ISOLATION, PURIFICATION AND CHARACTERIZATION OF PROTEASE INHIBITOR FROM *MORINGA OLEIFERA* LAM.

Thesis submitted to the
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In
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by

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CERTIFICATE

This is to certify that the research work presented in the thesis entitled "**Isolation, purification and characterization of Protease Inhibitor from *Moringa oleifera* Lam.**" is based on the original research work carried out by Miss. Bijina B under my guidance and supervision at the Department of Biotechnology, Cochin University of Science and Technology, in partial fulfillment of the requirements for the degree of Doctor of Philosophy, and that no part thereof has been presented for the award of any other degree.

M. CHANDRASEKARAN

DECLARATION

I hereby declare that the work presented in this thesis entitled "**Isolation, purification and characterization of Protease Inhibitor from *Moringa oleifera* Lam.**" is based on the original research carried out by me at the Department of Biotechnology, Cochin University of Science and Technology, Cochin under the guidance of Dr. M Chandrasekaran, Professor in Biotechnology and the thesis or no part thereof has been presented for the award of any degree, diploma, associate ship or other similar titles or recognition.

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Dedicated to my Parents

ABBREVIATIONS

%	-	Percentage
°C	-	Degree Celsius
A_{280}	-	Absorbance at 280nm
AIDS	-	Aquired Immunodeficiency Syndrome
Arg	-	Arginine
Asn	-	Asparagine
Asp	-	Aspartate
ATP	-	Adenosine tri phosphate
BAPNA	-	Nα-Benzoyal-arginine-4-nitro aniline
BSA	-	Bovine serum albumin
CM Cellulose	-	Carboxy methylcellulose
Cys	-	Cysteine
Da	-	Dalton
DEAE Cellulose	-	Diethyl amino ethyl cellulose
DEPC	-	Diethyl pyrocarbonate
DMSO	-	Dimethyl sulphoxide
DW	-	Distilled water
EDTA	-	Ethylene diamine tetra acetic acid
FPLC	-	Fast protein liquid chromatography
FT-IR	-	Fourier transform infra red spectroscopy
g	-	grams
Glu	-	Glutamic acid
Gly	-	Glycine
HCl	-	Hydrochloric acid

His	-	Histidine
Hrs	-	Hours
IC_{50}	-	Molar concentration of the inhibitor that gives 50% of the target enzyme activity
ICP-AES	-	Inductively coupled plasma atomic emission spectroscopy
K_{av}	-	Partition coefficient
kDa	-	Kilo Dalton
K_i	-	Dissociation constant
K_m	-	Substrate concentration at which the reaction velocity is maximum
Lys	-	Lysine
M	-	Molar
mg	-	milligram
ml	-	milliliter
mm	-	millimeter
M_r	-	Relative molecular weight
NBS	-	N-bromo succinamide
nM	-	nanomole
OD	-	Optical density
PAGE	-	Polyacrylamide gel electrophoresis
PEG	-	Polyethylene glycol
pI	-	Isoelectric point
PI	-	Protease Inhibitor
PMSF	-	Phenyl methyl sulphonyl fluoride
rpm	-	Rotations per minute

SDS	-	Sodium dodecyl sulphate
Ser	-	Serine
sp.	-	Species
TCA	-	Trichloro acetic acid
TEMED	-	N-N-N'-N'-tetramethyl ethylene diamine
Thr	-	Threonine
V_{max}	-	Maximal velocity
V_e	-	Elution volume
V_o	-	Void volume
µg	-	microgram
µl	-	microlitre
µM	-	micromole

CONTENTS

Chapter 1

INTRODUCTION

Enzyme inhibitors have received increasing attention as useful tools not only for the study of enzyme structures and reaction mechanisms but also for potential utilization in pharmacology (Bode and Huber, 1992; Cyran, 2002; Imada, 2005; Robert, 2005) and agriculture (Ahn et al., 2004; Terashita et al., 1980). Specific and selective protease inhibitors are potentially powerful tools for inactivating target proteases in the pathogenic process of human diseases such as emphysema, arthritis, pancreatitis, thrombosis, high blood pressure, muscular dystrophy, cancer and AIDS (Demuth, 1990; Johnson and Pellecchia, 2006).

Enzyme inhibitors are molecules that interact in some way with the enzyme to control the metabolic processes of a normal cell. They control almost all of the enzymatic metabolic processes that take place in and around the cell, to maintain normal biological function. Basically inhibitors are artificial or natural substances, which act by changing the conformation or the turn over number of the enzyme thereby altering the rate of the specific reaction that they catalyze.

Biological processes like signal transduction, cell cycle regulation, gene regulation etc. rely on protein-protein interactions (Changhui et al., 2004). These interactions also have an unavoidable role in cell dysfunction and may lead to the so called diseased condition of the

organism. In other words, the very same enzymes, which play many important biological roles in cell regulation, could also be harmful if left unchecked. For this reason, the system seeks the help of inhibitors, giving the organism both temporal and spatial control of the enzymatic activity (Fitzpatrick, 2004). The study of enzyme inhibitors for blocking particular protein-protein interactions would enable exquisite control of cellular processes and provide potential leads for novel chemotherapeutic agents.

The existence of specific naturally occurring enzyme inhibitors, like antithrombin, antipepsin and antitrypsin, controls the enzyme activity in human body and under physiological circumstances assures their intracellular and extracellular action (Gordana et al., 2002).

Proteases are single class of enzyme, which occupy the pivotal position with respect to their physiological role and commercial application. They represent one of the largest groups of industrial enzymes and account for about 60% of total worldwide sale of enzymes. They play widespread role in a variety of essential biological processes, both as nonspecific mediators of protein degradation and in the catalysis of specific cleavage events. Proteases have wide range of functions in nature like, in the regulation of biological metabolic processes such as spore formation, spore germination, protein maturation in viral assembly, activation of certain viruses in pathogenicity, various stages of mammalian fertilization process, blood coagulation, fibrinolysis, complement activation, phagocytosis and blood pressure control (Johnson and Pellecchia, 2006). Misregulation of proteolysis has been implicated in neoplastic, autoimmune and infectious

diseases, and a number of pathogens carry proteases essential for viability or infectivity (Rao et al., 1998).

Proteases have a long history of application in various biotechnological industries. They are extensively used mainly in the detergent and food industries. In view of the recent trend of developing environment friendly technologies, proteases are envisaged to have extensive applications in leather treatment and in several bioremediation processes. Their application in the leather industry for dehairing and bating of hides to substitute currently used toxic chemicals is a relatively new development and has conferred added biotechnological importance (Rao et al., 1998).

Besides being necessary from the physiological point of view, proteases are potentially hazardous to their proteinaceous environment and the respective cell or organism must precisely control their activity. When uncontrolled, proteases can be responsible for serious diseases. While these proteases are highly beneficial, they are also very dangerous if left unchecked. To limit these dangers, they must be strictly controlled both in time and place. Nature therefore, evolved controls for proteases. The control of proteases is generally achieved by regulated expression, secretion, activation and degradation of mature enzymes, and by the inhibition of their proteolytic activity. Protease inhibitors play very important role in the regulation of protease activity. The scientific study of the protease inhibitors is nearly as old as that of the proteases themselves. Hundreds of inhibitors of proteases are now known. Since the protein inhibitors of proteolytic enzymes are, indeed, proteins, they should be substrates for proteolysis. The

elucidation of this paradox remains a central focus for much of the work on the structure and function of protein inhibitors of proteases. Inhibitor proteins have therefore, been studied as model systems for elucidation of the mechanism of inhibition of proteases and also for studies of protein-protein interactions. The recognized importance of proteolytic processes in the regulation of post-translational processing of precursor proteins, and the involvement of proteases in intracellular protein metabolism and in various pathological processes has recently stimulated tremendous interest in naturally occurring, target oriented, protease inhibitors. They are exploited as valuable tools in medical research by virtue of their unique pharmacological properties that suggest clinical application (Hsu et al., 2006).

Proteins that form complexes with proteases and inhibit their proteolytic activity are wide spread in nature (Ryan. 1990). In addition to their roles in regulating proteolytic activities, they are important for protecting fluids or tissues from degradation by unwanted or foreign proteolytic activities (Neurath, 1984). Protease inhibitors are an important class of regulatory proteins that control the proteolytic events in all living organisms and are ubiquitously present in all life forms (Kassel, 1970; Umezawa, 1982). They have evoked tremendous interest because of their pivotal role in the regulation of various physiological and pathological processes involving cellular transformation, blood-clotting disorders, osteoporosis. in retroviral diseases and also in many protozoan diseases (Billing et al.. 1987).

Inhibitor families have been found that are specific for each of the mechanistic classes of proteolytic enzymes, i.e. serine, cysteine, aspartic and metalloproteases. Several nonhomologous families of protease inhibitors are recognized among microorganisms, plant and animal kingdom (Laskowski and Kato, 1980; Seelmeir et al., 1988). High concentrations of protease inhibitors are often found in fluids and tissues that are particularly vulnerable to foreign proteases (Neurath, 1984), such as blood serum (Travis and Salvesen, 1983), pancreatic acinar cells (Neurath, 1984), and storage tissues of plants (Richardson, 1980; Ryan, 1973).

Plants are important source of easily available proteases and protease inhibitors. Possibly ten protease inhibitor families have been recognized in plants and mostly they are located in seeds and leaves (Garcia-Oimedeo et al., 1987). In tuberous plants, they are present in the leaves and tubers. Among the cereals such as corn, barley, wheat and rye, they are primarily present in the endosperm. Plants contain a variety of serine protease inhibitors, which can be divided into at least 12 sub-families. Serine protease inhibitors have been the most studied protein inhibitors up to now and recently a considerable advance has been made in the study of the natural inhibitors of cysteine proteases (cystatins). In contrast, knowledge of inhibitors of both aspartyl and metalloproteases is very limited. The plant protease inhibitors differ in specificities and in their ability to inhibit one or more proteases at the same time. Majority of them inhibit trypsin and many inhibit chymotrypsin. Inhibitors of elastase, kallikrein, plasmin, subtilisin and thrombin have also been found (Ryan, 1990).

The protease inhibitors have been thought to play a vital role in the arsenal of defense mechanisms that plants use to protect against environment hazards during germination and seed growth. Pest and pathogens are major constraints to plant growth and development, resulting in heavy losses in crop yield and quality (Leo and Gallerani, 2005). Plant proteinase inhibitors have been well established to play a potent defensive role against predators and pathogens. Agricultural industries employ several chemical pesticides to solve this problem. Since the use of these chemical pesticides has a deleterious effect on human health, a recent trend is to use other safer strategies to enhance the defense mechanism of crops. Plant defense against herbivores and fungal pathogens involves the expression of a variety of bioactive secondary metabolites and defensive proteins. These defensive proteins include protease inhibitors, which can act as insect antifeedants and as antifungal proteins (Haq et al., 2004; Ryan, 1990).

Plants are known to accumulate protease inhibitors in their storage organs in amounts much more than required to inhibit endogenous proteases. This is apparent by their specific inhibitory activity towards pest proteases and little if any, against endogenous plant proteinases (Lawrence and Koundal, 2002). Further more, it has been demonstrated that wounding of plant tissue by insect chewing or by microbial infection significantly enhances the level of protease inhibitors in local as well as in the remote tissues (Green and Ryan, 1972). Inhibition of enzymes in the alimentary tract of insects is not the main adverse effect. Depletion of essential amino acids due to over secretion of digestive enzymes in the presence of inhibitors is thought to cause most of the toxicity signs observed, but there

are also other targets of toxicity (Kleter et al, 2000). The mechanism of action of protease inhibitors is not fully understood.

The major cause of food spoilage is microbial growth and metabolism resulting in the formation of amines, sulfides, alcohols, aldehydes, ketones and organic acids with unpleasant and unacceptable off-flavors. Microbial food spoilage is an area of global concern as it has been estimated that as much as 25% of all food produced is lost post-harvest owing to microbial activity (Parker, 2003). Thorough understanding of the growth and activity of spoilage microflora in seafood as well as any other food is crucial for the development of effective preservation techniques and subsequent reduction of losses due to spoilage. The use of enzyme inhibitors may prove to be yet another solution for the spoilage problem. Several antagonistic compounds have been isolated from microorganisms for use in fish preservation. The use of an adequate amount of natural protease inhibitors could be an effective way to extend the shelf life of many proteinaceous seafoods such as salted fish products. In this approach the inhibitors can retard the aging and other deteriorative processes caused by the action of endogenous and exogenous proteases, during the food processing and preservation. Ofcourse there is dearth of information in respect of application of enzyme inhibitors for seafood preservation. Protease inhibitors from plants also have a major role in food industry during bread making (Fernando et al., 2000).

Protease inhibitors used as antiviral agents have a wide range of market potential. The pharmaceutical industry accounts for only 1% of the total global sale. Out of this, HIV antiretroviral have experienced rapid

growth between 2000-2006, accounting for $6.6 billion in global sales. Norvir, a HIV protease inhibitor with high market value, is produced by Abott Laboratories, U.S.A. Tripanavir, another protease inhibitor in combination with Norvir is the most expensive antiretroviral drug ever developed (over $20,000 a year) (Gardiner, 2004). The anti-infectives market is poised to experience considerable growth in the next few years, with a forecast market value that is expected to double in size to more than $44 billion by 2010. In India, Ranbaxy Laboratories, Dr Reddy's and Cipla Laboratories are the pioneers in the production of protease inhibitors. Aprotinin is a protease inhibitor that has uses in biochemical research, medicine, and potentially in agriculture and is sold by Bayer under the name of Trasylol. It is best known as a clotting agent used to reduce blood loss in heart surgery (Landis, 2001), and has also been administered for over three decades in the treatment of acute pancreatitis (Belorgey, 1996). There are eight FDA approved protease inhibitors so far, they are: Amprenavir (Agenerase), Fosamprenavir (Lexiva), Indinavir (Crixivan), Lopinavir/ritonavir (Kaletra), Ritonavir (Norvir), Saquinavir (Fortovase), and Nelfinavir (Viracept) (Hanson and Hicks, 2006; Hsu et al., 2006).

In this context, in the present study, an attempt was made to screen effective inhibitors for serine proteases, from different plants which are locally available in Kerala, that might have the potential for application in pharmaceutical and food industry.

OBJECTIVES OF THE PRESENT STUDY

To date, several hundred bioactive compounds have been isolated from plant sources. Among them, protease inhibitors have drawn the attention recently owing to their pivotal role in pharmaceutical, agricultural and in industrial field. Inspite of the reports available on the scope for utilizing plants as useful source for deriving protease inhibitors all the commonly and locally available plant sources have not been explored as potential source. With the anticipation that commonly and widely available plants could return industrially and pharmacologically important protease inhibitor, an attempt was made to screen local plants for serine protease inhibitors and select a potential candidate for possible applications.

Thus, the primary objectives of the present study included

1. Screening of plants for serine protease inhibitor
2. Selection of potential source and isolation of the inhibitor
3. Purification of protease inhibitor
4. Characterization of protease inhibitor
5. Evaluation of protease inhibitor for various applications.

Chapter 2

REVIEW OF LITERATURE

2.1 Proteases

Proteases, also denominated as proteinases or peptidases, constitute one of the largest functional groups of proteins, with more than 560 members actually described (Barrett et al., 1998). By hydrolyzing one of the most important chemical bonds present in biomolecules, i.e., the peptide bond, proteases play crucial functions in organisms all over the phylogenetic tree, starting from viruses, bacteria, protozoa, metazoa, or fungi, and ending with plants and animals. Proteolytic enzymes are essential for the survival of all kinds of organisms, and are encoded by approximately 2% of all genes (Barrett et al., 2001).

Proteases play a critical role in many complex physiological and pathological processes such as protein catabolism, blood coagulation, cell growth and migration, tissue arrangement, morphogenesis in development, inflammation, tumor growth and metastasis, activation of zymogens, release of hormones and pharmacologically active peptides from precursor proteins, and transport of secretory proteins across membranes (Chambers and Laurent, 2001). In general, extracellular proteases catalyze the hydrolysis of large proteins to smaller molecules for subsequent absorption by the cell whereas, intracellular proteases play a critical role in the regulation of metabolism. Since proteases are physiologically necessary for living

organisms, they are ubiquitous, being found in a wide diversity of sources such as plants, animals, and microorganisms. Besides being necessary from the physiological point of view, proteases are potentially hazardous to their proteinaceous environment and the respective cell or organism must precisely control their activity. When uncontrolled, proteases can be responsible for serious diseases. The control of proteases is generally achieved by regulated expression/secretion and/or activation of proproteases, by degradation of mature enzymes, and by the inhibition of their proteolytic activity (Fitzpatrick, 2004).

2.1.1 Classification of proteases

Proteases are grossly subdivided into two major groups, i.e., exopeptidases and endopeptidases, depending on their site of action. Exopeptidases cleave the peptide bond proximal to the amino or carboxy termini of the substrate, whereas endopeptidases cleave peptide bonds distant from the termini of the substrate. Based on the functional group present at the active site, proteases are further classified into four prominent groups, i.e., serine proteases, aspartic proteases, cysteine proteases, and metalloproteases (Barrett et al., 1998; Hartley, 1960). There are a few miscellaneous proteases that do not precisely fit into the standard classification, e.g., ATP-dependent proteases which require ATP for activity (Menon and Goldberg, 1987). Based on their amino acid sequences, proteases are classified into different families (Argos, 1987) and further subdivided into "clans" to accommodate sets of peptidases that have diverged from a common ancestor (Rawlings and Barrett, 1993). Each family of peptidases have been assigned a code letter denoting the type of

catalysis, i.e., S, C, A, M, or U for serine, cysteine, aspartic, metallo, or unknown type, respectively (Rao et al., 1998).

2.2 Protease inhibitors

Proteases in biological systems are controlled by various mechanisms. They can be inactivated by proteolytic degradation or by binding with inhibitor molecules. The inhibitor can bind at the active site by mimicking the structure of the tetrahedral intermediates that occurs in the enzyme-catalyzed reaction (Bode and Huber, 2000). So the study of enzyme inhibitors provide valuable information on the mechanism and pathway of enzyme catalysis, the substrate specificity of the enzyme, the nature of the functional group of active site and the participation of the certain functional group in maintaining the active site conformation of the enzyme molecule. Proteases and their specific inhibitors are ubiquitously distributed in the plant, animal and microbial kingdoms, and play a key regulatory roles in many biological processes, including the blood coagulation system, the complement cascade, apoptosis and the hormone processing pathways (Neurath, 1989). Naturally occurring protease inhibitors are essential for regulating the activity of their corresponding proteases within these pathways.

Protease inhibitors, broadly distributed in nature, are proteins that form very stable complexes with proteolytic enzymes. The number of protease inhibitors isolated and identified so far is extremely large and hence form a good system to study aspects of molecular evolution and structure function relationships. Most of these inhibitors are small molecules with relative molecular masses ranging from 5-25 kDa, with compact

structures and many cases with a high content of disulphide bridges, characteristics that might contribute to their high thermal stability (Singh and Rao, 2002). The recent interest in the study of protease inhibitors is based on the fact that they can be a valuable tool in biochemical and biomedical studies (Umezawa, 1982). Protease inhibitors which specifically inhibit the proteases, that are essential in the life cycle of organisms that cause mortal diseases such as Malaria, Cancer and AIDS, can be used as strategy for drug design for the prevention of propagation of these causative agents (Johnson and Pellecchia, 2006).

2.3 Sources of protease inhibitors

Proteins that form complexes with proteases and inhibit their proteolytic activity are wide spread in nature controlling the proteolytic events in all living organisms (Laskowski and Kato, 1980; Neurath, 1984). Protease inhibitor from a variety of sources like plants, animals and microorganisms have been purified and characterized. Most of the protease inhibitors found are well characterized in plants and belongs to the group of serine protease inhibitors, which include trypsin (Richardson, 1991). The physiological significance of protease inhibitors has been extensively investigated in plants but little is known about in animals and microorganisms.

2.3.1 Plants as the source of protease inhibitors

A large number of protease inhibitors have been isolated and identified from plants (Tamir et al., 1996). Plant protease inhibitors are small proteins, generally present at high concentrations in storage tissues

(up to 10 % of total protein content), but also detectable in leaves in response to the attack of insects and pathogenic microorganisms (Ryan, 1990). Plant protease inhibitors continue to attract the attention of researchers because of their increasing use in medicine and biotechnology (Dunaevsky et al., 1998). In the course of evolution, plants have elaborated protective mechanisms that allow them to successfully resist different kinds of unfavorable conditions including insects and phytopathogenic microorganisms (Jackson and Tailor, 1996; Malek and Dietrich, 1999; Stotz et al., 1999). The defensive capacities of plant protease inhibitors rely on inhibition of proteases present in insect guts or secreted by microorganisms, causing a reduction in the availability of amino acids necessary for their growth and development (Lawrence and Koundal, 2002). Plant protease inhibitors have been mainly described in storage tissues such as tubers and seeds, but their occurrence in the aerial part of plants, as a consequence of several stimuli has also been widely documented (Lopes et al., 2004).

In plants at least 10 protease inhibitor families have been recognized (Garcia-Oimedeo et al., 1987). Plant protease inhibitors active towards the four mechanistic classes of proteases (serine, cysteine, aspartic and metalloproteases) have been described. The activity of protease inhibitors is due to their capacity to form stable complexes with target proteases, blocking, altering or preventing access to the enzyme active site. Protease inhibitors active towards serine proteases, the most widespread in nature, act as a potential substrate for proteases (Rawlings et al., 2004).

The possible role of protease inhibitors (PIs) in plant protection was investigated as early as 1947. Subsequently the trypsin inhibitors present in

soybean were shown to be toxic to the larvae of flour beetle, *Tribolium confusum* (Lipke et al., 1954). Following these early studies, there have been many examples of protease inhibitors active against certain insect species, both in *in vitro* assays against insect gut proteases (Koiwa et al., 1997; Pannetier et al., 1997) and in *in vivo* artificial diet bioassays (Samac and Smigocki, 2003; Urwin et al., 1997; Vain et al., 1998).

Plant protease inhibitor genes encode proteins that can inhibit insect digestive enzymes, resulting in starvation and even death of the insect. As their role of inhibitors is simply achieved by the activation of single genes, several transgenic plants expressing protease inhibitors have been produced in the last two decades and tested for enhanced defensive capacities, with particular efforts against pest insects (Michaud, 2000).

A protease inhibitor CpTi, exhibited a very broad spectrum of activity including suppression of pathogenic nematodes like *Globodera tabaccum*, *G. pallida*, and *Meloidogyne incognita* (Williamson and Hussey, 1996). The spore germination and mycelium growth of the fungus *Alternaria alternata* was inhibited by buckwheat trypsin/chymotrypsin inhibitor (Dunaveski et al., 1997). A cysteine protease inhibitor from Pearl millet inhibited growth of many pathogenic fungi including *Trichoderma reesei* (Joshi et al., 1998).

Members of the serine class of protease inhibitor have been the subject of extensive research than any other class of protease inhibitors. Such studies have provided a basic understanding of the mechanism of action that applies to the most serine protease inhibitor families and probably to the cysteine and aspartyl protease inhibitor families as well

(Barrett and Salvesan, 1986; Greenbaltt et al., 1989; Huber and Carrell, 1989). The role of serine protease inhibitors as defensive compounds against predators is well established. Most of the serine protease inhibitors families from plants are competitive inhibitors (Garcia-Oimedeo et al., 1987; Laskowski and Kato, 1980) and all apparently inhibit proteases with a similar standard mechanism (Laskowski and Kato, 1980). Additionally, serine proteinase inhibitors have anti-nutritional effects against several Lepidopteran insect species (Bown et al., 1998).

Isolation of the midgut proteinases from the larvae of Cowpea weevil, *C. maculatus* (Campos et al., 1989; Kitch and Mudrock, 1986) and bruchid *Zabrotes subfaceatus* (Lemos et al., 1987) confirmed the presence of cysteine mechanistic class of proteinase inhibitors. Cysteine proteinases isolated from insect larvae are inhibited by both synthetic and naturally occurring cysteine proteinase inhibitors (Wolfson and Murdock, 1987). The rice cysteine proteinase inhibitors are the most studied of all the cysteine protease inhibitors which are proteinaceous in nature (Abe and Arai, 1985) and highly heat stable.

Aspartic protease inhibitors have been recently been isolated from Sunflower (Park et al., 2000), Barley (Kervinen et al., 1999) and Cardoon (*Cyanara cardunculus*) flowers named as cardosin A (Frazao et al., 1999). A protein with a molecular weight of 10 kDa has been recently extracted from Pumpkin fruit phloem exudation (*Cucurbita maxima* L.). It acted as an aspartic proteinase inhibitor. Besides pepsin, it also suppressed activity of extracellular aspartic proteinase of the fungus *Glomerella cingulata* (the causative agent of anthracnose) (Christeller et al., 1998).

Plants have also evolved at least two families of metalloproteinase inhibitors, the metallo-carboxypeptidase inhibitor family in potato (Rancour and Ryan, 1968) and in tomato plants (Graham and Ryan, 1997) and a cathepsin D inhibitor family in potatoes (Keilova and Tomasek, 1976). The cathepsin D inhibitor (27 kDa) is unusual as it inhibits trypsin and chymotrypsin as well as cathepsin D, but does not inhibit aspartyl proteases such as pepsin, rennin or cathepsin E. The inhibitors of the metallo-carboxypeptidase from tissue of tomato and potato are polypeptides (4 kDa) that strongly and competitively inhibit a broad spectrum of carboxypeptidases from both animals and microorganisms, but not the serine carboxypeptidases from yeast and plants (Havkioja and Neuvonen, 1985). The inhibitor is found in tissues of potato tubers where it accumulates during tuber development along with potato inhibitor I and II families of serine proteinase inhibitors. The inhibitor also accumulates in potato leaf tissues along with inhibitor I and II proteins in response to wounding (Graham and Ryan, 1997). Thus, the inhibitors accumulated in the wounded leaf tissues of potato have the capacity to inhibit all the five major digestive enzymes *i.e.* trypsin, chymotrypsin, elastase, carboxypeptidase A and carboxypeptidase B of higher animals and many insects (Hollander-Czytko et al., 1985). Some reported protease inhibitors from variety of plants and its inhibitory properties are listed in the Table 2.1.

Table 2.1 Protease inhibitors from plants

Plant Source	Target Enzyme	Properties	References
Hyptis suaveolens	Trypsin	8.7kDa protein, strongly inhibits serine proteases	Cesar et al., 2004
Momordica charantia	Trypsin/ Chymotrypsin	Strong inhibitors of *Helicoverpa armigera* gut proteases	Manasi et al., 2003
Archidendron ellipticum	Serine proteinase	Very potent insect antifeedant.	Arindam et al., 2006
Solanum tuberosum	Chymotrypsin	Suppress the growth and development of the oomycete *P. infestans*	Valueva et al., 2003
Oryza sativa	Cysteine proteinase	Heat stable	Abe and Arai, 1985 ; Abe et al., 1987
Pennisetum glaucum L.	Cysteine proteinase	High antifungal activity	Joshi et al., 1998
Sunflower Barley	Aspartic protease "	High antifungal activity "	Park et al., 2000 Kervinen et al., 1999
Cyanara cardunculus	Aspartic protease	Antifungal activity	Frazao et al., 1999
Cucurbita maxima	Aspartic protease	10kDa protein	Christeller et al., 1998
Solanum tuberosum	Metallo-carboxypeptidase	4kDa, strongly and competitively inhibit a broad spectrum of carboxypeptidases	Rancour & Ryan, 1968
Potato	Metallo carboxypeptidase	Inhibits carboxypeptidases	Graham and Ryan, 1997
Chestnut fruit	Cysteine protease	Antifungal activity	Pernas et al., 1999
Pennisetum glaucum L	Cysteine protease	Antifungal activity	Joshi et al., 1998
Helianthus annuus	Trypsin	16kDa protein with antifungal activity	Marcela et al., 2000

2.3.2 Animals as the source of protease inhibitors

A major role for protease inhibitors in animals is to block the activity of endogenous proteinases in tissues where this activity would be harmful, as in case of pancreatic trypsin inhibitors found in mammals. Most animal species synthesize a variety of protease inhibitors with different specificities, whose function is to prevent unwanted proteolysis. They generally act by enabling access of substrates to the proteases' active site through steric hindrance. The serine classes of proteinases such as trypsin, chymotrypsin and elastase, which belong to a common protein superfamily, are responsible for the initial digestion of proteins in the gut of higher animals (Garcia-Oimedeo et al., 1987). *In vivo* they are used to cleave long, essentially intact polypeptide chains into short peptides, which are then acted upon by exopeptidases to generate amino acids, the end products of protein digestion. These three types of digestive serine proteinases are distinguished based on their specificity, trypsin specifically cleaving the C-terminal to residues carrying a basic side chain (Lys, Arg), chymotrypsin showing a preference for cleaving C-terminal to residues carrying a large hydrophobic side chain (Phe, Tyr, Leu), and elastase showing a preference for cleaving C-terminal to residues carrying a small neutral side chain (Ala, Gly) (Ryan, 1990).

Moreover, some of the protease inhibitors isolated from invertebrate sources are quite specific towards individual mammalian serine proteases. This also offers huge opportunities for medicine. Thus, the development of non-toxic protease inhibitors extracted from invertebrates for *in vivo* application may be quite important (Roston, 1996).

Cysteine proteinases are common in animals, eukaryotic microorganisms and bacteria as well as in plants (Barrett and Salvesan, 1986). In animals, they are sequestered lysosomal compartments or in the cytoplasm, where they are thought to be involved in intracellular protein turnover (Hershko and Ciechanover, 1982). Cysteine proteinases are not secreted as intestinal digestive enzymes in higher animals, but are found in midguts of several families of Hemiptera and Coleoptera where they appear to play important roles in the digestion of food proteins. These particular insects characteristically have mildly acidic pHs in their midguts near the pH optima of cysteine proteinases (pH-5) (Barrett, 1986).

Endogenous tight-binding inhibitors from the cystatin superfamily regulate lysosomal cysteine proteases from mammalian cells and plants. The presence of cystatin-like inhibitors in lower eukaryotes such as protozoan parasites has not yet been demonstrated, although these cells express large quantities of cysteine proteases and may also count on endogenous inhibitors to regulate cellular proteolysis.

Cystatins are known to act as defensive agents against bacteria (Blankenvoorde et al., 1998), viruses (BjoErck et al., 1990), and plant-eating insects. They can also regulate cell death (Erklund et al., 1997), antigen presentation (Pierre and Mellman, 1998) and their expression is changed in malignant processes (Kos and Lah, 1998). Some of the protease inhibitors reported in animals are detailed in Table 2.2.

Table 2.2 Protease inhibitors from animals

Animals	Inhibitor	Properties	Referenc
Onchocerca volvulus	Ov-SPI-1	Serine protease inhibitor	Louise et al., 2005
Anisakis simplex	*Anisakis simplex* inhibitor	Serine protease inhibitor	Stephen & Ju 1994
Ancylostoma ceylanicum	AceKI	Trypsin, Chymotrypsin and Pancreatic elastase inhibitor	Daniel et al., 2004
Trypanosoma cruzi	Chagasin	Endogenous cysteine protease inhibitor	Ana et al., 20
Schistocerca gregaria	SGTI	Canonical inhibitor of Bovine trypsin	Andras et al., 2002
Rhipicephalus sanguineus	RsTI	Strong trypsin inhibitor	Anna et al., 2
Crassostrea virginica	CVPI	Strong thermolysin inhibitor	Faisal et al.,
Crassostrea gigas	CGPI	Strong thermolysin inhibitor	Faisal et al.,
Phyllomedusa sauvagii	PSKP 1 & 2	Endo peptidase inhibitor	Leopoldo et 2004
Lymnaea	LTI	Trypsin inhibitor	Gregg et al.,

2.3.3 Microorganisms as the source of protease inhibitors

The marine environment exhibits considerably different characteristics than the terrestrial environment. Marine microorganisms have potential as important new sources of enzyme inhibitors (Chiaki, 2004). These microbial inhibitors are low molecular weight compounds derived from the hydrolysis of macromolecular substances. Presence of protease inhibitors in microorganisms is known since it was recognized to

the studies on antibiotics as they act as inhibitors of enzymes which are involved in growth and multiplication. Extracellular proteolytic enzymes, which hydrolyze organic nitrogen compounds in the medium, are thought to be harmful to cells. The production of inhibitors of the proteolytic enzymes by microorganisms has probably evolved as a mechanism to provide cell protection. Majority of the microbial protease inhibitors are produced extracellularly by various *Streptomyces* sps. The microbial serine protease inhibitors include leupeptin, inhibiting plasmin, trypsin, papain and cathepsin B; antipain, inhibiting trypsin, papain and cathepsin B; chymostatin inhibiting chymotrypsin and papain; elastinal, that inhibits pancreatic elastase; and elasnin, a strong inhibitor of human granulocyte elastase. All of these inhibitors have α-amino aldehyde group in the C-terminal part of the peptide. Several applications for medicinal purpose have been found for these inhibitors. Specific inhibitors of microbial origin have been used as useful tools in biochemical analysis of biological functions and diseases. The list of protease inhibitors reported from microbial source are listed in Table 2.3.

