STUDIES ON PHYSICOCHEMICAL AND BIOLOGICAL PROPERTIES OF PROTEASE INHIBITOR FROM EDIBLE MUSHROOM *PLEUROTUS FLORIDANUS*

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IN

BIOTECHNOLOGY

By

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DECLARATION

I hereby declare that the thesis entitled "**Studies on physicochemical and biological properties of protease inhibitor from edible mushroom *Pleurotus floridanus*"** is the authentic record of research work carried out by me at the Department of Biotechnology, Cochin University of Science and Technology for my doctoral degree, under the supervision and guidance of Dr. Elyas K K, Professor, Department of Biotechnology, University of Calicut and that no part thereof has previously formed the basis for the award of any degree or diploma, associateship or other similar titles or recognition.

Cochin-22 **Manzur Ali P P**

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LIST OF ABBREVIATIONS

%	-	Percentage
°C	-	Degree Celsius
µg	-	microgram
µL	-	microlitre
µM	-	micromole
nM	-	nanomole
A_{280}	-	Absorbance at 280 nm
A_{253}	-	Absorbance at 253 nm
BLAST	-	Basic Local Alignment Search Tool
BAEE	-	*N*α-Benzoyl-L-Arginine Ethyl Ester
bp	-	base pair
AIDS	-	Acquired immunodeficiency syndrome
Arg	-	Arginine
Asn	-	Aparagine
Asp	-	Aspartate
ATP	-	Adenosine tri phosphate
BAPNA	-	α-*N*-benzoyl-DL-arginine-*p*-nitroanilide
BSA	-	Bovine serum albumin
C18	-	Octadecyl bonded Silica
Cys	-	Cysteine
Da	-	Dalton
DEAE	-	Diethyl amino ethyl
DMSO	-	Dimethyl sulphoxide
DNA	-	Deoxyribonucleic acid

dNTP	-	Deoxyribonucleotide triphosphate
DEPC	-	Diethyl pyrocarbonate
DW	-	Distilled water
EDTA	-	Ethylene diamine tetra acetic acid
EtBr	-	Ethidium bromide
Fig.	-	Figure
FPLC	-	Fast protein liquid chromatography
g	-	gram
Glu	-	Glutamic acid
Gly	-	Glycine
HPLC	-	High performance liquid chromatograpghy
HCl	-	Hydrochloric acid
His	-	Histidine
h	-	Hours
IC_{50}	-	Molar concentration of the inhibitor that gives 50% of the target enzyme activity
ICP-AES	-	Inductively coupled plasma atomic emission spectroscopy
kDa	-	Kilo Dalton
K_m	-	Michaelis-Menten constant
K_i	-	Dissociation constant
Lys	-	Lysine
M	-	Molar
(M_r)	-	Relative molecular weight
mg	-	milligram
mL	-	millilitre

mm	-	millimetre
NBS	-	*N*-Bromo succinamide
OD	-	Optical density
PAGE	-	Polyacrylamide gel electrophoresis
PEG	-	Polyethylene glycol
pI	-	Isoelectric point
PI	-	Protease inhibitor
PMSF	-	Phenyl methyl sulphonyl fluoride
rpm	-	Revolutions per minute
RP	-	Reverse phase
SDS	-	Sodium dodecyl sulphate
Ser	-	Serine
sp.	-	Species
TCA	-	Trichloro acetic acid
TPCK	-	*N*- tosyl-L-phenylalanyl chloromethyl ketone
TEMED	-	N-N-N'-N'-Tetramethyl ethylene diamine
Thr	-	Threonine
V_{max}	-	Maximal velocity

CONTENTS

1 Introduction

Protease inhibitors are found abundantly in numerous plants, animals and microorganisms, owing their significance to their application in the study of enzyme structures, reaction mechanisms and also their utilization in pharmacology and agriculture. They are (synthetic/natural) substances that act directly on proteases to lower the catalytic rate. Although most of these inhibitory proteins are directed against serine proteases, some target cysteine, aspartyl or metalloproteases (Bode and Huber, 1992). Protease inhibitors are essential for regulating the activity of their corresponding proteases and play key regulatory roles in many biological processes. Applications of protease inhibitors are intimately connected to the proteases they inhibit; an overview of proteases with the modes of regulation of their proteolytic activity is discussed.

Proteases are classified as hydrolases, enzymes that catalyze the hydrolytic cleavage of specific peptide bonds in their target proteins. These enzymes are extensively distributed in nearly all plants, animals and microorganisms (Christeller, 2005; Haq et al., 2004; Joanitti et al., 2006; Lawrence and Koundal, 2002; Mosolov et al., 2001a; Mosolov and Valueva, 2005; Neurath, 1989; Ryan, 1990; Supuran et al., 2002; Valueva and Mosolov, 2004). In higher organisms, nearly 2% of the genes code for these enzymes (Barrett et al., 2001), which are classified based on the functional group present at the active site into four i.e., serine proteases, aspartic proteases, cysteine proteases, and metalloproteases (Hartley, 1960).

Proteases are indispensable to the maintenance and survival of organisms and play key roles in many biological processes, where the proteolytic events catalyzed by these enzymes serve as mediators of signal initiation, transmission and termination in many of the cellular events such as inflammation, apoptosis, blood clotting and hormone processing pathways (Ivanov et al., 2006). Proteases

are responsible either directly or indirectly for all bodily functions including cell growth, differentiation, death (apoptosis), cell nutrition, intra- and extra-cellular protein turnover (house-keeping and repair), cell migration and invasion, and fertilization and implantation. These functions extend from the cellular level to the organ and organism level to produce cascade systems such as homeostasis and inflammation and complex processes at all levels of physiology and pathophysiology.

Activation and inactivation of protease cascades are closely controlled at different regulatory levels such as protease gene transcription, mRNA translation, zymogen activation (all proteases are biosynthesized as large inactive precursors called proproteins or zymogens), substrate specificity, enzyme kinetics and by means of enzyme inhibitors. Most animal species synthesize a variety of protease inhibitors with different specificities, whose function is to prevent unwanted proteolysis. Any system that encompasses normal and abnormal bodily functions must have effective regulatory counterparts, i.e. protease inhibitors. Hence, the research interest in protease inhibitors has evoked tremendous attention in many disciplines. Protease inhibitors have been traditionally developed by natural product screening for lead compounds with subsequent optimization or by empirical substrate-based methods (West and Fairlie, 1995).

Proteases are also involved in various disease states. For instance, the destruction of the extracellular matrix of articular cartilage and bone in arthritic joints is thought to be mediated by excessive proteolysis activity (Royston, 1996). In emphysema, gingivitis, tumor invasion and inflammatory infections, it is suggested that tissue destruction is by proteases (Royston, 1996; Salzet, 2002). Among the enzymes involved in extracellular matrix degradation, a few serine proteases (elastase, collagenase, cathepsin G) are able to solubilise fibrous proteins such as elastin and collagen (Berquin and Sloane, 1996; Sloane et al., 1986). Given the specific recognition by proteases of defined amino acid sequences, it may be possible to inhibit these enzymes when they are involved in

pathological processes. Hence, potent inhibitors have to be developed as new therapeutic agents. Moreover, some of the protease inhibitors isolated from invertebrate sources are rather specific towards individual mammalian serine proteases offering huge opportunities for medical applications (Royston, 1996).

In mammals, approximately 3–5% of the plasma proteins are protease inhibitors (Laskowski and Kato, 1980). The protease inhibitors are of two basic functional classes, the active-site inhibitors that bind to the active site of the target protease and inactivate its ability to hydrolyze all substrates and the α2-macroglobulins which operate by the unique process of molecular entrapment. In American horseshoe crab, *Limulus polyphemus*, the plasma protease inhibitor, α2-macroglobulin, is the third most abundant protein in the plasma (Enghild et al., 1990). α2-macroglobulin at concentrations of 2–4 mg/mL is one of the most abundant proteins of human plasma, (Sottrup-Jensen, 1989) and is also the second-most abundant protein in plasma of cephalopods like Sepia (Vanhoorelbeke et al., 1993). Inhibitors of serine proteases are reported to block entry of merozoites of *Plasmodium* into erythrocytes (Breton et al., 1992). Low molecular mass protease inhibitors directed against key proteases responsible for virulence have shown promising results as therapeutic agents in a variety of conditions of parasitic diseases (Cohen et al., 1991; Engel et al., 1998a; McKerrow, 1999; Wasilewski et al., 1996).

During cell and tissue development and organism homeostasis, the protease signaling pathways work normally and are tightly controlled. Inappropriate proteolysis has a major role in cancer as well as cardiovascular, inflammatory, neurodegenerative, bacterial, viral and parasitic diseases. Because excessive proteolysis can be prevented by blocking the appropriate proteases, this area is widely explored by pharmaceutical companies (Abbenante and Fairlie, 2005). The history of drugs designed to suppress protease activity dates back to the 1950s. It has been more than 50 years since the first two drugs that affect protease signaling: heparin and the vitamin K analogue warfarin, which target the

blood coagulation cascade, were brought into clinical practice to treat thrombosis. Many new protease inhibitors are currently in development, with at least 50 different proteases being considered as potential targets. Several targets being identified using animal models, particularly rodent knockouts, and then used to screen for inhibitors, whereas others were rationally developed using structure-based drug design. Judging by the new compounds currently being tested in advanced clinical trials and the number of New Drug Application (NDA) for agents that are directed at protease targets, a boom of new therapies based on protease inhibition can be expected in the coming years (Turk, 2006).

In plants, protease inhibitors are accumulated in response to mechanical wounding (Valueva et al., 2001), UV-radiation (Conconi et al., 1996), lesion by insects (Bergey et al., 1996) or phytopathogenic microorganisms (Valueva et al., 2003). Many phytopathogenic fungi produce extracellular proteinases (Kalashnikova et al., 2003), suggesting an active role in the development of diseases (Sara and Heale, 1990). One of the important defense strategies found in plants to combat predators involves protease inhibitors, effective in particular against phytophagous insects and microorganisms. The defensive capabilities 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). Present methods of crop protection rely mainly on the use of chemical pesticides. Recent studies focused on exploiting protease inhibitors as it confers some protection to plants and crops. Studies using artificial diets containing plant derived protease inhibitors targeting particular class of proteinases in the insect gut have demonstrated that these proteins retard growth and development in a wide range of insect pests. Disrupting ability of insects to digest protein by transforming plant genomes with proteinaceous protease inhibitors provides an alternative approach to pest control.

To limit the harmful effects of synthetic molecules on environment and human health, plant genetic engineering was proposed as an alternative to create insect-resistant plants. Among the proteins exhibiting insecticidal effects originating from plants, protease inhibitors (PIs) emerged as an interesting alternative strategy for insect pest control (Lawrence and Koundal, 2002; Reeck et al., 1997) using genetic engineering. Through binding to digestive proteases of phytophagous insects, PIs impair protein digestion (Broadway and Duffey, 1986).

Rawlings assigned protease inhibitors in to families and clans based on the similarity of amino acid sequence or comparison of tertiary structure (Rawlings, 2010) and this classification is implemented in MEROPS database of inhibitors (www.merops.sanger.ac.uk). The classification of protein peptidase inhibitors is continually revised and currently the inhibitors are grouped into 71 families based on comparisons of protein sequences. These families can be further grouped into 38 clans based on the highest level of evolutionary divergence such that all the sequences in the same clan are evolutionarily related despite there being limited sequence similarity. It has been shown that structural similarities persist between related proteins despite there being no significant sequence similarity (Chothia and Lesk, 1986).

The microorganisms of prokaryotic domains of archaeabacteria and eubacteria and the kingdom of fungi, including higher fungi or mushrooms, constitute important sources of protease inhibitors. The number and diversity of proteases found in microorganisms (Rao et al., 1998) and higher fungi (Sabotič et al., 2007b) make them an inexhaustible source of novel protease inhibitors with unique features. Microorganisms that are generally recognized as safe and edible mushrooms offer a valuable source of such novel protease inhibitors that would also be acceptable for use in crops for human consumption. Microbial protease inhibitors are versatile in their structure and mechanism of inhibition in ways that differ from those of other sources.

Mushrooms are nutritionally functional food as well as a source of physiologically beneficial and nontoxic medicines. In order of decreasing cultivated tonnage of mushrooms, *Lentinus* (shiitake), *Pleurotus* (oyster), *Auricularia* (mu-er), *Flammulina* (enokitake), *Tremella* (yin-er), *Hericium* and *Grifola* (maitake) have various degrees of immunomodulatory, lipid-lowering, antitumor, and other beneficial or therapeutic health effects without any significant toxicity. Although the data for this functional food classes are not as strong as those for other functional foods such as cruciferous vegetables, they deserve serious investigation due to their potential usefulness in preventing or treating serious health conditions such as cancer, acquired immunodeficiency syndrome (AIDS) and hypercholesterolemia. Additionally, there is a need for epidemiological evidence of the role of this functional food class.

There are numerous studies on inhibitors in other life forms but the study in fungi is limited to the yeast inhibitors of endogenous proteases A and B (Biedermann et al., 1980; Maier et al., 1979), low molecular inhibitors of *Pleurotus ostreatus* (Dohmae et al., 1995), serine protease inhibitor from *Lentinus edodes* (Odani et al., 1999), proteinase inhibitor from *Trametes versicolor* (Zuchowski and Grzywnowicz, 2006), trypsin specific inhibitors from *Clitocybe nebularis,* CnSPIs (Avanzo et al., 2009) and Cospin (PIC1) from *Coprinopsis cinerea* (Sabotič et al., 2012). Structural and mechanistic studies of mycocypins, clitocypins and macrocypins, a group of cysteine protease inhibitors isolated from the mushrooms *Clitocybe nebularis* and *Macrolepiota procera* are also reported (Renko et al., 2010).

In this context, an attempt was made to screen for the presence of proteinaceous protease inhibitors from the fruiting body of mushrooms not hitherto subjected to this study.

Objectives of the present study

Mushrooms have been used in folk medicine throughout the world since ancient times. Attempts have been made in many parts of the world to explore the use of mushrooms and their metabolites for the treatment of a variety of human ailments. A mushroom characteristically contains many different bioactive compounds with diverse biological activity, with the content and bioactivity of the compounds depending on the preparation and consumption of mushroom. It is estimated that approximately 50% of the annual 5 million metric tons of cultivated edible mushrooms contain functional "nutraceutical" or medicinal properties. In this context, an attempt was made to screen locally available mushrooms for protease inhibitor with the anticipation of its use in possible therapeutic and biocontrol applications.

Thus, the primary objectives of the present study included

- **Screening of mushrooms for protease inhibitors.**
- **Isolation and purification of the inhibitor.**
- **Biophysical and biochemical characterization of protease inhibitor.**
- **Application of protease inhibitor in inhibition of lepedopteran and microbial proteases.**

2 Review of literature

2.1 Proteases

Proteolytic enzymes are degradative enzymes that catalyze the cleavage of peptide bonds in proteins. Proteases are physiologically important group of enzymes and extensively explored its potential in commercial fields. Being essentially indispensable to the maintenance and survival of their host organism, proteases play key roles in many biological processes. These enzymes are widely distributed in nearly all plants, animals and microorganisms, and in higher organisms, proteases have adapted to the wide range of conditions (variations in pH, reductive environment and so on) and use different catalytic mechanisms for substrate hydrolysis (Joanitti et al., 2006; Lawrence and Koundal, 2002; Ryan, 1990). Advances in analytical techniques have demonstrated that proteases perform highly specific and selective modifications of proteins such as activation of proenzyme by limited proteolysis, blood clotting and lysis of fibrin clots, and processing and transport of secretory proteins across the membranes.

Their involvement in the life cycle of disease-causing organisms make them ideal targets for developing therapeutics against fatal, dreadful diseases like malaria, cancer and AIDS. Proteolytic enzymes participate in essential physiological functions (digestion, embryonic development, cell-cycle regulation, wound healing, angiogenesis, apoptosis, antigen presentation and leukocyte migration) and human pathological process, such as arthritis, cardiovascular disorders, cancer blood clotting and hormone processing pathways (Ivanov et al., 2006). Proteases of microorganisms are involved in protein turnover, microconidial germination, hyphal fusion and gene expression (Chung and Goldberg, 1981; Lecadet et al., 1977; Leighton and Stock., 1970).

Despite the fact that these enzymes are indispensable to the cells and organisms that host them, they may cause serious damage when over expressed or present in higher concentrations. For this reason the activities of these enzymes need to be strictly regulated and controlled (Rawlings, 2010; Rawlings et al., 2004a). One important control mechanism involves interaction of the active enzymes with proteins that form less active or fully inactive complexes with their cognate enzymes, and are called protease inhibitors (PIs).

2.2 Classification of proteases

Proteases are classified in subgroup 4 of group 3 (hydrolases) by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology. 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 (3.4.21), aspartic proteases (3.4.23), cysteine proteases (3.4.22), and metalloproteases (3.4.24) (Hartley, 1960). There are a few miscellaneous proteases which do not precisely fit into the standard classification, e.g., ATP-dependent proteases which require ATP for activity (Menon and Goldberg, 1987). Each family of peptidases has 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.

2.2.1 Serine proteases

Serine proteases are characterized by the presence of a serine group in their active site. They are abundant among viruses, bacteria, and eukaryotes, suggesting that they are crucial to the organisms. The group shares a common

reaction mechanism consisting of a common catalytic triad of the three amino acids, serine (nucleophile), aspartate (electrophile), and histidine (base). Although the geometric orientations of these residues are similar, the protein folds are quite different, forming a typical example of a convergent evolution. Based on their structural similarities, serine proteases have been grouped into 20 families. Alkaline protease and subtilisin are the largest families of serine proteases.

2.2.2 Aspartic proteases

Aspartic acid proteases or acidic proteases depend on aspartic acid residues for their catalytic activity. Generally aspartic proteases show maximal activity at low pH (pH 3 to 4) and have isoelectric points in the range of pH 3 to 4.5. They have been grouped into three families, namely, pepsin (A1), retropepsin (A2), and enzymes from pararetroviruses (Barett, 1995). The members of families A1 and A2 are known to be related to each other, while those of family A3 show some relatedness to A1 and A2. The members of the pepsin family have a bilobal structure with the active site cleft located between the lobes (Sielecki et al., 1991). The active-site aspartic acid residue is situated within the motif Asp-Xaa-Gly, in which Xaa can be Serine or Threonine.

2.2.3 Cysteine/thiol proteases

Cysteine proteases occur in both prokaryotes and eukaryotes. About 20 families of cysteine proteases have been recognized. The activity of all cysteine proteases depends on a catalytic dyad consisting of cysteine and histidine. The order of Cys and His (Cys-His or His-Cys) residues differs among the families (Barett, 1994). Generally, cysteine proteases are active only in the presence of reducing agents. Based on their side chain specificity, they are broadly divided into four groups: (i) papain-like, (ii) trypsin-like with preference for cleavage at the arginine residue,(iii) specific to glutamic acid, and (iv) others. Papain is the best-known cysteine protease.

2.2.4 Metalloproteases

Metalloproteases are the most diverse of the catalytic types of proteases. They are characterized by the requirement for a divalent metal ion for their activity. They include enzymes from a variety of origins such as collagenases from higher organisms, hemorrhagic toxins from snake venoms, and thermolysin from bacteria (Hibbs et al., 1985; Okada et al., 1986; Shannon et al., 1989; Weaver et al., 1977; Wilhelm et al., 1987). About 30 families of metalloproteases have been recognized, of which 17 contain only endopeptidases, 12 contain only exopeptidases, and 1 (M3) contains both endo and exopeptidases. Based on the specificity of their action, metalloproteases can be divided into four groups, (i) neutral, (ii) alkaline, (iii) *Myxobacter* I and (iv) *Myxobacter* II. Thermolysin, a neutral protease, is the most thoroughly characterized member of clan MA. Histidine residues from the HEXXH motif serve as Zn ligands, and Glu has a catalytic function (Weaver et al., 1977).

2.3 Protease inhibitors

Proteases are involved in highly sophisticated essential biological processes, both in prokaryotic and eukaryotic cells. Proteolysis in physiological conditions is an irreversible reaction; the need for precise control of peptidases is obvious. The proteolysis regulation occurs at multiple levels, including protease gene transcription and translation, and, most importantly, at the protein level as zymogen activation and the most importantly by inhibitors of peptidases.

Protease inhibitors are one of the most abundant classes of proteins that are found in numerous plants, animals and microorganisms (Laskowski and Kato, 1980) and they have received greater significance owing to their potential as useful tools for the study of enzyme structures, reaction mechanisms, and their utilization in pharmacology and agriculture (Ahn et al., 2004; Imada, 2005; Robert, 2005). They are recognized as defense proteins since they control protease activities and are of vital importance in not only regulating many proteolytic

processes involved in the mobilization of tissue proteins but also in the processing precursors of proteins. Specific and selective protease inhibitors are powerful tools for inactivating target proteases in the pathogenic processes of human diseases such as emphysema, arthritis, pancreatitis, thrombosis, high blood pressure, muscular dystrophy, cancer and AIDS (Bijina et al., 2011a). Protease inhibitors which specifically inhibit the essential proteases in the life cycle of organisms causing mortal diseases such as malaria, AIDS and cancer can be used in drug design to prevent propagation of these causative agents (Johnson and Pellecchia, 2006). Protease inhibitors can be employed effectively as defence tools by virtue of their anti-nutritional interaction against insects that possess alkaline guts and which depend predominantly on serine proteases for digestion of plant material (Ryan, 1990). Microbial food spoilage is yet another area of global concern and it has been estimated that as much as 25% of all food produced is lost post-harvest owing to microbial activity. Application of natural protease inhibitors could be an effective means to extend the shelf life of proteinaceous foods including milk, meat and seafood, by preventing or retarding proteolysis caused by the action of endogenous tissue proteases and exogenous microbial proteases during food processing and preservation (Bijina et al., 2011b).

2.4 Source of protease inhibitors

Protease inhibitors are of very common occurrence, having been isolated and characterized from a large number of organisms, including plants, animals and microorganisms (Habib and Fazili, 2007). Naturally occurring PIs are essential for regulating the activity of their corresponding proteases and play key regulatory roles in many biological processes. The importance of the control of proteolytic activity by inhibitors in physiological processes is demonstrated by the fact that inhibitor molecules exceed 10% of the total protein in human plasma.

2.4.1 Prokaryote-derived protease inhibitors

Protein inhibitors of peptidases are grouped in clans and families based on the evolutionary and structural relationship among, inferred from the comparison of tertiary structure and/or amino acid sequence all modern-day inhibitors. Out of 71 families of proteinase inhibitors listed in the MEROPS database (www.merops.ac.uk, updated on Jan. 25, 2010) only 18 families were recognized in prokaryotes (Table 2.1). Significantly, with some remarkable exceptions, the occurrence of individual types of inhibitors is limited to few bacterial species scattered among phylogenetically distinct orders or even phyla of microbiota. The most abundant peptidase inhibitors in prokaryotic cells are homologous to α2-macroglobulin (family I39), serine carboxypeptidase inhibitor (family I51), α-1-peptidase inhibitor (family I4) and ecotin (family I11). Analysis of prokaryotic genomes available in the MEROPS database indicates that serpins are the third most abundant family of protease inhibitors in microbiota. They are found in 17 out of 55 and in 88 out of 662 fully sequenced genomes of Archaea and Bacteria, respectively. That means serpins are far more prevalent in the former super kingdom (31% of total gene count) than the latter (13%). Serpins are scattered among three (Korarchaeota, Crenarchaeota, Euryarchaeota) out of five evolutionary lineages of Archaea and in twelve phyla (out of 28) of Bacteria, including Acidobacteria, Aquificae, Bacteroidetes, Chlamydiae, Chloroflexi, Cyanobacteria, Dictyoglomi, Gemmatimonadetes, Firmicutes, Proteobacteria, Thermotogae and Verrucomicrobia. Interestingly, with the exception of three species of the Bacteroidetes phylum (*Bacteroides ovatus, Bacteroides uniformis and Prevotella copi*) which are human commensal bacteria, serpins are almost exclusively present in benign environmental microbiota (Kantyka et al., 2010).

Ecotin, a serine protease inhibitor found in the periplasm of *Escherichia coli* is a competitive inhibitor that strongly inhibits trypsin, chymotrypsin and elastase, and many other serine proteases with comparable potencies (Chung et al., 1983). The inhibitor was purified and its reactive site P1 residue was determined

to be Met84, which lies within a disulfide-bonded protein segment (McGrath et al., 1991b). *Streptomyces* subtilisin inhibitor (SSI) is the first proteinaceous protease inhibitor isolated from the culture filtrate of *Streptomyces albogriseolus* S-3253 and shows strong inhibitory activity against alkaline serine proteases such as subtilisin (Murao et al., 1972). Most of the extracellular inhibitory proteins discovered to date have been isolated from *Streptomyces* species and classified as members of the *Streptomyces* subtilisin inhibitor (SSI) family on the basis of their similar structures and protease inhibitor specificities (Laskowski and Kato, 1980). Although its structure-function relationship has been studied extensively by various physicochemical techniques, its biological significance in nature is little known (Hiromi et al., 1985).

There are a number of prokaryote proteins claimed to be peptidase inhibitors, but have not been fully characterized. The sequence of a proteinase inhibitor unrelated to that of any other peptidase inhibitor from *Bacillus subtilis* inhibited the intracellular subtilisin ISP-I from the same organism was isolated and characterized its *K*i (Nishino and Murao, 1986; Shiga et al., 1993). Similarly an extracellular inhibitor of trypsin, chymotrypsin and subtilisin was isolated from *Bacillus brevis* and named BbrPI (Shiga et al., 1992). The sequence contained no cysteine residues and was unrelated to that of any other peptidase inhibitor. A bacterial mutant strain deficient in BbrPI showed higher sensitivity to trypsin which suggests that the inhibitor may have a protective role from attack by exogenous proteinases (Shiga et al., 1995). Sporulation in *B. subtilis* is governed by one of the sigma factors, in particular sigma factor K which is synthesized as a precursor and processed by the membrane metallopeptidase SpoIVFB (family M50). By associating with two other proteins, SpoIVFA and BofA, the peptidase is inactive. SpoIVFB can cleave SpoIVFA at multiple sites,which leaves the possibility that BofA is a peptidase inhibitor (Zhou and Kroos, 2004).

Presence of small molecule inhibitors may partially explain the paucity of protein inhibitors in prokaryotes. Besides inhibitors that are proteins, some

bacteria synthesize peptides and derivatives of peptides that are efficient peptidase inhibitors. Perhaps the best known of these is leupeptin (*N*-acetyl-L-leucyl-L-leucyl-D,L-argininaldehyde) from *Streptomyces exfoliates* inhibits a wide range of serine, cysteine and threonine-type peptidases, including trypsin , neuroendocrine-specific mammalian subtilisin-related endoproteases (PACE4), calpain, clostripain and the trypsin-like activity of the proteasome. Other small molecule inhibitors produced by actinomycetes include bestatin and amastatin (inhibitors of aminopeptidases) and tyrostatin, which inhibits sedolisin (family S53) (Kantyka et al., 2010).

Table 2.1 Distribution of protein inhibitors of proteases from different clans and families of prokaryotes.

Clan	Family	Type of Inhibitor	Homologous genes		Reference
			Bacteria (out of 662 fully sequenced genomes)	Archaea (out of 55 fully sequenced genomes)	
IA	I1	Ovomucoid	11	1	(Laskowski and Kato, 1980; Lu et al., 2001)
IB	I2	Aprotinin	8	0	(Ascenzi et al., 2003; Beierlein et al., 2005)
ID	I4	α1-peptidase inhibitor	88	17	(Roberts et al., 2004)
JC	I9	Peptidase B inhibitor	6	0	(Kojima et al., 1999)
I-	I10	Marinostatin	10	0	(Rawlings et al., 2004b)
IN	I11	Ecotin	76	0	(Rawlings et al., 2004b)
IY	I16	*Streptomyces* subtilisin inhibitor	43	0	(Taguchi et al., 1977)

IX	I31	Equistatin inhibitor	1	0	(Novinec et al., 2006)
IU	I36	*Streptomyces* metallopeptidases inhibitor	1	0	(Rawlings et al., 2004b)
IK	I38	Metallopeptidases inhibitor	17	0	(Rawlings et al., 2004b)
IL	I39	α2-macroglobulin	184	1	(Rawlings et al., 2004b)
I-	I42	Chagasin	35	13	(Wang et al., 2007)
JE	I51	Serine carboxypeptidase Y inhibitor	138	19	(Mima et al., 2005)
IK	I57	Staphostatin B	2	0	(Filipek et al., 2005)
IK	I58	Staphostatin A	2	0	(Wladyka et al., 2005)
JB	I63	Pro-eosinophil major basic protein	4	0	(Glerup et al., 2005)
I-	I75	Bacteriophage lambda CIII protein	2	0	(Halder et al., 2007)
I-	I78	*Aspergillus* elastase inhibitor	33	0	(Okumura et al., 2008)

2.4.2 Fungal derived inhibitors

Numerous individual peptidase inhibitors and inhibitor families have been reported from practically all eukaryotic organisms except fungi, where their number is still limited (Rawlings et al., 2004a). Higher fungi or basidiomycetes, commonly called mushrooms, have been largely overlooked in the search for peptidase inhibitors, except for the detection of inhibitors of serine and metallo-peptidases in basidiocarp extracts (Rennert and Melzig, 2002; Wang et al., 2002). Specific protease inhibitors are essential in regulating endogenous proteolytic processes and protecting against exogenously introduced proteases of pathogens (Vandeputte-Rutten and Gros, 2002). The protective role of protease inhibitor in

mushroom is demonstrated against pathogenic proteases. For the mushroom pathogen *Verticillium fungicola*, subtilisin-type serine proteases are important for the attack of hyphal cell walls (Leger et al., 1997). Furthermore, dipteran larvae are major mushroom insect pests, and have serine proteases as the predominant digestive proteolytic enzymes (Coles et al., 2002; Terra and Ferreira, 1994). Initial studies on fungal protease inhibitors were limited to long-known yeast inhibitors of endogenous proteinases A and B (Biedermann et al., 1980; Maier et al., 1979) and reported low-molecular mass inhibitors of endogenous serine proteinase A from a mushroom *Pleurotus ostreatus* (Dohmae et al., 1995). Clitocypin was the first inhibitor of cysteine proteases isolated from the fruiting bodies of mushroom *Clitocybe nebularis* and displayed no significant similarity at the level of its amino acid sequence to any known peptidase inhibitor or other proteins in the databases, including genomic (Sabotic et al., 2006). In addition to a defensive role, a regulatory role in mushroom endogenous proteolytic systems was proposed, based on the specific inhibition of several putative fungal cysteine proteases (Brzin et al., 2000; Sabotic˘ et al., 2007a; Sabotic˘ et al., 2006). Clitocypin is a 16.8-kDa protein lacking cysteine and methionine residues, which, on account of its unique characteristics, was assigned to a new family I48, inhibitor of cysteine protease in the MEROPS inhibitor classification (Rawlings et al., 2004b).

The thiol-protease specific inhibitor, E-64, originally isolated from *Aspergillus japonicus* (Hanada et al., 1978), has been studied extensively as a potential antitumour agent in cell culture and animal models. Derivatives of E-64, displaying selectivity between different cysteine proteases (Frlan and Gobec, 2006), represent the next step towards their application in treating cancer and other diseases. A novel Lentinus proteinase inhibitor, purified from the fruiting bodies of the edible mushroom *Lentinus edodes*, inhibits bovine β-trypsin and α-chymotrypsin at independent sites isolated. Lentinus proteinase inhibitor showed activity against human enzymes of blood coagulation and fibrinolysis, activated factor XI. The complete primary structure composed of 142 amino acids with an

acetylated N-terminus was determined and exhibited striking similarity to the propeptide segment of a microbial serine proteinase, as well as to the N-terminal region of the mature enzyme (Odani et al., 1999). A trypsin specific inhibitor, CnSPIs have isolated from the basidiomycete *Clitocybe nebularis* showed resemblance to the primary structure and biochemical properties of *Lentinus edodes* was isolated. Cnispin is highly specific towards trypsin, with *K*i in the nanomolar range (Avanzo et al., 2009).

Cospin (PIC1), a serine protease inhibitor from *Coprinopsis cinerea* with biochemical properties similar to those of the previously characterized fungal serine protease inhibitors, cnispin from *Clitocybe nebularis* and LeSPI from *Lentinus edodes* was reported. Cospin exhibited specific inhibitory profile as a very strong inhibitor of trypsin with *K*i in the picomolar range. Structural studies revealed that the cospin has a β-trefoil fold (Sabotič et al., 2012). Macrocypins are cysteine protease inhibitors from the edible parasol mushroom *Macrolepiota procera* encoded by a family of genes that is divided into five groups with more than 90% within-group sequence identity and 75–86% between-group sequence identity. High yields of three different recombinant macrocypins were produced by bacterial expression. Macrocypins are effective inhibitors of papain, cathepsins B and H and exhibits weak inhibition of serine protease trypsin Macrocypins exhibit similar basic biochemical characteristics, stability against high temperature and extremes of pH, and inhibitory profiles similar to those of clitocypin from *Clitocybe nebularis* (Sabotic˘ et al., 2009). The anti nutritional effect of macrocypins (family I85) showed stunted growth and delayed development of Colorado potato beetle larvae (Sabotič and Kos, 2012). Protease inhibitors from liquid-cultured mycelia of the white rot fungus *Trametes versicolor* isolated and suppressed the activity of proteinase K and to a smaller extent, of Carlsberg subtilisin, whereas trypsin and chymotrypsin were not inhibited. The inhibitors were acidic proteins and showed remarkable heat stability and demonstrated the

presence of three proteins with molecular masses of 14.5, 16.6, and 20 kDa, respectively in SDS-PAGE (Zuchowski and Grzywnowicz, 2006).

