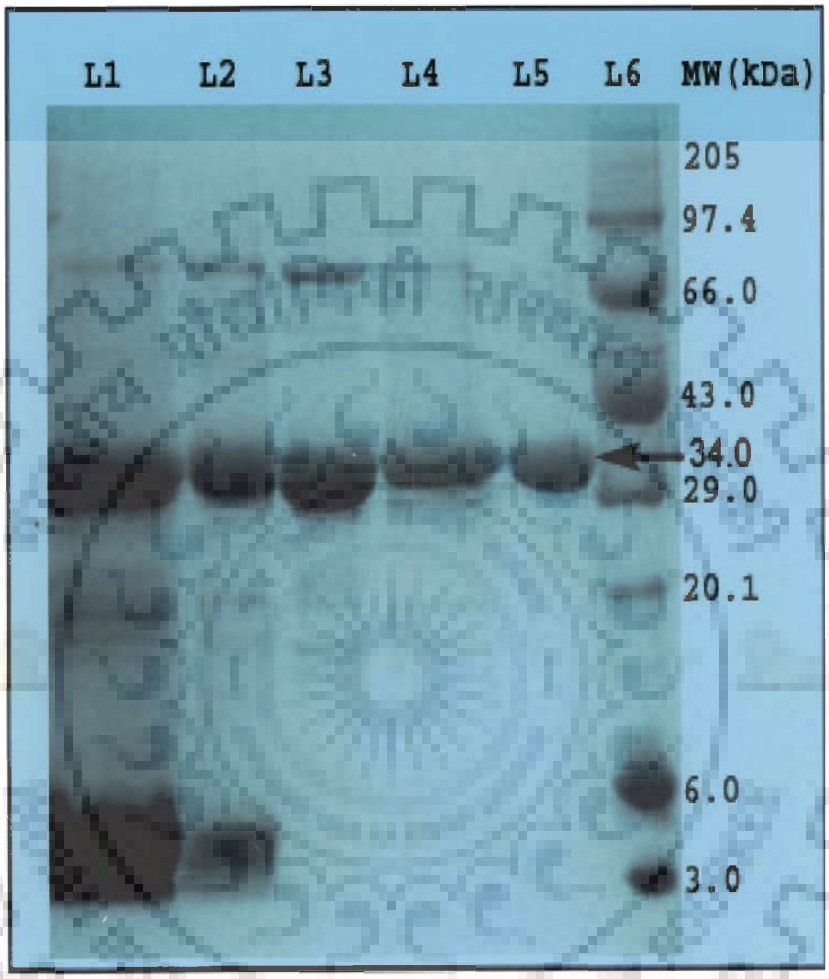

Fig. 2.1 SDS-PAGE analysis of the protein. L1, crude extract; L2, supernatant after acid precipitation step; L3, CM flow-through; L4, 50 mM NaCl fraction after anion exchange column; L5, purified protein in 100mM NaCl fraction after anion exchange chromatography ; L6, molecular weight markers.

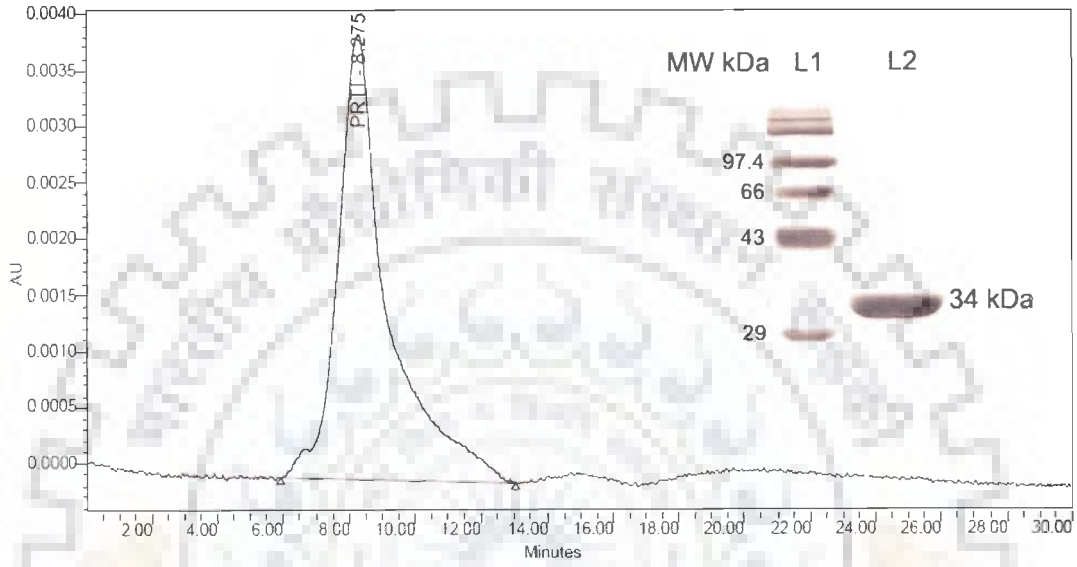

Fig 2.2 The HPLC elution profile of purified protein on gel-filtration column. Insert: SDS-PAGE analysis of HPLC purified PRTI.

2.4 Discussion

Putranjiva roxburghii belonging to *Euphorbiaceae* family is an ornamental tree of tropical India known as child life tree. Deseeded fruits are used against cough, cold, fever and sprue. Rosaries of hard stones are used for protecting children from infections. Roxburghonic keto acid and some flavonoids, terpenoids and triterpines has been purified and characterized from the leaf and trunk bark of this plant (Garg & Mitra, 1968, 1971a, b; Sengupta et al., 1967). The leaf extract of the *Putranjiva roxburghii* is found to be active against some bacterial and fungal strains (Kumar et al, 2006). Most serine proteinase inhibitors from seeds have been isolated and characterized from *Leguminosae*, *Cucurbitaceae*, *Solanaceae* and *Graminae* families (Garcia-Olmeda et al., 1987). There are not many reports of purification and characterization of these inhibitors from other plant families. Other families where an inhibitor has been purified and characterized include *Rutaceae* (Shee & Sharma, 2007) and *Euphorbiaceae* (Sritanyarat et al. 2006) families. Especially for the *Euphorbiaceae* family there are not many reports of isolation and purification of inhibitors, only a trypsin inhibitor was purified from the non rubber fractions of *Havea brasiliensis* latex (Sritanyarat et al. 2006).

To date, no protein has been characterized from *Putranjiva roxburghii*. This chapter describes the isolation and purification of a highly stable and potent trypsin inhibitor from the seeds of *Putranjiva roxburghii*. PRTI was purified by different protein purification steps acid precipitation, cation-exchange chromatography, anion-exchange chromatography and size-exclusion chromatography on HPLC. In first step of acid precipitation many low molecular mass proteins were precipitated along with some other proteins. This step is very useful in getting rid of many unwanted proteins in supernatant. Acid precipitation has been earlier used for the purification of *Brassica nigra* seed trypsin and subtilisin inhibitor by Genov et al. (1997).

In second step PRTI was further purified by ion exchange chromatography both cation and anion exchange. Ion exchange chromatography is widely used in protein purification due to its dynamic range and high resolution capabilities. Protein separation by this method is based on differences in electric charge (Roe, 2001). Since proteins fundamentally differ in their amino acid sequence, they vary in their net charge at any given pH other than their iso-electric point. Purification profile of ion exchange chromatography reveals that PRTI is negatively charged at pH 6.0 and remain unbound to CM column which is negatively charged. At pH 8.0 PRTI bound strongly to DEAE column which was positively charged due to presence of highly negative charge on PRTI at pH 8.0 in Tris-HCl buffer. Many other inhibitors also purified by ion-exchange chromatography (Macedo et al., 2003; Gomes et al., 2005; Azarkan et al., 2006; Macedo et al., 2007). The trypsin inhibitory activity was quite stable at each step of purification and PRTI protein also retain its activity after acid precipitation at very acidic pH. The SDS-PAGE analysis under both reducing (Fig. 2.2, inset) and non-reducing (data not shown) conditions showed that PRTI is a single polypeptide chain with a molecular mass of approximately 34 kDa.

Interestingly, the molecular mass of PRTI is significantly higher than the typical Kunitz type inhibitor (20 kDa). The first report demonstrating the existence in plants of high-molecular-weight serine proteinase inhibitors similar to serpins was published in 1972 (Mosolov and Valueva, 2004). A 43.5 kDa trypsin inhibitor was found in the seeds of common oat (*Avena sativa* L.) (Mikola & Kirsi, 1972). A serpin with a molecular weight of 42 kDa was also isolated from the phloem of pumpkin fruit. The protein acts as an efficient inhibitor of pancreaticand leukocyte elastases (Yoo et al, 2000). Earlier, trypsin inhibitors of 32.5, 33, 41 and 43.5 kDa has been reported from *Crotalaria pallida* seeds (Gomes et al., 2005), *Ipomoea batatas* (sweet

potato) root (Hou et al., 2001), *Solanum tuberosum* tubers (Balls and Ryan, 1963) and *Avena sativa* L. (Mikola and Kirsi, 1972) respectively.

In summary the results in this chapter clearly indicates that PRTI is an approximately 34 kDa protein with a single polypeptide chain. PRTI retained its inhibitory activity after each purification steps, this demonstrate the very stable nature of the *Putranjiva roxburghii* seed trypsin inhibitor.

CHAPTER 3

BIOCHEMICAL CHARACTERIZATION

3.1 Introduction

Proteins are the agents of biological function. Virtually every cellular activity is dependent on one or more proteins. Therefore, they can be classified functionally as catalytic proteins (enzymes), regulatory proteins (e.g. proteinase inhibitors), transport proteins, storage proteins (e.g. many seed protein in plants), structural proteins, contractile proteins, scaffold proteins, protective proteins and others. 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). 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 characterized, 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). The Kunitz-type inhibitor family 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).

Amino acid sequence determination is very essential to identify and characterize an unknown protein. There are many techniques available to determine the sequence, like Edman degradation method, mass spectroscopic analysis and c-DNA cloning. The Edman degradation method generally applies for N-terminal amino acid sequence determination. The mass spectroscopic technique applies for internal sequence analysis. The experiment can be performed by different instrument like ESI-MS/MS, MALDI-TOF/TOF etc.

The denaturation of protein leading to aggregation occurs due to the environmental changes such as temperature, pH and addition of denaturants like urea and guanidine hydrochloride (Tanford 1968, 1970) at lower pH, carboxyl groups are protonated and hydrophobic interaction dominate, this leads to precipitation of the polymer (Roy and Gupta, 2003). Proteolysis is an important means to study structural stability of a protein. It has been reported that protein resistant to proteolytic attack increases with its conformational rigidity and susceptibility to proteolysis reflects the segmental mobility (Fontana, 1988; Fontana et al, 1997; Hubbard, 1998).

3.2 Materials and Methods

3.2.1. Materials

Trypsin, chymotrypsin, pepsin, papain, N-benzoyl-L-arginine p-nitroanilide (BAPNA), N-benzoyl-L-tyrosine ethyl ester (BTEE) and BSA were purchased from Sigma-Aldrich Pvt. Ltd., PVDF membrane (Immobilon-P^{SQ} Millipore, USA), Reagents for SDS-PAGE were purchased from Sigma-Aldrich Pvt. Ltd. Molecular weight standards were obtained from Bio-Rad, Australia and Bangalore Genei^{TM}. India. Proteinase K was purchased from Bangalore Genei^{TM}, India. DTT and all other chemicals were purchased from Himedia chemicals. All other reagents were of analytical or HPLC grade.

3.2.2 *N-terminal and partial internal sequence determination*

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

3.2.3. Assay of inhibitory activity

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

Chymotrypsin inhibitory activity assay: - For chymotrypsin inhibitory assay, different concentrations of PRTI were incubated with 10 µg of chymotrypsin dissolved (final concentration is 4.0×10^{-9} M in 1.0 ml assay volume) in 1.0 mM HCl containing 20 mM $CaCl_2$ at 30 °C for 15 min. Enzyme-protein mixtures (100 µl) were added to a solution of 900 µl in a cuvette containing a 1.0 mM final concentration of substrate N-benzoyl-L-tyrosine ethyl ester (BTEE, molar extinction coefficient of 964 M-1 cm-1) in 50 mM Tris-HCl, pH 8.0 containing 10% methanol. The substrate hydrolysis was monitored by measuring the absorbance at 256 nm for 5 min with a Varian Cary 100 spectrophotometer.

3.2.4. Dissociation constant (Ki) Determination

Kinetic studies over a range of PRTI concentrations were performed to determine the inhibition constant (Ki) from Dixon plot using BAPNA as a substrate (Dixon, 1953; Segel, 1975). Studies were performed by adding a range of inhibitor concentrations to a fixed amount of trypsin (final concentration was 0.0032 nM) at two different substrate concentrations of 1.0 mM and 5.0 mM. The final inhibitor concentrations used were 0.033, 0.056, 0.070, 0.084, 0.098, 0.11, and 0.21 nM. All the reactions were performed as described earlier. The reciprocal velocity (1/v) versus inhibitor concentrations [I], for each substrate concentration, $[S_1]$ and $[S_2]$ were plotted (Dixon plots). A single regression line for each [S] was obtained, and the Ki was calculated from the intersection of the two lines.

3.2.5. Stability studies of PRTI

In stability studies, effect of temperature, pH and DTT was examined on inhibitory activity of PRTI.

Effect of temperature on PRTI activity: - For thermal stability studies, purified protein (1 mg/ml) in 50 mM Tris–HCl buffer, pH 8.0 was incubated at various temperatures (30°–100 °C) for 30 min. After incubation, all the samples were kept on ice for 15 min. and then centrifuged. Supernatants were taken for the estimation of trypsin inhibitory activity.

Effect of pH on PRTI activity: - In pH stability, trypsin inhibition assays under a range of pH (2-12) conditions were performed to assess the pH stability and pH optima of PRTI. Purified PRTI was incubated with buffers of pH 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 to a final concentration of 1 mg/ml. The buffers used were 0.1 M each of glycine-HCl (pH 2, 3), Na-acetate (pH 4, 5), Na-

phosphate (pH 6, 7), Tris-HCl (pH 8, 9), glycine-NaOH (pH 10, 11, 12). After incubation of 30 min, the trypsin inhibitory activity was determined at pH 8.0.

Effect of DTT on PRTI activity: - The effect of DTT on inhibitory activity was determined by incubating purified PRTI (1.0 mg/ml) with DTT at final concentrations of 1, 10 and 100 mM for 10–120 min at 37 °C. The reaction was stopped by adding twice the amount of iodoacetamide to each DTT concentration and then the residual trypsin inhibitory activity was measured. All experiments were done in triplicate and the results are the mean of three assays.