Table 2.3 Protease inhibitors from microbial source

Microorganisms	Inhibitor	Properties	Referenc
Pseudoalteromonas sagamiensis sp.	PSI	Anti-microbial agent	Takeshi et al.,
Alteromonas sp.	Marinostatins	Cysteine protease inhibitors	Imada et al., 1
Alteromonas sp.	Monastatin	Serine protease inhibitors	Imada et al., 1
Pleurotus ostreatus	POI	Serine proteinase A inhibitor	Dohmae et al., 1995
Lentinus edodes	Lentinus proteinase inhibitor	Trypsin inhibitor	Shoji et al., 19
Streptomyces lividans 66	SLPI	Strong inhibitory activity toward subtilisin BPN'	Ueda et al., 19
Streptomyces nigrescens TK-23	SMPI	Metalloprotease inhibitor	Murao et al., 1
Serratia marcescens	Sma PI	Metalloprotease inhibitor	Suh and Bene 1992

2.4 Classification of protease inhibitors

Naturally occurring proteinaceous protease inhibitors are primarily classified, based on the type of the enzyme they inhibit, into classes of protease inhibitors, such as serine protease inhibitors (Bode and Huber, 2000; Ryan, 1990). The classification is also performed based on sequence homology such as is done for the Kunitz-type inhibitors. A further classification can be done on the basis of their molecular mass, their protein architecture (monomeric or multimeric), the number of disulphide bridges present and their isoelectric points. These criteria determine in which family the protease inhibitor can be classified (Mosolov and Valueva, 1993). An

overview of the known families of protease inhibitors in animals, plants and microorganisms classified according to these criteria (Rawlings et al., 2004) is presented in Table 2.4.

Generally, Protease inhibitors can be grouped under two classes (PROLYSIS A protease and protease inhibitor web server)

i) Low molecular weight inhibitors

ii) Proteinaceous inhibitors

2.4.1 Low molecular weight protease inhibitors

The inhibitors belonging to this class are either synthetic or of bacterial and fungal origin, these small inhibitors irreversibly modify an amino acid residue of the protease active site. For example, phenyl methane sulfonyl fluoride (PMSF) inactivates the serine proteases, which react with the active serine whereas the chloromethylketone derivatives react with the histidine of the catalytic triad (Umezawa, 1982).

2.4.2 Proteinaceous inhibitors

Over hundred naturally occurring protein protease inhibitors have been identified so far. They have been isolated in a variety of organisms from bacteria to animals and plants (Leo et al., 2002). They behave as tight-binding reversible or pseudo-irreversible inhibitors of proteases preventing substrate access to the active site through steric hindrance. Their size is also extremely variable from 50 residues (e.g. BPTI: Bovine Pancreatic Trypsin Inhibitor) to up to 400 residues (e.g. alpha-1PI: alpha-1 Proteinase Inhibitor). They are strictly class-specific except proteins of the alpha-

macroglobulin family (e.g. alpha-2 macroglobulin) that bind and inhibit most proteases through a molecular trap mechanism. Based on the target enzyme, proteinaceous protease inhibitors are classified into four groups. They are serine, cysteine, aspartate and metalloprotease inhibitors (Rawlings et al., 2004).

Serine protease inhibitors have been the most studied protein inhibitors and are the largest class of protease inhibitors when looking at the number of families. Recently a considerable advance has been made in the study of the natural inhibitors of cysteine proteases (cystatins). In contrast, knowledge of inhibitors of both aspartyl and metalloproteases is very limited.

Rawlings et al., (2004) assigned proteinaceous protease inhibitors to 48 families on the basis of similarities detectable at the level of amino acid sequence. Then, on the basis of three-dimensional structures, 31 of the families are assigned to 26 clans. An 'inhibitor unit' was defined as the segment of the amino acid sequence containing a single reactive site (or bait region, for a trapping inhibitor) after removal of any parts that are known not to be directly involved in the inhibitory activity. A protein that contained only a single inhibitor unit was termed a simple inhibitor, and one that contained multiple inhibitor units was termed a compound inhibitor.

Table 2.4 Families of proteinaceous protease inhibitors

Family/ subfamily	Common name	Type –Example	Source	Families of peptid inhibited
I1	Kazal	Ovomucoid unit 3	*Meleagris gallopavo*	S (Laskowski and K 1980)
I2	Kunitz (animal)	Aprotinin	*Bos tauru*	S1(Laskowski and I 1980)

Kunitz (plant)	Soybean trypsin inhibitor	*Glycine max*	Mainly S1 (Laskowski and Kato, 1980), but also C1 (Oliveira et al., 2001) and A1 (Mares et al., 1989)
	Protease inhibitor B	*Sagittaria sagittifolia*	S1 (Laskowski and Kato, 1980)
Serpin	α_1-proteinase inhibitor	*Homo sapiens*	Mainly S1 (Huntington et al., 2000), but also S8 (Dufour et al., 1998), C1 (Al-Khunaizi et al., 2002) and C14 (Komiyama et al., 1994)
Ascidian	Ascidian trypsin inhibitor	*Halocynthia rorefzi*	S1 (Kumazaki et al., 1994; Kumazaki et al., 1993)
Cereal	Ragi seed trypsin/ α-amylase inhibitor	*Eleusine coracana*	S1 (Hojima et al., 1980)
Squash	Trypsin inhibitor MCTI-I	*Momordica charantia*	S1 (Wieczorek et al., 1985)
Ascaris	Nematode anticoagulant inhibitor	*Ascaris suum*	S1 (Bernard and Peanasky, 1993), but also M4 (Griesch et al., 2000)
YIB	Protease B inhibitor	*Saccharomyces cerevisiae*	S8
Marinostatin	Marinostatin	*Alteromonas* sp.	S1 (Takano et al., 1991)
Ecotin	Ecotin	*Escherichia coli*	S1 (Chung et al., 1983)
Bowman-Birk	Bowman-Birk plant trypsin inhibitor	*Glycine max*	Mainly S1 (Odani and Ikenaka, 1973), but also C1 (Hatano et al., 1996)
Pot 1	Eglin C	*Hirudo medicinalis*	Mainly S1 (Heinz et al., 1991)
Hirudin	Hirudin	*Hirudo medicinalis*	S1 (Bode and Huber, 1992)
Antistasin	Antistasin unit 1	*Haementeria officinalis*	S1 (Rester et al., 1999)
SSI	Subtilisin inhibitor	*Streptomyces albogriseolus*	Mainly S8 (Mitsui et al., 1979), but also S1 (Taguchi et al., 1998)and M4 (Kumazaki et al., 1993)
Elafin	Mucus proteinase inhibitor unit 2	*Homo sapiens*	S1 (Tsunemi et al., 1993)
Mustard	Mustard trypsin inhibitor	*Sinapis alba*	S1 (Menegatti et al., 1992)
Pacifastin	Proteinase inhibitor LCMI I	*Locusta migratoria*	S1 (Eguchi et al., 1994)
Pot 2	Proteinase inhibitor II	*Solanum tuberosum*	S1
7B2	Secretogranin V	*Homo sapiens*	S8 (Lindberg et al., 1995)
Pin A	PinA endopeptidase La inhibitor	Bacteriophage T4	S16 (Hilliard et al., 1998)

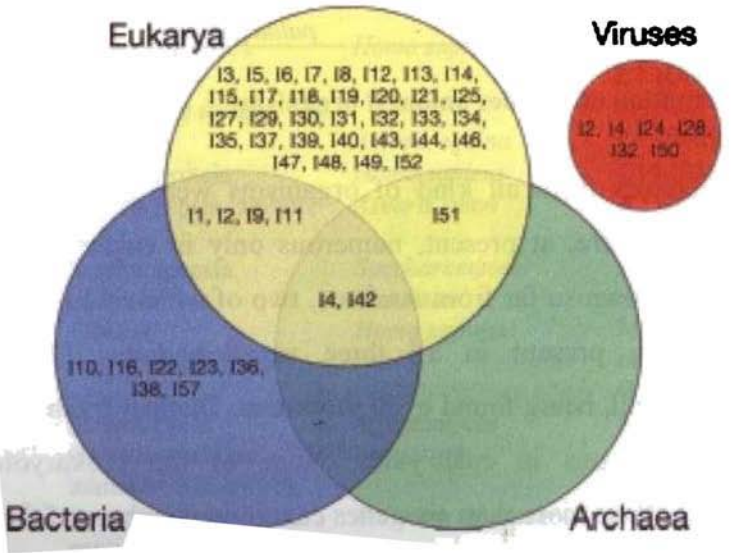
I25A	Cystatin 1	Cystatin A	*Homo sapiens*	C1 (Green et al., 198
I25B	Cystatin 2	Ovocystatin	*Gallus gallus*	Mainly C1 (Bode et 1988), but also C13 (Alvarez-Fernandez 1999)
I25C	Cystatin 3	Metalloprotease inhibitor	*Bathrops jararaca*	Not C1, but S8 (Cor al., 2003), M12 (Val al., 2001)
I27	Calpastatin	Calpastatin unit 1	*Homo sapiens*	C2 (Todd et al., 200
I29	CTLA	Cytotoxic T-lymphocyte antigen		C1 (Guay et al., 200
I31	Thyropin	Equistatin	*Actinia equina*	C1 (Strukelj et al., 2
I32	IAP	BIRC-5 protein	*Homo sapiens*	C14 (Riedl et al., 20
I33	Ascaris P13	Ascaris pepsin inhibitor PI-3	*Ascaris suum*	A1 (Ng et al., 2000)
I34	IA3	Saccharopepsin inhibitor	*Saccharomyces cerevisiae*	A1 (Phylip et al., 20
I35	TIMP	TIMP-1	*Homo sapiens*	Mainly M10 (Gomi et al., 1997), but als (Lee et al., 2003)
I36	SMI	Streptomyces metalloproteinase inhibitor	*Streptomyces nigrescens*	M4(Hiraga et al., 19
I37	PCI	Potato carboxy peptidase inhibitor	*Solanum tuberosum*	M14 (Bode and Hu 1992)
I38	Aprin	Metalloproteinase inhibitor	*Erwinia chrysanthemi*	M10 (Feltzer et al.,
I39	α_{2M}	α_2-macroglobulin	*Homo sapiens*	Numerous families including aspartic, c metallo and serine c types (Barrett, 1981
I40	Bombyx	Bombyx subtilisin	*Bombyx mori*	S8 (Pham et al., 199
I42	Chagasin	Chagasin	*Leishmania major*	C1 (Monteiro et al.,
I43	Oprin	Oprin	*Didelphis marsupialis*	M12 (Neves-Ferrei 2002)
I44		Carboxypeptidase A inhibitor	*Ascaris suum*	M14 (Homandberg 1989)
I46	LCI	Leech carboxypeptidase inhibitor	*Hirudo medicinalis*	M14 (Reverter et a
I47	Latexin	Latexin	*Homo sapiens*	M14 (Normant et a
I48	Clitocypin	Clitocypin	*Lepista nebularis*	C1 (Brzin et al., 20
I49	ProSAAS	ProSAAS	*Homo sapiens*	S8 (Basak et al., 20
I50	P35	Baculovirus p35 caspase inhibitor	*Spodoptera litura* nucleopolyhedrovirus	C14 (Xu et al., 200 also C25 (Snipas et 2001)
I51	IC	Carboxypeptidase Y inhibitor	*Saccharomyces cerevisiae*	S10 (Bruun et al., 1

TAP	Tick anticoagulant peptide	*Ornithodorus moubata*	S1 (Charles et al., 2000)
	Staphostatin B	*Staphylococcus aureus*	C47 (Rzychon et al., 2003)
	Staphostatin A	*Staphylococcus aureus*	C47 (Rzychon et al., 2003)
Triabin	Triabin	*Triatoma pallidipennis*	S1 (Fuentes-Prior et al., 1997)

2.4.3 Distribution of families amongst organisms

Inhibitors from all kind of organisms were characterized but the known families are, at present, numerous only in eukaryotes. Only three families are known so far from Archaea, two of which [I4 (serpins) and I42 (chagasin)] are present in all three superkingdoms; I4 is the most widespread of all, being found even in viruses. Distinct inhibitors, however, are most numerous in eukaryotes. None of the prokaryote genomes examined contains more than six genes encoding members of the families of peptidase inhibitors so far recognized, whereas all of the eukaryotic genomes contain tens or hundreds of them (MEROPS database). The Figure 2.1 summarises data for the distribution of the families that can be found in the MEROPS database (Rawlings et al, 2004). Although, for clarity, no intersection is shown between viruses and the other groups, it should be noted that family 14 occurs in the all four groups.

Figure 2.1 Distribution of inhibitor families throughout the superkingdoms of cellular organisms (MEROPS Database).



2.5 Mechanism of action of protease inhibitors

The mechanism of action of the proteinase inhibitors has been a subject of intense investigation (Barrett and Salvesan, 1986; Greenblat et al., 1989; Macphalen and James, 1987). Knowledge on mechanisms of protease action and their regulation *in vitro*, and *in vivo*, in animals, plants, microorganisms and more recently in viruses have contributed too many practical applications for inhibitor proteins in medicine and agriculture.

Proteins representing different inhibitor families are completely different and comprise α-helical proteins, β-sheet proteins, α / β -proteins and different folds of disulfide rich proteins. Protease inhibitors adopt

various structures ranging from mini proteins to large macromolecular structures, much larger than the target enzyme. From the structural point of view blocking of the enzyme active site is almost always achieved by docking of exposed structural elements, like loops or protein termini, either independently or in combination of two or more such elements. Besides recognition of different surfaces in the active site area, some inhibitors directly utilize the mechanism of protease action to achieve inhibition. The majority of the known protease inhibitors were reported to be substrate-like-binding molecules directed towards serine proteases blocking the enzyme at the distorted Michaelis complex reaction stage (Bode and Huber, 1992).

Based on the mechanism of inhibition the serine protease inhibitors can be distinguished into canonical inhibitors and non-canonical inhibitors.

2.5.1 Canonical inhibitors

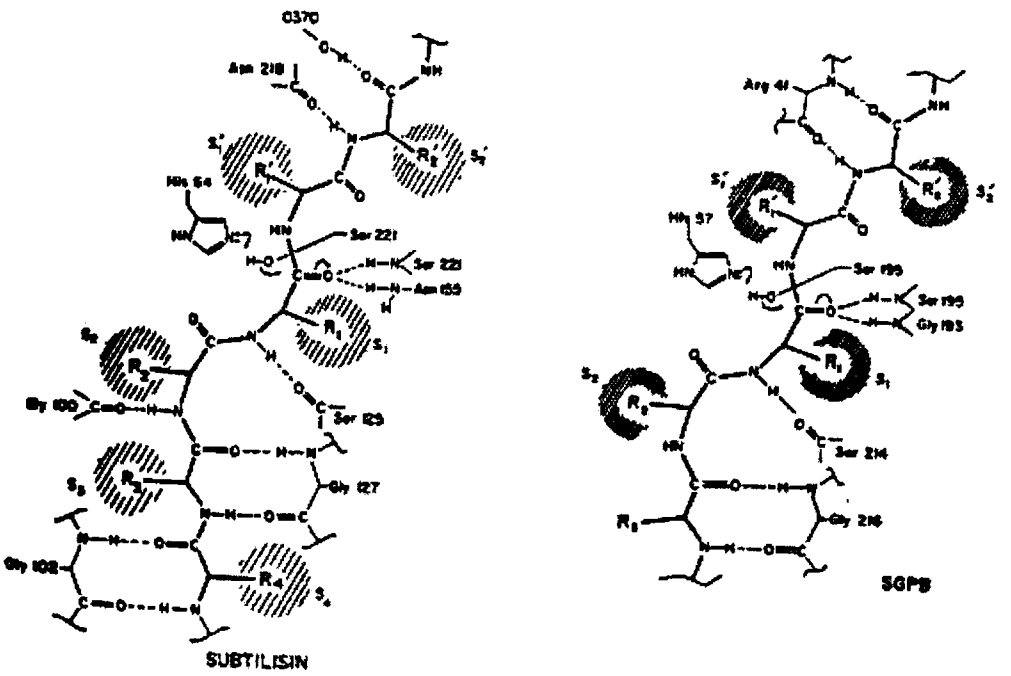
Canonical protein inhibitors of serine proteases interact with the enzymes by a common, generally accepted mechanism, "The Standard Mechanism" (Laskowski and Kato, 1980). Inhibitors obeying the standard mechanism are highly specific substrates for limited proteolysis by their target enzymes. They bind to enzymes in the manner of a good substrate very tightly, and are cleaved very slowly. Generally the standard mechanism inhibitors exhibit canonical conformation of the binding loop. The binding loop is in similar, so called canonical, conformation in inhibitor structures representing different inhibitor families (Bode and Huber, 1992; Apostoluk and Otlewski, 1998). On the surface of each inhibitor molecule lies at least one peptide bond called the reactive site which is defined as the part of the inhibitor molecule that enters into direct molecular contact with the active

center of the protease upon formation of the protease-inhibitor complex. Inhibition occurs as a consequence of binding of the active site substrate-binding region of a protease to the corresponding substrate-like reactive site on the surface of the inhibitor (Silverman et al., 2001).

Structural studies on several free inhibitors and on truly numerous enzyme-inhibitor complexes confirmed and extended the early observations that all inhibitors have a largely exposed combining loop surrounding the P1 residue, which in free inhibitor is hyper exposed. The combining loop serves as one of the two strands of the distorted antiparallel β-sheet of the complexes with the enzymes. The reactive site contains P1- P1' peptide bond located in the most exposed region of the protease binding loop (P1, P2 and P1', P2' designate inhibitor residues amino- and carboxy-terminal to the scissile peptide bond; S1, S2 and S1', S2' denote the corresponding subsites on the protease (Schechter and Berger, 1967) which can be cleaved by a serine protease. The inhibitor is converted from a 'virgin" (peptide bond intact) to a 'modified' (peptide bond hydrolyzed) inhibitor. Generally inhibitors obeying the standard mechanism show the presence of at least one disulfide linkage near the reactive site peptide that ensures that during conversion of virgin to modified inhibitor the two peptide chains are unable to dissociate. Therefore, conformation of the cleaved inhibitor is very similar to that of its intact form. The K_{cat}/K_m value for the hydrolysis of the reactive site peptide bond at neutral pH is very high typical for normal substrates, however, the individual values of K_{cat} and K_m for the inhibitors are several orders of magnitude lower than those for normal substrates, leading to an extremely slow hydrolysis of the reactive site peptide bond (Annedi et al., 2006).

In the interaction with subtilisin clan enzyme, the enzyme contributes two outside strands and the resultant distorted β-sheet is triple stranded (McPhalen and James, 1988). Aside from the residues contiguous to the reactive site peptide bond in most inhibitors, several discontiguous residues also contact the enzyme both by main chain and side chain interactions. These interactions are not as general for all the inhibitors as the contiguous one. They are probably idiosyncractic to the individual inhibitor families (Annedi et al., 2006). The mechanism of inhibition of serine protease with canonical inhibitor is illustrated in Figure 2.2.

Fig: 2.2 Diagrammatic representation of the interaction between the active sites of serine proteinases interacting with standard mechanism of canonical protein inhibitors (McPhalen and James, 1988).



2.5.2 Non-canonical inhibitors

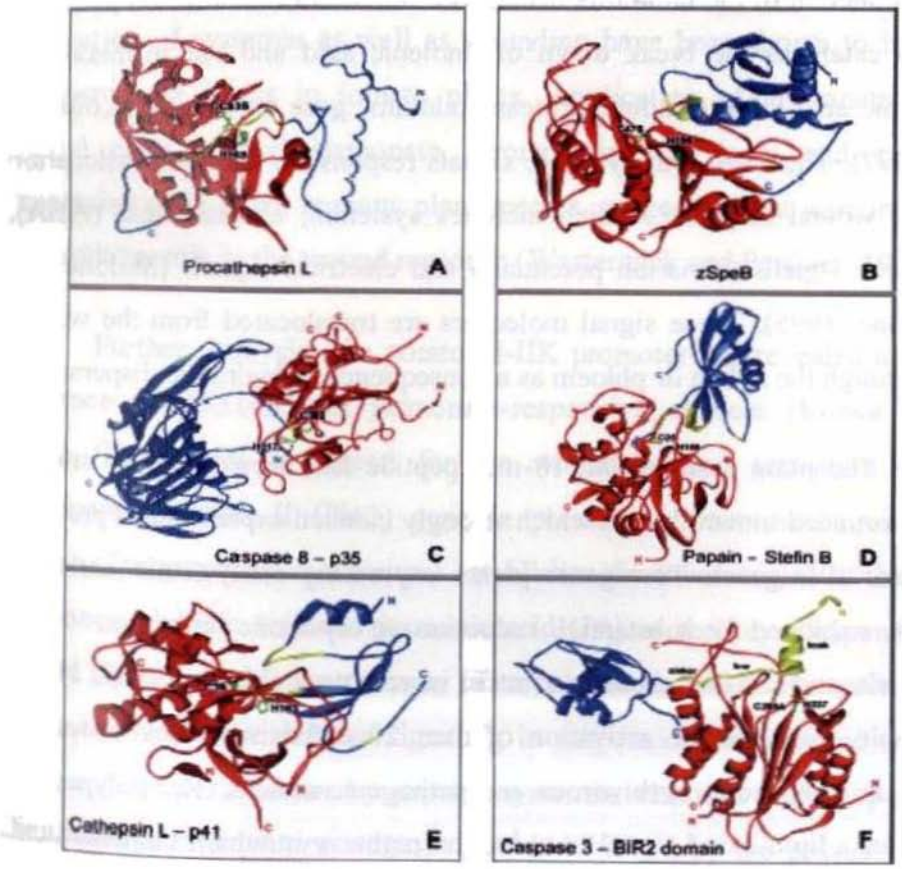
Inhibitors originating from blood sucking organisms are of the non-canonical type. They specifically block enzymes of the blood-clotting cascade, particularly thrombin or factor Xa. The interaction is mediated mainly through inhibitor N-terminus, which is disordered in solution and rearranges upon binding in the active site of an enzyme (Szyperski et al., 1992). There are also extensive secondary interactions, which provide an additional buried area and contribute significantly to the strength and specificity of interaction. In the studied cases there is two-step kinetics of association, the initial slow binding step occurs at the secondary binding site, then the N-terminus locks in the active site of protease. The classic example is recognition of thrombin by hirudin.

2.5.3 Cystatins - exosite binding inhibitors

Cystatins constitute the largest and best described group of natural cysteine proteinase inhibitors. The mechanism of cystatin action was elucidated by numerous kinetic and crystallographic studies (Figure 2.3) (Bode et al., 1988; Hall et al., 1995; Masson et al., 1998). Cystatins are exosite binding inhibitors and they bind adjacent to the protease active site, obstructing the access of substrate, but do not interact with the enzyme catalytic centre directly (Bode and Huber, 2000). The three-dimensional structure of the complex formed between human stefin B and papain conveyed the full understanding of cystatin inhibitory mechanism. The two hairpin loops, typical for cystatins, interact with the enzyme surface at the S1' through S4' binding sites, whereas the N-terminal portion of the cystatin

molecule runs through the S3–S1 subsites. Although the polypeptide chain binds in a substrate-like manner, at the P1 position it points away from the enzyme active site and so avoids cleavage. The cystatin molecule remains intact, but still prevents interactions with substrates, being non productively bound to the enzyme (Stubbs et al., 1990).

Figure 2.3. Ribbon drawing of inhibitory complexes formed by A, procathepsin L; B, SpeB zymogen; C, p35 and caspase 8; D, stefin B and papain; E, p41 and cathepsin L; F, XIAP and caspase 3 (Rzychon et al., 2004) .



2.6 Molecular genetics of protease inhibitors in plants

Plant proteinase inhibitor proteins that are known to accumulate in response to wounding have been well characterized. Earlier research on tomato inhibitors has shown that the protease inhibitor initiation factor (PIIF), triggered by wounding/injury switches on the cascade of events leading to the synthesis of these inhibitor proteins (Bryant et al., 1976; Melville and Ryan, 1973), and the newly synthesized protease inhibitors are primarily cytosolic (Hobday et al., 1973). The current evidence suggests that the production of the inhibitors occurs *via.* the octadecanoid (OD) pathway, which catalyzes the break down of linolenic acid and the formation of jasmonic acid (JA) to induce protease inhibitor gene expression (Koiwa et al., 1997). There are four systemic signals responsible for the translocation of the wound response, which includes systemin, abscisic acid (ABA), hydraulic signals (variation potentials) and electrical signals (Malone and Alarcone, 1995). These signal molecules are translocated from the wound site through the xylem or phloem as a consequence of hydraulic dispersal.

The plant systemin an 18-mer peptide has been intensely studied from wounded tomato leaves which strongly induced expression of protease inhibitor (PI) genes. Transgenic plants expressing prosystemin antisense cDNA exhibited a substantial reduction in systemic induction of PI synthesis, and reduced capacity to resist insect attack (McGurl et al., 1994). Systemin regulates the activation of over 20 defensive genes in tomato plants in response to herbivorous and pathogenic attacks. The polypeptide activates a lipid-based signal transduction pathway in which linolenic acid,

[redacted]eased from plant membranes and converted into an oxylipin-signaling [redacted]ecule, jasmonic acid (Ryan, 2000). A wound-inducible systemin cell [redacted]face receptor with an M(r) of 160,000 has also been identified and the receptor regulates an intracellular cascade including, depolarization of the plasma membrane and the opening of ion channels thereby increasing the intracellular Ca^{2+}, which activates a MAP kinase activity and a phospholipase A(2). These rapid changes play a vital role leading to the intracellular release of linolenic acid from membranes and its subsequent conversion to JA, a potent activator of defense gene transcription (Ryan, 2000). The oligosaccharides, generated from the pathogen-derived pectin degrading enzymes i.e. polygalacturonase (Bergey et al., 1999) and the application of systemin as well as wounding have been shown to increase the jasmonate levels in tomato plants. Application of jasmonate or its methyl ester, methyl jasmonate, strongly induces local and systemic expression of PI genes in many plant species, suggesting that jasmonate has a ubiquitous role in the wound response (Wasternack and Parthier, 1997).

Further, analysis of a potato PI-IIK promoter has revealed a G-box sequence (CACGTGG) as jasmonate-responsive element (Koiwa et al., 1997). The model developed for the wound-induced activation of the proteinase inhibitor II (Pin2) gene in potato (*Solanum tuberosum*) and tomato (*Lycopersicon esculentum*) establishes the involvement of the plant hormones, abscisic acid and jasmonic acid (JA) as the key components of wound signal transduction pathway (Titarenko et al., 1997). Recently, it has been shown that the defense signaling in suspensions of cultured cells of *Lycopersicon peruvianum* by peptide systemin, chitosan and by β-glucan elicitor from *Phytophtora megasperma*, is inhibited by the polysulfonated

naphtyl urea compound suramin, a known inhibitor of cytokine and growth factor receptor interactions in animal cells (Staratman et al., 2000). Levels of ABA have been shown to increase in response to wounding, electrical signal, heat treatment or systemin application in parallel with PI induction (Koiwa et al., 1997). Abscisic acid originally thought to be involved in the signaling pathway is now believed to weakly induce the mRNAs of wound response proteins and a concentration even as high as 100 mM induced only low levels of proteinase inhibitor as compared to systemin or jasmonic acid, suggesting the localized role of ABA (Birkenmeir and Ryan, 1998).

2.7 Protease inhibitor purification methods

For assessing the physiological function of newly isolated protease inhibitors, the purification of the protein to homogeneity is highly recommended. Once inhibitory activity is detected in a crude sample, it is important to perform an initial simple fractionation of the inhibitor activity: e.g. ammonium sulphate, organic solvent and polyethylene glycol fractionation. This is to determine whether the specific activity of the inhibitor increases after such fractionation.

Most of the natural inhibitors of proteases are proteins with molecular size ranging from 3000-80,000 Da. The most relevant method of protease inhibitor purification is the use of affinity ligands. In the absence of affinity purification, conventional purification procedures like Ion exchange chromatography, gel filtration chromatography, high performance liquid chromatography, reversed-phase high-performance liquid chromatography, fast protein liquid chromatography, isoelectric focusing and preparative gel electrophoresis are used. To establish effective purification steps of the

[redacted]tor sample it is important to determine the specific activity of the [redacted]bitor sample in each step of purifications (Hideaki and Guy, 2001).

2.7.1 Affinity chromatography

Recombinant proteases mutated at the active site, or natural ones with a covalent modification to the catalytic nucleophile, can serve as ideal ligands for purification of inhibitors. In this case, a crude sample can be applied directly to an inactive protease coupled to an insoluble support (e.g. Affi-Gel 10, BioRad labs), and the bound material can be eluted by a buffer at low or higher pH, or by chaotropic agent (Anastasi et al., 1983). The common affinity ligands used for the protease inhibitor purification includes, S-carboxymethyl-papain-sepharose (Gounaris et al., 1984), trypsin-Sepharose (Alinda et al., 2003), chymotrypsin-Sepharose (Gennis and Cantor, 1976) and trypsin- agarose (Sivakumar et al., 2005).

2.7.2 Ion exchange chromatography

The matrices for ion exchange chromatography contain ionizable functional groups such as Diethyl amino ethyl (DEAE) and Carboxy methyl (CM), which gets associated with the charged protein molecule, thereby adsorbing the protein to the matrices. The adsorbed protein molecule is eluted by a gradient change in the pH or ionic strength of the eluting buffer or solution (Arindam et al., 2006; Shrivastava and Ghosh, 2003).

2.7.3 Reversed-phase high-performance liquid chromatography

In recent years, reversed-phase high-performance liquid chromatography with porous, microparticulate, chemically bonded

alkylsilicas has emerged as a very rapid and selective method for peptide and protein purification (Hearn, 1982). The most efficient strategy for successful reversed-phase HPLC purifications of proteins is one that continuously redefines the practical limitations of the elution conditions as the molecular features of the protein of interest become more evident. Recovery for many proteins from alkylsilicas can be improved by the addition of stabilizing cations or cofactors to the eluent. The most commonly used reverse phase HPLC column includes, octadecylsilane column (Shoji et al., 1999), Vyda C $_{18}$ HPLC column (Cesar et al., 2004), Vydac 218 TP 1022 C-18 (Sivakumar et al., 2005) and μ-Bondapak C_{18} column (Ligia et al., 2003).

2.8 Characteristics of protease inhibitors

The characters of natural proteinaceous protease inhibitors isolated from plants, animals and microbes were thoroughly studied based on their physiological roles in various metabolic processes and also their properties used in various industries. The important properties looking for various industrial applications are presented in the following sections. The determination of the physicochemical parameters characterizing the structural stability of the inhibitors is essential to select effective and stable inhibitors under a large variety of environmental conditions. Moreover, the knowledge of their structural features is fundamental to understand the inhibitor-enzyme interactions and allow novel approaches in the use of synthetic inhibitors aiming for drug design.

2.8.1 pH and temperature stability

The intramolecular disulphide bridges are presumably responsible for the functional stability of most of the inhibitors in the presence of physical and chemical denaturants such as temperature and pH and reducing agents. Most of the protease inhibitors were not sensitive to pH over the range 2.0-10.0 and have varying thermo stabilities up to 70°C (Ligia et al., 2003). The stability towards pH and temperature is a crucial factor, which signifies the various industrial applications of proteinaceous protease inhibitors (Jui et al., 2002). The pH stability dictates the affinity of the inhibitor with different digestive proteases of the pest and insects those having neutral proteases like trypsin and chymotrypsin as their digestive enzymes, which implies the role of inhibitors as biopesticides (Joshi et al., 1999). The thermostability of protease inhibitor signifies its role as a stabilizer for many commercially important proteases, which has a major role in detergent industries to withstand higher temperature (Jui et al., 2002).