2.4.3 Plant-derived protease inhibitors

Endogenous plant protease inhibitors drawn attention in plant defense strategies as they target proteolytic virulence factors of phytopathogenic bacteria, fungi, parasites and viruses, preventing their roles in nutrient acquisition and evasion of host defense. They inhibit digestive proteases of herbivorous pests (e.g. insects, mites, slugs), preventing the utilization of food-derived organic nitrogen for their growth and development (Haq et al., 2004; Ryan, 1990). Therefore, the search for novel protease inhibitors offers development of environmentally friendly pest and pathogen management strategies. Another strategy involves augmenting crop endogenous resistance by genetically modified crop plants with protease inhibitors of plant origin (Ferry and Gatehouse, 2010).

Agricultural pests causing significant economic losses belong to orders Lepidoptera (butterflies' and moths'), Coleoptera (beetles), Diptera (true flies), Hemiptera (e.g. aphids), Orthoptera (e.g. locust) and Thysanoptera (thrips), of which lepidopteran larvae are considered the most destructive. They cause either direct damage to crops by feeding or indirect damage by transmitting viral diseases or secondary microbial infections. The most notable for their destructive capacity are the migratory locust (*Locusta migratoria*), several beetles, including Colorado potato beetle (*Leptinotarsa decemlineata*), boll weevil (*Anthonomus grandis*), Japanese beetle (*Popillia japonica*), the western corn rootworm (*Diabrotica virgifera*) and many species of aphids belonging to all families of the superfamily Aphidoidea. Different catalytic types of proteases provide the predominant proteolytic activity in different groups of insect pests. While serine proteases are predominant in digestive proteolysis in most insect species (e.g. Lepidoptera, Diptera), cysteine proteases predominate in Hemiptera, Coleoptera and Thysanoptera. In addition, aspartic and metalloproteases complement protein

digestion to different degrees in most insect orders. Therefore, protease inhibitors targeting different groups of proteases have shown variable antinutritional effects when fed to different insect pests (Sabotič and Kos, 2012).

Plant protease inhibitors are widely distributed among different botanical families, which inhibit different types of proteases. In plants, 1 to 10% of their total proteins are protease inhibitors, mostly found in storage organs such as seeds and tubers. The physiological significance of protease inhibitor on the digestive physiology of animals, occurrence, classification, and the role of inhibitors as defensive proteins in plants against insects and micro-organisms has been extensively studied. Protease inhibitors are over expressed in wounding, suggesting their role in protecting plants from insect attack and microbial infection (Jouanin et al., 1998; Pearce et al., 1982; Ryan, 1981; Ryan, 1989).

Expression of the protease inhibitor genes is usually limited to specific organs or to particular phases during plant growth, such as germination, early leaf senescence, drought and in wounding of plant parts. The expression of rice Bowman–Birk inhibitor (BBI) from *Oryza sativa* is upregulated and induced by pathogens or insects during germination of rice seeds (Lin et al., 2006). The buckwheat (*Fagopyrum sculentum*) trypsin/chymotrypsin inhibitor interferes with spore germination and mycelium growth of the tobacco brown-spot fungus *Alternaria alternata* (Dunaevskii et al., 1997). It has been found that potato tubers treated with elicitors, jasmonic, salicylic or arachidonic acids are able to excrete potatin and three chymotrypsin inhibitors. Wounding and water stress prompts the secretion of two kinds of Kunitz-type protease inhibitors by potato tubers. These inhibitors are closely associated with other secreted polypeptides and would protect them against degradation by extracellular chymotrypsin like protease. The secreted inhibitors could therefore interact with plant defense system (Ledoigt et al., 2006; Valueva et al., 2001). Systemic expression of mRNA of protease inhibitor-II (PI-II) in potato has been studied. Constitutive expression in tubers and floral buds and wound-inducible expression

in leaves have been reported and no PI-II mRNA was detected after the dormancy period (Peña-Cortés et al., 1988).

Insecticidal effects of protease inhibitors have been studied by diet incorporation assays or by *in vitro* inhibition studies resulted delayed growth and development, reduced fecundity and sometimes increased mortality (Annadana et al., 2002; Edmonds et al., 1996; Oppert et al., 2003). The effects of protease inhibitors towards aphid parasitoids, bioassays using soybean Bowman–Birk inhibitor (SbBBI) or oryzacystatin I (OCI) on artificial diet were performed on *Macrosiphum euphorbiae– Aphelinus abdominalis* system. Enzymatic inhibition assays showed that digestive proteolytic activity of larvae and adults of *Aphelinus abdominalis* predominantly relies on serine proteases and especially on chymotrypsin-like activity. Bioassays using *M. euphorbiae* reared on artificial diets supplemented with both OCI and SbBBI showed a fitness impairment of *Aphelinus abdominalis* that developed on intoxicated aphids (Azzouz et al., 2005). Trypsin inhibitors present in soybean were shown to be toxic to the larvae of the flour beetle (*Tribolium confusum*) (Lawrence and Koundal, 2002). Cowpea trypsin inhibitor (CpTi) inhibits the growth of nematodes, *Globodera tabacum*, *Globodera pallid* and *Meloidogyne incognita* (Williamson and Hussey, 1996).

Protease inhibitors an ideal choice to be used in developing transgenic crops resistant to insect pests. Numerous transgenic plants expressing entomotoxic proteins of various origins have thus been engineered. Among the proteins exhibiting insecticidal effects originating from plants, protease inhibitors emerged as an interesting strategy for insect pest control (Lawrence and Koundal, 2002; Reeck et al., 1997) using genetic engineering. The CpTI gene isolated from the cowpea plant (*Vigna unguiculata*), a member of the Bowman-Birk superfamily of protease inhibitors has been extensively used in the generation of insect resistant plants. This is the first plant-originated insect resistance gene to be successfully transferred into other plants species (Hilder et al., 1987). CpTI possesses the insecticidal properties against the insect groups of Lepidoptera, Coleoptera and

Orthoptera (Gatehouse et al., 1997). Cowpea trypsin inhibitor gene CpTI has also been introduced into *Brassica oleracea* var. *capitata* cultivars Yingchun and Jingfeng (Fang et al., 1997). The transformed plants showed resistance to *Pieris rapae* in laboratory tests. Transgenic tobacco expressing high levels of Kunitz type of trypsin inhibitor from soybean demonstrated resistance *Helicoverpa virescens* (Sharma et al., 2000). Some reported examples of plant protease inhibitors are listed in the Table 2.2.

Table 2.2 Plant protease inhibitors with some examples (Habib and Fazili, 2007)

Common name	MEROPS **Family/ subfamily**	**Type example**	**Source**	**References**
Kunitz (plant)	13A	Soybean Kunitz trypsin inhibitor Kunitz cysteine peptidase inhibitor 1	*Glycine max* *Solanum tuberosum*	(Laskowski and Kato, 1980) (Gruden et al., 1997)
Kunitz (plant)	I3B	Proteinase inhibitor A inhibitor unit Kunitz subtilisin inhibitor	*Sagittaria sagittifolia* *Canavalia lineata*	(Laskowski and Kato, 1980) (Terada et al., 1994)
Cereal	I6	Ragi seed trypsin/amylase inhibitor Barley trypsin/factor XIIa inhibitor	*Eleusine coracana* *Hordeum vulgare*	(Hojima et al., 1980) (Lazaro et al., 1988)
Squash	I7	Trypsin inhibitor MCTI-II Macrocyclic squash trypsin inhibitor	*Momordica charantia* *Momordica cochinchinensis*	(Huang et al., 1992) (Hernandez et al., 2000)
Potato type I	I13	Subtilisin-chymotrypsin inhibitor CI-1A Wheat Subtilisin/chymotrypsin inhibitor	*Hordeum vulgare* *Triticum aestivum*	(Greagg et al., 1994) (Poerio et al., 2003)
Mustard	I18	Mustard trypsin inhibitor Mustard trypsin inhibitor-2 Rape trypsin inhibitor	*Sinapis alba* *Brassica hirta* *Brassica napus*	(Menengatti et al., 1992) (Ceci et al., 1995) (Ceciliani et al., 1994)

Cystatin	I25B	Onchocystatin Ovocystatin Oryzacystatin II	*Onchocerca volvulus* *Gallus gallus* *Oryza sativa*	(Lustigman et al., 1992) (Laber et al., 1989) (Ohtsubo et al., 2005)
Kininogen	I25C	Metalloprotease Sarcocystatin	*Bothrops jararaca* *Sarcophaga peregrina*	(Cornwall et al., 2003) (Saito et al., 1989)
Bowman-Birk	I12	Bowman–Birk plant trypsin inhibitor unit 1 Bowman-Birk trypsin/chymotrypsin inhibitor Sunflower cyclic trypsin inhibitor	*Glycine max* *Arachis hypogaea* *Helianthus annuus*	(Odani and Ikenaka, 1976) (Suzuki et al., 1987) (Mulvenna et al., 2005)
Potato type II	I20	Proteinase inhibitor II Tomato peptidase inhibitor II inhibitor unit 2	*Solanum tuberosum* *Solanum lycopersicum*	(Greenblatt et al., 1989) (Barrette-Ng et al., 2003)

2.4.4 Animal-derived protease inhibitors

Uncontrolled proteolysis as a result of imbalance between active proteases and their endogenous inhibitors has been associated with different diseases such as Alzheimer's, cancer, rheumatoid arthritis and osteoarthritis, multiple sclerosis, muscular dystrophy, pancreatitis, liver disorders, lung disorders, lysosomal disorders, inflammation, Batten's disease, diabetes, pycnodysostosis, periodontitis, myocardial disorders, and many others (Tizon and Levy, 2007).

Bikunin, a Kunitz-type protease inhibitor, found in human serum, amniotic fluid and urine is a serine protease inhibitor with anti metastatic activity. It was subsequently demonstrated that bikunin inhibits tumor invasion and metastasis, at least in part, by a direct inhibition of plasmin activity as well as by inhibiting urokinase plasmin activator (Kobayashi et al., 1995; Kobayashi et al., 2002; Kobayashi et al., 2001). Three different proteinase inhibitors from human plasma has been tested for its ability to inactivate a purified *Aspergillus melleus* serine proteinase (Korzus et al., 1994).

α2-macroglobulin (α2-M) is the most extensively studied nonspecific protease inhibitor belonging to the group of nonspecific protease inhibitors. It is a major serum glycoprotein composed of four identical subunits of approximately 185 kDa each (Chaudhuri, 1993). The major function of α-macroglobulins, a group of high-molecular weight nonspecific protease inhibitors, is rapid inhibition of excess proteolytic activity due to either endogenous or exogenous proteases. Active and inactive forms of serpins are present in biological fluids. They are very abundant in mammalian plasma and play an important role in many physiologic processes, primarily to neutralize over expressed serine proteinase activity (Travis and Salvesen, 1983). The proteases and their serpin inhibitors were found at sites of neuronal injury and it was suggested that serpin levels affect the outcome of brain injury (Buisson A, 1998; Smith-Swintosky et al., 1995). Serine proteases and their inhibitors are extensively involved in inflammatory processes related to neurodegenerative diseases.

α1-Antichymotrypsin (ACT) was detected in activated astrocytes during normal aging of humans and monkeys (Abraham et al., 1989) and in several neurodegenerative diseases (Abraham et al., 1990). Neuroserpin is a member of the serpin family that is closely related in structure to another serpin, α1-antitrypsin (Briand et al., 2001; Silverman et al., 2001). Plasminogen activator inhibitor (PAI) and its target proteases such as plasminogen activators and thrombin are involved in a variety of physiological and pathological processes in the brain. Ulinastatin, an acid glycoprotein with a high molecular weight of 67000 Dalton, is contained in the fresh urine of healthy humans, and is well known to inhibit the activity of polymorphonuclear leukocyte elastase (PMNE) (Nishiyama and Hanaoka, 2001; Nishiyama and Hirasaki, 1994). Ulinastatin also suppresses the production of tumor necrosis factor-α (TNF-α) (Aosasa et al., 2001) and interleukins 6 and 8 (Sato et al., 2000).

A 6 kDa inhibitor has been structurally characterized from the haemolymph and integument of the silk worm *Bombyx mori* and shown to inhibit

extracellular proteinases from *Beauveria bassiana* and to inhibit conidial germination (Yoshida et al., 1990). Three inhibitors were purified from locust, *Locusta migratoria* designated as *Locusta migratoria* chymotrypsin inhibitor 1 and 2 (LMCI-1 and 2) and hemolymph inhibitor and five in *Schistocerca gregaria* designated as SGPIs or *Schistocerca gregaria* protease inhibitors. The locust peptide inhibitors display sequence similarities with nine cysteine rich domains in the light chain of pacifastin, a heterodimeric serine protease inhibitor isolated from the hemolymph of the crayfish *Pacifastacus leniusculus* (Clynen et al., 2005). Besides locusts and crayfish, a pacifastin-related precursor sequence was also identified in the endoparasitoid wasp *Pimpla hypochondriaca.* A 23 kDa inhibitor against subtilisin type proteinases has been purified from the blood cells and cuticle of the cray fish species *Astacus astacus* and *Pacifastacus leniusculus*. The *Astacus astacus* 23 kDa inhibitor was demonstrated to be effective against extracellular proteinases from *Aphanomyces astaci,* commonly known as crayfish plague but less effective against the saprotrophic *Aphanomyces laevis* when casein was used as a substrate (Dieguez-Uribeondo and Cerenius, 1998).

Extensive studies on protease inhibitors of hematophageous leeches have identified two groups of serine protease inhibitors, the first with specific inhibitors interfering in the activation of the blood clotting system and the second group with inhibitors that act on the extracellular matrix (Salzet, 2001). The development of new natural anticoagulants to overcome the pharmacological and/or biophysical limitations of heparin and warfarin is in progress. A thrombin inhibitor, Hirudin has been developed to inhibit thrombin, the final effector in coagulation which converts soluble fibrinogen into insoluble fibrin. Hirudin is a 65 amino acid peptide with three intramolecular disulfide bridges and a sulfated Tyr residue, isolated from the salivary glands of the medicinal leech with a *K*i of 0.2 pM (Salzet, 2002).

2.5 Classification of protease inhibitors

Protease inhibitors are grouped broadly in to two

i) Small molecule inhibitors, and ii) Proteinaceous inhibitors.

2.5.1 Small molecule inhibitors

Small molecule inhibitors (SMIs) are inhibitors that are not proteins, including peptides and synthetic inhibitors, laboratory reagents used in the characterization of peptidases, and others that are drugs such as the inhibitors of the retropepsin of the HIV virus. Although many SMIs are synthesized in the laboratory, a number also occur naturally and have been isolated from actinomycetes and fungi. SMIs have been grouped in to a) those that inhibit peptidases of more than one catalytic type, b) those that inhibit peptidases of a single catalytic type, c) those that inhibit peptidases from a single family, and d) those that are known to inhibit only a single peptidase (Rawlings, 2010). A small molecular weight inhibitor, epoxomicin from actinomycete strain No.Q996-17 has been shown to inhibit proteasome catalytic subunit 3, similarly fumagillin from *Aspergillus* sp. inhibits methionyl aminopeptidase 2 (Liu et al., 1998; Meng et al., 1996). SMIs include a number of general purpose inhibitors. General purpose cysteine peptidase inhibitors include the free thiol alkylators iodoacetamide, iodoacetate, NEM (*N*-ethylmaleimide) and PCMB (*p*-chloromercuribenzoate), metallopeptidase inhibitors include the metal chelators EDTA (ethylene diamine tetraacetic acid), EGTA (ethylene glycol tetraacetic acid) and 1, 10-phenathroline. General purpose serine peptidase inhibitors include DCI (3,4-dichloroisocoumarin), DFP (diisopropyl fluorophosphates) and PMSF (phenylmethanesulfonyl fluoride).

2.5.2 Proteinaceous inhibitors

Over 634 protein peptidase inhibitor species are distributed throughout the organisms from viruses to animals. The classification of protein peptidase

inhibitors is continually being revised by MEROPS database and is currently grouped into 71 families based on comparisons of protein sequences and include 17451 inhibitor sequences. The molecular weight and mechanism of inhibition varies from inhibitor to inhibitor. The family of protein peptidase inhibitor is depicted in the Table 2.3. These families can be further grouped into 38 clans based on comparisons of tertiary structure. Each clan, family and biochemically characterized peptidase inhibitor is given a unique identifier. A family identifier consists of the letter "I" followed by a number and two-letter clan identifier starts with "I" or "J" (Rawlings, 2010).

Table 2.3 Families of proteinaceous inhibitors

Family or subfamily	Inhibitor and unit name (source)	Reference
I1	Ovomucoid unit 3 (*Meleagris gallopavo*)	(Laskowski and Kato, 1980)
I2	Aprotinin (*Bos taurus*)	(Laskowski and Kato, 1980)
I3A	Soybean trypsin inhibitor (*Glycine max*)	(Laskowski and Kato, 1980), (Oliveira et al., 2001)
I3B	Protease inhibitor B (*Sagittaria sagittifolia*)	(Laskowski and Kato, 1980)
I4	α_1-proteinase inhibitor (*Homo sapiens*)	(Huntington et al., 2000), (Al-Khunaizi et al., 2002)
I5	Ascidian trypsin inhibitor (*Halocynthia rorefzi*)	(Kumazaki et al., 1994)
I6	Ragi seed trypsin/ α-amylase inhibitor (*Eleusine coracana*)	(Hojima et al., 1980)
I7	Trypsin inhibitor MCTI-1 (*Momordica charantia*)	(Wieczorek et al., 1985)
I8	Nematode anticoagulant inhibitor (*Ascaris suum*)	(Bernard and Peanasky, 1993), (Griesch et al., 2000)
I9	Protease B inhibitor (*Saccharomyces cerevisiae)*	(Kojima et al., 1999)
I10	Marinostatin (*Alteromonas* sp.)	(Takano et al., 1991)
I11	Ecotin (*Escherichia coli*)	(Chung et al., 1983)

I12	Bowman-Birk plant trypsin inhibitor (*Glycine max*) unit 1	(Odani and Ikenaka, 1973), (Hatano et al., 1996)
I13	Eglin C (*Hirudo medicinalis*)	(Heinz et al., 1991)
I14	Hirudin (*Hirudo medicinalis*)	(Bode and Huber, 1992)
I15	Antistasin unit 1 (*Haementeria officinalis*)	(Rester et al., 1999)
I16	Subtilisin inhibitor (*Streptomyces albogriseolus*)	(Mitsui et al., 1979), (Taguchi et al., 1998)
I17	Mucus proteinase inhibitor unit 2 (*Homo sapiens*)	(Tsunemi et al., 1993)
I18	Mustard trypsin inhibitor (*Sinapis alba*)	(Menegatti et al., 1992)
I19	Proteinase inhibitor LCMI I (*Locusta migratoria*)	(Eguchi et al., 1994)
I20	Proteinase inhibitor II (*Solanum tuberosum*)	(Barrette-Ng et al., 2003)
I21	Secretogranin V (*Homo sapiens*)	(Lindberg et al., 1995)
I24	PinA endopeptidase La inhibitor (bacteriophage T4)	(Hilliard et al., 1998)
I25A	Cystatin A (*Homo sapiens*)	(Green et al., 1984)
I25B	Ovocystatin (*Gallus gallus*)	(Bode et al., 1988), (Alvarez-Fernandez et al., 1999)
I25C	Metalloprotease inhibitor (*Bathrops jararaca*)	(Cornwall et al., 2003), (Valente et al., 2001)
I27	Calpastatin unit 1 (*Homo sapiens*)	(Todd et al., 2003)
I29	Cytotoxic T-lymphocyte antigen	(Guay et al., 2000)
I31	Equistatin (*Actinia equina*)	(Strukelj et al., 2000)
I32	BIRC-5 protein (*Homo sapiens*)	(Riedl et al., 2001)
I33	Ascaris pepsin inhibitor PI-3 (*Ascaris suum*)	(Ng et al., 2000)
I34	Saccharopepsin inhibitor (*Saccharomyces cerevisiae*)	(Phylip et al., 2001)
I35	Timp-1 (*Homo sapiens*)	(Gomis-Ruth et al., 1997), (Lee et al., 2003)

I36	Streptomyces metalloproteinase inhibitor (*Streptomyces nigrescens*)	(Hiraga et al., 1999)
I37	Potato carboxy peptidase inhibitor (*Solanum tuberosum*)	(Bode and Huber, 1992)
I38	Metalloproteinase inhibitor (*Erwinia chrysanthemi*)	(Feltzer et al., 2003)
I39	α_2-macroglobulin (*Homo sapiens*)	(Barrett, 1981)
I40	Bombyx subtilisin inhibitor (*Bombyx mori*)	(Pham et al., 1996)
I42	Chagasin (*Leishmania major*)	(Monteiro et al., 2001)
I43	Oprin (*Didelphis marsupialis*)	(Neves-Ferreira et al., 2002)
I44	Carboxypeptidase A inhibitor (*Ascaris suum*)	(Homandberg et al., 1989)
I46	Leech carboxypeptidase inhibitor (*Hirudo medicinalis*)	(Reverter et al., 2000)
I47	Latexin (*Homo sapiens*)	(Normant et al., 1995)
I48	Clitocypin (*Lepista nebularis*)	(Brzin et al., 2000)
I49	ProSAAS (*Homo sapiens*)	(Basak et al., 2001)
I50	Baculovirus p35 caspase inhibitor (*Spodoptera litura* nucleopolyhedrovirus)	(Xu et al., 2003)
I51	Carboxypeptidase Y inhibitor (*Saccharomyces cerevisiae*)	(Bruun et al., 1998)
I52	Tick anticoagulant peptide (*Ornithodorus moubata*)	(Charles et al., 2000)
I57	Staphostatin B (*Staphylococcus aureus*)	(Rzychon et al., 2003)
I58	Staphostatin A (*Staphylococcus aureus*)	(Rzychon et al., 2003)
I59	Triabin (*Triatoma pallidipennis*)	(Fuentes-Prior et al., 1997)
I63	Pro-eosinophil major basic protein (*Homo sapiens*)	(Glerup et al., 2005)
I64	Thrombostasin (*Haematobia irritans*)	(Cupp et al., 2004)
I66	Lentinus peptidase inhibitor (*Lentinula edodes*)	(Sabotic et al., 2012)

I67	Bromein (*Ananas comosus*)	(Sawano et al., 2005)
I68	Tick carboxypeptidase inhibitor (*Rhipicephalus bursa*)	(Arolas et al., 2005)
I69	Streptopain inhibitor (*Streptococcus pyogenes*)	(Kagawa et al., 2005)
I71	Falstatin (*Plasmodium falciparum*)	(Pandey et al., 2006)
I72	Chimadanin (*Haemaphysalis longicornis*)	(Nakajima et al., 2006)
I73	Veronica trypsin inhibitor (*Veronica hederifolia*)	(Conners et al., 2007)
I74	Variegin (*Amblyomma variegatum*)	(Koh et al., 2007)
I75	Bacteriophage lambda CIII protein (bacteriophage lambda)	(Halder et al., 2007)
I76	Thrombin inhibitor (*Glossina morsitans*)	(Cappello et al., 1996)
I77	Anophelin (*Anopheles albimanus*)	(Francischetti et al., 1999)
I78	Aspergillus elastase inhibitor (*Aspergillus fumigatus*)	(Okumura et al., 2008)
I79	AVR2 protein (*Passalora fulva*)	(Rooney et al., 2005)
I81	Toxostatin-1 (*Toxoplasma gondii*)	(Huang et al., 2009)
I83	AmFPI-1 (*Antheraea mylitta*)	(Roy et al., 2009)
I84	cvSI-2 (*Crassostrea virginica*)	(Xue et al., 2009)
I85	Macrocypin 1 (*Macrolepiota procera*)	(Sabotic et al., 2009)
I87	HflC (*Escherichia coli*)	(Akiyama, 2009)
I88	Oryctin (*Oryctes rhinoceros*)	(Horita et al., 2010)
I90	Trypsin inhibitor (*Mirabilis jalapa*)	(Kowalska et al., 2007)
I91	F1L protein (vaccinia virus)	(Zhai et al., 2010)

2.6 Mechanism of inhibition

Three types of inhibitors can be distinguished based on their mechanism of action: canonical (standard mechanism) and non-canonical inhibitors, and the serpins. Canonical inhibitors are small proteins showing substrate-like-binding and blocking the enzyme at the distorted Michaelis complex reaction stage (Bode and Huber, 1992; Laskowski and Kato, 1980; Otlewski et al., 1999), serpins on the other hand are much larger, typically 350–500 amino acids in size, distributed from viruses to mammals (Gettins, 2002; Silverman et al., 2001) with extremely strong and specific interaction known so far only for factor Xa and thrombin.

2.6.1 Canonical inhibitors

The largest group of protein inhibitors are canonical inhibitors that act according to the standard mechanism of inhibition (Laskowski and Kato, 1980). They often accumulate in high quantities especially in plant seeds, avian eggs, and various body fluids and comprise proteins from 14 to about 200 amino acid residues. The segment responsible for protease inhibition, called the protease-binding loop, surprisingly has always a similar, canonical conformation in all known inhibitor structures (Bode and Huber, 1992; Schechter and Berger, 1967). This convex, extended and solvent-exposed loop is highly complementary to the concave active site of the enzyme. The standard mechanism implies that inhibitors are peculiar protein substrates containing the reactive site P1-P1' peptide bond located in the most exposed region of the protease-binding loop (P1, P2 and P1',P2' specify inhibitor residues amino and carboxy terminal to the scissile peptide bond, respectively; S1, S2 and S1', S2' denote the corresponding subsites on the protease (Jackson and Russell, 2000).

A classification of canonical inhibitors was originally proposed by Laskowski and Kato in 1980. Currently, 18 inhibitor families are recognized (Laskowski et al., 2000). Most often they comprise either purely β sheet or mixed α/β proteins; they can also be α-helical or irregular proteins rich in disulfide cross-

links. In several inhibitor families like bovine pancreatic trypsin inhibitor (BPTI), Kazal, potato 1 and 2 and ecotin, typical secondary-structure elements together with the presence of a hydrophobic core are found. Bowman-Birk is essentially a lack of both hydrophobic core and extensive secondary structure. For these inhibitors, disulfide bonds, which are usually buried inside the molecule, are the major determinant of protein stability and/or rigidity. Inhibitors are often heavily cross-linked with conserved disulfide bonds. The topology of the disulfide bonds is usually well preserved within a single family. Selective reduction or elimination of disulfide bond(s) in inhibitors belonging to different families usually leads to a significant destabilization of the inhibitor molecule, to a lower association-energy and larger sensitivity to proteolysis (Krokoszynska et al., 1998; Rolka et al., 1992; Tamura et al., 1991; Yu et al., 1995). Representative set of canonical inhibitor structures are shown in Fig. 2.1.

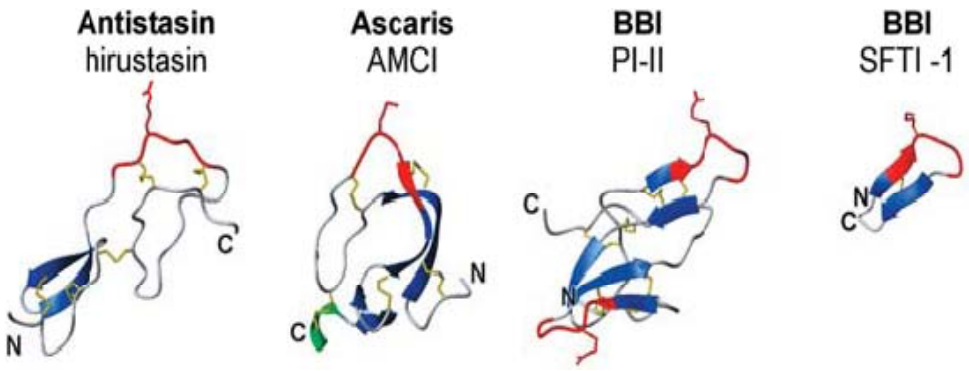

Fig. 2.1 Canonical inhibitor structures. Hirustasin (PDB: 1bx7); AMCI (1ccv); PI-II, protease inhibitor-II (1pi2); STFI-1 (1jbl).

The convex protease-binding loop exhibits an extended conformation, significantly protruding from the protein scaffold and serves as a rather simple recognition motif. The loop forms a sequential epitope spanning positions P3 to P3'. Residues that precede or follow this segment (e.g., P6-P4 or P4') and residues from a sequentially remote region, called the secondary contact region, can also

contact the enzyme and influence the association energy (Ardelt and Laskowski, 1991; Buczek et al., 2002; Laskowski and Qasim, 2000). The central section of the loop contains a solvent-exposed P1-P1' peptide bond, called the reactive site.

The amino acid sequences of the binding loops show many clear amino acid preferences in different families. For example, half cystine is present either at P3 (the Kazal, Bowman-Birk, grasshopper, silkworm, squash, potato 2, and Ascaris families) or at P2 (the bovine pancreatic trypsin inhibitor (BPTI), antistasin, arrowhead, hirustasin, and chelonianin families). Threonine is often observed at P2 (the Kazal, potato 1, Bowman-Birk, ecotin, and Ascaris families) and proline is conserved at P3 in the STI family (Schechter and Berger, 1967). A conserved isoluecine is always present at the P1' position in squash inhibitors (Brauer et al., 2002). In Bowman-Birk inhibitors, prolines are frequently observed at P3' and P4'.The Proline at P3' in geometry is required for strong inhibition while that at P4' stabilizes the P3' configuration (Cierpicki and Otlewski, 2000). The canonical loop conformation results from a rather extensive system of disulfide bond(s), hydrogen bonds, and/or hydrophobic interactions, which involve residues both from the loop and the inhibitor scaffold. The canonical inhibitor-cognate protease interaction is preserved in all cases tested and called the standard mechanism (Laskowski and Kato, 1980). The complex EI is much more stable than the Michaelis ES complex. Typical inhibition constant (*K*i) values are 10^6 to 10^9 fold lower than *K*m values. Often, complex can be easily crystallized and shows all typical features of protein-protein recognition (Apostoluk and Otlewski, 1998; Jackson and Russell, 2000).

Other very important features of the complex include: a short (usually about 2.7 Å) contact between the P1 carbonyl carbon and the catalytic serine residue (significantly shorter in rhodniin-thrombin (McGrath et al., 1994) and mung bean trypsin inhibitor-trypsin complexes (Bateman et al., 2001), and two hydrogen bonds between the carbonyl oxygen of P1 and Gly193/Ser195 amides of the oxyanion binding hole, and the hydrogen bonds between the P1 HN group and

the side chain of Ser195 and the carbonyl of Ser214. Conversion of the P2-P1 amide bond to an ester bond reduces the association free energy by about 1.5 kcal/mol (McPhalen and James, 1988; Peng and Wagner, 1992). The reactive-site peptide bond remains intact in all crystallographically studied complexes.All the above-mentioned hydrogen bonds and the shape complementarity of interacting areas ensure very similar recognition modes between different proteases and inhibitors.

In the complex, about 10–18 amino acid residues of the inhibitor and 17–30 residues of the protease make numerous interactions–mainly van der Waals (typically more than 100) and hydrogen bonds (about 8–15). The total area of the two components buried in the interface is about 1400 Å. According to NMR relaxation studies, the protease-binding loop, which is often poorly structured in free inhibitors (Bode and Huber, 1978; Krishnamoorthi et al., 1992; Liu et al., 1996), becomes significantly rigidified in the complex. There are no significant conformational changes on either the enzyme or inhibitor part accompanying complex formation, with the exception of zymogen complexes. In the trypsinogen-inhibitor complex, major structural rearrangements are observed in the activation domain comprising the active-site region (Bode, 1979). In canonical inhibitors, position P1 determines to a large extent the protease-inhibitor association energy. With the exception of Trp, Ile and Cys, all amino acids have been observed at this position in inhibitors representing different families (Kojima et al., 1991). P1 Gly and particularly P1 Pro are very disfavored for binding with most of the proteases tested (Hohenester et al., 1997; Qasim et al., 1995). Also the charged P1 side chains of Asp, Glu and His (but not their uncharged forms), The P1 side chain is fully exposed in all free inhibitor structures and becomes embedded in the S1 pocket upon complex formation.

2.6.2 Non-canonical inhibitors

The non-canonical inhibitors are much less abundant than canonical inhibitors or serpins. Very few of them have been characterized in terms of structure and kinetics of interaction with the target protease. Non-canonical inhibitors interact through their N-terminal segment which binds to the protease active site forming a short parallel β sheet (Szyperski et al., 1992). These inhibitors also form extensive secondary interactions with the target protease outside the active site, which provide additional buried area and contribute significantly to strength, speed, and specificity of recognition. Interestingly, such interactions are also formed by proteins possessing canonical-inhibitor-like folds or by Kazal-type inhibitors but with a distorted conformation of the binding loop. They are often found in blood-sucking organisms and inhibit proteases involved in clot formation–thrombin or factor Xa (Stubbs et al., 1995). Non-canonical inhibitors are small in size with a molecular weight of 6-8 kDa.

Ornithodorin and tick anticoagulant peptide (TAP) are non-canonical inhibitors of coagulation proteases from the soft tick show a scaffold of the archetypical canonical inhibitor BPTI Ornithodorin contains two BPTI-like domains containing insertion/deletion in the binding-loop segments, which lead to their major distortion (Waxman et al., 1990). In fact, this binding loop does not contact the protease, but as in the hirudin-thrombin complex, the N-terminal tail of ornithodorin penetrates the thrombin active site and forms a parallel β sheet with the thrombin Ser214-Gly219 segment. Similarly, TAP, which is a strong inhibitor of factor Xa (Wei et al., 1998), interacts through the N terminus with the active site of factor Xa (Moses and Hinz, 1983).