3.2.6. Proteolysis studies of PRTI

Purified PRTI proteins were used for proteolysis studies with different proteases. Purified protein was incubated with five different proteinases, namely trypsin, chymotrypsin, papain, pepsin and proteinase K, separately using a proteinase/PRTI molar ratio of 1:50 for different time periods ranging from 30 min to 24 h at room temperature. Trypsin, chymotrypsin and proteinase K were incubated with PRTI in 50 mM Tris–HCl, pH 8.0. Enzyme papain was incubated with PRTI in 50 mM sodium phosphate buffer, pH 6.9 and pepsin was incubated with PRTI in 100 mM sodium acetate buffer, pH 3.5. BSA was taken as a positive control and incubated with proteases in respective conditions at 1:100 molar ratios for 30 min. Time dependant proteolytic cleavage studies were also performed in narrow range of 30 sec, 1 min, 2 min, 5 min and 10 min incubation of PRTI with all five protease to determine the any structural domain. The proteolysis reaction was quenched by adding SDS-containing denaturing buffer and immediately heated at 95 °C for 5 min to denature the enzymes. All samples were analyzed on a 15% SDS-PAGE

3.3 Results

3.3.1 N-terminal and partial internal sequencing

In N-terminal sequencing of PRTI, first 10 residues from the N-terminal were obtained. The sequence determined was Arg-Pro-Pro-Gln-Ala-Gly-Tyr-Ile-Gly-Val. The N-terminal sequence of PRTI showed no similarities with any of the known trypsin inhibitors. However, two peptides of 13 and 16 amino acids obtained from partial internal sequencing by mass spectrometry analysis showed significant identity to Kunitz-type inhibitors. Peptide1 showed 100% identity to winged bean chymotrypsin inhibitor-3 (Shibata et al., 1988) and Peptide2 showed 100, 73 and 73% identity to *Acasia confussa* trypsin inhibitor (Wu and Lin, 1993), *Prosopsis juliflora* trypsin inhibitor (Negreiros et al., 1991) and trypsin isoinhibitors DE5 of *Adenanthera pavonina* L. (Richardson et al., 1986) respectively (Fig. 3.1). These results suggest that PRTI may belong to Kunitz type inhibitor family.

3.3.2 Inhibitory properties and Ki determination

The inhibitory activity of PRTI against trypsin and chymotrypsin were determined by measuring the hydrolytic activity toward BAPNA and BTEE respectively. The protein completely inhibited trypsin at a molar ratio of 1:1 but did not show any significant inhibition against α-chymotrypsin (Fig. 3.2). The dissociation constant (Ki) value and mode of inhibition of PRTI were determined from Dixon plot using BAPNA as a substrate (Fig. 3.3). The analysis of Dixon plot showed that the PRTI is a competitive inhibitor where two lines corresponding to each substrate intersect above the x-axis, a characteristic of competitive inhibition.

(A)

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

(B)

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

Figure 3.1: - Internal sequencing peptide was searched for homology against NCBI BLAST short sequence protein data base. Peptide1 shows 100% homology with WCI-3 (Winged bean chymotrypsin inhibitor-3; (Shibata et al., 1988). Peptide2 shows 100% homology with ACTI (*Acasia confussa* trypsin inhibitor; (Wu and Lin, 1993), and 73% homology with PJTI (*Prosopsis juliflora* trypsin inhibitor; (Negreiros et al., 1991) and trypsin isoinhibitors DE5 of *Adenanthera pavonina* L. (Richardson et al., 1986).

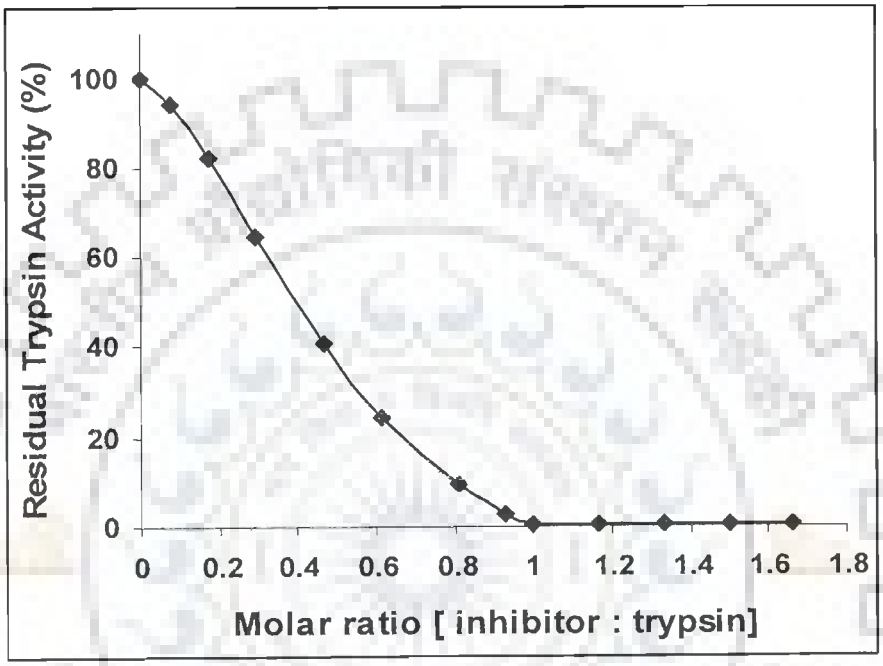

Figure 3.2: - Titration curve of trypsin inhibition by PRTI. Increasing concentration of PRTI was added to a fixed concentration of trypsin (0.0032 nM). Residual trypsin activity was measured by using BAPNA as substrate. Each point is the mean of three assays. Complete inhibition was found at 1:1, enzyme: inhibitor ratio. Each point is the mean of three assays.

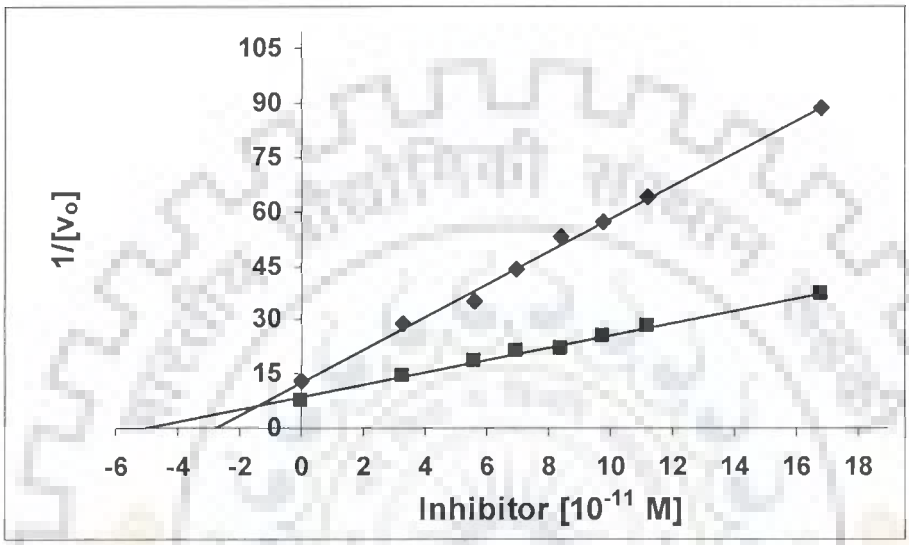

Figure 3.3: - Dixon plot for the determination of the dissociation constant (Ki) value of PRTI at two different concentration of BAPNA. Final concentrations of substrate were 0.001M (■) and 0.005M (♦). The reciprocals of velocity were plotted against different concentrations of PRTI. The dissociation constant (Ki) value was found to be 1.4×10^{-11} M.

The dissociation constant (Ki) value was found to be 1.4×10^{-11} M which clearly indicates that PRTI is a highly potent inhibitor of bovine trypsin.

3.3.3 Stability studies

In thermo stability studies, trypsin inhibitory activity of PRTI was determined at temperatures ranging from 20 to 100 °C. In thermal stability studies, trypsin inhibitory activity of PRTI was completely retained up to 70 °C. Above 70 °C, there was a slight decrease in the inhibitory activity with PRTI retaining almost 85% inhibitory activity up to 80 °C. The inhibitory activity of PRTI fell sharply above 80 °C with a loss of almost 80% inhibitory activity at 90 °C (Fig. 3.4). In pH stability studies, PRTI was highly stable under conditions ranging from highly acidic to highly alkaline. PRTI showed maximum inhibition at pH 8.0 and maintained over 95% of its inhibitory activity through a pH gradient of 2-12 (Fig. 3.5). The inhibitory activity of PRTI was examined in the presence of DTT. PRTI was found completely stable with no loss in inhibitory activity when incubated for 1 h up to 100 mM DTT. Only a slight decrease of 5% in inhibitory activity was observed when PRTI was incubated for 2 h at 100 mM DTT (Fig. 3.6).

3.3.4 Proteolysis study of PRTI

Proteolytic activity of different proteanases was examined on PRTI and results were obtained on SDS-PAGE. Observation of SDS-PAGE analysis suggests that PRTI is proteolytically stable against various proteinases. PRTI is very stable against trypsin, chymotrypsin and pepsin and the results obtained did not show any cleavage against these enzymes (Fig. 3.7 & 3.8). Although, PRTI is a serine proteinase inhibitor but proteolytically it is

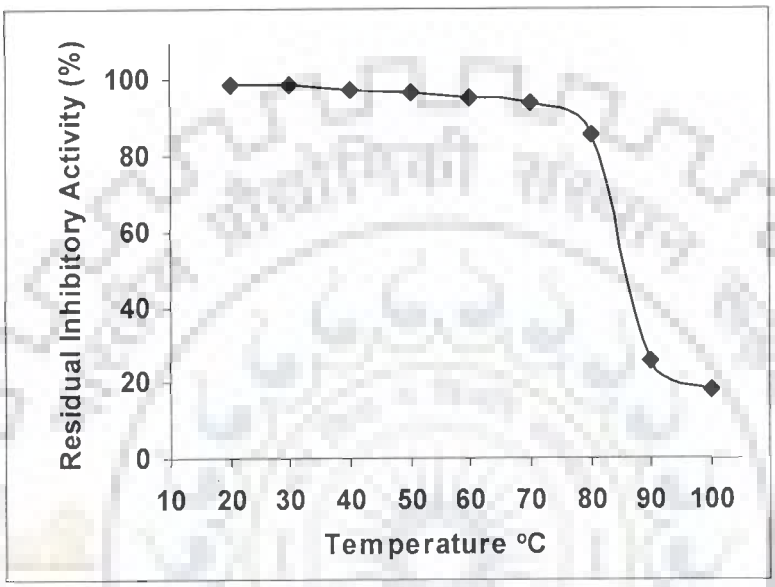

Figure 3.4: - Temperature stability of the inhibitory activity of PRTI after incubation for 30 min at the indicated temperatures. PRTI activity is highly stable up to 70 °C and retained its 85% activity at 80 °C. Each point is the mean of three assays.

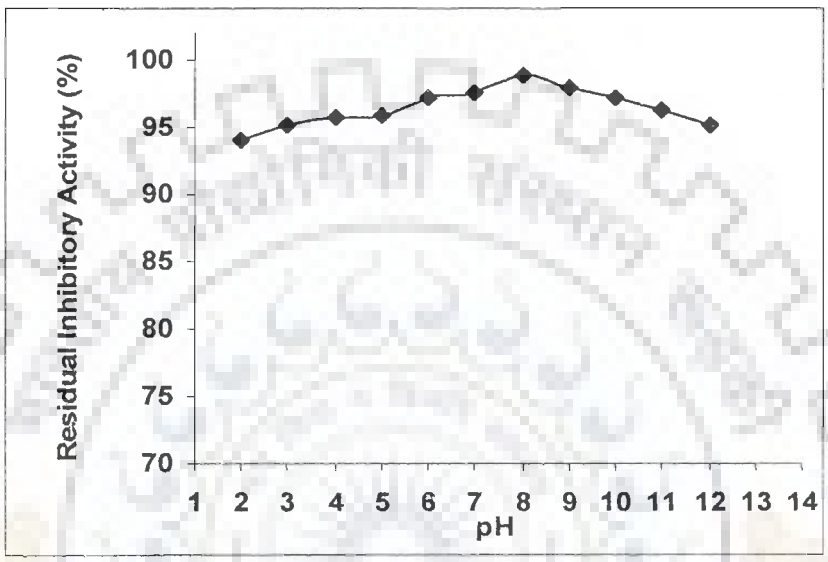

Figure 3.5: - pH stability of PRTI, after incubation at the indicated pH for 30 min at 37 °C. PRTI activity is quite stable in pH range 2-12 and shows maximum activity at pH 8.0. Each point is the mean of three assays.

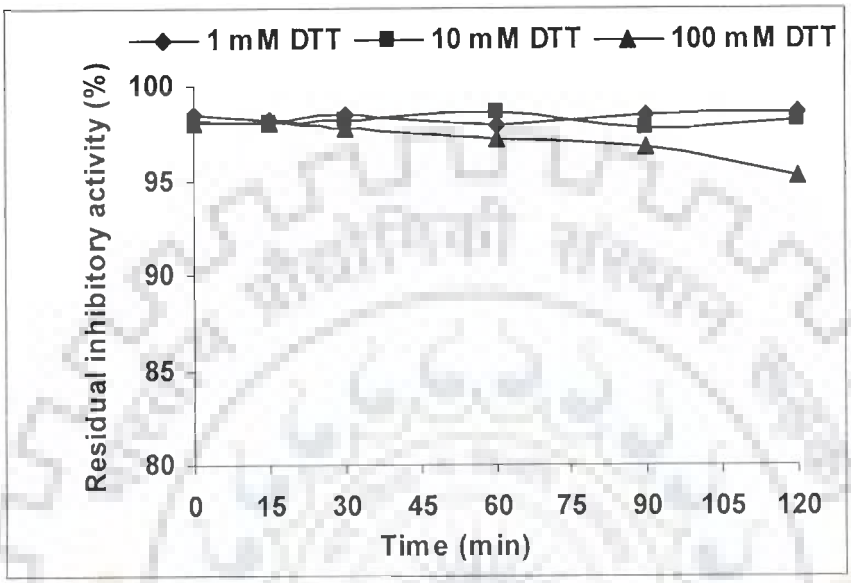

Figure 3.6: - Effect of DTT on the stability of PRTI. The inhibitor was treated with different final concentrations (1, 10 and 100 mM) of DTT for 15–120 min at 37 °C. The reaction was interrupted with iodoacetamide (two-fold molar excess relative to DTT), and residual trypsin inhibitory activity was measured using BAPNA as substrate. Each point is the mean of three assays.

very stable against aspartate proteinases like pepsin. When PRTI is incubated with proteolytic enzyme papain and proteinase k it has been observed that PRTI is proteolytically susceptible against aspartate proteinase papain and non-specific proteinase proteinase k (Fig. 3.7 & 3.8). PRTI is partially cleaved by papain and completely cleaved by proteinase k enzyme. Time dependant proteolytic cleavage studies were also performed to determine any domain structure of PRTI but there was no such difference observed in the cleavage pattern.