2.8.2 Effect of stabilizers/metal ions

Enhancement of thermal stability is beneficial for most of the biotechnological applications of proteins. Naturally occurring osmolytes such as amino acids, polyols and salts are known to protect proteins against thermal inactivation by stabilizing the thermally unfolded proteins (Gupta, 1991; Yancy et al., 1982). Glycine, PEG and cysteine hydrochloride conferred a complete protection towards thermal inactivation of protease inhibitors isolated from actinomycetes (Jui et al., 2002).

The presence of some metal ions is necessary for the activity of some protease inhibitors. Pearl millet cysteine protease inhibitor needs the Zn^{2+} for the protease inhibitory and antifungal activity of the protein. Metal chelation led to the complete loss of both protease inhibitory and antifungal activities, indicating a key role for Zn^{2+} in these activities. Analysis of metal chelated CPI using CD spectroscopy indicated a significant change in the secondary structure (Bimba et al., 1999).

2.8.3 Kinetic parameters

The importance of a protease inhibitor is directly related to the efficiency with which it inhibits the protease. A given inhibitor may inhibit several proteases and the relative efficiency is described by the kinetics of the interaction. Analysis of the kinetics of the reaction delineates the likely control point in complex biological media and gives crucial insights into the mechanism by which the inhibitor operates. The potency of an inhibitor is often described as IC_{50} (the molar concentration of the inhibitor that gives 50% inhibition of the target enzyme activity) or simply the percentage inhibition of the enzymatic activity of a fixed concentration of the inhibitor (Hideaki and Guy, 2001).

The two kinetic constants that define protease-inhibitor interactions are the speed (usually described as association rate constant, K_{ass}) and strength (usually described as the binding constant K_i) of the interaction. In principle a good, or physiological inhibitor should react rapidly with its target enzyme to form a tight complex, with K_{ass} greater than 10^5 /M.S, or K_i below 10^{-9} M (Hideaki and Guy, 2001). Most protein inhibitors of proteases

reactive site directed, i.e. they combine with the catalytic and substrate binding site of the proteases to form a tight and stable complex (Laskowski and Kato, 1980). The kinetics of inhibition of papain by cysteine protease inhibitor revealed that it has a reversible mechanism of action and the apparent K_i was found to be 6.5nM under the assay conditions. The low K_i indicates that it is a powerful inhibitor of papain (Bimba et al., 1999).

2.8.4 Reactive site and chemical modification

The localization and characterization of the amino acids comprising the reactive center and their correlation with the inhibitory function are essential for understanding the mechanism of action of the inhibitor. The first attempt to determine the amino acid residues involved in the reactive site of *Streptomyces* subtilisin inhibitor (SSI) was carried out by (Aoshima, 1976). They established that the photooxidation of SSI resulted in the destruction of three Met and one His residues with concomitant loss of inhibitory activity. Also the modification of Met by chemical oxidation by H_2O_2 or Cl_2 led to the loss of inhibition, therefore it was concluded that at least one Met residue is involved in the reactive site.

The hydrophobic amino acids such as Tyr, Trp and Lys were not essential for inhibition was confirmed by modification of SSI by diazonium-1-H-tetrazole. The carboxy-terminal four amino acid residues were shown to be important for maintaining the tertiary structure and the inhibitory activity of SSI. The modified SSI retained complete activity and its interaction with subtilisin was studied (Sakai et al., 1980).

Modifications of arginine and histidine resulted in the activation of serine protease inhibitory activity. Modifications in the serine residue of the same resulted in the loss of protease inhibitory activity (Bimba et al., 1999). The amino acid modification influences the antifungal properties of the Pearl millet cysteine protease inhibitor. Modifications of the cysteine, aspartic/glutamic acids or arginine residues resulted in a loss of antifungal activity of CPI as monitored by the inability to inhibit the growth of *Trichoderma reesi* (Bimba et al., 1999).

2.8.5 Protein-protein interactions

Interactions between proteins play a critical role in cell function and dysfunction. Blocking particular protein-protein interactions with specific ligands would enable exquisite control of cellular processes and provide potential leads for novel chemotherapeutic agents (Changhui et al., 2004). The interactions between serine protease inhibitors and their target enzymes have received increased attention since their complexes represent excellent model systems for investigating the fundamental biochemical and biophysical principles of protein-protein recognition. There are several reports on the interaction of protease inhibitor with its target protease. The binding and kinetics of the interaction between SSI and subtilisin has been studied by single photon counting technique and stopped-flow fluorescence spectroscopy (Uehara et al., 1980).

Goumarin, an antistatin type serine protease inhibitor isolated from *Hirudo nipponia*, which is composed of 57 aminoacid and contain 10 cysteine residues, showed an inhibitory spectrum towards chymotrypsin and

[redacted]. Amino acids at the P1 site acts as the key determinators of the [redacted] specificity of serine protease inhibitors (Ryoung et al., 2004). The crystallographic analysis of the complex formed by elastase or chymotrypsin and goumarin may provide detailed information upon the protease-inhibitor interaction, which is essential for the thorough understanding of the molecular recognition process and for the engineering of goumarin for medical applications (Ryoung et al., 2004).

2.8.6 Denaturation studies

Delineating the mechanism of protein denaturation and renaturation has been an interesting field of research. Kinetic investigations on denaturation of proteins whose three-dimensional structures have been elucidated by X-ray crystallography are especially useful for understanding the detailed mechanisms of conformational transitions of proteins (Tsunemi et al., 1993). Denaturation in proteins can be triggered by various methods like oxidations, reductions, temperature and pH. A kinetic study with the stopped flow method on the pH induced denaturation and renaturation of subtilisin inhibitor with intact disulfide linkages was carried out by monitoring the change in Trp fluorescence (Uehara et al., 1983). The thermal unfolding of SSI and its complex with subtilisin has been studied by circular dichroism (CD), differential scanning calorimetry (DSC), SDS-PAGE and 1H NMR spectroscopy (Arakawa and Horan, 1990; Tamura et al., 1991). The thermal denaturation of SSI was reversible and cooperative, proceeding in a two-state transition and leads to the dissociation of the dimers.

Soybean Kunitz trypsin inhibitor (SKTI) has played a key role in elucidating the mechanism of protease-protease inhibitor interactions thus helping in better understanding of the action of proteases. SKTI has two well-conserved disulfide bridges that play an important part in its structure. Proteolysis studies on reduced SKTI were performed with different reducing agents and its stability assessed in time course experiments. Thermal denaturation studies were done on SKTI due to its refractoriness to conventional chemical denaturants and also since foods generally undergo heat treatment and processing. During the process of thermal denaturation at both controlled and different rates, the different conformations of SKTI were probed using proteases like chymotrypsin and pepsin. This was done to assess their similarity with the native conformation that is protease resistant. Conformational transitions during thermal denaturation were monitored using 8-anilino-1-naphthalene sulfonic acid (ANS) fluorescence, intrinsic tryptophan fluorescence, CD and UV absorbance spectroscopy. Structure-function relation studies were also done on SKTI during thermal denaturation of native and reduced inhibitor (Song and Suh, 1998).

2.8.7 Protein engineering

Protein engineering through site directed mutagenesis allows the introduction of predesigned changes into the gene for the synthesis of a protein with an altered function.

Generally the properties of an amino acid residue at the reactive site (especially its center, the P1 site) of a protease inhibitor correspond to the specificity of the cognate protease. *Streptomyces* subtilisin inhibitor (SSI) is known to specifically inhibit bacterial subtilisins, and it has been

demonstrated that a functional change in subtlisin inhibitor was possible by replacing the amino acids at the reactive site (Met73) of SSI (Kojima et al., 1990). Replacement by Lys or Arg resulted in trypsin inhibition, replacement only by Lys gave inhibition of lysyl endopeptidase, and replacement by Tyr or Trp resulted in inhibition of alpha-chymotrypsin. The four mutant SSIs retained their native activity against subtilisin. Additional effects of replacing the Met70 at the P4 site of mutated SSI (Lys73) by Gly or Ala resulted in increased inhibitory activity towards trypsin and lysyl endopeptidase, while replacement with Phe weakened the inhibitory activity towards trypsin (Kojima et al., 1990). The role of amino acid residues involved in these interactions can be conveniently studied by protein engineering.

A recombinant human serine protease inhibitor known as Kunitz protease inhibitor (KPI) wild type has functional similarities to the Bovine Kunitz inhibitor, Aprotinin, and had shown a potential to reduce bleeding in an ovine model of cardiopulmonary bypass (CPB). KPI-185, a modification of KPI-wild type that differs from KPI-wild type in two amino acid residues and which enhances anti-kallikrein activity in an ovine model of CPB (Ohri et al., 2001).

Novel types of protease inhibitors with multi inhibitory activity were generated by phytocystatin domains in sunflower multi cystatin (SMC) by the serine protease inhibitor BGIT from bitter gourd seeds (Hideko et al., 2001). Two chimeric inhibitors SMC-T3 and SMC-T23, in which the third domain in SMC and the second and third domain in SMC were replaced by BGIT, acquired trypsin inhibitory activity (K_i: 1.46 X 10^{-7} M and

1.75 X 10^{-7} M) retaining inhibitory activity toward papain (K_i: 4.5 X 10^{-8} M and 1.52 X 10^{-7} M) respectively.

2.9 Application studies of protease inhibitors

2.9.1 Defense tools for plant protection

Pests and pathogens are major constraints to plant growth and development, resulting in heavy losses in crop yield and quality. Crop protection plays an integral role in modern-day agricultural production where the ever increasing demands on yield and the intensification of farming practice have increased the problem of pest damage, and hence control. Since the use of chemical fungicides has a deleterious effect on human health, a recent trend is to use other, safer strategies to enhance the defense mechanisms of crops. Extensive use of many chemical and biological pesticides has been able to provide only partial protection against the insect pest attacks. Therefore, biotechnological strategies for the pest control have been lately taken up as a field of active research. Anti-fungal proteins such as chitinases, glucanases, ribosome-inactivating proteins (RIPs), protease inhibitors and permatins play an important role in the defense of a plant against pathogen invasion (Lawrence and Koundal, 2002).

Protease inhibitors (PIs) are one of the prime candidates with highly proven inhibitory activity against insect pests and also known to improve the nutritional quality of food. Protease inhibitors regulate the action of proteases and play a significant role in the protection of plants from pest and pathogen invasion. Insects that feed on plant material possess alkaline guts and depend predominantly on serine proteases for digestion of food material

therefore protease inhibitors by virtue of their antinutritional interaction can be employed effectively as defense tools (Ryan, 1990). Some inhibitors are constitutively expressed in seeds and storage organs while others are induced on wounding in leaves (Green and Ryan, 1972). The plant protease inhibitors possessing insecticidal activities are listed in Table 2.5.

The introduction of various insect resistance genes into plant species can also be used as an effective component of Integrated Pest Management (IPM). Such insecticidal transgenic combined with other chemical, biological and agronomic control measures is expected to provide a reliable crop protection strategy (Pulliamt et al., 2001). Overexpression of heterologous inhibitors in transgenic plants has been shown to reduce the growth rates of several insect larvae (Gatehouse and Gatehouse, 1998; Hilder et al., 1987; Jounain et al., 1998). Further, transformation of plant genomes with PI-encoding cDNA clones appears attractive not only for the control of plant pests and pathogens, but also as a means to produce protease inhibitors, useful in alternative systems and the use of plants as factories for the production of heterologous proteins (Sardana et al., 1998). Transgenic crop plants expressing protease inhibitor genes are listed in Table 2.6.

Table: 2.5 Pesticidal activity of plant protease inhibitor

Inhibitor	Pest
Soybean trypsin inhibitor Bowman-Birk	*Tribolium castaneum* (Oppert et al., 1993) *Teleogryllus commodus* (Burgess et al., 1991)
Kunitz	*Helicoverpa armigera* (Ishikawa et al., 1994; Johnston et al., 1993) *Spodoptera litura* (Mcmanus and Burgess, 1995) *S.exigua* (Broadway, 1995; Broadway et al., 1986)
Potato protease inhibitors	*Sesamia inferens* (Duan et al., 1996) *Chrysodeixus erisoma* (Mcmanus et al., 1994) *T. commodus* (Burgess et al., 1991)
Potato multicystatin	*Diabrotica virgifera* (Orr et al., 1994) *D. undecimpunctata* (Orr et al., 1994)
Tomato protease inhibitor II Cowpea trypsin inhibitor	*Heliothis armigera* (Johnson et al., 1989) *Chilo suppressalis* (Xu et al., 1996) *C.inferens* (Xu et al., 1996) *Heliothis armigera*(Lawrence et al., 2001)
Squash trypsin inhibitor Cabbage protease inhibitor Manduca Sexta inhibitor	*H.Virescens* (Macintosh et al., 1990) *Trichoplusia ni* (Broadway, 1995) *Bemisia tabaci* (Thomas et al., 1995) *Frankliniella spp* (Thomas et al., 1994)
Soybean cysteine PI Soy cystatin Oryza cystatin I	*D. virgifera* (Zeng et al., 1988) *C. maculates* (Koiwa et al., 1997) *Otiorynchus suculatus* (Michaud et al., 1995) *C.chinensis* (Abe et al., 1992) *T. castaneum* (Chen et al., 1992) *Leptinorsa decemlineata* (Michaud et al., 1995) *Caenorhabditis elegans* (Urwin et al., 1995) *Chrysomela tremula* (Leple et al., 1995) *Riptortus clavatus* (Abe et al., 1992) *D. undcimpunctata* (Edmonds et al., 1996) *Eterodera schachtii* (Urwin et al., 1997) *Anthinomous grandis* (Pannetier et al., 1997) *Meloidogyne incognita* (Vain et al., 1998)
Oryzacystatin II	*C. chinensis* (Abe et al., 1992) *R. clavatus*
Wheat germ cysteine+ serine PI	*T.castaneum* (Oppert et al., 1993)

Table-2.6 Transgenic crop plants expressing protease inhibitor genes

Inhibitor	Crop plant	Crop pest
Cowpea trypsin inhibitor (CPTi)	Tobacco	*Heliothis virescens* (Hilder et al., 1987)
	Rice	*Chilo superssalis* (Xu et al., 1996)
		Sesamia inferens (Gatehouse and Gatehouse, 1998)
	Potato	*Lacanobia oleraceae* (Graham and Ryan, 1997)
	Strawberry	*Otiorynchus susucatus* (Sane et al., 1997)
	Tobacco	*Spodopteura litura* (Li et al., 1998)
	Cotton	*Helicoverpa armigera* (Alpteter et al., 1999)
	Wheat	*Sitotroga cerealla* (Lawrence et al., 2001)
CpTi + Snow drop lectin	Sweet potato	*Cyclas formicarius* (Newell et al., 1995)
		Manduca Sexta (Johnson et al., 1989)
Potato inhibitor I	Tobacco	*Chrysodeisus eriosoma* (Mcmanus et al., 1994)
Cystatin	Rice	*Sesamia Inferens (Duan et al., 1996)*
Tomato inhibitor I and II	Tobacco	*Manduca Sexta* (Johnson et al., 1989)
Sweet potato trypsin inhibitor (TI)	Tobacco	*Manduca Sexta* (Yeh et al., 1997)
Soybean Kunitz TI	Rice	*Nilaparvata lugens* (Lee et al., 1999)
Barley TI	Tobacco	*Agrotis ipsilon* (Carbonero et al., 1993)
		Spodoptura litura (Ussuf et al., 2001)
Nicotiana alta protease inhibitor	Tobacco	*Helicoverpa punctigera* (Health et al., 1997)
	Peas	*Plutella xylostella* (Charity et al., 1999)
Serpin type serine protease inhibitor	Tobacco	*Bemisia tabaci* (Thomas et al., 1995)

2.9.2 Therapeutic agents

Proteases are responsible either, directly or indirectly for all bodily functions including cell growth, nutrition, differentiation, and protein turn over. They are essential in the life cycles of organisms that cause mortal diseases such as malaria, cancer and AIDS (Demuth, 1990). The specific inhibition of these proteases can be used as a strategy for drug design for the prevention of propagation of the causative agents thus their effective regulators, i.e., protease inhibitors, are very essential, because of their pivotal role in the regulation of various physiological and pathological processes involving the mobilization of tissues proteins and in the processing of precursors of proteins (Gordana et al., 2002; Imada, 2005). Some of the proteinaceous protease inhibitors possessing therapeutic applications are presented in the Table 2.7.

Table 2.7 Protease inhibitors used in pharmaceutical industry

Protease Inhibitor	Source	Property	Reference
Bowman Birk type inhibitor (BBI)	*Glycine max*	Anticarcinogenic activity against prostate, colon, breast, and skin cancer	Kennedy, 19
Bowman Birk type inhibitor	*Pisum sativum*	Growth regulation on human colorectal Adenocarcinoma HT29 cells	Alfonso et al. 2005
BBI	Mung bean	Inhibitory activity against NS3 serine protease of Dengue virus	Murthy et al., 2000
Protease inhibitor	*Bauhinia forcata*	Anti coagulant activity	Clayton et al. 2005

Cystatin	Rice	Inhibition of *Leishmania* cysteine protease activity	Paul et al., 1999
Aspartate protease inhibitor	Plants	Antimalarial activity	Philip, 1998
SSI	*Streptomyces* sp.	Activity against Aspergillosis	Markaryan et al., 1996

2.9.3 Protease inhibitors in food processing

Food technology is a market driven activity. The current generation of food technologists is looking for the added value for the consumer, better profit margins and more efficient utilization of resources. Enzymatic modification of food proteins has an important role in the food industry with respect to traditional and high technology food processing as well as food spoilage.

Proteases are used by the food industry to control the viscosity, elasticity, cohesion, emulsification, foam stability, flavour development, texture modification, nutritional quality, solubility, digestibility and extractability (Lee, 1992; Morrissey et al., 1993). Applications include process of meat flavour development and tenderization, continuous bread making and modification of cracker and cookie texture, malt suppplimentation and chill proofing in brewing industry and hydrolysis of protein gels to lower viscosity for concentration or filtration. The desired degree of hydrolysis and percentage of peptide bonds hydrolyzed varies considerably with the different food processing. In many food processing there is a balance act in which there is a limited protein hydrolysis. This "limited hydrolysis" is a technique receiving considerable attention because it can yield products with improved properties and added value. Thus this

limited hydrolysis can only be mediated by the action of protease inhibitors. Thus they take an important role in the field of food processing (Fernando et al., 2000).

Microbial food spoilage is an area of global concern as it has been estimated that as much as 25% of all food produced is lost post-harvest owing to microbial activity (Baird-Parker, 2003). The use of an adequate amount of natural protease inhibitors is an effective way to extend the shelf life of many types of seafood such as salted fish products. This is because the inhibitors can retard the aging and other deteriorative processes like protein degradation caused by the action of endogenous and exogenous proteases, during the food processing and preservation (Reppond and Babbitt, 1993).

2.9.4 Protease inhibitors as stabilizers

Besides their applications in therapeutics and as potential biocontrol agents, protease inhibitors play an important role in basic research. Protease inhibitors and their enzymes have been excellent model systems to study protein-protein interactions. The properties of interfaces between polypeptide chains during interaction have been carried out using SSI as a model protein (Valdar and Thornton, 2001).

Protein protease inhibitors could potentially be used to stabilize proteases in commercial products such as liquid laundry detergents. However, many protein protease inhibitors are susceptible to hydrolysis inflicted by the protease. Philip et al. (2004) engineered *Streptomyces* subtilisin inhibitor (SSI) to resist proteolysis by adding an interchain

disulfide bond and removing a subtilisin cleavage site at leucine 63. When these stabilizing changes were combined with changes to optimize the affinity for subtilisin, the resulting inhibitor provided complete protease stability for at least 5 months at 31°C in a subtilisin-containing liquid laundry detergent and allowed full recovery of the subtilisin activity.

2.10 *Moringa oleifera*

Moringa oleifera is a panotropical multi purpose tree with a high biomass yield and can tolerate unfavourable environmental conditions (Foidl et al., 2001). Almost every part of the plant is of value for food. The flowers, leaves, and roots are used in folk remedies for tumors and the seeds for abdominal tumors. Bark regarded as antiscorbic, and exudes a reddish gum with properties of tragacanth; sometimes used for diarrhea. Roots are bitter, act as a tonic to the body and lungs, and are expectorant, mild diuretic and stimulant in paralytic afflictions, epilepsy and hysteria (Hartwell, 1971). No reports are available on the isolation of protease inhibitor molecule from *Moringa oleifera*. In addition, several low molecular weight bioactive compounds have been reported from *Moringa* seeds with bacterial and fungicidal activity and some anti inflammatory agents (Caceres et al., 1991). In addition to the pharmaceutical importance, water extracts of *Moringa oleifera* seeds was used to enhance a start up of a self inoculated upflow of anaerobic sludge blanket reactor (UASB) for treating domestic waste water (Kalogo et al., 2001). Seeds also contain small proteins that are able to flocculate particles in suspension in water. *Moringa oleifera* has very advantageous agronomic features, such that it should be possible to develop a natural commercial product without side effects (Broin et al., 2002).

Chapter 3

MATERIALS AND METHODS

3.1 SCREENING OF PLANTS FOR PROTEASE INHIBITOR

3.1.1 Plants for the study

Plants, which are locally available, and belonging to the families of Leguminosae, Malvaceae, Graminae, Rutaceae and Guttiferae were used as the source material to screen for protease inhibitory activity. Different plant parts including seeds, leaves, flowers and bark were used for the study.

3.1.2 Extraction and recovery of protease inhibitor

Plant materials for the study were washed thoroughly in distilled water and air-dried. A buffer extract was prepared in a 500 ml conical flask by homogenizing 25 g of plant materials in 100 ml of 0.1M phosphate buffer with pH 7.0 in an electrical blender. The homogenate was further mixed thoroughly by incubating the contents at room temperature in a rotary shaker for 30 minutes at 150 rpm. The slurry was then filtered through cheesecloth and the filtrate was centrifuged at 10,000 rpm for 15 minutes at 4°C for removing any cell debris that remains in the preparation (Pichare and Kachole, 1996). The clear supernatant obtained represented the crude extract, and was assayed for protease inhibitory activity and protein content as described in section 3.1.3.1 and 3.1.3.3 respectively.

3.1.3 Analytical methods

3.1.3.1 Protease inhibitor assay

Activity of protease inhibitor against protease was assayed according to the procedure described by Kunitz with slight modifications (Kunitz, 1947). In this method, the TCA soluble fractions formed by action of trypsin on the protein substrate Hammerstein casein was measured by the change in absorbance at 280nm. The residual caseinolytic activity of the trypsin in the presence of inhibitor, at 37°C, was used as a measure of inhibitory activity. Appropriate blanks for enzyme, inhibitor, and substrate were also included in the assay along with the test.

Reagents

1) Trypsin (SRL, India) - 0.5mg/ml (1000 units/mg)
   (Prepared in 0.1M phosphate buffer pH 7.0)
2) Hammerstein Casein
   in 0.1M phosphate buffer - 1%
3) Trichloro acetic Acid - 0.44M in Distilled Water
4) Phosphate Buffer (pH-7.0) - 0.1M

The assay procedure included the following steps:

a. One ml aliquot of trypsin was preincubated with 1 ml of suitable dilution of protease inhibitor at 37°C for 15 minutes.
b. To the above mixture 2ml of 1% Hammerstein casein was added and incubated at 37°C for 30 minutes.
c. The reaction was terminated by the addition of 2.5ml of 0.44 M trichloroacetic acid (TCA) solution.

d. The reaction mixture was transferred to centrifuge tube and the precipitated protein was removed by centrifugation, at 10,000rpm for 15 minutes (Sigma, Germany).

e. The absorbance of the clear supernatant was measured at 280nm in UV-Visible spectrophotometer (Shimadzu, Japan) against appropriate blanks. The TCA soluble peptide fractions of casein formed by the action of trypsin in the presence and absence of inhibitor was quantified by comparing with tyrosine as standard.

f. One unit of trypsin activity was defined as the amount of enzyme that liberated 1 μg of tyrosine per milliliter of the reaction mixture per minute under the assay conditions.

g. One unit of inhibitor activity was defined as the decrease by one unit of absorbance of TCA soluble casein hydrolysis product liberated by trypsin action at 280nm per minute at 37°C in the given assay volume.

The protease inhibitory activity was expressed in terms of percent inhibition and it was calculated as

$$\text{Inhibitory activity (\%)} = \frac{\text{Amount of tyrosine released without inhibitor} - \text{Amount of tyrosine released with inhibitor}}{\text{Amount of tyrosine released without inhibitor}} \times 100$$

3.1.3.2 Residual inhibitory activity

Residual inhibitory activity is the percent inhibitory activity of the sample with respect to the percent inhibitory activity of the control.

$$\text{Residual Inhibitory Activity} = \frac{\text{Inhibitory Activity of the sample (\%/ml)}}{\text{Inhibitory Activity of the control (\%/ml)}} \times 100$$

3.1.3.3 Protein

Protein content was determined according to the method of Lowry et al., (1951) using Bovine Serum Albumin (BSA) as the standard and the concentration was expressed in milligram per milliliter (mg/ml).

Reagents

a) Solution I: A stock solution containing 2% (w/v) sodium carbonate (w/v) in 0.1 N sodium hydroxide in distilled water.
b) Solution II: A stock solution of 0.5 % (w/v) cupric sulphate in distilled water.
c) Solution III: A stock solution of 1% (w/v) sodium potassium tartarate in distilled water.
d) Solution IV: Working reagent: To 100 ml of solution (I), 1 ml each of solution (II) and solution (III) was added and mixed well.
e) Solution V: 1:1 Folin and Ciocalteau's phenol reagent diluted with distilled water was prepared fresh just before use.

Estimation

The sample was made up to 500 µl with distilled water and added with 2 ml freshly prepared working reagent (Solution IV), mixed thoroughly, and incubated for 10 minutes. Later, 250µl of solution (V) was added, incubated for 30 minutes and the absorbance was measured at 750nm in a UV-Visible spectrophotometer (Shimadzu, Japan).

3.1.3.4 Specific activity

Specific activity of the sample was calculated by dividing the percent inhibitory activity with the protein content and was expressed as percent specific activity/mg protein.

$$\text{Specific activity} = \frac{\text{Inhibitory activity (\%)}}{\text{Protein (mg/ml)}}$$

3.2 SELECTION OF POTENTIAL SOURCE AND ISOLATION OF PROTEASE INHIBITOR FROM *MORINGA OLEIFERA*

Those protease inhibitors from various sources that could be precipitated by ammonium sulphate salts were selected for further screening and selection of potential source. For this, the strategy adopted was that the plant samples with more than 60% protease inhibition were selected and the crude protein inhibitor extract was prepared as described in the following sections.

3.2.1 Extraction and recovery of protease inhibitor

The crude buffer extract from the selected plants was prepared as described under section 3.1.2. The samples were assayed for protease inhibitory activity and protein content as described earlier under sections 3.1.3.1 and 3.1.3.3 respectively.

3.2.2 Ammonium sulphate precipitation

Ammonium sulphate precipitation of the prepared sample was done according to the method described by Englard and Seifter (1990). The fractionation using ammonium sulphate precipitation has the advantage of intermediate removal of unwanted proteins and simultaneously the protein of interest could be concentrated. Ammonium sulphate (SRL, India) required to precipitate the protease inhibitor was optimized by adding varying concentrations (30%, 60% and 90%) to the crude extract as detailed below.

1) To precipitate the protein, ammonium sulphate was slowly added initially at 30% (w/v) saturation to the crude extract while keeping in ice with gentle stirring.
2) After complete dissolution of ammonium sulphate, the solution was kept at 4°C for over night precipitation.
3) Protein precipitated was collected by centrifugation at 10,000 rpm for 15 minutes at 4°C.
4) To the supernatant, required ammonium sulphate for next level of saturation was added and the procedure mentioned above was repeated. The precipitation was continued up to 90% (w/v) of ammonium sulphate saturation.

3.2.3 Dialysis

The precipitate obtained after ammonium sulphate precipitation was further dialyzed against 0.01M phosphate buffer (pH 7.0), in order to remove the ammonium sulphate from the precipitate as detailed below.

3.2.3.1 Pretreatment of dialysis tube

Dialysis tube (Sigma-Aldrich) was treated to remove the humectants and protectants like glycerin and sulfur compounds present in it, and to make the pores of the tube more clear. The treated tube retain most of the proteins of molecular weight 12 kDa or greater. The method followed for the treatment of the dialysis tube was as follows.

a) Washed the tube in running water for 3-4 hrs.
b) Rinsed in 0.3% (w/v) solution of sodium sulfide, at 80°C for 1 minute.
c) Washed with hot water (60°C) for 2 minutes.
d) Acidified with 0.2% (v /v) sulphuric acid.
e) Rinsed with hot water (60°C).

3.2.3.2 Dialysis procedure

a) The precipitated protein was resuspended in minimum quantity of 0.1M phosphate buffer (pH 7.0).
b) The solution was taken in the pretreated dialysis tube (Section 3.2.3.1) (Sigma-Aldrich, cut off value 12 kDa) against 0.01M solution of phosphate buffer pH 7.0 for 24 hrs, at 4°C with frequent changes of buffer and assayed for protease inhibitory activity, protein content and specific activity as described under sections 3.1.3.1, 3.1.3.3 and 3.1.3.4 respectively.

3.2.4 Distribution of protease inhibitor in different plant parts of *Moringa oleifera*

Distribution of protease inhibitor in various parts of the mature plant was evaluated by preparing buffer extracts of seeds, leaf, flower, root and bark. Buffer extracts were prepared as described in section 3.1.2 and were assayed for protease inhibitory activity, protein content and specific activity as described under section 3.1.3.1, 3.1.3.3 and 3.1.3.4 respectively.

3.2.5 Selection of suitable solvent for extraction of protease inhibitor from *Moringa oleifera*.

Ideal solution that enables maximal extraction of the protease inhibitor from the sample was optimized by preparing crude extract of leaves with different solutions. 25 g of fresh leaves from the mature plant was dissolved in 100 ml each of sodium chloride 15% (w/v) (Wu and Whitaker, 1990), sodium hydroxide 0.2% (w/v), hydrochloric acid 0.05M (Tawde, 1961) , phosphate buffer 0.1M (pH 7.0) (Wu and Whitaker, 1990) and distilled water as described in section 3.1.2. The extracts thus prepared were assayed for protease inhibitory activity, protein content and specific activity as described in section 3.1.3.1, 3.1.3.3 and 3.1.3.4 respectively.

3.3 PROTEASE INHIBITOR PURIFICATION

Protease inhibitor, proteinaceous in nature, isolated from *Moringa oleifera* was purified by standard protein purification methods which included ammonium sulphate precipitation, followed by dialysis, Ion exchange chromatography and preparative polyacrylamide gel

electrophoresis as detailed below. All purification steps were carried out at 4°C unless otherwise mentioned.

3.3.1 Ammonium sulphate precipitation

Ammonium sulphate precipitation (Englard and Seifter, 1990) was done as described earlier under section 3.2.2.

3.3.2 Dialysis

The precipitate obtained after ammonium sulphate precipitation was further dialyzed against 0.01M-phosphate buffer (pH 7.0) as described previously under section 3.2.3.

3.3.3 Ion exchange chromatography

The active protease inhibitor fraction obtained after the dialysis of ammonium sulphate precipitation was further purified by Ion exchange chromatography, using DEAE cellulose as the anion exchanger (Rossomando, 1990). Proteins, due to surface charge, bind to ion-exchangers. These reversibly adsorbed proteins were eluted out either using a pH or a salt gradient.

3.3.3.1 Activation of DEAE Cellulose

The anion exchanger, DEAE Cellulose was activated as described below.

I. Ten gram of DEAE Cellulose (SRL, India) was soaked in citrate buffer (pH 3.0, 0.01M), allowed to settle, and the fine particles were removed by decanting.

II. It was then suspended in 1M NaCl for overnight.

III. Decanted the sodium chloride solution and washed several times with distilled water in sintered glass funnel using vacuum filtration, until the pH of the washings became neutral.