2.6.3 Serpins (*ser*ine *p*rotease *in*hibitors).

The serpins are a superfamily of proteins, typically 350–400 amino acids in length and the molecular weight of 40–50 kDa, with a diverse set of functions including, but not limited to, inhibition of serine proteinases in the vertebrate

blood coagulation cascade (Huber and Carrell, 1989; Marshall, 1993). Serpins are of clinical interest because mutations cause a number of disease states—for example, blood clotting disorders, emphysema, cirrhosis, and dementia—many of which are consequences of polymerization (Carrell and Lomas, 1997). Serpins are also of interest in the context of general protein structure and folding studies because of their dramatic conformational changes and the existence of metastable states. The serpin fold is comprised of 3-*β* sheets (A, B, C) and 7–9 α helices (Fig.2.2). A solvent exposed stretch of amino acids termed as reactive centre loop (RCL) contains the protein recognition site which forms a flexible stretch of ~17 residues between *β* sheets A and C. The reactive center loop (RCL), is crucial for the function of inhibitory serpins undergoing large structural changes that alter the folding topology of the molecule. In α1-antitrypsin, the RCL comprises residues P17–P4' in the notation of Schechter and Berger (1967), and contains the scissile bond between residues P1 and P1' cleaved by the target proteinase. In the native state, the RCL is exposed and, for inhibitory serpins, accessible for interaction with a proteinase. Upon cleavage of the scissile bond, the reactive center loop forms an additional strand inserted into the A β sheet, with concomitant conformational changes elsewhere in the molecule cleavage is typically associated with an increase in stability. The native to cleaved change is called the "stressed to relaxed" (S→R) transition (Carrell and Owen, 1985).

The S→R transition is integral to the function of inhibitory serpins. The mechanism of inhibition involves the formation of a stable complex between the proteinase and the cleaved form of the inhibitor, analogous to an enzyme-product complex modulating serpin conformational changes. The hinge, the P15–P9 portion of the RCL (Hopkins et al., 1993) provides mobility essential for the conformational change of the RCL in the S→R transition. Hinge region contains many conserved residues between P15–P10, towards the N terminal of the RCL and the amino acid at P14 (Ser-380) is of critical importance as its insertion in the *β*-sheet A is a prerequisite for inhibitory activity (Chang et al., 1996).

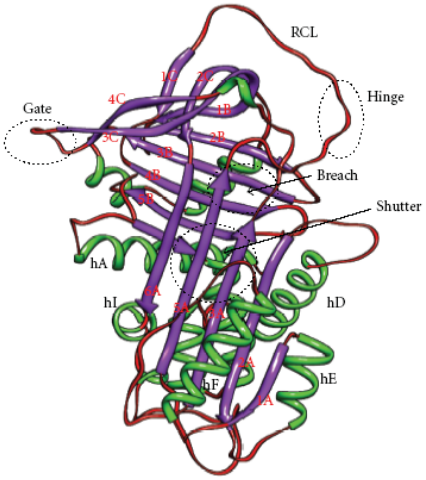

Fig. 2.2 Important domains in serpin conformations.

The mechanism of inhibition of serpin has been demonstrated biophysically and structurally as suicide substrate-like inhibitory mechanism where after binding to protease it is partitioned between cleaved serpin and serpin-protease complex (Horvath et al., 2005). Initially serpins bind to protease through a noncovalent Michaelis-like complex by interactions with residues flanking the scissile bond (P1-P1') (Gettins, 2002). The attack of the active- site serine on the scissile bond leads to a covalent ester linkage between Ser-195 of the protease and the backbone carbonyl of the P1 residue, resulting in the cleavage of the peptide bond. Protease specificity is determined by the P1-P1' bond, positioned for its ready accessibility to proteases. RCL inserts into the β sheet A and transports the covalently bound protease with it, thereby translocating the protease by over 70 Å and distorting its active site. (Gettins, 2002; Silverman et al., 2001). Distortion of the active site prevents the final hydrolysis events and the result is an irreversible covalent serpin-enzyme complex.

2.7 Purification of protease inhibitors

Purification of protein to homogeneity is an essential requirement for the characterization and property studies. There are several reports on purification of protease inhibitor from plants, animals and microbes. Protein purification methods like ammonium sulfate precipitation, ion exchange chromatography, affinity chromatography, gel filtration chromatography and RP-HPLC were regularly employed. The homogeneity is confirmed by methods like SDS-PAGE, Isoelectric focusing, MALDI-TOF, etc. Ammonium sulphate precipitation of the inhibitors was employed commonly to precipitate proteins. Alternatively, protease inhibitors were precipitated using acetic acid, ethanol, acetone, or combination of ethanol and acetone (Bhattacharyya et al., 2006; Haq et al., 2005; Kubiak et al., 2009; Macedo et al., 2000; Tian and Zhang, 2005).

Most purification protocols require more than one step to achieve the desired level of product purity. High purity and yield can be achieved by adding or repeating steps. Ion exchange chromatography is one of the chromatographic procedures employed in purification of protease inhibitors, wherein anion or cation exchangers are used according to the binding characteristic of protease inhibitor on the ion exchange resin. DEAE cellulose has been employed for the purification of a novel bi-functional protein proteinase/amylase inhibitor from the dietary leguminous pulse *Phaseolus aureus* Roxb (Haq et al., 2005). Anion exchanger Source 30Q is used for the purification of proteinase inhibitor GLPIA2 from *Ganoderma lucidum* (Tian and Zhang, 2005). Cation exchange chromatography on SP-Sepharose and anion exchange chromatography on Q-Sepharose are employed for the purification of trypsin-chymotrypsin inhibitors from black gram (*Vigna mungo*) (Cheung et al., 2009).

Affinity chromatography is another highly selective procedure that enables the purification of a biomolecule on the basis of its biological function, hence high resolution, and usually high capacity for the purification of protein of interest. Purification can be in the order of several thousand-fold and recoveries of

active material are generally very high. A range of trypsin bound resins were used for the purification of trypsin inhibitors. Trypsin inhibitors (TI) from wild-type soybean (Glycine soya) (WBTI) and domesticated soybean (*Glycine max*) (SBTI) were purified using prepared chitosan resin-trypsin as filler on the affinity chromatography column (Zhang et al., 2009). Serine protease inhibitor (AmPI) was purified from larval hemolymph of tasar silkworm *Antheraea mylitta* using trypsin-sepharose CL-6B affinity column (Rai et al., 2010). Affinity chromatography on Affi-gel blue gel has been used for the purification of a 20 kDa Kunitz-type trypsin inhibitor from *Gymnocladus chinensis* (Yunnan bean) seeds (Zhu et al., 2011). It was also reported that a Kunitz-type trypsin inhibitor with high stability from *Spinacia oleracea* L. seeds purified by Sepharose 4B-trypsin affinity chromatography (Kang et al., 2009).

Though affinity chromatography is an effective method to purify trypsin inhibitors, the possibility of limited proteolysis of proteinaceous inhibitor by trypsin cannot be ruled out. Gel filtration chromatography with urea or without urea is often employed to get proteinaceous inhibitor up to homogeneity. A serine proteinase inhibitor with high inhibitory activities against human neutrophil elastase (HNE) found in seeds of the Tamarind tree (*Tamarindus indica*) has been purified by Sephacryl S-300 and Sephadex G-50 gel filtration chromatography (Fook et al., 2005). Sephadex G-50 gel filtration has been employed for the purification of a novel serine protease inhibitor named bungaruskunin from venom of *Bungarus fasciatus* (Lu et al., 2008).

Reversed-phase high-performance liquid chromatography has emerged as a very rapid and selective method for peptide and protein purification which uses porous, microparticulate, chemically bonded alkylsilicas (Hearn, 1982). The most commonly used reverse phase HPLC column includes, octadecylsilane column (Shoji et al., 1999), Vydac C18 HPLC column (Cesar et al., 2004), Vydac 218 TP 1022 C18 (Sivakumar et al., 2005) and µ-Bondapak C18 column (Ligia et al., 2003). Low-molecular-mass trypsin inhibitor (clTI-1; chicken liver Trypsin

Inhibitor-1) from chicken liver and equine seminal plasma proteinase inhibitors were purified by RP-HPLC on a C18 column (Kubiak et al., 2009; Vasconcelos et al., 2009). Preparative HPLC has been used in the purification of Clitocybin D, a novel human neutrophil elastase inhibitor, from the culture broth of *Clitocybe aurantiaca* (Kim et al., 2009).

2.8 Characterization of protease inhibitors

2.8.1 pH and temperature stability

It was reported that 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). Extreme pH conditions will alter the structure of the inhibitor such that they no longer bind with the enzymes or with their substrates. Under strong acidic or alkaline conditions, the proteinaceous inhibitors get denatured and as a consequence they lose their activity partially or completely. 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). Insects that feed on plant material possess alkaline guts and depend predominantly on serine proteases for digestion of food material.

A novel thermostable protein inhibitor of trypsin and subtilisin, called BN, was isolated from the seeds of *Brassica nigra* (Genov et al., 1997). The purified protein gave a single band on SDS-PAGE, composed of two disulfide-linked polypeptide chains, consisting of 39 and 90 residues respectively. BN exhibits an extremely high thermostability and CD measurements showed that during heating to 97°C a considerable part of the polypeptide backbone folding is preserved. *Serratia marcescens* ATCC 27117 produced very small amounts of an inhibitor protein (SmaPI) that shows an inhibitory activity against extracellular 50 kDa metalloprotease (Kim et al., 1995). The inhibitor was stable in boiling water for up

to 30 min. The thermostability of SmaPI can be attributed to its reversible denaturation.

Three actinomycetes strains producing alkaline protease inhibitors API-I, API-II and API-III respectively, exhibited different properties in their molecular nature and in their pH and temperature stabilities (Pandhare et al., 2002). API-I and API-II exhibited stability over a pH range of 5-12 whereas API-III displayed wide pH stability from 2-12. API-I was stable at 60°C with a half-life of 2 h but API-II showed a half-life of 1 h at 45°C. API-III exhibited the least thermal stability with complete loss of activity at 37°C after 1 h.

Preparations of new low molecular weight protein inhibitors of serine proteinases have been obtained from buckwheat *Fagopyrum esculentum* seeds possessed high pH-stability in the pH range 2-12 and thermostability (Tsybina et al., 2004). Protease inhibitor (PISC-2002) isolated from culture supernatants of *Streptomyces chromofuscus* is stable over a large range of pH (2–10) and at high temperatures (80 °C/30 min), mainly because of the presence of proline and of a high content in hydrophobic amino acids (Angelova et al., 2006).

2.8.2 Effect of metal ions

Divalent metal ions serve to enhance the structural stability of a protein in a conformation that is critical for its biological function. 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 main amino acid residues that serve as ligands to divalent ions are Asp, His, Thr, and Glu (Kim and Wyckoff, 1991; Sowadski et al., 1985).

The inactivation of human coagulation factor Xa by the plasma proteinase inhibitors α_1-antitrypsin, antithrombin III and α_2-macroglobulin in purified systems was found to be accelerated by the divalent cations Ca^{2+}, Mn^{2+} and Mg^{2+}, whilst the decrease in rate constant at higher concentrations of Ca^{2+} and Mn^{2+} may

be due to factor Xa dimerization (Vincent Ellis et al., 1983). Studies on the impact of metal ions on the activity of *M. oleifera* protease inhibitor illustrated that addition of divalent ions such as Zn^{2+} at a concentration of 1 mM enhanced the protease inhibitor activity up to 31% and Hg^{2+} at a concentration of 10 mM enhanced up to 64% of protease inhibitor activity. Ca^{2+} and Mg^{2+} at higher concentration (10 mM) enhanced the protease inhibitor activity only to a marginal level (Bijina et al., 2011a).

2.8.3 Effect of oxidizing and reducing agents

Oxidation can occur when proteins are exposed to oxidizing agents such as hydrogen peroxide (H_2O_2), periodate, dimethyl sulfoxide, chloramine-T, *N*-chlorosuccinamide and *in vivo* in response to oxidants released by neutrophils (*e.g.* superoxide, hydroxyl radical) (Brot and Weissbach, 1983; Vogt, 1995). Oxidation of methionine residues is common and it has been shown to cause a decline in the biological activity of the protein. The specificity pattern of chymotrypsin was changed significantly upon oxidation of methionine to its sulfoxide (Weiner et al., 1966).

One of the most thoroughly studied examples of methionine oxidation and its effect on activity involves the α1-protease inhibitor (α1-PI). This protein can be oxidized on two of its eight methionine residues, and oxidation of one of these residues (Met358) causes an almost complete loss of inhibitory activity of α1-PI toward its primary biological target, elastase (Johnson and Travis, 1979). A similar oxidation in α2-macroglobulin is reported, but a more detailed investigation correlated loss of activity to oxidation of a single tryptophan residue (Reddy et al., 1994). Inhibitory activity of protease inhibitor isolated from *M. oleifera* is declined by the increase in the concentration (from 1% to 5%) of oxidizing agents DMSO and H_2O_2 (Bijina et al., 2011a).

The covalent link of cysteine residues by disulfide bonds is vital for the proper folding, stability and function of many proteins of prokaryotes and

eukaryotes (Creighton et al., 1995; Frand et al., 2000; Hogg, 2003). Significant conformational destabilization of the protein is resulted by the removal of a disulfide bond by reduction or substitution for another amino acid residue (Kawamura et al., 2008; Zavodszky et al., 2001). Reductive unfolding of mutant Leech carboxypeptidase inhibitor (LCI) has been studied using dithiothreitol (DTT) as reducing agent (Arolas et al., 2009). Lower concentration of dithiothreitol (DTT) had no effect on protease inhibitor isolated from *Peltophorum dubium* was observed. A Kunitz type trypsin inhibitor from *Erythrina caffra*, retained its inhibitory activity even after reduction with dithiothreitol (Lehle et al., 1996; Macedo et al., 2003).

2.8.4 Effect of detergents

Detergents (cationic, anionic, zwitterionic and non-ionic) are used for solubilizing proteins from lipid membranes and for improving the solubility of certain proteins in solution without compromising the native structure of proteins (Cardamone et al., 1994). Triton X-100, Tween 20 and Tween 80 are nonionic polyoxyethylene detergents and the major interaction with proteins are hydrophobic (Salameh and Wiegel, 2010). In fact, the detergents mimic the native, hydrophobic environment of the phospholipid bilayer, potential application in protein chemistry and in biotechnological process are explored (Israelachvili, 1991; Jones and Chapman, 1995; Tanford, 1980).

Kunitz inhibitors have a loop and bend structure conformation, stabilized chiefly by hydrophobic interactions. Studies on the effect of sodium dodecyl sulfate on soybean Kunitz inhibitor changed its three dimensional conformation significantly (Jirgensons, 1973). Conformation of *Cajanus cajan* inhibitor changed in the presence of SDS and deoxycholate (DOC), accompanied by loss in inhibitory activity in the presence of anionic bile salt, deoxycholate (Haq and Khan, 2005). Whereas the inhibitory activity of protease inhibitor isolated from *M. oleifera* showed an increase in the inhibitory activity in the presence SDS (Bijina

et al., 2011a). SDS and Tween 80 conferred thermal stability to the alkaline protease inhibitor of actinomycetes (Pandhare et al., 2002).

2.8.5 Effect of chemical modifiers

Chemical modification studies using amino acid side chain-specific chemical reagents is an important tool for studying structure, function relationship of enzymes and other biologically active proteins. In contrast to site-directed mutagenesis which make use of information regarding three dimensional structure of protein and cloning of the gene, chemical modification can be done without knowing the protein structure (Gote et al., 2007). Amino acid side chain specific chemicals like *N*-Ethylmaleimide, *N*-Bromosuccinanmide (NBS), PMSF, diethylpyrocarbonate (DEPC), trinitrobenzene-sulfonic acid (TNBS) are commonly employed for this purpose.

The amino acid of pearl millet cysteine protease inhibitor is modified using amino acid specific chemical modifiers. Modification of histidine using 10 mM Diethylpyrocarbonate and arginine with 10 mM *p*-Nitrophenylglyoxal enhanced protease inhibitory activity, whereas cysteine, glutamic/aspartic acid resulted in abolition of the anti-fungal activity of CPI (Joshi et al., 1999). The role of amino acids Lys, Arg, His in the structure and stability of the legume Bowman-Birk inhibitor is studied by chemical modifications and found state of association influence the physiological and functional role of these inhibitors (Kumar et al., 2004).

Chemical modification studies of a novel proteinase inhibitor, Lentinus proteinase inhibitor, purified from the fruiting bodies of the edible mushroom, *Lentinus edodes*, suggest involvement of one or more arginine residues in the inhibition of trypsin. Modification of arginine residues with cyclohexane-1,2-dione completely abolished the inhibitor activity, while modification of three lysine residues out of the eight with sodium 2,4,6-trinitrobenzene-1-sulfonate did not significantly affect the inhibitory activity (Odani et al., 1999).

Trypsin inhibitor purified from the seeds of *Peltophorum dubium*, PDTI was inactivated by lysine and arginine modifying reagents such as trinitrobenzene-sulfonic acid (TNBS) and 1, 2-cyclohexanedione (CHD). Inactivation of the inhibitor by CHD was greatest compared to TNBS, which suggests the involvement of arginine in the reactive site (Macedo et al., 2003).

2.8.6 Fluorescence binding studies

Fluorescence spectroscopy, which is an extremely sensitive technique that can be used to follow both protein folding and binding events. Proteins contain three aromatic amino acid residues (tryptophan, tyrosine and phenylalanine) which may contribute to their intrinsic fluorescence. The fluorescence of a folded protein is a mixture of the fluorescence from individual aromatic residues. Protein fluorescence is generally excited at 280 nm or at longer wavelengths, usually at 295 nm. Most of the emissions are due to excitation of tryptophan residues, with a few emissions due to tyrosine and phenylalanine. The tryptophan free variant α1-antitrypsin, is fully active as an inhibitor of thrombin. Thrombin has a fluorescence emission maximum of 340 nm which blue shifts to 346 nm, concomitant with a 40% increase in intensity, upon formation of the serpin proteinase complex indicative of substantial conformational change within the proteinase (Tew and Bottomley, 2001). Static fluorescence spectra of the three isoforms from *Acacia plumosa* Lowe seeds measured after excitation at 280 and 295 nm, gave the same λ_{max} emission value at 341 nm (Lopes et al., 2009); this value corresponds to the position of spectra of class II chromophores, in which tryptophan residues are exposed to the surface of the compact native protein molecule (Burstein et al., 1973).

2.8.7 Kinetics of inhibition

The inhibitor constant, *K*i, is an indication of how potent an inhibitor to its target enzyme. Enzyme inhibition studies are routinely conducted to assess the

presence and magnitude of protein-drug interactions. Characterization of inhibitor includes understanding the nature of inhibition process (i.e., competitive, noncompetitive, or uncompetitive) and determination of the inhibition constant (*K*i). A given inhibitor may inhibit several proteases, and 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.

Recombinant cnispin (rCnp) from basidiomycete *Clitocybe nebularis* which inhibits trypsin, with a *K*i value of 3.1 nM, was shown to be a fast-acting and tight-binding inhibitor of this enzyme. The *K*i value of cnispin for the inhibition of chymotrypsin is in the micromolar range, while those for subtilisin and porcine kallikrein are significantly higher (Avanzo et al., 2009). Protease inhibitor isolated from culture supernatants of *Streptomyces chromofuscus* strongly inhibited subtilisin, proteinase K, trypsin and proteinase of *S.albovinaceus*, with *K*i of micromolar range. The inhibitor weakly inhibited pepsin, but did not inhibit chymotrypsin and cathepsin D (Angelova et al., 2006).

In lentil, two BBI gene classes have been reported; one codes a trypsin/trypsin inhibitor, the other encoding a trypsin/chymotrypsin inhibitor. A full-length cDNA, encoding a Bowman-Birk protease inhibitor (BBI) from lentil immature seeds proved to be active against trypsin and chymotrypsin, showing *K*i values at the nanomolar level, comparable to those previously reported for trypsin/chymotrypsin BBI extracted from lentil seeds (Caccialupi et al., 2010; Ragg et al., 2006).

The quantitative relationship of proteases to protease inhibitors determined stoichiometrically. Serine proteinase inhibitor from the leguminous plant seeds of *Archidendron ellipticum* (AeTI), inhibited trypsin in the stoichiometric ratio of 1:1, but lacked similar stoichiometry against chymotrypsin (Bhattacharyya et al., 2006). Titration of trypsin with recombinant cnispin (rCnp) from basidiomycete *Clitocybe nebularis*, inhibitor from *Leucaena leucocephala*

(LlTI) (Oliva et al., 2000) and from *Dimorphandra mollis* (DmTI) (Macedoz et al., 2000) showed similar 1:1 stoichiometry.

2.9 Application studies

2.9.1 Pesticidal protease inhibitors

Since the introduction of DDT in the 1940s, pest control has relied heavily upon chemical insecticides. However, the development of insect resistance, an increased awareness of the environmental and health impacts of these chemicals, and the need for systems with a smaller environmental footprint has stimulated the search for new insecticidal compounds, novel molecular targets, and alternative control methods. In recent decades a variety of biocontrol methods employing peptidic or proteinaceous insect-specific toxins, protease inhibitors derived from microbes, plants and animals have been examined in the laboratory and field with varying results (Whetstone and Hammock, 2007).

Protease inhibitors prevent proteolysis in the insect gut leading to poor nutrient uptake, retarded development and eventually, death by starvation (Gatehouse et al., 1999). Much research has focused on protease inhibitors exploiting defense mechanism for crop protection. Studies using artificial diets containing plant-derived protease inhibitors targeting a particular class of proteases in the insect gut have demonstrated that these proteins retard growth and development in a wide range of insect pests. Disruption of the ability of insects to digest protein by transforming plant genomes with proteinaceous inhibitors provide an alternative approach to pest control (Reckel et al., 1997). Protease inhibitors supplemented in the diet resulted in the reduction of growth and survival rate of insect pests. The class of protease in use for the proteolysis by the Coleoptera species and lepidopterans are different. Cysteine proteinases account for most of the proteolytic activity in the gut of many Coleoptera species, whereas serine proteinases are the major digestive enzymes in many lepidopterans larvae.

Cysteine protease inhibitors and serine protease inhibitors were effective in controlling these two pest species (Macedo et al., 2003). Trypsin-specific inhibitors from the basidiomycete *Clitocybe nebularis*, CnSPIs incorporated into rearing medium and their insecticidal activity against *D. melanogaster* indicated the deleterious effect of CnSPIs against predatory insects (Avanzo et al., 2009). The protease inhibitors possessing insecticidal activities are listed in Table 2.4.

Several transgenic plants expressing protease inhibitors have been produced and tested for their efficacy in defensive capabilities, with particular efforts directed against insect pests (Valueva et al., 2001). Developing of transgenic plants expressing protease inhibitors directed at genes encoding inhibitors active against economically important orders of insect pests namely Lepidoptera, Diptera and Coleoptera, use serine and cysteine proteases. The PI genes have been particularly useful in developing transgenic plants resistant to insect pests, as they require the transfer of a single defensive gene, and can be expressed from the wound-inducible or constitutive promoters of the host (Boulter, 1993). The first PI gene to be successfully transferred was that coding for CpTi and produced transgenic tobacco with significant resistance against tobacco hornworm, *Manduca sexta* (Hilder et al., 1987).

Sugarcane productivity is challenged by a wide array of biotic and abiotic stresses, with insects being one of the major causes of economic losses, where the sugarcane borer *Diatraea saccharalis* is an insect pest causing stalk damage to sugarcane plants resulting in production loss for both the sugar and alcohol industries. The introduction of cDNAs of proteinase inhibitor encoding genes of soybean (*Glycine max* L.), Kunitz trypsin inhibitor (SKTI) and soybean Bowman–Birk inhibitor (SBBI) into sugarcane (*Saccharum officinarum* L.) genome conferred partial resistance to the sugarcane borer *Diatraea saccharalis* (Lepidoptera: Crambidae). The growth of neonate larvae of sugar-cane borer (*Diatraea saccharalis*) feeding the leaf tissues was significantly retarded as compared to larvae feeding on leaf tissues from untransformed plants (Falco and

Silva-Filho, 2003). Transgenic tobacco plant is made by over expressing a serine proteinase inhibitor gene, SaPIN2a, demonstrated more resistance to two devastating pests of important crops, cotton bollworm (*Helicoverpa armigera*) and tobacco cutworm (*Spodoptera litura*) larvae (Luo et al., 2009). The combined inhibitory effect of *Nicotiana alata* proteinase inhibitor (NaPI), multidomain potato type II inhibitor (pin II) and *Solanum tuberosum* potato type I inhibitor (StPin1A) on *H. armigera* reflected in the increased yield of cotton bolls in field trials of transgenic plants expressed both inhibitors (Dunse et al., 2010). Transgenic plants expressing protease inhibitor genes are listed in Table 2.5.

Table 2.4 List of protease inhibitor with pesticidal activity.

Source	Inhibitor	Target proteases	Target herbivore(s)	Reference
Cowpea	Cowpea trypsin inhibitor (CpTI)	Trypsin	*Lacanobia oleracea*	(Bell et al., 2001)
Rice	Oryzacystatin	Cysteine	*Diabrotica undecimpunctata howardi*	(Edmonds et al., 1996)
	Oryzacystatin	Cysteine	*A. abdominalis*	(Rahbe´ et al., 2003a)
	Oryzacystatin	Cysteine	*Sitophilus oryzae*	(Hosoyama et al., 1994)
	Oryzacystatin	Cysteine	*Callosobruchus chinansis* and *Rhiptorus clavatus*	(Kuroda et al., 1996)
Soybean	Trypsin inhibitor	Trypsin	*Tribolium confusum*	(Lawrence and Koundal, 2002)
Soybean	Soybean Bowman–Birk inhibitor (SbBBI)	Trypsin and Chymotrypsin	*Acyrthosiphon pisum*	(Rahbe´ et al., 2003b)
	Soybean Bowman–Birk inhibitor (SbBBI)	Trypsin and Chymotrypsin	*Spodoptera littoralis*	(Faktor and Raviv, 1997)
Mustard	Mustard-type trypsin-chymotrypsin variant Chy8	Trypsin and Chymotrypsin	*Acyrthosiphon pisum*	(Ceci et al., 2003)

Peltophorum dubium seeds	Kunitz	Trypsin	*Anagasta kuehniella*	(Macedo et al., 2003)
Tomato	Trypsin Inhibitor	Trypsin	*Helicoverpa armigera*	(Damle et al., 2005)
Archidendron ellipticum seeds	Kunitz	Trypsin	*Spodoptera litura*	(Bhattacharyya et al., 2006)
Potato	Potato multicystatin	Cysteine	*Frankliniella occidentalis*	(Annadana et al., 2002)
Potato and sea anemone	Cysteine Inhibitor	Cysteine	*Tribolium castaneum*	(Oppert et al., 2003)
Potato	Potato protease inhibitor	Trypsin and Chymotrypsin	*Sesamia inferens Chrysodeixus erisoma*	(Duan et al., 1996) (Mcmanus et al., 1994)
Cabbage protease inhibitor	Trypsin and Chymotrypsin	Trypsin and Chymotrypsin	*Trichoplusia ni*	(Broadway, 1995)

Table 2.5 List of examples for protease inhibitor expressing transgenic plants resistant to pests and pathogens.

Plant	Recombinant Inhibitor	Target proteases	Target herbivore(s)	Reference
Tobacco	Bovine spleen trypsin inhibitor	Serine	*Helicoverpa armigera*	(Christeller et al., 2002)
	Sporamin+Taro Cystatin	Serine+ Cysteine	*H. armigera*	(Senthilkumar et al., 2010)
	Tobacco trypsin protease inhibitor	Serine	*S. litura*	(Srinivasan et al., 2009)
Alfalfa	Oryzacystatin II	Cysteine	*Phytodecta fornicata*	(Ninkovic et al., 2007)
Apple	Nicotiana alata proteinase inhibitor	Serine	*Epiphyas postvittana*	(Maheswaran et al., 2007)
Potato	Multidomain cystatin fusions	Cysteine+ Aspartate	*Frankliniella occidentalis*	(Outchkourov et al., 2004a)

	Barley cystatin HvCPI-1 C68	Cysteine	*Leptinotarsa decemlineata*	(Alvarez-Alfageme et al., 2007)
Rice	Barley trypsin inhibitor	Serine	*Sitophilus oryzae*	(Alfonso-Rubi et al., 2003)
	Cowpea trypsin inhibitor (+Bt toxin Cry1Ac)	Serine	*C. medinalis*	(Han et al., 2007)
Sugarcane	Bovine pancreatic trypsin inhibitor (aprotinin)	Serine	*Scirpophaga excerptalis*	(Christy et al., 2009)
			Root parasitic nematodes	
Tomato	Taro cystatin	Cysteine	*M. incognita*	(Chan et al., 2010)
Wheat	Potato proteinase inhibitor 2	Serine	*Heterodera avenae*	(Vishnudasan et al., 2005)
			Pathogens	
Potato	Buckwheat serine proteinase inhibitor	Serine	*Pseudomonas syringae Clavibacter michiganensis*	(Khadeeva et al., 2009)
Rice	Potato carboxypeptidase inhibitor	Carboxype-peptidase A	*Magnaporthe oryzae*	(Quilis et al., 2007)
			Fusarium verticillioides	(Quilis et al., 2007)
	Sporamin+Taro cystatin	Serine+ Cysteine	*Pythium aphanidermatum*	(Senthilkumar et al., 2010)

2.9.2 Control of proteases in pathogenesis

Proteases have evolved to adapt to wide range of conditions found in complex organisms and function catalytically in different substrate hydrolysis. Excessive or inappropriate proteolysis is seldom a result of genetic aberrations but often an outcome of numerous endogenous and/or exogenous factors, effecting unwanted activation of protease signaling pathways of important physiological processes like cell-cycle progression, cell proliferation and cell death, DNA replication, tissue remodeling, haemostasis (coagulation), wound healing and the

immune response (Turk, 2006). Successful examples of therapeutic intervention using protease inhibitors include angiotensin converting enzyme (ACE) inhibitors for the treatment of hypertension, HIV aspartyl protease inhibitors to prevent development of AIDS and application of proteosome inhibitors in the treatment of multiple myeloma (Scott and Taggart, 2010).

Serine protease inhibitor Kazal type 1 (SPINK1) of human is synthesized in acinar cells of the pancreas secreted from the pancreas and regulate trypsin in certain circumstances to inhibit its activity. *SPINK1* gene has a coding sequence of approximately 7.5 kb consisting of 4 exons located on chromosome 5 and showed that mutations of the gene is associated with chronic pancreatitis (Witt et al., 2000). The plasminogen activator (PA) system includes four forms of serine protease inhibitors (SERPIN), SERPINA5 (also called protein C inhibitor, PCI), SERPINB2 (also called plasminogen activator inhibitor 2, PAI-2), SERPINE1 (also called plasminogen activator inhibitor 1, PAI-1), and SERPINE2 (also called protease nexin-1, PN-1). SERPINB2 and SERPINE1 are present in the human endometrium, SERPINE1 and mouse uteri during implantation indicating that the PA inhibitor is involved in implantation. SERPINE2 is widely expressed in various tissues and shows broad anti-protease activity specific to serine proteases, including trypsin, thrombin, plasmin and prostasin (Ohmuraya and Yamamura, 2011).

Administration of recombinant secretory leukocyte protease inhibitor, a serine protease inhibitor produced by various cell types, including neutrophils and activated macrophages is studied in spinal cord injury of mice. Recombinant secretory leukocyte protease inhibitor injected intraperitoneally localizes to the nucleus of circulating leukocytes, is detected in the injured spinal cord of mice, and reduces activation of nuclear factor-κB and expression of tumor necrosis factor-α (Ghasemlou et al., 2010). The coronavirus associated with severe acute respiratory syndrome, SARS, encodes a chymotrypsin-like cysteine protease M^{Pro} that is similar to picornavirus 3C protease. Since the 2003 SARS global outbreak,

several strategies of structure-based design of low molecular weight protease inhibitors have been applied in the search for antiviral drugs against SARS (Anderson et al., 2009; Sirois et al., 2007). Due to the obvious relevance of protease inhibitors, they have been studied extensively with the intent to develop therapeutic drugs according to their catalytic type, for each group of disease-causing organisms and for other human diseases. (Drag and Salvesen, 2010; Haq et al., 2010; Turk, 2006).

Parasites produce a diverse array of secreted and surface-bound proteases. The proteases of parasites are important virulence factors which help them in invading host by attacking the keratinocyte layers of the epidermis, the basal lamina, interstitial extracellular matrices, and the blood vessel walls (Table 2.6). Metalloproteases, aspartyl proteases, and serine proteases produced by *Porphyromonas gingivalis*, and different types of proteases produced by *Plasmodium falciparum* are studied in host pathogen interaction. A number of different experimental approaches and variety of pathogens and disease models have revealed a strong correlation of virulence and protease expression. Clinical isolates of *Entamoeba histolytica* correlates well with the level of activity of a secreted cysteine protease and mutational screens for virulence factors have identified protease-encoding genes necessary for virulence in *Streptococcus pneumoniae* and *Salmonella* (Armstrong, 2006). Protease inhibitors targeted against the viral aspartic protease used in treating HIV infection. Aspartic protease inhibitors also inhibited candida and reduced occurrence of candidiasis in patients. Secreted aspartic proteases are thus an important target for the development of new protease inhibitor based compounds for treating candidiasis (Braga-Silva and Santos, 2011; Dash et al., 2003; Naglik et al., 2004). In this context, protease inhibitors directed against key proteases responsible for virulence have shown promise as therapeutic agents in a variety of conditions of parasitic disease (Cohen et al., 1991; Engel et al., 1998a; McKerrow, 1999; Wasilewski et al., 1996).