3.3.5 Storage stability of PRTI activity

Purified PRTI was stored at -20 °C for one year period and inhibitory activity was checked after three, six and one year interval, found that PRTI retained ~100% activity after six month storage and retained over 90% inhibitory activity after one year storage. These observations clearly suggest that PRTI is very stable for long time storage at -20 °C and does not loss inhibitory activity significantly.

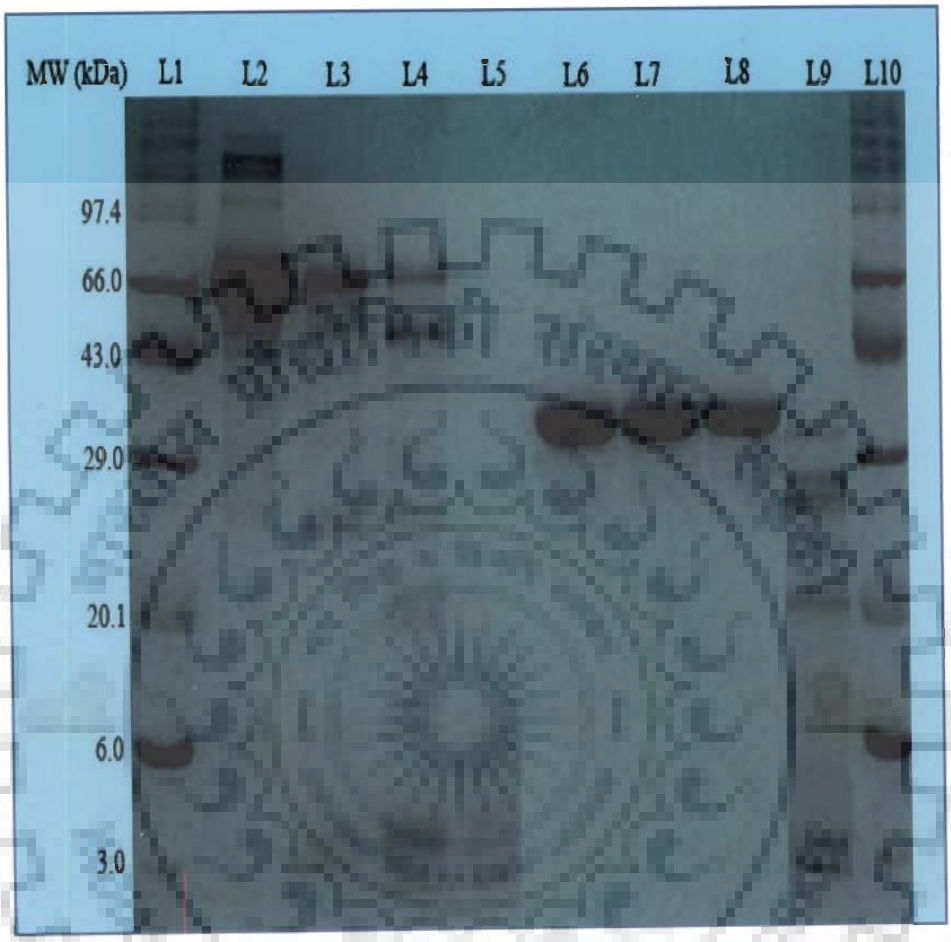

Figure 3.7: - SDS-PAGE analysis of effect of proteolysis on PRTI. L1 & L10- showed the molecular weight marker; L2- BSA native as a positive control; L3, L4 and L5- BSA with trypsin, chymotrypsin and proteinase K respectively as a positive control; L6; PRTI native without any proteinase; L7- PRTI with trypsin; L8- PRTI with chymotrypsin; L9-PRTI with proteinase K. PRTI is stable against the proteolytic activity of trypsin (Lane L7) and chymotrypsin (Lane L8) and susceptible against the activity of proteinase K, showing full cleavage (Lane L9).

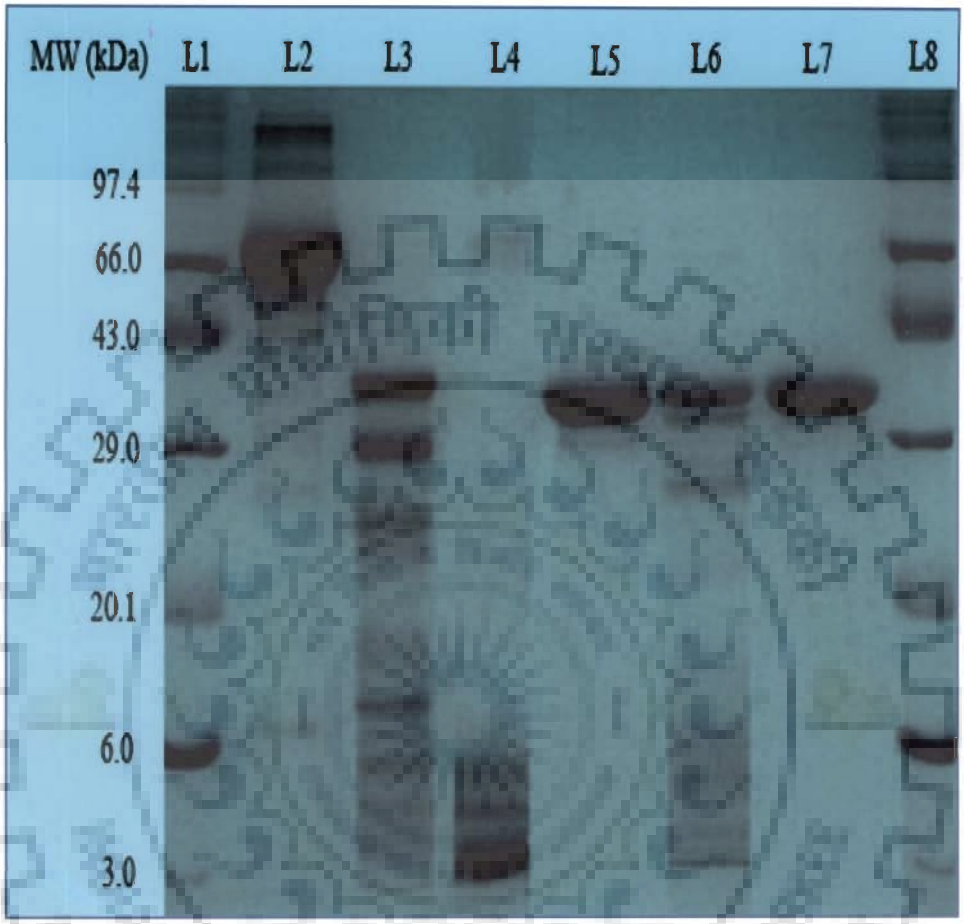

Figure 3.8: - SDS-PAGE analysis of effect of proteolysis on PRTI. L1 & L8- showed the molecular weight marker; L2- BSA native; L3 and L4- BSA with papain and pepsin as a positive control; L5-PRTI native without any proteinase; L6- PRTI with Papain; L7- PRTI with Pepsin. PRTI is stable against the cleavage of pepsin but showed the cleavage against the papain enzyme.

3.4 Discussion

The N-terminal sequence of 10 amino acids (Arg-Pro-Pro-Gln-Ala-Gly-Tyr-Ile-Gly-Val) of PRTI showed no similarities with any of the known trypsin inhibitors. However, two peptides of 13 and 16 amino acids obtained from partial internal sequencing by mass spectrometry analysis showed significant identity to Kunitz-type inhibitors. Peptide1 showed 100% identity to winged bean chymotrypsin inhibitor-3 (Shibata et al., 1988) and Peptide2 showed 100, 73 and 73% identity to *Acasia confussa* trypsin inhibitor (Wu and Lin, 1993), *Prosopsis juliflora* trypsin inhibitor (Negreiros et al., 1991) and trypsin isoinhibitors DE5 of *Adenanthera pavonina* L. (Richardson et al., 1986) respectively. These results suggest that PRTI may belong to Kunitz type inhibitor family because all above mentioned inhibitors are the Kunitz-type trypsin inhibitor which sequence showed the significant identity to the *Putranjiva roxburghii* trypsin inhibitor (PRTI).

The *Putranjiva roxburghii* trypsin inhibitor (PRTI) completely inhibited trypsin at a molar ratio of 1:1 but did not show any significant inhibition against α-chymotrypsin. The PRTI inhibited trypsin at enzyme: inhibitor molar ratio of 1:1. The stoichiometric ratio of 1:1 agree with those for other inhibitors (Richardson 1991; Souza et al., 1995; Sampio et al., 1996; Batista et al., 1996; Oliva et al., 1999; Macedo et al., 2000). The analysis of Dixon plot showed that the PRTI is a competitive inhibitor of bovine trypsin. The dissociation constant (Ki) value was found to be 1.4×10^{-11} M which clearly indicates that PRTI is a highly potent inhibitor of bovine trypsin. Earlier, high Ki values of 5.3×10^{-10}, 4.0×10^{-10}, 2.5×10^{-10}, 1.7×10^{-9} M have been reported for plant trypsin inhibitors from *Dimorphandra mollis* (Macedo et al., 2000), *Peltophorum dubium* (Macedo et al., 2003), *Archidendron ellipticum* (Bhattacharyya et al., 2006) and *Dimorphandra mollis* (Mello et al., 2001).

In thermal stability studies, trypsin inhibitory activity of PRTI was completely retained up to 70 °C. Above 70 °C, there was a slight decrease in the inhibitory activity with PRTI retaining almost 85% inhibitory activity up to 80 °C. The inhibitory activity of PRTI fell sharply above 80 °C with a loss of almost 80% inhibitory activity at 90 °C. Similar results have been reported for trypsin inhibitors from seeds of *Carica papaya* (Azarkan et al., 2006) and *Peltophorum dubium* (Macedo et al., 2003). The transition midpoint for papaya trypsin inhibitor is approximately 85 °C and it retains almost 71% of inhibitory activity at 80 °C whereas *Peltophorum dubium* inhibitor retains more than 80% of activity at 80 °C. As compare to PRTI, CpaTI also showed some similar results that the molecular weight is approximately similar and the inhibitory activity was stable at 80 °C and losing only 40 % of activity at 100 °C. Pre-incubation of the PRTI and CpaTI in the pH range (2–12) did not affect trypsin inhibitory activity (Gomes et al., 2005). In pH stability studies, PRTI was highly stable under conditions ranging from highly acidic to highly alkaline. PRTI showed maximum inhibition at pH 8.0 and maintained over 95% of its inhibitory activity through a pH gradient of 2-12. Similar results has been reported for other trypsin inhibitors including from *Igna laurina* (Macedo et al., 2007), *Crotalaria pallida* (Gomes et al., 2005), *Carica papaya* (Azarkan et al., 2006) and *Peltophorum dubium* (Macedo et al., 2003) and *Enterolobium contortisiliqumm* (Batista et al., 2001). The inhibitory activity of PRTI was examined in the presence of DTT. PRTI was found completely stable with no loss in inhibitory activity when incubated for 1 h up to 100 mM DTT. Only a slight decrease of 5% in inhibitory activity was observed when PRTI was incubated for 2 h at 100 mM DTT. This was in contrast to most trypsin inhibitors which lose inhibitory activity to different extent at high DTT concentration (Azarkan et al., 2006; Macedo et al., 2007). Similar results have been earlier reported for a Kunitz-type trypsin inhibitor from *Erythrina caffra* (ETI)

which retained inhibitory activity after reduction with DTT (Lehle et al., 1996). This clearly suggests that the inhibitory activity of PRTI like ETI is not affected by the presence of disulfide bridges. The crystal structure of ETI showed that the disulfide bridges are far apart from the active site residues involved in protease binding. The reactive loop in ETI is stabilized by hydrogen bonds and is not constrained by secondary structural elements or disulfide bridges (Onesti et al, 1991). The stability of PRTI as well as PDTI, ILTI, DMTI-Π is apparently unrelated to the presence of disulphide bridges since recent reports have described inhibitor isolated from *Bauhinia sp.* Seeds which are devoid of disulphide bridges and cysteine residues (Oliva et al., 2001).

Proteolytic activity of different proteinases was examined on native PRTI and results showed that it is highly resistant to proteolytic cleavage against some proteases. Observation of SDS-PAGE analysis suggests that PRTI is proteolytically very stable against trypsin, chymotrypsin and pepsin and the results obtained did not show any cleavage against these enzymes. Although, PRTI is a serine proteinase inhibitor but proteolytically it is very stable against aspartate proteinases like pepsin. When PRTI is incubated with proteolytic enzyme papain and proteinase k it has been observed that PRTI is proteolytically susceptible against aspartate proteinase papain and non-specific proteinase proteinase k. PRTI is partially cleaved by papain and completely cleaved by proteinase k enzyme. Similar kinds of results were also obtained for *Murraya koenigii* trypsin inhibitor (MKTI) where this trypsin inhibitor was found to be very stable against all five proteinases (Shee et al., 2007). Proteolytic stability against serine proteinase inhibitors i.e. trypsin, chymotrypsin and pepsin showed that PRTI have very compact and rigid structure and does not provide the access for the proteinase activity. Time dependant proteolytic cleavage studies were also performed to determine any domain structure

of PRTI but there was no such difference observed in the cleavage pattern. Purified PRTI was stored at -20 °C for one year period and inhibitory activity was checked after three, six and one year interval, found that PRTI retained ~100% activity after six month storage and retained over 90% inhibitory activity after one year storage. These observations clearly suggest that PRTI is very stable for long time storage at -20 °C and does not loss inhibitory activity significantly. Similar results were obtained by (Belew et al, 1975), for the trypsin and chymotrypsin inhibitors of *Cicer arietinum* and (Bhattacharyya et al, 2006), for the trypsin inhibitor from *Archidendron ellipticum* seeds.