IV. Equilibrated the resin in appropriate buffer by repeated washing with the same buffer.

3.3.3.2 Standardization of binding pH of protease inhibitor to DEAE Cellulose

The pH at which the protease inhibitor binds at its maximum to the anion exchanger was determined by eluting the protease inhibitor solution after incubating with DEAE Cellulose equilibrated to different pH. DEAE Cellulose was activated as per the method described under section 3.3.3.1. The resin was resuspended in deionised water and equilibrated separately in 0.01 M glycine-HCl buffer (pH 2.0-2.5), citrate buffer (pH 3.0-5.5), phosphate buffer (pH 6.0-8.0), and carbonate–bicarbonate buffer (pH 9.0-10.5). One milliliter of diluted sample of ammonium sulphate precipitated fraction dialysed in the above buffers was mixed with 2 ml slurry of DEAE Cellulose equilibrated in each pH buffer. Incubated at 4°C for overnight, and the supernatant was collected by decanting without disturbing the DEAE particles. Washed with appropriate buffer, added 2 ml of 0.4 M NaCl, and incubated overnight to get eluted the bound proteins from the DEAE Cellulose. Supernatant collected was centrifuged at 10,000 rpm for

15 minutes to remove fine particles and assayed for protease inhibitory activity and protein content as described under sections 3.1.3.1 and 3.1.3.3 respectively.

3.3.3.3 Purification using DEAE Cellulose column

DEAE Cellulose, activated as described under section 3.3.3.1, was carefully packed in XK16/26 column (30cm height) (Amersham Pharmacia) without any air bubble and the column was equilibrated with citrate buffer (0.01M) pH 3.0 overnight.

Fifteen milliliter of dialyzed sample, prepared as mentioned in section 3.3.2, with a protein content of 3.5 mg/ml was applied to the pre equilibrated DEAE Cellulose column. After the complete entry of sample into the column, the column was connected to the reservoir containing the above said buffer (citrate buffer 0.01M, pH 3.0) with a flow rate of 2 ml/ minute. The unbound proteins were washed out until the absorbance at 280nm reached near to zero. Elution was done at a flow rate of 2ml/minute using stepwise gradients of sodium chloride ranging from 0.1, 0.2, 0.3, 0.4 and 0.5 M, prepared in citrate buffer (0.01M) pH 3.0. Five milliliter fractions were collected and the protein content of each fraction was estimated by measuring the absorbance at 280nm. Peak fractions from the column were pooled and dialyzed against the phosphate buffer (0.01M) pH 7.0 as described under section 3.2.3. The dialyzed fractions were assayed for protease inhibitory activity, protein content and specific activity as described under sections 3.1.3.1, 3.1.3.3 and 3.1.3.4 respectively. The yield and fold of purifications was calculated as described below.

Calculation of yield of protein, yield of protease inhibitor activity and fold of purification

Yield of protein and yield of protease inhibitory activity of each fraction during purification is the percent activity obtained by dividing the total protein content or activity of that fraction with the total protein content or activity of the crude extract as the case may be. Fold of purification in each step was calculated by dividing the specific activity of the respective fraction with that of the crude extract.

$$\text{Yield of protein} = \frac{\text{Total protein content of the purified fraction}}{\text{Total protein content of the crude extract}} \times 100$$

$$\text{Yield of activity} = \frac{\text{Total activity of the purified fraction}}{\text{Total activity of the crude extract}} \times 100$$

$$\text{Fold of purification} = \frac{\text{Specific activity of the purified fraction}}{\text{Specific activity of the crude extract}} \times 100$$

3.3.4 Preparative polyacrylamide gel electrophoresis

Active fractions pooled from ion exchange chromatography were lyophilized in 1ml aliquot and resuspended in 0.1ml of sample buffer (0.0625M Tris-HCl, 2% SDS, 10% sucrose, 0.01% bromophenol blue, pH 6.8). Aliquots of three tubes were loaded on to a polyacrylamide gel prepared as described under section 3.4.1.3.2 and subjected to electrophoresis (Tarsons Dual mini vertical electophoretic unit). Low molecular weight markers of Amersham Pharmacia were used as standard. After electrophoresis, a portion of the gel with marker was stained, and

compared with the original gel, and the portion of the gel with protease inhibitor band was cut out and made into small pieces. The protein bands were eluted using an Electro elutor (FINE PCR Electro elutor) by applying current 25V for 10 minutes at 4°C into 200 µl of reservoir buffer for Native-PAGE (section 3.4.1.1). The eluted proteins in buffer was collected out and dialysed against phosphate buffer (0.01M) pH 7.0. The protein samples were lyophilized in aliquots, and stored.

3.4 CHARACTERIZATION OF PROTEASE INHIBITOR

Purified inhibitor was further subjected to characterization for their biophysical and physicochemical properties like molecular weight, isoelectric point, amino acid analysis, optimal temperature and pH for maximal activity, stability at different temperature and pH, and inhibition kinetics to determine the type of inhibition as described in the following sections.

3.4.1 Electrophoretic methods

The crude buffer extract of protease inhibitor prepared from fresh leaves, ammonium sulphate purified fraction and protease inhibitor obtained after ion exchange chromatography were all subjected to electrophoretic analysis by non denaturing Native–PAGE and denaturing SDS-PAGE in a vertical slab electrophoresis (Tarsons Dual mini vertical electophoretic unit) Electrophoresis was carried out in a 10% polyacrylamide gel according to the method described by Laemelli (1970). SDS–PAGE analysis of the purified inhibitor was carried out both under reducing and non-reducing conditions, i.e., with and without β-mercaptoethanol respectively.

3.4.1.1 Reagents for polyacrylamide gel electrophoresis

1) Stock acrylamide solution

Acrylamide (30%)	-	60.0 g
Bis-acrylamide (0.8%)	-	1.6 g
Distilled water (DW)	-	200.0 ml

Stored at 4°C in amber coloured bottle

2) Stacking gel buffer stock

Tris buffer (0.5M) - 6 g in 40 ml DW
Titrated to pH 6.8 with 1 M HCl (~ 48 ml) and made up to 100 ml with DW. Filtered with Whatman No: 1 filter paper and stored at 4°C.

3) Resolving gel buffer stock

Tris buffer (3 M) - 36.3 g
Titrated to pH 8.8 with 1M HCl (~ 48 ml) and made up to 100 ml with DW.
Filtered with Whatman No: 1 filter paper and stored at 4°C.

4) Reservoir buffer for Native-PAGE (pH 8.3)

Tris buffer	-	3.0 g
Glycine	-	14.4 g

Dissolved and made up to 1L with DW.
Prepared in 10X concentration and stored at 4°C.

5) Reservoir buffer for SDS-PAGE (pH 8.3)

Tris buffer	-	3.0 g
Glycine	-	14.4 g
SDS	-	1.0 g

Dissolved and made up to 1L with DW.
Prepared in 10X concentration and stored at 4°C

6) Sample buffer for Native-PAGE

Tris-HCl (pH 6.8)	-	0.0625 M
Glycerol (optional)	-	10% (v/v)
Bromophenol blue	-	0.01%

Prepared in 2X concentrations and stored at 4°C.

7) Sample buffer for Reductive SDS-PAGE

Tris-HCl (pH 6.8)	-	0.0625 M
Glycerol (optional)	-	10% (v/v)
SDS	-	2% (w/v)
Dithiothreitol	-	0.1M
Bromophenol blue	-	0.01%

Prepared in 2X concentrations and stored at 4°C

8) Sample buffer for Non-reductive SDS-PAGE

Tris-HCl (pH 6.8)	-	0.0625 M
Glycerol (optional)	-	10% (v/v)
SDS	-	2% (w/v)
Bromophenol blue	-	0.01%

Prepared in 2X concentrations and stored at 4°C

9) SDS (10%)	-	1 g in 10 ml DW
10) Sucrose (50%)	-	5 g in 10 ml DW (autoclaved at 121°C for 15 minutes and stored at 4°C.)

11) Protein staining solution

Coomassie brilliant blue (0.1%)	-	100 mg
Methanol (40%)	-	40 ml
Glacial acetic acid (10%)	-	10 ml
DW	-	50 ml

12) Destaining solution

Methanol (40%)	-	40 ml
Glacial acetic acid (10%)	-	10 ml
DW	-	50 ml

13) Protein markers for Native-PAGE

Separate markers from Sigma-Aldrich were used

Components		Volume	MW (M_r)
Bovine Serum Albumin	-	10 µl	66,000
Chickalbumin	-	10 µl	45,000
Carbonic anhydrase	-	5 µl	29,000
Lactalbumine	-	10 µl	14,200

Markers were prepared in Native 1X sample buffer, and 30 µl of marker mix was loaded to the gel.

14) Protein Markers for SDS-PAGE

Low molecular weight marker mix of Amersham Pharmacia was used. Lyophilized marker mix was reconstituted in 1X sample buffer for reductive SDS-PAGE, boiled for 5 minutes, and 5µl of marker was loaded on to the gel. The composition of the marker mix is as given below.

Components		MW (M_r)
Phosphorylase b	-	97,000
Bovine Serum Albumin	-	66,000
Ovalbumin	-	45,000
Carbonic anhydrase	-	29,000
Trypsin inhibitor	-	20,100
α-Lactalbumin	-	14,400

3.4.1.2 Native polyacrylamide gel electrophoresis

3.4.1.2.1 Gel preparation

Resolving gel (10%)

Acrylamide: bis-acrylamide (30:0.8)	-	10.00 ml
Resolving gel buffer stock	-	3.75 ml
Ammonium persulphate (APS)	-	a pinch
Water	-	16.25 ml
TEMED	-	15.00 µl

Stacking Gel (2.5%)

Acrylamide: bis-acrylamide (30:0.8)	-	2.5 ml
Stacking gel buffer stock	-	5.0 ml
Ammonium persulphate (APS)	-	a pinch
Water	-	12.5 ml
TEMED	-	15.0 µl

Sample buffer (1X)

Native-PAGE sample buffer (2X)	-	1.0 ml
50% Sucrose	-	0.4 ml
DW	-	0.6 ml

Sample preparation

Added 100 µl of 1X sample buffer to lyophilized sample, mixed well and 25µl sample and 5 µl marker mix was loaded to the gel.

Procedure

(a) Cleaned and assembled the gel plates.

(b) **Resolving gel** – Added all the components except APS in to a beaker, mixed gently and finally added APS. Immediately poured the mixture into the cast and poured a layer of butanol over the gel and allowed to polymerize at least for one hour.

(c) **Stacking gel** - Added the components of stacking gel except APS into a beaker, mixed gently and finally added APS. Poured the contents into the cast above the resolving gel and immediately inserted the comb between the glass plates. Allowed to polymerize at least for 1 hour.

(d) Gel was placed in the electrophoresis apparatus, and upper and lower reservoir was filled with reservoir buffer for Native-PAGE.

(e) The gel was pre run for 1 hr at 80 V.

(f) Loaded the gel with the protein sample.

(g) The gel was run at 80V till the sample entered the resolving gel.

(h) When the dye front entered the resolving gel, increased the current to 100V.

(i) The run was stopped when the dye front reached 1 cm above the lower end of the glass plate.

(j) Removed the gel from cast and stained for at least one hr in the staining solution.

(k) Destained the gel till the bands became clear and observed the protein bands under a transilluminator.

3.4.1.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The purified inhibitor protein was subjected to reductive and non reductive SDS-PAGE for evaluating the nature of polypeptide. Low

molecular weight marker of Amersham Pharmacia was used as standard and molecular weight of protease inhibitor was determined using Quantity One Software from Biorad.

3.4.1.3.1 Reductive SDS –PAGE

Gel preparation

Resolving gel (10%)

Stock Acrylamide solution	-	10.00 ml
Resolving gel buffer stock	-	3.75 ml
10% SDS	-	0.3 ml
Ammonium persulphate (APS)	-	a pinch
Water	-	15.95 ml
TEMED	-	15.0 µl

Stacking Gel (2.5%)

Stock Acrylamide solution	-	2.5 ml
Stacking gel buffer stock	-	5.0 ml
10% SDS	-	0.2 ml
Ammonium persulphate (APS)	-	a pinch
Water	-	12.3 ml
TEMED	-	15.0 µl

Sample buffer(1X)

SDS-PAGE sample buffer (2X)	-	1.0 ml
50% Sucrose	-	0.4 ml
DW	-	0.6 ml

Sample preparation

Added 100 µl of 1X sample buffer to pure lyophilized sample, mixed well, boiled for 5 minutes in a water bath, cooled

to room temperature, and 25 µl sample and 5 µl low molecular weight marker mix was loaded to the gel.

Procedure

Procedure followed for SDS polyacrylamide gel electrophoresis was essentially the same as that of Native-PAGE which was described under section 3.4.1.2 with the exception that the reservoir buffer used was that of SDS-PAGE.

3.4.1.3.2 Non-reductive SDS–PAGE

Gel preparation

Resolving and stacking gel was prepared as described under section 3.4.1.3.1.

Sample buffer (1X)

Sample buffer for Non-reductive SDS-PAGE (2X)	-	1.0 ml
50% Sucrose	-	0.4 ml
DW	-	0.6 ml

Sample preparation

Added 100 µl of 1X sample buffer to pure lyophilized sample, mixed well, and 25 µl sample and 5µl low molecular weight marker mix was loaded to the gel.

Procedure

Procedure followed for SDS-polyacrylamide gel electrophoresis was essentially same the as that of Native-PAGE

which was described under section 3.4.1.2 with the exception that the reservoir buffer used was that of SDS-PAGE.

3.4.1.4 Analysis of protease inhibitor by Dot - Blot method

The purified fraction collected from ion exchange chromatography, was analyzed for its protease inhibitory activity according to the method of Veerappa et al., (2002) as described below.

I. 3µl of protease inhibitor was mixed with 3µl trypsin (0.5mg/ml) and spotted on to a strip of X-ray film.

II. 3µl of trypsin was mixed with 3µl phosphate buffer 0.1M (pH 7.0) as the control and spotted on to the X-ray film.

III. Incubated the X-ray film at 37°C for 10 minutes.

IV. Washed the film under tap water till the zone of gelatin hydrolysis by trypsin was visualized.

V. Where the inhibitor is present, the trypsin does not degrade the gelatin on the x-ray film. If the inhibitor is absent, a clear zone is formed at the site of sample application on the X-ray film.

3.4.1.5 Reverse zymography

Protease inhibitory activity of the purified protein was further confirmed by Reverse Zymogram on Gelatin-PAGE, performed by adding gelatin (0.1% final concentration) to the poly acrylamide prepared according to the method of Felicioli et al. (1997).

3.4.1.5.1 Gel preparation

Resolving gel (10%)

Acrylamide: bis-acrylamide (30:0.8)	-	10.0 ml
Resolving gel buffer stock	-	3.75 ml
Gelatin (1%)	-	3.0 ml
SDS (10%)	-	0.3 ml
Ammonium persulphate (APS)	-	a pinch
Water	-	12.95 ml
TEMED	-	15.0 µl

Stacking gel and sample buffer was prepared as described under section 3.4.1.3.2.

Sample preparation

Added 100 µl of 1X sample buffer to pure lyophilized sample, mixed well, and 25µl sample was loaded to the gel.

Procedure

Procedure followed for gelatin-polyacrylamide gel electrophoresis was essentially the same as that of SDS-PAGE, which was described under section 3.4.1.3.2.

After the electrophoretic run, the gel was washed with 2.5 % (v/v) Triton X 100 for 30 minutes followed by rinsing the gel in phosphate buffer pH 7.0 (0.1M) and incubated at 37°C for 15 minutes in a bath containing 0.5mg/ml trypsin in phosphate buffer pH 7.0 (0.1M).

After the gelatin hydrolysis, the gel was washed with distilled water, stained with the Coomassie Brilliant Blue (Sigma Aldrich) for 1 hr and destained.

3.4.2 Molecular weight determination of protease inhibitor by gel filtration chromatography on Sephadex G75

Gel filtration chromatography was performed for the ammonium sulphate precipitated fraction of crude protease inhibitor using Sephadex G75 (Sigma Aldrich) in order to determine the molecular weight of protease inhibitor.

3.4.2.1 Preparation of column

a) 23g of Sephadex G75 (Sigma Aldrich) was suspended in distilled water and allowed to hydrate for 3 hrs at 100°C in a water bath, and fine particles were removed by decantation.

b) Hydrated gel suspension was degassed under vacuum to remove the air bubbles.

c) Filled the column with distilled water or eluent without air bubble. Gel suspension was carefully poured into the column (Amersham Pharmacia XK 26/70 column) without air bubbles and allowed to settle under gravity while maintaining a slow flow rate through the column.

d) Column was stabilized by allowing two times the bed volumes of eluent (0.1 M phosphate buffer, pH 7.0) to pass through the column bed in a descending eluent flow.

e) The position of the flow adapters are re-adjusted as required to maintain contact of the plungers with the gel bed.

3.4.2.2 Sample preparation and application

Two milliliter of dialyzed sample, prepared as described under section 3.3.2, with a protein content of 18.6 mg/ml was applied to the column. Care was taken to make sure that the sample was completely free of undissolved substances. After the complete entry of sample to the column, the proteins were eluted using 0.1M phosphate buffer pH 7.0, with a flow rate of 1ml/minute. One millilter fractions were collected and the protein content was estimated by measuring the absorbance at 280nm in a UV-visible spectrophotometer (Shimadzu, Japan). Peak fractions from the column were pooled and assayed for protease inhibitory activity and protein content as described under sections 3.1.3.1 and 3.1.3.3 respectively.

3.4.2.3 Calculation of molecular weight

The molecular weights of the eluted proteins were calculated by calibrating the column with low molecular weight gel filtration protein markers from Amersham Pharmacia.

3.4.2.3.1 Column calibration

The gel filtration column prepared by Sephadex G 75 was calibrated with low molecular weight gel filtration protein markers from Amersham Pharmacia.

Protocol

a) Prepared a fresh solution of Blue Dextran 2000 (0.1mg/ml) (Sigma Aldrich) in the eluent buffer (0.1M phosphate buffer, pH 7.0).

b) Applied the Blue Dextran (1-2% of the total gel bed volume) to the column to determine the void volume (V_0).

c) Dissolved the proper combination of the calibration kit proteins in the eluent buffer. The concentration of each protein was between 5-20 mg/ml.

The calibration markers used included the following:

Components		**MW (M_r)**
Ribonuclease A	-	13,700
Bovine Serum Albumin	-	67,000
Ovalbumin	-	45,000
Chymotrypsinogen A	-	25,000

d) Applied the calibration kit proteins to the column. The volume of calibration solution was 1% of the total gel bed volume.

e) Determined the elution volume (V_e) for each kit proteins by measuring the volume of the eluent from the point of application to the center of the elution peak.

f) Calculated the K_{av} value (Partition coefficient) for each protein and prepared a calibration curve of K_{av} versus log molecular weight.

K_{av} for each protein was calculated by the formula,

$$K_{av} = \frac{V_e}{V_0}$$

where V_e is the elution volume of each protein and V_0 is void volume of the column, which was calculated by running the column with Blue Dextran 2000.

Molecular weight of the protease inhibitor was calculated from the calibration curve prepared using the calibration kit proteins.

3.4.3 Amino acid analysis

Amino acid analysis of the purified inhibitor protein sample was done using Shimadzu High-Performance liquid chromatography (LC - 4A) "Amino Acid Analysis System" (Ammu et al., 2001) at Central Institute of Fisheries Technology (CIFT), Kochi.

3.4.4 Optimal pH for protease inhibitor activity

Optimum pH for the maximal activity of the protease inhibitor was determined by performing protease inhibitor assay at different pH ranging from 2.0-12.0 as described under section 3.1.3.1 with minor modifications. The substrate 1% casein was prepared in the respective buffer for each pH. The buffer systems used were, glycine-HCl Buffer (pH 2-3.5), citrate buffer (pH 4-6), phosphate buffer (pH 6-8), Tris-HCl buffer (pH 8-9), carbonate-bicarbonate buffer (pH 9.5-10.5), boric acid/potassium chloride/ sodium hydroxide (pH 11.0), disodium hydrogen phosphate/sodium hydroxide (pH 12.0). Protease inhibitory activity was calculated as described under section 3.1.3.1.

3.4.5 Stability of protease inhibitor at different pH

The stability of protease inhibitor over a range of pH was determined by evaluating the inhibitor activity at pH 7.0, after incubating the purified protease inhibitor in different buffers of pH ranging from 2.0-12.0 for 24 hrs, at 4°C. 1.2 ml of purified inhibitor was incubated with 10.8 ml of different buffer systems, which included, glycine-HCl buffer (pH 2-3.5), citrate buffer (pH 4-6), phosphate buffer (pH 6-8), Tris-HCl buffer (pH 8-9), carbonate-bicarbonate buffer (pH 9.5-10.5), boric acid/potassium chloride/ sodium hydroxide (pH 11.0) and disodium hydrogen phosphate/sodium hydroxide (pH 12.0). After incubation, 1 ml of sample was assayed for protease inhibitory activity as described under section 3.1.3.1 and was expressed as percent inhibition of protease activity.

3.4.6 Optimal temperature for protease inhibitor activity

Optimum temperature for the maximal activity of protease inhibitor was determined by assaying the inhibitor activity at different temperatures ranging from 10°C-80°C. The assay method followed was essentially the same as that described under section 3.1.3.1.

3.4.7 Stability of protease inhibitor at different temperatures

Temperature stability of purified inhibitor at different temperatures was evaluated by incubating 1.2 ml of purified protease inhibitor at different temperatures ranging from 30°C-70°C. The sample was drawn at different time intervals: 30 minutes, 1hr, 2hrs, 4hrs, 8hrs and 12hrs, and was further incubated at 4°C for 15 minutes. The protease inhibitory activity of each

sample was assessed by conducting the assay as described under section 3.1.3.1.

3.4.8 Effect of stabilizers on thermal stability of protease inhibitor

Effect of stabilizers on protease inhibitor against thermo inactivation was determined at 50° and 60°C, respectively, at which they were found to loose activity. The effect of various additives on the thermal stability was determined by incubating the inhibitor in the presence of an additive at the desired temperature for a stipulated period of time. Sample was drawn at different time intervals of 1hr, 2hrs, 4hrs and 8 hrs. At the end of incubation, the inhibitor was further incubated on ice for 15 min and the residual activity was determined. Stabilizers added included glycine (1M), cysteine hydrochloride (10mM), PEG 8000 (10mM), glycerol (10%), sorbitol (10%), casein (1%), $CaCl_2$ (10mM), urea (10mM), sucrose, BSA and starch (at 1% level). Protease inhibitor assay was carried out as described under section 3.1.3.1.

3.4.9 Effect of various metal ions on protease inhibitor activity

Effect of various metal ions on activity of protease inhibitor was evaluated by incubating the protease inhibitor along with different concentrations of various metals ions in the inhibitor solution for 30 minutes followed by measuring the protease inhibitory activity as described under section 3.1.3.2. The metals studied included sodium chloride, calcium chloride, magnesium sulphate, cupric sulphate, sodium molybdate, zinc sulphate, ferric chloride, manganese chloride, nickel chloride, mercury chloride, barium chloride, cadmium sulphate, and aluminum sulphate which

contributes the metal ions, Na^{+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Fe^{3+}, Mn^{2+}, Ni^{2+}, Hg^{2+}, Ba^{2+}, Cd^{2+}, Mo^{6+} and Al^{3+} each at 1 and 10 mM final concentrations respectively.

3.4.10 Metal chelation of protease inhibitor using EDTA

The metal ion concentration of purified protease inhibitor and its effect on inhibitory properties in its native state was determined by metal chelation using 30mM EDTA (SRL, India) according to the method described by Jack et al. (2004). Purified protease inhibitor (2.7mg/ml) was dialyzed extensively against 30mM EDTA for over night at 4°C for chelation of metal ions. The EDTA was removed further by dialyzing over night with frequent changes of deionised water. The inhibitory activity of the demetallized protease inhibitor was determined by conducting protease inhibitor assay as described in section 3.1.3.2.

3.4.11 Metal ion concentration of protease inhibitor

Metal ion (Ca^{2+}, Mg^{2+} and Zn^{2+}) concentrations of the protease inhibitor was determined as follows:

I. An aliquot of protease inhibitor was dialyzed extensively against distilled water and was used for mineral analysis by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES).
II. Another aliquot of protease inhibitor (2.7mg/ml) was dialyzed extensively against deionised water and was used for mineral analysis by ICP-AES.
III. A third aliquot was dialyzed extensively against 30mM EDTA for over night at 4°C for chelation of metal ions and then the EDTA

was removed by dialyzing against deionised water over night with frequent changes in deionised water. The mineral concentration of dialyzed sample was also determined by ICP-AES.

3.4.12 Effect of additional supplementation of Zn^{2+}, Ca^{2+} & Mg^{2+} to demetallized protease inhibitor

Impact of additional supplementation of different concentrations of metals to the demetallized protease inhibitor, in order to contribute Zn^{2+}, Ca^{2+} & Mg^{2+} metal ions towards regaining its inhibitory property was evaluated. One and 10mM final concentrations of calcium chloride, magnesium sulphate and zinc sulphate was incubated along with protease inhibitor for 30 minutes, followed by measuring the residual protease inhibitory activity as described under section 3.1.3.2.

3.4.13 Effect of various detergents on protease inhibitory activity

Effect of various non-ionic and ionic detergents such as Triton X-100, SDS, Tween-80, Tween-20, and Brij-35 (1% each w/v) on protease inhibitory activity was determined by incubating the protease inhibitor in each detergents for 30 minutes, dialyzed against 0.01M phosphate buffer pH 7.0 and estimated the residual inhibitory activity as described under section 3.1.3.2.

3.4.14 Effect of oxidizing agents on protease inhibitory activity

Impact of oxidizing agents on the activity of protease inhibitor was studied by incubating the protease inhibitor with 1, 2, 3, 4 & 5 % (v/v) of hydrogen peroxide and dimethyl sulfoxide for 30 minutes and measuring the residual inhibitory activity as described under section 3.1.3.2.

3.4.15 Effect of reducing agents

The effect of reducing agents on the activity of protease inhibitor was studied by incubating the protease inhibitor with 0.2, 0.4, 0.6, 0.8 and 1% (v/v) of dithiothretol, β-mercaptoethanol and sodium thioglycolate for 30 minutes and measuring the residual inhibitory activity as described under section 3.1.3.2.

3.4.16 Chemical modifications of amino acids in protease inhibitor

To determine the impact of chemical structure of the amino acids at the reactive sites of inhibitor molecule on its inhibitory activity, selected amino acids of the inhibitor molecules were chemically modified. Thus five different amino acids were individually modified using specific chemical modifiers and the effect of modifiers on the anti proteolytic activity of the inhibitor molecule was determined. Chemical modifications of amino acids of purified inhibitor was carried out using different chemical modifiers under their respective reaction conditions. 2 ml of purified inhibitor (2.7mg/ml) was used for this study. After the incubation with different concentrations ranging from 5, 10, 15, 20 and 25 mM of each modifier, the

sample was dialyzed against phosphate buffer and the residual protease inhibitory activity was estimated as described under section 3.1.3.2.

Chemical modifier	Amino acid Modified	Reaction conditions	
N-Ethylmaleimide	Cysteine	30°C	0.1M Tris/HCl buffer (pH-7.0) for 60 min (Colman & Chu, 1970)
Succinic anhydride	Lysine	30°C	0.1M Sodium carbonate buffer (pH-8.0) for 120 min (Habeeb etal., 1958)
Diethyl pyrocarbonate	Histidine	30°C	0.1 M Tris/HCl buffer (pH-7.0) for 30 min (Ovaldi et al., 1967)
PMSF	Serine	25°C	0.05M Tris/HCl buffer (pH-7.8) for 120 min (Gold & Farney, 1964)
N- Bromosuccinamide	Tryptophan	30°C	0.01M Tris/HCl buffer (pH-7.0) for 30 min (Spande & Witkop, 1967)

3.4.17 Effect of acid treatment on protease inhibitor

Sensitivity of protease inhibitor in an acidic environment was evaluated by incubating purified protease inhibitor with different

concentrations of HCl ranging from 0.02, 0.04, 0.06, 0.08 & 1M (pH 2.0) for 30 minutes. After the incubation, the pH was neutralized with 1 ml of 0.1M Tris-HCl buffer pH 9.0. The residual protease inhibitory activity was estimated as described under section 3.1.3.2.

3.4.18 Effect of protease treatment on protease inhibitor

Sensitivity of protease inhibitor to gastric enzymes like trypsin was assessed by incubating the purified protease inhibitor with different concentrations of trypsin (from Bovine pancreas, SRL, India) ranging from 0.2, 0.4, 0.6, 0.8 and 1% for 30 minutes at 37°C. The residual protease inhibitory activity was estimated as described under section 3.1.3.2.

3.4.19 Stoichiometry of protease-protease inhibitor interaction

The molar concentration of the purified protease inhibitor for the complete inactivation of the trypsin was determined by preincubating 1 nM trypsin (based on M_r 23,800) in 100µl of 0.1M phosphate buffer pH 7.0 with different amounts of chromatographically purified protease inhibitor (0.25 - 2.0 nM, based on M_r 23,600) at 37°C for 60 minutes. The long incubation time was necessary to ensure that the reaction was complete. The remaining activity of the trypsin was determined by the addition of 1% casein, followed by incubation and spectroscopic examination according to Kunitz method as described under section 3.1.3.1.

3.4.20 IC_{50} value of protease inhibition

The amount of protease inhibitor needed for 50% inhibition of protease activity was determined by conducting the assay as described under section 3.1.3.1.

3.4.21 Kinetic studies of inhibition of trypsin by protease inhibitor

Using the enzyme rate data a double reciprocal-plot was prepared and analyzed to determine whether the nature of protease inhibition is competitive, uncompetitive or non competitive. Protease inhibition kinetics was studied using various concentrations of substrate ranging from 0.001mM - 2.0mM. The assay was carried out (Erlanger et al., 1961), as detailed below:

a) 500µl of one nM trypsin was preincubated in 0.1M phosphate buffer alone for 20 minutes at 37°C.
b) 500µl of different concentrations of purified protease inhibitor (4, 6 & 8 nM) was preincubated with 100µl of one nM trypsin for 20 minutes at 37°C.
c) The pre-incubated mixtures were then added separately to the substrate solution 0.001mM-2.0 mM Nα-Benzoyal-Arginine-4-nitroanilide (BAPNA) (Sigma Aldrich) at 37°C for 10 minutes.
d) The reaction was arrested by adding 200µl of 30% (v/v) acetic acid.
e) The liberated p-nitro aniline was measured at 410 nm in a UV-visible spectrophotometer (Schimadzu, Japan).
f) One protease unit was defined as the amount of enzyme that increased absorbance by 1 OD/min and one protease inhibitory unit

is defined as the amount of protease inhibitor that inhibited one unit of protease activity and was expressed as percent inhibition as described under section 3.1.3.1.

The velocity of the enzymatic reaction (v) based on the rate of change in absorbance (A_{410}) of the reaction mixture was determined for each concentrations of BAPNA used.

The initial velocity data was plotted as the function of the concentration of substrate by the linear transformation of the Michaelis-Menten Equation and usual non-linear curve fitting of the Michaelis-Menten equation for the calculation of K_m and V_{max} of the reaction.

A Lineweaver-Burk curve, 1/v versus 1/[s] was plotted and the dissociation constant (K_i) and maximum Velocity (V_{max}) were calculated (Dixon, 1953).

3.5 APPLICATION STUDIES

3.5.1 Specificity with different pharmaceutically important proteases

The affinity of purified inhibitor with different classes of proteases having roles in much pharmaceutical and agricultural industry was tested. The proteases tested were, Cathepsin-B (Sigma Aldrich), Thrombin (Sigma Aldrich), Elastase (Sigma Aldrich), Chymotrypsin (Sigma Aldrich), Collagenase (Sigma Aldrich) and Papain (Sisco Research Laboratories Pvt. Ltd, India).

3.5.1.1 Assay of Cathepsin-B inhibitory activity

Cathespin-B inhibitory activity was assayed using a solution containing 1.25-2.5 units/ml of Cathepsin B (Sigma Aldrich) in cold deionised water described by Barrett (1981a). 25µl of Cathepsin B was preincubated with 25µl of purified inhibitor solution (0.27mg/ml) at 30°C for 10 minutes. After the incubation, added 200 µl of 20 mM N-Succinyl-Ala-Ala-Pro-Phe-P-Nitroanilide in dimethyl sulphoxide and incubated at 30°C for 5 minutes. One unit of enzyme will release one micromole of p-nitro aniline per minute from N-Succinyl-Ala-Ala-Pro-Phe-P-Nitroanilide at pH 7.0 at 30°C and one cathepsin inhibitory unit is defined as the amount of protease inhibitor that inhibited one unit of cathepsin activity and was expressed in percent inhibition as described under section 3.1.3.1.