Table 2.6 Proteases as virulence factors in pathogenic parasites

Role in pathogenesis	Pathogen	Protease	Reference
Direct tissue destruction, fibrinolysis in host invasion			
Fibrinolysis	*S. pyogenes, Y. pestis, B. burgdorferi*	Plasminogen activator (pla) Outer specific protein A (OspA)	(Fuchs et al., 1994; Poon-King et al., 1993; Sodeinde et al., 1992)
Corneal keratinitis	*Pseudomonas, Serratia*	Protease IV	(Engel et al., 1998b; Engel et al., 1998c)
Epidermal invasion	*S. stercoralis, S. mansoni*	Ss40	(Brindley et al., 1995; Cohen et al., 1991)
Peridontal disease	*P. gingivalis*	Cysteine protease	(Kuramitsu, 1998)
Cytolysis	*Serratia, Pseudomonas*	Serratia 56 K; Pseudomonas alkaline Protease	(Maeda et al., 1987; Molla et al., 1987)
Housekeeping functions			
Protein digestion	*S. mansoni; P. falciparum*	Cysteine protease	(Francis et al., 1997; Rosenthal et al., 1998; Wasilewski et al., 1996)
Molting	*O. volvulus, T. cruzi*	Cysteine protease	(Lustigman et al., 1996; Meirelles et al., 1992)
Proteolytic destruction of defense mediators			
Depletion of protease inhibitors	*Pseudomonas, Serratia,Candida C. salmositica*	Elastase, Serratia 56 kDa Protease	(Khan et al., 1994; Rasmussen et al., 1999; Zuo and Woo, 1997)
Inactivation of complement	S*treptococcus, Serratia, Pseudomonas, Candida, E. histolytica*	Streptococcal C5a Protease, Candida acid Protease, Entamoeba Cysteine protease	(Cutler et al., 1993; Reed et al., 1989b; Schenkein et al., 1995)
Activation of host protease zymogens			
Activation of host matrix metalloprotease	*P. gingivalis*	40 kDa trypsin-like protease	(DeCarlo et al., 1998; Sorsa et al., 1992)
Plasmin-catalyzed plasmin generation	*S. pyogenes, S. aureus*	Streptokinase-activated autoproteolytic conversion	(Lottenberg et al., 1994)

Protease-dependent microbial toxin systems			
Proteolytic activation of microbial toxins	*B. anthracis, P. aeruginosa*	Host cell furin/PACE4	(Gordon et al., 1995; Gordon et al., 1997) (Inocencio et al., 1994)
Microbial toxins that are proteases	*Clostridium botulinum, Clostridium tetani*.	Botulinus toxin, Tetanus toxin	(Montecucco and Schiavo, 1993; Schiavo et al., 1992)

2.10 *Pleurotus floridanus*

Mushrooms are fruiting bodies of higher fungi classified in the phylum basidiomycota. Edible and medicinal mushrooms produce compounds including polysaccharides and proteins without overt cytotoxicity and they represent a resource for seeking new natural drugs. Attempts have been made in many parts of the world to explore the use of mushrooms and their metabolites for the treatment of a variety of human ailments. Mushrooms contain more digestible protein than that of plants; very little fat and no cholesterol and provide valuable minerals like potassium and iron. Most importantly, mushrooms are rich in B group vitamins niacin, folic acid and riboflavin (Bano and Rajarathnam, 1982). Involvement of mushroom metabolites as adaptogens, immunostimulants and as antitumor agents are studied clinically (Franz, 1989).

Pleurotus species are commonly called oyster mushrooms. Cultivation of *Pleurotus* sp. is the second largest in amount after *Agaricus bisporus* in the world (Chang, 1991; Sivrikaya and Peker, 1999). There are about 40 species of this mushroom found in temperate and tropical parts of the world. *P. floridanus* is cultivated on a commercial scale in many parts of the world, including India. The anti inflammatory and platelet aggregation inhibiting activities of the methanol extract of *Pleurotus floridanus* Eger has been investigated. The extract ameliorated acute inflammation induced by carrageenan and chronic inflammation by formalin (Jose et al., 2004). *Pleuorotus* species are reported to be the best known source for correcting hypercholesterolemia (Endo, 1988). Antitumor and

antioxidant property of *P. floridanus* has been demonstrated. Methanol extract of *P. floridanus* has remarkable capacity to inhibit the growth of solid tumor induced by EAC cell line in a dose-dependent manner in experimental animals (Jose and Janardhanan, 2000). From the literature available on oyster mushrooms, only *Pleurotus ostreatus* is reported to yield low molecular inhibitors (Dohmae et al., 1995).

3 Materials and methods

3.1 SCREENING OF MUSHROOMS FOR PROTEASE INHIBITOR

3.1.1 Mushrooms for the study

Mushrooms, *Pleurotus floridanus*, *Agaricus bisporus* obtained from Kerala Agriculture University, Trichur, India and the wild mushroom *Phellinus* sp. from Cochin University of Science and Technology, Cochin-22 premises were used as the source material to screen for protease inhibitory activity. The fruiting body of mushrooms were aseptically transferred to an ice box and transported. Stock cultures were maintained at -70^{o}C.

3.1.2 Extraction and recovery of protease inhibitor

Frozen *P. floridanus* fruiting bodies (250 g) were homogenized with 0.01 M phosphate buffer pH 7.5 (500 mL) in a blender for 5 min. The homogenate was centrifuged (Sigma3K30, Germany) at 10,000 rpm for 15 min at 4^{o}C. The crude extract obtained as clear supernatant after centrifugation was used to assay protease inhibitor activity, protein content and specific activity as described in section 3.1.3.1.1 and 3.1.3.2 respectively.

3.1.3 Analytical methods

3.1.3.1 Protease inhibitor assay

Presence of protease inhibitor in the extract of mushroom was determined by assaying residual activity of trypsin (EC 3.4.21.4) that was *N*-tosyl-L-phenylalanyl chloromethyl ketone (TPCK) treated from Sigma-Aldrich using Hammerstein casein (SRL, India) and α-*N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA, Sigma) as substrates.

3.1.3.1.1 Caseinolytic assay

Protease inhibitor activity was assayed against trypsin according to the method described by Kunitz with slight modifications (Kunitz, 1947). Hundred microlitre of trypsin (0.1 mg/mL) was preincubated with suitable dilution of protease inhibitor at 37°C for 15 min and made up to 500 µL with 0.01 M phosphate buffer pH 7.5. To this mixture 100 µL of 1% Hammerstein casein (SRL, India) prepared in 0.1 M phosphate buffer was added and incubated at 37°C for 30 min. The reaction was terminated by the addition of equal volume of 0.44 M trichloroacetic acid (TCA) solution. The reaction mixture was centrifuged at 10,000 rpm for 15 min. The absorbance of the clear supernatant was measured at 280 nm 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 were quantified by comparing with tyrosine as standard. Appropriate blanks for the enzyme, inhibitor and the substrate were also included in the assay along with the test.

One unit of trypsin activity was defined as the amount of enzyme that liberated 1µmol of tyrosine per mL of the reaction mixture per min under the assay conditions. One unit of protease inhibitor activity (u) was defined as the decrease by one unit of trypsin activity. For easy computation and understanding, the protease inhibitor activity of certain experiments were expressed in terms of percent inhibition of trypsin activity.

The protease inhibitory activity was expressed in percentage inhibition and calculated as

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

3.1.3.1.2 Assay using BAPNA

Protease inhibitor activity was also measured using the synthetic substrate BAPNA (Kakade et al., 1974). Three seventy-five microlitre of the inhibitory protein diluted with phosphate buffer (pH 7.5) was incubated with 25 µL of 0.1 mg/mL trypsin in phosphate buffer pH 7.5 for 10 min at 37^{o}C. Then 50 µL, 2 mM freshly prepared BAPNA was added and incubated at 37^{o}C for 30 min. The reaction was stopped by the addition of 500 µL of 30% acetic acid. The optical absorbance of *p*-nitroaniline released by the reaction was read at 410 nm. The difference in OD was calculated by assaying trypsin activity in the absence and presence of inhibitor.

One unit of inhibitory activity (U) was defined as the amount of inhibitor required to inhibit the release of 1 µmol of *p*-nitroaniline per mL per min at pH 7.5 and at 37^{o}C. The amount of protein present in each step was estimated.

3.1.3.2 Protein estimation

Protein content was estimated according to the method of Bradford (1976) using bovine serum albumin (BSA) as the standard and the concentration was expressed in mg/mL.

Bradford reagent

Hundred milligram Coomassie Brilliant Blue G-250 (Sigma-Aldrich) was dissolved in 50 mL 95% ethanol, added 100 mL of 85% (w/v) phosphoric acid and diluted to 1 L when the dye has completely dissolved, followed by filtration through Whatman #1 paper.

Estimation

a. Prepared standards containing a range of 5 to 25 µg of bovine serum albumin (BSA) in 100 µL volume.

b. Diluted unknowns if necessary to a final volume of 100 µL. Added 1 mL Bradford reagent and incubated for 5 min at 28±2°C.
c. Measured the absorbance at 595 nm in a UV-Visible spectrophotometer (Shimadzu, Japan).

3.1.3.3 Specific Activity

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

$$\text{Specific activity} = \frac{\text{Inhibitory activity (U/mL)}}{\text{Protein (mg/mL)}}$$

3.2 SELECTION OF POTENTIAL SOURCE AND ISOLATION OF PROTEASE INHIBITOR

Protease inhibitors from mushrooms that could be precipitated by ammonium sulphate were selected for further screening and selection of potential source. For this samples with 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 mushroom was prepared as described under section 3.1.2. The samples were assayed for protease inhibitory activity and protein content as described earlier under section 3.1.3.1 and 3.1.3.2 respectively.

3.2.2 Ammonium sulphate precipitation

Ammonium sulphate precipitation of the prepared sample was done according to the method described by Englard and Siefter (1990). The fractionation using ammonium sulphate precipitation has the advantage of

intermediate removal of unwanted proteins with the simultaneous concentration of the protein of interest. Ammonium sulphate (SRL, India) required to precipitate the protease inhibitor was optimized by adding varying concentrations (30%, 60% and 90% saturations) to the crude extract as detailed below.

a. To precipitate the protein, ammonium sulphate was slowly added initially at 30% saturation to the crude extract while keeping in ice with gentle stirring.
b. After complete dissolution of ammonium sulphate, the solution was kept for precipitation at 4°C for 4 h.
c. Protein precipitated was collected by centrifugation at 10,000 rpm for 15 min at 4°C.
d. 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% of ammonium sulphate saturation.

3.2.3 Dialysis

The precipitate obtained after ammonium sulphate precipitation was further dialyzed against 0.01 M phosphate buffer (pH 7.5), 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 clear the pores of the tube. 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 h.
b. Rinsed in 0.3% (w/v) solution of sodium sulfide, at 80°C for 1 min.

c. Washed with hot water (60°C) for 2 min.
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 0.1 M phosphate buffer (pH 7.5).
b. The solution was taken in the pretreated dialysis tube (3.2.3.1) (Sigma-Aldrich, cut off value 12 kDa) against 0.01 M solution of phosphate buffer pH 7.5 for 24 h at 4°C with frequent changes of buffer and assayed for protease inhibitory activity, protein content and specific activity as described under section 3.1.3.1, 3.1.3.2 and 3.1.3.3 respectively.

3.3 IDENTIFICATION OF THE SELECTED MUSHROOM

Molecular identification of the selected mushroom was carried out using ITS1 and ITS4 primer pair to amplify internal transcribed spacers (ITS1 and ITS2) and 5.8S rDNA of the genomic DNA. PCR amplicon was subjected to sequencing, followed by homology analysis (Nazar, 2003).

The mushroom DNA isolation, the primer sequences and PCR conditions were as follows.

3.3.1 Template preparation for PCR (Doyle and Doyle, 1987)

a. One gram of fresh mushroom was ground to fine powder in pre cooled mortar using liquid nitrogen.
b. Transferred the powder to a sterile oak-ridge tube containing 16 mL CTAB extraction buffer and incubated at 65^{o}C for 1 h.
c. Added equal volumes of chloroform-isoamyl alcohol and mixed by gentle inversion.
d. Centrifuged at 10,000 rpm for 15 min at 4^{o}C.

e. The aqueous layer formed on top was transferred to a fresh sterile microfuge tubes using sterile cut tips.
f. Then added ice cold isopropanol (600 µL to 1 mL aqueous layer transferred) and gently inverted several times and kept at 4°C for 30 min. Centrifuged at 10,000 rpm for 15 min at 4°C.
g. The supernatant was removed and the pellet was washed with 70% ethanol.
h. Centrifuged at 10,000 rpm for 15 min at 4°C.
i. The pellet was allowed to air dry to remove traces of ethanol.
j. Resuspended the pellet in 100 µL Tris-EDTA buffer (pH 8.0) and stored at -20°C.

3.3.2 Primer sequence

Primer	Sequence (5'-→3')
ITS1	**TCC GTA GGT GAA CCT TGC GG**
ITS4	**TCC TCC GCT TAT TGA TAT GC**

3.3.3 PCR Mix composition

10X PCR buffer	2.5 µL
2 mM dNTP mix	2 µL
Forward primer (10 µM)	2 µL
Reverse primer (10 µM)	2 µL
Taq DNA polymerase	1 U
Magnesium Chloride (25 mM)	1.8 µL
Template DNA (40 ng/µL)	4 µL
Sterile Milli Q water to a final volume of	25 µL

3.3.4 PCR conditions

Initial denaturation	95°C	for 3 min.
Denaturation	95°C	for 30 sec.
Annealing	57°C	for 30 sec.
Primer extension	72°C	for 90 sec.
Repeated 30 times		
Final extension	72°C	for 10 min.

PCR was performed in a thermal cycler (Bio-Rad, USA).

3.3.5 Agarose gel electrophoresis (Sambrook and Russell, 2001)

a. Agarose gel with a concentration of 0.8% was prepared for electrophoresis of the PCR products.
b. 5 µL of the PCR products was loaded on to the gel and electrophoresed at 80 V until the migrating dye (Bromophenol blue) had traversed two-thirds distance of the gel. 100 bp DNA ladder (Fermentas, India) was used as the marker.
c. The gel was stained in freshly prepared 10 µg/mL ethidium bromide solution for 10 min.
d. The gel was viewed on a UV- Transilluminator, and image captured with the help of Digi Doc system (Bio-Rad).

3.3.6 DNA sequencing

Nucleotide sequences determined by the ABI Prism 310 genetic analyzer by using the big dye Terminator kit (Applied Biosystems) at BioServe India Ltd., Hyderabad. The sequenced PCR products were analyzed online using BLAST software (http://www.ncbi.nlm.nih.gov/blast) and the identity of the sequences were determined (Altschul *et al.,* 1980).

3.4 PROTEASE INHIBITOR PURIFICATION

Protease inhibitor, proteinaceous in nature, isolated from *P. floridanus* was purified by standard protein purification methods which included ammonium sulphate precipitation, followed by dialysis, DEAE sepharose ion exchange chromatography, Trypsin sepharose affinity chromatography and sephadex G-100 gel filtration chromatography. The purity was further confirmed by reverse phase HPLC. All purification steps were carried out at 4°C unless otherwise mentioned.

3.4.1 Ammonium sulphate precipitation

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

3.4.2 Dialysis

The precipitate obtained after ammonium sulphate precipitation was further dialyzed against 0.01 M phosphate buffer (pH 7.5) as described previously under section 3.2.3.

3.4.3 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 sepharose (Sigma-Aldrich) as the anion exchanger. Proteins bind to ion-exchangers due to surface charge. These reversibly adsorbed proteins were eluted out using a salt gradient.

3.4.3.1 Purification using DEAE sepharose column

DEAE sepharose was carefully packed in Bio-Rad econo column (25 X 1.5 cm) without any air bubble and the column was equilibrated with five column volumes of 0.01 M phosphate buffer pH 7.5. Five millilitre of dialyzed sample, prepared as mentioned in section 3.2.2, with a protein content of 20 mg 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 0.01 M phosphate buffer, pH 7.5 with a flow rate of 1 mL/min which was set up in an FPLC system (Bio-Rad), reading absorbance at 280nm. The bound fraction was eluted by a step gradient of 0.5 M NaCl in 0.01 M phosphate buffer. Peak fractions from the column were pooled and dialyzed against the phosphate buffer pH 7.5 (0.01 M) as described under section 3.2.3. The dialyzed fractions were concentrated using amicon UF-10 kDa membrane (Millipore, USA) and assayed for protease inhibitory activity, protein content and specific activity as described under section 3.1.3.1.2, 3.1.3.2 and 3.1.3.3 respectively.

3.4.4 Affinity chromatography

CNBr-activated Sepharose 4B was coupled to trypsin was used for the affinity purification of protease inhibitor from *P. floridanus.*

3.4.4.1 Preparation of CNBr-activated sepharose 4B

The lyophilized CNBr-activated Sepharose 4B (Sigma-Aldrich) was supplied in the presence of additives. These additives were washed away at low pH (pH 3) before coupling the trypsin. The use of low pH (pH 3) preserves the activity of the reactive groups, which otherwise hydrolyze at high pH. Three gram of lyophilized powder was (1 g lyophilized powder gives about 3.5 mL final volume of medium) suspended in 1 mM HCl. The swelled medium was washed for 15 min with 1 mM HCl on a sintered glass filter (porosity G3). Two hundred millilitre, 1 mM HCl was used per gram freeze-dried powder and added in several aliquots.

3.4.4.2 Coupling of trypsin

a. Thirty milligram of trypsin was dissolved in coupling buffer, 0.1 M $NaHCO_3$ pH 8.3 containing 0.5 M NaCl in a stopper vessel. Five millilitre coupling solution/g lyophilized powder was used.

b. Rotated the mixture end-over-end for 1 h at room temperature.
c. Washed away excess trypsin with 5 medium (gel) volumes of coupling buffer.
d. The free active groups were blocked by transferring the medium to 0.1 M Tris-HCl buffer, pH 8 and allowed to stand for 2 h. Washing was performed with 5 medium volumes of each buffer with three cycles of alternating pH (Each cycle consisted of a wash with 0.1 M acetic acid/sodium acetate, pH 4 containing 0.5 M NaCl followed by a wash with 0.1 M Tris-HCl, pH 8 containing 0.5 M NaCl).

3.4.4.3 Purification using trypsin-affinity chromatography

The concentrated ion exchange fraction (2 mg) was applied to the trypsin affinity column (15 X 1 cm) which was equilibrated with 0.01 M phosphate buffer pH 7.5. The experiment was performed on an FPLC system with a flow rate of 0.5 mL/min. The bound protein was eluted using 0.5 M NaCl in 0.01M HCl. The bound peak was immediately dialyzed against 0.01 M phosphate buffer pH 7.5 and concentrated using amicon UF-10 kDa membrane. Protease inhibitory activity, protein content and specific activity found as described under section 3.1.3.1.2, 3.1.3.2 and 3.1.3.3 respectively. The yield and fold of purifications were calculated.

3.4.5 Protease inhibitor purification by sephadex G-100 gel filtration chromatography

Gel filtration chromatography was performed using the concentrated active fraction purified from ion exchange chromatography to obtain homogenous protease inhibitor.

3.4.5.1 Preparation of column

a. Ten gram of sephadex G-100 (Sigma-Aldrich) was suspended in distilled water and allowed to hydrate for 5 h at 90°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. Gel suspension was carefully poured into the column (75 X 1.5 cm) without air bubbles and allowed to settle under gravity while maintaining a slow flow rate through the column.
d. Column was equilibrated with two times the bed volume of eluent (0.01 M phosphate buffer, pH 7.5) passed through the column bed in a descending eluent flow.
e. The position of the flow adapters are re-adjusted as required maintaining contact of the plungers with the gel bed.

3.4.5.2 Sample preparation and application

Two millilitre of dialyzed, concentrated ion exchange fraction with a protein content of 15 mg/mL was applied to the column. Care was taken to make sure that sample was completely free of undissolved substances. After the complete entry of sample to the column, the proteins were eluted using 0.01 M phosphate buffer pH 7.5, with a flow rate of 10 mL/h. One millilitre 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, concentrated using amicon UF-10 kDa membrane and assayed for protease inhibitory activity, protein content and specific activity as described under sections 3.1.3.1.2, 3.1.3.2 and 3.1.3.3 respectively.

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

Yield of protein and yield of protease inhibitory activity after each step of purification were calculated. The percentage 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.

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

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

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

3.4.7 Reverse-phase HPLC

The purified active fraction after gel filtration (20 μL, 0.1 mg/mL) was subjected to reversed phase HPLC (Schimadzu LC 2010) using Phenomenex C18 HPLC column (22.5 mm ID X 250 mm length) at a flow rate of 1 mL/min with 100% solvent A (0.1% trifluoroacetic acid (TFA) in water) for 10 min and a linear gradient (0–100%) of solvent B (0.09% TFA in 60% acetonitrile) over 60 min. Proteins were detected by monitoring the absorbance at 220 nm.

3.5 CHARACTERIZATION OF PROTEASE INHIBITOR

Purified inhibitor was further subjected to characterization for their biophysical and physicochemical properties like molecular weight, isoelectric

point, stability at different temperature and pH, binding studies and inhibition kinetics to determine the type of inhibition as described in the following sections.

3.5.1 Electrophoretic methods

The active fractions of protease inhibitor from *P. floridanus*, after ion exchange chromatography, affinity chromatography and gel filtration chromatography were subjected to electrophoretic analysis by non denaturing Native-PAGE and denaturing SDS-PAGE in a vertical slab electrophoresis (Mini-PROTEAN Tetra cell, Bio-Rad). Electrophoresis was carried out in a 16% polyacrylamide gel according to the method described by (Laemmli, 1970).

Reagents for polyacrylamide gel electrophoresis

1. **Stock acrylamide solution (30% T and 2.6% C)**

Acrylamide	-	29.22 g
Bis-acrylamide	-	0.78 g
Distilled water (DW)	-	100 mL

   Stored at 4°C in amber coloured bottle

2. **Stacking gel buffer stock**

   Tris buffer (0.5 M) - 6.05 g in 40 mL DW

   Titrated to pH 6.8 with 1 M HCl and made up to 100 mL with DW. Filtered through Whatman No: 1 filter paper and stored at 4°C.

3. **Resolving gel buffer stock**

   Tris buffer (1.5 M) - 18.15 g

   Titrated to pH 8.8 with 1M HCl and made up to 100 mL with DW. Filtered through Whatman No: 1 filter paper and stored at 4°C.

4. **Running buffer for Native-PAGE (pH 8.3)**

Tris buffer	-	3 g
Glycine	-	14.4 g

Dissolved and made up to 1L with DW.

Prepared in 10X concentration and stored at 4°C.

5. Running buffer for SDS-PAGE (pH 8.3)

Tris buffer	-	3 g
Glycine	-	14.4 g
SDS	-	1 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	-	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)	-	1.25 mL
Glycerol	-	2.5 mL
SDS (10%, W/v)	-	2 mL
Deionised water	-	3.55 mL
Bromophenol blue (0.5%, w/v)	-	0.2 mL

Prior to use added 50 µL β-mercaptoethanol to 950 µL sample buffer. Samples were diluted with sample buffer in 1:2 ratios and heated at 95^{o}C for 4 min.

8. SDS (10%) - 1 g in 10 mL DW

9. Ammonium persulfate (10%, w/v) - 0.1 g of ammonium persulfate was dissolved in 1 mL DW (prepared freshly).

10. Protein staining solution

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

11. Destaining solution

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

Silver staining

Silver staining of the gel after electrophoresis was performed by the method of Blum (1987) with slight modifications.

Reagents

1. Fixer

Methanol (50%, v/v)	-	50 mL
Acetic acid (5%, v/v)	-	5 mL
Milli Q water	-	45 mL

2. Wash

Methanol (50%, v/v)	-	50 mL
Milli Q water	-	50 mL

3. Sensitizing solution

Sodium thiosulfate (0.02%, w/v)	-	20 mg
Milli Q water	-	100 mL

4. Silver nitrate solution

Silver nitrate (0.2%, w/v)	-	200 mg
Milli Q water	-	100 mL

5. Developer

Sodium carbonate (6%, w/v)	-	3 g
Formaldehyde	-	12.5 µL
Milli Q water	-	100 mL

6. Stop solution

Sodium-EDTA	-	1.4 g
Milli Q water	-	100 mL

Procedure

a. The SDS-PAGE gel was incubated in fixer for 30 min.
b. Washed the gel in wash solution for 15 min followed by five washes in five min interval with Milli-Q water.
c. Incubated the gel in sensitizer for exactly 60 sec and washed twice in 60 sec interval with Milli-Q water.
d. The gel was immersed in chilled silver nitrate solution for 25 min and washed two times for 60 sec with Milli-Q water.
e. The gel was transferred to developer solution and kept until protein bands were developed.
f. Reaction was arrested by adding stop solution.

12. Protein Markers for SDS-PAGE

a. Wide range Sigma-Aldrich marker was used for ion exchange fractions of *P. floridanus*. Lyophilized marker reconstituted with 100 µL of deionized water resulted in a solution containing 2-3.5 mg of protein per mL of 62 mM Tris-HCl, pH 6.8, 1 mM EDTA, 4% sucrose, 0.5% dithiothreitol, 2% SDS, and 0.0005% bromophenol blue.

Components		**MW (M_r) in Da**
Aprotinin	-	6,500
α-Lactalbumin	-	14,200
Trypsin inhibitor	-	20,000
Trypsinogen	-	24,000
Carbonic anhydrase	-	29,000
Glyceraldehyde-3-phosphate dehydrogenase	-	36,000
Ovalbumin	-	45,000
Glutamic dehydrogenase	-	55,000
Albumin, bovine serum	-	66,000
Phosphorylase B	-	97,000
β-Galactosidase	-	116,000
Myosin	-	205,000

b. Low range molecular weight marker mix of Bio-Rad was used for the fractions obtained after affinity chromatography and gel filtration chromatography. Lyophilized marker mix was reconstituted in 1X sample buffer for reductive SDS-PAGE, heated for 5 min at 95^{o}C, and 5 µL of marker was loaded on to the gel. The composition of the marker mix is as given below.

Components		**MW (M_r) in Da**
Phosphorylase b	-	97,000
Bovine Serum Albumin	-	66,000
Ovalbumin	-	45,000
Carbonic anhydrase	-	29,000

Trypsin inhibitor	-	20,100
α-Lactalbumin	-	14,400

3.5.1.1 Native polyacrylamide gel electrophoresis

3.5.1.1.1 Gel preparation

Resolving gel (10%)

Stock acrylamide: bis-acrylamide	-	3.3 mL
Resolving gel buffer stock	-	2.5 mL
Water	-	4.2 mL
Ammonium persulphate (APS)	-	75 µL
TEMED	-	5 µL

Stacking Gel (4%)

Stock acrylamide: bis-acrylamide	-	1.34 mL
Stacking gel buffer stock	-	2.5 mL
Water	-	6 mL
Ammonium persulphate (APS)	-	50 µL
TEMED	-	10 µL

Sample preparation

Samples were prepared in 1X sample buffer up to a concentration of 25 µg and loaded 25 µL sample to the gel.

Procedure

a. The gel plates were cleaned and assembled.
b. **Resolving gel –** Prepared resolving gel solution combining all reagents except APS and TEMED in a beaker. Degassed the solution, added APS and TEMED. Immediately poured the mixture into the cast and poured a layer of water over the gel and allowed to polymerize at least for 45 min.
c. **Stacking gel** - Added the components of stacking gel except APS and TEMED into a beaker, mixed gently and finally added APS and TEMED.

Poured the contents into the cast above the resolving gel and immediately inserted the comb between the glass plates. Allowed to polymerize for at least for 45 min.

d. Gel was placed in the electrophoresis apparatus, and reservoir was filled with running buffer for Native-PAGE.
e. Protein samples were loaded to the gel.
f. The gel was run at 80 V till the sample entered the resolving gel.
g. When the dye front entered the resolving gel, increased the current to 120 V.
h. The run was stopped when the dye front reached 1 cm above the lower end of the glass plate.
i. Removed the gel from cast and stained for at least 1 h in the staining solution.
j. Destained the gel till the bands became clear and observed the protein bands under a transilluminator.

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

The purified inhibitor protein was subjected to SDS-PAGE for evaluating the nature of polypeptide. Low range molecular weight marker of Bio-Rad or broad range marker from sigma was used as standard molecular weight markers.

Reductive SDS–PAGE gel preparation

Resolving gel (16%)

Stock acrylamide: bis-acrylamide	-	5.333 mL
Resolving gel buffer stock	-	2.5 mL
10% SDS	-	0.1 mL
Water	-	1.987 mL
Ammonium persulphate (APS)	-	75 µL
TEMED	-	15 µL

Stacking Gel (5%)		
Stock acrylamide: bis-acrylamide	-	1.665 mL
Stacking gel buffer stock	-	2.5 mL
10% SDS	-	0.1 mL
Water	-	5.6675 mL
Ammonium persulphate (APS)	-	50 µL
TEMED	-	15 µL

Sample preparation

Prepared samples in 1X SDS-PAGE sample buffer to a concentration of 25 µg, mixed well, boiled for 5 min 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-PAGE was essentially the same as that of Native-PAGE which was described under section 3.5.1.1 with the exception that SDS was incorporated in gel preparation and running buffer used was that of SDS-PAGE. The gels were either coomassie or silver stained.

3.5.2 Mass by MALDI-TOF

The purified protease inhibitor from gel filtration chromatography was subjected to intact mass analysis. The protease inhibitor sample was desalted with ZipTip-C18 (Millipore, Billerica; MA). The intact molecular mass of the purified inhibitor was determined by MALDI TOF/TOF (ABI 4800).

3.5.3 Isoelectric focusing

Isoelectric point (pI) of the purified inhibitor protein was determined by isoelectric focusing which was performed in a Bio-Rad isoelectric focusing unit.

Immobilized pH gradient (IPG) strip of pH 3-10 (Bio-Rad) was used for the purpose. The detailed procedure is as given below.

3.5.3.1 Rehydration of sample with IPG strip

a. The lyophilized protease inhibitor sample (0.1 mg/mL) was prepared in 2 mL rehydration buffer.
b. Loaded 125 µL of this prepared sample to the equilibration tray.
c. Immobilized pH gradient (IPG) strip of pH 3-10 (Bio-Rad) was gently placed gel side down in the equilibration tray. Removed air bubbles if any. (Two IPG strips were used, one for isoelectric focusing and the other for 2-D electrophoresis).
d. The strips were overlaid with 2-3 mL of mineral oil to prevent evaporation during rehydration process.
e. Covered the equilibration tray and left the tray for overnight to rehydrate IPG strips.

3.5.3.2 Isoelectric focusing of the inhibitor

a. Paper wicks were placed at both ends of the clean, dry IEF focusing tray covering the wire electrodes.
b. IPG strips were taken out from the rehydration tray and drained the mineral oil by holding the strip vertically for 8 sec.
c. Paper wicks were made wet with Milli-Q water and placed the IPG strips in the focusing tray. Placed in the PROTEAN IEF cell and closed the cover.
d. Programmed the IEF cell as given below and run the electrophoresis.

	Voltage	Time	Volt-Hours	Ramp
Step 1	250	20 min	---	Linear
Step 2	4000	2 h	---	Linear
Step 3	4000	---	10,000 V-h	Rapid

(Maintained the cell temperature at 20^{o}C with maximum current of 50 µA/strip and no dehydration in all steps).

3.5.3.3 Staining and 2-D electrophoresis

a. IPG strips were removed from focusing tray after electrophoresis. Drained mineral oil and pressed the strip against a wet blotting paper.
b. One of the strips were transferred to coomassie stain tray and kept for 1 h.
c. Destained the gel for 10 min.
d. The other IPG strip was rinsed in a 1X Tris-glycine buffer and placed on the precast 16% SDS-PAGE gel (A portion of the gel was left at the top, according to the dimension of IPG strip during the casting of SDS-PAGE gel).
e. Overlaid molten agarose and allowed to set the agarose.
f. The electrophoresis was carried out at 200 V. The gel was silver stained after electrophoresis.

3.5.4 Activity staining

Inhibitory activity of the purified protease inhibitor was visualized by the method of Uriel and Berges (1968) with slight modification.

a. Native PAGE (10%) was performed (25 mA for 90 min) with 20 µg of purified inhibitor samples. One portion of the gel was coomassie stained.
b. The other portion of the gel was incubated in freshly prepared solution containing trypsin (0.04 mg/mL) in 0.1 M phosphate buffer pH 7.5 at 37°C for 30 min after electrophoresis.

c. The gel was immersed in Fast Blue (Sigma-Aldrich) solution containing *N*-acetyl-DL-phenylalanine-β-naphthyl ester (Sigma-Aldrich) prepared as follows (5 mg of *N*-acetyl-DL-phenylalanine-β-naphthyl ester was dissolved in 2 mL dimethyl formamide and made up to 20 mL with 0.05 M phosphate buffer, pH 7.4 and added 10 mg of Fast Blue).
d. Incubated for 60 min at 28^{o}C and washed the gel in 2% acetic acid for 30 min.

3.5.5 Analysis of protease inhibitor by Dot-Blot method

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

a. Three microlitre of protease inhibitor (0.1 mg/mL) was mixed with 3μL trypsin (0.1 mg/mL) and spotted on to a strip of X-ray film.
b. Three microlitre of Soya bean trypsin inhibitor (0.1 mg/mL) was mixed with 3 μL trypsin (0.1 mg/mL) as positive control and spotted on to a strip of X-ray film.
c. Three microlitre of trypsin was mixed with 3 μL phosphate buffer 0.01 M (pH 7.5) as negative control and spotted on to the X-ray film.
d. Incubated the X-ray film at 37°C for 10 min.
e. Washed the film under tap water till the zone of gelatin hydrolysis by trypsin was visualized.
f. Inference was made by observing the zone of hydrolysis indicating degradation of gelatin by trypsin. In the absence of the inhibitor a clear zone is formed at the site of trypsin application on the X-ray film due to gelatinase activity. Whereas, trypsin cannot degrade gelatin on the X-ray film in the presence of the inhibitor.