In summary the results in this chapter demonstrated that PRTI is a very stable and highly potent trypsin inhibitor and may belong to Kunitz family of trypsin inhibitors. The Ki value of 1.4×10^{-11} M demonstrated a high affinity between enzyme and inhibitor. PRTI displayed a remarkable stability at a wide range of pH (pH 2–12) and temperature (up to 80 °C). PRTI retained almost 95% of inhibitory activity when incubated with 100 mM DTT for 2 h which demonstrated that disulfide bridge do not have any direct role on inhibitory activity of PRTI. PRTI is proteolytically very stable against trypsin, chymotrypsin and pepsin and PRTI is proteolytically succeptible against aspartate proteinase papain and non-specific proteinase proteinase k. PRTI is partially cleaved by papain and completely cleaved by proteinase k enzyme.

CHAPTER 4

BIOPHYSICAL CHARACTERIZATION

4.1. Introduction

PIs are ubiquitous, small regulatory proteins generally present at high concentration (up to 15% of total protein), and are particularly distributed in the seeds of *Leguminoceae*, *Brassicaceae*, *Poaceae* as well as in tubers of *Solanaceae* family (Ryan, 1981; Oliva et al., 2000). These proteins play significant role in the control and the protection against proteolytic action of the digestive enzymes of seed predators (Batista et al., 1996; Shewry and Lucas, 1997). The inhibitory activity of these inhibitors is largely brought about by intra-molecular interactions such as, disulfide bond, hydrophobic interaction and hydrogen bond, which are involved in stabilization of the primary binding loop (reactive site loop) structure, enabling a stable complex with a cognate protease (Bode and Huber, 1992). To date, 10 families of plant PIs are recognized based on the protein primary and spatial structure including the number and position of disulphide bridges and active site, include Kunitz-type, Bowman-Birk type, potato I, potato II, squash, and cereal super-family (Laskowski and Kato, 1980; Richardson, 1991). The best-known groups of enzyme inhibitors from seeds are those which affect the activities of serine proteinases such as trypsin, chymotrypsin and subtilisin (Richardson, 1991). Apart from the serine PIs, inhibitors of the sulphydryl, acidic and metallo-proteinases have also gained prominence for their functional significance. The proteinase and proteinase inhibitor interaction is one of the most important targets to study the specificity of protein-protein interaction and molecular recognition. The proteinase inhibitors play an important role in regulating many physiological processes by inhibiting proteinases, such as inflammation, coagulation, fibrinolysis, intracellular protein breakdown, cell cycle, transcription and apoptosis (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 understand structure-activity relationship, it is important to study different aspects of inhibitor in relation to structural and functional stability in different physiological conditions and their three-dimensional structures. The stability of inhibitor can be studied as a function of temperatures, pH, salts and detergents using various biophysical methods. Circular dichroism and fluorescence spectroscopy are very important tools to evaluate the secondary structure and conformational stability under various physichochemical conditions. To understand the structure-function relationship of *Putranjiva roxburghii* trypsin inhibitor (PRTI) circular dichroism and fluorescence studies were performed under different physicochemical conditions. In CD studies secondary structure analysis were performed with native PRTI protein and at different temperatures. Fluorescence spectroscopy is a very powerful and sensitive technique to monitor conformational changes in proteins. The fluorophores can be either intrinsic (tryptophan) or extrinsic (ANS: 8-anilino-1-naphthalene sulfonate) probes. In proteins, the three aromatic amino acids, phenylalanine, tyrosine, and tryptophan are all fluorescent. These three amino acids are relatively rare in proteins. The fluorescence property of tryptophan has long been utilized to understand the folding/unfolding, substrate binding and conformational heterogeneity in different physicochemical conditions. A protein may possess just one or a few tryptophan residues, facilitating interpretation of the spectral data. A valuable feature of intrinsic protein fluorescence is the high sensitivity of tryptophan to its local environment. Changes in the emission spectra of tryptophan often occur in response to conformational transitions, subunit association, substrate binding, or denaturation. A complicating factor in the interpretation of

protein fluorescence is the presence of multiple fluorescent amino acids in most proteins. The environment of each residue is distinct and the spectral properties of each residue are generally different. Therefore we studied both intrinsic (tryptophan) and extrinsic fluorescence with the help of ANS (8-anilino-1-naphthalene sulfonate) dye probe. ANS binds non-covalently to exposed hydrophobic areas in protein mainly through its hydrophobic naphthalene moiety (Matilus et al, 1999). There are a number of dyes that can be used to non-covalently label proteins. These are typically naphthylamine sulfonic acids, of which 1-anilinonaphthalene-6-sulfonic acid (ANS) and 2-(p-toluidinyl) naphthalene-6-sulfonic acid (TNS) are most commonly used (Slavik, 1982) Dyes of this class are frequently weakly or nonfluorescent in water, but fluoresce strongly when bound to proteins (Daniel and Weber, 1996) or membranes. In fluoresecence studies, both ANS (8-anilino-1-naphthalene sulfonate) and tryptophan fluorescence were performed at different temperature, pH, denaturants, and detergents to understand the folding/unfolding nature of this protein. The conformational changes occurring at different physiological conditions, as analyzed by circular dichroism and fluorescence helped in correlating structure to the function of this bovine trypsin inhibitor. *Putranjiva roxburghii* although widely used as an ornamental tree, along the road side, gardens and homes. Little is known about the properties of the proteins contained in its seeds. In previous chapters, we reported the isolation and biochemical characterization of some properties of the trypsin inhibitor from *P. roxburghii* seeds (Chaudhary et al, 2008). The aim of work in this chapter is to describe the structural and conformational stability of PRTI in different chemicals and denaturating agents with the help of CD and fluorescence spectroscopy.

4.2. Materials and Methods

4.2.1. Materials

Protein was purified as described earlier. Trypsins, N-benzoyl-L-arginine p-nitroanilide (BAPNA), ANS (8-anilino-1-naphthalene sulfonate) were purchased from Sigma-Aldrich Pvt. Ltd. All other chemicals were purchased from Qualigens fine chemicals and Himedia chemicals.

4.2.2 Circular Dichroism spectroscopy studies

Circular dichroism measurements were carried out on a JASCO J-715 spectropolarimeter, equipped with a peltier-type temperature controller (PTC-348 WI) and a thermostat cell holder, interfaced with a thermostatic bath. CD instrument was routinely calibrated with D-10-camphorsulfonic acid. All solutions were filtered with a 0.22 μm pore nylon filter before performing the experiments. Far-UV CD spectra, (200 - 250 nm), were recorded in 1 cm path length quartz cell at a protein concentration of 0.35 mg/ml in 20 mM sodium phosphate buffer at pH 7.0. The effect of temperature on conformational stability of PRTI was determined by measuring CD spectra at temperatures ranging from 20 to 100 °C. Three consecutive scans were accumulated and the average spectra stored. CD values (MRE) also collected at 222 nm to determine the transition midpoint of the native PRTI in 20 mM sodium-phosphate buffer pH 7.0 in the temperature ranging from 20 -100 °C. The results of all the CD measurements are expressed as mean residue ellipticity (MRE).The results of all the CD measurements are expressed as mean residue ellipticity [θ] in deg cm^2 $dmol^{-1}$ using the relation: $[\theta] = [\theta] Mo / 10cl$, where [θ] is the observed ellipticity in millidegrees, *M*o is the mean residue weight of the protein (*M*o = 110), c is the protein concentration (mg/cm^3), and l is the path length (cm).

4.2.3 Fluorescence spectroscopy studies

Fluorescence measurements studies were made on a Cary Eclipse fluorescence spectrophotometer, model serial number EL06093734, at constant room temperature and emission scan mode (scan software version 1.1) at data interval of 1.0 nm using a 1.0 cm pathlength rectangular quartz cuvette. All the fluorescence spectra were measured at PRTI protein concentration of 2.0 μM. Stability studies of PRTI protein was performed with thermal denaturation at different temperature ranging from 30-100 °C and chemical denaturants guanidinium hydrochloride (GuHCl), SDS, Urea, reducing agent DTT and at different pH ranging from pH 2-12.

For ANS binding experiments, ANS (8-anilino-1-naphthalene sulfonate) dye was dissolved in 50 mM Tris–HCl, pH 8.5, to a final concentration of 50 mM. The final concentration of protein in the reaction mixture was 2.0 μM and ANS concentration was 100 μM. The molar ratio of protein and ANS was 1:50. The excitation was set at 390 nm and the emission spectra were measured in the range of 400–700 nm using an excitation and an emission slit widths of 5.0 nm.

For the study of the PRTI stability in the presence of guanidinium hydrochloride (GuHCl) and urea the stock solutions were prepared in a 50 mM Tris-HCl buffer pH 8.5 and a stock solution of 1 mg/ml of PRTI protein were made in same buffer. The different molar concentration of 2M, 4M, 6M, 7M and 8M of GuHCl and urea were incubated with PRTI for 02 to 22 h at room temperature before the spectra were recorded. The excitation wavelength was set at 290 nm for the intrinsic tryptophan fluorescence and for the ANS fluorescence excitation wavelength was set at 390 nm. For Tryptophan fluorescence emission spectra recorded between

300 and 550 nm and for ANS fluorescence emission spectra recorded between 400 and 700 nm. The excitation and the emission slit widths were fixed at 5 nm.

The effect of detergent SDS on conformational stability was studied by incubating purified PRTI (1.0 mg/ml) with SDS at final concentration of 0.2%, 0.4%, 0.6%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9% and 10% SDS for 2.0 h duration at 37 °C. The excitation wavelength was set at 290 nm for the intrinsic tryptophan fluorescence and for the ANS fluorescence excitation wavelength was set at 390 nm.

For tryptophan and ANS fluorescence measurement at different pH, protein solutions were prepared in 50 mM buffers of different pH values ranging from 2.0 to 12.0. The buffers used were glycine-HCl (pH 2.0, 3.0), Na-acetate (pH 4.0, 5.0), Na-phosphate (pH 6.0, 7.0), Tris-HCl (pH 8.0, 9.0), glycine-NaOH (pH 10, 11, 12). PRTI was incubated in each buffer for 2-20 h duration at 25 °C prior to measure the fluorescence. Tryptophan fluorescence emission spectra were recorded between 295 and 550 nm using an excitation wavelength of 290 nm and ANS fluorescence emission spectra were recorded between 400 and 700 nm using an excitation wavelength of 390 nm. The excitation and the emission slit widths were 5 nm. For the study of thermal denaturation PRTI (1.0 mg/ml) in 50 mM Tris-HCl buffer, pH 8.0 was incubated at various temperatures (30, 50, 70, 80, 90, 100 °C) for 30 min. After incubation all the sample were cooled to room temperature and then centrifuged. Supernatant were taken for the fluorescence measurement and estimation of trypsin inhibitory activity. Both intrinsic (tryptophan) and extrinsic (ANS) fluorescence measurements were taken.

The effect of DTT on conformational stability was studied by incubating purified PRTI (1.0 mg/ml) with DTT at final concentration of 5, 10, 20, 50,100 and 200 mM for 2.0 h duration at 37 °C. DTT stock solution of 1.0 M was prepared in 50 mM Tris-HCl buffer. The reaction

was stopped by adding twice the amount of iodoacetamide to each DTT concentration and then the residual trypsin inhibitory activity was measured. ANS was added 5.0 min prior to take the fluorescence measurement and emission spectra were recorded between 400 nm and 700 nm using an excitation wavelength of 390 nm.

4.2.4 PRTI inhibitory Activity assay

Trypsin inhibitory activity on bovine pancreatic trypsin was measured by estimating the remaining hydrolytic activity towards the substrate N-benzoyl-L-arginine p-nitroanilide (BAPNA) (Shibata et al, 1986). The purified PRTI was incubated with trypsin at a molar ratio of 1:1, at 30 °C for 15 min in 50 mM Tris-HCl buffer, pH 8.0 containing 20 mM $CaCl_2$ and 0.001 N HCl. After 15 min incubation, 1.0 ml of 0.5 mM BAPNA was added. After incubation of another 10 min at 30 °C, the reactions were stopped by adding 200 µl of 10% acetic acid. The changes in absorbance at 410 nm were recorded at 30 °C against a blank solution containing 1.5 ml of the substrate solution in same buffer with a Varian Cary 100 spectrophotometer. The amount of substrate (BAPNA) hydrolysis by the enzyme was calculated using the molar extinction coefficient of 8800 M^{-1} cm^{-1} at 410 nm.

4.3 Results

4.3.1 CD spectroscopy studies

Far-UV CD spectroscopy studies (240 -200 nm wavelength range) were carried out to analyze the secondary structure and conformational stability of PRTI at different temperatures from 20 to 100 °C (Fig. 4.2). Analysis of CD spectra of native PRTI showed that it is an α, β

protein with negative peaks at around 217 nm and 208 nm (Fig. 4.1.). Although, negative ellipticity was present but no clear negative peak characteristic of α-helical structures was observed at 222 nm. These results strongly suggest that PRTI is α, β protein rather than predominantly β protein. CD studies at increasing temperature demonstrated the thermo stability of PRTI structure. The PRTI retained the back bone protein folding with no significant change in CD spectra up to 70 °C. A significant loss in ellipticity was observed at and above 90 °C. This correlates well with the results of inhibitory activity where 15% loss was observed at 80 °C and 80% at 90 °C. The inhibitory activity and CD studies at increasing temperatures showed that transition midpoint for PRTI lies close to 88 °C (Fig. 4.3)

4.3.2 Fluorescence spectroscopy studies

For the conformational study of purified PRTI, 2 μM final concentrations used both for tryptophan and ANS fluorescence. Effect of Guanidinium Hydrochloride (GuHCl) and Urea were studied and found that both GuHCl and Urea show similar effect on the conformation of PRTI after incubation for 2 h. As the concentration of both denaturants increases the PRTI unfold slowly and above 7M and at 8M, protein completely unfolds and above 8M concentration (data not shown) did not found any increased changes in fluorescence intensities (Fig. 4.4 & 4.6). These observations suggest that PRTI is not very stable in the Urea and GuHCl medium and protein completely denature and unfold at 8M. After 22 h incubation of PRTI with Urea and GuHCl also shows the similar and did not observe much change in emission spectra. Tryptophan fluorescence measurement were also taken for both denaturants and clearly observed that PRTI unfolds as the concentration increases (Fig. 4.5 & 4.7). Both the tryptophan

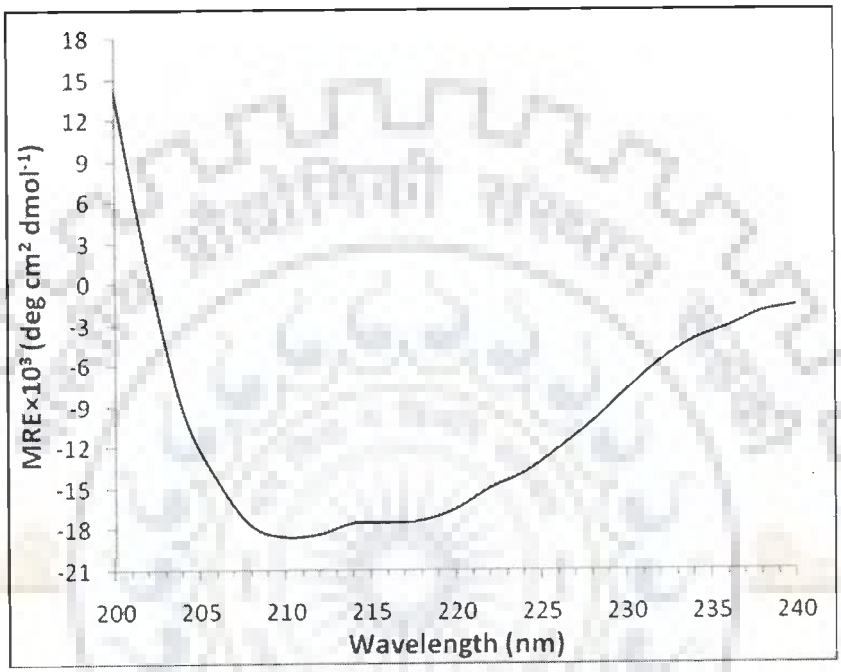

Figure 4.1: - Circular Dichroism (CD) spectra of native PRTI in 50 mM sodium-phosphate buffer pH 7.0. CD spectra were taken in the range of far UV wavelength (200-240 nm).