3.5.1.2 Assay of thrombin inhibitory activity

The thrombin inhibitory activity of protease inhibitor was evaluated. Prepared a stock solution containing 1mg/ml thrombin (Sigma Aldrich) in 0.1M Tris-HCl buffer pH 7.5, and 100 µl of 10µg/ml solution was preincubated with 100µl of purified inhibitor solution (0.27mg/ml) at 37°C for 10 minutes. Added 200µl of 1% casein to the mixture and incubated at 37°C for 30 minutes. The thrombin inhibitory activity of purified protease inhibitor was measured as described under section 3.1.3.1.

3.5.1.3 Assay of elastase inhibitory activity

The elastase inhibitory activity was tested with 10µg/ml elastase (Sigma Aldrich) solution with 0.27mg/ml purified protease inhibitor

according to Kunitz caseinolytic (Kunitz, 1947) method as described under section 3.1.3.1.

3.5.1.4 Assay of collagenase inhibitory activity

The collagenase inhibitory activity of purified protease inhibitor was checked by using 1% gelatin as substrate according to Ian (2001). 1mg/ml collagenase (Sigma Aldrich) was prepared in cold deionised water prior to assay. Preincubated one millilitre of collagenase with 0.27mg/ml purified inhibitor at 37°C for 10 minutes. Added 2ml of 1% gelatin to the reaction mixture and further incubated for 30 minutes at 37°C. The reaction was terminated using 0.44M TCA and the released amount of peptides were measured as described under section 3.1.3.1.

3.5.1.5 Assay of papain inhibitory activity

Papain inhibitory activity was evaluated by Caseinolytic method according to Murachi (1970). Prepared 6mg/ml solution papain, and preincubated 0.1 ml of papain with 0.1 ml purified inhibitor solution at 37°C for 10 minutes. After incubation, 0.5 ml of casein solution (1%) was added to the solution and the reaction mixture was incubated for 10 minutes at 37°C.The reaction was interrupted by adding 1.5 milliliter of trichloroacetic acid (5%) (w/v). The precipitate was removed by centrifugation at 10,000 rpm for 15 minutes and the released amount of tyrosine was estimated as described under section 3.1.3.1.

3.5.1.6 Assay of chymotrypsin inhibitory activity

Chymotrypsin inhibitory activity was assayed according to the modified method of Fritz et al (1966). Chymotrypsin from Bovine pancreas (Sigma Aldrich) was prepared by dissolving freeze dried material in 0.001M HCl at a concentration of 1mg/ml. Standard assay mixture contained 0.05 M Tris-HCl buffer, pH 7.6, 20mM peptide substrate, N-Suc-Ala-Ala-Pro-Phe-p-nitroanilide, 0.27 mg/ml inhibitor solution and chymotrypsin (10µg/ml). One unit of enzyme is defined as the amount of enzyme that induces the conversion of 1 µmol substrate/min. One chymotrypsin inhibitory unit is defined as the amount of protease inhibitor that inhibited one unit of chymotrypsin activity and was expressed in percent inhibition as described under section 3.1.3.1.

3.5.2 Activity spectrum of purified protease inhibitor towards commercially important proteases

The activity spectrum of purified protease inhibitor with different commercially available proteases from different sources were tested. All the enzymes were purchased from Sigma Aldrich. One milliliter of proper dilutions of each enzyme was incubated with one milliliter of protease inhibitor (0.27mg/ml) to check the affinity of the inhibitor with the proteases and the assay for protease inhibition was carried out as described under section 3.1.3.1.

Serial No.	Enzyme	Source
1	Protease	*Bacillus amyloliquifaciens*
2	Protease	*Bacillus licheniformis*
3	Protease	*Bacillus* sp.
4	Protease	*Aspergillus oryzae*
5	Subtilisin	*Subtilisin calsberg*
6	Esperase	*Bacillus lentis*
7	Proteinase K	*Tritirachium album*
8	Pronase E	*Streptomyces griseus*
9	Protease	*Engyodontium album*

3.5.3 Role of protease inhibitor in seafood preservation

The activity of *Moringa oleifera* protease inhibitor towards the seafood spoiling microorganism and its effect on protein degradation of *Peneaus monodon* during preservation under different storage conditions like room temperature, 4°C and –20°C was evaluated.

For this, the sample was peeled and head removed. 10g sample (*Peneaus monodon*) was weighed and taken in a sterile plastic bag, sealed and kept at each storage condition as control. As the test experiment, the same weight of samples were taken in the same conditions as that of the control and incubated in a sterile polyethylene bag containing 10ml of (0.2mg/ml) purified protease inhibitor prepared as mentioned under section 3.3.3 at each temperature for 8hrs, 24hrs and 168hrs respectively.

After incubation, the sample was drawn and extract was prepared by homogenizing the samples in sterile distilled water using a mortar and pestle under sterile conditions and kept in a rotary shaker for 30 minutes at 150rpm. One milliliter of extract was taken under sterile condition, and serially diluted the sample in physiological saline and the total microbial population of each sample was analyzed by pour plating the samples on Casein agar plates prepared by incorporating 1% casein in Nutrient agar medium.

The complete protein of the samples were extracted using 5% NaCl in 0.02M sodium bicarbonate according to Chandrasekaran (1985) and the cell pellet were removed by centrifugation at 10,000 rpm for 15 minutes at 4°C. The clear supernatant was assayed for total protein content as described in section 3.1.3.3.

Chapter 4

RESULTS

4.1 SCREENING OF PLANTS FOR PROTEASE INHIBITOR

Plants belonging to different families and from different localities of Kerala were screened for their protease inhibitory activity. Results presented in Table 4.1 shows that the plants belonging to Leguminosae family have maximum percent of inhibition towards trypsin. Maximum inhibitory activity was shown by *Cicer arietinum* (98.21%) followed by *Momordica charantia* (86.77), *Moringa oleifera* (76.7) and *Adathoda vasica* (76.12).

Table 4.1 Screening of plants for protease inhibitor

Serial No	Name of plants	Inhibitory activity (%)	Specific inhibitory activity (% activity /mg protein)
1	*Adathoda vasica*	76.12	115.33
2	*Allium sepa*	0.00	0.00
3	*Azadirachta indica*	6.50	0.77
4	*Amaranthus viridis*	51.21	98.48
5	*Arachis hypogea*	39.90	62.34
6	*Beta vulgaris*	0.00	0.00
7	*Carica papaya*	17.84	20.74
8	*Cassia fistula*	30.00	40.54
9	*Catharanthus roseus*	46.64	466.40
10	*Cicer arietinum*	98.21	10.68
11	*Cucurbita pepo*	22.40	27.32

12	*Dolichos biflorus*	60.74	33.74
13	*Hibiscus esculantus*	17.49	97.17
14	*Momordica charantia*	86.77	74.16
15	*Moringa oleifera*	76.70	109.57
16	*Ocimum sanctum*	50.60	56.22
17	*Oryza sativa*	15.33	4.26
18	*Phaseolus mungo*	56.00	80.00
19	*Pisum sativum*	55.98	75.65
20	*Phaseolus aureus*	18.64	32.70
21	*Solanum tuberosum*	73.78	163.96
22	*Triticum vulgare*	42.34	49.23

4.2 SELECTION OF POTENTIAL SOURCE AND ISOLATION OF PROTEASE INHIBITOR FROM *MORINGA OLEIFERA*

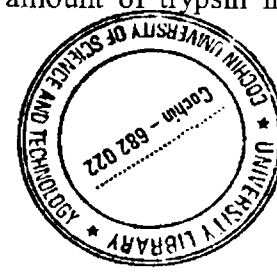
Plant species that showed more than 60% protease inhibitory activity were subjected to further screening. Crude sample prepared from these plants were subjected to partial purification by ammonium sulphate precipitation and evaluated for their inhibitory activity. Among the plants evaluated, *Moringa oleifera* showed maximal percent of protease inhibition (92%) compared to others (Table 4.2). Whereas, maximum specific activity of protease inhibition was recorded with *Momordica charantia* (1144) and *Solanum tuberosum* (192.36) compared to *Moringa oleifera* (76.66). Since the protease inhibitor from *Momordica charantia* and *Solanum tuberosum* were studied earlier and well characterized, in the present study, *Moringa oleifera* was selected as the source for the isolation of protease inhibitor, for no reports are available on this species.

Table 4. 2 Protease inhibition of ammonium sulphate precipitated fraction of different plant extracts

Serial No	Name of plants	Saturation of $(NH_4)_2 SO_4$ (%)	Protease inhibition (%)	Specific Activity (% activity/mg protein)
1	*Adathoda vasica*	30-60	31.16	103.86
2	*Cicer areitinum*	0-30	2.60	13.00
3	*Momordica charantia*	30-60	57.20	1144.00
4	*Moringa oleifera*	30-60	92.00	76.66
5	*Solanum tuberosum*	30-60	48.09	192.36

4.2.1 Distribution of protease inhibitor in different parts of *Moringa oleifera*

From the data presented in Fig 4.1, it is evident that among the different parts of *Moringa oleifera* tested, the crude extract isolated from the mature leaves and seeds showed highest level of inhibition against trypsin. The crude extract prepared from leaves showed maximum percent of inhibition (77%) followed by the seed extract (63%). The plant parts like bark, flowers and roots recorded negligible amount of trypsin inhibitory activity.



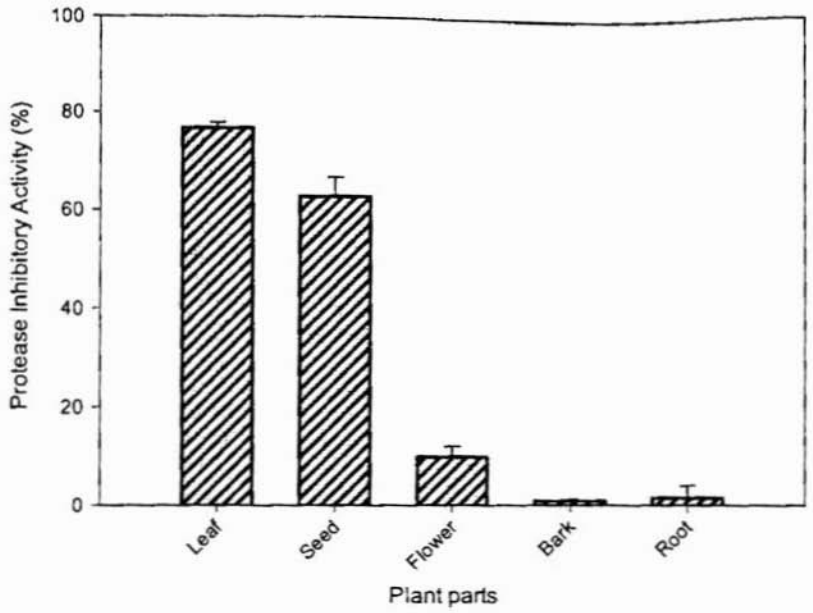


Fig 4.1 Distribution of Protease Inhibitor in different parts of *Moringa oleifera*

4.2.2 Extraction of protease inhibitor from *Moringa oleifera* using different solvents

Among the various extraction media evaluated for recovering protease inhibitory molecules from plant sources, the crude extract prepared in phosphate buffer showed maximum protease inhibitory activity (79%) followed by that prepared in distilled water (68%) (Fig 4.2). The inhibition of trypsin by the extract prepared in sodium chloride and sodium hydroxide was very less compared to that prepared in distilled water and phosphate buffer. Whereas, in terms of protein content in the crude extract, phosphate buffer enabled maximal protein content compared to other media, while distilled water extract contains very less protein concentration although showed high protease inhibitory activity. The specific protease inhibitory activity obtained for each extract is presented in Table 4.3.

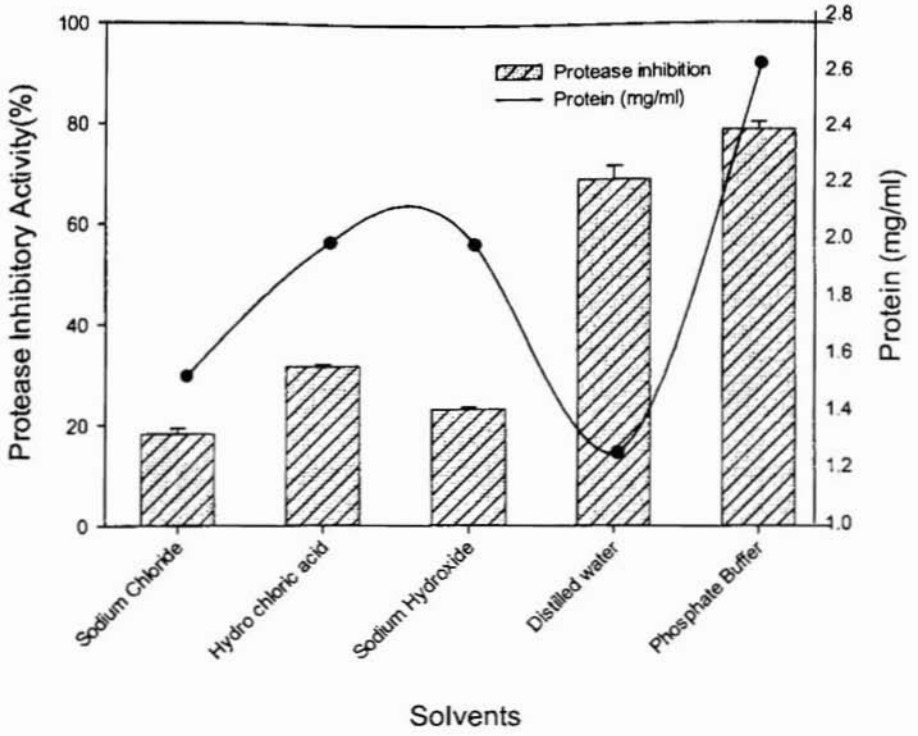


Fig 4.2 Extraction of Protease Inhibitor using different solvents

Table 4.3 Specific protease inhibitory activity of protease inhibitor extracted with different extraction media

Serial No	Extraction Medium	Specific protease inhibitory activity (% activity/mg protein)
1	Sodium chloride (15%)	12.20 ± 0.05
2	Hydrochloric acid (0.05M)	16.60 ± 0.04
3	Sodium hydroxide (0.2%)	11.60 ± 0.04
4	Distilled water	56.60 ± 0.23
5	Phosphate buffer (0.1M)	30.20 ± 0.10

4.3 PURIFICATION OF PROTEASE INHIBITOR

Following the standard protein purification methods including ammonium sulphate precipitation, dialysis, ion exchange chromatography using DEAE Cellulose, and polyacrylamide gel electrophoresis, purified the protease inhibitor present in the crude buffer extract prepared from the leaves of *Moringa oleifera*. The yield and fold of purification of protease inhibitor obtained in each step of purification is summarized in Table 4.4.

As a first step towards the purification of protease inhibitor, the crude buffer extract was subjected to ammonium sulphate precipitation and concentration of ammonium sulphate required for complete precipitation of inhibitor was standardized. The protease inhibitor could be precipitated at 0-90% (w/v) saturation of ammonium sulphate. However, the fraction, obtained with 60-90% (w/v) saturation was found to be efficient for precipitating the protease inhibitor compared to other fractions. The complete precipitation was done using ammonium sulphate concentration of 30-90% (w/v) saturation and the precipitated fractions were used for further studies. The fold of purification of protease inhibitor obtained for ammonium sulphate precipitation, ion exchange chromatography and polyacrylamide gel electrophoresis were 1.5, 2.5 and 41.4 respectively.

Table 4.4 Yield and fold of purification

Sample	Volume (ml)	Total protein (mg)	Inhibitor activity (%)	Specific inhibitor activity (%/mg)	Yield of protein (%)	Yield of activity (%)	Fold of purification
Crude extract	100	710	9400	13.2	100	100	1
Ammonium sulphate fraction (30-90%)	36	126	2520	20	17.7	26.8	1.5
Ion exchange chromato-graphy (DEAE)	56	116	3808	32.8	16.3	40.5	2.5
PAGE	0.5	0.095	52	547.4	0.013	0.6	41.4

4.3.1 Ion exchange chromatography

The dialysate obtained after ammonium sulphate concentration (30-90% fraction) was further subjected to ion exchange chromatography using DEAE Cellulose. The binding pH of the protein to the DEAE Cellulose was standardized. The binding affinity of the protein was maximum in the acidic pH. Results presented in Fig 4.3 evidence that bound protease inhibitor eluted from the DEAE Cellulose column, equilibrated with citrate buffer of pH 3.0 had maximum protease inhibitory activity, compared to other pH buffers.

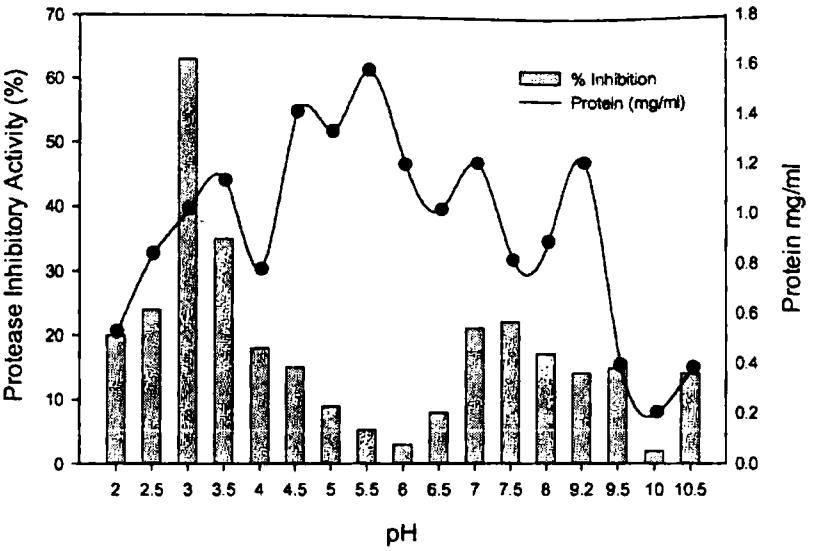
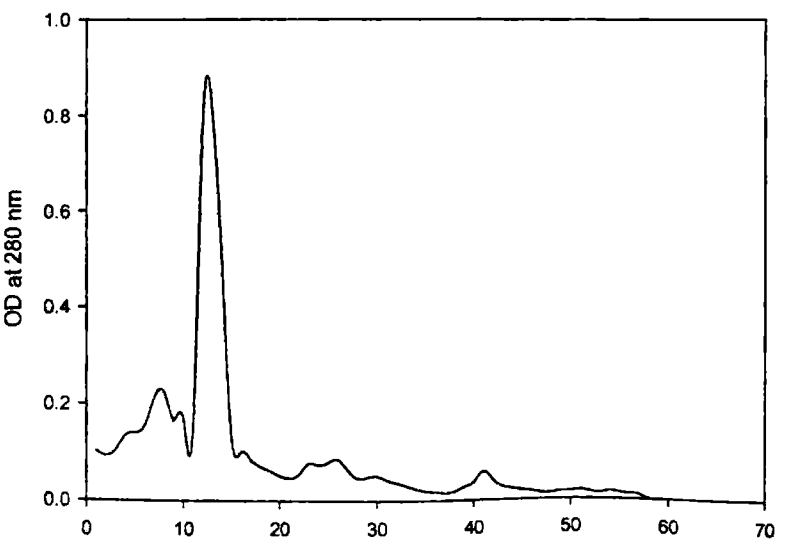


Fig 4.3 Standardisation of binding pH of *Moringa oleifera* Protease Inhibitor to the DEAE Cellu

The elution profile of protease inhibitor is depicted in Fig 4.4. A single protein peak demonstrating maximum inhibitory activity towards trypsin was eluted at 0.2M NaCl in citrate buffer of pH 3.0. This purification step resulted in the increase in purification fold up to 2.5 with a specific inhibitory activity of 32.8 mg protein.



4.4 CHARACTERIZATION OF PROTEASE INHIBITOR

4.4.1 Native polyacrylamide gel electrophoresis

The purified inhibitor obtained after ion exchange chromatography was analysed through native ploy acrylamide gel electrophoresis. The fraction with maximum protease inhibitory activity was visualized as a single protein band confirming their purity and homogeneity (Fig 4.5). The single protein eluted from the gel showed a protease inhibitory activity of 52 %.

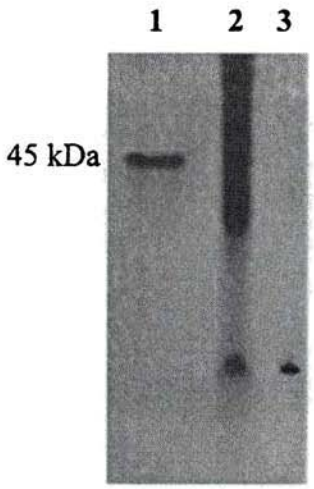
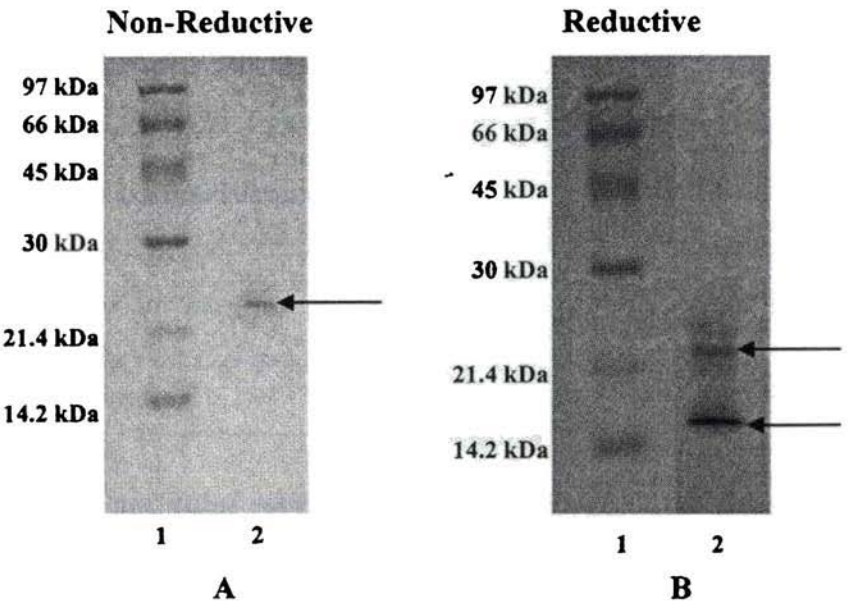


Fig 4.5 Native PAGE analysis of Protease Inhibitor

4.4.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The gel pattern of the purified inhibitor fraction when subjected to SDS-PAGE under reducing and non-reducing conditions is presented in the Fig 4.6. A single polypeptide band with a molecular weight of 23,600 Da in

non-reductive SDS-PAGE (Fig 4.6.A) testifies the purity of the fraction. Whereas, under reducing conditions, in the presence of β-mercaptoethanol, in addition to the protein band with 22,000 Da, another protein band with low molecular weight (14,000 Da) also appeared (Fig 4.6 B). The two bands obtained under reducing conditions were eluted out from the gel and evaluated for their protease inhibitory activity by reverse zymography. The results suggest that the two peptide bands after reduction possessed protease inhibitory activity.



1. **Marker**
2. **Purified Sample**

Fig 4.6 SDS PAGE analysis of purified sample

4.4.3 Analysis of protease inhibitor by Dot-Blot method

Protease inhibitory activity of the purified protein was checked on X- ray film by Dot-Blot method, where the purified protease inhibitor was able to block the gelatin hydrolysis caused by trypsin, to a greater extent. The presence of inhibitor was confirmed by comparing the clearing zone formed due to gelatin hydrolysis. While a clear zone was formed due to gelatin hydrolysis by trypsin, there was a reduction in clearing by the trypsin incubated with protease inhibitor (Fig 4.7).

1. **Trypsin with protease inhibitor**
2. **Trypsin alone**

Fig 4.7 Dot-Blot analysis of Protease Inhibitor

4.4.4 Reverse zymography

The protease inhibitory activity of the purified protein was confirmed by reverse zymogram analysis, which clearly indicated protease inhibitory activity of the purified protein band separated by polyacrylamide gel electrophoresis (Fig 4.8).

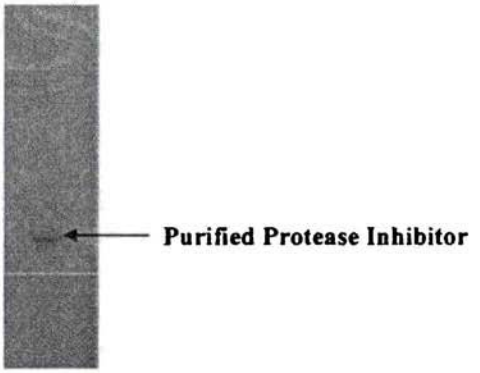


Fig 4.8 Reverse zymography of Purified Inhibitor from *Moringa oleifera*

4.4.5 Molecular weight determination of protease inhibitor by gel filtration chromatography using Sephadex G75

The molecular weight of the protease inhibitor was determined by gel filtration chromatography on Sephadex G75. The elution profile of protease inhibitor on Sephadex G75 column presented in the Fig 4.9, testifies a single major peak with maximum protease inhibitory activity. The molecular weight of the protein was calculated from the standard graph plotted for K_{av} versus log molecular weight of the standard proteins. From the K_{av} value, the molecular weight of the purified protein was found to be 29,000 Da.

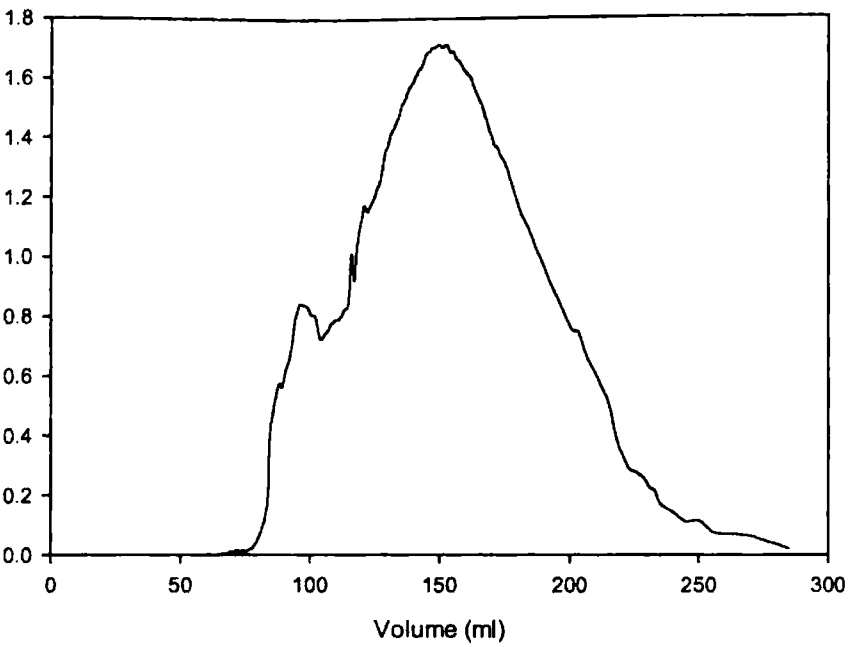


Fig 4.9 Elution profile of Protease Inhibitor on Sephadex G75

4.4.6 Amino acid analysis

Amino acid content of the purified protease inhibitor is presented in Table 4.5. The highest amount of amino acid represented in the protein was glycine (27.29%/g sample) and the lowest one was lysine (0.22%/g sample).

Table 4.5 Amino acid composition of *Moringa oleifera* protease inhibitor

Amino acid	(% g Amino acid)/g Inhibitor
Glycine	27.29
Glutamic acid	12.53
Alanine	11.19
Proline	10.74
Aspartic acid	8.95
Valine	5.15
Serine	4.25
Leucine	4.25
Arginine	4.02
Threonine	3.36
Isoleucine	3.13
Phenyl alanine	2.23
Histidine	1.79
Methionine	0.89
Lycine	0.22

4.4.7 Determination of optimal pH for protease inhibitor activity

The activity profile of protease inhibitor at different pH is depicted in Fig 4.10. From the results it is inferred that, the protease inhibitor was active over a wide range of pH 6.0-10.0 and the maximal activity was obtained at pH 7.0 (68% protease inhibition). The inhibitor lost its activity at highly acidic and highly alkaline pH conditions.

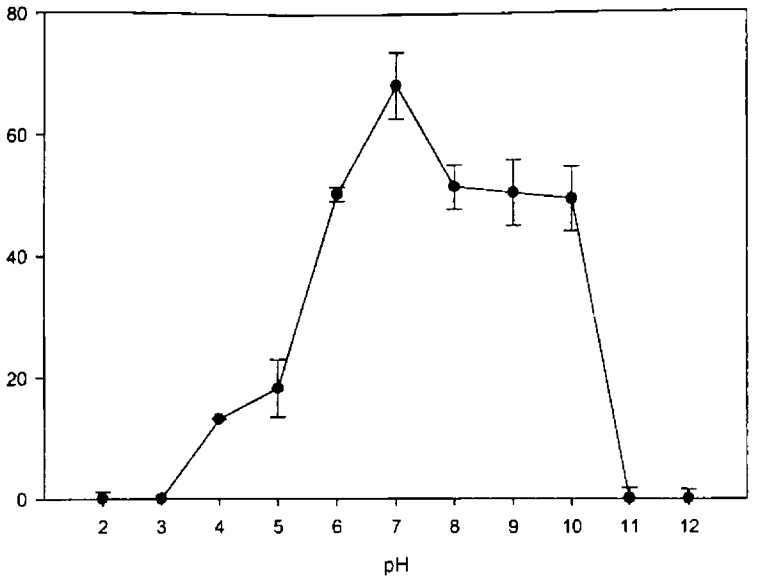


Fig 4.10 Activity profile of the Protease Inhibitor at different pH

4.4.8 Determination of stability of protease inhibitor at different pH

Stability studies conducted for a period of 24 hours in different buffer systems showed that the protease inhibitor had stability over a wide range of pH. From the result presented in Fig 4.11, it was observed that the protease inhibitor retained its activity over a pH range of 5.0-10.0, and the maximal inhibitory activity was obtained at pH 10.0 (67% protease inhibition). In the acidic pH 3.0 and alkaline pH 11.0, the inhibitory activity sharply declined recording 16 and 37% inhibition compared to the inhibitory activity at pH 10.0. It was also observed that the protease inhibitory activity was stable for 24 hours in the pH range of 5.0 - 10.0.

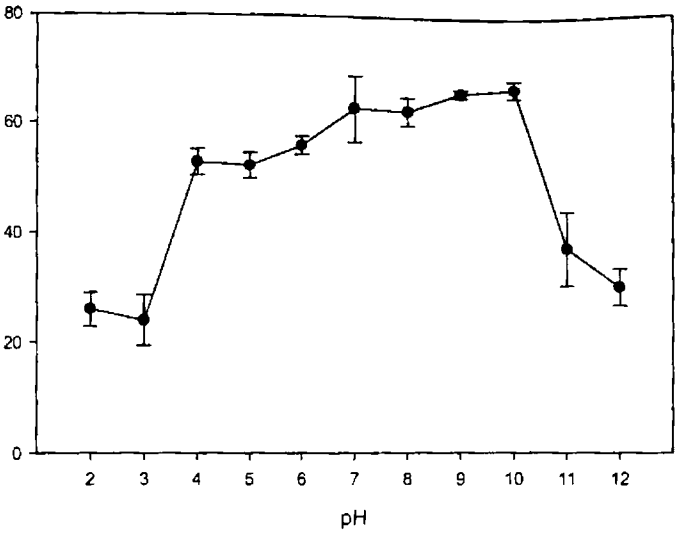
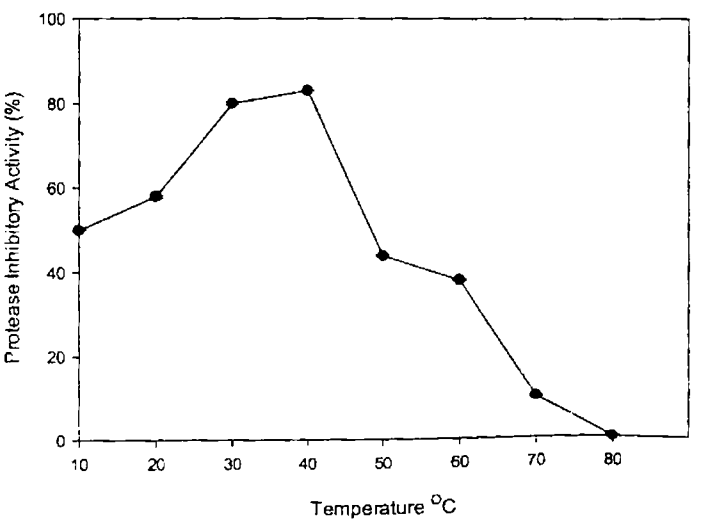


Fig 4.11 The stability of Protease Inhibitor at different pH

4.4.9 Optimal temperature for protease inhibitor activity

Results presented in Fig 4.12 suggest that the protease inhibitor is most active only up to 50° C with a maximal protease inhibitory activity of 83% protease inhibition in the range of 30°C - 40°C. The protease inhibitory activity declined at temperatures above 50°C, and the protein was totally inactive at 70°C (10.2 % protease inhibition).