3.5.6 Continuous rate spectrophotometric assay

The activity of protease inhibitor was determined by a continuous rate spectrophotometric assay and expressed as the inhibition of BAEE (*N*α-Benzoyl-L-Arginine Ethyl Ester) units. (Unit Definition: One BAEE unit will produce a ΔA_{253} of 0.001 per min at pH 7.5 and 25 °C using BAEE as a substrate).

a. Mixed 50 µL trypsin (0.1 mg/mL) with 50 µL purified protease inhibitor (0.15 mg/mL) and made up to 200 µL with 63 mM phosphate buffer, pH 7.5. Transferred the contents to a cuvette which was 25^{o}C thermostatted in a UV-Vis spectrophotometer (Schimadzu, Japan).
b. Added 1 mL 0.23 mM *N*α-benzoyl-L-arginine ethyl ester (BAEE), mixed by inversion and recorded the increase in ΔA_{253} for 5 min.
c. ΔA_{253}/min was obtained using the maximum linear rate for the test, blank and uninhibited solution.

The activity was calculated by the equation

$$\text{BAEE Units} = \frac{(\Delta A_{253}/\text{minute Test} - \Delta A_{253}/\text{minute Blank})(df)}{(0.001)}$$

3.5.7 Qualitative detection of inhibition by HPLC

Casein hydrolysis by trypsin, with inhibitor and without inhibitor was analyzed using reverse phase HPLC. Twenty-five microlitre of trypsin (0.1 mg/mL) was preincubated with 25 µL of protease inhibitor (0.1mg/mL) at 37°C for 15 min and made up to 500 µL with 0.01 M phosphate buffer pH 7.5. To this mixture 100 µL of 1% Hammerstein casein (SRL, India) prepared in 0.1 M phosphate buffer was added and incubated at 37°C for 30 min. The reaction was terminated by the addition of equal volume of 0.44 M trichloroacetic acid (TCA) solution. The reaction mixture was centrifuged (Sigma, Germany) at 10,000 rpm

for 15 min. Twenty microlitre of the sample was loaded to C18 column and the experiment was performed as described under section 3.4.7.

3.5.8 Effect of temperature on inhibitor stability

Thermal stability of inhibitor was studied by incubating purified protease inhibitor (0.1 mg/mL) at different temperatures ranging from 4°C-100^{o}C for 60 min. The sample was drawn and further incubated at 4°C for 15 min. The protease inhibitory activity of each sample was assessed by conducting the assay as described under section 3.1.3.1.2

3.5.9 Stability of protease inhibitor at different pH

The stability of protease inhibitor over a range of pH was determined by performing the inhibitor activity assay at pH 7.5, after incubating the purified protease inhibitor in different buffers of pH ranging from 2-12 for 4 h at 4°C. Ten microlitre (0.1 mg/mL) of purified inhibitor was incubated with 40 µL of different buffer systems, which included KCl-HCl buffer (pH 2), citrate buffer (pH 4-6), phosphate buffer (pH 7), Tris-HCl buffer (pH 8-9), borax/NaOH (pH 10), disodium hydrogen phosphate/ NaOH (pH 11) and potassium chloride/NaOH (pH 12). After incubation sample was assayed for protease inhibitory activity as described under section 3.1.3.1.2.

3.5.10 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 1 mM concentrations of various metals ions in the inhibitor solution for 30 min followed by measuring the protease inhibitory activity as described under section 3.1.3.1.2. The metals studied included sodium chloride, calcium chloride, magnesium sulphate, ferric chloride, manganese chloride, nickel chloride, mercury chloride, barium chloride, cadmium sulphate, sodium molybdate and aluminum sulphate which contributes

the metal ions, Na^{+}, Ca^{2+}, Mg^{2+}, Fe^{3+}, Mn^{2+}, Ni^{2+}, Hg^{2+}, Ba^{2+}, Cd^{2+}, Mo^{6+} and Al^{3+} respectively.

3.5.11 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 30 mM EDTA (SRL, India) according to the method described by Jack et al (2004). Purified protease inhibitor (0.1 mg/mL) was dialyzed extensively against 30 mM EDTA over night at 4°C for chelation of metal ions. The EDTA was removed further by dialyzing against deionised water over night with frequent changes of deionised water. The inhibitory activity of the demetallised protease inhibitor was determined by conducting protease inhibitor assay as described in section 3.1.3.1.2.

3.5.12 Metal ion concentration of protease inhibitor

Minerals concentrations of protease inhibitor were determined as follows:

I. An aliquot of protease inhibitor was dialyzed extensively against deionised water and was used for mineral analysis by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES).

II. Another aliquot was dialyzed extensively against 30 mM EDTA, overnight at 4°C for chelation of metal ions and then the EDTA was removed by dialyzing against deionised water overnight with frequent changes. The mineral concentration of dialyzed sample was also determined by ICP-AES.

3.5.13 Effect of various detergents on protease inhibitor activity

Effect of various non-ionic and ionic detergents such as Triton X-100, Tween-80, Tween-20, SDS and CTAB (1% each w/v) on protease inhibitory

activity was determined by incubating the protease inhibitor in each detergent for 30 min, dialyzed against 0.01 M phosphate buffer pH 7.5 and estimated the residual inhibitory activity as described under section 3.1.3.1.2.

3.5.14 Effect of oxidizing agents on protease inhibitor activity

Impact of oxidizing agents on the activity of protease inhibitor was studied by incubating the protease inhibitor with hydrogen peroxide (1-2%, v/v) and dimethyl sulfoxide (1-5%, v/v) for 30 min and measuring the residual inhibitory activity as described under section 3.1.3.1.2.

3.5.15 Effect of reducing agents

The effect of reducing agents on the activity of protease inhibitor was studied by incubating the protease inhibitor with 50, 100, 150, 200, 250, 300, 350 μM of β-mercaptoethanol and 20, 40, 60, 80, 100, 120 μM dithiothreitol for 30 min and measuring the residual inhibitory activity as described under section 3.1.3.1.2.

3.5.16 Chemical modification 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 was evaluated by effecting chemical modification in the selected amino acids of the inhibitor molecules. Thus four 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 were carried out using different chemical modifiers under their respective reaction conditions. Hundred microlitre of purified inhibitor (0.1 mg/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 3.1.3.1.2.

Chemical modifier	**Amino acid modified**	**Reaction conditions**	
N-Ethylmaleimide	Cysteine	30°C	0.1 M Tris/HCl buffer (pH-7.0) for 60 min(Colman and Chu, 1970).
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	0.05 M Tris/HCl buffer (pH-7.8) for 120 min (Gold and Farney, 1964).
N- Bromosuccinamide	Tryptophan	30°C	0.01 M Tris/HCl buffer (pH-7.0) for 30 min (Spande and Witkop, 1967).

3.5.17 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 & 0.1 M for 30 min. After incubation, the pH was neutralized with 0.1 M Tris-HCl buffer pH 9. The residual protease inhibitory activity was estimated as described under section 3.1.3.1.2.

3.5.18 Effect of protease treatment on protease inhibitor

Sensitivity of protease inhibitor to higher concentration of trypsin was assessed by incubating the purified protease inhibitor with different concentrations of trypsin (from Bovine pancreas, SRL, India) ranging from 0.02, 0.04, 0.06, 0.08 and 0.1% for 30 min at 37°C. The residual protease inhibitory activity was estimated as described under section 3.1.3.1.2.

3.5.19 Intrinsic fluorescence spectroscopy

Fluorimetry was performed on a Cary Eclipse spectrofluorimeter using a slit width of 5 mm and an excitation wavelength of 278 nm. The emission wavelength was observed from 290 nm. Phosphate buffer, pH 7.5, served as control. Protease inhibitor having an A_{280} value, 0.1 was used for the emission spectra. The emission spectra for inhibitor and trypsin alone were also performed. Trypsin was complexed with inhibitor in a ratio of 1: 1 concentration.

3.5.20 Peptide mass fingerprinting

Protease inhibitor was reduced, alkylated with iodoacetamide and trypsin digested. Peptides were extracted according to standard techniques and were analyzed by MALDI-TOF-TOF mass spectrometer using a 4800 Proteomics Analyzer (Applied Biosystems). Spectra were analyzed to identify protein of interest using Mascot sequence matching software (Matrix Science) with Swiss-Prot and NCBInr database.

3.5.21 Specificity of protease inhibitor

The specificity of the purified inhibitor was studied against commercially important proteases like chymotrypsin (Sigma-Aldrich), elastase (Sigma-Aldrich), proteinase K (Sigma-Aldrich) and thermolysin (Sigma-Aldrich).

3.5.21.1 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 0.001 M HCl at a concentration of 1 mg/mL. Standard assay mixture contained 0.05 M Tris-HCl buffer (pH 7.5), 20 mM peptide substrate, *N*-Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide, 0.1 mg/mL inhibitor solution and chymotrypsin (10 µg/mL).

One unit of chymotrypsin is defined as the amount of enzyme that induces the conversion of 1 µmol substrate/min. One chymotrypsin inhibitory unit (U) was defined as the amount of inhibitor needed to inhibit the release of 1 µmol of *p*-nitroaniline per mL per min at pH 7.5 and at 37^{o}C.

3.5.21.2 Assay of elastase, thermolysin and proteinase K inhibitory activity

The elastase, thermolysin and proteinase K inhibitory activity was tested taking 0.1 mg/mL of respective proteases with 0.1 mg/mL purified protease inhibitor according to Kunitz caseinolytic method as described under section 3.1.3.1.1.

3.5.22 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 100 µL of 0.01 M phosphate buffer pH 7.5 with different amounts of purified protease inhibitor (0.05-1 nM) at 37°C for 30 min (based on M_r 38,334). The long incubation time was necessary to ensure that the reaction had gone to completion. The remaining activity of the trypsin was determined according to the method described under section 3.1.3.1.2.

3.5.23 Kinetic studies of inhibition

Kinetics of trypsin inhibition by protease inhibitor was conducted with different concentrations of inhibitor [I] against various concentrations of substrate [S]. Lineweaver-Burk 1/v versus 1/ [s] was plotted, the apparent *K*m (*K*m') and maximum velocity (V_{max}) were calculated for each concentration of inhibitor and secondary plot was plotted by taking 1/*V*max versus [I] to determine dissociation constant of the inhibitor (*K*i).

a. Twenty-five microlitre of trypsin (0.1 mg/mL) was incubated with different concentrations of substrate (0.1 mM-2 mM) α-*N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) suitably diluted to 500 µL with 0.01 M phosphate buffer, pH 7.5 and kept at 37°C for 10 min. The reaction was arrested by adding 500 µL of 30% (v/v) acetic acid.

b. Lineweaver-Burk 1/v versus 1/ [s] was plotted, the *K*m and maximum velocity (V_{max}) were calculated (Dixon, 1953).

c. The apparent *K*m' and maximum velocity (V_{max}) in the presence of different purified protease inhibitor concentrations (0.014, 0.07 and 0.27 nM) was calculated similarly.

d. A secondary plot was plotted by taking 1/*V*max versus [I] to determine dissociation constant of the inhibitor (*K*i).

3.6 APPLICATION STUDIES

3.6.1 Inhibition of midgut proteases

The lime swallowtail *Papilio demoleus* (Lepidoptera: Papilionidae) larvae were collected from the citrus leaves from Kakkandu, Ernakulam. Midguts from

the larvae were extruded out and transferred into a microfuge tube kept on ice at 4^{o}C containing 0.01 M phosphate buffer, pH 7.5. The midguts were homogenized at 4^{o}C and then centrifuged at 12000 g for 10 min at 4^{o}C. The supernatants were used as the crude enzyme (trypsin) extract and were stored at -20^{o}C. Protease inhibitory activity and protein content and specific activity were found as described under sections 3.1.3.1.2, 3.1.3.2 and 3.1.3.2 respectively.

3.6.2 Evaluation of protease inhibitor activity against proteases derived from different microbial sources

The activities of purified inhibitor (~150 µg/mL) against proteases derived from different microbial sources were evaluated. Proteases obtained from *Aspergillus oryzae, Bacillus licheniformis, Bacillus* sp., esperase, *Bacillus amyloliquefaciens* were used for the study (All the proteases are from Sigma-Aldrich). A concentration of 0.1 mg/mL proteases was used and the assay was performed as described under section 3.1.3.1.1. For easy computation and understanding, the protease inhibitor activity of commercial proteases was expressed in terms of percent inhibition.

4 Results

4.1 SCREENING OF MUSHROOMS FOR PROTEASE INHIBITOR

Mushrooms, *Pleurotus floridanus*, *Agaricus bisporus* obtained from Kerala Agriculture University, Trichur, India and the wild mushroom *Phellinus* sp. from Cochin University of Science and Technology, Cochin-22 premises were screened for protease inhibitory activity using their crude extracts. Results presented in Table 4.1 show that *Pleurotus floridanus* has maximum inhibitory activity (512.92 u/mL) compared to *Phellinus* sp. (252 u/mL) and *Agaricus bisporous* (46.4 u/mL).

Table 4.1 Screening of mushrooms for protease inhibitor

Serial No.	Mushroom Extract	Inhibitory activity (u/mL)
1	*Agaricus bisporous*	46.4
2	*Pleurotus floridanus*	512.92
3	*Phellinus* sp.	252

4.1.1 SELECTION OF POTENTIAL SOURCE AND ISOLATION OF PROTEASE INHIBITOR FROM MUSHROOM

Mushrooms *P. floridanus* and *Phellinus* sp. which showed maximal protease inhibitory activities were subjected to further screening. Crude sample prepared from these mushrooms were subjected to partial purification by ammonium sulphate precipitation and evaluated for their inhibitory activity. Among the mushrooms *P. floridanus* showed maximal protease inhibition compared to *Phellinus* sp. *P. floridanus* showed a protease inhibitory activity of 581.24 u/mL with a specific protease inhibitory activity of 145.31 u/mg protein. In contrast, *Phellinus* sp. showed a protease inhibitory activity of 384.66 u/mL with a specific protease inhibitory activity of 113.95 u/mg protein (Table 4.2). Since the protease inhibitor from *P. floridanus* recorded maximum protease inhibitory activity, it was selected for the isolation of protease inhibitor and further studies.

Table 4.2 Protease inhibition of ammonium sulphate precipitated fraction of mushrooms

Serial No.	Extract	Saturation of ammonium sulphate (%)	Protease inhibitory activity (u/mL)	Specific protease inhibitory activity (u/mg protein)
1	*Pleurotus floridanus*	30-60	581.24	145.31
2	*Phellinus* sp.	30-60	384.66	113.95

4.2 IDENTIFICATION OF THE SELECTED MUSHROOM

Partial nucleotide sequences encoding 18S ribosomal RNA gene and internal transcribed spacer 1; 5.8S ribosomal RNA gene and internal transcribed spacer 2 complete sequence; and partial sequence of 28S ribosomal RNA gene, were amplified and resolved at a size of 750 bp on 1.2% agarose gel (Fig. 4.1). The amplicon was sequenced and identity was established using BLAST software. The obtained sequence showed 100% similarity with already available sequences of *P. floridanus* (NCBI GenBank accession No.GU7210580).

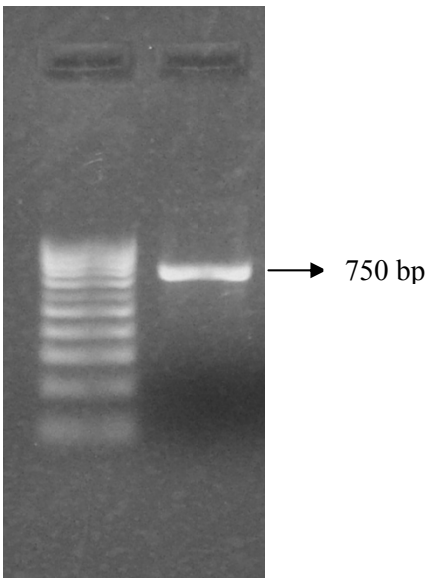

Fig. 4.1a

5’-GCTTCCGTAGGTGAACCTGCGGAAGGATCATTAATGAATTCA
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TTTCAACCACCTGTGAACTTTTGATAGATCTGTGAAGTCGTCTCTCAAG
TCGTCAGACTTGGTTGCTGGGATTTAAACGTCTCGGTGTGACTACGCAG

TCTATTTACTTACACACCCCAAATGTATGTCTACGAATGTCATTTAATG
GGCCTTGTGCCTTTAAACCATAATACAACTTTCAACAACGGATCTCTTG
GCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAA
TTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCCCCT
TGGTATTCCGAGGGGCATGCCTGTTTGAGTGTCATTAAATTCTCAAACT
CACTTTGGTTTCTTTCCAATTGTGATGTTTGGATTGTTGGGGGCTGCTG
GCCTTGACAGGTCGGCTCCTCTTAAATGCATTAGCAGGACTTCTCATTG
CCTCTGCGCATGATGTGATAATTATCACTCATCAATAGCACGCATGAAT
AGAGTCCAGCTCTCTAATCGTCCGCAAGGACAATTTGACAATTTGACC
TCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATA-3'.

Fig. 4.1b

Fig. 4.1 a) PCR amplicon of 18S rDNA segment. Lanes 1. 100 bp ladder
2. Amplicon. **b)** DNA sequence of amplicon.

4.3 PURIFICATION OF PROTEASE INHIBITOR

Standard purification methods were employed for the purification of protease inhibitor. The inhibitory protein was purified up to homogeneity employing $(NH_4)_2SO_4$ precipitation, DEAE sepharose chromatography, trypsin affinity chromatography and gel filtration chromatography. The yield and fold of purification of protease inhibitor extracted from the fruiting bodies of the edible mushroom *P. floridanus* is summarized in Table 4.3. It was found that 30-60% $(NH_4)_2SO_4$ saturation was efficient for precipitating the protease inhibitor molecules compared to other fractions. The fold of purification of protease inhibitor obtained for $(NH_4)_2SO_4$ precipitation, DEAE sepharose chromatography, trypsin affinity chromatography and gel filtration were 1.63, 3.61, 21.5 and 35.25 respectively. The net yield of protease inhibitor was 0.5 μg/g of mushroom.

TABLE 4.3 Yield of protein, Yield of protease inhibitor activity and Fold of purification in comparison with crude extract.

Purification step	Volume (mL)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield of protein (%)	Yield of activity (%)	Fold of purification
Crude Extract	500	2342	7450	3.18	100*	100*	1*
$(NH_4)_2SO_4$	30	94.92	493	5.19	21.05	6.617	1.63
DEAE Sepharose	1.2	1.836	21.1	11.49	0.0784	0.283	3.61
Trypsin affinity	0.6	0.202	13.83	68.47	0.0086	0.186	21.5
Gel Filtration	0.45	0.1	11.21	112.1	0.00427	0.15	35.25

*Values taken arbitrarily

4.3.1 Ion exchange chromatography

The dialysate obtained after ammonium sulphate saturation (30-60% fraction) was subjected to ion exchange chromatography using DEAE sepharose. Five millilitre (15 mg) of ammonium sulphate (30- 60%) fraction was loaded to DEAE sepharose column (25 X 1.5 cm). Peak fractions were pooled and concentrated using Amicon 10 kDa membrane. Inhibitory activity was shown by the peak V with 3.6 fold of purification (Fig. 4.2).

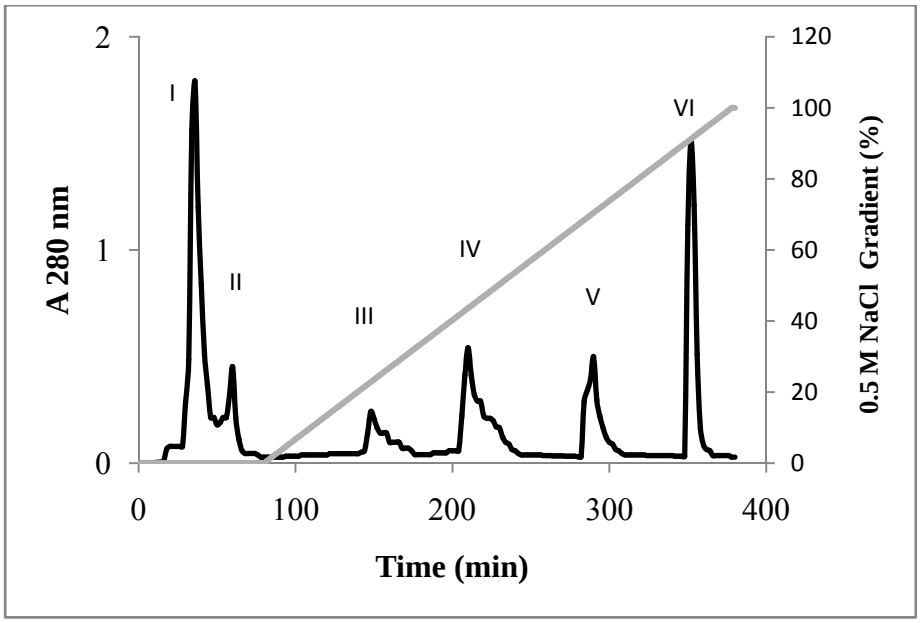

Fig. 4.2a

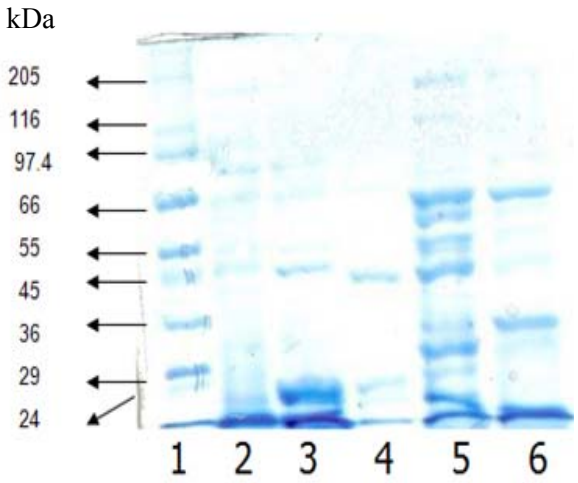

Fig. 4.2b

Fig. 4.2 a) Elution profile of protease inhibitor from DEAE Sepharose anion exchange chromatography column (25 X 1.5 cm) using NaCl gradient. **b)** SDS-PAGE profile of the peaks. Lanes 1. Molecular weight marker, 2. Peak I, 3. Peak II, 4. Peak III, 5. Peak 4, 6. Peak V.

4.3.2 Affinity chromatography

Active ion exchange chromatographic fraction (Peak V) which showed maximum inhibitory activity against trypsin was used for trypsin affinity chromatography. Three milligram of active ion exchange fraction was loaded on to trypsin affinity column (15 X 1 cm). The bound inhibitor from affinity column was eluted using 0.5 M NaCl in 0.01 M HCl as a single peak which resulted in 21.5 fold of purification with a specific protease inhibitory activity of 68.47 U/mg protein (Fig. 4.3).

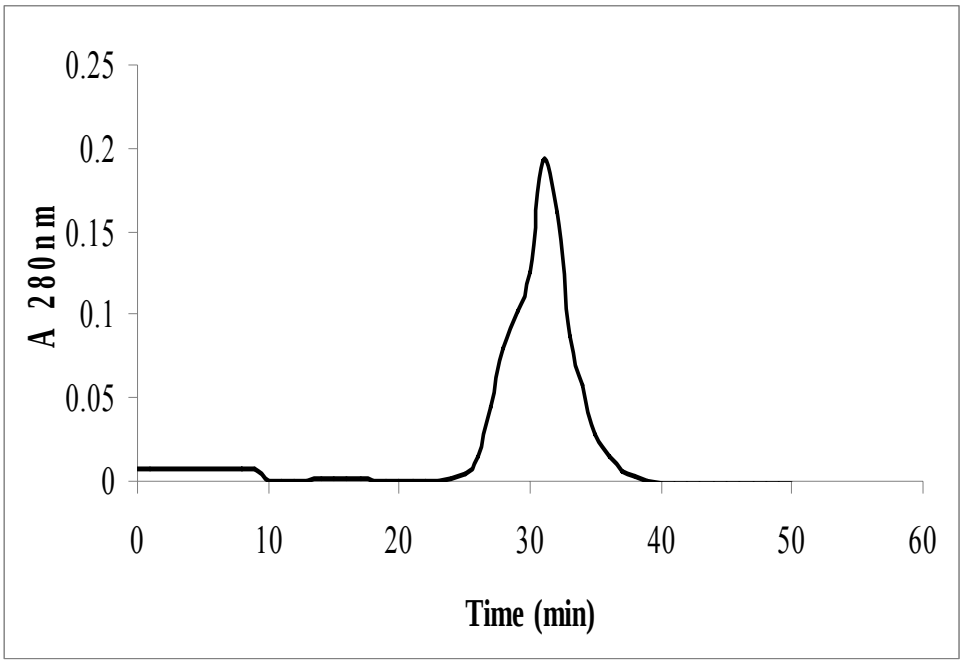

Fig. 4.3a

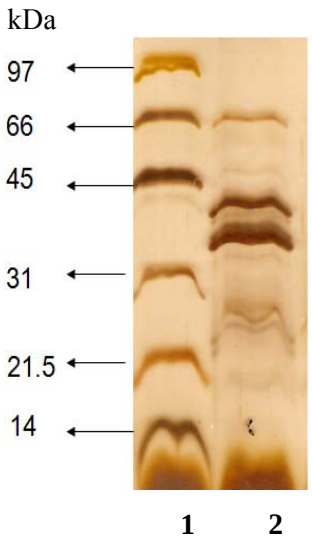

Fig. 4.3b

Fig. 4.3 Affinity chromatography of protease inhibitor. **a)** Elution profile of inhibitor from trypsin affinity chromatography column (15 X 1 cm). **b)** SDS-PAGE analysis of the affinity purified inhibitor. Lane 1. Molecular weight marker. 2. Affinity purified inhibitor.

4.3.3 Gel filtration chromatography

The inhibitory fraction obtained from affinity chromatography yielded multiple bands in SDS-PAGE, and a homogenous inhibitory protein was obtained by Sephadex-G100 gel filtration chromatography (peak V). The fraction with maximum protease inhibitory activity was visualized as a single protein band confirming its purity and homogeneity (Fig. 4.4). Gel filtration resulted in an increase in purification fold up to 35.25 with a specific protease inhibitory activity of 112.1 U/mg protein.

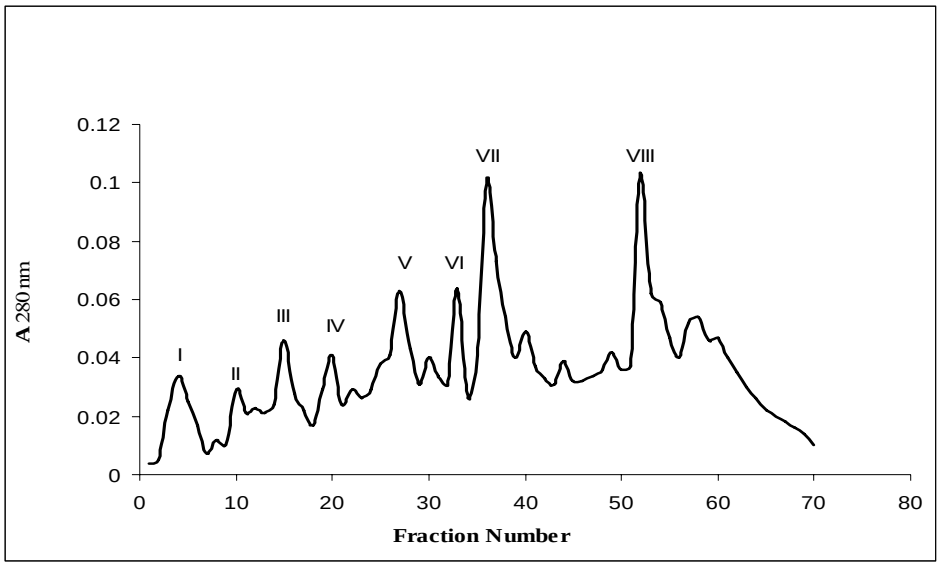

Fig. 4.4a

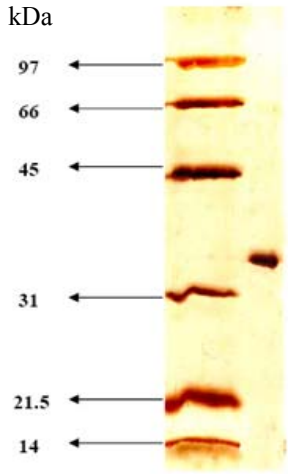

Fig. 4.4b

Fig. 4.4 Gel filtration chromatography of the inhibitor. **a)** Elution profile of the inhibitor **b)** SDS-PAGE analysis of the inhibitor. Lane 1. Molecular weight marker. 2. Purified protease inhibitor.

4.3.4 HPLC profile of the inhibitor

Gel filtration purified active fraction (20 µL, 0.1 mg/mL) was rechromatographed in a reversed phase HPLC system using Phenomenex C18 column. A single homogenous peak was observed in the chromatogram, eluted with a linear gradient (0–100%) of solvent (0.09% TFA in 60% acetonitrile) over 60 min (Fig. 4.5).

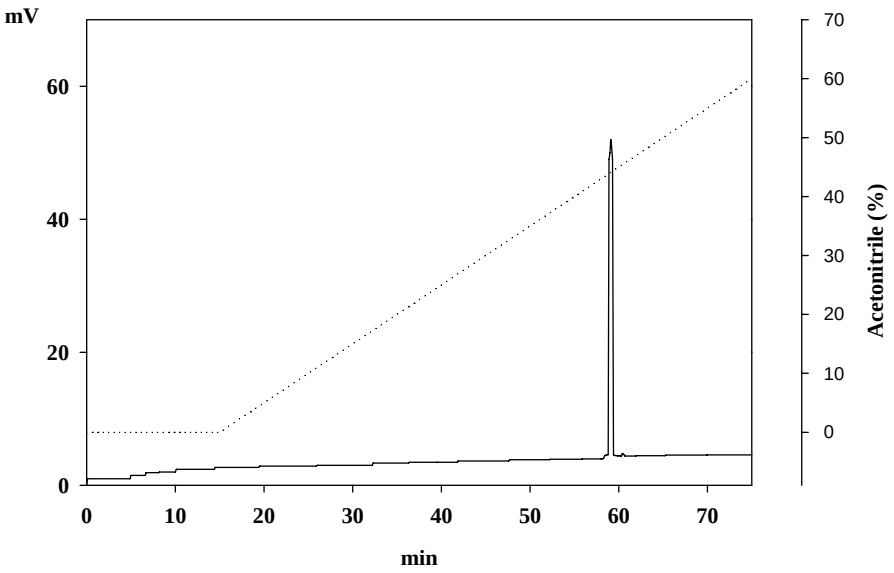

Fig. 4.5 Reverse phase HPLC profile of the purified inhibitor

4.4 CHARACTERIZATION OF THE INHIBITOR

4.4.1 Molecular mass determination by MALDI-TOF

The purified inhibitor sample was desalted with ZipTip -C18, performed MALDI TOF/TOF and intact molecular mass was determined. A single peak with molecular weight of 38334 Da was obtained (Fig. 4.6)

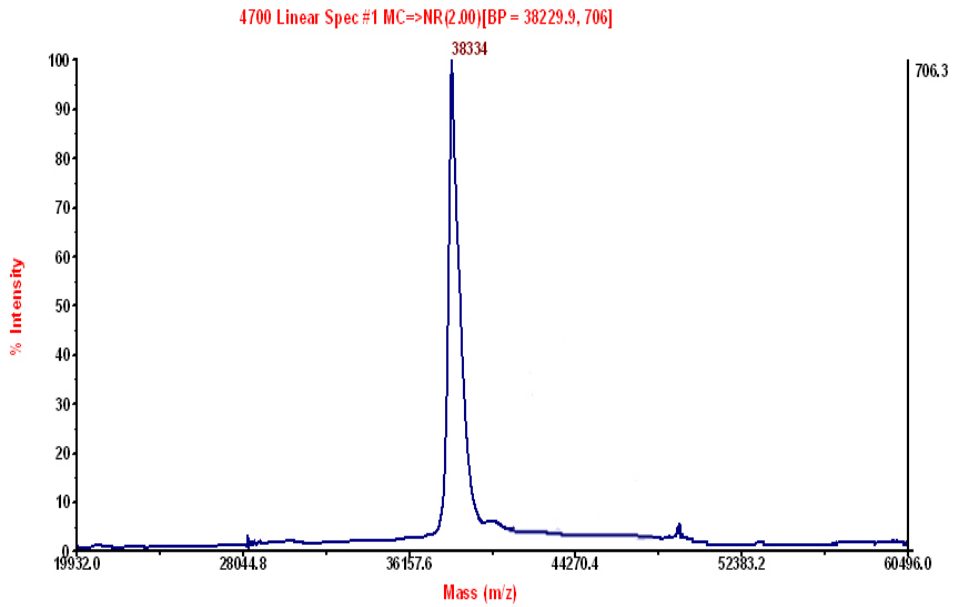

Fig. 4.6 Mass of the inhibitor by MALDI TOF/TOF

4.4.2 Isoelectric focusing and 2D electrophoresis

Isoelectric point (pI) of the purified inhibitor protein was determined using an immobilized pH gradient (IPG) strip of pH 3-10. The inhibitor was resolved at a pH of 4.4 (Fig. 4.7a). Another strip was subjected to 2D electrophoresis to check

the presence of multiple proteins with same pI. The result depicted in Fig. 4.7b showed a single band after silver staining.