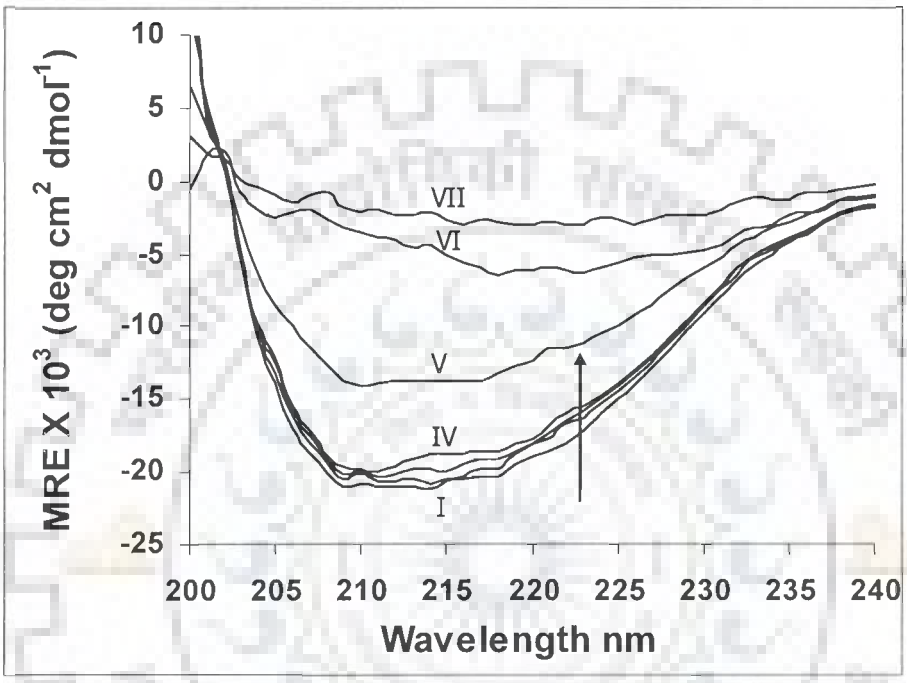

Figure 4.2: - Far-UV CD (200-240 nm) spectra measurements were carried out at PRTI concentration of 0.35 mg/ml with 1 nm bandwidth in 1 cm path length quartz cell in 50mM sodium phosphate buffer at pH 7.0 as a function of temperature are ranging from 20 °C to 100 °C (from bottom curve to the top curve temperature ranging from 20 °C, 30 °C, 50 °C, 70 °C, 80 °C, 90 °C and 100 °C).

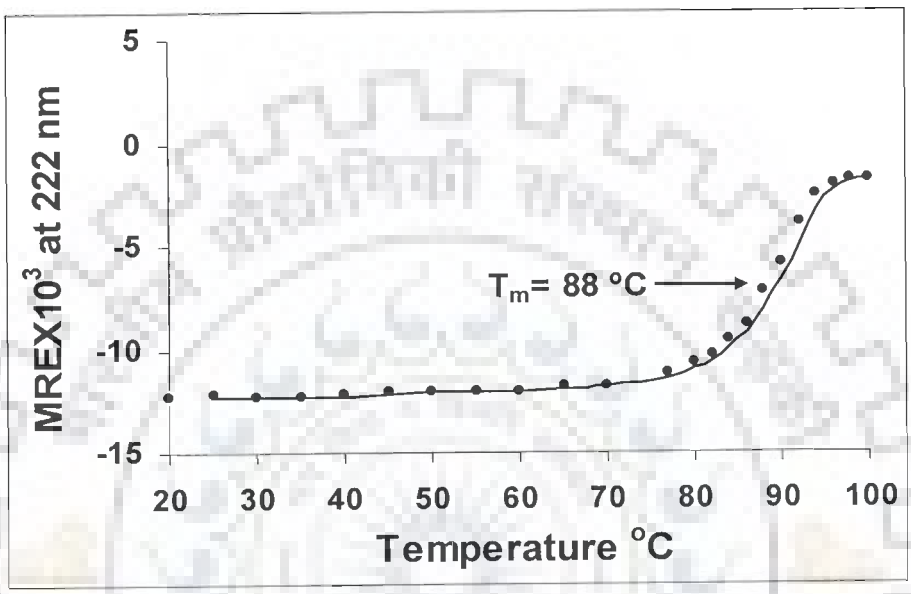

Figure 4.3: - CD values (MRE) spectra at 222 nm at temperature are ranging from 20-100 °C. The transition midpoint of native PRTI was determined to be 88 °C.

and ANS fluorescence emission of PRTI reveals that PRTI is not very stable in Urea and GuHCl, as the concentration increases gradually unfolding rises and at 8M and above, protein completely lose the native conformation and unfolded molten globule structure present.

In the presence of SDS (0.2-10%) detergent intensity of the ANS emission spectra of PRTI increases rapidly up to 2% SDS and above that (3-10%) increases slowly (Fig. 4.8), indicating that SDS work as a strong unfolding agent for PRTI. But in the tryptophan emission measurement, up to 5% SDS there was not much changes observed in the intensity. After 5% SDS and in between 6-10% SDS changes observed in the emission intensity with the λ_{max} at 325 nm (Fig. 4.9). The ANS fluorescence measured at various concentration of HCl showed a constant increase in ANS fluorescence with increasing concentration of HCl up to 1.0 M indicating a gradual unfolding of the native structure (Fig. 4.10). Tryptophan fluorescence spectra at various concentration of HCl showed sharp decrease in the intensity as concentration increases to 200 mM with fluorescence λ_{max} at 322 nm (Fig. 4.11). Temperature ANS fluorescence studies reveals that PRTI is very stable at temperature ranging from 20-100 °C and only slight changes occur in emission spectra between 20-50 °C range (Fig. 4.12) but the tryptophan fluorescence emission spectra showed the continuous increases in the intensity as the temperature increases from 20-100 °C (Fig. 4.13). Tryptophan fluorescence emission spectra of native PRTI at different temperature showed the similar conformational changes as was observed in the CD studies. ANS and tryptophan emission spectra showed that PRTI structural conformation unchanged at pH 6.0 to 9.0 and gradual changes occur at alkaline pH (pH 10, 11, 12), besides at acidic pH more conformational changes observed in an increasing order from pH 5.0 to pH 2.0 (Fig. 4.14). However PRTI retained more than 95% of trypsin inhibitory activity in the pH raging 2.0-12 and maximum activity was observed at pH 8.0. Relatively very high

λ_{max} at 335 nm was observed at pH 8.0 of Tris-HCl buffer compared to spectra at other pH values (pH 7.0 and 9.0) of the same buffer (Fig. 4.15), ANS fluorescence emission after 1 h incubation with DTT exhibited that up to 100 mM DTT concentration there was no significant changes occur only slight changes observed with 200 mM DTT (Fig. 4.16). Trypsin inhibitory activity of PRTI also retained up to 100 mM DTT and only 5-7% loss in activity was observed.

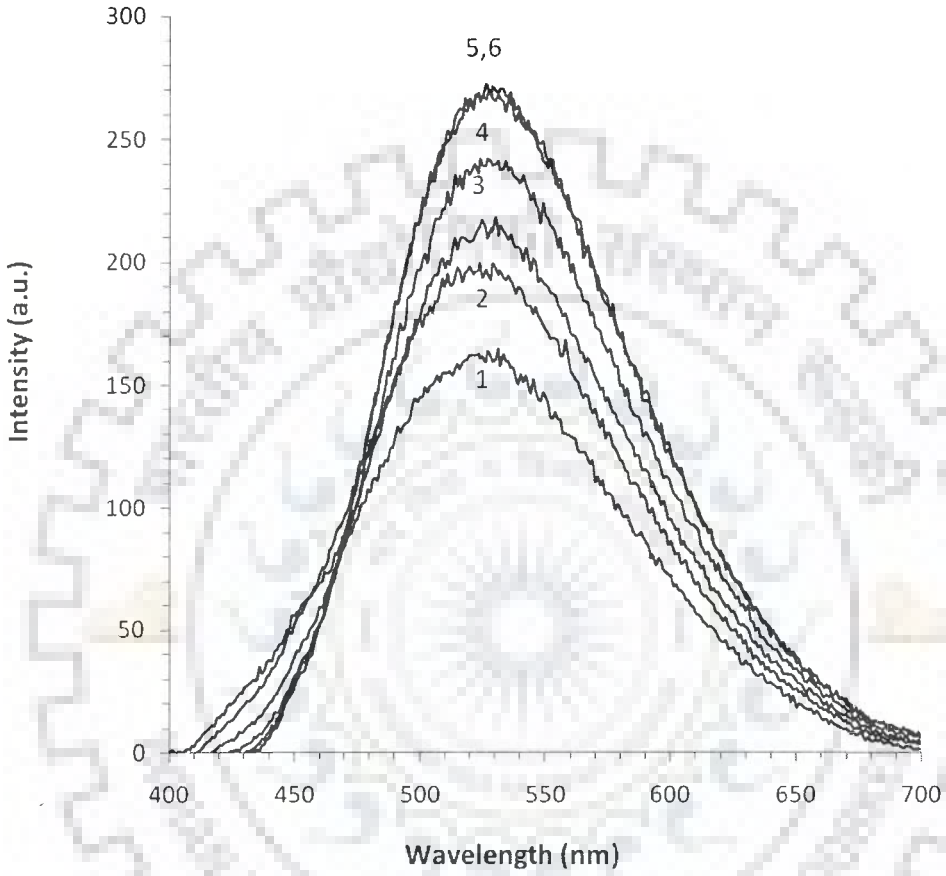

Figure 4.4: - ANS fluorescence emission spectra of PRTI (2×10^{-6} M) as a function of increasing concentration of GuHCl. Excitation wavelength was set at 390 nm. Spectra 1, 2, 3, 4, 5 and 6 shows PRTI native, 2M, 4M, 6M, 7M, and 8M GuHCl. Emission spectra shows that as the concentration of GuHCl increases, intensity is also increases and showing the gradual unfolding of PRTI

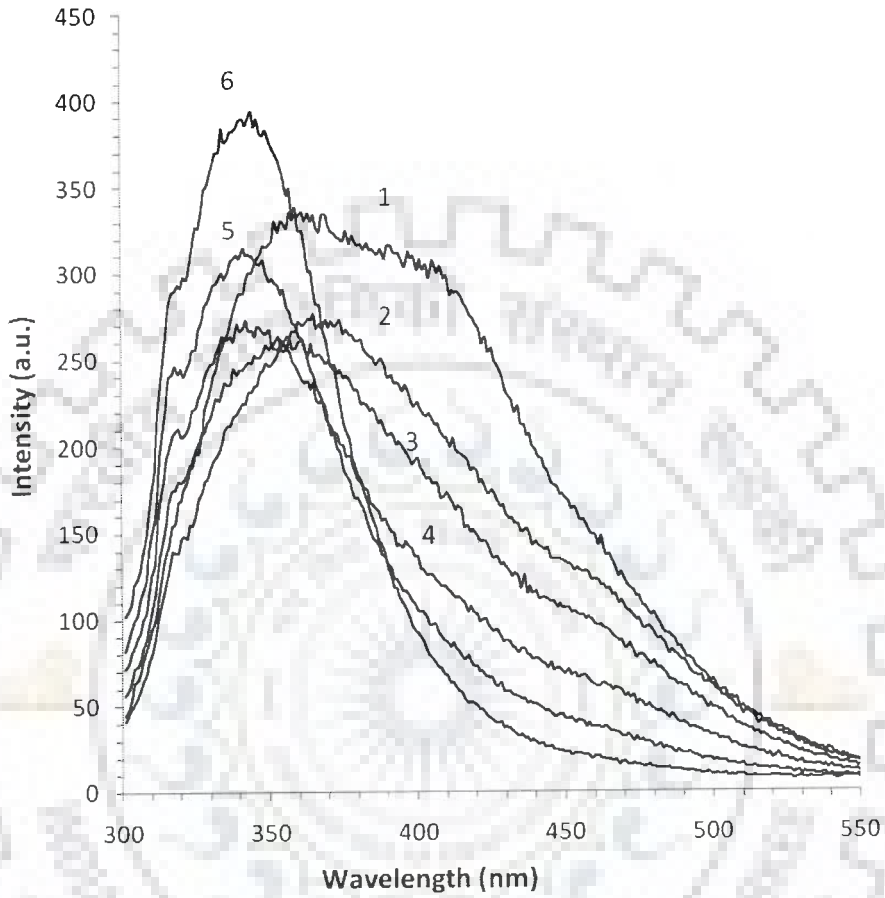

Figure 4.5: - Tryptophan fluorescence emission spectra of PRTI (2×10^{-6} M) as a function of increasing concentration of GuHCl. Excitation wavelength was set at 290 nm. Spectra 1, 2, 3, 4, 5 and 6 shows PRTI native, 2M, 4M, 6M, 7M, and 8M GuHCl. The emission spectra showed that as the concentration of GuHCl increases up to 6M, intensity is decreases (showing the gradual unfolding of PRTI) and after that at 7 & 8M intensity again increases (showing the complete unfolding or molten globule structure of PRTI).