4.4.10 Stability of protease inhibitor at different temperatures

The thermo stability profile of protease inhibitor presented in Fig. 4.13 evidence the temperature stability of protease inhibitor from *Moringa oleifera*. The purified inhibitor is moderately heat stable, as observed by the decrease in activity after preincubation at temperatures above 50°C. It showed stability for two hours at 30°C & 40°C with 54 & 53% protease inhibitory activity respectively. At 50°C the inhibitor retained only 46% inhibitory activity up to 2 hours. At temperatures above 50°C the protease inhibitor was inactive with a decline in inhibitory activity and was stable only for 30 minutes. This moderately heat stable nature of protease inhibitor indicates scope of its application in various industries.

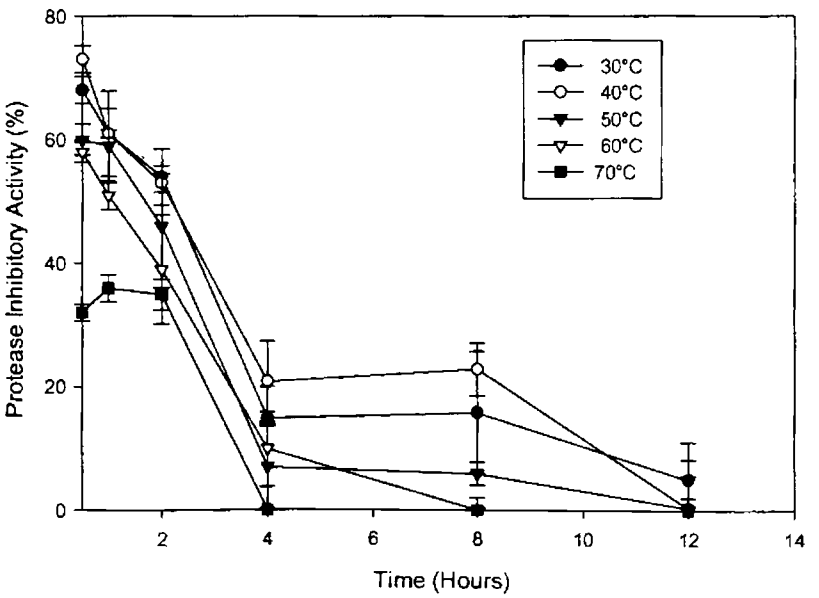


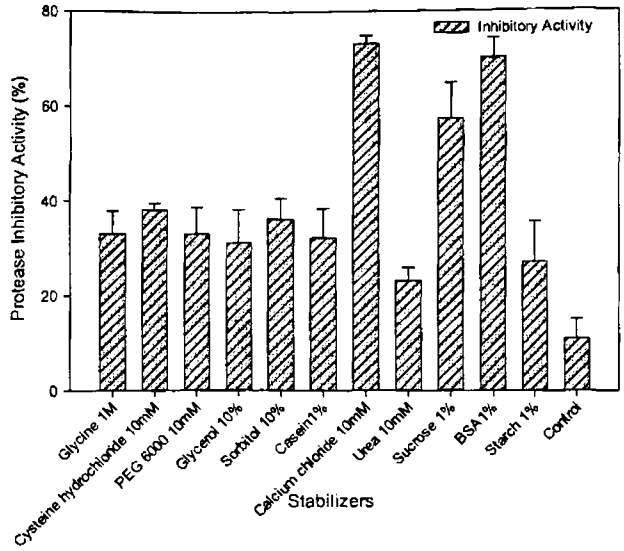
Fig 4.13 Temperature stability of Protease Inhibitor

4.4.11 Effect of stabilizers on thermal stability of protease inhibitor

Effect of additives as stabilizers on protease inhibitor against thermal inactivation was studied at 60 and 50°C at which the inhibitor was found to lose its activity. The results presented in Fig 4.14 showed that calcium chloride (10mM) effected complete protection for the protease inhibitor with 73% protease inhibitory activity upto four hours at 50°C. However it offered only 45% protease inhibitory activity at 60°C. BSA (1%) conferred complete stability with a 70% protease inhibition at 50°C. The addition of BSA (1%) to the protease inhibitor at 60°C enhanced the activity to 50% inhibition. The two polyols, glycerol and sorbitol, at a concentration of 10%, effected 31 & 36% activity, and urea and starch did not have any profound effect on thermal stability of protease inhibitor. Stabilizers like PEG 6000, casein, and cysteine hydrochloride supported partial stability to the protease inhibitor at 50 and 60°C. Sucrose, urea, starch and sorbitol did not enhance the thermal stability at 60°C.

In general, all the stabilizers promoted thermal stability and inhibitory activity compared to control at 50°C. Maximal stability was promoted by calcium chloride followed by BSA and sucrose. Whereas, at 60°C, BSA followed by calcium chloride, casein, cysteine hydrochloride and glycine alone supported stability compared to control. Sucrose and starch, which supported stability at 50°C, did not promote stability at 60°C.

(A) Incubation at 50°C



(B) Incubation at 60°C

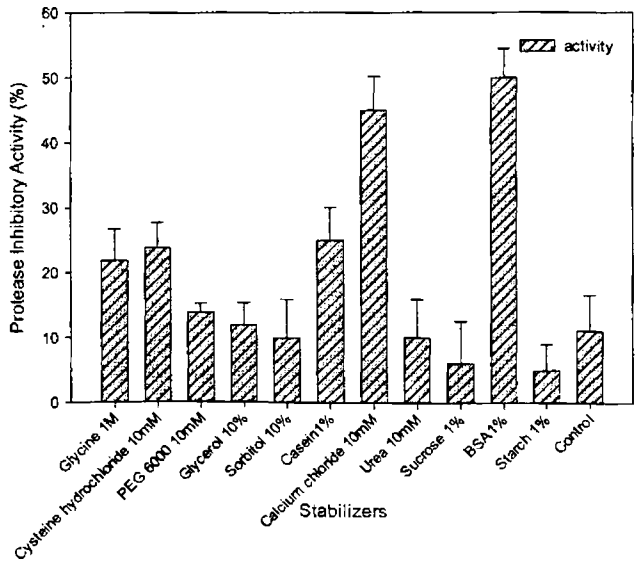


Fig 4.14(A&B) Effect of Stabilizers on thermal stability of Protease Inhibitor after four hours of Incubation at two different temperatures

Comparison at 50 & 60°C

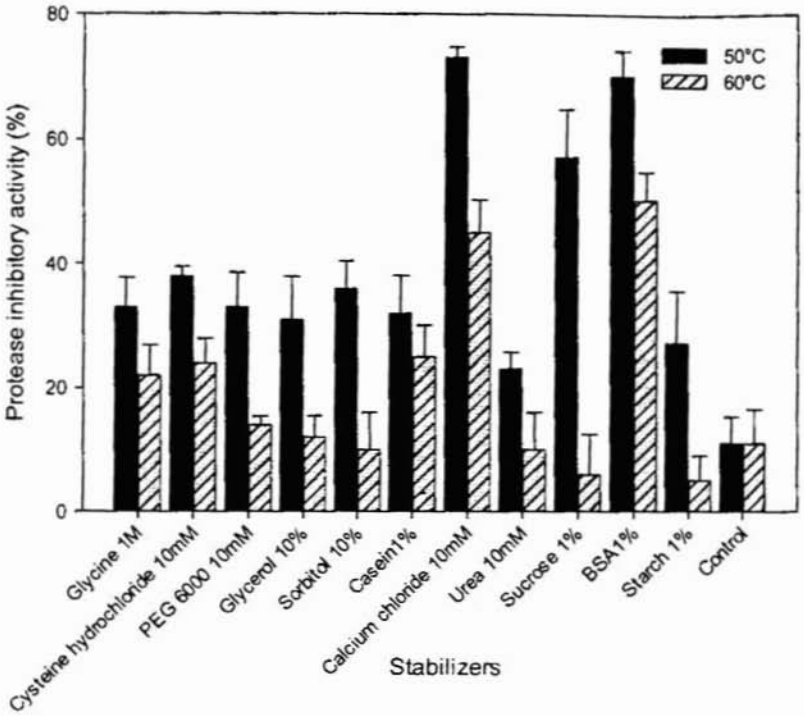


Fig 4.14 (C) Comparison of the effect of stabilizers on the activity of Protease Inhibitor at 50°C & 60°C

4.4.12 Effect of various metal ions on protease inhibitor activity

The activity profile of protease inhibitor in the presence of different monovalant and divalent metal ions was determined by incubating with different concentrations of metal ions. The results presented in Fig 4.15 illustrates that zinc sulphate which supplies divalent Zn^{2+} ions at a concentration of 1mM, enhanced the protease inhibitory activity up to 31% showing a residual inhibitory activity of 131% when compared to control. Whereas, 10mM mercuric chloride, which supplies Hg^{2+} ions enhanced

protease inhibitory activity to 164% residual protease inhibitory activity. Ca^{2+} and Mg^{2+} at 10mM concentration enhanced the protease inhibitory activity only up to a marginal level. Presence of Na^{2+}, Ba^{2+}, Ni^{2+} Cd^{2+}, Mo^{6+} and Al^{3+} did not support protease inhibitory activity when compared to control and instead had a negative effect.

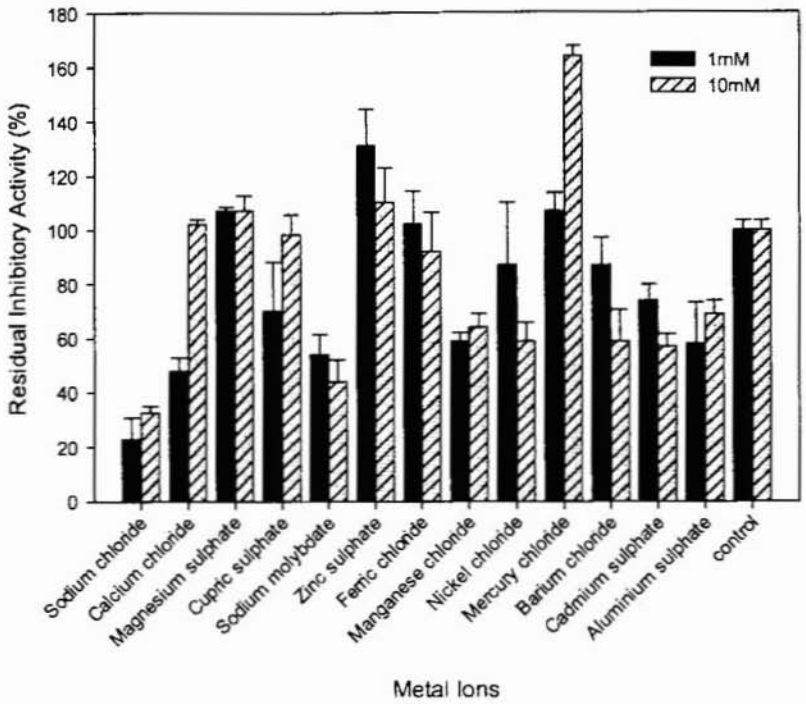


Fig 4.15 Effect of Metal Ions on Protease Inhibitory Activity

4.4.13 Metal chelation of protease inhibitor using EDTA

The presence of divalent cations in protease inhibitor was confirmed by ICP-AES analysis (Table 4.4.). The atomic emission spectrum showed the presence Ca^{2+}, Zn^{2+} and Mg^{2+} in the protease inhibitor. Protease inhibitor prepared in distilled water contained calcium, magnesium and zinc at 5.65, 0.52 1.85 ppm respectively. Protease inhibitor dialysed against

deionised water contained calcium, magnesium and zinc at 4.26, 0.31 and 0.33 ppm. Protease inhibitor dialysed against EDTA contained 0.16 and 0.05 ppm calcium and magnesium respectively and the concentration of zinc ion was not detectable in the sample. Results presented in Fig 4.16 indicated that metal chelation led to a 53% loss in the protease inhibitory activity.

Table 4.4 Mineral ion concentration of protease inhibitor

Sample	Concentration in (ppm)		
	Ca	Mg	Zn
Demetallized Protease inhibitor	0.16	0.05	ND
Protease inhibitor in Distilled water	5.65	0.52	1.85
Protease inhibitor in Deionised Water	4.26	0.31	0.33

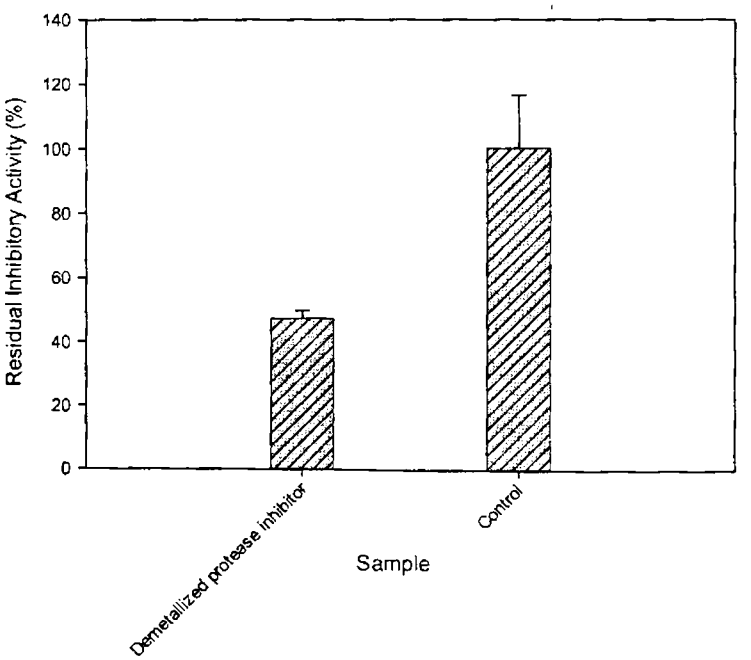


Fig 4.16 Effect of Demetallization on Protease Inhibitor

The results depicted in Fig 4.17 show the effect of the additional supplementation of Ca^{2+}, Mg^{2+} and Zn^{2+} on the demetallized protease inhibitor in its protease inhibitory activity. The addition of Zn^{2+} & Mg^{2+} at a concentration of 10mM enhanced the protease inhibitory activity to 27 & 46% respectively with a residual inhibitory activity of 126 &146% respectively than that of control. The Ca^{2+} ion at a concentration of 10mM also enhanced the protease inhibitory activity to a marginal increase at 10mM concentration.

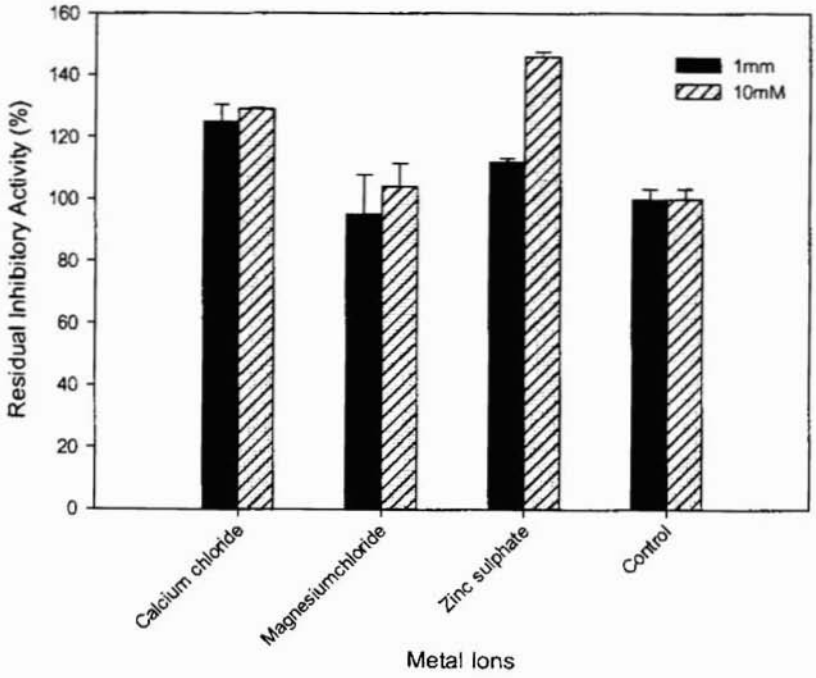


Fig 4.17 Effect of additional supplementation of metal ions to the demetallized Protease Inhibitor

4.4.14 Effect of various detergents on protease inhibitory activity

The result presented in Fig 4.18 concludes that all the ionic and non-ionic detergents except SDS have negative effects on the protease inhibitory activity. In the presence of SDS, the residual activity of the protease inhibitor is 162% and protease inhibitory activity was enhanced to 62% compared to control. Triton X 100, Tween 80 and Tween 20 have an inhibitory activity on protease inhibitor with a 50% loss in activity compared to the activity of control. Brij 20 had completely inactivated the inhibitor with 67% loss in inhibitory activity.

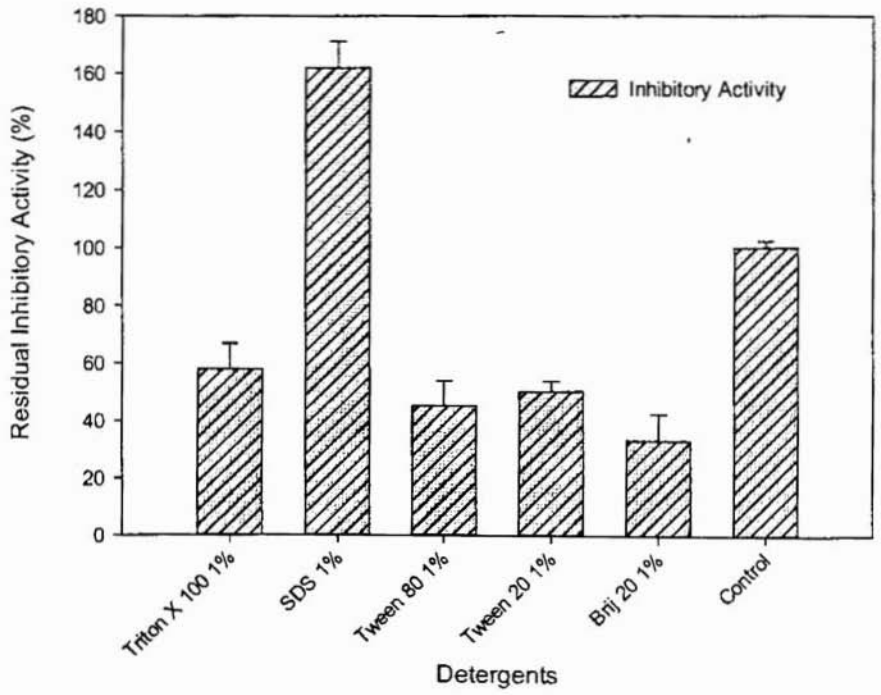


Fig 4.18 Effect of Detergents on Protease Inhibitory Activity

4.4.15 Effect of oxidizing agents on protease inhibitory activity

The results presented in Fig 4.19 indicated that the protease inhibitory activity decreased along with increase in the concentration of oxidizing agents. At 1% of DMSO, the residual inhibitory activity was decreased to 28% and at 5% it reached to residual inhibitory activity of 12%. The residual inhibitory activity of protease inhibitor in the presence of 1% H_2O_2 is 52% and decreased on increasing the concentration of H_2O_2. At 5% H_2O_2 it retained only 32% of protease inhibitory activity compared to control. Thus oxidizing agents inactivated the protease inhibitor from *Moringa oleifera*.

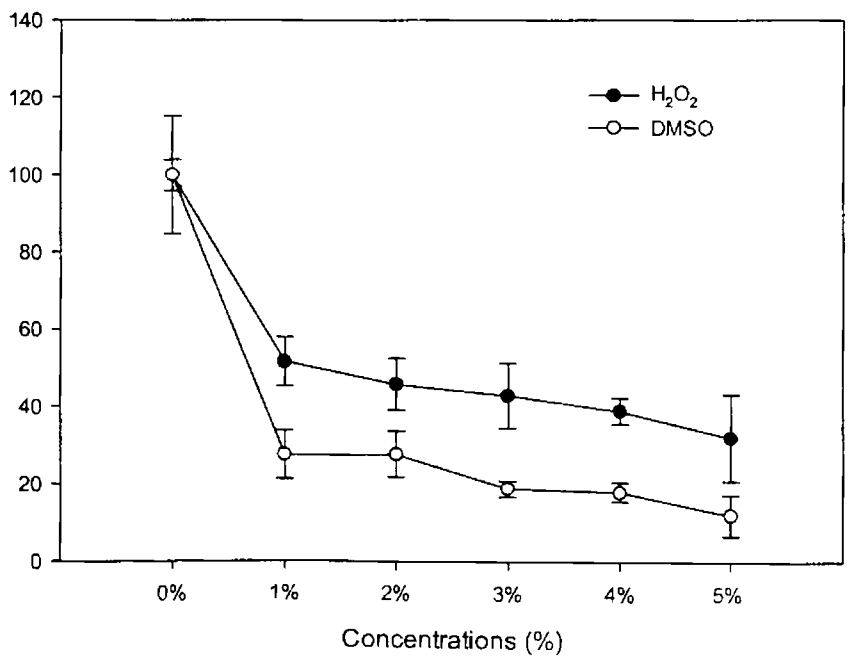


Fig 4.19 Effect of oxidizing agents on Protease inhibitory Activity

4.4.16 Effect of reducing agents on protease inhibitory activity

The result presented in Fig 4.20 testifies that the reducing agents have positive effects on the protease inhibitory activity. The residual inhibitory activity of the protease inhibitor increased along with increase in the concentrations of reducing agents. Thus Dithiothreitol and β-mercaptoethanol, up to a concentration of 1%, enhanced protease inhibitory activity to 49 and 41% respectively.

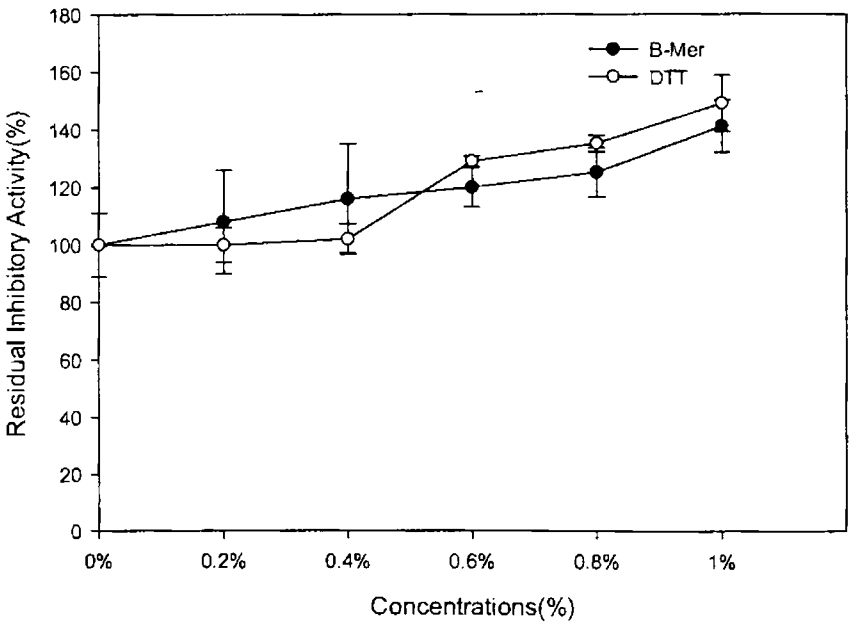


Fig 4.20 Effect of Reducing agents on Protease Inhibitory Activity

4.4.17 Chemical modifications of amino acids in protease inhibitor

Five different amino acids were individually modified using specific chemical modifiers and the effect of amino acid modifications on protease inhibitory activity was determined and the results are presented in Table 4.6 and Fig 4.21. Among the five chemical modifiers, NBS and PMSF followed by DEPC alone influenced positively by enhancing the residual inhibitory activity compared to others. Modification of tryptophan residue by N-bromosuccinamide resulted in the activation of protease inhibitory activity to a greater extent. Increase in concentration of PMSF resulted in the enhancement of protease inhibitory activity with a residual inhibitory activity of 280% at 25mM compared to control. Modification of cysteine by N-ethylmaleimide led to marginal enhancement in the protease inhibitory activity (10% enhancement at higher concentration (25mM)). Increased concentration of DEPC resulted in an increase in the protease inhibitory activity. At concentration of DEPC above 10mM there was an enhancement of protease inhibitory activity up to a level of 50% compared to control. In contrast, lysine modification by succinic anhydride resulted in the loss of protease inhibitory activity at increasing concentrations. The protease inhibitory activity of lysine modified inhibitor was only 9% with a residual inhibitory activity of 91% compared to control. On increase in the concentrations of succinic anhydride, there was a gradual decline in protease inhibitory activity, and at higher concentration the protease inhibitor was totally inactive.

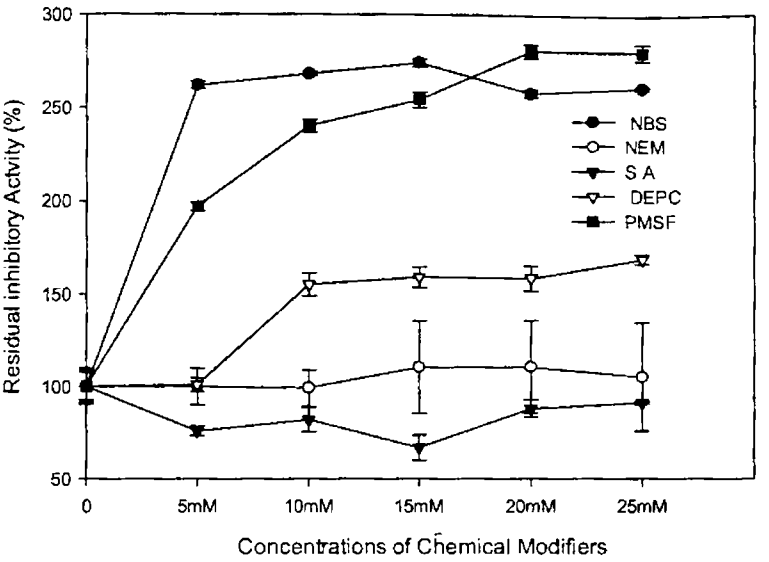


Fig 4.21 Effect of Chemical modifiers on the activity of Protease Inhibitor

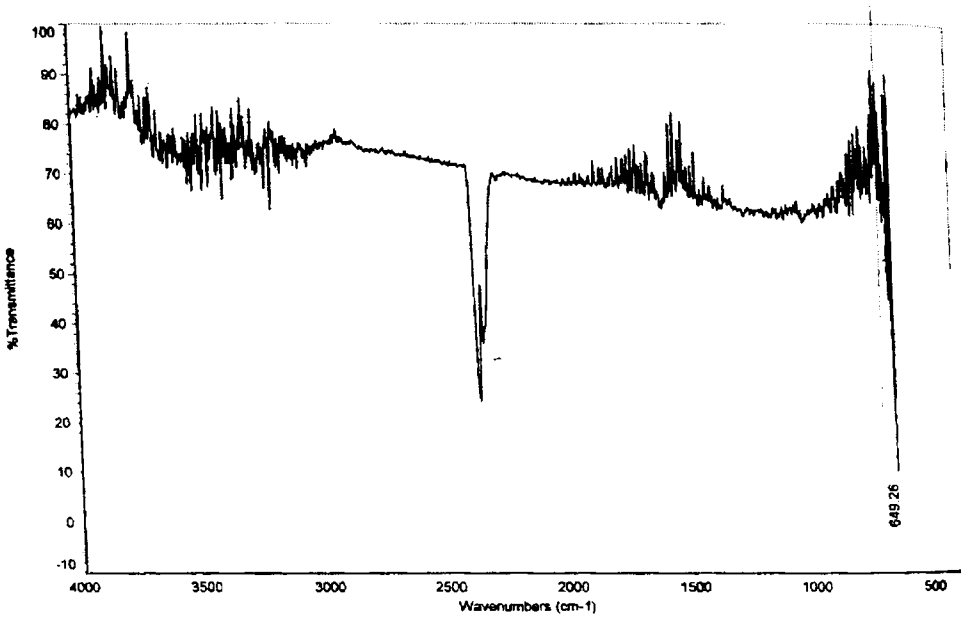
Infra red spectrum of native and modified protease inhibitor were analyzed to know whether the enhancement in protease inhibitory activity was due to the structural changes in the protein (Fig 4.22). The results illustrate a change in spectrum of modified protease inhibitor due to the change in the carbon- hydrogen bond present in the functional group present in the reactive site of the inhibitor.

Table 4.6 Effect of chemical modification on the activity of protease inhibitor

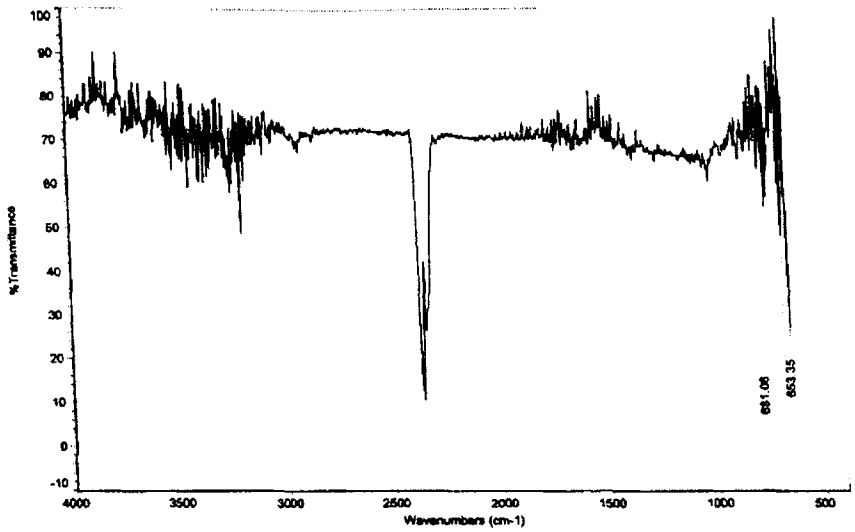
Chemical Modifier	Amino acid modified	Concentration mM	Effect on protease inhibition
N-Ethyl maleimide	Cysteine	5	No Effect
		10	No Effect
		15	No Effect
		20	+
		25	+
Succinic anhydride	Lysine	5	-
		10	-
		15	-
		20	-
		25	-
N- Bromosuccinamide	Tryptophan	5	+
		10	+
		15	+
		20	+
		25	+
Diethylpyrocarbonate	Histidine	5	No Effect
		10	+
		15	+
		20	+
		25	+
Phenyl methyl sulphonyl fluoride	Serine	5	+
		10	+
		15	+
		20	+
		25	+

Fig 4.22 (A, B & C) FT-IR Spectrum of native Protease Inhibitor, tryptophan modified Protease Inhibitor and serine modified Protease Inhibitor

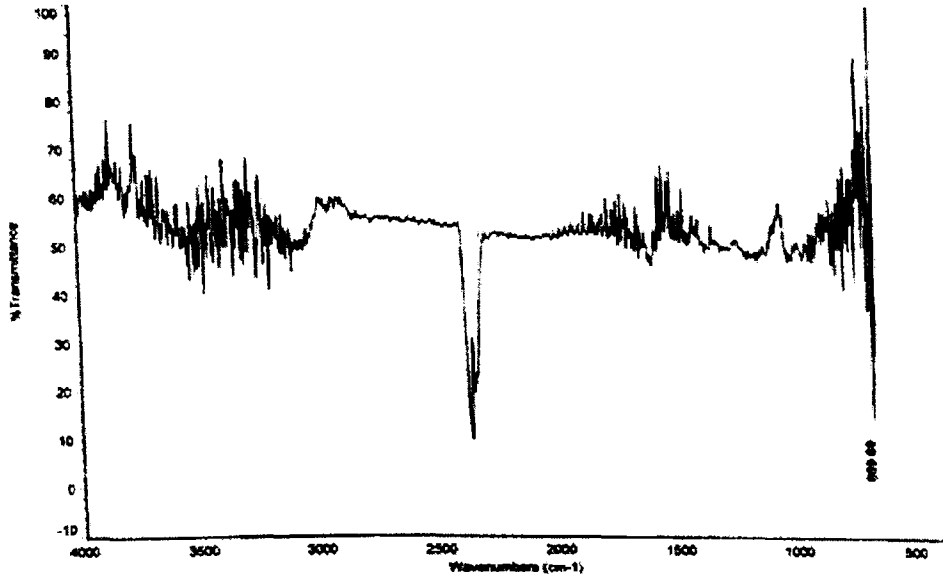
(A)



(B)


% Transmittance
Wavenumbers (cm-1)
681.06
853.35

(C)


% Transmittance
Wavenumbers (cm-1)

4.4.18 Effect of acid treatment on protease inhibitor

Impact of acid treatment, up to 0.02 M HCl, on protease inhibitor did not significantly reduce the inhibitory activity (Fig 4.23). There was a gradual decrease in the activity of protease inhibitor along with increase in the concentration of HCl, and the inhibitor showed a residual inhibitory activity of 68% at 0.1M HCl.