Fig. 4.7a

Fig. 4.7b

Fig. 4.7 a) Isoelectric focusing on pre-cast IPG strip. The protein band was visible on coomassie stained IPG strip and resolved at pI of 4.4.**b)** 2D gel of the protease inhibitor

4.4.3 Activity staining

Activity staining of the purified protease inhibitor was carried out on Native-PAGE and visualization of a clear band on a coloured background after staining confirmed that the purified protease inhibitor has inhibitory activity (Fig. 4.8). Further the inhibitory protein was found to be homogenous in Native-PAGE.

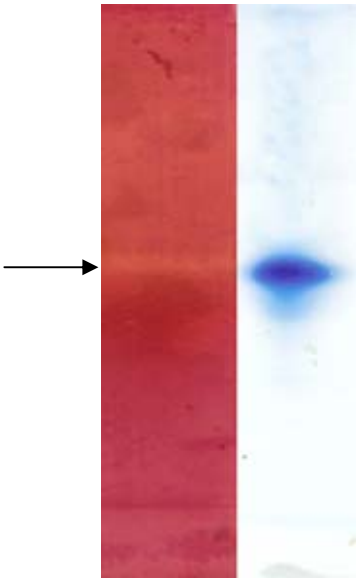

Fig. 4.8 Activity staining of purified protease inhibitor (Uriel and Berges, 1968) in 8% Native-PAGE. Lanes: 1.Activity stained inhibitor, 2. Inhibitor stained with coomassie.

4.4.4 Analysis of protease inhibitor by Dot-Blot method

Dot-blot analysis was performed on X-ray film indicated that the protease inhibitor blocked the gelatin hydrolysis by trypsin similar to that of the control protease inhibitor Soya bean trypsin inhibitor (SBTI). The presence of inhibitor was confirmed by comparing the clearing zone formed due to gelatin hydrolysis

by trypsin and a reduction in the size or absence of clearing zone by trypsin incubated with protease inhibitor (Fig. 4.9).

Fig. 4. 9 Dot-blot analysis of protease inhibitor on X-ray film. 1. Trypsin control 2. Trypsin with Soyabean trypsin inhibitor as positive control 3. Trypsin with purified inhibitor.

4.4.5 Continuous rate spectrophotometric assay

Results obtained for the activity of protease inhibitor by continuous rate spectrophotometric assay is depicted in the Fig. 4.10. The trypsin hydrolysis of the substrate BAEE was found to be decreased gradually over a period of 5 min time in the presence of protease inhibitor. The trypsin activity was 1850 BAEE units, which got decreased to 230 BAEE units in the presence of protease inhibitor.

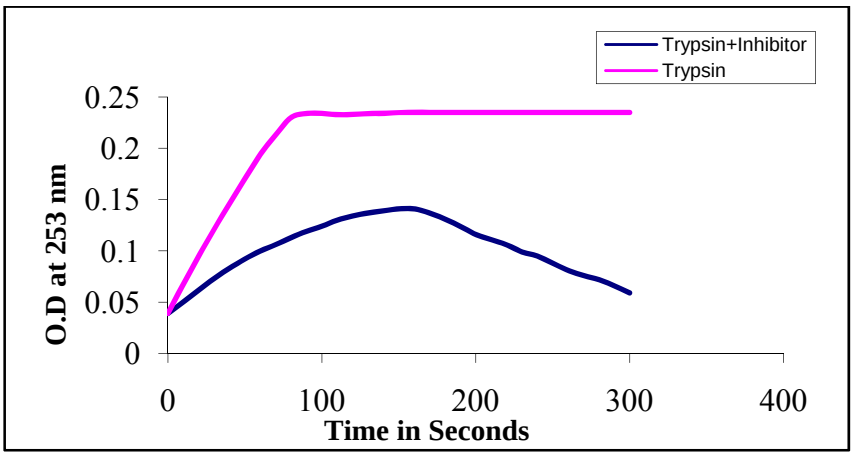

Fig. 4.10 Continuous rate spectrophotometric assay of protease inhibitor

4.4.6 Qualitative detection of inhibition by HPLC

Inhibition of casein hydrolysis by trypsin in the presence of inhibitor and without inhibitor was determined using reverse phase HPLC employing C18 column. The HPLC chromatogram depicted in Fig. 4.11 testified that the number and intensity of peptide peaks are decreased considerably in the presence inhibitor.

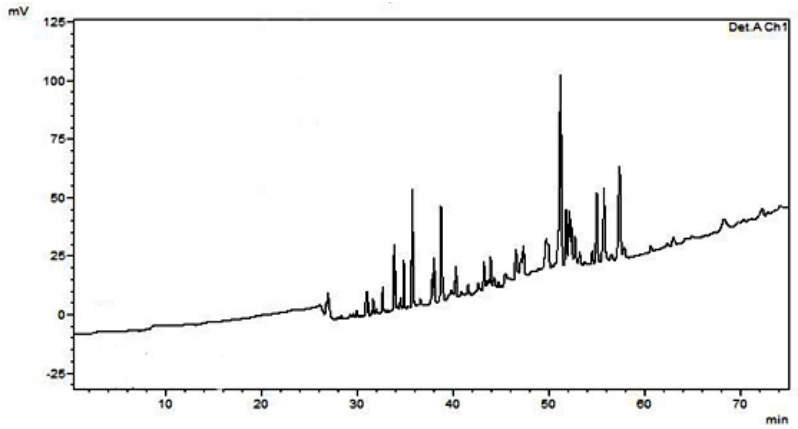

Fig. 4.11a

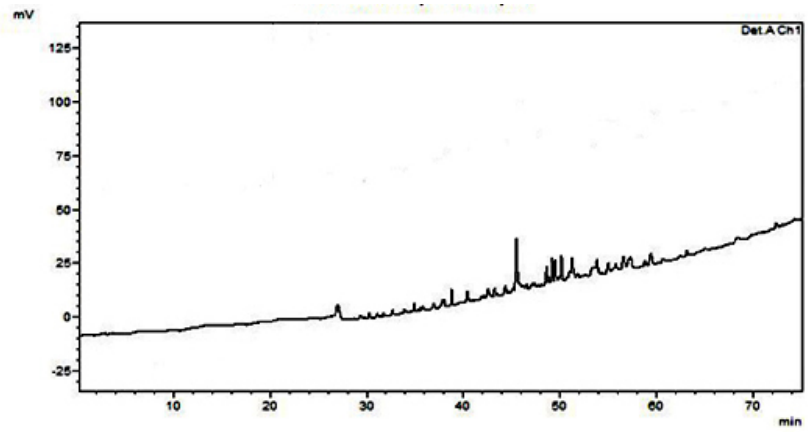

Fig. 4.11b

Fig. 4.11 HPLC profile of the casein hydrolysate **a)** by trypsin. **b)** trypsin in the presence of inhibitor

4.4.7 Effect of temperature

The protease inhibitor was evaluated for its activity and stability at wide range of temperatures varying between 4 to 100^{o}C and the results obtained are presented in the Fig. 4.12. The protease inhibitor was observed to demonstrate considerable stability over a broad range of temperature up to 90^{o}C although maximal activity of the protease inhibitor was observed around 30-40^{o}C. However, the purified inhibitor recorded a gradual decrease in activity after pre-incubation at 50-90°C for 60 min and a complete loss of activity at 100°C.

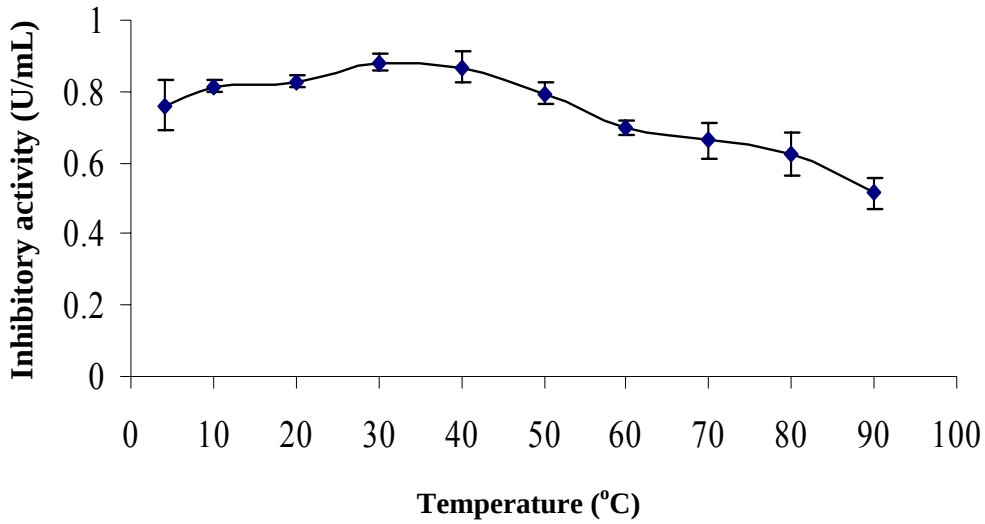

Fig. 4.12 Effect of temperature on activity of the protease inhibitor

4.4.8 Effect of pH

Studies conducted on the effect of pH on stability were evaluated in different buffer systems, showed that the protease inhibitor was stable over a wide range of pH. From the results presented in Fig. 4.13, it was inferred that there was considerable protease inhibitor stability over a pH range of 4-10; although a maximal inhibitory activity was recorded at pH 8. It was also noted that at high alkaline conditions of pH 11-12 and high acidic conditions of pH 2-3 the protease inhibitor was not stable.

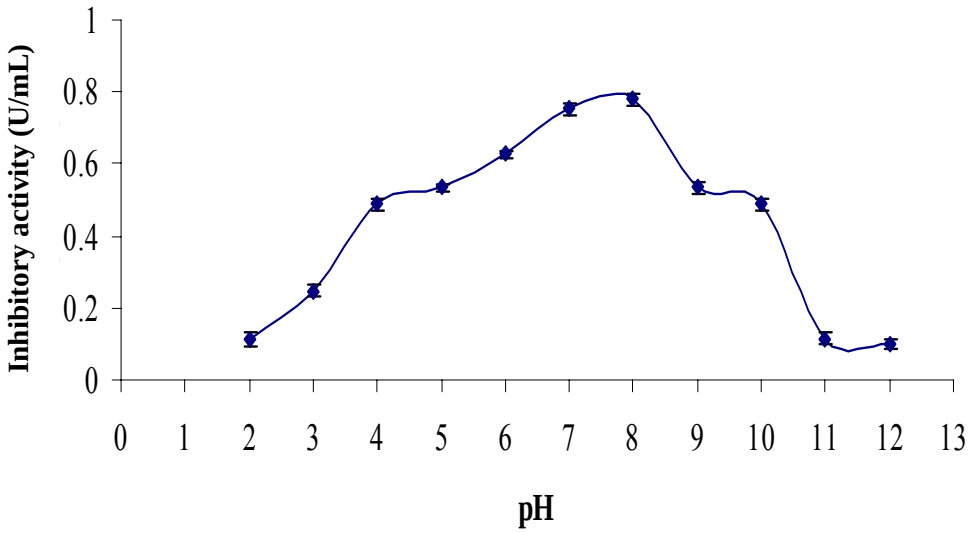

Fig. 4.13 Effect of pH on inhibitory activity of protease inhibitor

4.4.9 Effect of metal ions

Data obtained for the studies on the effect of metal ions such as Ca^{2+}, Mg^{2+}, Mn^{2+}, Hg^{2+}, Na^{2+}, Ba^{2+}, Ni^{2+}, Cd^{2+}, Fe^{3+}, Mo^{6+} and Al^{3+} on the activity of protease inhibitor presented in the Fig. 4.14 evidence that addition of divalent ions such as Mn^{2+}, Na^{2+}, Ba^{2+} and Ni^{2+} at a concentration of 1 mM enhanced the protease inhibitory activity. Further it was observed that 1 mM concentration of Mn^{2+} enhanced the inhibitory activity to 1.45 U compared to that of control (0.833 U). 1 mM concentration of Na^{2+}, Ba^{2+} and Ni^{2+} also led to an increase in protease inhibitory activity reasonably. It was also noted that none of the divalent ions other than Mn^{2+}, Na^{2+}, Ba^{2+} and Ni^{2+} enhanced protease inhibitory activity.

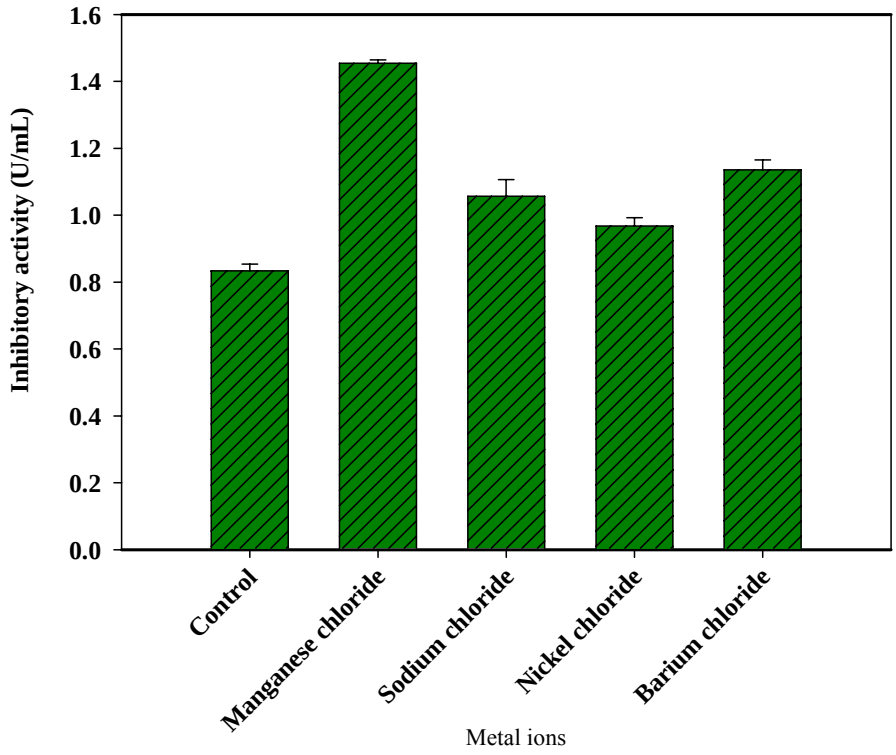

Fig. 4.14 Effect of metal ions on protease inhibitor activity

4.4.10 Metal chelation of protease inhibitor using EDTA

Results obtained for the ICP-AES analysis presented in Table 4.4 confirmed the presence of divalent cations in protease inhibitor. The atomic emission spectrum showed the presence of Ca^{2+}, Mg^{2+} and Zn^{2+} in the protease inhibitor. Protease inhibitor dialyzed against deionised water contained calcium, magnesium and zinc at 20.17, 30.47 and 17.78 ppm respectively. Protease inhibitor dialyzed against EDTA decreased the metal ion concentration significantly. Further, results presented in Fig. 4.15 indicated that metal chelation has no effect on protease inhibitory activity.

Table 4.4 Metal ion concentration of inhibitor

Sample	Concentration in ppm		
	Ca	**Mg**	**Zn**
Inhibitor dialyzed against deionised water	20.17	30.47	17.78
Demetallised inhibitor	4.66	3.08	4.17

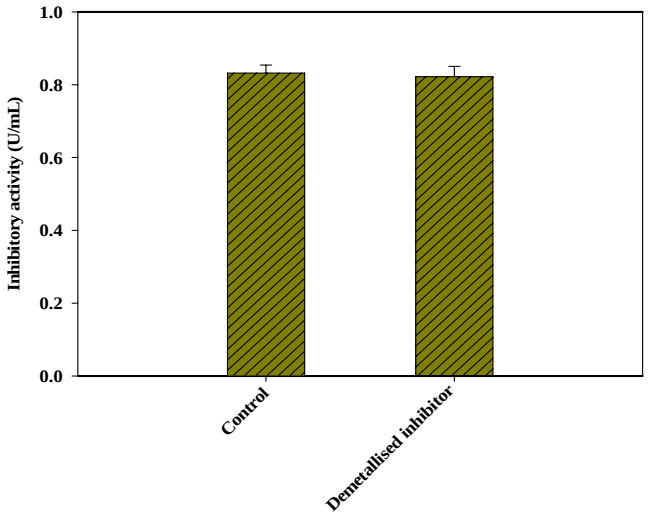

Fig. 4.15 Effect of demetallisation on protease inhibitor

4.4.11 Effect of detergents

The stability of inhibitor in the presence of detergents was investigated and the results obtained are depicted in Fig. 4.16. Nonionic detergents normally are considered as mild detergents and that they do not interact extensively with the protein surface, whereas ionic detergents, in particular SDS, generally bind non-specifically to the protein surface. From the results it could be noted that the anionic detergent SDS and cationic detergent CTAB at a concentration of 1% reduced the inhibitory activity significantly. Whereas, the nonionic detergents Triton X and Tween 80 increased the inhibitory activity more than 2.5 times.

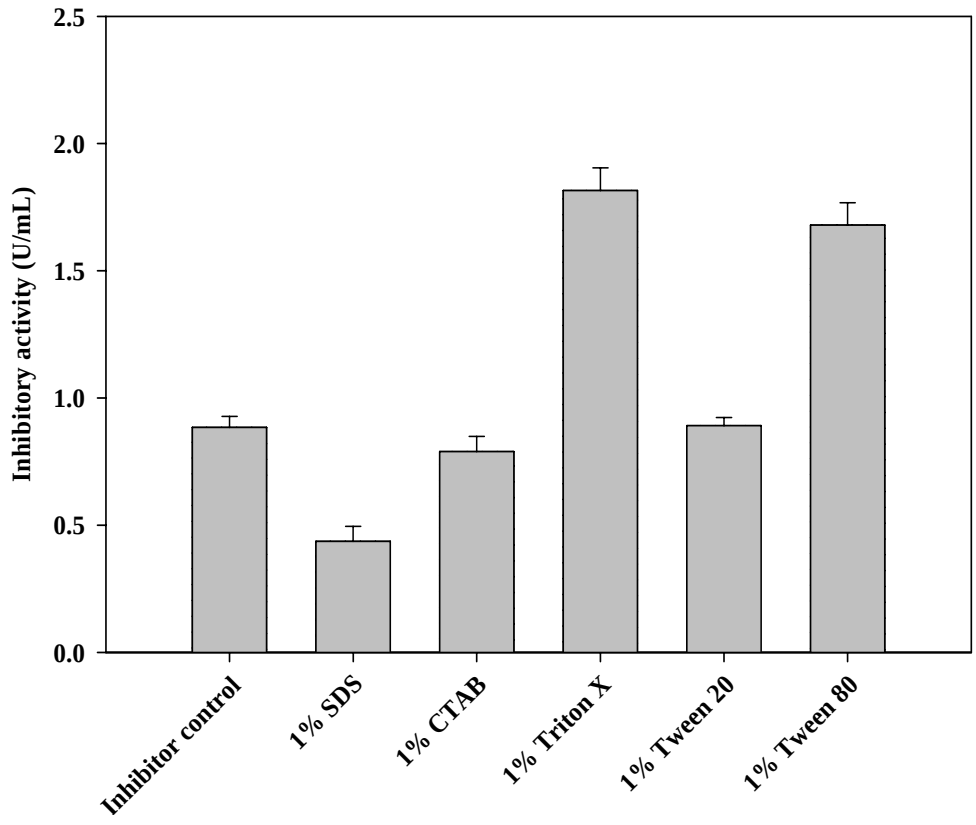

Fig. 4.16 Effect of detergents on protease inhibitory activity

4.4.12 Effect of oxidizing agents

The results depicted in the Fig. 4.17 testify that the protease inhibitory activity decreased along with increase in concentrations of oxidizing agents H_2O_2 and DMSO. It was observed that the protease inhibitory activity gradually decreased along with increase in concentration from 1% to 5% of DMSO and complete inactivation at 6% of DMSO. Whereas, oxidation of protease inhibitor by H_2O_2 was stronger than that of DMSO since complete inactivation of the inhibitor was noted at a concentration above 2% of H_2O_2.

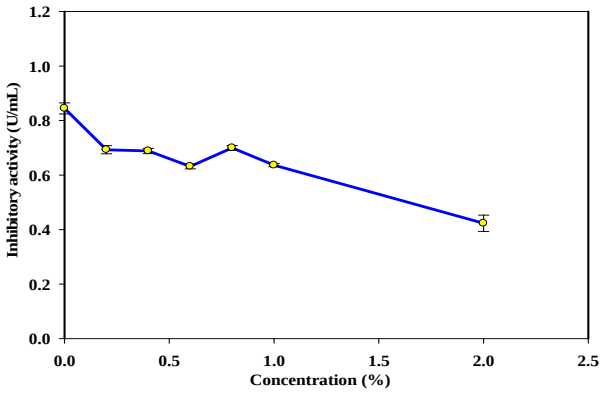

Fig. 4.17a

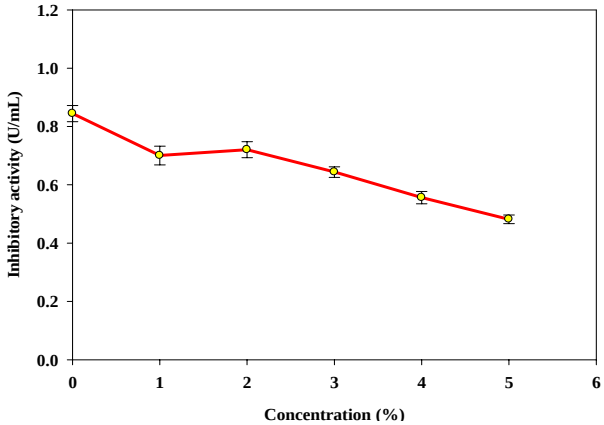

Fig. 4.17b

Fig. 4.17 Effect of oxidizing agents on protease inhibitor. **a)** In the presence of H_2O_2. **b)** In the presence of DMSO

4.4.13 Effect of reducing agents

The effect of reducing agents on protease inhibitory activity was studied using dithiothreitol and β-mercaptoethanol and the results are documented in Fig. 4.18. From the results it was inferred that the activity was increased by dithiothreitol up to a concentration of 80 µM, and a concentration of above 140 µM dithiothreitol resulted in complete inactivation of the inhibitor. Whereas in the case of β-mercaptoethanol inactivation occurred at a concentration of 400 µM when compared to dithiothreitol.

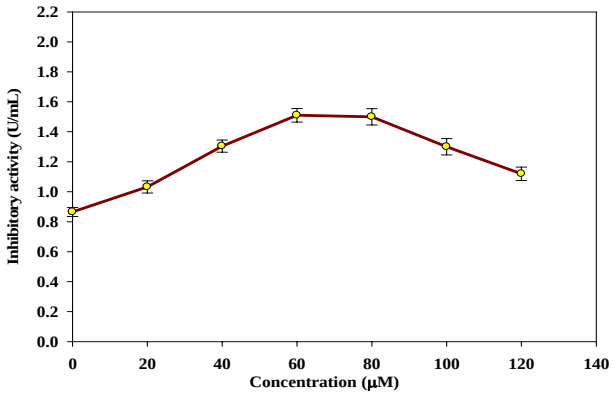

Fig. 4.18a

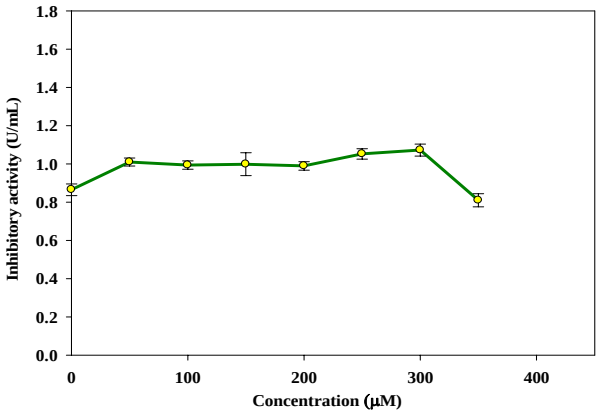

Fig. 4.18b

Fig. 4.18 a) Effect of dithiothreitol on protease inhibitor
b) Effect of β-mercaptoethanol on inhibitor

4.4.14 Chemical modifications of amino acids in protease inhibitor

Amino acids were individually modified using specific chemical modifiers and their effect on protease inhibitory activity was determined and the results are presented in Fig. 4.19. Among the four chemical modifiers used, PMSF inactivated the protease inhibitory activity even at lesser concentration of the modifier. Inhibitory activity of the PMSF modified inhibitor was drastically reduced to 0.234 U/mL at 4 mM concentration of the inhibitor compared to the control activity 0.892 U/mL. Similar results were obtained for modification of cysteine by *N*-Ethylmaleimide at slightly higher concentrations. An abrupt reduction in the inhibitory activity form 0.892 U/mL to 0.345 was observed at 10 mM concentration of *N*- Ethylmaleimide. Modification with 30 mM concentration of the *N*-Ethylmaleimide resulted a further reduction of the inhibitory activity to 0.155 U/mL.

Modification of histidine amino acid residue of the inhibitor with DEPC inactivated the inhibitor at concentrations of above 25 mM. The inhibitory activity declined initially to 0.68 U/mL at 5 mM concentration of the modifier and was found stable up to 20 mM concentration of DEPC. But at 25 mM concentration of DEPC the activity was reduced to 0.4768 U/mL followed by complete inactivation at 30 mM concentration. Modification of tryptophan residue by *N*-Bromosuccinamide had no effect on protease inhibitory activity. The protease inhibitor was modified with 5-25 mM concentration of *N*-Bromosuccinamide and the results of the study indicated that the inhibitory activity was not affected by the modifier.

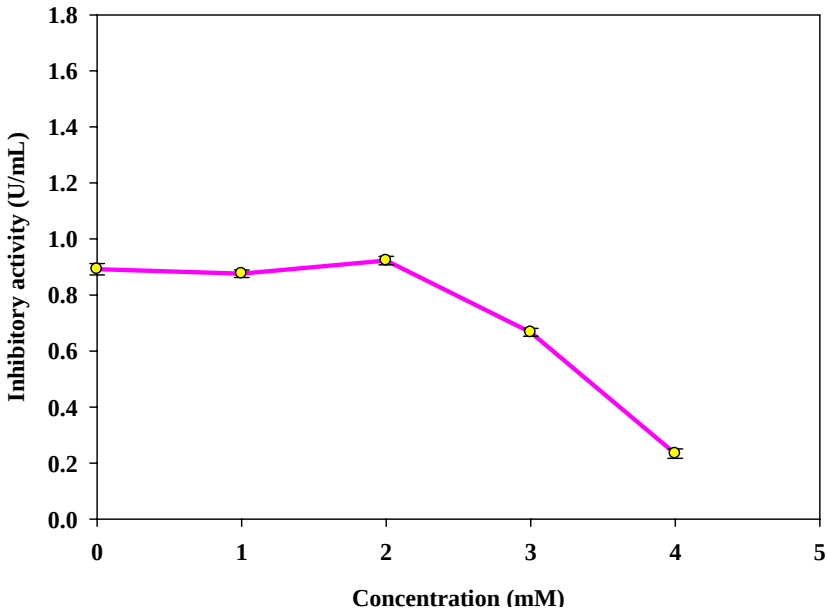

Fig. 4.19a

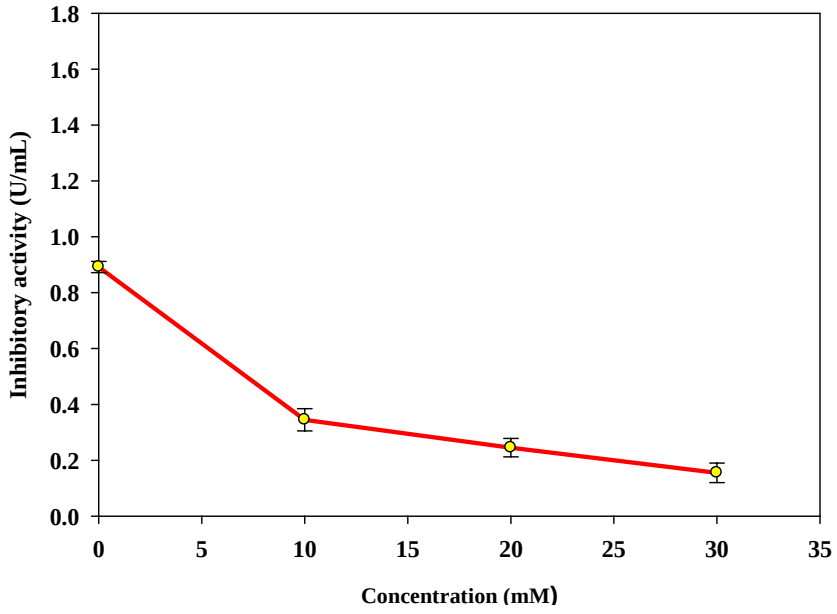

Fig. 4.19b

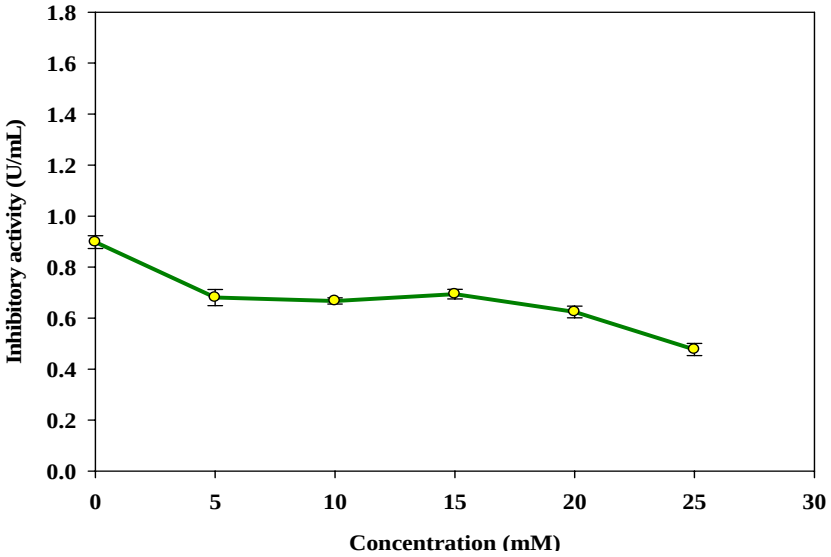

Fig. 4.19c

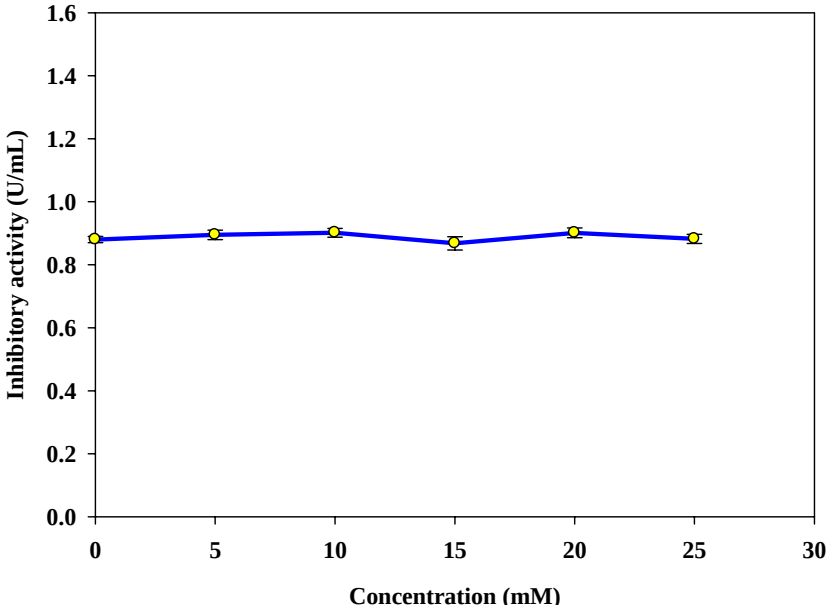

Fig. 4.19d

Fig. 4.19 Effect of chemical modification on protease inhibitory activity by **a)** PMSF. **b)** *N*-Ethylmaleimide. **c)** DEPC. **d)** *N*-Bromosuccinamide

4.4.15 Effect of acid treatment on protease inhibitor

Acid treatment of protease inhibitor showed a gradual decrease in the inhibitory activity along with the increasing concentration of HCl (Fig. 4.20). The inhibitory activity was stable in 0.04 M concentration of HCl (0.75 U/mL). However, a sharp decline in the activity was observed for the concentrations above 0.04 M HCl and complete inactivation was noted at 0.08 M HCl.