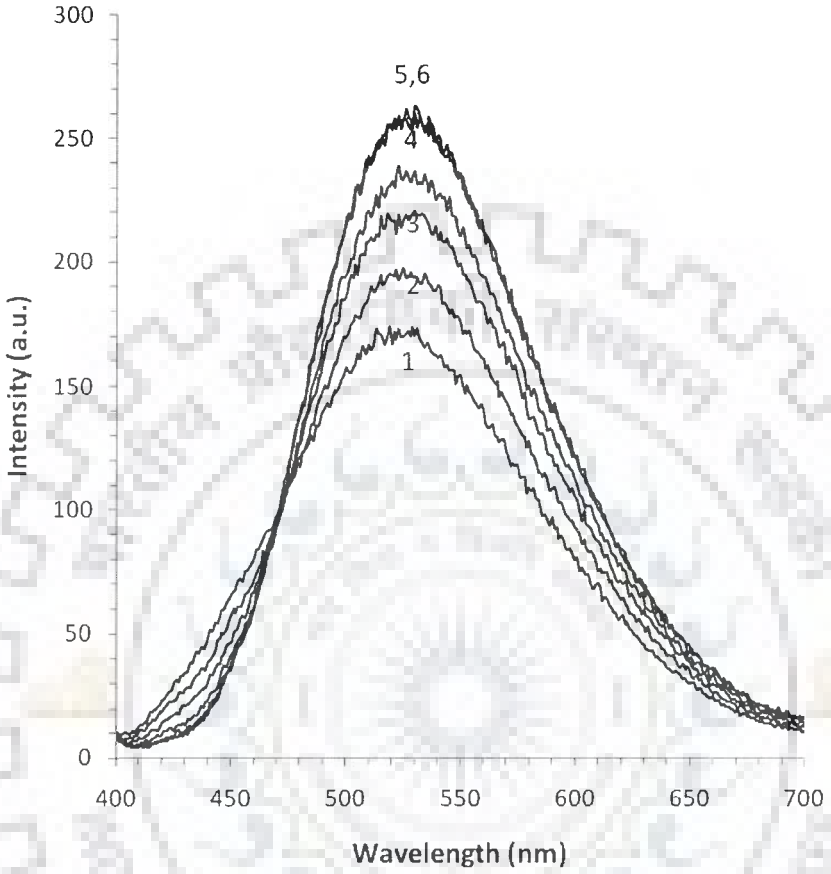

Figure 4.6: - ANS fluorescence emission spectra of PRTI (2×10^{-6} M) as a function of increasing concentration of Urea. Excitation wavelength was set at 390 nm. Spectra 1, 2, 3, 4, 5 and 6 shows PRTI native, 2M, 4M, 6M, 7M, and 8M Urea. Emission spectra showed that as the concentration of Urea increases, intensity is also increases and PRTI protein gradually unfolded.

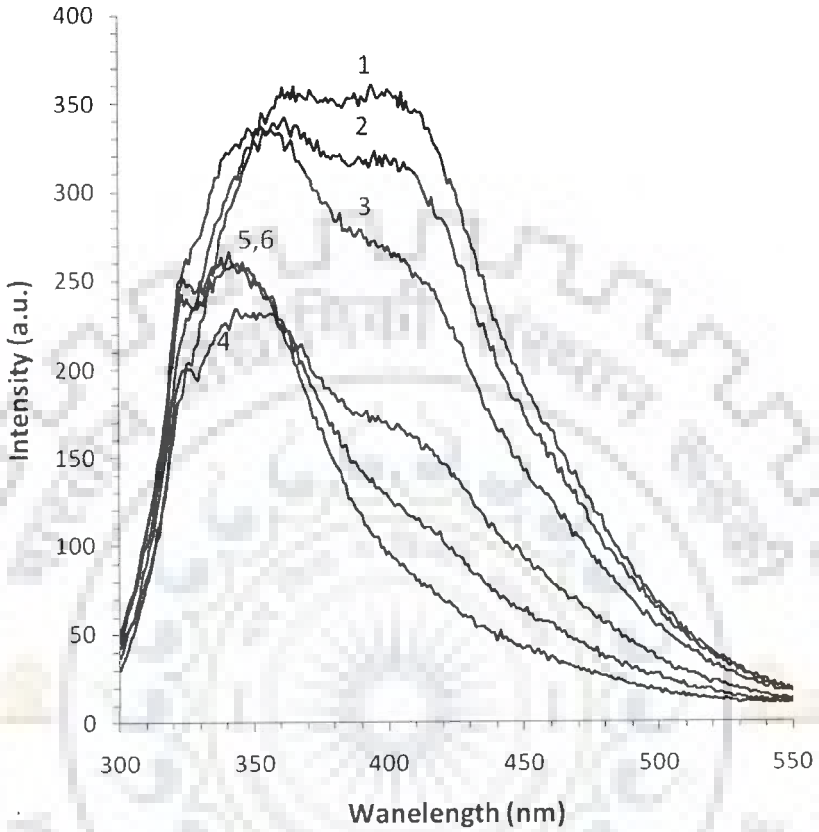

Figure 4.7: - Tryptophan fluorescence emission spectra of PRTI (2×10^{-6} M) as a function of increasing concentration of Urea. Excitation wavelength was set at 290 nm. Spectra 1, 2, 3, 4, 5 and 6 shows PRTI native, 2M, 4M, 6M, 7M, and 8M Urea. Emission spectrum shows that, as the concentration of Urea increases up to 6M, intensity is decreases (showing the gradual unfolding of PRTI) and after that at 7 & 8M, intensity again increases (showing the complete unfolding or molten globule structure of PRTI).

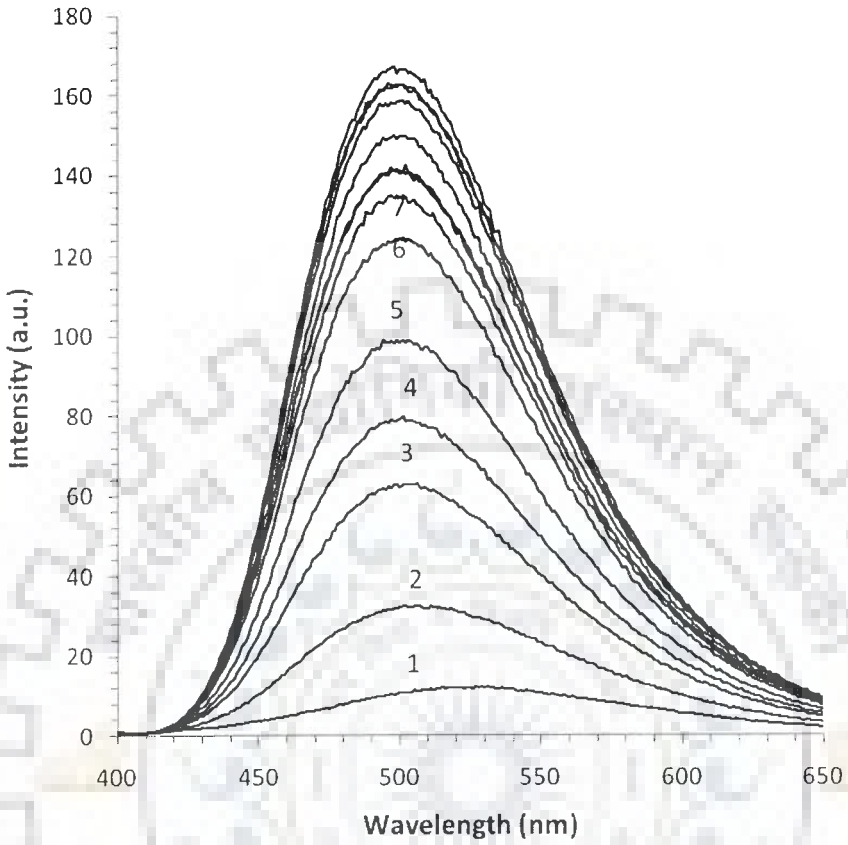

Figure 4.8: - ANS fluorescence emission spectra of PRTI as a function of increasing concentration of SDS (0-10%). Excitation wavelength was set at 390 nm. Emission Spectra 1, 2, 3, 4, 5, 6 and 7 shows PRTI native, 0.2%, 0.4%, 0.6%, 1%, 2%, and 3% SDS. Up to 3% SDS PRTI shows rapid unfolding with the sharp increase in intensity and above that, in the range of 4-10% SDS intensity increases gradually due to the complete unfolding.

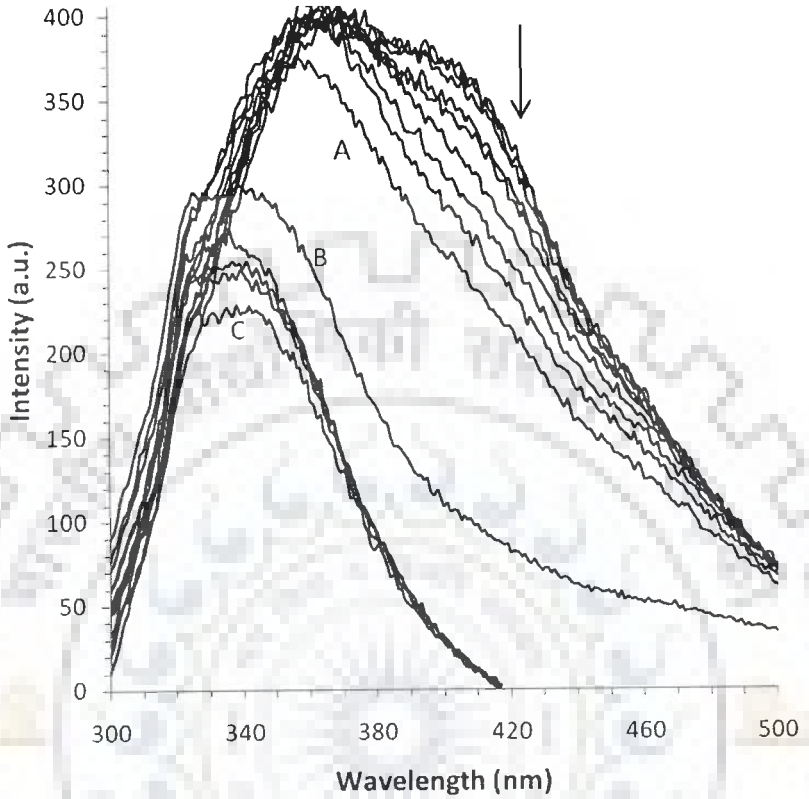

Figure 4.9: - Tryptophan fluorescence emission spectra of PRTI as a function of increasing concentration of SDS (0-10%). Excitation wavelength was set at 290 nm. Emission Spectra A, B, and C indicates 5%, 6% and 10% SDS. Up to 5% SDS, PRTI unfolds gradually. Between 5% and 6% PRTI unfolds rapidly and at 10% SDS PRTI completely unfolded.

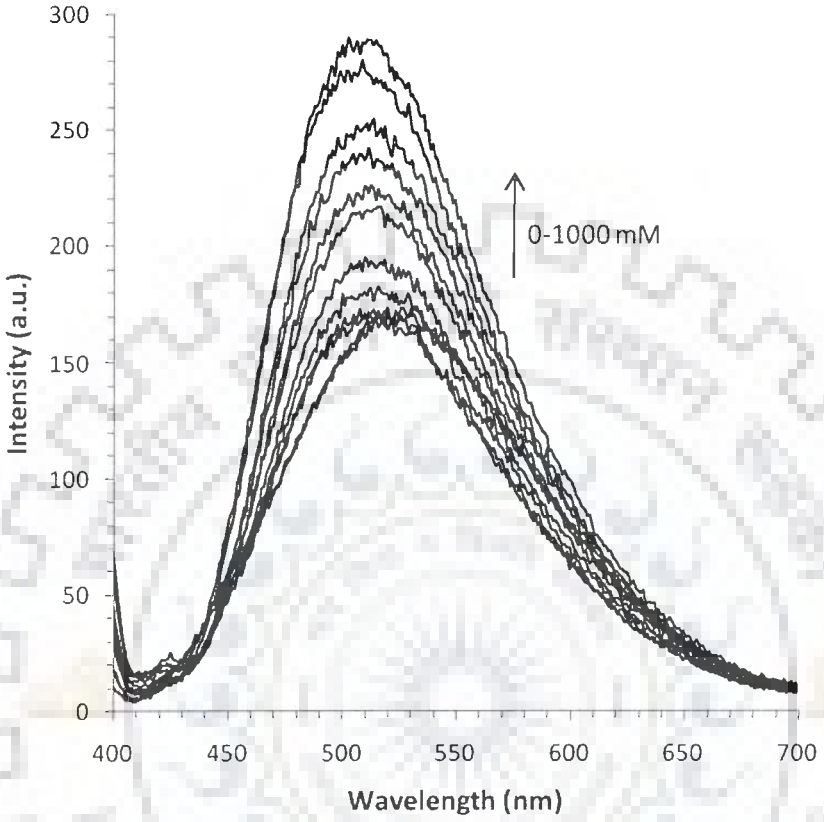

Figure 4.10: - ANS fluorescence emission spectra of PRTI as a function of increasing concentration of HCl (native PRTI, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 mM). Excitation wavelength was set at 390 nm.

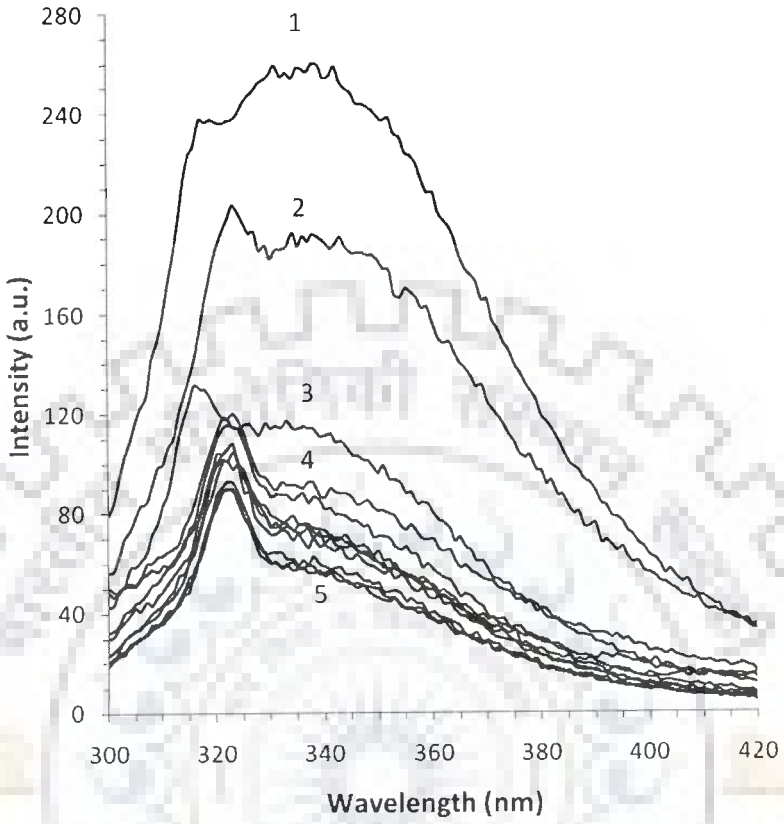

Figure 4.11: - Tryptophan fluorescence emission spectra of PRTI as a function of increasing concentration of HCl (native PRTI, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 mM). Excitation wavelength was set at 290 nm. Sharp decrease in the intensity up to 300 mM indicates the rapid exposed of the tryptophan residues of PRTI.