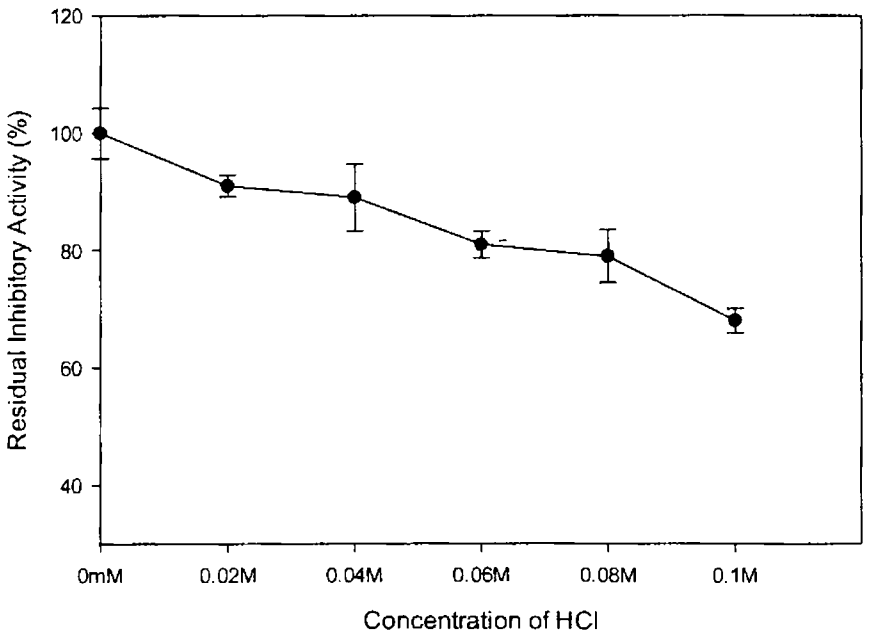


Fig 4.23 Effect of Increasing concentrations of HCl on the activity of Protease Inhibitor

4.4.19 Effect of protease treatment on protease inhibitor

The pretreatment of inhibitor with different concentrations of protease significantly reduced the protease inhibitory activity (Fig 4.24). The result suggests that the residual protease inhibitory activity decreased to 25% on pretreatment with 1% trypsin. It was observed that increase in concentration of trypsin resulted in gradual decline in residual inhibitory activity.

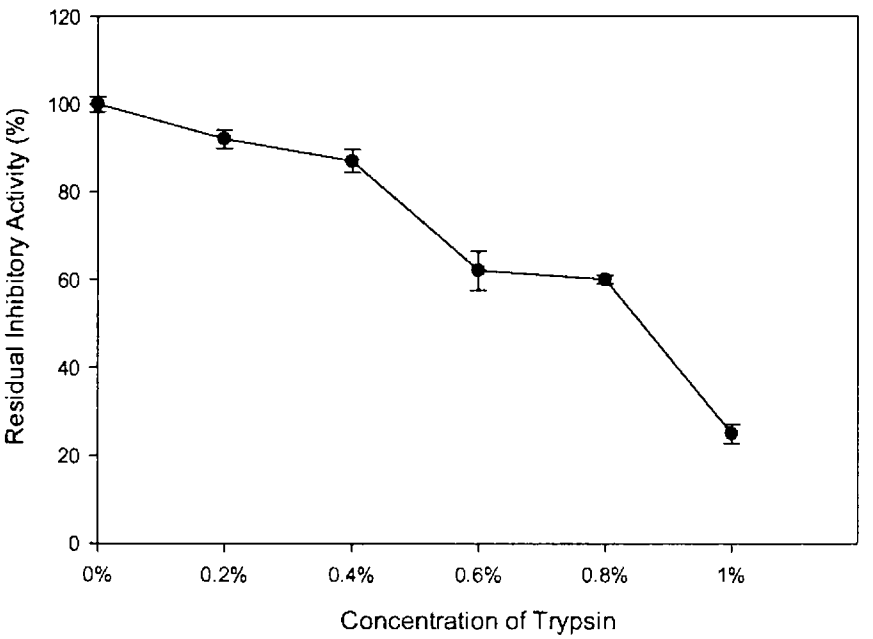


Fig 4.24 Effect of protease treatment on Protease Inhibitory Activity

4.4.20 Stoichiometry of protease-protease inhibitor interaction

The data obtained for the studies conducted on protease-protease inhibitor interaction is depicted in Fig.4.25. Extrapolation to zero protease activity corresponds to 1.5 nM of inhibitor. It is predicted that the stoichiometry of trypsin-protease inhibitor interaction is 1:1.5 and 35.4 μg of protease inhibitor is necessary to completely inactivate 23.8 μg of trypsin.

The amount of inhibitor needed for 50% inhibition (IC_{50}) of trypsin calculated from the graph was found to be 0.6nM.

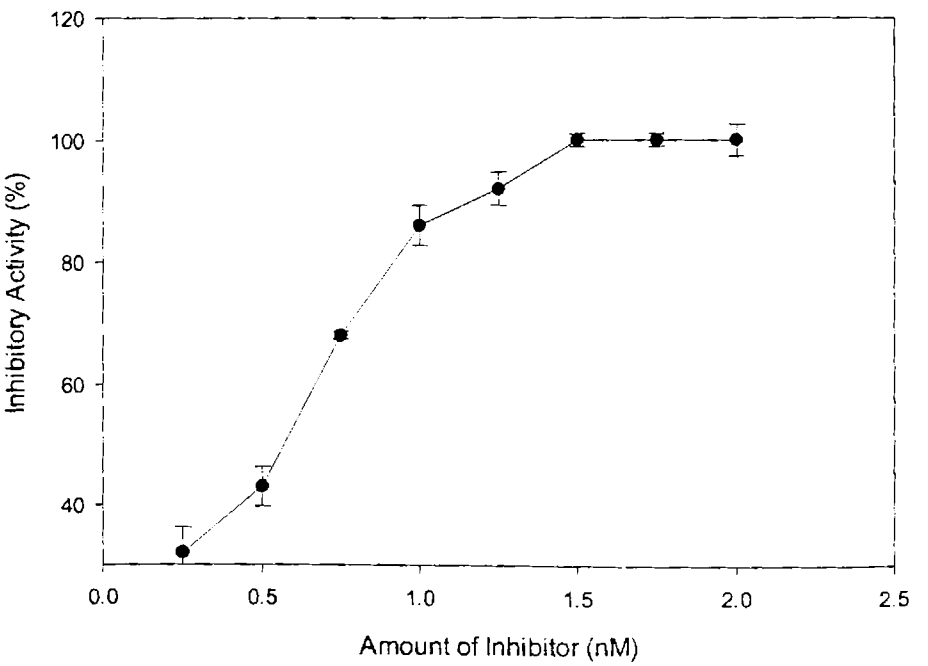


Fig 4.25 Stoichiometry of Protease-Protease Inhibitor Interaction

4.4.21 Kinetic studies of inhibition of trypsin by protease inhibitor

Kinetics of inhibition of trypsin by protease inhibitor revealed that it has a reversible mechanism of action. The same fixed concentration (1.0 nM) of trypsin, preincubated with enzyme buffer alone and different concentrations of inhibitor (4, 6 and 8 nM), yielded different slopes for plots 1/v versus 1/[S] for nine different [S] values (Fig 4.26). Inhibition of substrate hydrolysis occured at very low concentration of protease inhibitor and the K_i was calculated by plotting a Line Weaver-Burk plot, which was found to be 1.5 nM under the assay conditions. The low K_i value implies that it is a powerful inhibitor of serine proteases.

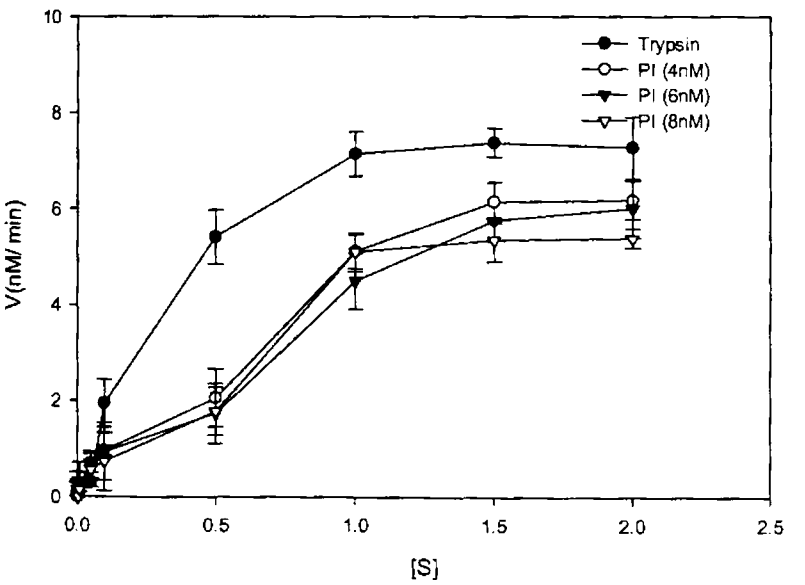


Fig 4.26 Michaelis-Menten plot showing the effect of Protease Inhibitor on trypsin

4.5 APPLICATION STUDIES

4.5.1 Specificity with different pharmaceutically important proteases

The inhibitory activity assessed with different pharmaceutically important proteases revealed that it is highly specific towards serine proteases like chymotrypsin, elastase and thrombin. The inhibitor also has affinity towards cysteine proteases like papain and Cathepsin B (Fig 4.27). Protease inhibitor showed stronger inhibition towards elastase and thrombin with 92 & 93 % inhibition respectively. It has higher percentage of inhibition towards Cathepsin B (79%) than towards papain (54%). There was no significant inhibition for *Moringa oleifera* protease inhibitor towards collagenase.

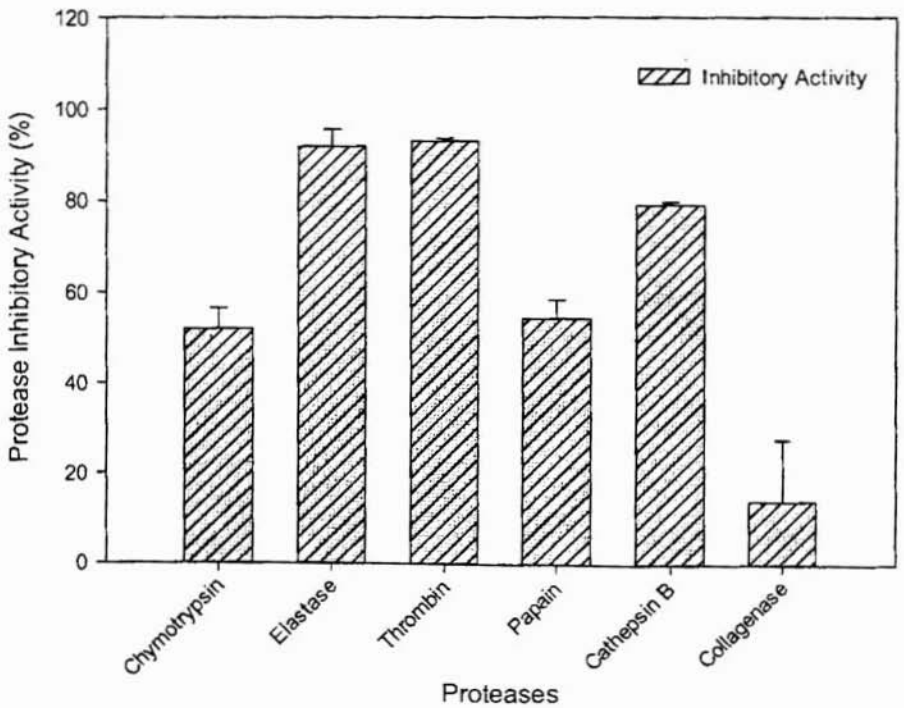


Fig 4.27 Inhibitory activity of *Moringa oleifera* Protease Inhibitor towards pharmaceutically important proteases

4.5.2 Activity spectrum of protease inhibitor towards commercially important proteases

Results presented in Fig 4.28 evidence the activity spectrum of protease inhibitor towards different commercially available industrially important proteases. The protease inhibitor has 100% inhibition towards the proteases isolated from *Bacillus* sp., *Bacillus licheniformis* and *Aspergillus oryza*. It showed 76% inhibition towards the protease isolated from *Engyodontium album*. Affinity of inhibitor towards subtilisin, esperase, pronase E and proteinase K is negligible compared to other proteases. The inhibitory activity of *Moringa oleifera* protease inhibitor towards subtilisin was 3.7% compared to other neutral proteases. From the results it is inferred that the protease inhibitor isolated from *Moringa oleifera* has more affinity towards neutral proteases.

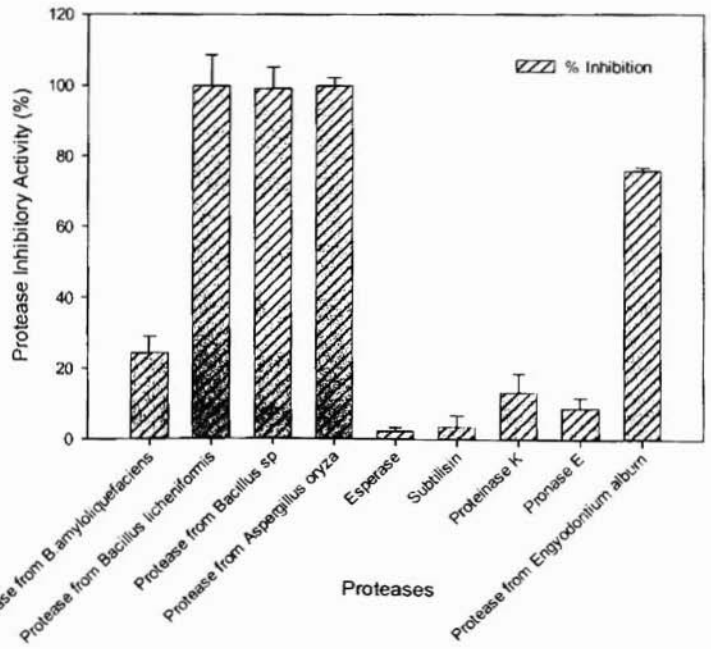


Fig 4.28 Activity spectra of Protease Inhibitor with different industrially important Proteases

4.5.3 Protease inhibitor as seafood preservative

The activity of protease inhibitor towards seafood preservation was evaluated by treating the shrimp, *Peneaus monodon* with protease inhibitor and incubating at different storage temperatures. It was found that protease inhibitor influenced the total viable microbial flora present on the *Peneaus monodon*. The result showed that there is a reduction in microbial population in the sample treated with protease inhibitor compared to the untreated sample (Fig 4.29).

(A) Untreated *Peneaus monodon* **B) Treated *Peneaus monodon***

Fig 4.29 Comparison of the microbial flora of the Protease Inhibitor treated and untreated *Peneaus monodon*

The protein degradation of both control and test samples were monitored by estimating the total protein concentration of each sample. From the result obtained, it is clear that, there is a 41% reduction in protein degradation of the untreated sample compared to control at room temperature after 8 hours of incubation (Table 4.6). But at 4°C & -20°C there was no considerable decrease in protein content compared to initial

untreated sample. Whereas, in protease inhibitor treated sample there was no loss in protein content and there was no detectable protein degradation at each storage temperature studied compared to control. They remain unchanged after incubation in each condition. The protein content of both treated and untreated sample are illustrated in Fig 4.30.

Table 4.6 Effect of protease inhibitor on the protein degradation of *Peneaus monodon*

Sample	Protein content (mg/ml)			
	Initial 0hr	**28± 2°C after 8hrs**	**4°C after 24hrs**	**-20°C after 168hrs**
Untreated *Peneaus monodon* peeled and undeveined	24.00	17.00	22.00	22.56
Treated *Peneaus monodon* peeled and undeveined	25.28	24.56	24.50	25.09

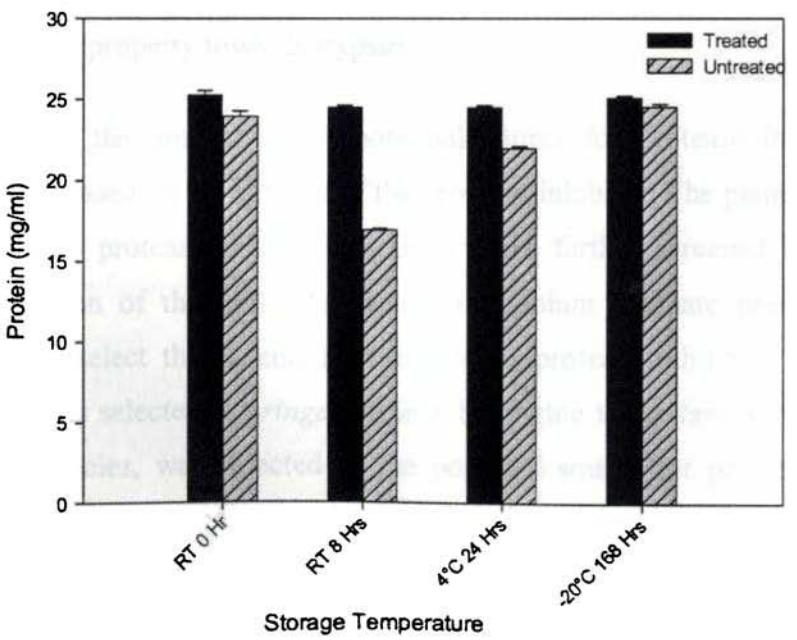


Fig 4.30 Effect of Protease Inhibitor on the Protein degradation of *Peneaus monodon*

Chapter 5

DISCUSSION

5.1 SCREENING OF PLANTS FOR PROTEASE INHIBITOR

Most of the natural protease inhibitors are proteinaceous in nature and are located mainly in seeds, leaves and tubers, which act as specific defense and regulatory proteins. Many reports are available on the isolation, purification and characterization of protease inhibitor from seeds of legume plants (Ryan, 1990). Hence, few plants belonging to different families of Leguminosae, Malvaceae, Rutaceae, Graminae and Moringaceae were screened for protease inhibitor. The screening revealed that most of the plants belonging to Leguminosae store molecules that have highest inhibitory property towards trypsin.

In the present study, potential source for protease inhibitor was selected based on the activity of the protease inhibitor. The plants with more than 60% protease inhibitory activity were further screened after partial purification of the molecules using ammonium sulphate precipitation in order to select the potential proteinaceous protease inhibitor. Among the five plants selected, *Moringa oleifera*, belonging to the family Moringaceae of 16 species, was selected as the potential source for protease inhibitor since those samples recorded high level of protease inhibitory activity after ammonium sulphate fractionation. Although *Moringa oleifera* is known for containing several low molecular weight bioactive constituents with

pharmaceutical and industrial applications (Kalogo et al., 2000), the presence of any protein inhibitors in the Moringaceae family is not reported so far.

Moringa oleifera is a panotropical multipurpose tree with high biomass yield and can tolerate unfavorable environmental conditions (Foidl et al., 2001). Since it can withstand many environmental unfavorable conditions, the presence of some important molecules like enzymes and enzyme inhibitors are involved in the physiology and biochemistry of the plant. Among them, protease inhibitors have a major role in regulation of plant metabolic processes. In plants the presence of protease inhibitors are mainly considered as storage proteins and as defense tools (Azzouz et al., 2005). Results obtained in the present investigation indicate that *Moringa oleifera* is a potential source for protease inhibitor since it recorded 76% protease inhibitory activity. The production of these molecules will depend on many factors. Mainly it is a regulatory and storage protein for the plant. In some cases, the distribution and accumulation of these proteins may vary upon wounding or due to some insect or pest attack (Ryan, 1990). On evaluation of the distribution of protease inhibitor in different plant tissues of the mature *Moringa oleifera* plant, it was observed that the mature leaves had maximum percent of protease inhibition with maximum specific inhibitory activity followed by the seeds. Further, the distribution of inhibitor in flowers, roots and bark were negligible when compared to the leaves and seeds. Since leaves are the major tissues attacked by pest and pathogens, the accumulation of this protease inhibitor is maximum in leaves, compared to other parts, indicating a tissue specific expression of these proteins. In winged bean plant, western blot analysis of the expression

of the protease inhibitor in different tissues suggested that the expression of the protease inhibitor is tissue specific and species specific (Datta et al., 2001). Hence, it may be presumed that leaves and seeds of *Moringa oleifera* are the rich source of protease inhibitor, which is mostly directed towards serine proteases such as trypsin and chymotrypsin.

The extraction medium has a major role in the complete extraction of the protein from any desired source. Hence different solvents were used for extracting proteinaceous protease inhibitors from the leaves. Phosphate buffer was selected as the potent extraction medium for maximal extraction of protease inhibitor from the leaves without any loss in activity. The protein concentration and the protease inhibitory activity were highest in the extract prepared with phosphate buffer. Phosphate buffer facilitated the complete release of proteins from the leaves into the solvent with maximum inhibitory activity. The protease inhibitory activity in the extracts prepared with sodium hydroxide and sodium chloride was very less compared to the other extracts prepared in phosphate buffer, distilled water and sodium chloride. Infact 0.1M phosphate buffer of pH 7.6 was reported to be a good extractant for the maximal extraction of proteins from *Cajanus cajan* seeds with high amount of trypsin inhibitory activity and protein concentration (Pichare and Kachole, 1996).

5.2 PURIFICATION AND CHARACTERIZATION OF PROTEASE INHIBITOR

Protease inhibitor, isolated from *Moringa oleifera* was purified by ammonium sulfate precipitation followed by ion exchange chromatography, and preparative polyacrylamide gel electrophoresis. Ion exchange

chromatography yielded a single fraction with maximum protease inhibitory activity, which was further analyzed by polyacrylamide gel electrophoresis to check its purity and homogeneity. A 2.5 fold purification with a 16.3% protein recovery obtained after ion exchange chromatography, was enhanced up to 41.4 fold with a protein recovery of 0.013% after preparative PAGE. It is clear that an increase in fold and recovery of protein yield can be obtained by repetitive purifications using combinations of many advanced purification methods.

Purification of protease inhibitor isolated from Indian tasar silk worm, *Antheraea mylitta* by ammonium sulphate fractionation showed a 1.56 fold purification. After ion exchange chromatography the fold of protein purification was increased up to 49.9 and increased to 200 fold by FPLC (Isel et al., 2004; Shrivastava and Ghosh, 2003). This suggests that the fold and recovery of protein can be increased by using different advanced combination of purification methods.

The purity and homogeneity of the single fraction with maximum inhibitory activity obtained after ion exchange chromatography were analyzed by SDS-PAGE. The results of SDS-PAGE analysis undoubtedly evidenced the homogeneity of protease inhibitor. An apparent molecular weight of 23.6 kDa was obtained in SDS-PAGE under non-reducing conditions. But under reducing conditions, in the presence of β-mercaptoethanol the protein was visualized as two-polypeptide band, one having molecular weight of 22 kDa and another protein with low molecular weight of 14 kDa. This result indicates that *Moringa oleifera* protease inhibitor is probably composed of two polypeptide chains linked by one or

more disulphide bonds. This type of two chain structure have been reported previously for a 20 kDa Kunitz type protease inhibitor found in seeds of *Adenanthera pavonia, Acacia elata, Albizia julibrissin* and *Prosopsis juliflora* (Andrea et al., 2001; Kortt and Jermyn, 1981; Richardson, 1991). The protease inhibitory activity of each band was confirmed by reverse zymography method on gelatin-incorporated polyacrylamide gel and by Dot-Blot assay method. The results obtained evidence that both the peptide bands inhibited gelatin hydrolysis to a greater extent. When the two peptide bands obtained after SDS-PAGE under reducing conditions were eluted out and rechecked for the protease inhibitory property of each band, it was observed that the protein band with a molecular weight of 22 kDa had the maximal protease inhibitory activity compared to the low molecular weight protein band. It is hence inferred that the larger protein is the major subunit responsible for the protease inhibitory activity.

The molecular mass obtained by gel filtration chromatography was approximately 29 kDa, which was in agreement to the molecular mass estimated by SDS-PAGE analysis. The molecular mass and the disulphide content of the polypeptide indicated that *Moringa oleifera* protease inhibitor has high homology with Kunitz type of inhibitors. It was reported earlier that the molecular mass of Kunitz type inhibitors are in the range of 18-26 kDa and are mostly monomeric or dimeric members, in which subunits are linked by a disulphide bridge (Ligia et al., 2003; Richardson, 1991). They are proteins with 170-190 amino acids, which usually contain four cysteine residues that form two disulphide bridges. Hence it is concluded that the protease inhibitor isolated from *Moringa oleifera* is a

small protein with a molecular weight of 23.6 kDa and belongs to the Kunitz type of serine protease inhibitor family.

Most of these inhibitors are small molecules with relative molecular masses ranging from 5-25 kDa, with compact structures and many cases with a high content of disulphide bridges, characteristics that might contribute to their high thermal stability (Singh and Rao, 2002). According to Mcmanus et al., (1994) a protease inhibitor isolated from potato tuber (PI-2) is a dimeric protein with two subunits that differ in size as determined by SDS-PAGE (15 kDa for larger subunit and 6 kDa for smaller subunit) belongs to Kunitz family of serine protease inhibitor. The molecular mass of *Peltophorum dubium* trypsin inhibitor (PDTI) by gel filtration and SDS-PAGE was ~ 20 kDa, which agreed with the amino acid composition of 185 residues, including four cysteine residues. The molecular mass, low disulphide content and N-terminal sequence indicated that PDTI is a Kunitz type inhibitor that shared high homology with soybean trypsin inhibitor and Kunitz inhibitors from the seeds of Mung bean, and Brazilian Carolina tree (Richardson, 1991). So based on the molecular nature of the novel protease inhibitor from *Moringa oleifera*, it is inferred that it could be placed under the family of Kunitz type in serine protease inhibitor class.

The protease inhibitor from *Moringa oleifera* has an optimum pH at 7.0 for its maximal activity, was active in the pH range of 6.0-10.0, and was totally inactive in the extreme acidic and basic pH. The extreme pH conditions could have totally altered the structure of the inhibitor making them completely inactive to bind with the enzymes or with their substrates. Under strong acidic or alkaline conditions, the proteinaceous inhibitors get

denatured and as a consequence they loose their activity partially or completely. In general, all the protease inhibitors isolated from plants have a wide pH range of 2.0-10.0. Many enzyme inhibitors in seeds are present in multiple molecular forms, which may differ considerably in their pI values. Most inhibitors in the Kunitz family are acidic and some are very sensitive to acidic pH and stable in the alkaline pH (Mello et al., 2002).

The intra molecular disulphide bridges are presumably responsible for the functional stability of Kunitz type protease inhibitors in the presence of physical and chemical denaturants such as temperature, pH and reducing agents (Kridric et al., 2002). The data obtained for the pH stability studies of the protease inhibitor from *Moringa oleifera* evidence that the protease inhibitor was stable over a wide range of pH from 5.0-10.0. However, the activity got decreased at extreme pH conditions of acidic and basic. The stability of protease inhibitor in a wide range of pH signifies its use as biopesticides, which can withstand highly alkaline conditions of insects' gut flora. Insects that feed on plant material possess alkaline guts and depend predominantly on serine proteases for digestion of food material. The majority of the protease inhibitors exhibiting anti-feedent properties reported so far are active against the neutral serine proteases such as trypsin and chymotrypsin (Ryan, 1990).

The conformational changes accompanying thermal denaturation under neutral, acidic and reducing conditions of *Cajanus cajan* proteinase inhibitor were investigated using near and far ultraviolet circular dichroism (CD) spectroscopy. The protein inhibitor shows a reversible N<-->D transition at neutral pH with a Tm approximately equal to 63°C. The effect

is more pronounced at low pH and in the presence of dithiothreitol. Only partial reversibility is observed under acidic conditions. Significant changes in the near as well as far-ultraviolet CD spectrum are observed in the presence of dithiothreitol suggestive of the importance of disulphide linkages in maintaining the structure of *Cajanus cajan* proteinase inhibitor (Haq and Khan, 2005).

Highest optimal temperature recorded for maximal activity of protease inhibitor isolated from *Moringa oleifera* was in the range of 30-40°C. Most of the inhibitors of Kunitz family plant protease inhibitors are active at temperatures up to 50°C (Hamato et al., 1995). However, the protease inhibitory activity declined at temperatures above 50°C and the protease inhibitor was totally inactive at 80°C. Whereas it was active at 10° and 20°C. The activity increased along with increase in the temperature and was maximum at 40°C.

Thermal inactivation of protease inhibitor at different temperatures resulted in a progressive loss of activity at temperatures above 40°C. The inhibitor is moderately stable up to two hours at 30°C and 40°C with 50 % protease inhibitory activity. These observations indicate that the protease inhibitor has high intrinsic stability in its native state, which gives a high degree of thermal stability. This property of protease inhibitor is typical of all trypsin inhibitor family. Soybean trypsin inhibitor, purified by gel permeation chromatography, had much thermal stability and the presence of a protein substance accelerated the thermal inactivation of the inhibitor (Ellenreider et al., 1980). The high thermal and pH stability of protease inhibitor testifies its applications in various industries. The thermal

inactivation was due to folding and unfolding of proteins due to heat, resulting from the disturbance of covalent and non-covalent interactions (James and Shawn, 1998). The *Schizolobium parahyba* chymotrypsin inhibitor, a Kunitz type inhibitor, lost its activity to 40% at 70°C after one hour of incubation and the thermal inactivation was due to the partial unfolding of protein structure, which resulted in a disturbance in covalent and non-covalent interactions (Souza et al., 1995). The thermal stability of the *Schizolobium parahyba* chymotrypsin inhibitor as a function of pH has been investigated by fluorescence and CD spectroscopy and the studies suggested its high thermal stability in a wide range of pH. The low histidine content (~1.7%) and the high acidic residue content (~22.5%) of the inhibitor suggested a flat pH dependence of thermal stability in the region 2.0-8.8 and that the decrease in thermal stability at low pH can be due to the differences in pK values of the acidic groups (Teles et al., 2005). A number of protease inhibitor from plants has been reported to have high optimal temperatures up to 70°C and are stable at temperatures in the range of 50-70°C (Huang et al., 1981).