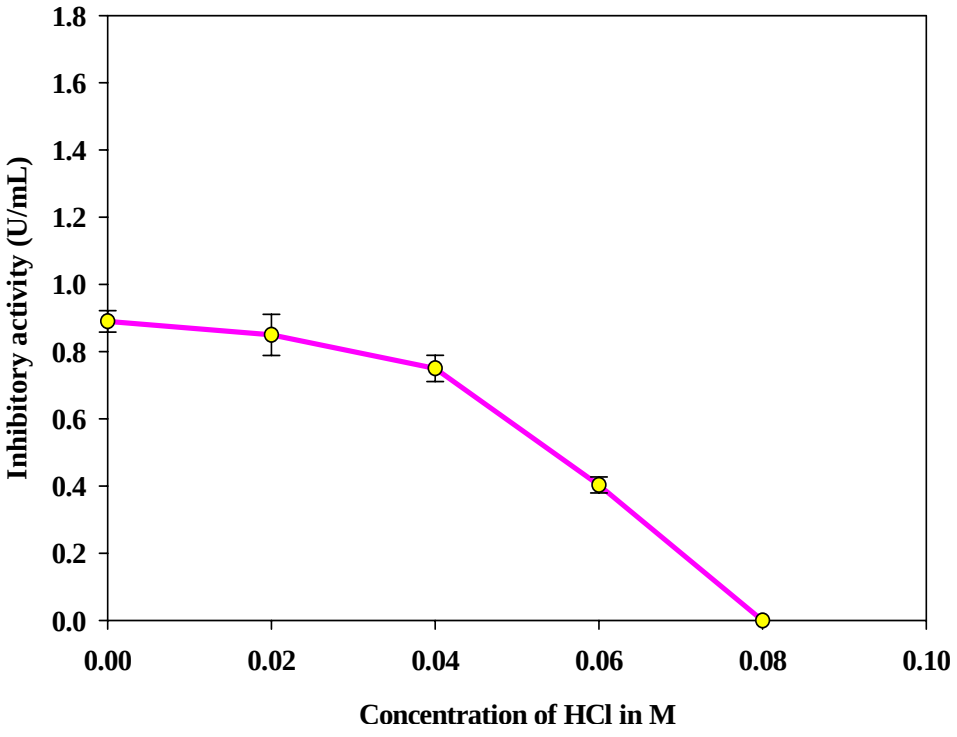

Fig. 4.20 Effect of increasing concentration of HCl on the activity of protease inhibitor

4.4.16 Effect of protease treatment on inhibitor

The result presented in the Fig. 4.21 suggests that pretreatment of inhibitor with increasing concentration of protease trypsin reduced the protease inhibitory activity. It was observed that the activity of the inhibitor was not affected by trypsin up to a concentration of 0.06%. However the protease inhibitory activity was observed to get declined for the concentrations above 0.06% trypsin. The inhibitory activity was reduced to 0.4134 U/mL at 0.1% of trypsin from 0.8943 U/mL.

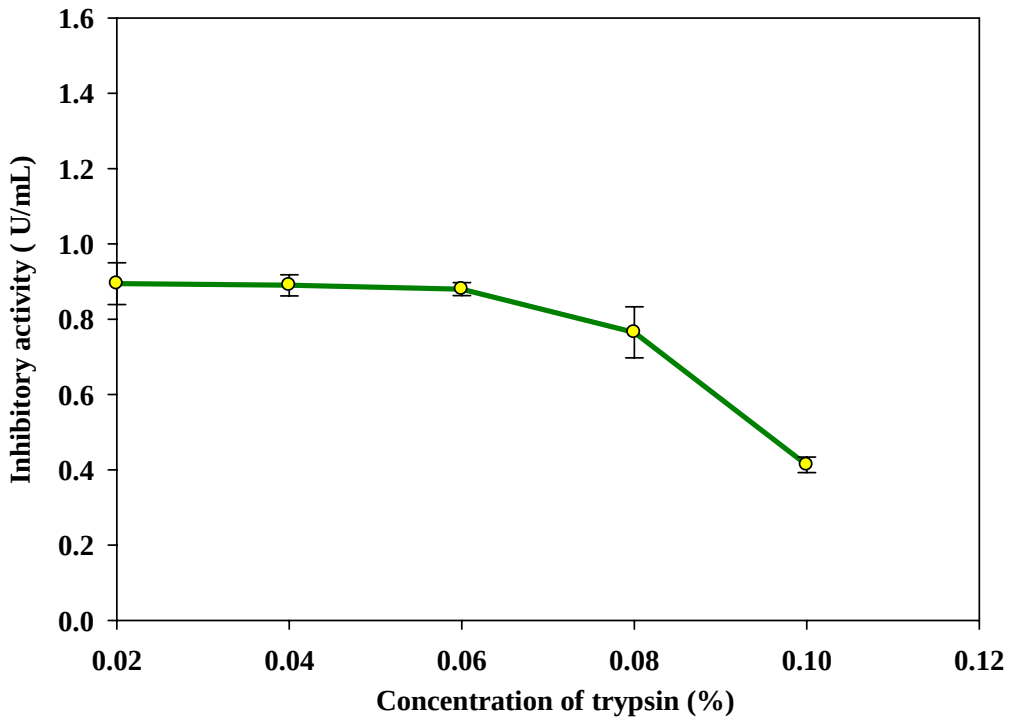

Fig. 4.21 Effect of protease treatment on protease inhibitory activity

4.4.17 Binding studies of inhibitor using flourimetry

Results depicted in the Fig. 4.22 suggest that the binding of inhibitor isolated from *P. floridanus* with trypsin was strong. The inhibitor and trypsin were mixed in 1:1 ratio and excited at 278 nm. The emission spectrum of trypsin at an excitation wavelength of 278 nm revealed a major peak at 343 nm indicating the intrinsic fluorescence of tryptophan amino acid residue. The emission spectrum of the complex of trypsin with protease inhibitor of *P. floridanus* showed complete loss in fluorescence intensity at 343 nm compared with inhibitor alone, suggesting that binding of inhibitor results in the quenching of tryptophan fluorescence.

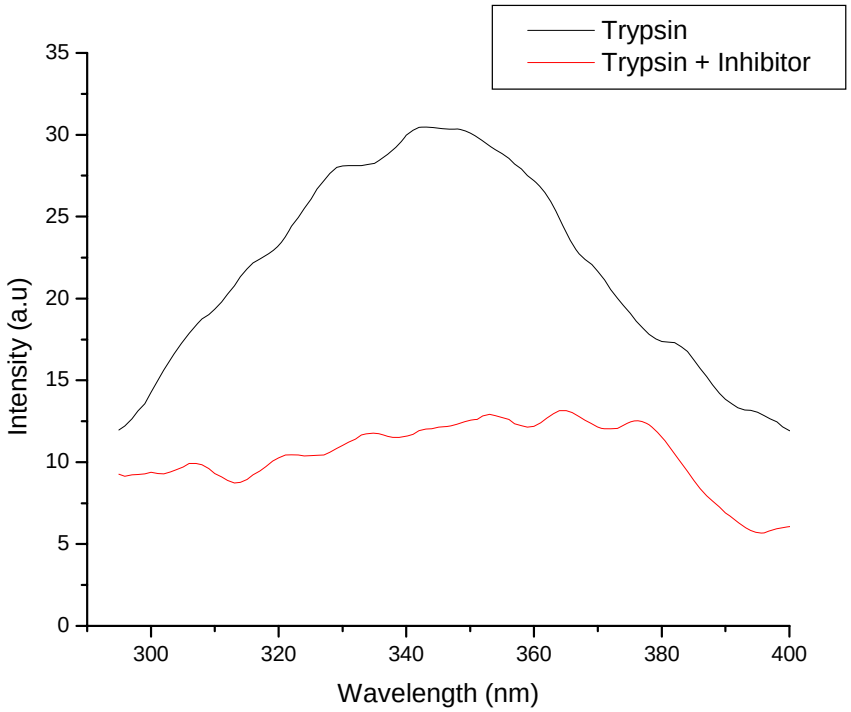

Fig. 4.22 Fluorescence analysis of trypsin and trypsin-inhibitor complex

4.4.18 Peptide mass fingerprinting

Peptide mass fingerprint generated by MALDI-TOF-TOF of protease inhibitor was analyzed with the MASCOT search tool in Swiss-Prot database did not match any of the inhibitors (Fig. 4.23). However, one of the fragments of the inhibitor searched with NCBInr database showed similarity to serine proteinase inhibitor from *Momordica charantia* (gi|61213645|Q9S747.1) and trypsin inhibitor of *Cucumis melo* seeds (gi|1246050|AAB35852.1-B).

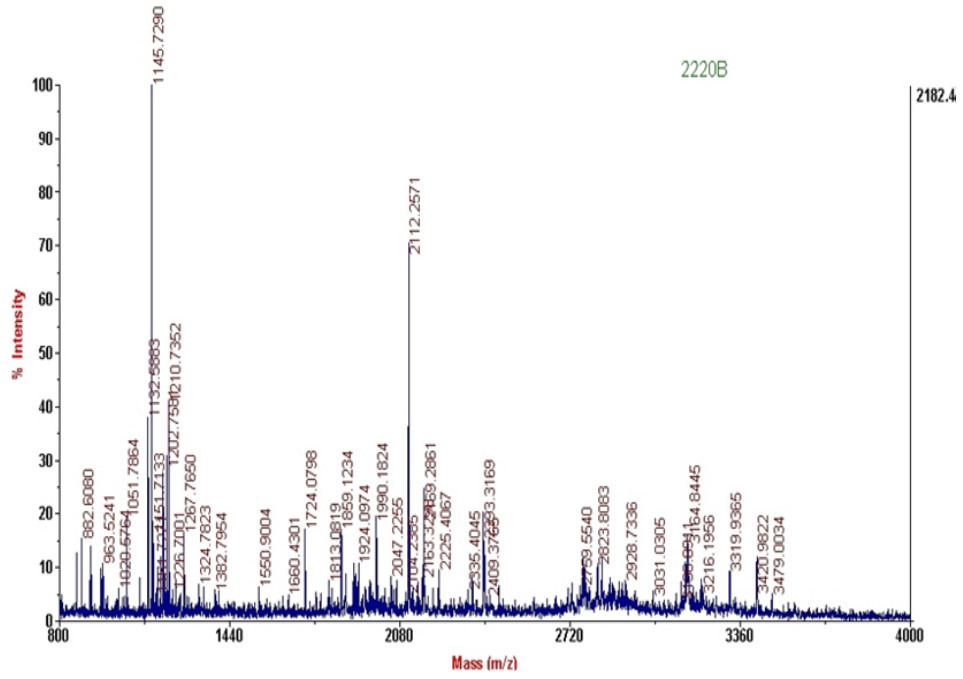

Fig. 4.23 Peptide mass finger print of the inhibitor

4.4.19 Specificity of the inhibitor

Specificity studies conducted for the inhibitor depicted in the Fig. 4.24 showed a striking specificity towards trypsin. The inhibitor showed not much activity towards thermolysin and proteinase K compared to trypsin inhibition. Further, the inhibitor showed no detectable inhibition against chymotrysin, subtilisin and papain.

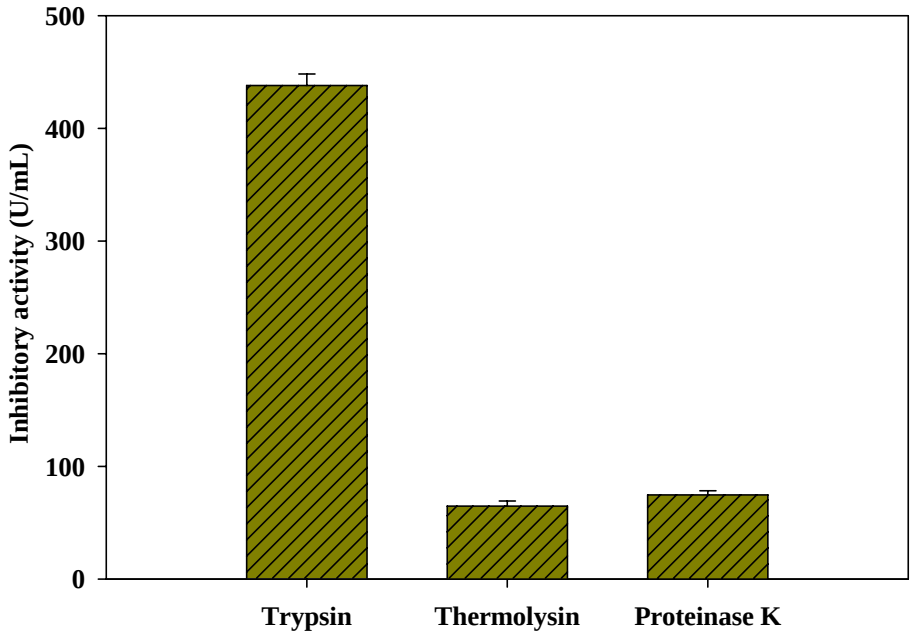

Fig. 4.24 Specificity of the inhibitor against proteases

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 (100% inhibition) corresponds to 1 nM of inhibitor. It is predicted that the stoichiometry of trypsin–protease inhibitor interaction is 1:1 and 38.334 g of protease inhibitor is necessary to completely inactivate 23.4 g of trypsin. It was also found that the amount of inhibitor needed for 50% inhibition (IC_{50}) of trypsin calculated from the graph was 0.5 nM.

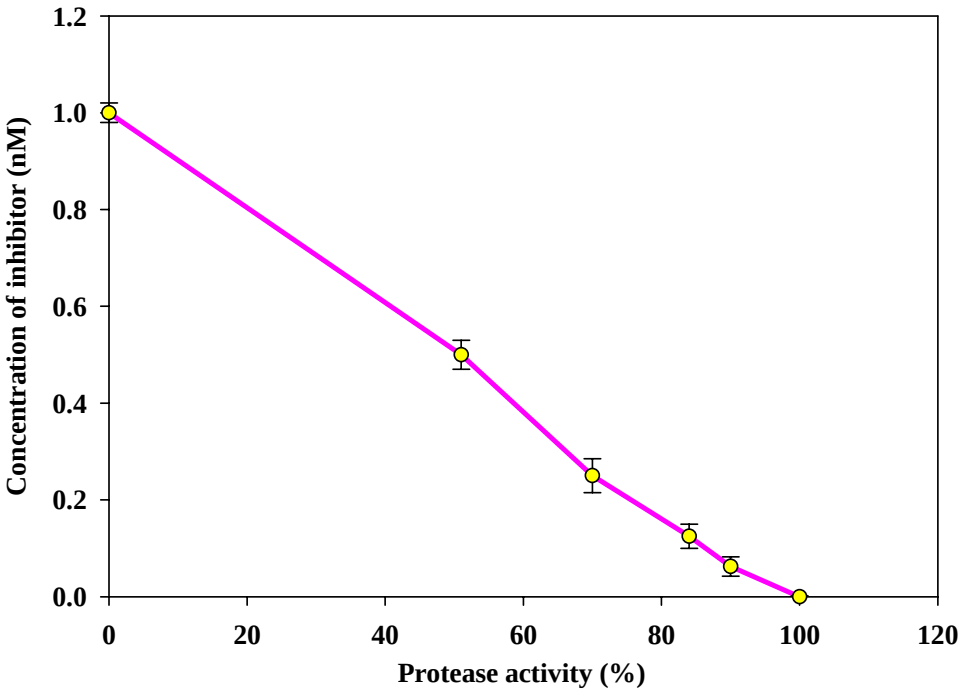

Fig. 4.25 Stoichiometry of protease-protease inhibitor interaction

4.4.21 Kinetic studies of inhibition

From the date presented for the secondary plot of the inhibition kinetics in the Fig. 4.26, it was inferred that identical concentration of trypsin (1 nM) preincubated with different concentrations of inhibitor (0.014, 0.07 and 0.27 nM) yielded different slopes for plots 1/*v* versus 1/[s] for six different [s] values. It was observed that inhibition of substrate hydrolysis occurred at very low concentration of protease inhibitor and the *K*i calculated from the secondary plot was found to be $1.043X10^{-10}$ M under the assay conditions. The sub-nano molar range of *K*i value implies that it is a powerful inhibitor of serine proteases.

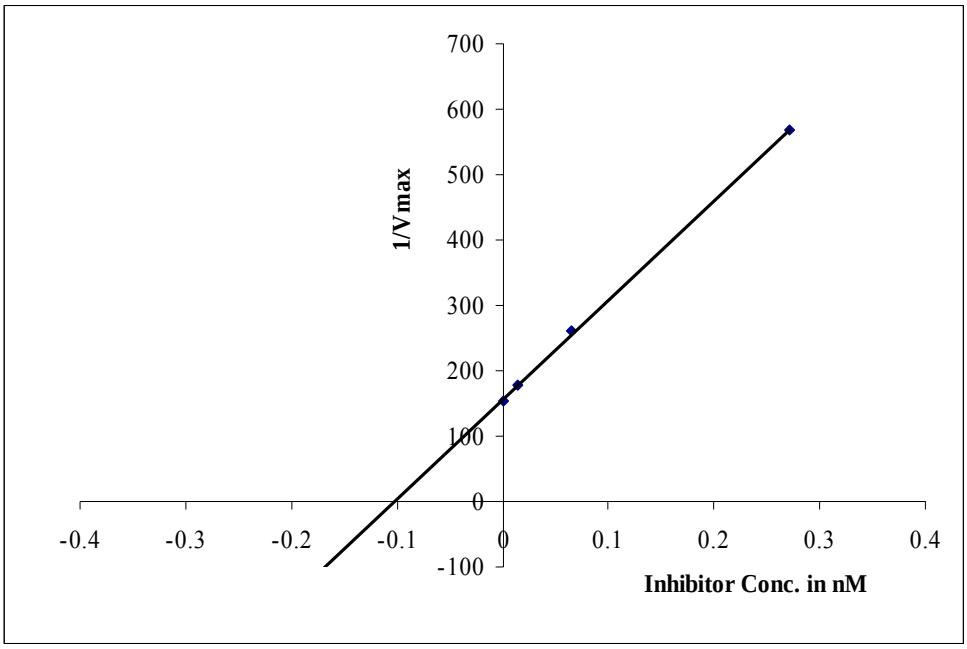

Fig. 4.26 Secondary plot of the inhibitor

4.5 APPLICATION STUDIES

4.5.1 Inhibition of midgut protease of *Papilio demoleus* (Lepidoptera: Papilionidae)

Results obtained for the *in vitro* inhibition studies of inhibitor with midgut proteases isolated from citrus pest, the lime swallowtail *Papilio demoleus* (Lepidoptera: Papilionidae) is presented in the Fig. 4.27. Twenty five microlitre (0.1 mg/mL) of the midgut proteases isolated from citrus pest was pre incubated with 25 µL and 50 µL of the inhibitor (0.1 mg/mL). Considerable inhibition of the midgut protease was observed with 50 µL of the inhibitor purified from *P. floridanus.* Midgut protease of the *Papilio demoleus* showed a protease activity of 1.236 U which was decreased to 0.613 U by 50 µL of the inhibitor.

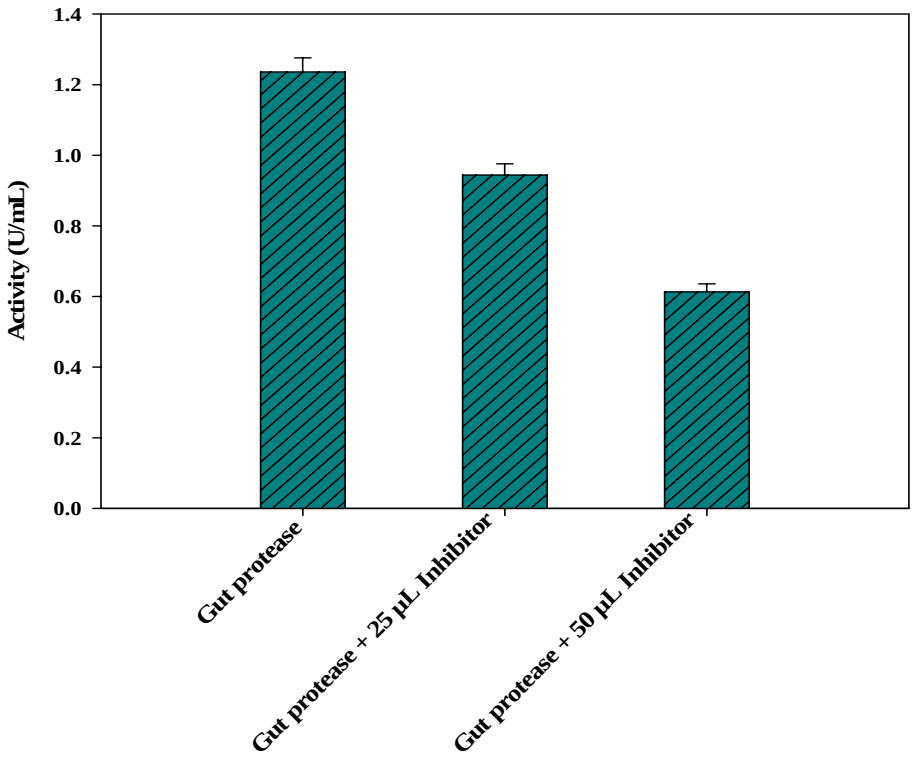

Fig. 4.27 Inhibition of midgut protease of *Papilio demoleus*

4.5.2 Evaluation of protease inhibitor activity against proteases derived from different microbial sources

Inhibitory activity of *P. floridanus* protease inhibitor with different industrially important proteases was evaluated. From the results presented in Fig. 4.28, it was inferred that the inhibitor could inhibit industrially important microbial proteases and thus the commercially available protease esperase was completely inhibited compared to significant levels of inhibition of protease of *A. oryzae, B. licheniformis, Bacillus* sp. *and B. amyloliquefaciens*. The activity spectrum indicated that the interactions of inhibitor with different proteases are by a common and generally accepted mechanism.

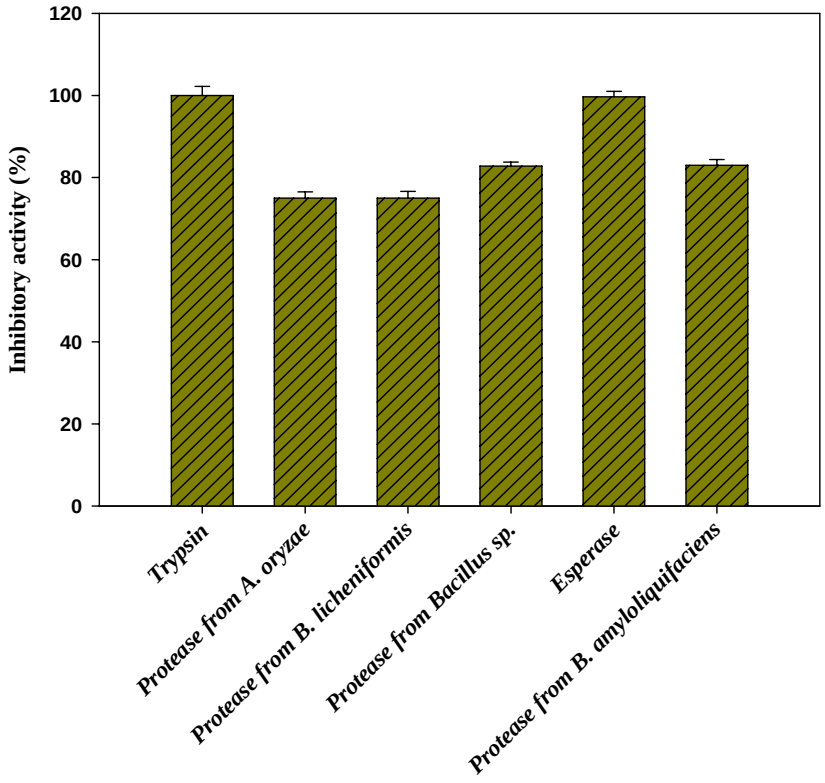

Fig. 4.28 Inhibitory activity against proteases of microbial sources

5 Discussion

5.1 SCREENING OF MUSHROOMS FOR PROTEASE INHIBITOR

Potential applications of protease inhibitor in the fields of medicine, crop protection and biotechnology were described, based on their target proteases. Reports available on protease inhibitors from microorganisms (prokaryotes, yeasts and filamentous fungi) are rather very few when compared to proteinaceous inhibitors isolated from plants. Recently higher fungi, basidiomycete have emerged as a valuable source of new protease inhibitors with unique characteristics and therefore offer great potential for future applications. Further it was inferred that literature available on protease inhibitors from basidiomycete, mushroom are very much limited. In this context in the present study, mushrooms were screened based on activity of protease inhibitor towards recognition of potential source for deriving protease inhibitor. Among the mushrooms screened after partial purification with ammonium sulphate saturation, maximum activity was demonstrated by *Pleurotus floridanus* extract compared to *Agaricus bisporous* and *Phellinus* sp. which showed much lesser activity. Hence *P. floridanus* was selected as the potential source. The identity of the mushroom was reconfirmed by molecular ribotyping.

The proteinaceous nature of the protease inhibitor was further confirmed after partial purification using ammonium sulphate precipitation. The phosphate buffer of pH 7.5 was used for the extraction of protease inhibitor as it was reported to be a good extractant for the maximal extraction of proteins from *Cajanus cajan* seeds and *Moringa oleifera* with high amount of trypsin inhibitory activity and protein concentration (Bijina et al., 2011a; Pichare and Kachole, 1996). The pH of the phosphate buffer was selected to be 7.5 as the optimum protease activity of trypsin is at pH 7.5.

P. floridanus is well known as an edible mushroom, found in temperate and tropical parts of the world, and is consumed as delicious food worldwide. Several bioactive compounds were isolated from *Pleurotus* sp. and tested its efficacy in anti inflammation, platelet aggregation, hypercholestremia, anti tumor and anti oxidant activity (Jose and Janardhanan, 2000). But the presence of any protease inhibitors in the bacidiomycete, *P. floridanus* was not reported so far. In this context the results of the present study indicated potential of this mushroom as source of protease inhibitor.

5.2 PURIFICATION AND CHARECTERIZATION OF PROTEASE INHIBITOR

The inhibitory protein obtained from *P. floridanus* was purified up to homogeneity by $(NH_4)_2SO_4$ precipitation, followed by DEAE sepharose chromatography, trypsin affinity chromatography and gel filtration chromatography. A single peak was resulted in reverse phase high pressure liquid chromatography. It was found that 30-60% $(NH_4)_2SO_4$ saturation was efficient for precipitating the protease inhibitor compared to other fractions. The fold of purification of protease inhibitor obtained for trypsin affinity chromatography and gel filtration were 21.5 and 35.25 respectively. The bound inhibitor from affinity column was eluted as a single peak demonstrating maximum inhibitory activity towards trypsin which resulted in 21.5 fold of purification with a specific inhibitory activity of 68.47 units/mg protein. Fold and recovery of protein can be increased by combination of purification methods. Trypsin affinity chromatography was reported to increase the fold of purification of trypsin inhibitors (TI) from wild-type soybean (*Glycine soya*) (WBTI) and domesticated soybean (*Glycine max*) (SBTI) 718 and 279-fold, with the activity recovery of 62% and 59% respectively (Zhang et al., 2009). Trypsin affinity chromatography is proved to be an efficient method in purifying trypsin specific inhibitors and successfully employed in purifying Trypsin inhibitors (TI) from wild-type

soybean (*Glycine soya*) (WBTI) and domesticated soybean (*Glycine max*) (SBTI) using chitosan resin-trypsin as filler on the affinity chromatography column (Zhang et al., 2009). Similarly trypsin agarose affinity chromatography was employed for the isolation of a 16 kDa protein from *Helianthus annuus* flowers, causes the complete inhibition of *Sclerotinia sclerotiorum* ascospores germination (Giudici et al., 2000). Though affinity chromatography is an efficient method to purify inhibitor up to a certain extent, the homogenous inhibitory protein obtained by gel filtration chromatography resulted in an increase in purification fold up to 35.25 with a specific inhibitory activity of 112.1 units/mg protein. The purity of inhibitor was further refined by reverse phase HPLC.

The limitation of trypsin affinity chromatography was observed, exhibiting multiple bands in SDS-PAGE. Partial proteolysis of inhibitor in its lower concentrations by trypsin can be inferred from the SDS-PAGE analysis. Similar pattern of purification steps was used in the isolation of a cysteine proteinase inhibitor from fruit bodies of a mushroom *Clitocybe nebularis* (Brzin et al., 2000). The net yield of protease inhibitor was 0.5 µg/g of mushroom.

The SDS-PAGE analysis of the purified proteinaceous protease inhibitor molecule indicated the homogeneity of protease inhibitor. Single bands obtained in both reductive SDS-PAGE and Native-PAGE, evidenced the single polypeptide nature of the inhibitor. The molecular mass of the proteinaceous protease inhibitor was estimated based on SDS-PAGE analysis. Appearance of a single polypeptide band with a molecular mass of 37 kDa in reductive SDS-PAGE testified the purity of the fraction. Moreover, the intact molecular mass was confirmed to be 38.334 kDa by MALDI-TOF. The molecular mass of the inhibitor ranges from less than 10 kDa to 50 kDa and depends on inhibitor source and inhibitor family. The pacifastin family of serine protease inhibitors isolated from silk worms have molecular weight of 4 kDa (Clynen et al., 2005). Kunitz inhibitors are in the range of 18-26 kDa. Serpins (serine proteinase inhibitors) are the largest super family of protease inhibitors with 350–400 amino acids and the molecular weight of 40–50

kDa (Khan et al., 2011). The only other inhibitor isolated and sequenced from basidiomycetes, *Pleurotus* sp. to date is from *Pleurotus ostreatus*. Endogenous inhibitors, IA-1 and IA-2, inhibit an intracellular serine proteinase (proteinase A) purified from the fruiting bodies of *P. ostreatus,* and inhibitors are acidic polypeptides with respective molecular masses of 8307 and 8244 Da (Dohmae et al., 1995). It may be noted that the molecular mass obtained using MALDI-TOF for *P. floridanus* in the present study did not show any similarity with those protease inhibitors isolated from other basidomycetes such as *Clitocybe nebularis* (Avanzo et al., 2009) and *Lentinus edodes* (Odani et al., 1999). Hence, the inhibitor isolated from *P. floridanus* is assumed to be a high molecular weight inhibitor and can be included in the Serpin family of inhibitors.

Isoelectric point (pI) values have long been a regular measure for distinguishing between proteins. Protease inhibitors show a range of pI from acidic to alkaline with respect to the source and type of inhibitor. pI of serpin-6 of *Manduca sexta* is 5.4 (Wang and Jiang, 2004). The acidic nature of the isoelectric point is generally found in the Kunitz type inhibitors. Three isoforms of protease inhibitors, ApTIA, ApTIB and ApTIC from *Acacia plumose* separated on isoelectric focusing gel gave isoelectric points of an acidic nature: 5.05, 5.25 and 5.55, respectively (Laber et al., 1989; Lopes et al., 2009) and similarly in *A. ellipticum*: 4.1, 4.55, 5.27 and 5.65 (Bhattacharyya et al., 2006). Serine protease inhibitors (ISPI-1, 2, 3) have been purified from larval hemolymph of greater wax moth larvae, *Galleria mellonella* with isoelectric points ranging between 7.2 and 8.3 (Andreas et al., 2000). Serine protease inhibitors from the basidiomycete *Clitocybe nebularis* CnSPIs, have isoelectric points 4.8 and 5.2 (Avanzo et al., 2009). Macrocypins from basidiomycete *Macrolepiota Procera* and clitocypins from *C. nebularis* exhibit similar isoelectric points of around 4.8 (Sabotic et al., 2009). In the present study isoelectric point of the inhibitory protein separated on the IPG strip was 4.4, suggesting unique isoelectric point of the inhibitor.

Activity staining of the purified protease inhibitor evaluated on Native PAGE and visualization of a clear band on a colored background after staining confirmed that the isolated protease inhibitor has inhibitory activity. Dot blot analysis of protease inhibitor activity performed on X-ray film, also indicated that the protease inhibitor blocked the gelatin hydrolysis by trypsin similar to that of the control protease inhibitor Soya bean trypsin inhibitor. Continuous rate spectrophotometric assay was carried out using BAEE as substrate. It was noticed that the rate of hydrolysis of BAEE by trypsin was reduced to 230 units from 1800 BAEE units in the presence of inhibitor. HPLC profile of casein hydrolysate by trypsin in the presence of inhibitor resulted significant reduction of peptide peaks.

Stability studies conducted in different buffer systems showed that the protease inhibitor was stable over a wide range of pH. It was observed that there is a considerable protease inhibitor activity over a pH range of 4-10; although a maximal inhibitory activity was recorded at pH 8. At high alkaline conditions of pH 11-12 and high acidic conditions of pH 2-3 the protease inhibitor was not stable. Intramolecular disulfide bridges are presumably responsible for the functional stability of the inhibitor at different temperature and pH. The inhibitory activity was stable in the pH range of 4-10, although the stability declined rapidly at pH levels above pH 8. In fact the protease inhibitor showed sharp decline in stability at pH conditions above pH 10.

Reports presume that intramolecular disulfide bridges are responsible for the functional stability of the inhibitor in the presence of physical and chemical denaturants such as temperature, pH and reducing agents (Oliveira et al., 2007). Under strong acidic or alkaline conditions, the protein inhibitors get denatured and as a consequence they lose their activity partially or completely. pH activity profiles of the gut lumen of the red flour beetle, *Tribolium castaneum* revealed the presence of proteinases with acidic (pH 4–5) and alkaline (pH 8.5–11) optima. The substrate BAPNA were preferentially hydrolyzed at the alkaline pH optima suggested trypsin-like proteinases (Oppert et al., 2003). Protease inhibitors

targeting proteases of different insect pests have shown anti-feedent properties. The alkaline gut of lepedopteran and dipteran larvae primarily relies on serine proteases like trypsin and chymotrypsin for the digestion of plant material, where as cysteine proteases predominate in Hemiptera, Coleoptera and Thysanoptera (Sabotič and Kos, 2012). Hence, the stability of protease inhibitor of *P. floridanus* in alkaline range of pH signifies its possible use as biopesticide, as one of the criterion to withstand the highly alkaline conditions of insect's gut flora is complied with.

Similarly when evaluated for activity and stability at wide range of temperatures varying between 4^{o}C to 100^{o}C, the protease inhibitor was observed to demonstrate considerable stability over a broad range of temperature up to 90^{o}C. A novel 8.7 kDa protease inhibitor from chan seeds (*Hyptis suaveolens* L.) inhibited proteases from the larger grain borer *Prostephanus truncatus* (Coleoptera: Bostrichidae) and was stable over the temperature range 4–95^{o}C (Aguirre et al., 2004). Inhibitors with compact structures and in many cases with a high content of disulfide bridges, characteristics that might contribute to their high thermal stability (Singh and Rao, 2002).