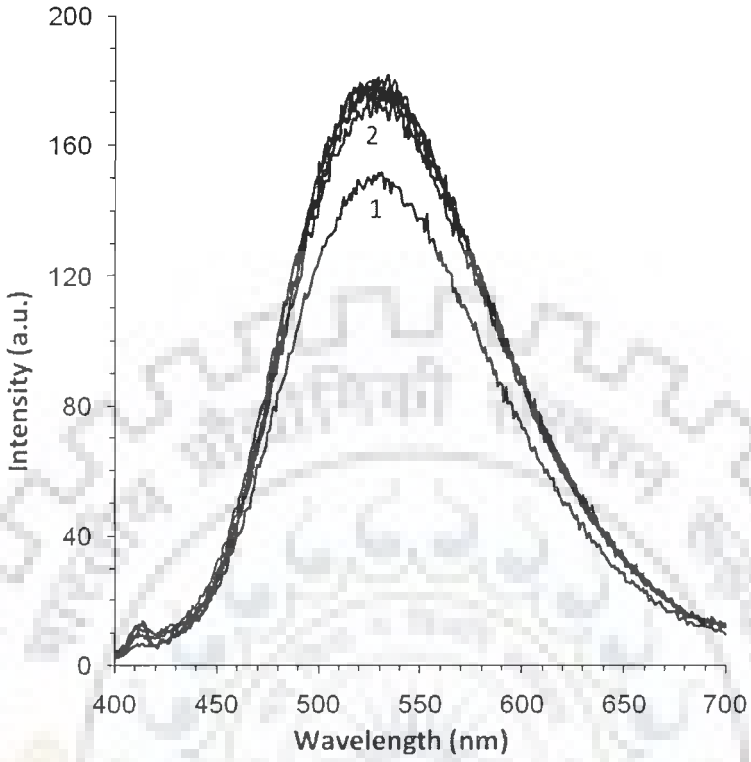

Figure 4.12: - ANS fluorescence emission spectra of PRTI as a function of temperature. Excitation wavelength was set at 390 nm.

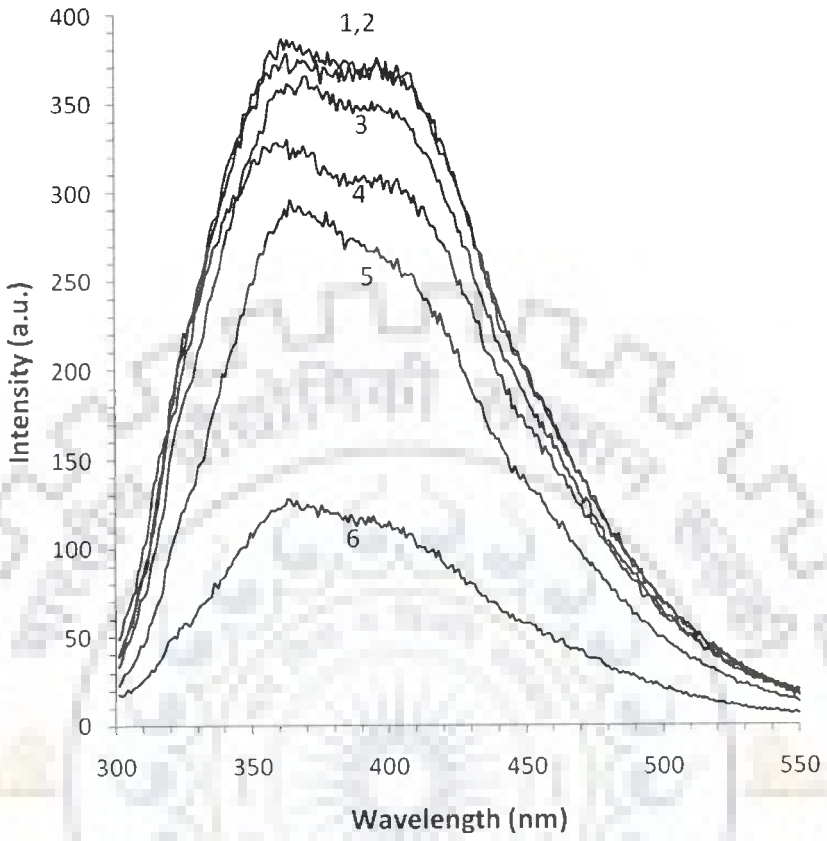

Figure 4.13: - Tryptophan fluorescence emission spectra of PRTI as a function of temperature. Excitation wavelength was set at 290 nm. Spectra 1, 2, 3, 4, 5 and 6 show the PRTI denaturation at 30, 50, 70, 80, 90 and 100 °C respectively.

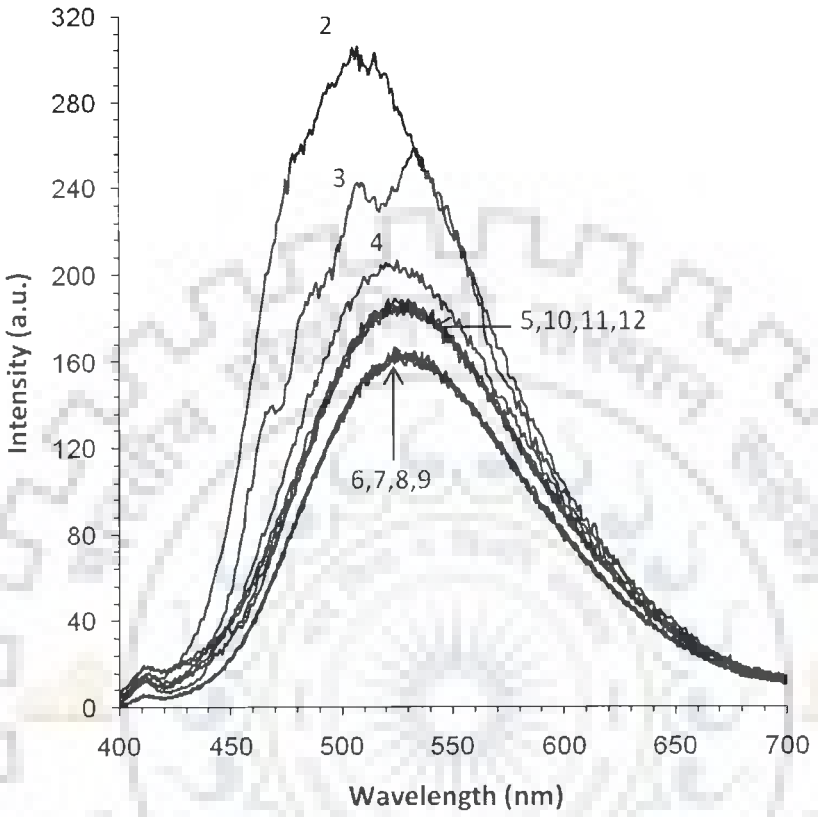

Figure 4.14: - ANS fluorescence emission spectra of PRTI as a function of different pH values. PRTI is quite stable at pH ranging from 6-8 (spectra 6, 7, 8, and 9) and show little unfolding in pH 5, 10, 11, 12 (Spectra 5, 10, 11, 12). At extreme of acidic condition PRTI shows little high unfolding at pH values pH 2, 3 and 4 (Spectra 2, 3 and 4).

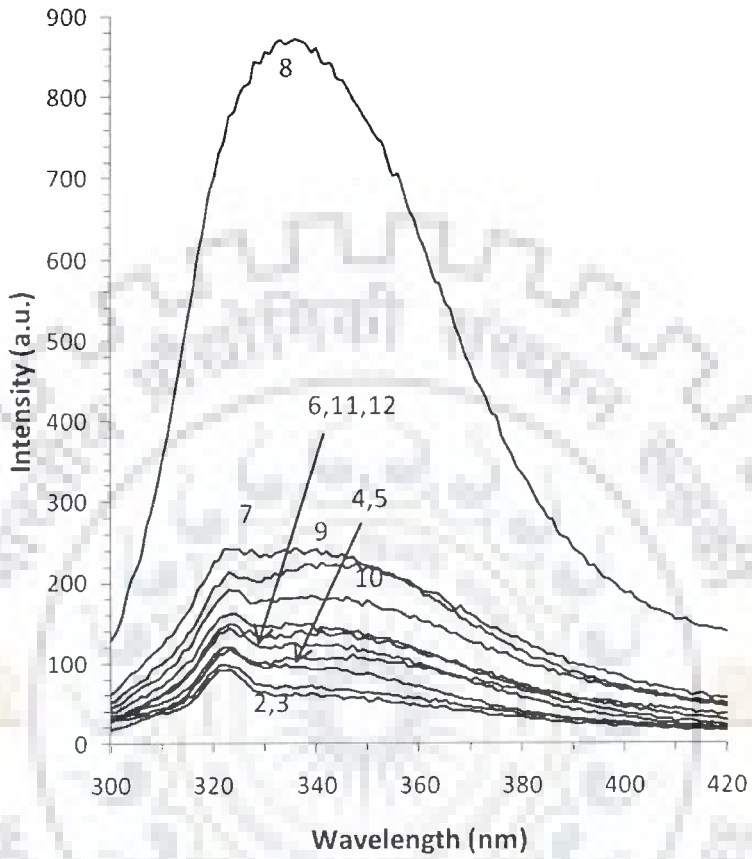

Figure 4.15: - Tryptophan fluorescence emission spectra of PRTI as a function of different pH values. Excitation wavelength was set at 290 nm. Numbers given to the spectra in the figure also indicate the pH values.

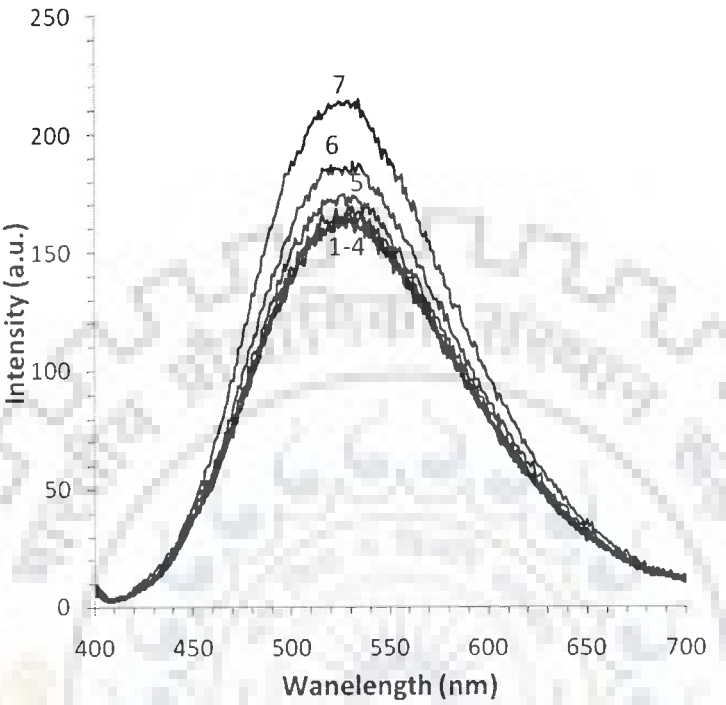

Figure 4.16: - ANS fluorescence emission spectra of PRTI as a function of DTT concentration. PRTI is quite stable at DTT concentration ranging from 5 mM to 200 mM (1-7, shows 05, 10, 20, 50, 100 and 200 mM DTT).

4.4 Discussion

Far-UV CD spectroscopy studies (240 -200 nm wavelength range) were carried out to analyze the secondary structure and conformational stability of PRTI at different temperatures from 20 to 100 °C. Analysis of CD spectra of native PRTI showed that it is an α, β protein with negative peaks at around 217 nm and 208 nm. Although, negative ellipticity was present but no clear negative peak characteristic of α-helical structures was observed at 222 nm. These results strongly suggest that PRTI is α, β protein rather than predominantly β protein. Most trypsin inhibitors particularly Kunitz family inhibitors are predominantly β-sheet proteins with little helical content (Sweet et al., 1974; Onesti et al, 1991; Azarkan et al., 2006). CD studies at increasing temperature demonstrated the thermo stability of PRTI structure. The PRTI retained the back bone protein folding with no significant change in CD spectra up to 70 °C. A significant loss in ellipticity was observed at and above 90 °C. This correlates well with the results of inhibitory activity where 15% loss was observed at 80 °C and 80% at 90 °C. The inhibitory activity and CD studies at increasing temperatures showed that transition midpoint for PRTI lies close to 88 °C. The results were similar to other trypsin inhibitors which exhibited significant structural stability at high temperatures (Roychaudhuri et al., 2003; Azarkan et al., 2006; Macedo et al., 2003) The structural composition of the PRTI was determined by deconvolution of the spectra using the program K2D (Yang et al., 1986; Andrade et al., 1993). The results obtained at 20 °C indicated that the protein has a low content of the secondary β-sheet (7%) structural elements, and a high content α-helix (61%) and unordered structure (32%). Temperature denaturation study at 20, 30, 50, 70, 80, 90, 100 °C showed that at 80 °C protein has >80% activity with decrease in α-helical contents from 61% to 37%, at 90 °C protein lost ~80% inhibitory activity with sharp decrease in α-helical contents to 16%. These results suggest

helix to coil transition at high temperature and possibly α-helices may play an important role in inhibitory activity of PRTI. Also, the possibility of a relatively large error in determining secondary-structure content by deconvolution of CD spectra (K2D) remains very high. Most trypsin inhibitors particularly Kunitz-type inhibitors are predominantly β-sheet proteins with little helical content. These studies have shown that *Erythrina* trypsin inhibitor (ETI), approximately >35% β-sheet, 18% turn and 42% random coil; Papaya proteinase inhibitor (PPI), approximately >35% β-sheet, 18% turn and 42% random coil; and Soybean trypsin inhibitor (STI) contains approximately >25% β-sheet, 16% turn and 50% random coil (Sweet et al., 1974; Onesti et al, 1991; Azarkan et al., 2006). On comparing the structure of PRTI with other Kunitz type inhibitors, it becomes clear that PRTI is a α-β protein with higher α-helical contents rather than a predominantly β-sheet protein.