Enhancement of thermal stability is desirable for most of the biotechnological applications of proteins. Naturally occurring osmolytes such as amino acids, polyols and salts are known to protect proteins against thermal inactivation by stabilizing the thermally unfolded proteins (Yancy et al., 1982). Thermal stability increases the efficiency of proteins and is one of the essential features for their commercial exploitation (Jui et al., 2002). Effect of stabilizers on the thermal stability of protease inhibitor was studied by the addition of different stabilizers and incubating the inhibitor at 50 & 60°C where it lost its activity. Among the stabilizers tested almost all of the

stabilizers gave stability at 50°C and promoted the inhibitory activity compared to the control. Maximal stability was provided by calcium chloride (73%) followed by BSA (70%). At 60°C the stabilizers such as sucrose and starch, which supported the stability at 50°C, did not promote the inhibitory activity. BSA promoted protease inhibitory activity at 60°C with maximal inhibitory activity than calcium chloride.

The role of electrostatic interactions also plays a major role in thermal stability of protease inhibitors. Calcium chloride provided a significant increase in the stability of protease inhibitors at higher temperatures. Calcium ions stabilize the protein through specific and non-specific binding sites, and may also allow for additional binding within the protein molecule preventing the unfolding at higher temperatures. High content of charged residues present in the protease inhibitors may contribute considerably towards maintaining stability by electrostatic interaction in neutral pH (Souza et al., 1995). These interactions also play important roles in maintaining the structural stability of enzymes of thermophilic organisms. Hydrophobic protein interactions are intensified in the presence of sugars and polyhydric alcohols. Glycerol and some carbohydrates stabilize intra molecular interactions by preferential hydration of the polypeptide chain (Timasheff and Arakawa, 1989). Protein stability is related to the increase of hydrophobic forces inside these molecules (Yutani et al., 1980). Solvents with a low dielectric constant strengthen the hydrophobic interactions among non-polar residues (Gekko and Ito, 1990). In the presence of such solvents, several proteins show a greater resistance to thermal denaturation processes (Cordt et al., 1994; Geanfreda and Scarfi, 1991).

The improvement of thermal stability of protease inhibitor by protein engineering might be a valuable tool for various industrial applications. The use of protease inhibitor as thermal stabilizers for protease enzyme is a novel tool developed in modern biotechnology industries. It is reported that a protease inhibitor (API-1) isolated from actinomycete has improved the thermal stability of protease enzyme used for detergent industries. The fungus *Conidiobolus macrosporous* produced high yield of extracellular alkaline protease that has potential biotechnological applications in both detergent and leather industries. The alkaline protease was stable only up to 40°C and lost its activity on increasing the temperature. It was found that the binding of API-1 enhanced the stability of enzyme at 50°C up to one hour (Jui et al., 2002). Thus thermal inactivation of detergent proteases can be prevented by binding of protease inhibitor. Consequently there is a scope for a novel strategy for stabilization of proteases through the formation of reversible enzyme-inhibitor complexes.

Detergents are used extensively for solubilizing protein from lipid membranes and other biological materials and for maintaining the solubility of certain proteins in the solution. Protease inhibitors and detergents are routinely used together in cell lysis buffers to inhibit unwanted proteolysis and facilitate membrane protein solubilization in protein purification procedures. Hence the effect of detergents on the activity of protease inhibitor from *Moringa oleifera* was also studied. From the results it is inferred that all the detergents studied except SDS have inhibitory effect on protease inhibitory activity. SDS enhanced 62% protease inhibitory activity compared to control and hence it is concluded that SDS can act as stabilizer for the protease inhibitor under unfavorable conditions like thermal

denaturation. The reduction in the protease inhibitory activity in the presence of detergents may be attributed to the combined effect of factors such as reduction in the hydrophobic interactions that play a crucial role in holding together the protein tertiary structure and the direct interaction with the protein molecule. The hydrophobic nature of SDS apparently caused rearrangement of peptide backbone conformation leading to helix-formation with more hydrophobic residues exposed and consequently available to associate with the detergent (Bressollier et al., 1999).

Earlier studies on the effect of SDS on soybean Kunitz inhibitor showed that the protein was highly sensitive to this detergent, with respect to its conformation (Jirgensons, 1973). Absence of characteristic features in the far-ultraviolet region (190–250 nm) representative of hydrogen bonded α- helical or β-structures in soybean Kunitz inhibitor leads to the conclusion that the inhibitor is stabilized chiefly by hydrophobic interactions and the major conformation in this protein is the loop and bend structure. CD spectroscopic analysis of the Kunitz-type pigeon pea inhibitor has revealed the absence of any α-helix and the CD spectrum is representative of the β-II class of polypeptides (Haq and Khan, 2003; Wu et al., 1992). Upon binding with their target proteases, most serpins form complexes that are stable to denaturation in SDS. This stability indicates the presence of covalent bond between enzyme and inhibitor.

From the studies reported, three distinct modes of interaction of detergents with proteins has been proposed (Nozaki et al., 1974): (a) association with specific binding sites of native proteins (b) cooperative association between protein and a large number of detergent molecules

without major conformational change (c) cooperative association with conformational changes in the protein such that the native structure is destroyed and replaced by an extended rod-like conformation with a moderately high content of α helix, in which most of the hydrophobic residues are presumably exposed for association with the detergent. Loss of activity (function) without a concomitant loss or change in structure suggests that certain key amino acids required for inhibiting enzyme activity and the reactive site residues are affected.

Addition of metal ions such as Ca^{2+}, Mg^{2+}, Hg^{2+}, Na^{2+}, Ba^{2+}, Ni^{2+}, Cd^{2+}, Mo^{6+} and Al^{3+} on the activity of protease inhibitor from *Moringa oleifera* was studied. The results illustrate that addition of divalent ions such as Zn^{2+} at a concentration of 1mM enhanced the protease inhibitory activity up to 31% and Hg^{2+} at a concentration of 10mM enhanced up to 64% of protease inhibitory activity compared to that of control. Ca^{2+} and Mg^{2+} at higher concentration (10mM) enhanced the protease inhibitory activity only to a marginal level. Metal ions have a major role in maintaining the structural integrity of protease inhibitor. The side chain carboxylates of glutamate and aspartate residues can participate in binding of divalent cations to metalloproteins. The presence and role of metal ions in the protease inhibitor was studied by chelating the protease inhibitor with EDTA. The metal ion concentration of both native and demetallized protein was estimated using inductively coupled atomic emission spectroscopy and revealed the presence of Zn^{2+}, Ca^{2+} and Mg^{2+} in the protease inhibitor. The removal of these ions resulted in the loss of protease inhibitory activity. There was a 53% loss in protease inhibitory activity compared to control. Removal of Zn^{2+} ion from Pearl millet cysteine protease inhibitor resulted in

the loss of protease inhibitory activity and antifungal activity (Joshi et al., 1998). Disruption in the secondary structure of the demetallized inhibitor implied the role of Zn^{2+} in maintaining the structural integrity of protein. Demetallization results in the amino acid modifications and thus make the inhibitor unable to bind with the active site of the enzyme. Thus the metal ions are important in maintaining the biologically active conformation of protease inhibitor to bind with enzyme or substrate to exhibit the anti protease activity.

The removal of metal ions not only decreases the biological activity of the proteins but also enhance the biological activity. Soybean Bowman Birk inhibitor is a metalloprotein and removal of metal bound to BBI enhances BBI inhibitory activity against matrix metalloproteases-1 (MMP-1). The potential metal binding capability of BBI as well as the ability of BBI to inhibit the activation of Pro MMP-1 will provide new insights into the potential use of BBI in food products and the potential *in vivo* health enhancing abilities of BBI in degenerative angiogenic diseases onset and/or progression. The presence of $Ca^{2+}$and Mg^{2+} ions are detectable in native BBI and the removal of these ions enhanced the protease inhibitory activity of soybean Bowman Birk inhibitor (Jack et al., 2004).

Additional supplementation of Zn^{2+}, Ca^{2+} and Mg^{2+} ions to the demetallized protein increased the protease inhibitory activity at higher concentrations. The addition of $Zn^{2+}$and Mg^{2+} enhanced the activity to 27 & 46% respectively. Ca^{2+} at 10mM concentration enhanced the protease inhibitory activity to a marginal level. This observation implies the presence and role of metal ions in the activity of protease inhibitor from *Moringa*

oleifera. The metal ions will bind to the protein in a reversible mechanism and they retain their conformational stability for their biological activity.

Results obtained for the effect of various oxidizing agents on protease inhibitor activity, showed that the protease inhibitory activity decreased along with an increase in the concentration of oxidizing agents. At 1% dimethyl sulphoxide, the protease inhibitor lost 72% of inhibitory activity and at 5% the residual inhibitory activity was only 12% compared to control. Whereas, in the presence of H_2O_2 the *Moringa oleifera* protease inhibitor retained 52% at 1% H_2O_2 and 32% at 5% H_2O_2 concentration. The loss in activity was due to the oxidation of methionine residue present at the reactive site of the inhibitor.

It is reported that oxidation of methionine residues has been shown to cause a decrease in the biological activity of the protein. Thus α-1 protease inhibitor, a serine protease inhibitor can be oxidized on two of its eight methionine residues and oxidation of one 'Met^{358}' located in the reactive site causes an almost complete loss of inhibitory activity towards its primary target elastase. It was proposed that methionine oxidation is a general means for regulating the activity of proteins. Thus oxidation could be a major factor for the regulation of protease inhibitory activity (Johnson and Travis, 1979).

The effect of reducing agents on the activity of protease inhibitor illustrates that the residual inhibitory activity increased along with increase in concentrations of reducing agents. The intra molecular disulphide bridges are presumably responsible for the functional stability of Kunitz type protease inhibitor in the presence of reducing agents. Dithiothreitol at a

concentration of 1mM had no effect on the activity or stability of *Peltophorum dubium* protease inhibitor (Ligia et al., 2003). *Erythrina caffra* trypsin inhibitor, a Kunitz type trypsin inhibitor from *Erythrina caffra* retained its inhibitory activity after reduction with dithiothreitol (Lehle et al., 1996).

Acid treatment on protease inhibitor up to 0.02M HCl did not reduce the inhibitory activity significantly. Whereas, increase in the concentration of HCl led to a gradual decrease in protease inhibitory activity. The pretreatment of protease inhibitor with digestive protease like trypsin resulted in a gradual decrease in inhibitory activity on increasing the concentration of trypsin. This suggests the sensitivity of protease inhibitor isolated from *Moringa oleifera* towards higher concentrations of HCl and digestive proteases.

Results obtained for the effect of chemical modifiers on the activity of protease inhibitor from *Moringa oleifera* testify that, modification of amino acids, tryptophan, serine and cysteine enhanced the protease inhibitory activity to a great extent. In contrast lysine modification by succinic anhydride reduced the protease inhibitory activity at higher concentrations. The IR spectrum obtained for native and modified protease inhibitor evidence that some modification has occurred on the amino acid present at the reactive site of protease inhibitor. The loss of activity after modification of lysine may be due to the change in conformation of the inhibitor molecule during binding of the modifying agents. Alternatively, one of the modified residues, though not being involved in the reactive site of the inhibitor, may disturb the interaction of the inhibitor with the target

enzyme. There was a change in inhibitory activity of protease inhibitor from buckwheat seeds on modifications of lysine and arginine residue present at the reactive site of the inhibitor (Tsybina et al., 2001).

In proteins, the arrangement of functional groups in a particular manner is an essential requirement for their activity. Knowing the reactive site and the functional group involved for the particular biological activity, it will be a valuable tool for designing novel protease inhibitors as drugs for blocking specific protease actions and thus aiming protease inhibitors as a target for the pharmaceutical industry. The modification of amino acids by chemicals is a valuable tool for knowing the reactive site and also to understand the chemical group involved in specific protein-protein interactions.

In chemical modification, a chemical reagent binds covalently to specific amino acid side chains of a protein and may produce changes in the properties/activity of a protein. Attempts to correlate these changes with catalytic activity have been made previously (Jonossen and Svendson, 1982; Yang et al., 1998). It is reported that chemical modification of Pearl millet cysteine protease inhibitor provided evidence for the presence of two distinct sites responsible for antifungal and antifeedant activities (Joshi et al., 1998). Antifungal activity was lost after modification of cysteine, arginine or aspartic/glutamic acid residues, whereas cysteine protease inhibitory activity was selectively enhanced by modification of histidine or arginine residues. In the majority of specific serine protease inhibitors, the inhibitory action is localized to a specific reactive site situated within a loop closed by a disulphide bridge (Ozawa and Laskowski, 1966).

It is reported that *Peltophorum dubium* protease inhibitor, a Kunitz type serine protease inhibitor was inactivated by lysine and arginine modification with tri-nitrobenzene-sulfonic acid and 1,2-cyclohexanedione respectively (Ligia et al., 2003). The inactivation was more with arginine modification.

Chemical modification of actinomycete protease inhibitor (API) by a tryptophan specific modifier NBS, resulted in the loss of its functions as a consequence of its inability to form a functional enzyme-inhibitor complex. Thus the tryptophan of API plays an essential role in maintaining the hydrophobicity of the binding pocket and in its efficient binding to the target enzyme (Jui et al., 2002).

Protease inhibitors from plants and microorganisms are characterized by either a reversible or irreversible mechanism (Polgar, 1989). Kinetic studies of trypsin by *Moringa oleifera* protease inhibitor revealed that it has a reversible mechanism of action. It was observed that the stoichiometry of trypsin and protease inhibitor interaction takes place in a 1:1.5 molar ratio. The amount of protease inhibitor needed for the 50% trypsin inhibition was 0.6 nM. Results of the kinetic studies of protease inhibition indicate that trypsin inactivation occurs by uncompetitive inhibition. The inhibition of substrate hydrolysis occurred at very low concentrations of protease inhibitor and the K_i was calculated to be 1.5×10^{-9} M under the assay conditions. The low K_i value indicates a relatively high affinity of *Moringa oleifera* protease inhibitor for the trypsin.

The inhibitory activity of Kunitz type protease inhibitor varies. A few members of this family are specific for chymotrypsin but do not inhibit

trypsin. Some are specific for both trypsin and chymotrypsin with high affinity. *Peltophorum dubium* protease inhibitor inhibited bovine and porcine trypsin stoichiometrically (K_i of 4 X 10^{-10} M and 1.6 X 10^{-10} M respectively) but affected bovine chymotrypsin only weakly (K_i of 2.6 X 10^{-7}M) (Ligia et al., 2003).

Results of kinetic studies of inhibition of *Coccidioides immitis* protease indicates the inhibition occurs through competitive inhibition with a K_i value of 2.3 X 10^{-8} (Ling and Garry, 1989). The kinetics of inhibition of papain by Pear millet cysteine protease inhibitor revealed that it has a reversible mechanism of action with an uncompetitive mode of inhibition. Inhibiton of casein degradation by papain occurred at a very low concentration (6.5 X 10^{-9} M) which illustrated its high affinity towards papain (Joshi et al., 1998).

5.3 APPLICATION STUDIES

The high affinity of *Moringa oleifera* protease inhibitor with serine protease inhibitor signifies its importance in various pharmaceutical and agricultural industries. The results presented in this study depicts that the protease inhibitor isolated from *Moringa oleifera* has high affinity towards serine proteases like trypsin and chymotrypsin, elastase, and thrombin. It showed an inhibitory activity of more than 90% towards thrombin and elastase, two serine proteases, which has more importance in pharmaceutical industry. The novel protease inhibitor also had affinity towards cysteine protease like Cathepsin B with an inhibitory activity of 76% and papain (54%).

The protease inhibitor isolated from *Moringa oleifera* has more than 90% inhibition towards elastase. Human leukocyte elastase (HLE) is capable of degrading a variety of proteins. Under normal circumstances, its natural inhibitors effectively control the proteolytic activity of HLE. However, an imbalance between elastase and its endogenous inhibitors may result in several pathophysiological states such as chronic inflammatory diseases, cardiovascular disorders and chronic obstructive pulmonary disease (including emphysema and chronic bronchitis). Hence there is a scope for the use of this novel elastase inhbitor from *Moringa oleifera* as a drug in pharmaceutical industries. It is reported that bitter gourd, *Momordica charantia* contains both trypsin and elastase inhibitory protein (Hamato et al., 1995). Similarly the high affinity towards thrombin also signifies its use as anticoagulant agent for pharmaceutical industry. The *Moringa oleifera* protease inhibitor also showed affinity towards cysteine protease like Cathepsin B, the major virulent proteases present in many protozoan diseases like Leishmaniasis. An attractive target for new chemotherapy for Leishmaniasis is two cysteine proteases: Cathepsin B-like and Cathepsin L-like, which are required for parasitic growth and virulence (Mottram et al., 1998). It is reported that peptide based cysteine protease inhibitors alter the Golgi complex ultra structures and function in *Trypanosoma cruzi* (Juan et al., 1998).

Serine protease inhibitors modulates protease activities and control a variety of the critical protease mediated processes like coagulation, fibrinolysis and tissue remodeling (Laskowski and Kato, 1980), and also in the neurobiology of aging (Higgins et al., 1990) and the development of cancer (Koivunen et al., 1991). Earlier reports evidence that consumption of

seeds containing protease inhibitors lowers the incidence of breast, colon, prostatic, oral and pharyngeal cancers (Correa, 1981). Several studies performed indicated that soybean derived Bowman Birk inhibitor can prevent carcinogenesis *in vivo* and malignant transformation *in vitro* (Kennedy, 1998).

It has been suggested that a Cathepsin B-like protease, a secretory form of lysosomal Cathepsin B, present in some cancer exudates, is involved in the invasive process of cancer pathology. Cysteine protease inhibitor present in the same fluid strongly inhibited the action of Cathepsin B in the cancer cells. Thus the inhibitor could play a protective role in the tumor invasion (Keppler et al., 1985).

It is reported that a Kunitz type trypsin inhibitor from *Enterolobium contortisiliquum* seeds strongly inhibited Bovine trypsin and chymotrypsin and also some serine proteases involved in the blood clotting cascade and the fibrinogen proteolysis: human plasma kallikrein, Factor XIIa and plasmin (Isabel et al., 1996).

Maria et al., (2004) reported protein hydrolysates isolated from rapeseed as a source of HIV protease inhibitor. Thus the identification of inhibitory peptides derived from natural proteins is a novel approach towards the search for therapeutic drugs that is world while promising.

The inhibitor could be also explored for their potential applications in biocontrol agents and also in food industry. Based on the ability to inhibit proteases of insect digestive tracts, protease inhibitors have been shown to have potential usefulness as antifeedent agents. Results obtained in the

present study suggest that *Moringa oleifera* protease inhibitor can inhibit major digestive protease like trypsin and chymotrypsin. Most of the insect possesses trypsin or chymotrypsin as their digestive enzymes. Since the novel inhibitor has high affinity towards trypsin and chymotrypsin, the inhibitor can be used as a biocontrol agent for crop protection.

Insects that feed on plant material possess alkaline guts and depend predominantly on serine proteases for digestion of food material. The majority of the protease inhibitors exhibiting anti-feedent properties reported so far are active against the neutral serine proteases such as trypsin and chymotrypsin (Ryan, 1990). Studying plant defense responses and devising newer and ecofriendly strategies for plant protection against pests and pathogens is today's one of the most dynamic areas of research in plant science. The insecticidal effect of protease inhibitors, especially serine and cysteine protease inhibitors, have been studied by direct incorporation assays or by *in vitro* inhibition studies. They induced delayed growth and development, reduced fecundity and sometimes increased mortality (Annadana et al., 2002; Azzouz et al., 2005; Oppert et al., 1993; Oppert et al., 2003).

Plant genetic transformation with exogenous genes encoding factors of resistance to insects is a modern and attractive alternative to synthetic chemical insecticides in the control of several aggressive plant pests and pathogens. The antinutritive and antibiotic effect of protease inhibitors and their consequences on the growth and development of insects and pests have been recognized. So the genetic transformation encoding protease inhibitor

genes is the different alternative in increasing the plant resistance to herbivores and also to many fungal pathogens (Haq et al., 2004).

Identification of novel proteins, which can confer sustained resistance, is an essential prerequisite to the application of gene transfer techniques for development of pest and pathogen resistance in crop plants. There is a considerable interest in using the proteinase inhibitors as potential defense proteins. The proteinase inhibitors used so far in developing insect-resistant transgenic plants were active against the neutral proteinases such as trypsin whereas mid gut proteinases of major Lepidopteron insects such as *Helicoverpa armigera* have been shown to be highly active at pH 10.0 (Harsulkar et al., 1998). Several cysteine and serine protease inhibitors have been expressed in transgenic plants belonging to different families to enhance their resistance against Lepidoptera (Leo and Gallerani, 2002; Leo et al., 2002) and Coleoptera. The *Moringa oleifera* protease inhibitor described in this study is most active at pH 7.0 and is active over a wide range of pH 6.0-10.0. Its anti trypsin and chymotrypsin activity will make *Moringa oleifera* protease inhibitor a novel bifunctional defense protein for its potential use in the transgenic research.

The *Moringa oleifera* protease inhibitor is stable over a wide pH and temperature range. Therefore, one can envision the direct application of protease inhibitor as a biocontrol agent for the protection of plants against phytopathogenic fungi and to insects by encapsulation for surface application or can be sprayed directly. The seeds of plants can be protected from fungal pathogen attack during germination in soil by coating them with a formulated preparation of protease inhibitor.

The affinity of *Moringa oleifera* protease inhibitor with different industrially important proteases is also evaluated. The novel protease inhibitor has completely inactivated the protease from *Bacillus* sp. and also a fungal protease isolated from *Aspergillus oryzae*. It has 76% inhibitory activity towards a protease isolated from an entamopathogenic fungus *Engyodontium album*. Antifungal proteases have captured the attention on account of their economic implications associated with the protection of crops against fungal attack. Plants are the richest source of antifungal proteins. The potent antifungal protein isolated from broad bean is a trypsin-chymotrypsin inhibitor (Banks et al., 2002; Marcela et al., 2000).

The ability of *Moringa oleifera* protease inhibitor to bind reversibly with all detergent proteases and also the stability of inhibitor in a wide range of pH and temperature provide a new tool to use these inhibitors as thermal stabilizers to enhance the thermal stability of detergent proteases.

The use of protease inhibitor from *Moringa oleifera* as a shrimp preservative for long-term storage was evaluated. The results illustrate that the microbial population present on the shrimp *Peneaus monodon* that is protease inhibitor treated decreased compared to control, which is untreated. The protein hydrolysis, a crucial factor for the seafood spoilage was also analyzed for treated and untreated samples at various storage conditions. At room temperature, the *Moringa oleifera* protease inhibitor could prevent the protein degradation to a great extent with a 41% increase in protein content compared to the untreated sample. There was no considerable effect at 4°C on protein degradation. The protease-producing organisms are responsible for the fish and shrimp muscle degradation during preservation

(Chandrasekaran, 1985). The protease inhibitor isolated from *Moringa oleifera* could be used as a safe seafood preservative preventing proteolysis and consequent spoilage. At present, chemical preservatives and antibiotics are used, but the use of enzyme inhibitor, which is a part of edible plant, could be the best alternative and safe preservative.

The protein hydrolysis in fish and shrimp muscle is generally an undesirable process. The presence of proteases causes the softening of some fish muscle and also causes gel weakening in 'Surimi'. So the use of protease inhibitor will be important for the control of proteolysis. Many legume protease inhibitors have been found to have inhibitory effects on the extracts of fish enzymes (Soottawat et al., 1999).

Moringa oleifera protease inhibitor together with its broad pH and temperature stability make it an ideal candidate for its exploration in various biotechnological applications especially in pharmaceutical, food industry and also as a biocontrol defense protein for the protection of plants against pest and pathogen infestations. Being of plant origin it can be conveniently subjected to various recombinant techniques with minimum genetic manipulations.

Chapter 6

SUMMARY AND CONCLUSION

Twenty-two plants belonging to different families and from different localities of Kerala were screened for their protease inhibitory activity. Many leguminous plants producing protease inhibitor was reported earlier. Plant species with more than 60% protease inhibitor was further selected on the basis of proteinaceous nature of protease inhibitor. *Moringa oleifera*, the panotropical tree with high biomass yield which can withstand many unfavorable environmental conditions was selected as the potential source for protease inhibitor.

Protease inhibitor content was maximum in the leaves (77 %) in mature *Moringa oleifera* plant followed by the seed extract (63 %). The plant parts like flowers, bark and roots were having negligible amount of trypsin inhibitor.

Phosphate buffer was the efficient extracting medium for the complete extraction of the protease inhibitor with a protease inhibitory activity of 79% from mature leaves with maximum protein concentration. Distilled water as extractant showed maximum specific inhibitory activity. The trypsin inhibition was very less in the extract prepared in sodium chloride and sodium hydroxide compared to that prepared in phosphate buffer and distilled water.

Protease inhibitor from *Moringa oleifera* was purified using standard protein purification methods, which included ammonium sulphate precipitation, followed by dialysis, ion exchange chromatography and preparative polyacrylamide gel electrophoresis (PAGE). It was observed that 30-90% saturation of ammonium sulphate is needed for the complete precipitation of plant protease inhibitor. On further purification by ion exchange chromatography, a single peak with maximum protease inhibitory activity was obtained with a 2.5 fold of purification. The purity of the fraction was further analyzed by electrophoresis, which furnished a single peptide band with 41.4 fold of purification.

The purity and homogeneity was confirmed by polyacrylamide gel electrophoresis. The protein eluted from 0.2M NaCl yielded a single protein band under Native-PAGE. In SDS-PAGE under non-reducing conditions, the protein yielded a single polypeptide band with a molecular weight of 23.6 kDa. But under reducing conditions, it yielded two bands with molecular weight of 22 kDa and 14 kDa. This signifies the dimeric nature of the polypeptide. The protease inhibitory activity of the protein band was confirmed by reverse zymography on gelatin incorporated gel and also by doing the *in vitro* inhibitor assay. It was observed that the protease inhibitory activity was highest in the larger fragment compared to the smaller one.

The molecular weight of the protease inhibitor was also confirmed by gel filtration chromatography on Sephadex G75. From the graph obtained for K_{av} versus log molecular weight of standard proteins, it was

found that the molecular weight of protease inhibitor was approximately 29 kDa.

Based on the amino acid composition, it was inferred that the purified protease inhibitor contained highest amount of glycine (27.29 %/g sample) and the lowest one was lysine (0.22 %/g sample).

Moringa oleifera protease inhibitor had an optimum pH 7.0 for its activity and they were active over a pH range of 6.0-10.0. The stability of the protease inhibitor at different pH testifies that it is stable over a pH range of 5.0-11.0. The protease inhibitor was active over a temperature up to 50°C and the maximum activity was obtained in a temperature range of 30-40°C. The protease inhibitor was inactive at temperatures above 50°C. It showed stability at 30 & 40°C for two hours with more than 50% protease inhibitory activity.

All the stabilizers promoted the thermal stability and inhibitory activity compared to control at 50°C. 10mM calcium chloride, 1% BSA and 1% sucrose offered complete protection for thermal denaturation of protease inhibitors at 50°C. Two polyols, glycerol and sorbitol enhanced the protease inhibitory activity compared to control. Whereas at 60°C, BSA followed by calcium chloride, casein, cysteine hydrochloride and glycine alone supported stability compared to control. Sucrose and starch, which supported stability at 50°C, did not promote stability at 60°C.

Higher concentration of Zn^{2+} and Hg^{2+} enhanced the protease inhibitory activity to 31% and 64% respectively compared to control. Ca^{2+} and Mg^{2+} at a concentration of 10mM enhanced the protease inhibitory

activity to a marginal level. Presence of Na^{2+}, Ba^{2+}, Ni^{2+}, Cd^{2+}, Mo^{6+} and Al^{3+} did not support protease inhibitory activity when compared to control and instead they had a negative effect of protease inhibitory activity. The metal chelation of *Moringa oleifera* protease inhibitor led to a loss in protease inhibitory activity to 53% compared to control. The presence of 4.26, 0.31 and 0.33ppm of Ca^{2+}, Mg^{2+} and Zn^{2+} was detected in the native protease inhibitor. The additional supplementation of Mg^{2+} and Zn^{2+} on the demetallized protease inhibitor enhanced the protease inhibitory activity to 27 & 47% respectively compared to demetallized protease inhibitor. The additional supplementation of Ca^{2+} enhanced the protease inhibitory activity to a marginal increase at 10mM concentration.

The protease inhibitor retained 62% inhibitory activity in the presence of SDS compared to control. All the other detergents like Tween 80, Tween 20, Triton X 100 and Brij 20 had negative effect on protease inhibitory activity.

In the presence of oxidizing agents, protease inhibitory activity decreased on increasing the concentrations of oxidizing agents. But the reducing agents had a positive effect on the protease inhibitory activity. The residual activity of protease inhibitor increased on increasing the concentrations of oxidizing agents. DTT and β-mercaptoethanol up to a concentration of 1% enhanced the protease inhibitory activity to 49 & 41% respectively.

The modification of tryptophan residue by N-bromosuccinamide resulted in the activation of protease inhibitor to a great extent. Modification of serine residue by PMSF also enhanced the protease inhibitory activity

with a residual inhibitory activity of 280% at 25mM compared to control. Modification of cysteine by N-ethylmaleimide resulted an enhancement in protease inhibitory activity only to a marginal level. The higher concentration of DEPC, which resulted in the modification of histidine residue, also enhanced the protease inhibitory activity to a 10% compared to control. Modification of lysine by succinic anhydride resulted in a loss of protease inhibitory activity on increasing the concentration. Lysine modification resulted in a 91% residual inhibitory activity compared to control.

The impact of HCl on protease inhibitor up to 0.02M did not decrease the protease inhibitor activity. But there was a gradual decrease on increasing the concentration of HCl. The pretreatment of protease inhibitor with digestive protease also reduced the protease inhibitory activity on increasing the concentration of trypsin.

The complete inactivation of trypsin by protease inhibitor is in the ratio 1:1.5. At 1.5 nM concentration of protease inhibitor, there was complete inactivation of 1nM trypsin. It is calculated that 35.4 μg of protease inhibitor was necessary to completely inactivate 23.8μg of trypsin. The concentration of protease inhibitor required for the 50% trypsin inhibition was 0.6nM. The kinetics of trypsin inhibition by protease inhibitor from *Moringa oleifera* revealed a reversible mode of uncompetitive protease inhibition. The K_i value was found to be 1.5 X 10^{-9} M and the very less K_i value reports the high affinity of *Moringa oleifera* protease inhibitor to trypsin.

The protease inhibitor showed high affinity towards most of the serine proteases such as trypsin, chymotrypsin, elastase and thrombin. This illustrates the use of protease inhibitor in both pharmaceutical and agricultural industries. The protease inhibitor also had affinity towards cysteine proteases like Cathepsin B and papain and the inhibitor did not show any affinity towards collagenase.

Moringa oleifera protease inhibitor possessed a high activity spectrum towards many commercially available industrially important proteases. The protease inhibitor completely inactivated proteases isolated from *Bacillus* sps. and *Aspergillus oryzae.* It also showed 76% inhibitory activity towards the protease isolated from *Engyodontium album.* The activity towards subtilisin was very less (3.7%) compared to the other proteases.

The use of protease inhibitor towards seafood preservation was also demonstrated. The *Moringa oleifera* protease inhibitor could be able to regulate the microbial growth present on the shrimp *Peneaus monodon* during the preservation at various conditions compared to the control. The protease inhibitor could prevent the protein degradation to a greater extent. They retained the total protein of the sample throughout their storage time on comparing with control. Thus protease inhibitor could be used as preservative to prevent proteolytic degradation of shrimp during storage.

Conclusion

Protease inhibitors are one of the most important tools of nature for regulating the proteolytic activity of their target proteases. They are synthesized in biological systems and they play a critical role in controlling a number of diverse physiological functions. The current investigation focused on the isolation, purification and characterization of a novel protease inhibitor from *Moringa oleifera*. The results obtained during the course of study opens new perspectives for the utilization of protease inhibitor from *Moringa oleifera* for various pharmaceutical, agricultural and food industries. The biological and physicochemical properties exhibited by the novel protease inhibitor from *Moringa oleifera* clearly testify its suitability for the development as a drug for application in pharmaceutical industries such as anticoagulant agent or biocontrol agent in agriculture and even as a food preservant. There is a scope for further research on the structure elucidation and protein engineering towards a wide range of further applications. Detailed structure/function analysis of these proteins is important to facilitate their use in genetic engineering for various applications.

Chapter 7

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