Maximal activity of the protease inhibitor was observed at temperatures around 30-40^{o}C. Whereas, the purified inhibitor recorded a gradual decrease in activity after pre-incubation at 50-90°C for 60 min and a complete loss of activity at 100°C. These observations indicate that the protease inhibitor has high intrinsic stability in its native state, which gives a high degree of thermal stability. Three actinomycetes strains producing alkaline protease inhibitors API-I, API-II and API-III respectively, exhibited different properties in their molecular nature and in their pH and temperature stabilities (Pandhare et al., 2002). API-I and API-II exhibited stability over a pH range of 5-12 whereas API-III displayed wide pH stability from 2-12. API-I was stable at 60°C with a half-life of 2 h but API-II showed a half-life of 1 h at 45°C. Preparations of new low molecular weight protein inhibitors of serine proteinases obtained from buckwheat *Fagopyrum*

esculentum seeds possessed high pH-stability in the pH range 2-12 and thermostability (Tsybina et al., 2004). Protease inhibitor (PISC-2002) isolated from culture supernatants of *Streptomyces chromofuscus* was stable over pH (2–10) and at high temperatures (80 °C/30 min), mainly attributed to the presence of proline and of a high content of hydrophobic amino acids (Angelova et al., 2006). The high thermal and pH stabilities of *P. floridanus* inhibitor testified its possible applications in various industries. Enhancement of thermal stability is desirable trait for most of the biotechnological applications of proteins and for their commercial exploitation (Pandhare et al., 2002), as it increases the efficiency of proteins and is therefore one of the essential requirements.

In the present study among the metal ions Ca^{2+}, Mg^{2+}, Mn^{2+}, Hg^{2+}, Na^{2+}, Ba^{2+}, Ni^{2+}, Cd^{2+}, Fe^{3+}, Mo^{6+} and Al^{3+} evaluated for their impact on the activity of protease inhibitor. Addition of Mn^{2+}, Na^{2+}, Ba^{2+} and Ni^{2+} at 1 mM concentration, led to an enhancement in the protease inhibitory activity. It was noted that 1 mM of Mn^{2+} enhanced the inhibitory activity considerably compared to that of control and 1 mM of Na^{2+}, Ba^{2+} and Ni^{2+} also enhanced protease inhibitory activity reasonably. None of the other divalent ions could enhance protease inhibitory activity. Involvement of heavy metal ions Cd^{2+}, Zn^{2+}, Fe^{2+}, Cu^{2+}, Hg^{2+} and Pb^{2+} in the inhibitory activity of cysteine proteases, papain and clostripain have been reported earlier (Schirmeister and Peric, 2000). Inductively coupled atomic emission spectroscopy (ICP-AES) estimation of the metal ion concentration of both native and demetallized protein revealed the presence of Zn^{2+}, Ca^{2+} and Mg^{2+}. It was observed that the demetallized inhibitor retained protease inhibitory activity indicating no role for the metal ions Zn^{2+}, Ca^{2+} and Mg^{2+} in protease inhibitory activity. Instead they may be involved in maintaining structural integrity of the inhibitor.

The removal of a Zn^{2+} atom from cysteine protease inhibitor of pearl millet (CPI) was reported earlier to result in the loss of anti-fungal and protease inhibitor activity. The disruption in the secondary structure of the demetallized

inhibitor implied the role of Zn^{2+} in maintaining the structural integrity of CPI (Joshi et al., 1999). Similarly, additional supplementation of Zn^{2+} was found to enhance the inhibitory activity of protease inhibitor isolated from *Moringa oleifera* (Bijina et al., 2011a). The plasma proteinase inhibitors α_1-antitrypsin, antithrombin III and α_2-macroglobulin in purified systems were also reported to be accelerated by the divalent cations Ca^{2+}, Mn^{2+} and Mg^{2+} (Vincent Ellis et al., 1983).

Detergents act as surfactants since they decrease the surface tension of water and mimic the native, hydrophobic environment of the phospholipid bilayer *in vivo*. Detergents are used extensively in protein extraction procedures for solubilizing protein from lipid membranes and other protein bound membranes. Detergents also maintain the solubility of certain proteins in the solution. Protease inhibitors and detergents are routinely used together in cell lysis buffers to inhibit proteolysis and facilitate membrane protein solubilization in protein purification procedures. Nonionic detergents normally are considered as mild detergents and that they do not interact extensively with the protein surface, whereas ionic detergents, in particular SDS, generally bind non-specifically to the protein surface, which usually lead to protein unfolding (Mogensen et al., 2005). In the present study the anionic detergent SDS and cationic detergent CTAB at 1% reduced the inhibitory activity significantly probably due to unfavorable electrostatic interactions that might have caused unfolding and/or disrupt trypsin binding. Whereas, the nonionic detergents Triton X and Tween 80 contributed to an increase, more than 2.5 times in the inhibitory activity. The interaction of mild detergents with hydrophobic amino acids of the inhibitor probably might have changed its conformation in such a way to facilitate strong binding with the trypsin.

Phaseolus aureus inhibitor exhibited a cooperative transition in the presence of SDS leading to increased α-helical structure. The hydrophobic nature of SDS results in the rearrangement of peptide backbone conformation leading to

helix-formation with more hydrophobic residues exposed and consequently available to associate with the detergent. *Cajanus cajan* inhibitor showed any major conformational change in the presence of either sodium dodecyl sulfate (SDS), sodium deoxycholate (DOC). However, the inhibitory activity of the PI was decreased significantly in the presence of DOC. Loss of activity (function) without a concomitant loss or change in structure suggests that certain reactive site amino acids are required for inhibiting enzyme activity (Haq and Khan, 2005). Conformational sensitivity of protease inhibitors to the detergents studied with Soybean Kunitz inhibitor showed that the protein was highly sensitive to sodium dodecyl sulfate. The absence of hydrogen bonded α-helical or β-structures leads to the conclusion that the inhibitor is stabilized chiefly by hydrophobic interactions and that the major conformation in this protein is the loop and bend structure (Jirgensons, 1973).

Results obtained for the effect of oxidizing agents, H_2O_2 and DMSO on protease inhibitory activity showed that the inhibitory activity decreased along with an increase in concentration of oxidizing agents. The protease inhibitory activity gradually decreased along with increase in concentrations and complete inactivation was noted at 6% of DMSO. Whereas, oxidation of protease inhibitor by H_2O_2 was stronger than that of DMSO and complete inactivation of the inhibitor was observed at concentrations above 2% of H_2O_2. The results indicated the presence of methionine residue at the reactive site of the inhibitor isolated from *P. floridanus.* Oxidation of one of the eight methionine residues of α1-protease inhibitor (α1-PI) (Met358) caused an almost complete loss of inhibitory activity of α1-PI toward its primary biological target, elastase (Johnson and Travis, 1979). Similarly, inhibitory activity of protease inhibitor isolated from *M. oleifera* was declined in response to an increase in the concentration (from 1% to 5%) of oxidizing agents DMSO and H_2O_2 (Bijina et al., 2011a).

Intra disulfide bonds are vital for the proper folding and stability of many proteins. The effect of reducing agents on protease inhibitory activity studied

using dithiothreitol and β-mercaptoethanol indicated complete inactivation of the inhibitor at concentrations above 140 μM of dithiothreitol. Compared to dithiothreitol, in the case of β-mercaptoethanol inactivation was noted at 400 μM. Leech carboxypeptidase inhibitor (LCI) is a 67-residue, tight-binding metallocarboxypeptidase inhibitor composed of a compact domain with a five-stranded β-sheet and a short α-helix that are strongly stabilized by four disulfide bonds. The contribution of each particular disulfide to the folding, stability and function of LCI was investigated by constructing a series of single and multiple mutants lacking one to four disulfide bonds. The designed multiple mutants of LCI were unable to fold correctly, displaying a highly unstructured conformation and a very low inhibitory capability indicated the importance of disulfide bonds in LCI for both correct folding and achievement of a functional structure (Arolas et al., 2009). In contrary, lower concentration of dithiothreitol (DTT) had no effect on protease inhibitors isolated from *Peltophorum dubium* and *Erythrina caffra*, a Kunitz type trypsin inhibitor (Lehle et al., 1996; Macedo et al., 2003).

Results obtained for the effect of chemical modifiers on the activity of *P. floridanus* protease inhibitor confirmed that modification of amino acid serine led to a drastic reduction in the activity even at a smaller concentration of chemical modifier. An abrupt reduction in the activity of inhibitor was noticed at 10 mM concentration of *N*-Ethylmaleimide whereas; the modification histidine resulted in a reduction in the activity at slightly higher concentrations of the modifier. The results indicated further the presence of serine, cysteine and histidine residue in the reactive site of the inhibitor. In contrast, the modification of tryptophan with *N*-Bromosuccinamide had no effect on the activity of the inhibitor.

Reactive site modification of protease inhibitor by amino acid specific chemicals proved to be a valuable tool in specific protein–protein interactions. Chemical reagents bind to amino acid side chains covalently and may produce changes in the properties/activity without knowing the protein structure. In Kunitz-domains, primary sites interacting with the target proteases (and

determining their protease-specificity) are found as a short segment containing conserved cysteine, and also a secondary site contacting the target proteases includes residues adjacent to the fourth conserved cysteine (Scheidig et al., 1997). Anti-fungal activity of pearl millet cysteine protease inhibitor (CPI) was abolished after modification of cysteine, arginine or aspartic/glutamic acid residues, while CPI activity was selectively enhanced by modification of histidine or arginine residues (Joshi et al., 1999). Involvement of an arginine residue in amylase inhibitor activity in the case of barley has been reported (Abe et al., 1993). Lentinus proteinase inhibitor, purified from the fruiting bodies of the edible mushroom*, Lentinus edodes*, suggested involvement of one or more arginine residues in the inhibition of trypsin (Odani et al., 1999).

Protease inhibitor stability in acid illustrated that the inhibitor was stable up to a concentration of 0.04 M HCl. The activity showed gradual decrease with increasing concentration of HCl and a complete loss of activity was recorded at 0.08 M HCl. The pretreatment of inhibitor with increasing concentration of trypsin exhibited a decrease in inhibitory activity. It was inferred that the inhibitor isolated from *P. floridanus* is sensitive towards higher concentration of HCl and trypsin.

Results of fluorescence binding studies of the inhibitor isolated from *P. floridanus* with trypsin were obtained. The emission spectrum of trypsin revealed a major peak at 343 nm indicating the intrinsic fluorescence of tryptophan amino acid residue. The emission spectrum of the complex of trypsin with protease inhibitor of *P. floridanus* showed complete loss in fluorescence intensity at 343 nm compared with inhibitor alone, suggesting that binding of inhibitor results in the quenching of tryptophan fluorescence. The removal of a Zn^{2+} atom from pearl millet cysteine protease inhibitor resulted in the loss of anti-fungal and protease inhibitor activity with concomitant decrease in the fluorescence intensity of CPI, suggesting that binding of the metal ion results in the quenching of tyrosine fluorescence. The increase in the tryptophan fluorescence of papain at 343 nm

indicates the inability of demetallated CPI to bind with the active site of the enzyme (Joshi et al., 1999).

Peptide mass fingerprint of protease inhibitor isolated from *P. floridanus* was analysed with the MASCOT search tool in Swiss-Prot database did not match any of the inhibitors. Whereas, one of the fragment of the inhibitor searched with NCBInr database showed similarity to serine proteinase inhibitor from bitter guards (*Momordica charantia*) and of trypsin inhibitor *Cucumis melo* seeds. Since, the protease inhibitor from *P. floridanus* showed limited homology with existing protease inhibitors; the inhibitor may be a novel one, which may have distinct structure and sequence homology compared to other reported protease inhibitors. Peptide mass fingerprinting (PMF) by MALDI-MS and sequencing by tandem mass spectrometry have evolved as the desired and primary methods for identification of proteins following separation by two-dimensional gel electrophoresis, SDS–PAGE or liquid chromatography. Protein identification and differentiation by peptidemass fingerprinting (PMF) has been an excellent tool to differentiate proteins with very similar physicochemical and functional properties. The mass spectra obtained after tryptic digestion (peptidemass fingerprint) of inhibitor isolated from *Solanum tuberosum* cv. Desirée was analysed with the "MASCOT search tool" also did not match to any of the inhibitors of other plants (Obregón et al., 2012).

Specificity studies conducted for the isolated inhibitor from *P. floridanus* showed striking specificity towards trypsin. The inhibitor showed not much activity towards thermolysin and proteinase K compared to trypsin inhibition, while no detectable inhibition was observed against chymotrysin, subtilisin and papain. Trypsin-specific inhibitors obtained from the basidiomycete *Clitocybe nebularis,* Cnispin inhibited trypsin with high specificity. Cnispin inhibited chymotrypsin with *K*i in the micromolar range, and showed weaker inhibition of subtilisin and kallikrein, and no inhibition of the other serine proteases tested

(Avanzo et al., 2009). Similarly, Kunitz-type protease inhibitor domain of the human WFIKKN-KU2 protein displayed striking specificity for trypsin, but no detectable inhibition was observed in the case of plasmin, lung tryptase, plasma kallikrein, thrombin, urokinase, tissue plasminogen activator, pancreatic kallikrein, chymotrypsin or elastase (Nagy et al., 2003).

The inhibitory constant *K*i of the *P. floridanus* inhibitor was found to be $1.043X10^{-10}$ M from secondary plot. The *K*i was in sub-nanomolar range and quite similar to the protease inhibitors from mushroom *Clitocybe nebularis* (Avanzo et al., 2009) and *Lentinus edodes* (Odani et al., 1999). The *K*i value of serine protease inhibitor from the basidiomycete *Clitocybe nebularis*, CnSPIs, for the inhibition of trypsin was 3.1 nM (Avanzo et al., 2009). Similarly, lentinus proteinase inhibitor purified from the fruiting body of edible mushroom *Lentinus edodes* shown apparent dissociation constant of $3.5X10^{-10}$ M (Odani et al., 1999). Some were specific for both trypsin and chymotrypsin with high affinity. *P. dubium* protease inhibitor inhibited bovine and porcine trypsin stoichiometrically (*K*i of 4×10^{-10} M and 1.6×10^{-10} M respectively) but affected bovine chymotrypsin only weakly (*K*i of 2.6×10^{-7} M) (Macedo et al., 2003).

Data obtained from the kinetic studies performed with trypsin indicated that the *P. floridanus* inhibitor has a reversible mechanism of action. A Lineweaver–Burk curve, 1/*v* versus 1/[s], was plotted to study the pattern of inhibition (competitive, uncompetitive or non-competitive). It was observed that identical concentration of trypsin (1 nM) preincubated with enzyme buffer alone and with different concentrations of inhibitor (0.014, 0.07 and 0.27 nM) yielded different slopes for plots 1/*v* versus 1/[s] for various concentrations of substrate (BAPNA) ranging from 0.05 to 0.4 mM. Inhibition of substrate hydrolysis occurred at very low concentration of protease inhibitor and *K*i was calculated from the secondary plot as $1.043X10^{-10}$ M under the assay conditions. The low *K*i values indicated a relatively high affinity of the inhibitor for the enzyme. Protease inhibitors from plants and microorganisms were characterized by either a

reversible or irreversible mechanism (Polgar, 1989). Kinetic studies of trypsin by *P. floridanus* inhibitor revealed that it had a reversible mechanism of action. The kinetic studies of inhibitor also revealed that trypsin inactivation occurs by uncompetitive inhibition during which the inhibitor binds only to the enzyme–substrate complex and as a result both affinity of the enzyme (*Km*) and *V*max undergoes change.

The data obtained for the studies conducted on trypsin-protease inhibitor interaction illustrated that extrapolation to zero protease activity (100% inhibition) corresponding to 1 nM of inhibitor, suggests that 1 nM trypsin was completely inhibited by 1 nM of the inhibitor. The amount of inhibitor required for 50% inhibition (IC_{50}) of trypsin calculated from the graph, was 0.5 nM. The stoichiometry of trypsin-inhibitor interaction was similar to other trypsin inhibitors. Titration of trypsin with protease inhibitor from the basidiomycete *Clitocybe nebularis,* inhibitor purified from the fruiting body of edible mushroom *Lentinus edodes* and with serine proteinase inhibitor from the leguminous plant seeds of *Archidendron ellipticum* (AeTI), inhibited trypsin in the stoichiometric ratio of 1:1 (Avanzo et al., 2009; Bhattacharyya et al., 2006 ; Odani et al., 1999).

5.3 APPLICATION STUDIES

The midgut proteases isolated from citrus pest, the lime swallowtail *Papilio demoleus* (Lepidoptera: Papilionidae) was inhibited by the protease inhibitor isolated from *P. floridanus, in vitro*. The inhibitory activity of the inhibitor was stable in the pH range of 4-10 and the remarkable specificity against trypsin signified its possible use as biopesticide. The alkaline gut of lepedopteran and dipteran larvae primarily relies on serine proteases like trypsin and chymotrypsin for the digestion of plant material (Sabotič and Kos, 2012). Serine proteases have been identified in extracts from the digestive tracts of insects from many families, particularly those of lepidoptera and many of these enzymes are

inhibited by protease inhibitors (Houseman et al., 1989). The caterpillars of *Papilio demoleus* can completely defoliate young citrus trees and devastate citrus nurseries. In mature trees, caterpillars may prefer young leaves and leaf flush. The mean leaf area consumed by all instars of a single larva was 247.6 cm^2; and 83.6% of this area was consumed in the fifth instar, 12.7% in the fourth, 2.3% in the third and less than 2% in the first two instars (Badawi, 1981). Hence, the *in vitro* inhibition study of the inhibitor against *Papilio demoleus* suggested the potential of *P. floridanus* inhibitor for use as a biocontrol agent against many lepidopteran insect pests.

There are many reports on protease inhibitor's function as plant defense molecules and were considered for use in preventing insect predation. Numerous biochemical tests and inhibitor incorporated feeding assays demonstrated that these inhibitors impaired insect digestion, and could be good candidate proteins to be used in transgenic plants resistant to insect pests (Amirhusin et al., 2007; Mosolov et al., 2001b; Srinivasan et al., 2005). The order lepidoptera, which includes a number of crop pests, that have their guts pH optima of the guts are in the alkaline range of 9-11 (Applebaum, 1985). These insects' gut dominated by serine proteases and metalloexopeptidases. Additionally, serine proteinase inhibitors have anti-nutritional effects against several lepidopteran insect species (Applebaum, 1985; Shulke and Murdock, 1983). Purified Bowman-Birk trypsin inhibitor at 5% of the diet inhibited growth of the *Culex nigripalpus* larvae (Brovosky, 1986). The only report available on a protein protease inhibitor of microbial or fungal origin as an effective antinutritional agent is the cysteine protease inhibitors macrocypins (family I85) obtained from the edible parasol mushroom (*Macrolepiota procera*), which have been shown to be detrimental to the growth and development of Colorado potato beetle larvae (Sabotič and Kos, 2012).

Inhibitory activity of *P. floridanus* protease inhibitor was evaluated with different industrially important proteases. The inhibitor showed inhibition against

many industrially important microbial proteases apart from its remarkable specificity towards trypsin. From the results obtained, it was inferred that the protease inhibitor could completely inhibit the commercially available protease esperase compared to significant levels of inhibition of proteases of *A. oryzae, B. licheniformis, Bacillus* sp*. and B. amyloliquefaciens*. The activity spectrum indicated that the interactions of inhibitor with different proteases are by a common and generally accepted mechanism as the inhibitory activities are almost similar. It was also noted that the commercial chymotrypsin and elastase were not inhibited by *P. floridanus* protease inhibitor may be due to the lack of binding site for the inhibitor, whereas reasonable inhibition was showed against thermolysin and proteinase K. The results obtained for the inhibition studies against these commercial enzymes indicated probable application for the *P. floridanus* protease inhibitor in regulating their activity. Further this protease inhibitor has potential for use as a defensive mechanism against attacking insects or for the endogenous protease regulation (Dohmae et al., 1995). The specificity of the inhibitor was high towards trypsin and trypsin like proteases, and also has potential for use in therapeutics (Ramachandran and Hollenberg, 2008).

Excessive proteolysis plays an important role in cancer and in cardiovascular, inflammatory, neurodegenerative, bacterial, viral and parasitic diseases. Protease inhibitors have been explored with the intent to develop therapeutic drugs against proteases that have a potential as therapeutic targets, according to their catalytic type, for each group of disease-causing organisms and for other human diseases (Sabotič and Kos, 2012). A potent peptidic inhibitor of HIV-1 protease of bacterial origin (ATBI) has been found in an extremophilic *Bacillus* sp. (Dash and Rao, 2001; Vathipadiekal et al., 2010). Proteases are important virulence factors of many pathogenic bacteria, which play roles in acquiring nutrients by direct degradation of host tissue components. Bacterial type I signal peptidases, SPase are serine proteases that are widespread among Gram-

negative and Gram-positive bacteria and are important antimicrobial drug targets as they are involved in the secretion of many virulence factors (Paetzel et al., 2000). Several proteases of the serine catalytic type have also been targeted for the design of specific protease inhibitors for use in cancer treatment, including the urokinase plasminogen activator and matriptase (Abbenante and Fairlie, 2005; Bialas and Kafarski, 2009; Ulisse et al., 2009). In this context, the inhibitor isolated from the edible mushroom *Pleurotus floridanus* has immense potential for use in therapeutics.

6 Summary and conclusion

Edible mushrooms were screened as source for deriving protease inhibitors. Among the mushrooms evaluated *Pleurotus floridanus*, an edible mushroom obtained from Kerala Agriculture University, Trichur, India; was selected as the potential source of protease inhibitor based on its highest activity. The selected mushroom was identified based on its molecular characterization using ITS1 and ITS4 primers followed by sequencing of amplicon, BLAST search and confirmation. The sequence was deposited in the NCBI GenBank and was assigned with the accession no GU7210580.

The protease inhibitor isolated from *P. floridanus* was purified up to homogeneity employing conventional purification strategies by $(NH_4)_2SO_4$ precipitation, followed by DEAE sepharose chromatography, trypsin affinity chromatography and gel filtration chromatography. The fold of purification of protease inhibitor obtained for $(NH_4)_2SO_4$ precipitation, DEAE sepharose chromatography, trypsin affinity chromatography and gel filtration were 1.63, 3.61, 21.5 and 35.25 respectively. Gel filtration chromatography yielded a homogenous inhibitory protein which also resulted in an increase in purification fold up to 35.25 with a specific inhibitory activity of 112.1 units/mg protein. The purity of the protease inhibitor was further confirmed by reverse phase high pressure liquid chromatography which showed a single peak.

Electrophoreses analyses by Native-PAGE and reductive SDS-PAGE showed single band indicating single polypeptide nature of the inhibitor. The intact molecular mass of the inhibitor protein was confirmed as 38.334 kDa by MALDI-TOF which correlated with 37 kDa molecular mass obtained with reductive SDS-PAGE. Activity staining of the purified protease inhibitor evaluated on Native PAGE and visualization of a clear band on a colored

background after staining enabled confirmation of the inhibitory activity by the isolated protease inhibitor. Continuous rate spectrophotometric assay was carried out using BAEE as substrate. It was noticed that the rate of hydrolysis of BAEE by trypsin was reduced to 230 units from 1800 BAEE units in the presence of inhibitor. HPLC profile of casein hydrolysate by trypsin in the presence of inhibitor resulted significant reduction of peptide peaks.

The isoelectric point of the isolated inhibitory protein separated on the IPG strip was found to be 4.4 and the protein was separated as a single band in 2-D electrophoresis. Dot blot analysis performed on X-ray film indicated that the protease inhibitor blocked the gelatin hydrolysis by trypsin similar to that of the Soya bean trypsin inhibitor used as control.

The protease inhibitor was found to be stable over a wide range of pH although considerable levels of protease inhibitor activities were observed over a pH range of 4-10 with maximal inhibitory activity at pH 8. The protease inhibitor was observed to have considerable stability over a broad range of temperature up to 90^{o}C while maximal activity of the protease inhibitor was observed at 30-40^{o}C. It was also noted that the activity of the purified inhibitor decreased gradually after pre-incubation at 50-90°C for 60 min. The inhibitor was completely inactivated at 100°C.

The anionic detergent SDS and cationic detergent CTAB, at 1% concentration reduced the inhibitory activity significantly while the nonionic detergents Triton X and Tween 80 led to an increase of more than 2.5 times in the inhibitory activity.

Additional supplementation of 1 mM Mn^{2+} enhanced the inhibitory activity (1.35 U) compared to that of control (0.733 U). Na^{2+}, Ba^{2+} and Ni^{2+} also enhanced protease inhibitory activity reasonably at 1 mM concentrations. Whereas it was observed that metal ions such as Ca^{2+}, Mg^{2+}, Hg^{2+}, Cd^{2+}, $Fe3^{+}$, Mo^{6+} and Al^{3+} had no effect on the activity of protease inhibitor. Further inductively coupled atomic emission spectroscopy (ICP-AES) revealed the presence of Zn^{2+}, Ca^{2+} and Mg^{2+} in the inhibitor and indicated that demetallisation did not affect the activity of the inhibitor.

The activity of inhibitor decreased along with an increase in the concentration of oxidizing agents and thus the protease inhibitory activity was observed to get gradually decreased in response to increase in concentration from 1% to 5% of DMSO, and complete inactivation at 6% of DMSO. Whereas, oxidation of protease inhibitor by H_2O_2 was observed to be stronger than that of DMSO; and complete inactivation of the inhibitor was noted at concentrations above 2% of H_2O_2.

Reducing agents dithiothreitol (DTT) and β-mercaptoethanol led to decreased protease inhibitory activity. Complete inactivation of the inhibitor was observed at concentrations above 140 µM dithiothreitol. Whereas inactivation occurred at 400 µM of β-mercaptoethanol, when compared to dithiothreitol.

Chemical modification of amino acids cysteine and serine in the inhibitor protein molecule led to a drastic reduction in the activity even at a lesser concentration of the chemical modifier. In contrast modification of histidine led to reduced activity at higher concentrations of the modifier, whereas the modification of tryptophan with *N*-Bromosuccinamide did not show any effect on the inhibitory activity of the inhibitor.

Binding studies of inhibitor with trypsin using flourimetry revealed that the emission spectra of trypsin was quenched, indicated there was a strong binding of protease inhibitor with trypsin. The inhibitor was found to be stable in 0.04 M HCl and pretreatment of the inhibitor with trypsin decreased the activity of inhibitor in response to increasing concentration of trypsin.

Peptide mass fingerprint of protease inhibitor did not match any of the inhibitors in Swiss-Prot database. Nevertheless one of the fragment of the inhibitor searched with NCBInr database showed similarity to serine proteinase inhibitor from bitter guards (*Momordica charantia*) and of trypsin inhibitor *Cucumis melo* seeds.

The protease inhibitor showed remarkable specificity towards trypsin. It was observed that it did not show much activity towards thermolysin and proteinase K when compared to trypsin. Further no detectable inhibition was observed against chymotrysin, subtilisin and papain. The inhibitory constant *K*i of the inhibitor was found to be $1.043X10^{-10}$ M. The predicted stoichiometry of trypsin–protease inhibitor interaction was observed as 1:1 and 38.334g of protease inhibitor was necessary to completely inactivate 23.4g of trypsin. It was also found that the amount of inhibitor needed for 50% inhibition (IC_{50}) of trypsin was 0.5 nM.

The purified protease inhibitor was also observed to inhibit the proteases isolated from citrus pest, the lime swallowtail *Papilio demoleus* (Lepidoptera: Papilionidae). The inhibitory activity of the inhibitor was stable in the pH range of 4-10 and had a remarkable specificity against trypsin indicating its possible use as biopesticide.

The protease inhibitor was found to be active against the commercially available protease esperase compared to the significant level of inhibition of proteases of *A. oryzae, B. licheniformis, Bacillus* sp. *and B. amyloliquefaciens.* Further the high specificity of the inhibitor towards trypsin and trypsin like proteases indicated its potential for use in therapeutics.

Conclusion

A serine protease inhibitor from basidiomycete *Pleurotus floridanus* was isolated and evaluated for the possible application against proteases of microbial origin. The physico chemical characterization of the inhibitor indicated that protease inhibitor is new with novel characteristics. The protease inhibitor was stable over a wide range of pH and its inhibitory activity against several serine proteases, inhibition of gut protease of citrus pest, the lime swallowtail *Papilio demoleus,* suggest its possible use as biopesticide, which can withstand alkaline conditions of insect's gut flora. Further there is scope for its application in the development of pharmaceuticals against diseases manifested by proteases and as preservative in controlling spoilage of foods caused by protease activity. There is scope for further research on structure elucidation and binding studies employing bioinformatics tools to facilitate their use in wide range of applications.

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LIST OF PUBLICATIONS

a) Peer Reviewed

Manzur Ali P. P, Sapna. K, Abraham Mathew, Rekha Mol K. R and Elyas K K. (2012). Screening and activity characterization of protease inhibitor isolated from mushroom *Pleurotus floridanus*. *Advanced Biotech*. **12 (04):**27-30.

Manzur Ali P. P, Sapna. K, Rekha Mol K.R, Chandrasekaran M and Elyas K. K "Trypsin Inhibitor from edible mushroom *Pleurotus floridanus* active against proteases of microbial origin" *Process Biochemistry* (Under Review).

Sapna. K, **Manzur Ali P. P**, Abraham Mathew, Rekha mol K. R, Sarita G. Bhat, Chandrasekaran. M and Elyas K. K. (2012). "Marine *Pseudomonas mendocina* BTMW 301 as a potential source for Extracellular Proteinaceous Protease Inhibitor" *Advanced Biotech.* **11(12)**:16-19.

Elyas K.K.; Abraham Mathew; Rajeev K Sukumaran; **Manzur Ali P.P**.; Sapna K.; Ramesh Kumar S.; Rekha Mol K.R.(2010) "Production optimization and properties of β-glucosidases from a marine fungus *Aspergillus* -SA 58". *New Biotechnology.* **27(4)**:347-351.

K. Sapna, **P.P Manzur Ali**, Rekha Mol K.R., Chandrasekaran M., Sarita. G Bhat and K. K. Elyas "Isolation, purification and characterization of a pH tolerant and temperature stable proteinaceous protease inhibitor from marine *Pseudomonas mendocina* BTMW 301" *Biotech Letters* (Under Review).

b) Full paper in proceedings of National/ International Symposiums/ Conferences/ Seminars

K. Sapna, **P. P Manzur Ali** and K. K. Elyas (2008) "Screening, isolation and application studies of protease enzyme inhibitors from marine microorganisms" Proceeding of the International Conference on

Biodiversity Conservation and Management (Biocam 2008) conducted by Rajeev Gandhi Chair, CUSAT, 3rd to 6th February, 2008. Natarajan et al (eds) **ISBN: 978-81-907269-7-9.**

Rekha Mol K. R, **Manzur Ali P.P** , Abraham Mathew, Smitha S, Sapna K, Sarita G Bhat and Elyas K.K (2011) "Screening of various biological sources for antibacterial peptides." Proceedings of National symposium on "Emerging trends in Biotechnology" conducted by Department of Biotechnology, CUSAT, 1st and 2nd September, 2011. Sarita G bhat (edr) **ISBN number: 978-93-80095-30-1.**

c) Oral/Poster presentations in National/ International Symposiums/ Conferences/ Seminars

K. Sapna, **P.P Manzur Ali**, Abraham Mathew and K. K "Serine protease enzyme inhibitor from marine bacteria: Implications for inhibition on microbial growth." Book of abstracts of MECOS '09, Cochin, Kerala. International symposium on Marine Ecosystems challenges and Opportunities, Cochin from 9-12 February, 2009.

Rekha Mol K.R, **Manzur Ali P.P** , Abraham Mathew, Smitha S, Sapna K, Sarita G Bhat and Elyas K.K "Isolation and purification of extracellular antibacterial protein from *Aspergillus* sp. MF 9". At International Conference on "Advances in Biological Sciences" (ICABS), Dept. of Biotechnology, Microbiology and Inter University Centre for Biosciences, Kannur University, from 15-17 March, 2012.

Sajimol Augustine, **P P Manzur Ali,** K Sapna, K K Elyas, S Jayalekshmi "Immobilization of trypsin with chitosan capped ZnS:Mn nanocrystals for therapeutic and diagnostic applications (2012) International conference on Nanotechnology at the Bio-Medical interface, NanoBio 2012 at Amrita centre for nanoscience and molecular medicine, 21-23 February, 2012 (2nd Best poster award)

GENBANK SUBMISSIONS

Pleurotus floridanus strain PF101 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence. **Manzur,P.A**., Elyas,K., Linda,L., Sapna,K., Rekha,K.M. and Chandrasekaran,M. **GenBank Acc No. GU7210580.**

Calvatia candida strain PB101 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence. **Manzur,P.P**., Elyas,K.K., Sapna,K., Rekha,K.R.M. and Chandrasekaran,M. **GenBank Acc No. GU939632.**

16S rRNA gene sequence of *Pseudomonas mendocina* partial. Sapna,K., Elyas, K.K., Chandrasekaran, M., Sarita,B.G. and **Manzur, A. GenBank Acc No. GU139342**.

Bacillus subtilis subsp. subtilis strain BTSB22 16S ribosomal RNA gene, partial sequence. Smitha,S., **Manzur,A.P.P.** and Sarita,B.G. **GenBank Acc No. GI:296044582.**

Rekha Mol,K.R., Elyas,K.K.,**Manzur Ali,P.P**., Sapna,K., Abraham,M.and Ramesh Kumar,S. *Aspergillus fumigatus* strain MF 9 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene,and internal transcribed spacer 2, complete sequence; and 28Sribosomal RNA gene, partial sequence. **GenBank Acc No. HQ285882.**