Fluorescence spectroscopy is a very powerful and sensitive technique to monitor conformational changes in proteins. The fluorophores can be either intrinsic (tryptophan) or extrinsic (ANS: 8-anilino-1-naphthalene sulfonate) probes. In proteins, the three aromatic amino acids, phenylalanine, tyrosine, and tryptophan are all fluorescent. These three amino acids are relatively rare in proteins. The fluorescence property of tryptophan has long been utilized to understand the folding/unfolding, substrate binding and conformational heterogeneity in different physicochemical conditions. A valuable feature of intrinsic protein fluorescence is the high sensitivity of tryptophan to its local environment. Changes in the emission spectra of tryptophan often occur in response to conformational transitions, subunit association, substrate binding, or denaturation. A complicating factor in the interpretation of protein fluorescence is the presence of multiple fluorescent amino acids in most proteins. The environment of each residue is distinct and the spectral properties of each residue are generally different. Therefore

we studied both intrinsic (tryptophan) and extrinsic fluorescence with the help of ANS (8-anilino-1-naphthalene sulfonate) dye probe. Effect of Guanidinium Hydrochloride (GuHCl) and Urea were studied and found that both GuHCl and Urea show similar effect on the conformation of PRTI after incubation for 2 h. As the concentration of both denaturants increases the PRTI unfold slowly and above 7M and at 8M, protein completely unfolds. These observations suggest that PRTI is not very stable in the Urea and GuHCl medium and protein completely denature and unfold at 8M. After 22 h incubation of PRTI with Urea and GuHCl also shows the similar and did not observe much change in emission spectra. Tryptophan fluorescence measurement were also taken for both denaturants and clearly observed that PRTI unfolds as the concentration increases. Both the tryptophan and ANS fluorescence emission of PRTI reveals that PRTI is not very stable in urea and GuHCl, as the concentration increases gradually unfolding rises and at 8M and above, protein completely loss the native conformation and unfolded molten globule structure present.

In the presence of SDS (0.2-10%) detergent, intensity of the ANS emission spectra of PRTI increases rapidly up to 2% SDS and above that (3-10%) increases slowly, indicates that SDS work as a strong unfolding agent for PRTI. But in the tryptophan emission measurement, up to 5% SDS there was not much changes observed in the intensity, after 5% SDS and in between 6-10% SDS changes observed in the emission intensity with the λ_{max} at 325 nm. The probable reason of these findings may be that tryptophan residues are buried tightly in the intrinsic core of the protein and could not expose with incubation of 5% SDS. As the SDS concentration increases PRTI unfolds and tryptophan residues exposed in the medium, due to the effect of fluorescence quenching by the aqueous medium, intensity decreases and the λ_{max} emission spectra shifted at 325 nm. The ANS fluorescence measured at various concentration of

HCl showed a constant increase in ANS fluorescence with increasing concentration of HCl up to 1.0 M indicating a gradual unfolding of the native structure. Tryptophan fluorescence spectra at various concentration of HCl showed sharp decrease in the intensity as concentration increases to 200 mM with fluorescence λ_{max} at 322 nm. Probably tryptophan moiety of protein exposed to the medium and fluorescence quenched, above 300 mM only gradual decrease in the intensity were observed. SKTI also exhibited the similar results where it showed maximum fluorescence at 190 mM HCl, reflecting the ANS ability to access the hydrophobic core of the molten state of SKTI (Roychaudhuri et al. 2004). Temperature ANS fluorescence studies reveals that PRTI is very stable at temperature ranging from 20-100 °C and only slight changes occur in emission spectra between 20-50 °C range. Earlier studies also confirmed that PRTI maintained its activity up to the 80 °C and only 15% loss observed and above 90 °C protein lost 80% inhibitory activity (Chaudhary et al. 2008). ANS and tryptophan emission spectra showed that PRTI remains conformationaly unchanged at pH 6.0 to 9.0 and gradual changes occur at alkaline pH (pH 10, 11, 12) besides at acidic pH more conformational changes observed in an increasing order from pH 5.0 to pH 2.0. However PRTI maintained above 95% of trypsin inhibitory activity in the pH raging 2.0-12 and maximum activity showed at pH 8.0. Relatively very high λ_{max} at 335 nm was observed at pH 8.0 of Tris-HCl buffer compared to spectra at other pH values (pH 7.0 and 9.0) of the same buffer. Possibly this reflects that particularly at pH 8.0 of Tris-HCl, the tryptophane residues of PRTI are buried inside the hydrophobic core and conformationally more stable than any other pH values. This finding is well correlates the maximum inhibitory activity of PRTI at pH 8.0. The quenching in the emission intensity at extremes of acidic and alkaline pH indicated that the range of tested pH condition affects the PRTI conformation, but was not enough to induce complete denaturation. Similar results

showed by the SPCI where conformational changes occur although activity remains stable (Teles et al., 2004). ANS fluorescence emission after 1 h incubation with DTT exhibited that up to 100 mM DTT concentration there was no significant changes occur only slight changes observed with 200 mM DTT. This structural stability in DTT is also well related with the inhibitory activity stability, where it is found that PRTI retain 95% inhibitory activity in 100 mM DTT against trypsin. This suggests that both conformation and inhibitory activity are not affected by the reduction of disulphide bridges. Similar results have been earlier reported for a trypsin inhibitor from *Erythrina caffra* (ETI) which retained inhibitory activity after reduction with DTT (Lehle et al., 1996).

In summary the work done in this chapter demonstrate that CD analyses of PRTI revealed that it is a α, β protein rather than predominantly β-sheet protein and the polypeptide backbone folding of the protein was retained up to 80 °C. The transition midpoint of native PRTI lies close to 88 °C. Far UV CD spectra of PRTI of native and temperature denaturation and CD spectra analysis with the K2D program server showed that PRTI is a α- β protein with negative peaks at around 217 nm and 208 nm with higher α-helical (61%) contents. Analysis of CD spectra of temperature denaturation exhibited that PRTI having helix to coil transition at high temperature and possibly α-helices may play an important role in inhibitory activity of PRTI. ANS and intrinsic tryptophan fluorescence monitoring studies of conformational stability exhibited that PRTI gradually unfolds as the concentration of GuHCl and Urea increases and above 8M, completely unfolded molten globule structure present. PRTI lost its native conformation after incubation with 3-5% SDS and 200-1000 mM HCl concentration. Fluorescence emission spectra analysis significantly correlates the structure-activity relationship when studied as a function of DTT, pH and temperature denaturation. PRTI structure and

inhibitory activity was retained up to 100 mM DTT, 80 °C temperature and in highly alkaline and acidic pH ranging from 2.0-12.

CONCLUSIONS

CONCLUSIONS

Purification of *Putranjiva roxburghii* trypsin inhibitor (PRTI)

- *Putranjiva roxburghii* belonging to *Euphorbiaceae* family is an ornamental tree of tropical India known as child life tree. To date, no protein has been characterized from this plant. This research work describes the purification and characterization of a highly stable and potent trypsin inhibitor from the seeds of *Putranjiva roxburghii.*

- The *Putranjiva roxburghii* trypsin inhibitor (PRTI) was purified to homogeneity by acid precipitation, cation-exchange (CM sepharose) chromatography, anion-exchange (DEAE sepharose) chromatography and size-exclusion chromatography on HPLC.

- The SDS-PAGE analysis under both reducing and non-reducing conditions confirmed that PRTI is a single polypeptide chain with approximate molecular mass of 34 kDa.

- PRTI remains very active during the various purification procedures and does not lose any inhibitory activity during purification.

Biochemical characterization of PRTI

- N-terminal sequence (Arg-Pro-Pro-Gln-Ala-Gly-Tyr-Ile-Gly-Val) analysis of 10 amino acids, with a NCBI BLAST short sequence search, did not showed resemblance with any trypsin inhibitors.

- Two peptides of 13 and 16 amino acids obtained from partial internal sequencing by mass spectrometry (LC-MS/MS and MALDI-TOF/TOF) analysis showed significant identity to Kunitz-type inhibitors. Peptide1 showed 100% identity to winged bean chymotrypsin inhibitor-3 and Peptide2 showed 100, 73 and 73% identity to *Acasia confussa* trypsin inhibitor, *Prosopsis juliflora* trypsin inhibitor and trypsin isoinhibitors DE5 of *Adenanthera pavonina* L. respectively.

- The resemblance of partial internal sequencing peptides with some Kunitz-type inhibitor clearly suggests that PRTI may belong to Kunitz type inhibitor family.

- The PRTI protein completely inhibited trypsin at a molar ratio of 1:1 of inhibitor: trypsin, but did not show any significant inhibition against α-chymotrypsin.

- The analysis of Dixon plot showed that the PRTI is a competitive inhibitor where two lines corresponding to each substrate intersect above the x-axis, a characteristic of competitive inhibition.

- The dissociation constant (Ki) value was found to be 1.4×10^{-11} M which clearly indicates that PRTI is a highly potent inhibitor of bovine trypsin.

- In thermal stability studies, trypsin inhibitory activity of PRTI was completely retained up to 70 °C. Above 70 °C, there was a slight decrease in the inhibitory activity with PRTI retaining almost 85% inhibitory activity up to 80 °C.

- The inhibitory activity of PRTI fell sharply above 80 °C with a lose of almost 80% inhibitory activity at 90 °C.

- In pH stability studies, PRTI was highly stable under conditions ranging from highly acidic to highly alkaline. PRTI showed maximum inhibition at pH 8.0 and maintained over 95% of its inhibitory activity through a pH gradient of 2-12.

- The inhibitory activity of PRTI in the presence of DTT was found completely stable with no loss in inhibitory activity when incubated for 1 h up to 100 mM DTT. Only a slight decrease of 5% in inhibitory activity was observed when PRTI was incubated for 2 h at 100 mM DTT.

- PRTI is proteolytically resistant against trypsin, chymotrypsin and pepsin enzyme and susceptible against nonspecific proteinase proteinase-K and aspartate-proteinase papain.

- Proteolytic stability against serine proteinase inhibitors i.e. trypsin, chymotrypsin and pepsin showed that PRTI have very compact and rigid structure and does not provide the access for the proteinase binding and activity.

- PRTI is partially cleaved by papain and completely cleaved by proteinase k enzyme. Time dependant proteolytic cleavage studies were also performed to determine any

domain structure of PRTI but there was no such difference observed in the cleavage pattern.

- PRTI retained almost 90% inhibitory activity after one year storage at -20 °C.

Biophysical characterization of PRTI

- Analysis of CD spectra (240 -200 nm wavelength range) of native PRTI showed that it is an α, β protein with negative peaks at around 217 nm and 208 nm. Although, negative ellipticity was present but no clear negative peak characteristic of α-helical structures was observed at 222 nm.

- These results strongly suggest that PRTI is α, β protein rather than predominantly β protein. Most trypsin inhibitors particularly Kunitz family inhibitors are predominantly β-sheet proteins with little helical content.

- CD studies at increasing temperature demonstrated the thermo stability of PRTI structure. The PRTI retained the back bone protein folding with no significant change in CD spectra up to 70 °C. A significant loss in ellipticity was observed at and above 90 °C.

- This correlates well with the results of inhibitory activity where 15% loss was observed at 80 °C and 80% at 90 °C.

- The inhibitory activity and CD studies at increasing temperatures showed that transition midpoint for PRTI lies close to 88 °C.

- GuHCl and Urea show similar effect on the conformation of PRTI after incubation for 2 h. As the concentration of both denaturants increases the PRTI unfold slowly and above 7M and at 8M, protein completely unfolds and above 8M concentration did not found any increased changes in fluorescence intensities.

- Both the tryptophan and ANS fluorescence emission of PRTI reveals that PRTI is not very stable in urea and GuHCl, as the concentration increases gradually unfolding rises and at 8M and above, protein completely loss the native conformation and unfolded molten globule structure present.

- In the presence of SDS (0.2-10%) detergent intensity of the ANS emission spectra of PRTI increases rapidly up to 2% SDS and above that (3-10%) increases slowly, indicates that SDS work as a strong unfolding agent for PRTI. But in the tryptophan emission measurement, up to 5% SDS there was not much changes observed in the intensity. After 5% SDS and in between 6-10% SDS changes observed in the emission intensity with the λ_{max} at 325 nm.

- The ANS fluorescence measured at various concentration of HCl showed a constant increase in ANS fluorescence with increasing concentration of HCl up to 1.0 M indicating a gradual unfolding of the native structure.

- Tryptophan fluorescence spectra at various concentration of HCl showed sharp decrease in the intensity as concentration increases to 200 mM with fluorescence λ_{max} at 322 nm.

- Temperature ANS fluorescence studies reveals that PRTI is very stable at temperature ranging from 20-100 °C and only slight changes occur in emission spectra between 20-50 °C range.

- ANS and tryptophan emission spectra showed that PRTI remains conformationally unchanged at pH 6.0 to 9.0 and gradual changes occur at alkaline pH (pH 10, 11, 12) besides at acidic pH more conformational changes observed in an increasing order from pH 5.0 to pH 2.0. However PRTI maintained above 95% of trypsin inhibitory activity in the pH raging 2.0-12 and maximum activity showed at pH 8.0.

- Relatively very high λ_{max} at 335 nm was observed at pH 8.0 of Tris-HCl buffer compared to spectra at other pH values (pH 7.0 and 9.0) of the same buffer, possibly this reflects that particularly at pH 8.0 of Tris-HCl, the tryptophan residues of PRTI are buried inside the hydrophobic core and conformationally more stable than any other pH values. This finding is well correlates the maximum inhibitory activity of PRTI at pH 8.0.

- ANS fluorescence emission after 1 hr incubation with DTT exhibited that up to 100 mM DTT concentration there was no significant changes occur only slight changes observed with 200 mM DTT.

- This structural stability in DTT is also well related with the inhibitory activity stability, where it is found that PRTI retain 95% inhibitory activity in 100 mM DTT against trypsin. This suggests that both conformation and inhibitory activity are not affected by the reduction of disulphide bridges.

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